

Abstracts from
The 27th Annual Meeting of
The Japanese Society of Toxicologic Pathology

Osaka, January 27–28, 2011

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SL-1

ER Stress and Diseases

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Protein, a major component of the cell, is synthesized according to the central dogma (DNA → RNA → Protein). Proteins are polypeptides when synthesized and must therefore be correctly folded and assembled to fulfill their function as assigned by genetic code. Anfinsen's dogma telling that protein is folded spontaneously according to its primary amino acid sequence without energy consumption is basically still accepted; however, the extremely high concentration of proteins in the cell results in an environment which is unfavorable for spontaneous protein folding. Protein folding is productively assisted by a group of proteins inside the cells, termed molecular chaperones, which utilize ATP-derived energy.

Ribosomes synthesizing proteins are categorized into free ribosomes, which synthesize proteins localized in the cytosol and mitochondria, and membrane-bound ribosomes, which synthesize secretory and membrane proteins. Secretory and transmembrane proteins gain their correct tertiary and quaternary structures in the endoplasmic reticulum (ER), the first organelle they encounter after synthesis on membrane-bound ribosomes, which contains a number of molecular chaperones in abundance. Only proteins correctly folded in the ER are permitted to move along the secretory pathway, reaching their final destinations (Golgi apparatus, lysosome, plasma membrane and extracellular space). The ER is therefore considered to control the quality of secretory and transmembrane proteins.

The accumulation of unfolded proteins in the ER occurs under a variety of conditions, collectively termed ER stress. Examples include when the quality control system in the ER is compromised by environmental stress, when excessive amounts of proteins are delivered into the ER, and when a protein which cannot be properly folded due to genetic mutation is synthesized. ER stress exerts a detrimental effect on various cellular functions. If ER stress persists, the cell eventually suffers from a shortage of necessary proteins because unfolded proteins are retained in the ER and then degraded. Moreover, unfolding of proteins results in the exposure of hydrophobic amino acids and the resulting formation of aggregates with the protein itself as well as other cellular proteins gives rise to proteotoxicity.

The ER stress response, also called the unfolded protein response, is activated to protect the cell from ER stress and to maintain the homeostasis of the ER. This response results in a general attenuation of translation to decrease the burden on the ER. Further, the ER stress signal is transmitted to the nucleus to induce transcription of genes encoding ER-localized molecular chaperones, which results in the delivery of large amounts of induced molecular chaperones to the ER to cope with unfolded proteins.

Cells which are unable to cope with ER stress adequately

undergo apoptosis. Apoptosis of a small number of cells is beneficial to the organism, because it can minimize production of unfolded proteins. However, apoptosis of a large number of cells beyond a certain threshold may lead to the loss of function of an organ, which is detrimental to the multicellular organism. Recent findings have revealed links between ER stress and various diseases, such as diabetes, atherosclerosis, neurodegenerative diseases including Parkinson and amyotrophic lateral sclerosis, heart diseases, obese and metabolic syndrome, and inflammatory bowel disease, and have accordingly gained attention from a variety of research fields.

In my lecture I will talk on ER stress and diseases in depth.

SL-2

Foundation and Progress of Japanese Society of Toxicologic Pathology

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JSTP was founded in 1985 for the purpose of assessing the safety of environmental chemicals including pharmaceutical drugs. It was created subsequent to the formation of The Japanese Society of Toxicology (JST) in 1981 and The Japanese Society of Pathology (JSP) which will reach its 100-year anniversary this year. The conceptual framework of JSTP differs from JST and JSP and is based on the combined sciences of toxicology and pathology. Since its formation the JSTP has been active domestically and internationally and has increased the number of members from industry, regulatory agencies and academia with current membership at 950 in 2010. As authors and founding members of JSTP, our purpose is to provide JSTP historical background information and propose future directions for the JSTP.

JSTP was founded under the leadership of the first president, the late Dr. Yasukazu Nishiyama, with the cooperation of several executive committees (ECs) and the support of JSP. The first academic meeting was organized by Dr. Nishiyama with the participation of two speakers from the U.S. and Germany. There was active discussion at that meeting and the founding members were greatly encouraged to develop Toxicologic Pathology (TP) in Japan. The second meeting was held as a JSTP annual meeting organized by late Dr. Kosaku Fujiwara where the presentations were made by JSTP members with active discussions and exchange of information and ideas among JSTP members. Since then JSTP annual meetings have continued up to this year. At these annual meeting Dr. Nishiyama and the ECs developed the rules and activities of JSTP. The organizational structure of the society was established to consist of the president, several ECs, councilors and general members, and various committees. It was determined that society activities would include an annual meeting, publication of the journal, holding histopathology slides conference and continuing education programs based on the recent topics, establishing a certification system for toxicologic pathologists, and joining to International Federation Society Toxicologic Pathologists (IFSTP). These rules, guidelines, and activities have been maintained up to the present time.

Historically the JSTP has promoted and sponsored a laboratory animal histopathology seminar series. This has been done as one of activities of the non-profitable foundation of International Life Science Institute (ILSI) in Washington D.C., U.S.A. The planning and administration of the seminar series has been done by the same faculty. This seminar series started 1981 and ended 1999, for a total of 16 seminars. The seminar

was circulated in the U.S.A. (with the late Dr. Thomas C. Jones, Harvard Medical School as the organizer), in Germany (Dr. Ulrich Mohr, Hannover Medical School) and in Japan (Dr. Yoichi Konishi, Nara Medical School) and it has provided a tangible contribution to the international recognition of TP and contributed to the expertise of toxicological pathologists in general.

As new international regional STPs were established in North and South America, Britain, the European Union, Korea and India, an IFSTP was established with an EC consisting of the representatives from those regional STPs. This was followed by efforts to globally standardize diagnostic nomenclature, to foster high standards for pathologists through continuing education, and to develop an international certification system for toxicologic pathologists. JSTP has been actively participating in these activities.

In 21th century, the application of molecular biological technology to TP is an important subject. However, it is emphasized that it will always be necessary to have a broad knowledge and experience with morphology to be able to anchor the molecular findings to definitive structural and functional changes in order to place the results obtained by molecular biology into appropriate perspective.

The purpose of this presentation was to introduce some historical background and past activities of the JSTP and to encourage younger members of JSTP to build on this foundation and maintain the high professional standards and visibility of JSTP.

EL-1

Adverse versus Adaptive Hepatic Enzyme Induction (Hypertrophy)

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The liver is the major site of xenobiotic metabolism in mammals. The liver is also one of the primary organs responsible for maintenance of normal homeostasis and physiological functions and, as a dynamic system, is capable of relatively rapid responses to stimuli leading to enzyme induction.

There are two general categories of hepatic enzyme systems: (1) constitutively expression enzyme systems and (2) altered expression (increased or decreased) systems in response to xenobiotic or endobiotic stimuli. We are generally most concerned with increased enzyme induction since enzyme induction sufficient to disrupt homeostasis may be associated with undesired effects, including hepatotoxicity, drug-drug interactions, altered pharmacokinetics, and carcinogenesis.

Hepatic xenobiotic enzyme induction may be associated with changes in liver weight, histological evidence of hepatocellular hypertrophy or hyperplasia, and/or changes of serum clinical chemistry analytes. Within certain limits these responses represent normal physiological adaptive response to stimuli that serve to protect the host from undesirable effects. In that regard, enzyme induction is good. This form of enzyme induction is also common and desirable. However, depending upon the sex and species being exposed, the dose and frequency of exposure, diet and age of the animals and other factors, enzyme induction in preclinical animal studies can be associated with undesirable effects. When undesirable or adverse effects do occur, they are most often a consequence of high levels of exposure and species specificity.

For purposes of this presentation, the focus will be on hepatocellular hypertrophy associated with microsomal hepatic enzyme induction, primarily induction of CYP enzymes. The terms adaptive and adverse in the liver may be defined as follows:

Adaptive – A biological, morphological or physiological change in hepatic regulatory pathways in response to a stimulus that modulates (adapts) organ function to preserve homeostasis.

Adverse – A dose-related and reproducible biochemical, morphological or physiological change in the liver in response to a stimulus that either by itself or in combination adversely affects the performance of the liver or the whole organism or compromises the ability to respond to additional environmental challenge.

Morphological changes associated with hepatic microsomal enzyme induction include liver enlargement, increased liver weight, hepatocellular hypertrophy, and transient hepatocellular hyperplasia. The hypertrophic hepatocytes typically have a distinctive lobular distribution. The magnitude of these changes is primarily influenced by the sensitivity of the test species and the level of xenobiotic exposure. A 10 to 50% increase in liver weight is a typical response to a xenobiotic. Histological evidence of hypertrophy can be seen when absolute liver weight is increased 20% or more. When the dose of the xenobiotic is high or the exposure prolonged, the adaptive liver responses can be exceeded resulting in toxicity and carcinogenicity.

Hallmarks of an adverse response including hepatocellular necrosis, biliary stasis, and bile duct hyperplasia can be readily recognized histologically.

When histological effects are limited to hepatocellular hypertrophy associated with hepatic enzyme induction, serum clinical chemistry analytes such as alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transferase do not show consistent or substantial changes in activity. The same is true for dogs and monkeys. However, elevations of these and other serum or plasma markers of hepatobiliary injury are well known in studies where there is frank hepatotoxicity. Similarly, inconsistent elevations of alkaline phosphatase, and gamma glutamyl transferase seen in dogs exposed to phenobarbital or corticosteroids have been attributed to hepatobiliary insult and not microsomal enzyme induction. Glucocorticoid-induced increased alanine aminotransferase that is not associated with hepatic microsomal enzyme induction has been documented in rats.

Translation of preclinical animal studies associated with hepatic enzyme induction to humans may be unpredictable because of species differences in nuclear receptors leading to different induction responses between animals and humans. Consequently, assessment of the relevance of observed hepatic enzyme inductive effects in animals to humans must be made on a case-by-case weight-of-evidence basis.

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EL-2

Mechanistical Considerations on Induced Pathological Lesions

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The development of a pharmaceutical product bases on a hypothetical function of a molecule for an intended clinical indication. The screening of such a product includes the exposure of laboratory animals. Due to absorption, distribution, metabolism, storing and/or excretion, toxic metabolites may affect molecular targets causing cellular responses, and hence influence the organism.

Reasons for mechanistical considerations may be different. For example, for pesticides, marketing authorization may be risk cup based on the mode of action. For pharmaceuticals, the authorities want to get the information of the relevance of induced lesions for human. Important related issues are risk extrapolation and dose responses. Furthermore, mechanistical studies need to be performed for the candidate selection during development, to understand the mode of action of the test item, to optimize the product line, and mainly to understand the mechanism of induced tissue injury. The latter should provide arguments for the discussion for the decision of adverse vs non-adverse effects, species-specific lesions, and even possible ontogenetic potential. Under pre-designed experimental conditions, mechanistical investigations may be even the key to detect toxic lesions.

Most often, a problem is recognized during the dose range finding studies in rodents and non-rodents as well as in subacute toxicity studies. Under certain conditions, mechanistical studies may also be performed as alone-standing studies on products that were developed before.

Mechanistical investigations are in most cases a multidisciplinary approach. There is a broad range of tools necessary in most cases. The selection of methods bases on possible previous study, including the whole range of regulatory studies (e.g. in-silico toxicology, genotoxicity, safety pharmacology, acute studies, toxicokinetics etc.) and on available information on the test item under testing.

The selected methodology is mainly driven by the detection of functional or pathological lesions. Morphological evaluation (including immunohistochemistry, image analysis, electron microscopy etc.) can provide a proof of hypothesis only in a limited number of cases. Usually, it triggers further investigations that may include cytobiological or biochemical methods, i.e. testing on DNA-binding, DNA-adduct analysis or protein-binding. Special in-vivo or in-vitro metabolism studies for the comparison of species and investigations on the bioavailability of products may be necessary. Special markers may be applied to detect or explain target organ pathology, e.g. markers for renal toxicity, proliferation markers, enzyme markers for oxidative stress etc. Furthermore, biochemical investigations on proteins, DNA/RNA, hormones or enzyme measurements along with specific immunohistochemical or histochemical investigations or alone standing may provide insides on mechanisms of toxicity.

WS I-1

General Aspect of Wistar Hannover Rat

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In 2-year carcinogenicity studies with medicinal and chemical products, Sprague-Dawley(SD), Fischer344(F344) or Wistar strain is used as one of international standard rat strains. In May, 2007, National Toxicology Program (NTP) announced in its website that F344/N rats, which had been used in NTP toxicology studies for more than 30 years, were considered not to be appropriate for 2-year carcinogenicity studies due to inherent problems such as high incidence of mononuclear cell leukemia and testicular tumor, infertility, sporadic seizure, spontaneous chylothorax. NTP recommended using Wistar Hannover (Wistar Han) rats, instead of F344 rats, for NTP studies. Wistar Han rats had been used in toxicology studies in Europe with features of long survivability, moderate size, overall low incidence of spontaneous background tumors, which are favorable to general toxicity and carcinogenicity studies.

However, NTP concerned about reproductive capability (small litter size) of Wistar Han rats, and announced the change to using Harlan Sprague Dawley rats, in lieu of Wistar Han rats, in reproductive and development studies (2009) and then in carcinogenicity studies (2010).

In Europe, Wistar Han rats have been used in whole toxicology studies including general, carcinogenicity, reproductive and development studies. As in Europe, expectation and hope are increasing that using this strain of rat for the entire toxicology program, if possible.

Recently, a few breeders started supply of Wistar Han rats in Japan and background data have been collected and shown at workshops focused on this strain as a new standard strain candidate in toxicology studies in addition to rat strains currently used.

In this workshop, basic and background information on histopathology of Wistar Han rats are provided by the following presenters of WS1-2 to WS1-6.

WS I-2

In House Background Data of Wister-Hannover Rats

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Background and Purpose

Wistar-Hannover rats have been considered as a replacement for Sprague-Dawley (SD) rats because their body weight gain is gradual and slow and there are relatively few spontaneous tumors during long term study. In this study, rats were housed for 26 weeks without treatment and background data were collected. In addition, results obtained were compared with the same strain rats obtained from a different breeder and SD rats.

Materials and Methods

Six-week-old RccHanTM:WIST (Japan Laboratory Animals, Inc) rats, 55 animals per sex, were housed without any treatment for 26 weeks. General condition, body weight and food consumption were recorded and urinalysis, ophthalmologic examinations were conducted. At the 26th week (midterm examination), 15 animals per sex were necropsied and hematology, clinical biochemistry, organ weight measurements and pathological examination were conducted. For comparison, Slc:WistarHannover/Rcc from a different breeder (n = 15, Japan SLC, Inc.) and SD strain rats, CrI:CD (SD) (n = 36, Charles River Laboratories Japan, Inc.), were similarly examined.

Results and Discussion

There were no apparent differences between the Wistar-Hannover rats from the 2 different breeders in terms of the general condition, body weight gain, food consumption, hematological, biochemical and organ weight values. Compared to SD rats, food-intake showing an approximately constant value during the study period was smaller and body weight gain was slower. In the ophthalmological examinations, there was a high incidence of corneal and lenticular turbidity. Urine volume was tended to be larger in males and smaller in females. Hematological examinations showed that total white blood cell and neutrophil counts tended to be lower and ALP and IP values were also lower in biochemical examination. In histopathological examinations of the RccHanTM:WIST rats, there were no clear morphological changes in the lens, however, retinal atrophy was seen in 6 out of 15 females (40%), indicating a high incidence. Retinal atrophy in females seems to be a background pathological change in this strain. A long-term study is still continuing, this presentation will include the progress and further analysis.

WS I-3

Histopathological characteristics of Wistar Hannover rats [RccHanTM:WIST, BrlHan:WIST@Jcl(GALAS) and CrI:WI(Han)]

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【Purpose】To obtain the historical control data, pathological examination was performed in Wistar Hannover rats from three different suppliers. Their data was evaluated and compared with the historical control data of SD rats.

【Methods】RccHanTM:WIST [Rcc], BrlHan:WIST@Jcl (GALAS) [Jcl] and CrI:WI (Han) [CrI] rats were maintained without treatment and 20 male and 20 female rats from each suppliers were sacrificed at 10, 19 and 32 weeks of age.

【Results and Discussion】There were differences in the incidence of spontaneous occurring lesions between those rats as follows: Mineralization of the outer medulla in the kidneys only in female Jcl. Hyaline droplets in the proximal tubules in males at 32-week old (Rcc=CrI>Jcl). Thymic atrophy at 32 weeks of age (Rcc>CrI=Jcl). The splenic extramedullary hematopoiesis in males at 10-week old (CrI>Jcl>Rcc). Hemosiderin deposition in the spleen in females at 19 (CrI=Jcl=Rcc) and 32-week old (CrI=Jcl>Rcc). Mononuclear cell infiltration in the heart in males at 32-week old (Rcc>Jcl=CrI). Inflammatory cell infiltration in the cecum in CrI at 10-week old. Vacuolation in the ductal epithelium of the epididymes at 19 (Rcc=Jcl>CrI) and 32-week old (Jcl>Rcc=CrI). Degeneration/necrosis of corpus luteum (Rcc=Jcl>CrI) at older than 19-week old. Thyroid dysplasia, a genetical disorder, in a Jcl female (1/20) at 19-week old. In comparison with those in SD rats, the following findings were relatively frequently observed in Wistar rats: hemosiderin deposition in the hepatocytes and periportal area, lipofuscin deposition in the proximal tubules, erosion in the glandular stomach, vacuolation in the limiting ridge, hemosiderin deposition in the interstitium of the epididymes, mineralization in the cornea, retinal dysplasia and hemosiderin deposition in the retina. Whereas, fatty changes in the perilobular hepatocytes and follicular cell hypertrophy in the thyroid, which were very common in SD rats, were observed at a low incidence in Wistar rats.

WS I-4

Background Data on Hannover Wistar Rats (RccHanTM: WIST) in Shin Nippon Biomedical Laboratories

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For the 2-, 4-, 13- and 26-week repeated-dose toxicity studies, each consisting of 30 males and 30 females, background data on body weight, hematology, blood biochemistry, organ weights, and spontaneously occurring lesions were obtained from RccHanTM: WIST rats (WH rats) were fed a low protein commercial diet (CR-LPF) at Shin Nippon Biomedical Laboratories Ltd. Body weight gain of WH rats undergoes a lower transition than in Sprague-Dawley rats (SD rats). Ophthalmology showed corneal and optic media opacity more frequently than in SD rats. In hematology, clinical biochemistry, and organ weights, coefficients of variation for the various parameters were lower than in SD rats. Histopathology revealed corneal mineralization, retinal dysplasia, and brown pigmentation in the hepatocytes (hemosiderosis), which have been reported in Hannover Wistar rats, and similar lesions are reported in the histopathological data on SD rats. However, chronic nephrosis and fibrosis of the pancreatic islets, which were observed frequently in SD rats, were not observed. Moreover, mammary tumor, thyroid dysplasia, and hyperplasia were not observed.

The characteristics of the Hannover Wistar rat provide highly reliable toxicological data, contributing to the development of drugs and medicines.

WS I-5

Profile of Corneal Mineralization in Wistar Hannover Rats

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[Abstract] As there are only a few studies using Wistar Hannover rats in Japan, we are researching their background data. We investigated the incidences of histological corneal mineralization and ophthalmological corneal opacity as relatively common findings in RccHanTM:WIST (Rcc) and CrI:WI(Han) (CrI). For the histopathological examination, we used eyes from 120 rats at 8, 10, 19 and 32 weeks old in Rcc and 19 and 32 weeks old in CrI, respectively.

[Corneal opacity] In both strains, there was granular and/or macular opacity on the surface of the cornea unilaterally or bilaterally. Both strains indicated age-related increases of the incidence from 6 to 32 weeks old, from 5.0% to 20.0% in Rcc and from 8.3% to 38.3% in CrI, respectively. However, there were lot-to-lot variations in both strains, and it might have affected the incidence. Moreover, we found a higher incidence in male rats with many bilateral cases.

[Mineralization] In both strains, basophilic granular, linear, mottled or coarse deposits were observed between the corneal epithelium and stroma. Moreover, we could often see a space there and/or activated corneal cells in the stroma. The deposits were negative for von Kossa staining. The incidence was 37.5% and 35.9% in Rcc and 35.9% and 61.7% in CrI at 19 and 32 weeks old, respectively. There were lot-to-lot variations in both strains and the higher incidence was found in males.

WSI-6

[Conclusion] To confirm the usability of Wistar Hannover rats in toxicological studies, it is important to reveal their characteristics. In this study, it was found that corneal mineralization frequently occurs and there are lot-to-lot variations in the incidence in both strains.

Back Ground Data for General Toxicology in RCCHanTM:WIST Rats

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[Purpose] Wistar Hannover (RccHanTM:WIST) rats, a new strain of Wistar Hannover rats which maintains the advantage but no demerit of the established strain, have been examined for collection of background data in preparation for use in toxicity studies and carcinogenicity studies. In this paper, we mainly report the data of the 32-week age necropsy group from the background data we are collecting.

[Method] The animals were received at 4 weeks of age, quarantined/acclimated and divided into 4 groups (25 males and 25 females/group) — 8-, 10-, 19- and 32-week age necropsy groups — at 6 weeks of age. They were housed individually in stainless- steel cages which were placed in an animal room where temperature was kept at $23 \pm 3^{\circ}\text{C}$, relative humidity at $50 \pm 20\%$, air ventilation 10-15 times per hour and 12-hour lighting per day. They were allowed free access to feed (CR-LPF, radiation-sterilized, pelleted, Oriental Yeast Co., Ltd.) and tap water. The items examined during and at the end of the observation period were the same as for general toxicity studies, and thus the data of CrI:CD(SD) rats and F344/DuCrI:CrIj rats which were housed under the same environment were used to compare with this background data.

[Results] The body weight of this new strain was intermediate of the SD and F344 rats, and food consumption was lower than SD rats in males while it was comparable to SD rats in females. In ophthalmological examination, focal corneal opacity was observed at each examination stage at higher incidence than in SD and F344. In hematological examination, leukocyte count was lower than that of SD rats at each stage. Organ weight tended to be higher for the adrenal and thymus for both males and females and the testis and ovary in this strain than in SD. In histopathological examination, pigmentation in hepatocytes (without sex difference or week-age difference), presence of pigmented macrophages in the interstitium of epididymis (at higher incidence in 19-week old rats compared to that of 10-week old rats) and mineralization in the cornea of the eye (at high frequency especially in males, not related to week age) were observed. The incidence of age-related atrophy of the ovary and vagina was lower than in SD rats. Otherwise there were no remarkable changes in clinical observation, urinalysis, blood chemistry examination or at necropsy. In the experiment for 110-week age necropsy group, the survival rate was 68% in males and 77% in females.

WSII-1

INHAND Updates on Organ System Working Groups

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The project of International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) is a joint initiative of the Societies of Toxicologic Pathology from the United States (STP), Great Britain (BSTP), Japan (JSTP), and European countries (ESTP). This initiative is a 5-year project from 2008 to 2012 to create internationally standardized nomenclature and diagnostic criteria for neoplastic and non-neoplastic lesions in rats and mice.

Organ System Working Groups for 15 different organ systems of the rat and mouse have been established and now are working on preparation of manuscripts to be published in official journals of *Toxicologic Pathology* (STP), *Experimental and Toxicologic Pathology* (ESTP), and *Journal of Toxicologic Pathology* (JSTP). Publication of the manuscript will be rotated among the 3 official journals. Although progress on the work varies among Organ System Working Groups, the manuscript of the respiratory tract was first published in *Toxicologic Pathology* as a supplement in 2009 and subsequently the liver and gallbladder supplement in the same journal in 2010. Works on other organ systems are still underway and draft manuscripts of the nervous system, urinary system, immune system, mammary and other glands including Zymbal, preputial and clitoral glands will be prepared soon.

In principle, a draft manuscript of each organ system will be first reviewed by the members of the Global Editorial and Steering Committee (GESC). After reviewing by GESC, the manuscript will be uploaded to the goRENI Web site for 60 days to gather comments from members of STP, BSTP, ESTP, and JSTP. In consideration of all comments gathered, the manuscript will be revised and then finalized for publication as a supplement in an official journal. Images of lesions appearing in the supplement publication are all color. In this workshop, INHAND updates on the works by Organ System Working Groups will be reported.

WSII-2

INHAND Update on Respiratory System

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The current progress of the INHAND Project of the respiratory tract was reported. The INHAND Project of the respiratory was already completed. The standardized nomenclature and diagnostic criteria of the respiratory tract lesions are documented as “Proliferative and Nonproliferative Lesions of the Rat and Mouse Respiratory Tract” *Toxicologic Pathology*, Vol.37 Number 7 Supplement (Renne R et al., 2009), and are available electronically at the goRENI Web site (<http://www.goreni.org>).

The document presents pathogenesis/cell of origin, diagnostic features, differential diagnoses, comment and microscopic photograph for each lesion, and contains the following groups of lesions.

Nasal cavity: Congenital Lesions, Epithelial changes, Inflammation, Vascular changes, Non-neoplastic proliferative Lesions, and Neoplastic proliferative lesions.

Larynx, trachea, bronchi and bronchioles: Epithelial changes, Inflammation, Vascular changes, Non-neoplastic proliferative Lesions, and Neoplastic proliferative lesions.

Terminal bronchioles, alveoli and pleura: Congenital Lesions, Epithelial changes, Intra-alveolar accumulations, Inflammation, Abnormal dilatation/destruction of alveoli, Vascular changes, Non-neoplastic proliferative Lesions, and Neoplastic proliferative lesions.

In this presentation, we introduced the outline of the INHAND document in comparison with the current JSTP ‘s guidebook of toxicological histopathology (JSTP, 2000).

WSII-3

INHAND Update on Liver and Gallbladder

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In accordance with the guidance for Organ System Working Groups on the project of International Harmonization of Nomenclature and Diagnostic Criteria (INHAND), the manuscript of the liver and gallbladder lesions in rats and mice was prepared by the Liver Working Group and the draft was uploaded to the goRENI Web site to gather comments from members of the Societies of Toxicologic Pathology from the United States (STP), Great Britain (BSTP), and Japan (JSTP), and European countries (ESTP). Taking those comments into consideration, the manuscript was revised and the final draft was prepared in August 2010. The final draft was reviewed by the members of the Global Editorial and Steering Committee (GESC) and then finalized for publication as a supplement in *Toxicologic Pathology* in 2010.

A remarkable point in this document is that a new entity, “non-regenerative hepatocellular hyperplasia”, was added to non-neoplastic hepatoproliferative lesions, which represents a departure from traditional nomenclature schemes found in standard textbooks. The non-regenerative hepatocellular hyperplasia consists of a proliferative collection of hepatocytes spanning several lobules keeping a normal lobular architecture and without evidence of prior hepatic damage. There are basically two variations of this hyperplastic lesion. One is accompanied by angiectasis and/or spongiosis hepatis which may result in distortion of hepatic cords, while the other tends to be larger than several lobules and sometimes associated with a mild compression of adjacent hepatic parenchyma. The hepatocytes within the lesion tend to be larger in size but phenotypically similar to normal hepatocytes and have neither cytological atypia nor neoplastic growth pattern. Regenerative hepatocellular hyperplasia and focus of cellular alteration are essential differential diagnoses for non-regenerative hepatocellular hyperplasia. Non-regenerative hepatocellular hyperplasia can be distinguished from regenerative hyperplasia by absence of prior or ongoing parenchymal damage (necrosis) and from focus of cellular alteration by lacking phenotypical alterations.

In this workshop, differences in nomenclature or diagnostic criteria for liver and gallbladder lesions between this document and the Textbook of Toxicologic Pathology (JSTP, 2000) will be reported.

WSII-4

INHAND Update on Cardiovascular System

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The current status and the topics of concern were presented. Cardiovascular working group has largely obtained internal consensus with regard to the general outline of the list of terminology, synonym and modifier. Creating the text part has also been initiated, some of which are now under the process of internal review.

Rodent progressive cardiomyopathy: This is a disease entity specific to rodents, which is quite different from “cardiomyopathy” in humans and other animal species. The use of the term without consideration to species specificity should be avoided. With regard to the drug-induced lesions, use of this term may be appropriate even in a short-term study when acceleration of disease progression was occurred by the treatment. On the other hand, if the induced lesion is distinct from rodent spontaneous cardiac disease, more descriptive terms, *i.e.*, myocardial degeneration, necrosis, fibrosis or cellular infiltration, should be used to characterize the lesion. Nevertheless, it is noticeable that even unique drug-induced cardiac lesions may establish to a lesion indistinguishable from rodent progressive cardiomyopathy during a chronic treatment. Therefore, the severity of the lesion should carefully be evaluated in the chronic studies to detect cardiotoxic effects of the treatment.

Proliferative lesions: The terminology defined in the “Standardized System of Nomenclature and Diagnostic Criteria (SNNDC)” Guides is applied fundamentally to our proposal. Further discussion will be required for the decision on the propriety of including into the list some terms, including Schwannoma, atriocaval tumor, angioliipoma and hemangiopericytoma. Remaining topics are the discussion for the definition of criteria that should be considered in the differential diagnosis among preneoplastic lesions, benign and malignant tumors.

Cross-Section approach between working groups: Some of the vascular lesions which occur in any organs are described in overlap by each working group. The exchanges of information will be held between working groups, in order to ensure alignment in definitions, *e.g.* relationship between the principal terminology and synonyms, and the diagnostic criteria of proliferative lesions.

WSII-5

INHAND Update on mammary, Zymbal's, preputial, and clitoral Glands

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Our INHAND working group is focused on developing diagnostic criteria for the mammary and specialized sebaceous glands (Zymbal's gland, male preputial gland, female clitoral gland) all which originate from a thickening of the embryonic ectoderm which invades the underlying mesenchyme, and. The morphology of mammary gland depends on animal species, aging, and physiological condition, such as estrus cycle, pregnancy, and lactation. Also, the growth is regulated by numerous hormones including prolactin. Zymbal's gland is located beneath the squamous epithelium of the external ear canal, and the susceptibility of a species to the carcinogenic action of chemicals is related to cytochrome P-450 and peroxidase-dependent enzymatic pathways in Zymbal's gland. The growth and secretory activity of male preputial and female clitoral glands are regulated primarily by testosterone and the pituitary hormones (adrenocorticotrophic hormone, growth hormone, and prolactin).

Our group is using the diagnostic categories in these organs; degenerative, inflammatory, vascular, and miscellaneous changes, and neoplastic lesions. Scientific review for our draft paper has been already finished by GESC members on Oct 2010, and this paper is planning to be published in 2011. In this session, I will summarize both our draft paper and the special considerations and comments from working group and GESC members. The latter included the following: a- using one general diagnosis for mammary gland ductular dilation versus three (dilation, ectasia, and galactocoele) which are differentiated by specific diagnostic criteria; b- separating preputial / clitoral gland carcinoma diagnoses into subtypes by the presence of specific differentiating characteristics or simply diagnosing carcinoma and leaving subtypes as a descriptive component of report.

O-1

Identification of Ultimate Carcinogenic Metabolite of Arsenic in Bladder: Determination of Metabolic Pathway of DMMTA^V and its Effects in DMA^V-induced Rat Bladder Carcinogenesis

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【Background】 We have previously demonstrated the bladder carcinogenicity of dimethylarsinic acid (DMA^V) in rats. Dimethylmonothioarsinic acid (DMMTA^V) is a metabolite of DMA^V in rats, and recently is detected in the urine of humans exposed to arsenic. Our previous findings that DMMTA^V was much more toxic than DMA^V in both rat and human urothelial cells suggested that it may involve in the DMA^V-induced rat bladder carcinogenesis. The purpose of the present study were to determine the metabolic pathway of DMMTA^V and evaluate the its effects in DMA^V-induced bladder carcinogenesis in rats.

【Methods】 Experiment 1: 10-week-old male SD rats were treated with DMA^V (50 mg/kg) by single intragastric administration and arsenic concentrations in bile were examined. Experiment 2: 10-week-old male F344 rats were treated with DMA^V (100 ppm As) with or without pretreatment of antibiotic (Neomycin, 2 mg/ml) in drinking water as follows: Neomycin (3 days)→DMA^V (1 day); water (3 days)→DMA^V (1 day). At the end of day 4, arsenic concentrations in feces and urine were examined. Experiment 3: 6-week-old male F344 rats were divided into 3 groups and treated as follows: Neomycin (2 mg/ml)+DMA^V (100 ppm As), DMA^V(100 ppm As) alone, Neomycin (2 mg/ml) alone, respectively, for 2 weeks. Arsenic concentrations in feces and urine and cell proliferation activity in bladder urothelium were examined at the end of week 2.

【Results】 Experiment 1: DMMTA^V was detected 20 min, after DMA^V appeared in bile. Experiment 2: DMMTA^V was found in feces and urine of rats treated with DMA^V but not in rats pretreated with Neomycin. Experiment 3: Accompanying the significant decreases in the urinary concentrations of DMMTA^V, cell proliferation activities in bladder urothelium were significantly decreased in Neomycin+DMA^V group compared to DMA^V group. **【Conclusion】** These results indicate that DMMTA^V is produced from DMA^V by intestinal bacteria in colon and suggest that DMMTA^V is an ultimate carcinogen in DMA^V-induced rat bladder carcinogenesis.

O-2

Possible Involvement of Genotoxic Mechanisms in the Modes of Action Underlying Ochratoxin A-Induced Renal Carcinogenesis

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Ochratoxin A (OTA) is one of mycotoxin produced by fungal species, and often found as a contaminant in cereals and agricultural products. OTA targeting the proximal tubule S3 segment has been known as a nephrotoxicant and renal carcinogen in spite of its genotoxicities remaining undetermined. Therefore, the modes of action underlying OTA induced-renal carcinogenicity are poorly understood. In the present study, to explore a possible participation of genotoxic mechanisms in OTA induced-renal carcinogenesis, *in vivo* mutation frequencies (MFs) in the reporter genes and levels of 8-hydroxydeoxyguanosine (8-OHdG), one of oxidative DNA damages, were examined in the kidney of *gpt* delta rats given OTA at a carcinogenic dose. Exp 1. Groups of 4-5 male *gpt* delta rats were administered OTA at a concentration of 0 (control) or 5 ppm in the basal diet for 4 or 13 weeks. As a result, in the treatment group, apoptosis, karyomegaly, and vacuolation in proximal tubule epithelial cells in the outer stripe of the outer medulla (OSOM) were found with higher incidences. At 13 weeks, there were no significant differences in the *gpt* MFs indicating the point mutation, and Spi^- MFs showing the deletion mutation among the groups. 8-OHdG levels were no significant differences compared with the control values through the experimental period. Exp 2. Groups of 5 male *gpt* delta rats were fed 0 (control) or 5 ppm OTA in the basal diet for 4 weeks. Macroscopically, the harvested kidneys were divided into the cortex, outer medulla, and inner medulla using the curving scissors based on the anatomical characteristics. In the cortex, significant differences of the *gpt* and Spi^- MFs were not observed. In the outer medulla, although there were no significant changes in the *gpt* MFs, Spi^- MFs in the OTA treatment group ($0.45 \pm 0.15 \times 10^{-5}$, $P < 0.05$) were significantly higher than control group ($0.13 \pm 0.20 \times 10^{-5}$). In conclusion, there were no changes in the *gpt* and Spi^- MFs using genomic DNA extracted from whole kidneys (Exp 1.). In the light of the sight specific toxicity observed in OSOM, we carried out *in vivo* mutation analysis with genomic DNA in outer medulla, consequently confirming significant increases of Spi^- MFs (Exp 2.). Therefore, it was strongly suggested that the genotoxic mechanisms may play an important in the modes of action underlying OTA induced-carcinogenicity. For the future, quantitative analysis of 8-OHdG levels in the cortex or outer medulla will be performed.

O-3

The Suppression of Growth, Invasion and Metastasis Ability of Rat Hepatoma Cells by Glutathione Peroxidase 2 siRNA*

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We have established 6 rat hepatoma cell lines, which exerted different metastatic potential to the lung after inoculation into the tail vein of nude mice. Among them, the higher metastatic cell lines had highly expression of glutathione peroxidase 2 (GPx2) compared to lower metastatic cell lines and normal rat liver cells. In the present experiment, we investigated the influence of interference of GPx2 in L2 cells which have highly metastatic ability. GPx2 expression was suppressed by more than 50% by GPx2-siRNA transfection. The cell lines with either GPx2-siRNA or control-siRNA and the original cell line without transfection were analyzed 24 or 48 hrs after transfection, as follows. GPx2-siRNA significantly inhibited cell proliferation for 48 hrs. The *in vitro* migration and invasion ability was suppressed by GPx2-siRNA by 76% and 51% compared with control-siRNA transfected cell, respectively. The activity of Matrix metalloproteinase 9 was also reduced by GPx2-siRNA. Moreover, *in vivo* study, the both number and area of metastatic nodules per area in the lung of nude mice was significantly reduced by 77% and 74% compared with control-siRNA transfected cell line. In conclusion, the suppression of GPx2 expression in tumor cells significantly reduced cell growth, invasion ability *in vitro* and metastatic ability *in vivo*.

O-4

Distinct roles of Canonical Wnt signaling in the Stem Cell Expansion and the Active Proliferation of Colon Epithelium

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The canonical Wnt signaling pathway plays a central role in the homeostasis of intestinal epithelium and the disruption of this pathway is involved in the majority of colon cancers. In order to investigate the role of the canonical Wnt signaling in the control of proliferation of the colonic epithelial cells, we generated the doxycycline-inducible β -catenin mice controlled by a tetracycline-responsive regulatory element (TRE) with a ROSA26 promoter-driven reverse tet repressor-VP16 transgene (M2rtTA) allele. The induction of lower levels of β -catenin led to active proliferation of colonic epithelium. In contrast, the induction of higher levels of β -catenin did not result in the active proliferation of colonic epithelium, and cells expressing nuclear β -catenin were dividing slowly. Interestingly, the higher levels of β -catenin led to the frequent fission and/or branching phenotype of the colonic crypts, which is accompanied by the upregulation of intestinal stem cell markers, *Lgr5* and *Musashi-1*, suggesting that higher levels of β -catenin expands colonic stem cells. Subsequently, we analyzed transcriptional profiles of colonic crypts with β -catenin induction at the different levels to clarify the molecular mechanism underlying the cellular proliferation and the stem cell expansion of colon epithelium. The evaluation of transcriptional profiles demonstrated that there were the differences between lower and higher β -catenin induced colonic crypts. These results indicate that Wnt signaling plays distinct roles on the cellular proliferation and the stem cell expansion of colon epithelium by differently regulating the target genes.

O-5

Lac Color Targets Plasma Hyaluronan-binding Protein and Inhibits Induction of Thyroid Capsular Invasive Carcinomas

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Recently, we have shown involvement of PI3 kinase/Akt signaling for development of capsular invasive follicular cell carcinomas produced by promotion with sulfadimethoxine (SDM) in a rat two-stage thyroid carcinogenesis model. Induction of capsular inflammation is suggested in the cancer invasion mechanism. Inhibitors of plasma hyaluronan-binding protein (PHBP) are a new class of agents that are expected to be effective for amelioration of chronic tissue destructive diseases, such as chronic nephrosis and liver cirrhosis. The present study was performed to examine whether PHBP inhibitors are effective for suppression of cancer invasion, and rats were orally treated with coccid-derived natural food colorants that contain active ingredients of PHBP inhibitors in this thyroid capsular invasion model. One week after initiation with DHPN, male F344/NSIC rats were fed a powdered diet containing 5% lac color (laccic acid: 76.6%) or 3% cochineal extract (carminic acid: 35.6%) during the promotion with SDM for 8 or 13 weeks. Incidence of capsular invasive carcinomas (13 weeks) as well as tenascin-positive invasive foci (8 weeks) decreased in SDM+lac color compared to SDM-alone through inhibition of inflammatory responses including angiogenesis linked to PI3/PKB cascades in the initial phase of cancer invasion. Cochineal extracts, on the other hand, facilitated cancer progression through promoting angiogenesis during the late phase of cancer development. Capsular invasive carcinomas induced by SDM-promotion exhibited a strong PHBP-immunoreactivity, and lac color decreased the number of PHBP-positive carcinomas. Transcript levels of *HABP2*, *Plau*, and *Plat* also decreased with lac color. Thus, the inhibitory effect of laccic acid on invasive carcinomas was probably due to the inhibition of inflammatory responses mediated by inhibition of tissue proteolysis resulting from activation of plasma hyaluronan-binding protein.

O-6

Modifying Effects of Serum Adipokines on DMBA-induced Mammary Carcinogenesis in Ovariectomized Rats

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Over the past decades, breast cancer mortality in Japan has rapidly increased. Epidemiological studies have demonstrated that obesity is a risk factor of breast cancer in postmenopausal women. On the other hand, the reason for the increasing mortality rate from breast cancer in premenopausal women is uncertain. To assess serum adipokines related to mammary carcinogenesis in the early life-stages, we have used a leptin receptor mutant Zucker (+/*fa*) rat model, which shows hyperleptinemia during adolescent stage, with 7,12-dimethylbenz(*a*)anthracene (DMBA)-initiation. The incidence and multiplicity of mammary adenocarcinomas in (+/*fa*) rats were significantly higher than the wild type (+/+) rats. In addition, some carcinomas induced in short latency periods in Zucker (+/*fa*) rats were moderately/poorly differentiated and characterized with severe invasion and interstitial proliferation. Western blot analysis revealed STAT-3 phosphorylation, a marker for activation of leptin-signaling pathway, in the moderately/poorly differentiated carcinomas. Therefore, it was suggested that hyperleptinemia in the early life-stages might influence the promotion/progression of mammary carcinogenesis. The present studies examined, modifying effects of hyperleptinemia/insulinemia on DMBA-induced mammary carcinogenesis in ovariectomized rats, for clarification of the mechanisms-based risk factors of breast cancer in postmenopausal women. A total of 40 SD female rats in the preliminary experiment were divided into 4 groups. Group 1 and 2 rats were ovariectomized with and without high-fat diet, respectively, and group 3 and 4 ones were sham-operated with and without high-fat diet feeding, respectively, for 4 weeks each. At the end of the pre-experiment, groups 1 and 2 showed hyperinsulinemia and group 1 additionally showed hyperleptinemia. In the main study, a total of 96 DMBA-initiated SD rats were used, and the other treatments than DMBA-initiation were same with the pre-experiment. The high-fat diet did not show any effects on the incidence and multiplicity of mammary carcinomas both in the ovariectomized and sham-operated rats. All carcinomas in sham-operated rats were well-differentiated, while carcinomas found in 2/6 and 3/6 rats of groups 1 and 2, respectively, showed moderate/poor differentiation. In the latter types of carcinomas, ERK1/2 activation was prominent but STAT-3 was not affected. These results indicated that other adipokines/hormone status than hyperleptinemia, such as hyperinsulinemia, might affect the induction of moderately/poorly differentiated mammary carcinomas in the DMBA-initiated ovariectomized rats. Further studies were needed to more clearly identify the mechanisms-based risk factors of breast cancer in postmenopausal women.

O-7

Epigenetic alterations in the liver induced by phenobarbital sodium

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Phenobarbital (PB) is a non-genotoxic hepatocarcinogen in rodents but the detail mechanisms of hepatocarcinogenesis are still unclear. It is well known that epigenetic alterations are involved in genomic instability and epigenetic mechanisms contribute to carcinogenesis. To clarify the epigenetic alterations, PB was administered by gavage to female rats at doses of 0, 8 and 80 mg/kg/day for a period of 4 weeks. After 4 weeks of PB treatment, LPO levels increased significantly in the 80 mg/kg/day group compared with controls. Unexpectedly quantitative RT-PCR analysis for Dnmts revealed that *Dnmt1* and *Dnmt3a* in the 80 mg/kg/day group were down-regulated significantly, compared to the control. Furthermore, *Hdac1* and *Hdac3* in the 80 mg/kg/day group were down-regulated significantly. ChIP-PCR analysis revealed that E2f1 binding to its enhancer element in the genomic *Dnmt1* region and pCreb binding to cAMP responsive element in the genomic *Dnmt3a* region were significantly decreased in the 80 mg/kg/day, compared to the control. ChIP-PCR also revealed that histone H3 of these elements was significantly acetylated. These indicate that PB induced down-regulations of Dnmts via modulation of transcription factor in spite of histone acetylation. Further quantitative RT-PCR analysis revealed that *Hif1a* was down-regulated and *Myc* was up-regulated significantly in the 80 mg/kg/day group. Thus we conclude that these epigenetic alterations may induce genomic instability leading to hepatocarcinogenesis and *Hif1a* signaling pathway may contribute to the epigenetic alterations.

O-8

A practical threshold for 2-amino-3-methylimidazo[4,5-f]quinoline carcinogenesis in the liver and colon of male F344 rats

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The carcinogenicity of the low amounts of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and to investigate mechanisms by which IQ exerts its carcinogenic effects. A total of 1595 male F344 rats were divided into 7 groups and administered with IQ at doses of 0, 0.001, 0.01, 0.1, 1, 10 and 100 ppm in the diet for 16 weeks. We found that IQ doses of 1 ppm and below did not induce preneoplastic lesions in either the liver or the colon, while IQ doses of 10 and 100 ppm induced preneoplastic lesions in both these organs. These results demonstrate the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats. The finding that p21^{Cip/WAF1} was significantly induced in the liver at doses well below those required for IQ mediated carcinogenic effects, suggests that induction of p21^{Cip/WAF1} is one of the mechanisms responsible for the observed no-effect of low doses of IQ. Furthermore, IQ administration caused significant induction of CYP1A2 at doses of 0.01 to 10 ppm, but administration of 100 ppm IQ induced CYP1A1 rather than CYP1A2. This result indicates the importance of dosage and when interpreting data on the carcinogenicity and metabolic activation of IQ. Overall, our results suggest the existence of no-effect levels for the carcinogenicity of this genotoxic compound.

O-9

Induction of interstitial pneumonia by intra tracheal spray of nano size ZnO

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Nanotechnology has considerable global socioeconomic value, and the benefits afforded by engineered nano materials are expected to have significant beneficial impacts. However, there is an urgent need to determine potential human health hazards before wide spread introduction of nano materials into the market. Previously, we have demonstrated that carcinogenic activity of nano-scale titanium dioxide by a novel intra-pulmonary spraying (IPS)-initiation-promotion protocol in the rat lung. The present study was conducted to detect carcinogenic activity of nanoscale ZnO administered by IPS-initiation-promotion protocol in the rat lung. Female human c-Ha-ras proto-oncogene transgenic rat (Hras128) transgenic rats were treated first with N-nitrosobis(2-hydroxypropyl)amine (DHPN) in the drinking water and then with ZnO (mean diameter 30 nm, without coating) by IPS. Although ZnO-IPS did not increase the multiplicity of DHPN-induced alveolar lesions (hyperplasia + adenoma), interstitial pneumonia was observed with alternating zones of inflammation, fibrosis. Quantitative analysis of Azan-Mallory stainings revealed that ZnO-IPS significantly increased fibroblastic area in the lung, which was independent of DHPN treatment. *In vitro* mechanism analysis revealed that the media from the primary alveolar macrophage treated with ZnO significantly enhanced cell proliferation of fibroblasts (CCD34), but not lung alveolar (A549) and mesothelium cells (Meso1). These data indicate that ZnO-IPS treatment interstitial pneumonia but did not promote lung carcinogenesis. One of the mechanisms for interstitial pneumonia induction is mediated by secreted protein from ZnO-laden alveolar macrophages, which increase cell proliferation of fibroblasts in the lung.

O-10

Pulmonary effect of SWCNT by intratracheal instillation to rats.

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Nanoparticles have specific optical and electric properties. The production of highly functional materials by utilizing such properties has already begun; however, the effects of nanoparticles on humans have not been elucidated. Concerns about the biological effects of these nanoparticles being produced for use in industrial products have arisen since epidemiologic data have shown a correlation between airborne nanoparticles, as typified by PM2.5 derived from the combustion of fossil fuel, and cardiovascular diseases.

In the last JSTP meeting, we have reported about the pulmonary effects of multiwall carbon nanotube (MWCNT) by instillation to rats. In this study, we evaluated the pulmonary effects of single-wall carbon nanotube (SWCNT). SWCNT, one of manufactured nanomaterials, is a cylindrical nanostructure substance from wrapping a single graphene sheet. There is a little information on effect of SWCNT on the human health. As SWCNTs were reported to induce pulmonary inflammation and/or fibrosis in vivo studies and were reported not to do, the pulmonary toxicity of SWCNT is inconclusive. Despersation of the sample SWCNT was confirmed before experiment. SWCNT (The mean diameter based on volume and mass by dynamic light scattering technique was 720 nm.) was instilled to male Wistar male rats (9 weeks old). 0.2 mg (0.66 mg/kg) or 0.4 mg (1.32 mg/kg) of SWCNT was suspended in 0.4 ml of distilled water including 0.1% Triton X. The negative control group was exposed to distilled water including 0.1 % Triton X. The animals were dissected at 3 days, 1 week, 1 month, 3 months, and 6 months after instillation.

Total cell count in BALF was increased at 1 week and 1 month in high dose of SWCNT, at 1 week in low dose of SWCNT. Neutrophil count in BALF was also increased in high and low dose of SWCNT during observation period compared with negative control. The peak of neutrophil counts was observed at 1 month. Pointcounting evaluation of lung tissue showed that significant inflammatory changes were seen especially in 1 week, 1 month, and 3 months after instillation. At 3 months of high dose group, foamy macrophages with fibres, debris of macrophages in the alveoli, and slight thickness of alveolar wall were observed. Further observation would be needed in longer time period. No specific pathological changes were seen in other organs (brain, liver, kidney, spleen and testis).

This research was funded by a New Energy and Industrial Technology Development Organization of Japan (NEDO) grant "Evaluating risks associated with manufactured nanomaterials ; Developing toxicity evaluating methods by the inhalation exposure".

O-11

Dose-dependent Induction of Mesothelioma by Multi-wall Carbon Nanotube in Male Fischer 344 Rats with Elevated Serum N-ERC/mesothelin

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The present study examined a dose relationship of our previously reporting mesotheliogenicity of multi-wall carbon nanotube (MWCNT) in rats, with an influence of injection sites and a behavior of serum N-ERC/mesothelin (ERC/m) level. MWCNT (0.1, 0.3, 1.0 mg/kg bw, n=12 per group), crocidolite (1mg/kg bw, n=10) or vehicle (2% CMC, n=5) was singly administered either intrascrotally (is-group) or intraperitoneally (ip-group). The experimental period was originally set to be 52 weeks, but because of the considerable animal deaths due to mesotheliomas within 41 weeks, all survivors were sacrificed at the end of week 42. Mesotheliomas were observed only in MWCNT groups with a clear dose-dependency, occurring in a faster and severer manner in ip- than in is-group. Serum ERC/m level was elevated in MWCNT groups with a trend similar to mesotheliomas, and observed in mesothelial hyperplasia cases with the lesser grade. In crocidolite groups, such changes were not observed. It is thus indicated that the mesotheliogenicity of MWCNT is dose-dependent, and serum ERC/m level may serve as a biomarker to detect premalignant lesions and to monitor the progression of the mesotheliogenesis.

O-12

Lack of Tumor Promoting Potential of 2-Tetradecylcyclobutanone, a Radiolytic Product of Stearic Acid, in Azoxymethane-induced Carcinogenesis in F344 Rats

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The usefulness of food irradiation is acknowledged to prevent food-borne diseases, but the safety of irradiation is not fully proved. To evaluate the safety of 2-tetradecylcyclobutanone (2-TCB), a radiolytic product originated from stearic acid, a 90-day oral toxicity test in rats was performed. Six-week-old male F344 rats (n=12) were given 0, 0.001, 0.005 and 0.01% 2-TCB for 13 weeks in the powder diet. The medium dose, 0.005% 2-TCB, was chosen from the study of Raul, F., the dose being equivalent to nearly 200 times of the maximum daily intake in humans. Through the experimental period, average body weights and food intake in each group were not different. No toxic effects were observed in the serum data, organ weight and histological findings.

Next, azoxymethane (AOM)-induced carcinogenesis was examined. Six-week-old male F344 rats (n=30) were given 0, 0.001, 0.005 and 0.025% 2-TCB for 25 weeks after AOM treatment. Incidences of cecum/colon tumors were 31%, 34%, 37% and 37%, respectively, significance being not different. There is no dose-response relationship in the incidences of small intestine tumors. These data suggest that 2-TCB exerts no tumor promoting effect on the intestinal tract carcinogenesis in rats under the present conditions.

O-13

Effects of rennin-angiotensin system blockade on podocyte injury in Osborne-Mendel rats

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【Introduction】 Osborne-Mendel (OM) rats develop mild hypertension and progressive glomerular injury with early-onset proteinuria leading to end-stage renal disease. According to our previous study, podocyte injury was suggested to be a key determinant of the glomerulopathy in OM rats. In this study, we verified the hypothesis that the rennin-angiotensin system (RAS) might be involved in the pathogenesis of the podocyte injury in OM rats.

【Materials and Methods】 Male OM rats were administrated RAS blockers or a vasodilatory drug from 3 weeks of age. We used angiotensin-converting enzyme inhibitor (ACEi, lisinopril, 75 mg/L) or angiotensin II receptor blocker (ARB, losartan, 400 mg/L) as the RAS blockers and hydralazine (HYD, 0.5 mmol/L) as vasodilator. All drugs were daily administrated via drinking water. These rats were sacrificed and the kidneys were sampled for histological and immunohistological analyses at 13 or 20 weeks of age. Age-matched untreated male OM rats were examined as controls.

【Results】 In all drug-treated rats, systolic blood pressure was lower than that in controls during the experiment. Urinary protein excretion was significantly prevented in RAS blocker-treated rats, but not in HYD-treated rats. Glomerular sclerosis steadily developed with age in untreated OM rats, while the treatment with drugs significantly ameliorated the glomerular lesion, especially in RAS blocker-treated rats. Immunofluorescence study for nephrin, a constituent of podocyte slit diaphragm (SD), revealed that the expression of nephrin was decreased with age in untreated OM rats, but the reduction of nephrin expression was inhibited in all drug-treated rats. However, the inhibitory effect of nephrin reduction was less in HYD-treated rats than RAS blocker-treated rats. Desmin, a conventional marker of podocyte injury, was highly expressed in untreated and HYD-treated rats as compared to RAS blocker-treated rats. Ultrastructurally, effacement of podocyte foot processes and decreasing number of SDs were observed in untreated and HYD-treated rats, but these pathological changes of podocytes were prevented by RAS blocker-treatments.

【Conclusion】 These results indicated that the glomerulopathy in OM rats might be independent of systemic blood pressure and the activation of RAS, especially angiotensin II played a significant role in the pathogenesis of podocyte injury.

O-14

Luteal Effects of Ethylene Glycol Monomethyl Ether (EGME), Sulpiride, and Atrazine in Normal Cycling Rats

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[Introduction] Ethylene glycol monomethyl ether (EGME), Sulpiride, and Atrazine are known to induce common ovarian toxicity including the increase in progesterone (P4) secretion and luteal cell hypertrophy after repeated administration. The aim of this study was to define differential pathways of these chemicals to ovarian toxicity.

[Materials and Methods] Oral doses of EGME (300 mg/kg), Sulpiride (100 mg/kg), or Atrazine (300 mg/kg) were given daily for four times from proestrus to diestrus to normal cycling SD rats. Serum hormone levels were measured by radioimmunoassay. New and old corpora lutea (CL) were separated with laser microdissection and analyzed for mRNA expression of steroidogenic genes (*SR-BI*, *StAR*, *P450scc*, and *3 β -HSD*), and luteolytic gene (*20 α -HSD*) with real-time PCR. The immunohistochemistry of steroidogenic factors was also performed and their intensities were analyzed with ImageJ software.

[Results and Discussion] Treatment with all chemicals significantly increased serum P4 levels, and EGME as well as Sulpiride induced increases in prolactin (PRL) levels. Although there was no histopathological change in any treatment groups, all three chemicals upregulated steroidogenic factors and downregulated luteolytic factor expressions at gene or protein levels in new CL. In old CL, EGME significantly stimulated *3 β -HSD* expression. From these results, luteal stimulating effect in new CL of EGME and Sulpiride are considered to be consequential due to the activation of PRL secretion in the pituitary, whereas the possibility of luteal direct effect of EGME is remained. Atrazine may directly activate new CL by stimulating steroidogenic factor expressions. The present study provides new insights regarding the differential pathways mediating the stimulation of luteal P4 secretion by the ovarian toxicants EGME, Sulpiride, and Atrazine in female rats *in vivo*.

O-15

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Neuroblastic Apoptosis in the Subventricular Zone is Caused by 1-methyl-4-phenylpyridinium (MPP+) Converted from MPTP through MAO-B

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Intraperitoneal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration induces apoptosis of subventricular zone (SVZ) doublecortin (Dcx)-positive neural progenitor cells (migrating neuroblasts, A cells). Actually, a metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP+), is responsible for neural progenitor cell toxicity. In the present study, to examine whether the MPTP-induced SVZ cell apoptosis is caused directly by MPP+ metabolized through monoamine oxidase B (MAO-B), MPTP or MPP+ was intracerebroventricularly (icv) injected into C57BL/6 mice. At Day 1 postinjection, many terminal deoxynucleotidyl transferase-mediated dUTP endlabeling (TUNEL)-positive cells were observed in the SVZ of both low (36 μ g) and high (162 μ g) dose MPTP- and MPP+-injected mice. The number of Dcx-positive A cells showed a significant decrease following high dose of MPTP- or MPP+-injection on Days 1 and 3, respectively, whereas that of EGFR-positive C cells showed no change in mice with any treatment. In addition, prior icv injection of a MAO-B inhibitor, R(-)-deprenyl (deprenyl), inhibited MPTP-induced apoptosis, but not MPP+-induced apoptosis. MAO-B- and GFAP- double positive cells were detected in the ependyma and SVZ in all mice. It is revealed from these results that icv injection of MPTP induces apoptosis of neural progenitor cells (A cells) in the SVZ via MPP+ toxicity. In addition, it is suggested that the conversion from MPTP to MPP+ is caused mainly by MAO-B located in ependymal cells and GFAP- positive cells in the SVZ.

O-16

The impairment of metrial gland development in tamoxifen exposed rats

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Tamoxifen, a non-steroid selective estrogen receptor modulator, has been widely used for therapy of estrogen-receptor positive breast cancer. In the present study, tamoxifen was intraperitoneally administered at doses of 0 and 2 mg/kg/day during GDs 8 to 10, and the placentas were sampled on GDs 11, 13, 15, 17, and 21.

The fetal mortality rates in the tamoxifen-treated group were increased up to approximately 50% from GD 15 onward. However, there were no effects on the weights of live embryos/fetuses and their placentas at each sampling time, and no macroscopic abnormalities in the fetuses and placentas on GD 21. Histopathologically, the size of the metrial gland in the tamoxifen-treated group was reduced at all sampling times compared with the control group. The spiral arteries appeared less well developed in the hypoplastic metrial gland. The uNK cells around the spiral arteries were decreased from GD 13 onward in the tamoxifen-treated group. The number of mitotic cells which appeared to be uNK cells, was lower on GDs 11 and 13 in the tamoxifen-treated group. There were no obvious changes in the labyrinth zone, basal zone or decidua basalis.

It is known that the development of the metrial gland is a part of decidualization, which is a sequential process of growth and differentiation of uterine stromal cells and uNK cells, and remodeling of extracellular matrix and maternal vasculature. In NK gene knock-out mice (TgE26 mice), there was no development of mesometrial triangle area into the metrial gland and the reproductive performance is very poor (mortality: 40%), suggesting that uNK cells are necessary in the placental growth and gestational success. The uNK cells are involved in a role of regulation and restructuring of spiral arteries in the metrial gland and maternal immune tolerance form toward invading trophoblast cells at the maternal-fetal interface. Alterations of uNK cell function and inadequate remodeling of spiral arteries play an important role in preeclampsia, which leads to high maternal blood pressure, elevated concentrations of urinary protein and poor fetal growth.

Therefore, it is suggested that the anti-estrogen effect of tamoxifen inhibits the proliferation of decidualized endometrial stromal cells in the metrial gland, and leads to metrial gland hypoplasia, resulting from the inhibition of uNK cell proliferative activity and the defective development of spiral arteries. Tamoxifen-induced embryo/fetus-toxicity might be associated with the immune tolerance deficiency, caused by decreased uNK cells in metrial gland hypoplasia and/or the preeclampsia, caused by defective development of spiral arteries.

P-1

Histopathological Evidence for the Effects of BrdU on the Developing Olfactory System in a Rat Developmental Disorder Model

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Whereas 5-bromo-2'-deoxyuridine (BrdU), the thymidine analog, is a well-known marker for proliferating cells, prenatal exposure to BrdU (50 mg/kg, gestation days; GD, 9-15, ip) induces developmental neurotoxicity (DNT) in rat offspring, such as locomotor hyperactivity, impaired learning and memory, and lower anxiety levels.

Previously, we reported that BrdU induced excessive cell death in various GD16 fetal brain areas with different sensitivity. However, at this stage, we could not clarify the effects of BrdU on the olfactory bulb (OB). Therefore, to examine the effects of BrdU on OB development, we conducted a histopathological examination of the OB on later brain stages, GD20 fetal brain and postnatal day (PD)11 neonatal brain. On GD20 examination, the thinner mitral cell layer and fewer distributions of tyrosine hydroxylase (TH) immunoreactive cells in glomerular layer were observed in the BrdU group. In PD11 neonatal BrdU group, disruption of the mitral cell layer structure was observed. Neurites of TH-positive cells in the glomerular layer showed abnormal spindle extension, suggesting induction of disturbances in synaptogenesis. Furthermore, fewer distributions of parvalbumin (PV), a Ca-binding protein coexisting with GABA neurons, having underdeveloped neurites in external plexiform layer was also observed.

We already reported that BrdU affects GABAergic neurons in the PD11 neonatal cerebral and limbic cortex (the hippocampus, amygdale and entorhinal area). The present findings suggest that abnormal GABAergic neurons may contribute to BrdU-induced abnormal behaviors in rat.

P-2

SATRATOXIN G - INDUCED APOPTOSIS IN MOUSE OLFACTORY SENSORY NEURONS

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Satratoxins produced by *Stachybotrys chartarum*, are suspected to contribute etiologically to damp building-related illnesses. Acute intranasal exposure of mice to satratoxin G (SG) specifically induces apoptosis in olfactory sensory neurons (OSNs) of the nose and the olfactory bulb (OB). While the onset of OSN apoptosis and atrophy corresponds with increases of proapoptotic gene expression in the nasal turbinates, the upstream mechanisms remain unclear. In this study, to apoptotic mechanisms of SG, the development of apoptosis were investigated up to 24 h after treatment of SG (3 to 25 ng/ml) in the OP6 cell line, derived from the E10 mouse olfactory placode and differentiated to OSN-like cells. In addition, we also examined expression of apoptosis-related genes in SG-treated differentiated OP6 cells by real time PCR method. SG was found to induce cytotoxicity in OP6 cells as revealed by a cell death detection ELISA at 6 h and by MTT assay at 24 h. In SG-treated OP6 cells, dead cells were observed and were characterized by pyknosis or karyorrhexis. The nuclei of OP6 cells, which showed pyknosis or karyorrhexis, were stained by the modified TUNEL method. In addition, DNA ladder was detected from OP6 cell samples. These suggested that SG induced apoptosis in OP6 cells as well as in vivo. SG induced robust apoptosis-related genes at 1 to 6 h in SG-treated differentiated OP6 cells prior to the development of apoptosis after SG treatment. Taken together, these data suggested SG induced robust apoptosis on OP6 cells as well as the OSN of the mouse olfactory epithelium.

P-3

Role of TLR4 in Brain Lesions of Cuprizone-Treated Mice

1. Neurobehavioral study

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Pathobiological abnormalities of brain white matter have been recently found in several mental disorders including schizophrenia. Accumulating evidence suggests that microglia is involved in progression of the white matter lesions. Toll-like receptor 4 (TLR4) mediates cellular signal transduction by endotoxin lipopolysaccharide (LPS) and also responds to other ligands such as heat shock protein, oxidized lipid, and extracellular matrix. TLR4 is known to be mainly expressed in microglia in the central nervous system. In this study, demyelination inducer, cuprizone (CPZ), was administered to C3H/HeN (wild type) and C3H/HeJ (mutant of TLR4) mice in order to clarify the relationship between abnormal behaviors and TLR4-mediating microglial response. Male mice of two strains were fed diets containing 0- and 2000-ppm-CPZ for 6 weeks, and then were fed untreated diets for 6 weeks as a recovery period. Neurobehavioral examinations (open field, light-dark box, and Y-maze tests) were conducted periodically in both strains. As excess release of neurotransmitter dopamine relates to mental disorders, mRNA levels of dopamine receptors were measured in brain samples with real-time RT PCR. In open field test, CPZ increased motor activity and rearing in C3H/HeN mice, peaking at week 3; tremor and mild straub tail were found at week 6 and thereafter. C3H/HeJ mice delayed peaks of motor activity and rearing in at week 5; no evidences of tremor and straub tail were found. Light-dark box test revealed that anti-anxiety effect was observed in the CPZ-treated group in C3H/HeN, not C3H/HeJ mice. In Y-maze test, there were no impairments of short-term memory in either strain. As to mRNA levels of dopamine D1a and D2 receptors, different pattern of expressions between C3H/HeN and C3H/HeJ mice was observed. As described above, CPZ-mediated abnormal behaviors were obviously suppressed in C3H/HeJ mice compared to C3H/HeN mice; these were corresponding to the pathological findings, i.e. demyelination with activation of microglia, reported by Soma *et al.* (P-4 in this JSTP meeting). These results suggest that TLR4-mediating microglial response and dopamine signaling plays an important role in CPZ-induced abnormal behaviors.

P-4

Role of TLR4 in Brain Lesions of Cuprizone-Treated Mice - 2. Histopathological Study -

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Toll-like receptor 4 (TLR4) is a major component of signal transducer for lipopolysaccharide (LPS). TLR4 also responds to endogenous ligands such as heat shock protein, extracellular matrix and fibrin. In the CNS, TLR4 is known to be mainly expressed in microglia and the role of TLR4 and microglia has been studied in several neural diseases. However, it remains unknown how the TLR4 is involved in the demyelinating disease.

We exposed copper chelator, cuprizone (CPZ), to C3H/HeN (wild type) and C3H/HeJ (mutant of TLR4) mice to investigate the function of TLR4 and microglia in the demyelinating and remyelinating lesions. The male of two strains of mice was fed 0- and 2000-ppm-CPZ-supplemented diets for periods of 3 or 6 weeks. Remyelination was achieved by feeding normal diet for 1 or 6 weeks after administering CPZ for 6 weeks in both strains of mice.

Demyelination, together with aggregation of activated microglia, was observed in corpus callosum, white matter of cerebellum and optic thalamus, as demonstrated by immunohistochemistry (IHC) for myelin basic protein (MBP) and Kluver-Barrera's stain. Scoring of myelination in IHC for MBP revealed that the demyelination in corpus callosum was weaker in C3H/HeJ mice than C3H/HeN mice. In consistent with the pathological findings, increases of mRNA levels of Nrf2, TNF α and IGF-1 after CPZ treatment were reduced by the deficiency of TLR4.

The results suggested that TLR4 signaling accelerates activations of microglia, increases the production of cytokine and oxidative stress and progresses the demyelination in the brain.

P-5

Acute Toxicity of MPTP and MPP⁺ to the Brain of Embryonic and Newborn Mice

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1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) causes damage to dopaminergic neurons in the nigrostriatal system in adult C57BL/6J mice, similar to the damage seen in Parkinson disease. Recently, a few reports have confirmed neuroblastic apoptosis in the subventricular zone (SVZ) of the mouse strain by intraperitoneal injection of MPTP. While there have been many researches on the neurotoxicity of MPTP in adult mice, there have been only few in fetal mice. In the present study, we assessed the toxicity of MPTP and 1-methyl-4-phenylpyridinium (MPP⁺), a metabolite of MPTP, to embryonic and newborn mice after a single injection. The injection caused loss of tyrosine hydroxylase-positive cells and fibers and increased apoptotic cells in the nigrostriatal system. In addition, MPTP and MPP⁺ induced a marked increase of apoptotic cells in the SVZ. The present results may indicate that MPTP and MPP⁺ pass through the placenta and blood-brain barrier in embryonic and newborn mice and induce apoptosis in the SVZ as well as the nigrostriatal system.

P-6

Valproic Acid Exposure to Rat Affects the Fetal Cerebral Cortex Ultrastructure

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INTRODUCTION: Our preceding study on valproic acid (VPA, 800 mg/kg) exposure in pregnant female rats on gestation day (GD) 11 had revealed hypoplasia of the cortical plate (CP) and sub-plate in the fetuses at GD16. Here we examined the fetal cerebral cortex ultrastructure, post-VPA exposure at GD16, using a transmission electronic microscope (TEM).

METHOD: GD16 fetal brain was obtained after VPA exposure on GD11, and epoxy-embedded sections were prepared for TEM examination.

RESULTS: In the ventricular zone (VZ), multiple cell divisions were observed. Cells on bottom of the VZ were bound together by desmosome junction. The VZ consisted of two distinctive cell types, the cells having oval nucleus with moderate chromatin and cells having invaginated nucleus with electron dense cytoplasm. In the VPA group, both cell types were smaller and nuclear-cytoplasmic (N-C) ratio was higher than those of the control. In the intermediate zone (IZ), cells showing oval nucleus with long cytoplasmic processes were directed tangentially to the cortical surface, and microtubules were observed therein. Cell direction in the IZ was unordered in the VPA group as compared to the control. In the sub-plate zone, the vacuolar structures surrounded the unit membrane, and extracellular space and extended cell processes into the CP were observed. In the VPA group, small vacuolar structures, and extracellular spaces were noticed, resulting in a fuzzy sub-plate zone. The CP consisted of cells having large and pale stained nucleus that were lined up radially to the cortical structure. However, less numbers of the layers were observed in the VPA group. Moreover, in both the control and VPA groups the number of organelles such as ribosome, mitochondria, and endoplasmic reticulum in the CP was larger as compared to the VZ and IZ.

CONCLUSION: Cells constituting each layer showed a distinctive structure by TEM. Although organellar structures and their distribution were similar between the VPA and control groups, VPA exposure caused disruption of the cell position and symmetry. Please start the abstract body here. The TIMES and SYMBOL fonts of 9 point with a single spacing should be used throughout the abstract including title, authors' names and their institutional information.

P-7

Effect of Developmental Exposure to Manganese on the Neuron and Glia of the Hippocampal Dentate Gyrus in Rats

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Manganese (Mn) is known to exert neurotoxicity similar to parkinsonism. In the present study, the effect of developmental exposure to Mn on the neuronal and glial development were examined in rats. Pregnant SD rats were administered manganese chloride (MnCl₂·4H₂O) at 0, 32, 160, 800 ppm in diet from gestational day 10 to postnatal day (PND) 21. For the offspring at weaning on PND 21 and the adult stage on PND 77, immunohistochemical analysis in the hippocampal dentate gyrus was performed for Reelin, NeuN, glutamic acid decarboxylase 67 (GAD67), doublecortin (Dcx), T box brain 2 (Tbr2), glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1), and cyclooxygenase 2 (Cox2). Cellular proliferation by PCNA-immunohistochemistry and apoptosis by TUNEL-assay were also estimated in the subgranular zone (SGZ) of the dentate gyrus. Offspring showed no effect in the body weight, food consumption and clinical signs. Mn concentrations in the cerebellum of offspring at 160 and 800 ppm increased on PND 21, although there were no changes in those of offspring on PND 77 and dams. In the dentate gyrus of offspring, increases of Reelin-positive neurons in the hilus and the Dcx-positive neurons in the SGZ were observed at 800 ppm on PND 21. As for the glial cells, offspring displayed increases in Iba1- and Cox2-positive microglia in the dentate hilus in all treated groups on PND 21. Furthermore, offspring displayed increases in the mRNA levels of proinflammatory cytokines such as IL-1 α , IL-6, Nos2 and TNF- α in the hippocampus at 800 ppm on PND 21. Immunohistochemical distribution for other molecules and apoptosis index were unchanged on PND 21. There were no effects in any parameter in offspring on PND 77. Considering the role of Reelin for neuronal migration of the dentate granular cells, Mn may disrupt differentiation of type 3 and immature dentate granular cells resulting in increased Reelin-positive neurons. On the other hand, considering the activation of microglia by in vitro exposure to Mn, Mn increased microglia with increased neural Mn concentrations from low doses as with increases in proinflammatory cytokine mRNAs at 800 ppm and may affect neuronal immune function through activation of microglia.

P-8

Chronological Changes of Oligodendroglia Precursor Cells in Spongy Change of Central Nervous System Induced by Hexachlorophene (HCP) and Cuprizone (CPZ) in Rats

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Spongy change is observed in the white matter of central nervous system in weanling rats given HCP and CPZ, and this change recovered after cessation of the treatment. To identify the mechanism of recovery from the spongy change, we focused on the behavior of the oligodendroglia precursor cell under neurotoxic status. <Materials & Methods> Study 1: Eighteen 25-day-old female Crj:WI(Wistar) rats were treated orally with 35 mg/kg HCP for 5 days and recovered for 7 days. Study 2: Twenty-four 21-day-old male Crj:(CD)SD rats were fed powdered chow containing 1w/w% CPZ for 8 days and received normal diet for 16 days. The brain was removed and stained with HE, followed by immunostaining. For the immunohistochemistry, anti-NG2, to identify oligodendroglia precursor cells, was used as the primary antibody. <Results> Study 1: In HCP-treated rats, NG2-positive cells were detected on day 3, and peaked on day 5 in the corpus callosum and anterior commissure. However, the positive cells decreased on day 12. In the caudate putamen, NG2-positive cells were found on days 3, 5 and 12. Study 2: In CPZ-treated rats, NG2-positive cells were detected on day 3, and prominently increased on day 8 in the medial forebrain bundle. In the lateral septal nuclei, NG2-positive cells were found on days 6 and 8. However, the positive cells decreased on day 24 in both areas. Oligodendroglia precursor cells were found in the spongy change area. Our data suggest that oligodendroglia precursor cells have implications for efforts to enhance endogenous repair in the spongy change induced by HCP and CPZ.

P-9

Neurogenesis after Delayed Neuronal Death (DND) by Global Brain Ischemia in the Cynomolgus Monkey Hippocampus

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Neurogenesis following selective delayed neuronal death (DND) in the hippocampal CA1 region resulting from transient global brain ischemia, has been reported in the hippocampal dentate gyrus (DG) of rats and cynomolgus monkeys. However, neurogenesis in CA1 after DND has not been thoroughly investigated.

Juvenile (aged 0 or 1 years) and adult (aged 5 to 7 years) cynomolgus monkeys underwent surgery to induce transient global brain ischemia surgery by clipping both the vertebral and common arteries for 20 minutes, followed by reperfusion. As a control, one animal from each age group underwent sham surgery. All animals received repeated intravenous injections of 5-bromo-2-deoxyuridine (BrdU) from 3 to 10 days after ischemia induction. Brains were perfused transcardially with 4% paraformaldehyde 3, 6, 15, or 22 days after surgery. Antibodies against NeuN, BrdU, Reelin, S100B, GFAP, and Musashi 1 were used for immunohistochemistry, and chronological changes in the hippocampal CA1, DG, subgranular zone, and subventricular zone were investigated.

Neuronal death and loss were shown in CA1 of both juvenile and adult monkeys 3 days after ischemia. Neurogenesis was noted in the subgranular zone in both juvenile and adult monkeys 15 days after ischemia, and the number of newborn neurons was greater in juveniles than in adults. Increased BrdU-positive neuro-progenitors were noted in both the juvenile and adult CA1, and the number was greater in juveniles than in adults. In particular, juvenile monkeys showed BrdU-positive neuro-progenitors in the subventricular zone near CA1, and a few BrdU-positive mature neurons were confirmed in CA1 22 days after ischemia.

Neurogenesis was confirmed in and around the DG of both juveniles and adults from 15 days after ischemia, but not in the adult CA1, which had been injured by ischemia. In contrast, newborn mature neurons that were considered to be differentiated from neuro-progenitors and to have proliferated and migrated from the subventricular zone near the injured CA1, were noted in the juvenile CA1. These results suggested that regeneration from potential neuro-progenitors after ischemic injury differ by region and age.

P-10

Pathogenesis of Failed Closure of Optic Fissure in FLS Mice with Ocular Coloboma: Zymographic Analysis of Collagenase Activity in Normal Optic Fissure Margin

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The ocular coloboma is mainly found in the region of the embryonic optic fissure and is due to a disturbance of its closure mechanism. Our previous studies clarified that almost 70% of FLS mice showed coloboma caused by failure of optic fissure closure during pregnancy period. Electron microscopy revealed that basal lamina of both optic fissure margins remained in colobomatous fetal eyes, whereas it gradually disintegrated and disappeared in normally developing fetal eyes. To clarify the pathogenic mechanism of failure of optic fissure closure, serial coronal sections of eyes from F1 progeny fetuses between FLS and CBA mice were examined histopathologically, immunohistochemically and by in situ FITC-conjugated zymography. Ocular coloboma was not detected in any F1 fetuses, and the both margins of inner and external layers of the optic cup showed complete fusion at the optic fissure. By in situ FITC-conjugated zymography, apparently positive gelatinase activity was detected around the fusing optic fissure in fetuses at gestation day of 12.0-12.5 and disappeared at gestation day of 13.5, although the activities were not located right on basement membrane.

P-11

Macrophagic Cellular-Transition Associated with Retinal Atrophy in RCS (Royal Collage of Surgeons) Rat

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The RCS rat has its photoreceptor cells in the process of being destroyed with ageing, and succumbs to retinal atrophy due to having hereditarily dysfunctional retinal pigment epithelium. We have been analyzing various changes in the retina of the RCS rat. This time, we focused on macrophagic cells which infiltrated in the retina, and we examined the trend of the atrophying process by immunohistochemical methods.

Materials & Methods; Nine paraffin-embedded eyeball samples of two to 18 week-old RCS rats, which had been preserved for immunohistochemistry, were utilized in this study. The retinas were assayed by the polymer method using a commercial kit (Simple Stain MAX-PO (M) or (R), Nichirei) for anti-ED-1 (Chemicon), -Iba1 (Wako), and -GFAP (DAKO) antibodies.

Results; At two to three weeks old, the rod and cone layer (RCL) began to be destroyed, ED-1 positive macrophages often infiltrated from the sclera to choroid, but less were detected in the retina. On the other hand, Iba1 positive microglia were scattered in all the layers of the retina.

At six weeks old, the RCL was destroyed with degenerative and decreasing photoreceptor cells, ED-1 positive cells were observed both outside of and inside the retina at the same level. Iba1 positive cells increased from the photoreceptor cell layer (PCL) to the RCL and also in the optic nerve.

At 10 weeks old, showing marked retinal atrophy, ED-1 positive cells increased in the retina and optic nerve. Iba1 positive cells increased in the PCL, RCL, and optic nerve as well.

At 18 weeks old, the retinal layer disappeared due to severe atrophy, ED-1 and Iba1 positive cells decreased to less than the level at 10 weeks old. GFAP positive reactivity was progressed from 10 weeks old in the process of astrocytes and Müller cells. The reactivity was also remarkable in the inner reticular layer. Gemistocytic astrocytes were seen in the optic nerve.

Discussion; It is well known that microglia play an important role on the retinal atrophying process¹⁾. Moreover, this study shows monocyte-originated macrophages infiltrate progressively from the choroid to the retina. Namely, the choroid-retina barrier may be destroyed with the retinal atrophy process.

Reference; 1. Thanos S. (1992) *Brain Res* 588, 21-28.

P-12

Immunohistochemical Analyses of Ocular Lesions in the *hhy* Hydrocephalus Mutant Mouse

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Mutant animals with hydrocephalus are useful for studying the pathogenesis of refractory congenital hydrocephalus. Hemorrhagic hydrocephalus (*hhy*) mouse is a spontaneous hydrocephalic mouse which develops ventricular dilatation with dome-like appearance of the head, and frequent brain hemorrhage. The *hhy* mouse has heterotopic cerebral cortices around the ventricles. Previously we reported that the *hhy* mouse also develops a retinal dysplasia. A causative gene for *hhy* has been identified on the mouse Chr 12, however, detailed pathogenesis remains to be clarified. In this study, we conducted immunohistochemical analyses of ocular lesions in the *hhy* mouse.

We examined homozygous (*hhy/hhy*) and control (*hhy/+* or *+/+*) mice from embryonic day 15 (E15) to 3 weeks of age. Frozen and paraffin sections were made and processed for immunohistochemistry using anti-HHY peptide of the causative gene and various antibodies against cell adhesion molecules, proliferative, and stem cell markers.

In the control mice, HHY protein was expressed outer layer of the developing retina with liner pattern and co-localized with N-cadherin. N-cadherin expression was partly lacking in the *hhy* homozygous mice and laminar structure of the retina was disrupted in that regions. Abnormal distribution of PCNA- and Pax6-positive neuroblasts was found in the E15 mice. These data indicated that lack of HHY protein causes an aberrant positioning of the neuroblast in the developing retina, followed by a retinal dysplasia.

P-13

Busulfan-induced Ocular Toxicity in Neonatal Rats.

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[Introduction] Busulfan (BUS) is a bifunctional alkylating agent and has teratogenic potentials. In addition, it is known to induce cataract in human. Although we previously demonstrated BUS-induced fetal and neonatal neurotoxicities in rats, an ocular toxicity has not been fully clarified. In the present study, we demonstrated an ocular toxicity in neonatal rats treated with busulfan.

[Methods] Animals were male Crl:CD(SD) rats, 6 days of age, and were subcutaneously treated with a single dosing of BUS at 0 and 20 mg/kg, and were euthanized at 1, 2, 4, 7 and 12 days after treatment (DAT). The eyes were fixed with Davidson's solution and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and TUNEL method. In addition, an immunohistochemical staining using anti-Cleaved Caspase-3 and Phospho histone H3 antibodies was performed.

[Results] In the control group no histological changes were observed, except for a few cell deaths mainly in the inner nuclear layer. In the BUS group cell death characterized by pyknosis and karyorrhexis appeared in the outer nuclear layer of the ciliary marginal zone (CMZ) and the lens epithelium. These cells were considered to be apoptosis, because of positive reaction for TUNEL method and Cleaved Caspase-3 immunohistochemistry. The number of apoptotic cells in the retina was increased at 1 DAT, peaked at 2 DAT and almost disappeared at 12 DAT. In addition, retinal dysplasia was formed at 4 DAT in the same region where apoptosis was observed, and the severity was enhanced at 12 DAT. On the other hand, apoptosis of the lens epithelial cells was observed at 2 and 4 DAT. Such apoptosis was hardly to detect after 4 DAT, whereas the lens epithelial cells disappeared partially with no regeneration even at 12 DAT. In addition, degeneration of the lens fibers that was characterized by swelling and vacuolar changes at the equatorial zone to the posterior pole was observed 12 DAT and the lesion was drastically enhanced with time.

[Conclusion] The present study demonstrates that neonatal rats treated singly with busulfan are induced apoptosis in the outer nuclear cells in retina and lens epithelial cells in neonatal rats, retinal dysplasia and lenticular degeneration.

P-14

Nasal Lesion of Rats and Mice by 13-Weeks Inhalation Exposure to 1-Bromo-2-Chloropropane

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The annual production volume of 1-Bromo-3-chloropropane (BCP) amounts to 2000 tons in Japan (2004). Current knowledge of BCP toxicity is limited, and particularly, its toxic effects on the nasal cavity is hardly understood. In the present study, nasal lesions induced by 13-week inhalation exposure to BCP are compared in rats and mice.

[Method] F344/DuCrjCrj rats and B6D2F1/Crj mice (male and female, n=10 for each group) were whole-body exposed to 0, 25, 50, 100, 200, 400 ppm BCP, 6 hr/day, 5 day/wk, for a total of 13 wks. The nasal cavities of the rats and mice were decalcified and cut 3 cross section for histopathological examination.

[Results] In the rats, the hyperplasia of respiratory epithelia and goblet cells, and the desquamation and disarrangement of olfactory epithelia were observed in the nasal cavity. Also, the hyperplasia of goblet cells were observed in the nasopharynx.

In the mice, hyperplasia and eosinophilic change (eosinophilic globules) in the respiratory epithelia, and desquamation, atrophy and eosinophilic change in the olfactory epithelia were observed in the nasal cavity, and eosinophilic changes were observed in the nasopharynx.

In both rats and mice, lesions were observed in the nasal cavity and nasopharynx. These lesions were slightly different between rats and mice, namely, the goblet cell hyperplasia were observed in the rat nasal cavity and nasopharynx whereas an increase in eosinophilic changes were observed in the olfactory epithelium and respiratory epithelium of the mice nasal cavity, and in the epithelium of the mice nasopharynx. These lesions of the nasal cavity were observed at doses of more than 50 ppm in rats (goblet cell hyperplasia) and at a dose of 400 ppm in mice (eosinophilic change).

This study was carried out on a commission from the Ministry of Health Labour and Welfare.

P-15

The toxicity of nicotine by intratracheal instillation to the F344 rats

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There are many chemicals including carcinogen in cigarette smoke, and nicotine is one of the components. Nicotine is taken in the blood via lung by the inhaled smoke and binds nicotinic acetylcholine receptors at the central and peripheral nerves. *In vivo*, nicotine induces various effects not only respiratory system but also central and peripheral nerve systems, circulatory organs and digestive organs. In addition, according to the reports about proliferative stimulation to the non-neuronal cell by nicotine, there is a possibility that nicotine promotes lung tumorigenesis. However, there are few reports about *in vivo* toxicity and some changes by nicotine exposure in the respiratory organs. So, this experiment was conducted to examine the toxicity and cell proliferation of nicotine in the lung by intratracheal instillation (i.t.) *in vivo*.

6 week-old male F344 rats were administered nicotine by i.t. from week 4 every 3 weeks, total nine times of the administrations and they were sacrificed at week 30 and their lungs were examined histopathologically. The doses of nicotine were 0.05, 0.1 and 0.2 mg nicotine/rat, based on fatal dose in human (adult: 50~60 mg). In the groups of high doses, 0.1 and 0.2 mg nicotine/rat, third or fourth administration was terminated because of death of some rats following nicotine i.t. Total administration and rat were 0.05 mg nicotine/rat group, 9 times (5 rats) and 4 times (5 rats); 0.1 mg nicotine/rat group, 3 times (5 rats) or 4 times (3 rats); 0.2 mg nicotine/rat group, 3 times (3 rats). And, as a control group, 5 rats were administrated 5 times 0.2 ml saline/rat.

In the groups of 0.1 and 0.2 mg nicotine/rat, some rats caused convulsion as soon as i.t. administration. And then some of them died and the others recovered from acute symptom survived through the experiment. Histopathologically, though any proliferative changes of alveolar cells weren't observed, inflammatory change in the lung was promoted by nicotine i.t. From the results of this study, it was revealed that the group of 0.05 mg nicotine/rat administrated nine times induced the strongest inflammatory change. In conclusion, nicotine i.t. promoted nervous symptom in acute phase, and strong inflammation in lung in chronic phase by nicotine administration for a long time, even at low dose.

P-16

Gastric Mucosal Injury and Its Recovery after Treatment with Mycotoxin Fusarenon X in Rat

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Background: Cell lineages in the gastric mucosa are originated from stem/progenitor cells that are normally located at the isthmus of the gastric glands. Gastric mucosal damage such as ulceration or oxyntic atrophy is thought to cause altered differentiation of the gastric cell lineages during its recovery process. However, there are only a few reports regarding the recovery process after injury to the chief cells.

Methods: Crl:CD(SD) rats were administered a single gavage dose of 1.5 mg/kg of Fusarenon X, a potent chief cell toxic agent. The stomach was examined histopathologically 1, 2, 3, 10, 17 and 29 days after the treatment.

Results: The following findings were observed: Days 1-3 after the administration: atrophy and apoptosis of the chief cells and slight apoptosis of the mucous neck cells; Days 3-17: decrease of the chief cell number and weakened/decreased expression of chief cell markers (Mist1 and Intrinsic factor); Days 10-17: emergence of eosinophilic cells at the base of the glands which expressed trypsinogen but not amylase; Day 29: full recovery from the injury. Proliferative activity (Ki-67 positive cell number): transient and focal decrease on Day 1 and ectopic emergence of positive cells at the base of the glands on Days 3 and 10.

Conclusion: In the recovery process after injury to the chief cells, the presence of ectopic Ki-67 positive cells and the emergence of eosinophilic cells were observed at the base of the glands. These findings suggest recruitment of the second-line progenitor cell population distant from normal progenitor zone in the isthmus and transient disruption of regulation of the chief cell lineage differentiation.

P-17

Evaluation of Safety of *Lactobacillus casei* Shirota strain in a Mouse Model of Bacterial Translocation

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Introduction: Probiotics are defined as bacteria that have beneficial effects on human health. The probiotic *Lactobacillus casei* Shirota (LcS) strain regulates intestinal function and immunity of the host and has been eaten for 70 years or more. In recent years, probiotics have been used to alleviate adverse reactions to antineoplastic agents in cancer treatment or to treat gastrointestinal diseases, including inflammatory bowel disease. At the same time, bacterial translocation (BT) of probiotics has been reported in patients with immunodeficiency or other diseases. Therefore, using BT as a biomarker, we evaluated the safety of LcS in a mouse model of BT with intestinal mucosal injury and immunosuppression induced by an antineoplastic agent.

Methods: Seven-week-old male BALB/c mice were allocated to the saline group, 5-fluorouracil (5-FU) group, or 5-FU + LcS group. In the saline group, saline (0.2 mL) was administered orally for 7 days. In the 5-FU group, 5-FU (400 mg/kg) was administered orally once, after which saline was administered orally for 7 days. In the 5-FU + LcS group, after administration of 5-FU, an LcS suspension (more than 2.0×10^{10} CFU/kg) was administered orally for 7 days. At 3 days after administration of 5-FU, histological examination was performed to assess intestinal mucosal injury and bone-marrow damage. At 7 days after administration of 5-FU, bacteriological examination and histological examination were performed to assess BT in organs.

Results: Animals in the 5-FU group and 5-FU + LcS group showed histological findings suggesting intestinal mucosal injury and bone-marrow suppression, and also detected enteric bacteria in the blood, mesenteric lymph nodes and liver. No significant difference was noted in the frequency of detection or count of these bacteria between the two groups, while BT of LcS was not found in any of the animals in the 5-FU + LcS group.

Conclusion: The present study indicates that LcS did not undergo BT after its oral administration (2.0×10^{10} CFU/kg) while indigenous enteric bacteria did, and that it did not have an adverse effect on BT of other bacteria.

P-18

The Usage of Wistar Hannover GALAS Rats for Chronic Toxicity and Carcinogenicity Studies (Toxicity Assessment of *Aloe arborescens* Miller var. *natalensis* Berger)

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F344 rats, widely used as a suitable strain for rat carcinogenicity study, is well known to have a high incidence of testicular stromal tumor and large granular lymphocyte (LGL) lymphoma as spontaneously appearing tumors. In some cases, these tumors make difficult to give the conclusions for detail carcinogenicity assessment. With that, recently, the usefulness of Wistar Hannover rats is focused on. We performed one-year chronic toxicity and two-years carcinogenicity studies using Br/Han:WIST@Jcl (GALAS) rats (CLEA Japan, Inc.). The test material for the assessment was *Aloe arborescens* Miller var. *natalensis* Berger (designated as 'Aloe'), a member of the family *Liliaceae*, and called Kidachi Aloe in Japan. Though various pharmacological and therapeutic activities have been reported, no oral toxicity data with long administration have hitherto been available.

The spontaneously appearing tumors on 50 male and 50 female rats treated the MF basal diet (control groups) in two-years carcinogenicity study were summarized. The survival rates of rats for up to 2 years (104 weeks) were 67% (males) and 59% (females). The final body weights were 600.5±111.9g (males) and 400.1±88.4g (females). Histopathologically, some neoplastic lesions, pituitary tumor, thyroid tumor, mammary gland tumor and uterine tumor, were observed.

In the studies of one-year and two-years, the male and female rats of the groups treated 4.0% Aloe showed the symptom of diarrhea and lose their body weights slightly. Histopathologically, severe sinus dilatation of ileocecal lymph nodes, and yellowish pigmentation of ileocecal lymph nodes and renal tubules were observed in one-year study. And, in two-years study, Aloe exerted equivocal carcinogenic potential at 4.0% high dose level on colon. Aloe is not carcinogenic at non-toxic dose levels and that carcinogenic potential in at 4.0% high dose level on colon is probably due to irritation of the intestinal tract by diarrhea.

Wistar Hannover rats were concluded to be useful for long-term toxicity assessment of test substances, because they were observed high survivability and low incidences of the spontaneous appearing tumors in long-term carcinogenicity study.

P-19

Combined Effects of Caffeic Acid and Sodium Nitrite in *In Vivo* and *In Vitro*

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Caffeic acid (CA) is one of the components of coffee, which is known as an antioxidant and cancer chemopreventive agents. However, we confirmed that reaction of CA and NaNO₂ under acidic conditions generates reactive oxygen species (ROS). In addition, benzoxazine (BZX) derivative which is reaction product of CA and NaNO₂ was detected in the livers of rats treated with these two compounds. In this study, pro-oxidant potential of products in the reaction of CA and NaNO₂, and combined effects resulting from oxidative stress in rats treated with two compounds were investigated.

Pro-oxidant potential of BZX and OXZ derivatives were examined in *in vitro* experiment using DCFH-DA, as a fluorescence probe for ROS. Six-week-old male F344 rats were randomly divided into seven groups consisting of five animals each and treated with basal diet, 2.0% CA in the diet, 0.3% NaNO₂ in the drinking water and combination of 0.6 and 2.0% CA with 0.1 and 0.3% NaNO₂. All rats were sacrificed at 4 weeks after treatment. Livers and forestomach were used for the examination of histopathology, 8-hydroxydeoxyguanosine (8-OHdG) and TBARS formation.

Fluorescence intensities of DCFH-DA were more increased by BZX and OXZ derivatives than CA. Relative liver weight was significantly increased in the rats co-treated with 2.0% CA and 0.3% NaNO₂. Mild epithelial hyperplasia with hyperkeratosis was observed in the forestomach of rats in combined group. On the other hand, changes of 8-OHdG levels in the forestomach epithelium and liver DNA and TBARS levels in the liver were not observed.

While *in vitro* experiment revealed that reaction products in CA and NaNO₂ under acidic condition have pro-oxidant potential, combined effects resulting from oxidative stress were not observed in the liver and forestomach of rats. In addition, BZX and OXZ derivatives will be quantitatively analyzed by HPLC.

P-20

Carcinogenicity and chronic toxicity study of Diphenylarsinic acid in rat

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【Objective】 Diphenylarsinic acid (DPAA) which is an organic arsenic compound is exerted the neurotoxicity in human, but carcinogenicity of DPAA is not yet known. We reported rat liver carcinogenesis in medium-term bioassay, DPAA clarified that rat liver carcinogenesis promotion action. In the present study, to evaluate toxicity of DPAA, treated water to rats for chronic toxicity study and for carcinogenicity study.

【Method】 Male and Female F344 rats, at 8 weeks of age, were given 0, 5, 10, 20ppm DPAA in their drinking water until they were sacrificed. The number of the rats used male and female each 10 in the chronic toxicity test, and used each 51 in carcinogenicity study.

【Result】 As a result of chronic toxicity test, an increase restraint tendency in the last body weight was watched at 10, 20ppm male group, and 20ppm female group. Absolute weight and the relative weight of the liver increased significantly in female 20ppm group, in comparison with the control group. In the liver, a bile duct hyperplasia by the DPAA was shown in male and female 20ppm group. In addition, expansion of a common bile duct epithelium hyperplasia and the stenosis of an aperture in the Vater papilla were showed, and expansion of the common bile duct recognized in all examples that male and female 20ppm group. As a result of carcinogenicity test, survival rate significantly declined in comparison with the control group in female 20ppm. It was thought that the cause was high biliary tract system disorder by DPAA. The appearance of hepatocyte tumor did not recognize a difference in control group and 20ppm group. Moreover, the nervous system symptom by DPAA was shown in neither examination.

【Consideration】 DPAA showed the toxicity in biliary tract system in the rat, but it developed that did not cause liver cancer.

P-21

Involvement of macrophages and myofibroblasts in chronic α -naphthylisothiocyanate (ANIT)-induced peribiliary fibrosis in rat model

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To investigate the pathogenesis of post-bile duct (BD) injury fibrosis, BD epithelial injury was induced in 6-week-old male F344 rats by intraperitoneal injections of ANIT (75 mg/kg BW/week) for 19 weeks (W). Macrophages reacting to ED1 (CD68), SRA-E5 (CD204) and OX6 (MHC II) increased during almost entire experimental period (W3-19), whereas ED2 (CD163)-positive macrophages increased at late stages (W13-19). Macrophages reacting to OX6 were also reactive for ED1, ED2 and SRA-E5. α -smooth muscle actin-positive myofibroblasts began to be seen from W10, being associated with peribiliary fibrogenesis. Myofibroblasts were also positive for vimentin and desmin. Real-time PCR analysis revealed that mRNAs of MCP-1 and TGF- β 1 were significantly increased at W10-19. This study shows that macrophages with different immunophenotypes and myofibroblasts showing various cytoskeletons participate in post-BD injury fibrosis.

P-22

Inhibitory Effect of Hydrogen-Rich Water on TAA-Induced Hepatic Fibrosis in Rats

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Free iron-induced OH radical plays an aggravating role in the pathogenesis of chronic hepatitis C. OH radical are demonstrated to induce the DNA damage and lipid peroxidation. It has been reported that the acute and chronic liver damages induced by thioacetamide (TAA) were inhibited by administration of the iron-restricted diets. Therefore, we investigated the inhibitory effect of molecular hydrogen, known as the selective OH radical scavenger, on TAA-induced hepatic fibrosis in rats. Eight-week-old male Wistar rats were intraperitoneally-treated with TAA or saline 6 times at intervals of 4 days. Hydrogen-rich water or tap water was administered during the experiment from 2 days before treatment of TAA. Direct red staining for hepatic fibrosis and 8-hydroxy-deoxyguanosine (8-OHdG) level for oxidative stress were evaluated. TAA-induced hepatic fibrosis including bridging fibrosis in Glisson's capsule and nodules were decreased by administration of hydrogen-rich water. 8-OHdG levels in the liver were lower in TAA-hydrogen-rich water group than TAA-tap water group. These results indicate that hydrogen molecules scavenge the oxidative stress and inhibit the hepatic fibrosis induced by TAA.

P-23

Altered Expression and Distribution of ALT Isozymes during D-galactosamine-induced Liver Injury in Rats

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Serum alanine aminotransferase (ALT) has been widely used to access hepatic damage in clinical and pre-clinical studies, although it is not always a specific liver injury marker. Recently, two ALT isozymes (ALT1 and ALT2) were found. It is expected that safety evaluation based on these isozymes would enable a more precise interpretation of the elevation of serum ALT. This study aimed to identify the distribution of the ALT isozymes and the changes in these in the course of D-galactosamine HCl (D-gal) induced hepatotoxicity in the liver in rats.

At intervals of 6 hours, and 1, 3 and 7 days after the single intraperitoneal administration of 400 mg/kg D-gal or saline to male Crl:CD(SD) rats, histopathology and immunohistochemical analysis were performed.

In the control livers, the ALT1 positive hepatocytes were predominantly distributed in the periportal area rather than in the midzonal and centrilobular areas, whereas ALT2 positive hepatocytes were sparse without showing any characteristic distribution. The bile duct epithelia were positive for both ALT1 and ALT2.

Serum ALT increased from 6 hours to 1 day after the administration of D-gal. Histopathological examination revealed single cell necrosis of the hepatocytes and increased mitotic figures in the hepatocytes from 6 hours to 1 day and 3 days after administration, respectively. Immunohistochemically, from 6 hours to 1 day after the administration, the necrotic hepatocytes were negative for both ALT1 and ALT2. In intact hepatocytes, positive reaction for ALT2 increased in the periportal and midzonal areas while, on the other hand, that for ALT1 slightly increased.

The results provided the histological distribution of the ALT isozymes in the normal liver of rats and direct morphological evidence of leakage of ALT1 and ALT2 from necrotic hepatocytes resulting in the elevation of serum ALT levels during D-gal induced hepatotoxicity. The toxicological significance of intensified ALT immuno-staining in hepatocytes was not clear, especially with ALT2, although it might be related with the elevation of serum levels. In addition to the present data, further study of the ALT isozymes with other toxicants or analysis of the serum ALT isozymes could give a new insight for the safety evaluation of serum ALT elevations.

P-24

Distribution in expressions of heat shock protein 25 and adipophilin in carbon tetrachloride-induced rat liver injury

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In the last annual meeting, we reported that heat shock protein (Hsp) 25 has a function of anti-inflammation in thioacetamide-induced rat liver injury model. On the other hand, although adipophilin (Adp) is reported to be related to a function of lipid droplet formation, there is no report on relationship of Adp with hepatocyte injury.

We investigated expressions of Hsp25 and Adp with the immunohistochemistry and real time RT-PCR in carbon tetrachloride (CCl₄)-induced rat liver injury model. CCl₄ was administered at a dose of 2 mL/kg in male Crl:CD(SD) rats aged 6 weeks. Rats were sacrificed on 1, 2, 3, 5, 7 and 10 days after the dosing. Liver was removed, and examined.

Expression of Hsp25 was changed according to liver injury and subsequent recovery; Hsp25 was expressed strongly in necrotic hepatocytes on day 1, whereas Hsp25-positive hepatocytes were seen around macrophages which infiltrated in centrilobular lesions on day 2, although a few Hsp25-positive hepatocytes were still present in the centrilobular areas. On day 3, a small number of Hsp25-positive hepatocytes were observed around the centrilobular lesions. Expression pattern of Hsp25 mRNA accorded with that in the immunohistochemistry. Based on localization in Hsp25 expression pattern, it was considered that the expression of Hsp25 on day 1 might have been due to cytoprotective mechanism, whereas Hsp25 on days 2 and 3 might be expressed as the anti-inflammatory function.

Expression patterns of Adp in the immunohistochemistry and real time RT-PCR agreed generally with those of Hsp25 in this model. Therefore, Adp might have functions like Hsp25 under CCl₄-induced liver injury.

P-25

Qualitative and quantitative analyses of podocyte-associated molecules in canine renal glomeruli sampled by microdissection or sieving methods.

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[Background] The podocyte is a terminally-differentiated epithelial cell that plays a crucial role in the glomerular filtration. As the podocyte has poor ability of regeneration, its injury causes development and progression of glomerular damage. Podocyte-associated molecules which are expressed in the foot processes and slit diaphragms contribute to the maintenance of podocyte function and morphology. It has been indicated that these molecules might be useful biomarkers for evaluation and detection of early podocyte injury in humans and experimental podocytopathy models caused by chemicals. In dogs, podocyte injury has been examined by electron microscopy. However, alteration of podocyte-associated molecules accompanied by podocyte injury has not been investigated. The aim of the present study is to define the expression and localization of podocyte-associated molecules in canine renal glomeruli by qualitative and quantitative analyses. **[Materials and Methods]** Kidney tissue was obtained from five normal adult beagles and the glomeruli were isolated from cortical tissue by sieving method. In the present study, we examined four molecules as nephrin, podocin, α -actinin-4 and α 3-integrin. The expression and localization of these proteins were detected by western blotting and immunofluorescence. The gene expression of four molecules in the glomeruli was revealed by RT-PCR. Additionally, we quantitatively analyzed nephrin mRNA in the laser-dissected glomeruli by real-time RT-PCR. **[Results]** The expression and localization of nephrin, podocin, α -actinin-4 and α 3-integrin were detected in canine glomeruli. Quantitative analysis of nephrin mRNA was possible using 100 laser-dissected glomeruli. **[Discussion]** In this study, we revealed expression and localization of four podocyte-associated molecules in canine glomeruli. The qualitative and quantitative analysis podocyte-associated molecules in the isolated or laser-dissected glomeruli might be useful for early detection of podocyte injury.

P-26

tBHQ Attenuates Glomerular ROS Injury of Diabetic Mice via Activating Nrf2-dependent Antioxidant Genes

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【Objective】 Tert-butylhydroquinone (tBHQ) has been used as a synthetic food antioxidant. As an effective inducer of antioxidant, it mediates the antioxidant gene expressions by promoting ROS-mediated dissociation of Nuclear factor erythroid 2-related factor 2 (Nrf2) and Keap1, and improving its conjugation with Nrf2-antioxidant responsive element (ARE). In order to investigate the effects of tBHQ on ARE signaling pathway, Nrf2 and its target genes Heme oxygenase-1 (HO-1) and γ -glutamylcysteine synthetase (γ -GCS) were observed in the renal tissue of diabetic mice.

【Materials and Methods】 The uninephrectomized streptozotocin (STZ)-induced diabetic CD1 mice were treated with 1% tBHQ in food, the uninephrectomized diabetic mice induced by STZ were used as disease control. Urinary albumin, malondialdehyde (MDA) contents of serum and the glomeruli, renal extracellular matrix (ECM) and expressions of Nrf2, HO-1, γ -GCS were determined. **【Results】** Administration of tBHQ resulted in the increased accumulation of Nrf2 in the nucleus and up-regulation the expressions of Nrf2, HO-1, γ -GCS at levels of protein and mRNA than those of diabetic mice. There were significant increased quantifications of urinary albumin /24h, increased contents of MDA of serum and the glomeruli in diabetic mice compared with control mice at the corresponding time, and these parameters were markedly improved treated with 1% tBHQ. Reduced glomerular ECM deposition in diabetic mice treated with 1% tBHQ were observed **【Conclusion】** tBHQ may activate Nrf2-ARE pathway, up-regulate the expressions of protein HO-1 and γ -GCS, attenuate the ROS injury to the glomeruli and reduce ECM deposition.

P-27

Investigation of actin-cytoskeleton related proteins by proteome analysis with isolated glomeruli of diabetes rats

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[Background]

Recently, proteome analysis with human urine or serum has been applied to the study of diabetic nephropathy. On the other hand, proteome analysis with glomeruli of type 2 diabetes has not been reported. Further, proteome analysis with human renal biopsy specimens is difficult, because renal biopsy for diabetes patients is clinically rare and little specimens are available for proteome analysis. To this end, we performed proteome analysis with isolated glomeruli of type 2 diabetes mellitus rats.

[Materials and Methods]

We performed preliminary experiments on Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which are representative models for obesity and diabetes; Long-Evans Tokushima Otsuka (LETO) rats were used as controls. At week 27 (early-stage of diabetic nephropathy) and 38 (proteinuria stage), we isolated glomeruli from the OLETF and LETO rats using sieving method. We performed proteome analysis to investigate the intergroup differences in protein expression in the glomeruli. Further, we investigated the changes of cytoskeleton related proteins in both groups.

[Result]

We identified and quantified 192 and 218 proteins with 95% confidence by using QSTAR Elite LC-MS/MS system and Protein Pilot 2.0 software at week 27 and 38, respectively. A total of 87 proteins displaying significant quantitative changes comparing with control LETO rats were detected in the glomeruli of early and/or proteinuria stages of diabetic nephropathy and 25 of them were related to the actin-cytoskeleton. Those included proteins participating in the increase of stress fibers (ACTN4, ARHGDI1), impairment of actin polymerization (ARPC1B, ARPC5, ACTR3), microtubules (TUBA1C), and intermediate filaments (VIM, LMNA, NES), detachment from glomerular basement membrane (INTGB1), and disentanglement of actin bundles or networks (SPTAN1, PLS3).

[Summary]

Through the results of the present study, investigating changes in cytoskeleton related proteins of diabetic glomeruli by proteome analysis, changes in protein expression of the followings are suggested to be seen; 1) crease in stress fibers at 38 weeks, 2) impairment of actin polymerization at both 27 and 38 weeks, suggesting collapse or dysfunction of actin filaments, 3) decrease in proteins related microtubules at both 27 and 38 weeks. Our results also demonstrated the usefulness of proteome analysis with isolated glomeruli in mechanism analysis of diabetic nephropathy.

P-28

The effects of endogenous prostaglandin in cisplatin-induced rat renal failure

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PGE₂ is synthesized by one of the two cyclooxygenase (COX) isoforms, COX-1 or COX-2 and prostaglandin E synthase (PGES). The activity of PGE₂ is carried out via four different receptor subtypes: EP1, EP2, EP3, and EP4. PGE₂ plays important roles in maintaining homeostasis. However, roles of PGE₂ in renal lesions are still unknown. The purpose of this study is to investigate the roles of PGE₂ in cisplatin (CDDP)-induced rat renal failure. Renal lesions were induced in F344 rats by an intraperitoneal, single dose of CDDP (6 mg/kg body weight). On days 1, 3, 5, 7, 9, 12 and 15 after CDDP dosing, kidneys were removed. Histopathological changes were mainly observed in the proximal tubules in the cortico-medullary junction. There were renal epithelial necrosis, apoptosis, cell cycle arrest, regeneration and interstitial fibrosis. After injection, it was found by immunohistochemistry that the expression of COX-1 and mPGES was gradually increased in the affected tubules, whereas that for COX-2 was not seen. Among EPs, EP4 was observed in the affected tubules. By using NRK-52E cells, a rat renal proximal tubular cell line, the effects of PGE₂ on the cell proliferation, apoptosis cell cycle and differentiation were investigated *in vitro*. NRK-52E cells treated with 11-deoxy-PGE₁, an EP4 agonist, underwent the G₀/G₁ arrest and decreased apoptosis. To investigate the effects of 11-deoxy-PGE₁ on the differentiation, NRK-52E cells treated with TGF-β1, an inducer of epithelial-mesenchymal transition (EMT) were used. In the presence of 11-deoxy-PGE₁ decreased the mRNA expression of α-smooth muscle actin.

In conclusion, the present study for the first time shows that COX-1 plays more important roles than do COX-2 in the CDDP-induced acute renal failure. Furthermore, the product, PGE₂, may regulate renal epithelial regeneration via EP4 in the affected renal tubules through inhibition of apoptosis and EMT. Endogenous PGE₂ was clearly shown to participate in the complicated pathological conditions of regenerating renal epithelial cells in CDDP-induced rat renal failure.

P-29

Expressions of Oxidative Stress Markers in Cisplatin-Induced Nephrotoxicity in Rats

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The purpose of this study was to evaluate whether N^ε-hexanoyl lysine (HEL), acrolein, dityrosine and advanced oxidation protein products (AOPP) reflect the severity of cisplatin-induced nephrotoxicity. Immunoexpression of HEL, acrolein and dityrosine in kidneys, urinary HEL and acrolein concentration and plasma AOPP concentration were examined up to day 4 post-cisplatin injection in rats. Cisplatin-induced tubular injury was observed histopathologically on days 2-4 after injection and became more severe time-dependently. On days 2-4, HEL, acrolein and dityrosine were immunostained in the cytoplasm of damaged tubular cells and their immunostaining intensity increased as tubular injury became more severe. Urinary HEL and acrolein levels and plasma AOPP level showed a tendency to increase as tubular injury became more severe. These results suggest that expressions of HEL, acrolein, dityrosine and AOPP were associated with the pathogenesis of cisplatin-induced nephrotoxicity.

P-30

Morphological changes of papillary ducts induced by trimethyltin in rats

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[Purpose] We examined morphological changes of papillary ducts in rats exposed to trimethyltin chloride (TMT).

[Method] Male and female SD rats, 9 weeks old, were dosed per oral by a single administration of 10 mg/kg of TMT dissolved in distilled water. Animals were sacrificed at 7 days after dosing in the experiment 1, and at 1, 3, 6, 24 and 48 hours after dosing in the experiment 2. The kidneys were examined histopathologically and ultrastructurally.

[Results] In the experiment 1, numerous eosinophilic droplets were found in cytoplasm of the papillary ducts of all animals treated with TMT and were also observed in the papillary interstitial cells of animals sacrificed at 48 hours and 7 days after dosing. The eosinophilic droplets were positive for PAS reaction but negative for lysozyme in immunohistochemical staining. Corresponding to eosinophilic droplets, many autophagosomes were observed in the cytoplasm of papillary ducts in electron microscopical examination. Autophagosomes included concentric membranous structures and microvesicular structures. In the experiment 2, accumulation of the microvesicular structures with slightly dilated cisterna were sporadically observed in the cytoplasm of papillary ducts of the animals sacrificed at 1 hour after dosing.

[Conclusion] In this study, we demonstrated that autophagosomes were induced by TMT in rats. Further studies are needed to clarify the exact mechanisms of TMT induced autophagosomes in tubular epithelium in rats.

P-31

Histopathologic Examination of Renal Papillary Necrosis in an Oral Gavage Study of p-Cresidine in TSG-p53 Knockout Mice

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Histopathological examination was performed to clarify the incidence and its related-findings of the renal papillary necrosis (RPN) observed in p53 knockout (p53 KO) mice in a six-month oral gavage study of p-Cresidine (p-Cre). p-Cre (200 and 400 mg/kg/day) was given by gavage to each group consisting of 16 male and female TSG-p53 KO mice (heterozygote) for 6 months. RPN was observed in 1 male in the 200 mg/kg/day group and in 10 males and 3 females in the 400 mg/kg/day group. Chromophobe spherical substances and its fused substances were observed in the tubular lumen at the renal papilla in 6 males in the 200 mg/kg/day group and in all males and 13 females in the 400 mg/kg/day group, including the all animals exhibiting RPN. Eosinophilic granules in the tubular lumen at the renal papilla were observed in 5 males in the 200 mg/kg/day group and in 14 males and 9 females in the 400 mg/kg/day group. RPN regularly coexisted with both the chromophobe substances and eosinophilic granules, except for 1 male and female in 400 mg/kg/day group which had no eosinophilic granule. These findings were not observed in the control group. The chromophobe substances and eosinophilic granules were present with dose-related manner; therefore it was suggested that these substances could be p-Cre or its metabolites. RPN was also observed in the cases indicating the chromophobe substance only. Therefore, the chromophobe substance accumulated in the lumen of the renal tubules at the renal papilla might be one of the pathogenetic factors of the RPN observed in p53 KO mice treated with p-Cre by gavage.

P-32

Thy-1 expressing cells in rat renal interstitial fibrosis, in correlation with myofibroblasts

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Myofibroblasts play an important role in fibrosis by producing excessive amounts of extracellular matrices. In renal interstitial fibrosis, besides the pre-existing interstitial fibroblasts and perivascular undifferentiated mesenchymal cells, it has been considered that renal tubular cells may be a possible origin of myofibroblasts through the process known as the epithelial-mesenchymal transition (EMT). However, the detailed mechanism remains to be investigated. Recent studies suggest that Thy-1, a GPI-anchored cell surface protein, is involved in myofibroblast differentiation. In this study, we immunohistochemically investigated Thy-1 expressing cells in rat renal interstitial fibrosis, in correlation with cells expressing myofibroblastic markers such as vimentin, desmin and α -smooth muscle actin (α -SMA). Cisplatin (CDDP)- and unilateral ureteral obstruction (UUO)-induced renal fibrosis models were produced using male 6-week-old F344/DuCrj rats. Kidney samples were cryopreserved, or fixed in periodate-lysine-paraformaldehyde (PLP) fixative and embedded in paraffin by the AMeX method. In CDDP-induced model, Thy-1 expressing cells appeared around injured and dilated renal tubules in the cortico-medullary junction. Thy-1 expressing cells also showed vimentin and desmin expression, but did not colocalize with α -SMA expression which increased with the development of renal fibrosis. In UUO-induced model, in the cortex, Thy-1 expressing cells showed vimentin and desmin expressions, but did not colocalize with α -SMA expression; in contrast, in the medulla, the distribution of Thy-1 expressing cells coincided with that of vimentin, desmin and α -SMA. In these two models, Thy-1 did not label with regenerating tubules. This study shows the possibility that Thy-1 expression plays a role in myofibroblastic differentiation in renal interstitial cells; the functions of thy-1 may be different between the cortex and medulla.

P-33

Study on toxicological aspects of crystal-mediated nephrotoxicity induced by FYX-051, a xanthine oxidoreductase inhibitor, in rats

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To clarify the toxicological aspects of crystal-mediated nephrotoxicity, we performed analysis concerning the correlation between representative kidney-related parameters and renal histopathology using the individual data obtained from the 4-week toxicity studies of FYX-051, a xanthine oxidoreductase inhibitor, by oral administration at 1 and 3 mg/kg to SD rats and at 3 and 10 mg/kg to F344 rats.

In SD rats, the correlation coefficient on histopathology between the right and left kidneys was 0.7826 and remained within a lower range of strong correlation (the range: $\pm 0.7 \sim \pm 0.9$). The correlation coefficient between body weight gains, urinary volume, osmolarity, serum BUN, creatinine, and relative kidney weights and renal histopathology was -0.6648, 0.7896, -0.7751, 0.8195, 0.8479, and 0.8969, respectively, showing a strong correlation except a moderate correlation in body weight gains (the range: $\pm 0.4 \sim \pm 0.7$).

In F344 rats, the correlation coefficient on histopathology between the right and left kidneys was 0.8637, remaining within an upper range of strong correlation. The correlation coefficient between the above parameters and renal histopathology was -0.8175, 0.8616, -0.9045, 0.9010, 0.8991, and 0.9524, respectively, showing an extremely strong correlation in urinary osmolarity, serum BUN, and relative kidney weights (the range: $\pm 0.9 \sim \pm 1.0$).

Therefore, the present study suggests that FYX-051-induced nephrotoxicity may occur with more inconsistency in the degree of nephropathy between the right and left kidneys in SD rats than in F344 rats which would explain the above outcomes.

P-34

Pathological Changes of the Islet Induced by High-dose Sulfonylurea in Rats

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Sulfonylureas (SU) are oral hypoglycemic medications which have clinical experiences over half a century. Pathological changes of pancreatic islet, such as degranulation, are induced in laboratory animal species after repeated administration of high dose SU. However, there are no literatures which describe single cell vacuolation of pancreatic islet cells after a single gavage of SU at high dose levels to non-diabetic animals. In this study, histopathological changes of islet were examined after single dose and 4-day repeated dose administration of SU to rats. Single cell vacuolation was observed in the male F344 rats after single oral dose of glibenclamide, glimepiride, or tolbutamide (dose levels; 300 and/or 1000 mg/kg). Single cell vacuolation was considered to be correspondent to dilatation of rough ER adjacent to the nucleus in some islet cells under electron microscopic examination. Recovery of the changes was confirmed four days after single dose of 1000 mg/kg, and the C_{max} of glibenclamide was 6.3 µg/mL. Thus, recovery of the vacuolation was confirmed under higher plasma concentration compared to the C_{max} (0.082 µg/mL) of the Japanese clinical dose. In addition, after 4-day repeated dose gavages of glibenclamide (300 or 1000 mg/kg), single cell vacuolation was not observed. This study demonstrated single cell vacuolation of the islet after single dose administration of SU to rats. As the vacuolation was transient, we concluded that toxicological significance of the single cell vacuolation would be low.

P-35

Relationship between adrenal functions and histopathological changes in monkeys treated with ACAT inhibitor

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Acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor induces adrenal toxicity in several animals, though there is little reports that the relationship between adrenal functions and histopathological changes in monkeys treated with these drugs.

In the present study, we investigated that the hormonal examinations involved in adrenal (ACTH, cortisol and DHEA), ACTH stimulation test (cortisol and aldosterone) and histopathological examination of 13-week oral treatment with new ACAT inhibitor X in cynomolgus monkey.

As a result, vacuolation, eosinophilic change, necrosis and fibrous septum in zona fasciculata were observed in adrenal histopathology. No abnormal changes in hormonal examinations and ACTH stimulation test were observed in the animals which only vacuolation and/or eosinophilic changes were observed in adrenal. On the other hand, necrosis and/or fibrotic septum were observed in the animals with concomitant high ACTH and reduced response of cortisol after stimulation by ACTH. No abnormal changes in cortisol, DHEA and response of aldosterone after stimulation by ACTH were observed in any other animals.

In conclusion, it suggested that the vacuolation and eosinophilic changes in zona fasciculata dose not affect adrenal function in monkey receiving X although the necrosis and fibrotic septum in zona fasciculata affect it.

P-36

Histological Changes Observed in Miniature Pigs Fed a Diet Containing Vegetable Oil

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It has been shown that dietary administration of various vegetable oils to stroke-prone spontaneously hypertensive rats (SHRSP) causes shortening of the survival time and endocrine disruption. Histological examination showed necrosis of the myocardium, regeneration of renal tubules and cerebral hemorrhage.

In the present study, miniature pigs, being closer to the human model, were used to investigate the effects of dietary vegetable oils.

[Materials and Methods] Twenty-four miniature pigs (NIBS, Nisseiken, 4-weeks-old, male) were divided into 4 groups and fed a diet containing either, 9% canola oil + 1% soy oil (group A), 9% H₂-added soy oil + 1% soy oil (group B), or 10% soy oil (group C), or a basal diet for pigs (NS, Nisseiken, group D) for 18 months, and autopsied under deep anesthesia. Blood and blood biochemical analyses were carried out, and tissue samples were fixed in 10% buffered formalin, embedded in paraffin and examined under light microscopy. Testes were fixed in phosphate-buffered, 2.5% glutaraldehyde and examined under electron microscopy (EM).

[Results] No significant differences were observed in the blood and blood biochemical examinations. The mean weight of the testes differed between the groups (B>A>C, p<0.05). Atrophy of the seminiferous tubules, vacuolar changes of the Sertoli cells, and hypertrophy and hyperplasia of the Leydig cells were observed in all groups. However, these changes were less significant in group B. In the pancreas, single cell necrosis of the acinar cells was observed in group B at a high frequency. Under EM microscopy, large mitochondria with numerous vesicular cristae were observed in the cytoplasm of the Leydig cells in group C.

[Summary] The histological findings in miniature pigs fed a diet containing vegetable oil from weaning differed from those in SHRSP rats.

P-37

Dysregulated Maintenance of CpG Methylation in Rat Prostate Correlated with Aging

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In the 25th annual meeting of this society, we reported that perinatal exposure to a high dose of methoxychlor induced delayed-type prostatic enlargement in Sprague-Dawley rats after maturation. In order to elucidate the mechanism of the delayed occurrence, aging factors were explored by comparing the expression of genes related to prostatic enlargement and DNA methylation in the prostate between the 10- and 52-week-old untreated control rats. Quantitative analysis of genetic expression revealed significant up-regulation of *Clu*, *Plau*, and *Srd5a2* (genes involved in prostatic enlargement) and significant down-regulation of *Uhrfl* (a gene related to DNA methylation) at 52 weeks of age. Analysis on DNA methylation revealed a significant decrease in global methylation and significant hypomethylation of CpG islands at the transcription start site of *Clu*, *Plau*, and *Srd5a2* at 52 weeks of age. There are seven preferential androgen receptor (AR)-binding sites in the flanking region of the genomic *Uhrfl*. Chromatin immunoprecipitation (ChIP)-PCR assay showed a significant decrease in AR binding at three AR-binding sites of the 3'-side at 52 weeks of age. There was no significant change in expression of *Ar*. Since *Uhrfl* is the important factor which recruits Dnmt1 to DNA replication sites for the maintenance of DNA methylation, our experimental result suggested that a decrease in global methylation might be induced by down-regulation of *Uhrfl*. It also suggested that the expression of *Uhrfl* in the prostate may be controlled by AR. Although the detail is unclear, these indicate the possibility that dysregulated maintenance of DNA methylation with aging might be associated with delayed type prostatic enlargement induced by perinatal exposure to methoxychlor.

P-38

Piperonyl Butoxide Has Anti-estrogenic Activity, and Exerts Adverse Effects on Female Reproductive Organs of Rats

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[Introduction] A 13-week repeated oral toxicity study of piperonyl butoxide (PBO), a synergist for the insecticides, induced atrophy of the uterus in rats in our previous study. We investigated the effects of PBO on the female reproductive tract in rats, focusing on potency of anti-estrogenic activity of PBO.

[Materials and methods] <Exp. 1> 6-week-old female CrjDonryu rats were fed the diet containing 0, 5000, 10000 and 20000 ppm of PBO for 28 days. We added two food restricted groups relevant to similar growth to the PBO 10000 and 20000 ppm groups to investigate the effect of body weight decrease on the female reproductive tract. <Exp. 2> To detect anti-estrogenic activity in vivo, adult female rats, ovariectomized 2 weeks before, were fed the diet containing same doses of PBO in Exp. 1 for 14 days. Concurrently, the rats were subcutaneously injected 17 β -estradiol (E2) 1 μ g/kg BW every day. Uterine wet and blotted weights and uterine epithelial cell height were measured. <Exp. 3> To investigate a potency of anti-estrogen activity in vitro, reporter gene assay to human estrogen receptor α (ER α) was performed.

[Results] <Exp. 1> In PBO 20000 ppm group, body weight was decreased during first week, but slightly recovered thereafter. Continuous prolonged diestrus or abnormal cyclicity was observed in PBO 20000 ppm group after first week. In the food restricted group relevant to PBO 20000 ppm prolonged diestrus was detected during body weight reduction period only. Increases in large atretic follicles, decreases in new corpora lutea, vacuolation in interstitial cells in the ovary, uterus atrophy and vaginal mucinous degeneration were observed in PBO 20000 ppm group only. In other groups, no effect on the reproductive tract showed. <Exp. 2> Body weight decrease was observed in PBO treated groups. Absolute uterine weights were significantly decreased in PBO 10000 and 20000 ppm groups compared to those in E2 treated control group, but not significantly in relative weights. The uterine epithelial cell height was significantly decreased in PBO 20000 ppm group. <Exp. 3> PBO was ER α antagonist positive in this study, while it was weaker than 4OH-TAM.

[Discussion] These results indicate that PBO exerts anti-estrogenic activity on the female reproductive tracts, but not secondary effects related to decrease of body weight gain.

P-39

A Comparison among Effects of PPARs on Ovarian Follicles in Rats

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Di (2-ethylhexyl) phthalate (DEHP), a peroxisome proliferator activated receptor alpha and gamma, is known to be an ovarian toxicant. A mechanism of its toxicity has been reported to be a suppression of estradiol production in the ovary, leading to an ovulation (Lovekamp-Swan et al, 2003). Recently, Sato et al. (2009) reported that a PPAR alpha/gamma dual agonist induced unruptured preovulatory follicles in rats suggesting a possibility that PPARs have different toxic pathways to the ovary. The present study was conducted to investigate different effects of PPARs on follicle development in rat ovary. Seven week-old normal cycling Donryu female rats were treated with DEHP at 3000 mg/kg bw/day, di (2-ethylhexyl) adipate (DEHA) at 2000 mg/kg bw/day by gavage or clofibrate (CF) at 2500 ppm in diet for 2 or 4 weeks, and examined morphological changes in the female reproductive tracts and ovary related hormonal profiles.

As a result, DEHP and DEHA treatments increased abnormal vaginal cytology which is mostly 2-day estrus while regular 4 day estrous cyclicity was observed in the CF group throughout the treatment period. The ovarian weights were reduced in the DEHP group only after 2-week treatment, though the liver weights in all treated groups were significantly increased. Morphologically increased atretic large follicles, decreased Graafian follicles, unruptured follicles with luteinized granulosa cells or luteinized follicles were found in the DEHP and DEHA groups whereas their incidences were low. The latter two lesions suggested any disruption of follicle rupture at ovulation. No abnormalities were detected in the CF group. In hormonal assay, lower E2 levels were detected in the DEHP and DEHA groups at proestrus, but not in the CF groups.

These results indicate that DEHP and DEHA treatments have two types of ovarian toxicities characterized by lower estrogen production in antral follicles and disturbance of follicle rupture at ovulation. CF, a PPAR alpha, did not have any toxicity to the ovary. Main target of these effects to the ovary might be PPAR gamma. Further investigation related to prostaglandin or progesterone should be needed to clarify their mechanisms.

P-40

Epidermal and Skin Appendages Hyperplasia Through The Activation of Hedgehog Signaling

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We faced epidermal and skin appendages hyperplasia in short-term repeated dose toxicity studies in rats for a newly synthesized compound and investigated the mechanism of those hyperplasia with toxicogenomics approach. Compounds A and B which had induced hyperplasia in previously performed toxicity studies, and compound C which had not induced them were administered to CrI:CD(SD) rats respectively for 4 days. Total RNAs were extracted from the skin and subjected to GeneChip (Rat 230 2.0, Affymetrix) analysis. Comprehensive gene expression analysis revealed that the genes related to hedgehog signaling pathway were up-regulated in the skin obtained from rats given compounds A and B. These results suggested that activated hedgehog signaling might associated with induction of epidermal and skin appendage hyperplasia. *Gli1* is one of the target genes in hedgehog signaling activation, therefore *Gli1* expression would be an indicator of activation of the pathway. So, next we conducted *in vitro* experiments to investigate the effect of compounds on *Gli1* expression. Compounds (A to F) were exposed to NIH3T3 cells derived from mouse embryo skin and the changes of the gene expression were examined by real time-PCR using TaqMan probe. In *in vitro* exposure, compounds A and B increased *Gli1* mRNA but compounds C to F, which could not induced epidermal hyperplasia *in vivo*, did not. In addition, the up-regulation of *Gli1* mRNA by compounds A and B was inhibited in the presence of cyclopamine, a specific inhibitor of hedgehog signaling, clearly demonstrating that compounds A and B could activate hedgehog signaling. It has been reported that hedgehog signaling participated in development of hair follicle, sebaceous and mammary gland, and abnormal activation of the pathway could contribute to induce proliferative disorder such as basal cell carcinoma. Collectively, it was suggested that compounds A and B induced epidermal and skin appendages hyperplasia through the activation of hedgehog signaling.

P-41

Age-Related Susceptibility to Induction of Osteochondral and Vascular Lesions By Semicarbazide Hydrochloride in Rats.

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Semicarbazide (SEM) has been found in food in glass jars sealed with plastic gaskets manufactured using azodicarbonamide as a blowing agent, such as baby foods, jams and conserves. It is also known to act as an osteolathyrogen, inducing osteochondral and vascular lesions in young rats due to impaired cross-linking reactions of collagen and elastin. Since intake of SEM for infants is estimated to be much higher than for adults, we compared histopathological osteochondral and vascular lesions in young and adult female rats. 3- and 20-week-old female SD rats were given diet containing SEM-HCl at 0, 500 or 1,000 ppm, and 0 or 1,000 ppm, respectively, for 4 weeks. Half of the animals were then maintained on basal diet for a further 2 weeks as recovery groups. Only in young rats, deformation of the knee joints as well as thorax and tail was observed at 500 and 1,000 ppm. Histopathologically, severe osteochondral lesions, such as disarrangement and thickening of epiphyseal cartilage and deformation of articular cartilage, were observed, but the severity of these lesions became reduced during the recovery period. In the thoracic aorta, the edges of elastic laminae became roughened and the appearance of interlaminae spaces was altered in both the 4-week treatment and recovery groups. On the other hand, osteochondral lesions in adult rats were relatively mild. Fissures in the cartilage matrix of the tibia were characteristic of adult rats, and in these reduction of severity was not obvious in the recovery group. In the thoracic aorta of adult rats, histology of elastic laminae did not differ between the 0 and 1,000 ppm groups, both after the 4-week treatment and the recovery period. Although the intake of SEM-HCl per kg body weight in young animals was about twice as much as that in adult rats, the lesions in young animals at 500 ppm were clearly of greater intensity than in adult animals at 1000 ppm. Therefore, animals with growing processes are considered to be more susceptible than adults. The growth of bones reaches equilibrium by approximately 20 weeks of age, while elastic laminae of the aorta are known to be matured by 8 weeks. Accordingly, it is suggested that the severity of induced lesions and their reversibility depend on the developing stage of the target organ.

P-42

The Histopathological Difference between Femur and Sternum in Young Rats with Dexamethasone Treatment

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The femur and sternum are commonly examined in preclinical toxicity studies in rat. On the other hand, the morphology and maturation process of bone vary according to bone type, and we reported previously that the area of observation must be considered in evaluating bone toxicity in rat. The aim of the present study was to provide a method for evaluating bone toxicity induced by drugs in young rats, to investigate histopathologically femur and sternum, dosed with dexamethasone (DEX), known to retard the skeletal growth in young rats.

Male Crl:CD (SD) rats at 6 weeks of age (n=6/each group) were administered 0, 0.3, 1 and 3 mg/kg of DEX for 14 days. The animals were euthanized at 8 weeks of age at the end of the study period. At the necropsy, the distal femur and sternum were removed for histopathologic examination. Samples were fixed with 10% neutral buffered formalin, decalcified with Plank-Rychlo's solution, embedded in paraffin and sectioned at a thickness of 4-5 μ m. The specimens were stained with hematoxylin and eosin, and were histologically examined using a light microscope similar to the routine histopathological evaluations performed in toxicity studies. These were subjected to immunohistochemical identification of proliferating cell nuclear antigen (PCNA) and vascular endothelial growth factor (VEGF) for qualitative analysis of cellular proliferation and angiogenesis.

In the femur of DEX-treated group, it is observed that thinning of growth plate in proliferation, maturation, hypertrophy and calcification zone, thickening of growth plate in cartilage degeneration and osteogenic zone. On the other hand, thickening of chondrocyte of growth plate in hypertrophy and calcification zone was observed in the sternum of DEX-treated group. PCNA-positive chondrocytes were not obvious in femur and sternum. Immunohistochemistry it is revealed that VEGF-positive osteocytes in growth plate of osteogenic zone reduced only in the femur of DEX-treated group.

It is thus indicated that there are some differences in angiogenesis between the femur and sternum in the young rat treated with DEX.

P-43

28-day Repeated Dose Toxicity Study of Gellan Gum K3B646 in Crl:CD(SD)IGS Rats.

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Gellan gum is a straight chain extracellular polysaccharide produced by *Sphingomonas elodea* based on repeating β -D glucose, β -D glucuronic acid, β -D glucose and α -L rhamnose units. It has been widely used as a thickening, suspending/stabilizing, and gelling agent in foods but a production strain (S-60) of native gellan gum, containing arylsulfatase and β -glucuronidase, with UHT (ultra high temperature) dairy applications results in formation of *p*-cresol, which gives an off odor and taste. The *p*-cresol can be metabolized from natural conjugated components in milk by these enzymes. Use of gellan gum K3B646 produced by *S. elodea* strain GBAD-1 (featuring deletion of the arylsulfatase and β -glucuronidase genes) constructed by genetic engineering, prevents the unwanted odor and flavor. The present study was designed to evaluate and characterize any subacute toxicity when this form was given to both sexes of Crl:CD(SD) rats (6 animals/sex/group) at dietary levels of 0 (control), 0.5, 1.5 and 5.0% for 28 days.

During the study, the treatment caused no adverse effects on clinical signs, survival, body weights, and food and water consumption, or on findings of urinalysis, ophthalmology, hematology, blood biochemistry, gross pathology, or histopathology. Increased relative cecum (filled) weights, evident in males of 5.0% groups were considered to be a physiological adaptation.

Thus, the no-observed-adverse-effect level (NOAEL) for gellan gum K3B646 was concluded to be a dietary level of at least 5.0% (3687 mg/kg/day for males and 4074 mg/kg/day for females).

P-44

Effects of Repeated Intravenous Dose of Water-Soluble Large Molecules in Animals

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Purpose: We investigated a nonspecific effect of repeated intravenous dose of water-soluble large molecules in rats.

Materials and Methods: Dextran (DEX), a group of glucose polymers are traditionally used therapeutically as plasma volume expanders and as carriers of drug delivery systems. Small molecule DEX (40 kDa, the same molecular weight as commercially available plasma volume expander; small molecule DEX group) and large molecule DEX (200-300 kDa, large molecule DEX group) were given to 7-week-old SD rats (6 males per group) through an intravenous dosage of 5 mL/kg/day for 28 days. The concentration of each DEX was 10% as same as the plasma volume expander in both the treatment groups. Saline was administered in the same manner to the control group.

Results: Molecular weight related changes were noted in all rats in both the treatment groups. In organ weight measurements, lung, spleen and liver weight were increased. In histopathologic examinations, foam cell infiltration of lung and spleen and vacuolization of hepatocytes were noted. The vacuoles of hepatocytes were stained blue by colloidal iron staining indicating DEX accumulation in hepatocytes. These changes were more severe in the large molecule DEX group than small molecule DEX group. PAS-positive granules in glomeruli and dilation of peritubular capillaries in the renal cortex were noted in the kidneys of all rats in large molecule DEX group.

P-45

Histopathological Examination of Influences on Porcine Coronary Arterial Wall Caused by Implantation of Two Drug Eluting Stents Connected with Overlapping in their Boundary Portion

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[Background] Nowadays, stent implantation therapy for stenosed portion of coronary artery is frequently carried out. However, about 20 % of patients receiving implantation of the bare metal stent (BMS) suffer from re-narrowing in the implanted portion caused by excess vascular smooth muscle cell proliferation. At present, application of drug eluting stent (DES) coated with anti-proliferative drugs has become ordinary method to reduce the narrowing. Also, use of overlapped stents connected with border portion of 2 stents in case suffered from rather longer narrowing has become common at present. But, in case of application of overlapped stent, the amount of the drug elution increased two times around the local area. So, in this study we examined histopathologically possible influences of increased amount of drug elution from 2 types of DESs and one type of BMS in the intact porcine coronary arteries.

[Methods] We implanted Sirolimus eluting stents, Biolimus A9 (BA9) (Sirolimus analogue) eluting stents and BMS with 50% overlapping in porcine right coronary arteries. At either 2, 4 or 12 weeks after stent implantation, specimens were taken from stent-implanted arteries. Sections obtained from resin embedded specimens were observed histopathologically, and luminal surfaces of all types of the stent implanted arteries at 2 and 4 weeks after implantation were observed with a scanning electron microscope.

[Results] At 4 weeks, BMS implanted arteries showed good recovery of endothelial cells and formation of neointimal layer caused by smooth muscle cell proliferation, while DES implanted arteries were characterized by coverage with less numbers of endothelial cells, exudation of fibrinous material indicating poorer neointima formation. At 12 weeks, neointima formed with BA9 eluting stents consisted mainly of smooth muscle cells. Compared with Sirolimus eluting stents, BA9 eluting stents showed rather mild inflammatory change and well recovery of endothelial cells.

[Conclusion] Implantation of DES delayed healing process of arterial wall and which was more prominent in overlapped zone of both stents. The grade of inflammatory lesion and progress of recovery process of endothelium differed between two types of DES, though both of coating drugs belong to limus family. These results possibly suggested that not only pharmacological effects of coating drugs but also design of the stent play the key role for better results of coronary stent implantation therapy.

P-46

The safety evaluation of tissue adhesive for the optic nerve in rabbits

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【Introduction】 The biological tissue adhesive (hereinafter abbreviated as adhesive) might adhere to the optic nerve when the adhesive was used in the brain surgery. Because safety information of the adhesive was requested in clinical practice, the safety of the adhesive to the optic nerve was evaluated in rabbits.

【Methods】 Under anesthesia, Japanese white rabbits underwent a craniotomy procedure. A burr hole of 1cm in the diameter was made on the left coronary suture. The dura mater was incised, a cannula was inserted from this incision into the subarachnoid space in the skull base, and the adhesive or the physiological saline was administered. To confirm the adhesive came in contact with the optic nerve, a part of rabbits were sacrificed immediately after administering under the pentobarbital sodium anesthetizing. In the other animals, clinical signs and ophthalmological examination were performed. After 7 days of administration, their animals were sacrificed under the pentobarbital sodium anesthetizing and histopathological examination was performed. In the histopathological examination, paraffine-embedded sections of optic nerve, eyeball, brain and oculomotor nerve were routinely prepared and stained with hematoxyline and eosin, bodian, and luxol fast blue for the light microscopic observation.

【Results】 No abnormalities in clinical signs and ophthalmological examination were observed in any animals until the necropsy. In the adhesive group, although the contact between the adhesive and the optic nerve was confirmed, no abnormalities in necropsy were observed in any animals. In histopathological examination, although very slight lymphocyte infiltration in the optic nerve sheath was seen in the adhesive group, no abnormalities in the optic nerve were seen. And abnormalities were not observed in the oculomotor nerve also.

【Conclusion】 The method of administration that the adhesive came in contact with the optic nerve was able to be established. No abnormalities were observed in any examinations. In histopathological examination, lymphocyte infiltration in the optic nerve sheath was seen. However, the change was very slight. Therefore it was suggested that the possibility of defect in vision caused by inflammation and degeneration and necrosis in nerve tissue by the contact of the adhesive to optic nerve was low.

P-47

Histopathological Study Concerning the Effect of Covering of Fibrin Glue Combined with Polyglycolic Acid Felt on Wound Healing.

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【Purpose】 In operation of oral cancer, covering with fibrin glue and polyglycolic acid felt (PGA felt) to the cut surface of the cancer, which have been reported to be effective for suppress of wound contraction and relief of pain. In the present study, we examined histopathologically the effect on wound healing of covering of fibrin glue and PGA felt using an animal model.

【Methods】 Two full-thickness skin wounds (1.5×1.5-cm square) were prepared on the back of guinea pig. The wounds were covered with fibrin glue and PGA felt or collagen-based artificial dermis. In the control group, the wounds were not covered. The guinea pigs were euthanized at 1 and 2 weeks after wounding, and the wounds and surrounding tissue were removed. For histological examination, the collected tissues were embedded in paraffin and stained with hematoxyline and eosin. And the rate of test substances covering to the wounds was calculated. The preservation rate of wound area was measured by the image analysis software.

【Results】 The fibrin glue and PGA felt group showed remarkable high values of the test substance coverage rate and the preservation rate of the wound area in comparison with those of other groups. At 2 weeks after treatment, fibrin glue and PGA felt group showed 80% in the test substance coverage rate and showed high value in the preservation rate of the wound area. In other groups, there were many animals whose wound were not covered with test substances. In the histopathological examination, granulation tissue proliferation in the PGA felt, and the granulation tissue filled up the defect region.

【Conclusion】 It is considered that the space of granulation tissue proliferation is maintained in the wound by covering with fibrin glue and PGA felt, and the invasion of granulation tissue into PGA felt prevents the sheet from peeling off. These results suggest the effects of suppress of wound contraction and relief of pain are caused by covering of fibrin glue combined with PGA felt.

P-48

Histopathological Changes of the Skin (Treated Site) in Repeated Dose Dermal Toxicity Studies in Rats

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As a part of the toxicological evaluation of pharmaceutical agents (external preparations) and cosmetics, repeated dose toxicity studies by percutaneous administration are conducted to investigate their toxicity. To ensure precise histopathological evaluation of the skin (treated site), it is important to know the difference of tissue reactions related to the physical and chemical properties of the test articles. In this study, we have reviewed the findings on the treated site in repeated dose dermal toxicity studies previously carried out in our laboratory.

We reevaluated the HE histopathological specimens of the skin (treated site) in the sham treatment control groups, vehicle control groups (0.5%CMC-Na solution, olive oil), placebo control groups (embrocations such as lotion, ointment, and plaster [tape]) prepared in 4-week repeated dose dermal toxicity studies in CrI:CD (SD) rats. Animals in all studies were fitted with neck collars during the treatment period, and the clipping area was covered with cotton lint and fixed with elastic bandages.

As the main changes, acanthosis and inflammatory cell infiltration in dermis were observed at the skin (treated site). The degree of inflammatory cell infiltration was severer in the olive oil-treatment of the vehicle control group and in the placebo control groups in both sexes in comparison with the sham treatment group. The degree of acanthosis was severe in the ointment- and tape-treatment of the placebo control groups in males, and the olive oil-treatment of the vehicle control group and the placebo control groups in females in comparison with the sham treatment groups.

The hydrophobic agents such as olive oil and embrocations are difficult to remove from the skin, thereby these agents might change to the irritants due to oxidization or bacterial degradation. In addition, due to its adhesiveness, daily replacement of tapes might stimulate the skin (treated site). In repeated dose dermal toxicity studies, it is important to evaluate the histopathological examination of the treated site carefully because the change in the skin (treated site) might have been modified by the physical and chemical properties of the external preparation and cosmetics.

P-49

Histopathological Changes of The Skin (Treated Site) in Percutaneous Toxicity Studies in Dogs

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In recent years, the formulation forms of existing drugs have been shifted into external preparations for their little adverse effect and convenience in medication and maintenance of the blood concentrations. However, there have been almost no reports concerning the lesion of the skin (treated site) in safety studies. Therefore, we are reporting on the results of reevaluation in the skin in the repeated dose percutaneous toxicity studies previously carried out in our laboratory

We reevaluated the HE preparations of the skin (treated site) in 80 of males and females beagle dogs at 7- to 11-month old used for the sham treatment control groups (the dorsal hair was clipped-off and the animal was fitted with jacket and neck collar) or placebo control groups (in addition to the above-mentioned treatment, embrocations [lotion, creams, or ointment] or patches [tapes] were applied) in repeated dose percutaneous toxicity studies

Only slight acanthosis was observed in the sham treated groups. Moreover, no abnormal change was observed in the animals treated with lotion. In the other placebo control groups (creams, ointments, and tapes), in addition to acanthosis, inflammatory cell infiltration mainly consisted of neutrophil was observed in the superficial dermis and perifollicle. The incidence and degree of the acanthosis and inflammatory cell infiltration in the superficial dermis in the ointments and tapes were remarkable than that of the creams. Furthermore, the incidence of the inflammatory cell infiltration of perifollicle was also high in the ointments.

The degree of changes in the skin (treated site) in the percutaneous toxicity studies of dogs was in the order of the lotion \leq sham treated group $<$ creams $<$ tapes $<$ ointments. In particular, the degree of inflammatory cell infiltration in the perifollicle was strong in the ointments. Since the viscosity of the ointment was high compared to that of the creams and the ointments were difficult to remove, they might be changed to an irritant due to alteration by oxidization or bacterial degradation. Inflammatory cell infiltration in the superficial dermis was also observed in the tapes. As the tapes are adhesiveness, replacing them daily might stimulate the skin. In percutaneous toxicity studies, a careful examination of the skin is important because there is a possibility that the lesion in the skin is modified by the formulation of the test substance and their application methods.

P-50

Histopathological Changes of the Skin (Treated Site) in Cumulative Skin Irritation Studies in Rabbits

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[Background] Cumulative skin irritation studies in rabbits are carried out to investigate local toxicity caused by continuous contacts of pharmaceuticals and cosmetics to skin.

For a precise evaluation of the test articles influences on skin, it is important to consider the additional effects associated to the dosage form or administration methods.

In the present study, we investigated these additional effects using specimens from cumulative skin irritation studies previously performed in our laboratory.

[Methods] We reviewed the hematoxylin-eosin sections (87 animals) of the skin (treated sites). The sections were derived from the non treatment (only clipping of the back skin), comparative (open treatment of a physiological salt solution or injection water) and base control (open treatment of a lotion or an ointment, occlusive treatment of a tape or a poultice) groups in 14- or 28-day cumulative skin irritation studies in rabbits.

[Results] There were influences of the administration with the bases in one of two lotions, one of two ointments, four of five tapes and one of two poultices. Main histopathological findings included acanthosis, inflammatory cell infiltration in dermis, hemorrhage in dermis and hyperkeratosis. Severity of the inflammatory cell infiltration in dermis and acanthosis increased in the occlusive treatment more so than the open treatment in the base control groups. These findings tended to be more intense in lotions than in ointments by comparison in the open treatment, and in poultices than in tapes in the occlusive treatment.

[Discussion] The tissue reactions were of noteworthy differences among the studies even if the dosage form was the same, suggesting that the differences were associated with the ingredients of the bases. In addition, these reactions intensified in the occlusive treatment as compared to the open treatment, which was considered to be due to the physical stimulation, such as daily attachment and detachment, as well as the base ingredients. In conclusion, it is necessary to carefully ascertain the presence of the influences by the base ingredients or dosing methods when judging the local toxicity of the test articles to skin.

P-51

Combination of Histopathological Examination and Immunophenotyping Is Important in Immunotoxicological Study

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Immunotoxicity studies were brought to international attention in toxicology and proposed as guideline; ICH S8 (Immunotoxicity studies for human pharmaceuticals). We verified whether immunotoxic profile was accurately evaluated using combination of histopathological examination and flow cytometric immunophenotyping (FCI). In this study, we used Crl:CD(SD) rats administered cyclosporine A (CsA), cyclophosphamide (CPA) and azathioprine (AZA) as immunotoxicants, and we confirmed immunotoxicological mechanisms by hematology, histopathological examination, and FCI.

CsA decreased specifically T cell count in FCI, although total lymphocyte count did not change in peripheral blood. In the histopathological examination, lymphocyte count was decreased in periaarterial lymphoid sheath and paracortex of spleen and lymph node, respectively, and medulla area was decreased in thymus. In addition, FCI showed that T cell count or CD4 single positive cell count was selectively decreased in spleen and lymph node or thymus, and the alteration of T cell counts brought the histopathological changes to those lymphoid/hematopoietic organs. CPA induced atrophy of spleen, lymph node and thymus in histopathological examination. However, FCI showed that total lymphocyte counts was significantly decrease, and relative B cell count was specifically decreased at early phase. Those findings suggested that CPA suppressed B cell response earlier than T cell response. Although AZA induced no histopathological changes, decrease number of relative B cell and NK cell were confirmed by FCI.

Taken together these results, both hematology and histopathological examination were not sufficient, rather the combination of these exams and FCI is considered to be more reliable in the evaluation of drug-induced immunotoxic potential and its mechanisms.

P-52

Immuno-Toxicological Evaluation of Cyclosporine A, Prednisolone and Ibuprofen

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To investigate the immunotoxic potential of pharmaceuticals, the examinations prescribed by ICH S8 Guideline and the following additional toxicological, haematological, histopathological, and immuno-functional examinations were performed: 1) absolute and relative organ weights of spleen, thymus and adrenal gland, 2) histopathology of thymus, spleen, mesenteric lymph nodes, submandibular lymph nodes, Peyer's patches (ileum) and bone marrow (sternum and femur), 3) Immuno-phenotyping (peripheral blood, thymus and spleen), 4) T cell dependent antibody response (TDAR). Cyclosporine A, prednisolone and ibuprofen were selected as model compounds and administered orally for 28 days in SD rats. As the results, the cellularity of T-cell compartments in thymus (medulla), spleen (PALS), and paracortical zone of mesenteric and submandibular lymph nodes (LNs) were decreased in cyclosporine A-treated rats. The numbers of follicular germinal centers in the LNs were reduced in cyclosporine-treated rats. The cellularity in thymus (cortex and medulla), spleen (PALS, marginal zone and follicles), lymph nodes (paracortical zone and follicles in mesenteric, submandibular LNs and GALT) were reduced in prednisolone-treated rats. No abnormalities were detected in ibuprofen-treated rats. In the immuno-phenotyping, DN, CD4 and CD8 lymphocytes in the thymus and spleen were significantly decreased in cyclosporine A-treated rats, and DN, DP, CD4 and CD8 lymphocytes in peripheral blood, thymus and spleen were also significantly decreased in prednisolone-treated rats. In the TDAR, cyclosporine A inhibited T and B cell functions, prednisolone inhibited the B cell function, and ibuprofen likely activated the T cell function. The results suggest that cyclosporine A and prednisolone induced immuno-toxicological effects in SD rats, indicating the test battery employed in this study is very useful for immuno-toxicological evaluation for pharmaceuticals.

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Immunohistochemical Evaluation Method for Drug-induced Phospholipidosis in Mice

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Drug-induced phospholipidosis (PLP) is an abnormal accumulation of phospholipids in the lysosomes following administration of cationic amphiphilic drugs. The phospholipid accumulation is observed as a cytoplasmic vacuolation in the histopathological examination. However, it is difficult to discriminate between phospholipid accumulation and lipid (non-phospholipid) accumulation, because lipid accumulation is also observed as cytoplasmic vacuolation on a light microscopy.

In this study, using the mice administered orally ketoconazole (a well-known chemical to induce PLP), we investigated the usefulness of immunohistochemical staining for LAMP-2 (a lysosome-associated protein) and adipophilin (a protein that forms the membrane around non-lysosomal lipid droplets) to discriminate between PLP and lipid accumulation in the livers and kidneys.

Seven week-old Crlj:CD1(ICR) male mice were administered orally 300 mg/kg/day of ketoconazole or 0.25% tragacanth gum as a control for 7 days, and the livers and kidneys were removed on the following day after final administration. H.E. staining and immunohistochemical staining for LAMP-2 and adipophilin were performed using formalin-fixed paraffin sections. In addition, electron microscopic evaluation was performed.

As results, cytoplasmic vacuolation was observed in the centrilobular hepatocytes and renal tubular epithelia in the ketoconazole-treated group on light microscopy, which was revealed to be PLP-characteristic lamellar bodies on electron microscopy. In the immunohistochemistry, these vacuoles had positive reaction for LAMP-2 immunostaining, but negative reaction for adipophilin immunostaining. On the other hands, cytoplasmic vacuolation was observed in the perilobular hepatocytes and renal tubular epithelia in the control group and ketoconazole-treated group, which was revealed to be lipid droplets on electron microscopy. These vacuoles had positive reaction for adipophilin immunostaining, but negative reaction for LAMP-2 immunostaining.

In conclusion, the immunostaining for LAMP-2 and adipophilin using formalin-fixed paraffin sections could discriminate between PLP and lipid accumulation in the livers and kidneys of mice.

P-54

Histopathological Analysis of STAM Mice

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Non-alcoholic steatohepatitis (NASH) represents a histopathological spectrum of liver disease associated with obesity, diabetes and insulin resistance that extends from steatohepatitis to cirrhosis and hepatocellular carcinoma. STAM mice is a new model of non-genetically onset of NASH-HCC by streptozotocin and high fat diet. In this study, we examined histopathological analysis of STAM mice about NASH and proliferative lesions.

Twenty STAM mice, divided into STZ-alone (control) group and STZ+high fat diet (NASH) group, were sacrificed at weeks 10 and 18, and examined histopathological analysis of liver.

NASH groups at weeks 10 and 18 were observed histopathological features of human NASH including hepatic steatosis, ballooning, inflammation and fibrosis but not cirrhosis. In contrast, control groups at weeks 10 and 18 were not observed. NASH and control groups were shown hyperglycemia, and NASH groups were shown hyperleptinemia and hypoadiponectinemia compared with control groups, but 8-OHdG level was not differently with both groups. At weeks 10 and 18 in control groups, incidence of foci, adenoma and HCC were 67, 0, 0% and 100, 67, 0%, respectively. In NASH groups, incidence of foci, adenoma and HCC were 40, 40, 20% and 100, 100, 71%, respectively.

The present study suggested that STAM mice might be a model of NASH-HCC. And further studies are needed in order to elucidate the mechanisms of NASH and hepatocarcinogenesis.

P-55

A Preliminary Study on Establishment of Rheumatoid Arthritis Model Using SKG/Jcl Mice With a Special Reference to Histopathological Alterations

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Immunotoxic chemicals may influence the occurrence of autoimmune disease as a chronic effect in addition to an acute effect causing immunosuppression. However, adequate methods for evaluation of autoimmune diseases caused by chemicals have not been established yet. Human chronic rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of which the pathogenesis is unclear. Although a number of animal models showing similar lesions to RA have been established, they are mostly utilized to assess ameliorative effects of chemicals on arthritis because those models exhibit severe acute arthritis. To evaluate the influence of chemicals on chronic arthritis, an arthritis model with mild and persistent inflammation is necessary. From this point of view, this study was undertaken to establish a mild arthritis model using the SKG mouse that shows spontaneous arthritis similar to RA.

A single intraperitoneal injection of PBS or Curdlan (an immunoactivable agent) was given to female SKG/Jcl mice at 5 weeks of age and then arthritis score and the hind paw thickness were measured periodically. These animals were necropsied at 10 weeks of age and subjected to hematology and flow cytometry of peripheral blood. Histopathological examination was performed on systemic organs and tissues from all animals. In addition, grades of synovial cell hyperplasia, inflammatory cell infiltration, and bone erosion were scored for the joints of digits of forelimb and hindlimb, carpal, tarsal, and knee. The total score of these 3 factors was used as Histological score for evaluation of arthritis.

A slight and minimal spontaneous arthritis was observed in the control group, while the grade of arthritis was enhanced in the Curdlan-treated group. In the Curdlan-treated group, arthritis score and hind paw thickness were increased with time and Histological score was also increased as compared to the controls. In addition, hematology and flow cytometry revealed decreases in lymphocyte count, B cell count, and CD4⁺CD25⁺ regulatory T cell count in the peripheral blood from these treated animals.

The progression of arthritis in this model was found to be slower than other models. Therefore, this model would be available for detection of both promoting and inhibitory effects of chemicals on arthritis. In addition, it was suggested that the CD4⁺CD25⁺ regulatory T cell in the peripheral blood might have a role in enhancement of chronic arthritis in the Curdlan-treated group.

P-56

Analysis for induction and localization of intestinal cytochrome P450

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【Background】Cytochrome P450(CYP) which is distributed to the liver, intestine, kidney and brain is involved in many responses including drug metabolism. Although quantitative or histological evaluation for hepatic CYP have been conducted so far, but there is not enough report of histological study about intestinal CYP. Therefore, we confirmed expression and localization of intestinal CYP in rat that had been administered the CYP inducement drug (Dexamethasone: DEX) or food (St. John's wort: SJW).

【Method】 Male Sprague-Dawley rats, 7 weeks old, were orally administered SJW (1000 mg/kg, daily for 14 days), DEX (30 mg/kg, daily for 4 days) or vehicle (diluted water: 10 mL/kg, daily for 14 days). Animals were sacrificed under anesthesia and removed liver and small intestine day after last administration. Samples were routinely processed, and stained H&E. Immunohistochemical staining (anti CYP3A1/3A2 and anti CYP2B1/2B2 staining) and western blotting (CYP3A1/3A2) were performed to analyze expression and localization of CYP. In addition, anti Sarcoplasmic or Endoplasmic Reticulum Calcium (SERCA)2 ATPase staining and immunoelectron microscopic study were conducted to confirm expression site of CYP.

【Result】 Morphologic change was not found at the intestinal epithelium cells though enlarged hepatic cells were seen in the DEX group. In the control group, as a result of anti CYP3A1/3A2 or anti CYP2B1/2B2 staining, positive cells were detected at the upper small intestine, but the number of positive cells decreased from the middle to lower side. Slight inducement of only CYP3A1/3A2 was admitted at the upper small intestine of the SJW group, and highly inducement of CYP3A1/3A2 and CYP2B1/2B2 was admitted at the upper part of the DEX group. About the liver, the inducement of CYP3A1/3A2 and CYP2B1/2B2 was detected only in the DEX group. In the Western blotting, increase of CYP3A1/3A2 expression was detected in the small intestine of SJW and DEX group, and in the liver of the DEX group. In the immunoelectron microscopy, the positive reaction (deposition of DAB) was confirmed in the area of sER in the intestinal epithelial cell. Furthermore, in the anti-SERCA2 ATPase staining, the positive cells were detected in the same villus as the CYP-induced villus of the DEX group.

【Conclusion】 It was confirmed that intestinal CYP3A1/3A2 and CYP2B1/2B2 are expressed most strongly at the upper small intestine and there are a localization. Also, CYP3A1/3A2 or CYP2B1/2B2 were induced most strongly at the upper small intestine by administration of SJW or DEX, so it was thought that the evaluation at this part of intestine was useful to see the influence on these CYP. Furthermore, it was suggested that intestinal CYP is induced with increase and/or activation of sER.

P-57

Comparisons of properties of macrophages and myofibroblasts between two different cutaneous fibrosis rat models

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Interactions of macrophages with myofibroblasts play central roles in fibrogenesis. Characteristics of macrophages and myofibroblasts were investigated in punch-made wound healing (WH) and bleomycin-induced scleroderma (BS) models in rats. In both models, ED1+ and ED2+ macrophages were predominant at early and mid stages, whereas OX6+ macrophage appeared later. Galectin-3 (fibrogenic factor) in WH was expressed exclusively in ED1+ macrophages; conversely, the expression in BS was highly correlated with ED1+, ED2+ and OX6+ macrophages. In BS, macrophage appearance was correlated closely to myofibroblast formation, of which appearance patterns were similar to those in granulation tissue phase of WH. In both models, the immunohistochemical marker expression analyses showed that pericytes and hair follicle dermal sheath cells might be possible precursors of myofibroblasts. In BS, particularly, hair follicle loss due to apoptosis appeared to be associated intimately with macrophage recruitment and subsequent fibrosis in perifollicular areas; the perifollicular fibrosis was characteristic of BS, resulting in greater cutaneous fibrosis in BS than WH. This study showed similarities and differences in properties of macrophages and myofibroblasts between WH and BS models. More detailed comparisons are under way.

P-58

Research for Distribution and Differentiation of Preadipocytes Using Organotypic Culture System of Adipose Tissue Slices – Adipose Progenitor Cells Reside on Surface of Mature Adipocytes.

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Obesity plays a key role in the manifestation of metabolic diseases and is an important research field for the development of novel drugs. In obesity, an increase in the number of adipocytes derived from the adipose progenitor cells is a major contributing factor that ultimately results in an increased adipose tissue mass. Adipocytes develop in coordination with blood vessels, however, the precise localization and biologic characteristics of the adipose progenitor cells are still a focus of debate. In this study, the presence and localization of the adipose progenitor cells and preadipocytes were determined using a unique organotypic culture system of adipose tissue slices.

Tissue slices of subcutaneous white adipose tissue from 6-week-old Sprague-Dawley rats were cultured at the interface between the air and a culture medium for up to 5 days. Confocal laser microscopy of the tissue slices revealed that the capillaries made a complicated network among the mature adipocytes. After a 2-day incubation in an adipogenic medium, numerous preadipocytes containing fine lipid droplets appeared on the surface of the mature adipocytes showing no apparent connection to the capillaries whereas preadipocytes adjacent to the blood vessels also emerged. On Day 5 of incubation, the preadipocytes underwent further adipose differentiation, and many of these preadipocytes were surrounded by endothelial cells.

This organotypic culture system of adipose tissue slices was confirmed to be a useful model for adipose tissue research. The results of this study indicate that the adipose progenitor cells which reside on the surface of mature adipocytes could be a considerable source of cells for adipogenesis.

P-59

Investigation of a new pituitary tumor classification marker in rat (pituitary specific transcription factor 1)

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The expression of pituitary specific transcription factor 1 (Pit-1) was investigated immunohistochemically for 50 pituitary proliferative lesions (hyperplasia, n=6; adenoma, n=44) in 40 to 115-week old male CrI:CD(SD) rats, and its usability in the classification of rat pituitary tumors was examined.

Fifty proliferative lesions were classified into 4 categories by conventional immunohistochemistry, based on the hormones produced, as 1) Prolactin (PRL) positive, 2) LH positive, 3) PRL/LH double positive, and 4) Null cell type (negative for PRL, LH, GH, TSH and ACTH. Pit-1 was positive for both PRL positive lesions and a PRL positive area in the PRL/ LH double positive lesions. On the other hand, all LH positive lesions/areas and the null cell tumors were negative for Pit-1. Taken together the fact that steroidogenic factor-1 (SF-1), which is other transcriptional factor for the gonadotroph, was positive in not only the LH positive lesions/area but also in the null cell tumors (Yasuno et.al., 26th JSTP meeting, 2010), the null cell tumors in this study was considered to be committed to the gonadotroph cell lineage. In toxicologic pathology, there have been few reports that focused on transcriptional factors for the anterior hormones in pituitary tumors. The present study suggests that immunohistochemical investigation of transcriptional factors including Pit-1 and SF-1 would be useful for the precise classification of pituitary tumors in rats.

P-60

Immunohistochemical analysis of rat central nervous system tumors induced by *N*-ethyl-*N*-nitrosourea

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Although central nervous system (CNS) tumor occurs less frequently in human, high-grade tumor has poor prognosis. Immunohistochemical analysis has been used for histological classification, especially MIB-1 antibody to the Ki-67 antigen has been considered as a good marker for grading of the glial cell tumors.

In the experimental animal, *N*-ethyl-*N*-nitrosourea (ENU)-induced CNS tumor in rat is known as a useful model. However, detailed analysis for grading the rat CNS tumor has not been examined.

In this study, we administered ENU (20mg / kg b.w.) to pregnant F344 rats on the 17th day of gestational period. Then we sacrificed offspring rats at 35 weeks of age and evaluated cell density, mitotic figure, vascular endothelial proliferation figure and necrotic focus of the 66 ENU-induced rat CNS tumor on hematoxylin-eosin stained section. We also performed immunohistochemistry using GFAP (glial fibrillary acidic protein), S-100, NFP (neurofilament protein) and Ki-67 antibodies.

The glioblastoma classified as grade IV exhibited severe cellular atypism and necrotic focus. The rat malignant astrocytoma classified as grade III exhibited severe cellular atypism without necrosis. The astrocytoma classified as grade II exhibited cellular atypism weakly. These are some similarity, with human tumor. The percentage of Ki67 positive cells in glioblastoma was $8.10 \pm 1.79\%$, malignant astrocytoma was $9.28 \pm 2.24\%$ and astrocytoma was $3.50 \pm 1.28\%$. The value of glioblastoma and malignant astrocytoma was significant higher than that of astrocytoma ($p < 0.05$). The density of the vascular endothelial proliferation in the tumor did not affect on the Ki67 induces. All tumors exhibited positive-staining for both GFAP and S-100, and negative-staining for NFP.

In conclusion, it was suggested that the immunostaining of Ki67 was a valuable tool for the classification of rat astrocytoma.

P-61

Oxidative Stress Induced a Disturbance in Cell Polarity

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Polarized hepatocytes contain the intercellular junction, tight junction (TJ), that is one of the most important machinery for sealing the bile canalicular lumen from the sinusoidal space. Excessive productions of free radicals and oxidative stress are implicated in the pathogenesis of several diseases, including hepatitis, cirrhosis and also hepatocellular carcinoma. The molecular mechanisms of oxidative stress-induced hepatotoxicity and aberrations of hepatocytes polarity are still unknown. In normal rat liver, cell polarity proteins, Par3 and aPKC has co-localized with ZO-1, TJs constituted protein. The treatment of carbon tetrachloride (CCl₄), which strongly promotes lipid peroxidation in the liver, resulted in the disassembly of TJ and also changes the localization of Par3 and aPKC from TJs to cytosol. Golgi apparatus has an important role for the maintenance of cell polarity through the membrane traffic function. The Golgi marker GM130 is asymmetrically distributed above the apical side of the nuclei in normal liver. Asymmetric localization of GM130 has been disappeared and the disturbance of apico-basal cell polarity was observed in CCl₄-treated hepatocytes. Importantly, the immuno-precipitated analysis revealed that the Par3-aPKC interaction was inhibited by CCl₄ treatment. Furthermore, phosphorylation level of aPKC Thr410/403 increased and its regulating kinase, PI3-kinase signaling is activated. These results suggested that oxidative stress inhibit the interaction between Par3 and aPKC and consequently the hepatocyte polarity is disrupted.

P-62

Implication of The Results of Tissue Cross Reactivity Study in Therapeutic Antibody Development

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In order to reach the consensus for ICH S6 addendum which address about the pre-clinical development of biologics including therapeutic antibody, issues regarding the tissue cross reactivity (TCR) studies using normal human and preclinical animal model are still in active discussion.

In our development process for humanized anti-IL-6 receptor monoclonal antibody (ACTEMRA®), we adjusted the test system of the TCR study to the characteristics of the antibody, and reported several of these data 1,2).

In current report, we integrate these data, and discuss about the following points which need to be consider to evaluate the outcome of the TCR studies.

1)If the therapeutic antibody under development were not suitable for immunohistochemistry (IHC), it is possible to synthesize chemically leveled antibody for IHC detection. However, it is also important to know the effect of leveling procedure to the physicochemical characteristics of antibody such as affinity against target antigen.

2)If sensitive antibody for IHC are available other than the therapeutic antibody, it is possible to have the information regarding the target antigen distribution in wide range of normal tissues.

3)The risk assessment which only based on the antigen distribution data may lead to both over- and under-estimation of possible human risk, but if other preclinical and clinical results are took into consideration, the data of antigen distribution may provide useful information for the risk assessment.

Based on these view point, we considered the data from TCR study may effectively contribute for the development of antibody therapeutics.

1 : A.Kato et al, Regul Toxicol Pharmacol. 2009 53:46-51. IL-6R distribution in normal human and cynomolgus monkey tissues.

2 : H.Takai et al, Acta histochemica. On-line (2010 May 21). The importance of characterization of FITC-labeled antibodies used in tissue cross-reactivity studies.

P-63

Histopathology, Hematology and Blood Chemistry on Pregnancy and Lactation in Rats

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It is necessary to have detailed knowledge of changes caused by pregnancy, delivery and lactation in the pathological examination of combined repeated dose and reproductive/developmental toxicity screening tests (Reprotox study). However, there are few reports describing them. Hence, histopathological examination, hematology and blood chemistry measurements were performed in total 65 maternal rats on gestation day 17 (GD17), 20 (GD20), lactation day 1 (LD1) and 4 (LD4). Ten non-mated rats were used as age-matched controls for LD4. In addition, a retrospective examination using the specimens and data from 29 Reprotox studies conducted in our company was performed. In the histopathological examination, characteristic features as to the stage were observed in the ovary, uterus, and vagina at the time of the gestation, delivery and lactation. Moreover, extramedullary hematopoiesis in the spleen and increased erythrocytic hematopoietic cells in the bone marrow were observed in all stages accompanied by mild anemia (low red blood cell count, hemoglobin concentration and hematocrit). These changes were severe right after delivery (LD1). Increased lipid droplets in cortical cells of the adrenal glomerular zone were observed on all stages. These changes may be related with low total protein and albumin and unbalanced electrolyte. In the retrospective study, degeneration/necrosis of the renal proximal tubular epithelium and mucosal epithelium of the glandular stomach were observed in a few animals (respectively 25/136, 5/136). In addition, decreased lymphocyte proportion and increased neutrophil proportion, extended prothrombin time and activated partial thromboplastin time, increased platelet count and calcium (all stages) and high ALP value (GD17 and LD4) were observed; however, no related histological changes were observed. In conclusion, gestation, delivery and lactation caused variable effects to maternal body, and variable parameters and organs were changed.

P-64

Possible Involvement of Lysophosphatidic Acid Receptor-5 Gene in the Acquisition of Growth Advantage of Rat Tumor Cells

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Aberrant expressions of lysophosphatidic acid (LPA) receptor genes have been reported in tumor cells. In the present study, we measured the expression levels of Lpa5 gene and its DNA methylation status in rat tumor cells, and investigated cell growth effects of LPA in the Lpa5 expressed cells. Real time reverse transcription (RT)-polymerase chain reaction (PCR) analysis revealed that increased expressions of the Lpa5 gene were detected in rat liver-derived hepatoma RH7777 and lung-derived adenocarcinoma RLCNR cells but no expressions in normal liver and lung tissues. For the analysis of DNA methylation status, bisulfite sequencing was performed with RH7777 and RLCNR cells, comparing with other tumor cells and normal tissues of lung and liver. Lpa5 gene in Lpa5 unexpressed cells and normal tissues were highly methylated in the 5' upstream region. In contrast, Lpa5 gene in RH7777 and RLCNR cells was unmethylated, correlating with increased expressions of Lpa5. In the assays for cell growth effects of LPA, LPA enhanced cell proliferation and motility in RH7777 and RLCNR cells. LPA also stimulated cell invasion in RLCNR, but not in RH7777 cells. In rat liver and lung tumors induced by nitroso-compounds, 4 out of 6 hepatocellular carcinomas (HCCs) and 5 out of 6 lung adenocarcinomas indicated increased expressions of the Lpa5 with unmethylated status. These results suggest that increased Lpa5 expressions due to aberrant DNA methylation may involve in the acquisition of growth advantage of rat tumor cells.

P-65

Involvement of Constitutive Androstane Receptor in the Chemical-inducible Hepatocarcinogenesis in Mice

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[Introduction] Constitutive Androstane Receptor (CAR) is an orphan nuclear receptor playing an essential role for induction of liver hypertrophy and carcinogenesis in rodents after continuous administration of phenobarbital (PB). However, involvements of CAR in liver hypertrophy and hepatocarcinogenesis induced by other CYP2B-inducers with hepatocarcinogenic potential remains undetermined in rodents. In the 26th Annual Meeting of JSTP, we have reported that piperonyl butoxide (PBO, pesticide synergist) and (DBDE, polybrominated flame retardant), which are CYP2B-inducers and non-genotoxic hepatocarcinogens, induced liver hypertrophy via CAR-independent and CAR-mediated pathway, respectively. In this study, we investigated the involvement of CAR in liver tumor development processes of PBO and DBDE using 2-stage hepatocarcinogenesis model with CAR knock-out mice.

[Materials and Methods] 5-week-old male CAR+/+ (wild, C3H strain) and CAR-/- (KO) mice initiated by diethylnitrosamine (90 mg/kg, intraperitoneally) were treated with PBO, DBDE and PB for 27 weeks at doses of 5000, 50000 and 500 ppm in diet, respectively.

[Results and Discussion] PBO and PB treatments induced liver proliferative lesions (foci of hepatocellular alteration and adenomas), mainly eosinophilic type, in wild mice whereas these lesions in both groups were drastically reduced in CARKO mice. On the other hand, DBDE induced mainly basophilic liver proliferative lesions in both of wild and CARKO mice. These results clearly indicate that hepatocarcinogenesis process of PBO as well as PB is CAR-dependent, and that hepatocarcinogenesis process of DBDE is CAR-independent. There might be different pathways between CAR-mediated hepatocarcinogenesis and liver hypertrophy in mice.

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Mechanisms of Hepatocarcinogenesis through Constitutive Androstane Receptor (CAR) in Mice: Expression of Cell Proliferative-Related Factors in the Preneoplastic and Neoplastic Lesions in the Liver.

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In our previous study using wild C3H and CAR knock out (KO) mice, CAR might be involved in eosinophilic altered liver foci and adenomas (eosinophilic lesions) induced by phenobarbital (PB) and piperonyl butoxide (PBO), while an other mechanism might be related to the basophilic foci and adenomas (basophilic lesions) induced by decabromodiphenyl ether (DBDE). The present study was investigated what cell proliferative-related factors were involved in the mechanisms of CAR-dependent hepatocarcinogenesis in mice. 6-week-old male C3H and CARKO mice were fed diet containing 5,000 ppm PBO, 50,000 ppm DBDE or 500 ppm PB for 13 or 27 weeks after intraperitoneal injection of 90mg/kg diethylnitrosamine. Immunohistochemistry and real-time RT-PCR for cyclin D1, c-Myc, TGF β receptors (TGF β R) 1 and 2 and phosphorylated (p-)Smad2/3 (or Smad3 mRNA) were performed using liver tissues. At week 13, eosinophilic foci were induced by PBO or PB. Basophilic foci were induced in all groups including controls in wild mice. These lesions decreased or were not observed in CARKO mice. At 13 week, percentages of proliferative cell nuclear antigen-positive hepatocytes in the non-proliferative area did not increase in any groups. Immunohistochemically, cyclin D1 was positive in the cytoplasm and some of nucleus only in the eosinophilic foci at week 13. At 27 week, number of cyclin D1-positive nucleus increased in the eosinophilic lesions, while it was positive mainly in the cytoplasm in the basophilic lesions. At 13 week, c-Myc and TGF β R were also strongly positive only in the eosinophilic foci. On the other hands, these factors were negative or slightly positive in the basophilic foci. The c-Myc-positive level was much stronger in the eosinophilic lesions than in the basophilic ones at 27 week. TGF β R and p-Smad2/3 were positive in both eosinophilic and basophilic lesions. The levels of TGF β R were same in both types of lesions but that of p-Smad2/3 was slightly strong in the basophilic lesions. Remarkable changes of mRNA expression for *Cyclin D1*, *c-Myc*, *Tgfr1* and 2, and *Smad3* in the whole liver were not detected in any groups at week 13. These data suggest that cyclin D1 and c-Myc, down-stream factors of CAR, might be involved in the development process of eosinophilic lesions. In addition, CAR might be related to the expression of TGF β R in the early process of CAR-dependent hepatocarcinogenesis.

P-67

Molecular Pathological Analysis on Liver Tumor Promotion Mechanism of Omeprazole in Rats

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Omeprazole (OPZ), a CYP1A inducer, is known to cause preneoplastic lesion in the liver of rats in a two-year carcinogenicity study, but its mechanism is not clear. CYP1A induces, such as β -naphthoflavone and piperonyl butoxide, have been reported to have liver tumor promoting effects in rats, and oxidative stress resulting from the production of reactive oxygen species (ROS) is involved in their promoting effects. In order to clarify the OPZ's promotion mechanism, male F344 rats were subjected to a two-third partial hepatectomy, and treated with 276 mg/kg OPZ by oral gavage once a day for six weeks after an i.p. injection of N-diethylnitrosamine (DEN). In the DEN+OPZ group, the body weight increase was suppressed from 3 weeks after the start of experiment, and liver weights significantly increased. In addition, the number and area of glutathione S-transferase placental form (GST-P)-positive foci significantly increased in the DEN+OPZ group. Proliferating cell nuclear antigen (PCNA)-positive cells also significantly increased in the DEN+OPZ group. Real-time RT-PCR showed that the expression of Ahr gene battery such as Cyp1a1, Cyp1a2, Ugt1a6 and Ald1a1, and NF-E2-related factor 2 gene battery such as Ahr, Gpx2 and Yc2 significantly up-regulated in the DEN+OPZ group. TBARS also increased in the DEN+OPZ group. However, the production of ROS and 8OHdG was not altered by the treatment of OPZ. On the contrary, genes related to cell proliferation such as Myc, Raf-1 and Map2k2 were up-regulated in the DEN+OPZ group. These results suggest that the enhancement of cell proliferation related genes that is not related to oxidative stress is involved in the liver tumor-promotion of OPZ.

P-68

Modifying Effects of *N*-Acetyl-*L*-Cysteine (NAC) on Indole-3-Carbinol (I3C)-Induced Liver Tumor Promotion in Rats

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We previously reported that administration of I3C, an alkaloid in cruciferous vegetables, in rats enhanced tumor-promoting activity resulting from the oxidative stress such as oxidative DNA and lipid peroxidation due to reactive oxygen species (ROS) generated. Also the mRNA in phase I and II drug metabolic enzymes were upregulated by the I3C feeding. To clarify whether oxidative stress is involved in the liver tumor promoting effect of I3C, modifying effects of the antioxidant agent NAC, a precursor of glutathione, on I3C-induced liver tumor promotion were investigated in rats. Male rats were administered a single intraperitoneal injection of *N*-diethylnitrosamine (DEN) and were fed a diet containing 5,000 ppm of I3C for 8 weeks from 2 weeks with or without 3,000 ppm of NAC in the drinking water after DEN initiation. One week after the commencement of the administration of I3C, all rats were subjected to two-thirds partial hepatectomy.

The body weight gains of the DEN-I3C and DEN-I3C-NAC groups were significantly decreased compared to the DEN-alone group, but there were no significant changes between the DEN-I3C and DEN-I3C-NAC groups in body weight gains and food consumptions. Immunohistochemistry experiments of glutathione *S*-transferase placental form (GST-P) showed that the numbers and areas of the GST-P positive cells promoted by the I3C were significantly suppressed by the combination of the NAC administration. Quantitative real-time RT-PCR analysis showed that the mRNA of phase II enzymes such as *Nqo1*, *Gpx2* and *Ugt1a6* belonging to the Nrf2-gene batteries were down-regulated in the DEN-I3C-NAC group compared to the DEN-I3C group. On the other hand, *Cyp1a1* was not suppressed in the DEN-I3C-NAC group compared to the DEN-I3C group. There was no marked difference in the production of microsomal ROS and 8-OHdG, an oxidative DNA marker, between the DEN-I3C-NAC and DEN-I3C groups. Furthermore, real-time RT-PCR analysis showed that the expression levels of *Igf1*, *Fgf21* and *Mapkapk3* decreased by the NAC treatment. These results may suggest that coadministration of NAC suppresses the hepatocellular tumor-promoting activity of I3C in rats through not only the decrease of phase II enzymes but also the alterations of the signal cascade such as MAPK signaling.

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Promotion Effects of 2-amino-3-methylimidazo[4,5-f]quinoline in A Two-stage Hepatocarcinogenesis Model of B6C3F1 Mice

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2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is one of the genotoxic and carcinogenic heterocyclic amines (HCAs) produced during the cooking of meat and fish. This study was designed to study effect of IQ in a two-stage hepatocarcinogenesis model of B6C3F1 mice. 6-week-old male mice were subjected to two-thirds partial hepatectomy, followed by *N*-diethylnitrosamine (DEN) intraperitoneal injection and given a diet containing of 0, 30, 100 or 300 ppm IQ for 8 or 40 weeks. At 8 weeks, no obvious pathologic lesion was observed in all groups, although immunohistochemistry study of ki-67 revealed that proliferation of liver cells was significantly increased in DEN+300 ppm IQ group compared with DEN alone group. No cytokeratin (CK) 8/18-positive foci, which is a biomarker of mouse liver preneoplastic lesions, was detected in livers of all groups at 8 weeks. At 40 weeks, incidence of hepatocellular carcinoma (HCC) and CK8/18-positive HCC were significantly increased in DEN+100 ppm IQ and DEN+300 ppm IQ groups compared with DEN alone group. These results demonstrated that IQ exerts promotion effects at week 40 in this two-stage hepatocarcinogenesis model.

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Establishment of diethylnitrosamine-induced hepatocarcinogenesis model in postweaning C57BL/6 mice

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Introduction: The C57BL/6 strain is used as a background for many transgenic mouse models. Because of its low susceptibility to hepatocarcinogenesis, however, few studies have investigated the relationship between applied dosage of Diethylnitrosamine (DEN), a genotoxic carcinogen commonly used to initiate hepatic lesions in rodents, and preneoplastic/neoplastic lesions in the C57BL/6 strain. **Methods:** We treated C57BL/6 mice with 25, 50 and 75 mg/kg of DEN for 4 or 8 weeks by i.p. injection in C57BL/6 mice to investigate the formation of preneoplastic and neoplastic lesions in the liver at the end of 33 week duration. Histopathologic lesion type was assessed using H and E staining as well as cytokeratin 8/18 (CK8/18) and Cytokeratin 19 (CK19). **Results:** DEN induced preneoplastic lesions and cytokeratin8/18 positive foci in a dose dependent manner. In the 75 mg/kg for 8 weeks treatment group, hepatocellular adenoma, cholangioma and hemangioma and cytokeratin 19 positive foci were also induced, but significant decrease in body weight was observed. **Discussion:** We demonstrated that DEN induced preneoplastic lesions in dose dependent manner. In addition, immunohistochemically stained CK8/18 and CK19-positive foci positive foci were considered as a good marker for evaluation of hepatocarcinogenesis. We conclude that suitable DEN treatments range from 75mg/kg for 4 weeks (total = 300 mg/kg) to 50mg/kg for 8 weeks (total = 400 mg/kg). These results should prove useful for future studies investigating hepatocarcinogenesis in both the background C57BL/6 strain and other transgenic mouse models derived from it.

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Identification of New Potential Biomarker Molecules in Mice Hepatocarcinogenesis

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To identify the novel biological markers of mouse liver preneoplastic lesions, protein lysates from microdissected hepatocellular carcinomas (HCCs) in the liver of C57BL/6J and B6C3F1 mice treated with diethylnitrosamine DEN (10 mg/kg) were analysed by QSTAR Elite LC-MS/MS. From microdissected samples, 109 (C57BL/6J) and 61 (B6C3F1) proteins were identified with 66% confidence or higher and quantified with ProteinPilot 2.0 Software. Significant overexpression of cytokeratin (CK)18, apolipoprotein A-1 (APOA1), calreticulin (CALR), cingulin etc. and down-regulation of enzymes of urea cycle were detected in HCCs of those mice strains. Furthermore, overexpression of CK8/18 complex, prohibitin 1 (PHB1), prohibitin 2 (PHB2), septin 9 (SEPT9) and CALR were immunohistochemically confirmed in mice liver preneoplastic lesions and tumors. Our data imply that CK8/18, APOA1, CALR, PHB1, PHB2 and SEPT9 might become important novel protein biomarkers of mouse liver preneoplastic lesions developing into tumors.

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Possible involvement of genotoxic mechanisms in estragole-induced carcinogenesis in the mouse liver.

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【PURPOSE】Estragole (ES), a natural constituent of several herbs, has been shown to be hepatocarcinogenicity in mice. Although ES was not mutagenic in the Ames test, ES-specific DNA adducts were detected in mouse liver. Thus, ES-genotoxicity remains undetermined, which makes ES-induced hepatocarcinogenesis unclear. In the present study, to clarify the modes of action underlying ES-hepatocarcinogenicity, *in vivo* mutagenicity and quantitative analyses of the ES-specific DNA adducts have been performed using *gpt* delta miceth. 【METHOD】Male and female B6C3F1 *gpt* delta mice (6-week-old) were given ES (37.5, 75, 150 and 300 mg/kg b.w.) by gavage 5 days per week for 13 weeks. The highest dose in females was adjusted to 250 mg/kg b.w. from week 2 because one 300 mg/kg b.w. female died during week 1. At necropsy, bone marrows were sampled from the femurs for micronucleus (MN) test and the livers were sampled for *gpt* and *red/gam* (Spi⁻) reporter gene assay and quantitative analysis of the ES-specific DNA adduct by LC-MS/MS. 【RESULT】The liver weights were significantly increased at doses of 75 mg/kg b.w. and above in males, and at the highest dose in females. No significant differences between groups in both sexes were observed in MN test. The mutant frequencies (MFs) of *gpt* gene at the highest dose in males and from doses of 75 mg/kg b.w. in females were significantly higher than the relevant control values. In *gpt* mutation spectra at the highest doses in both sexes, GC:TA transversion was predominant in males and GC:TA and GC:CG transversions and GC:AT transition were predominant in females. The MF of Spi⁻ at the highest dose in females was significantly higher than the control value. ES-specific DNA adducts, ES-3'-8-dG, 3'-N²-dG and 3'-N⁶-dA, were detected dose-dependently in the all ES-treated groups.

【DISCUSSION】The present study clearly showed that ES has the *in vivo* mutagenicity in the liver. Since the amount of ES-specific DNA adducts were dose-dependently increased in both sexes, it was plausible that ES-specific DNA adduct formation may be responsible for the *in vivo* mutagenicity.

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Ethanol Promotes Diethylnitrosamine-induced

Hepatocarcinogenesis in Rats

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We previously established Cx32 dominant negative transgenic rats (Tg), which have much decreased formations for gap junctional intercellular communication (GJIC) in the liver, and are susceptible to diethylnitrosamine (DEN) induced hepatocarcinogenesis compared to littermate wild-type rats.

In the present study, to assess the influence of ethanol (EtOH) induced liver damage prior to carcinogen exposure with or without GJIC, 7 wk-old male Tg and littermate wild-type rats were treated with EtOH (3.75 mg/kg/day, i.g.), or water for 2 wks. DEN (50 ppm, drinking water) was then administered for 12 wks.

EtOH induced expression of CYP2E1, which is a central enzyme in a microsomal ethanol oxidizing system (MEOS), in the liver, however histological change was not clear in both Tg and wild rats.

In wild rat, EtOH pretreatment did not affect formation of DEN-induced GST-P positive area. On the other hand, EtOH increased induction of preneoplastic foci and adenomas in Tg rats. These results suggest that pretreatment of EtOH enhances DEN-induced hepatocarcinogenesis without existence of hepatitis, especially under decline of Cx32 related GJIC function.

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Preexistence of acetaminophen hepatic damage promotes diethylnitrosamine-induced hepatocarcinogenesis in rats

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Connexin 32 (Cx32) is a major gap junction protein in the liver. We previously established Cx32 dominant negative transgenic rats (Tg), which have much decreased capacity for gap junctional intercellular communication (GJIC), and are susceptible to diethylnitrosamine (DEN)-induced hepatocarcinogenesis compared to littermate wild-type rats (wild).

On the other hand, Tg rats are less sensitive to hepatotoxic agents containing acetaminophen (APAP), which is commonly used as antipyretic and analgesic agent.

In the present study, designed to assess the influence of APAP-induced liver damage prior to carcinogen exposure with or without GJIC, 7 wk-old male Tg and littermate wild rats were given a hepatotoxic dose of APAP (500 mg/kg, i.g.), or the vehicle alone, 10 times over 5 wks. DEN (50 ppm, drinking water) was then administered for 6 or 12 wks.

Centrilobular cell damage was observed in APAP-treated 12 wk-old Tg and wild animals, the hepatotoxicity appearing stronger in the wild case. Formation of GST-P positive area was accelerated by APAP pretreatment in both Tg and wild rats at both 6 and 12 wks, but this effect was more prevalent in Tg animals. The results suggest preexistence of hepatic damage enhances DEN-induced hepatocarcinogenesis.

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The Suppression of Metastasis and Invasion Ability of Rat Hepatoma Cells by Connexin 43-siRNA Transfection

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To reduce cancer mortality, understanding of mechanisms of cancer metastasis is very important. We have established 6 rat hepatoma cell lines, which exerted different metastatic potential to the lung after inoculation into the tail vein of nude mice. Our former cDNA array analysis showed that the higher metastatic cell lines tended to express more connexin 43 (Cx43) than the lower metastatic cell lines and normal rat liver tissue.

In the present experiment, we analyzed the influence of interference of Cx43 in the higher metastatic cell line by siRNA transfection. Cx43-expression was suppressed by more than 50% by siRNA transfection. Thus, the cell lines with either Cx43-siRNA or control-siRNA and the original cell line without transfection were analyzed 24 or 48 hrs after transfection, as follows. There were no differences in cell proliferation activity analyzed by WST-1 assay. The *in vitro* migration and invasion ability, using Boyden chambers and fibronectin as a chemoattractant, was suppressed by Cx43-siRNA by 36% (P<0.05) and 68% (P<0.001) compared with control-siRNA transfected cell, respectively. Secretion of active MMP-9 in the culture supernatant was significantly (P<0.05) reduced by Cx43 siRNA comparing with control siRNA. Moreover, the number and area of metastatic nodules in the lung of nude mice measured on the histological specimen per unit area were reduced by 66% (P<0.01) and 68% (P<0.05), respectively, compared with control-siRNA transfected cell line. The labeling indices of Ki67 and cleaved caspase 3 in the metastatic cells tended to be decreased without significant difference.

In conclusion, the suppression of Cx43 expression in tumor cells reduced *in vitro* migration and invasion ability and *in vivo* metastatic ability. Cx43 might be one of the regulating factors of tumor cell attachment to the endothelial cells at metastasizing site and a possible molecular target for the suppression of cancer metastasis in Cx43 over expressing tumor.

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Investigation of Spontaneous Lesions in Connexin32-Deficient Mice

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Connexin32 (Cx32), one of the components of gap junction (GJ), is the predominant liver connexin and is also expressed in various organs and tissues. Cx32 has important roles in the maintenance of tissue homeostasis through cell-cell communication and the control of cell growth, differentiation and tumor formation. It is known that Cx32 protein decreases in neoplastic cells. In the present study, Cx32 knockout mice (Cx32KO) and wild-type mice were bred for 24 months, and the incidence of neoplastic and non-neoplastic lesions was examined in various organs and tissues.

Fifty males and 50 females of Cx32KO mice (male: Cx32^{Y/-}, female: Cx32^{-/-}) and 50 males and 50 females of wild-type mice (C57BL/6, male: Cx32^{Y/+}, female: Cx32^{+/+}) were bred for approximately 24 months. All animals were euthanized at 24 months of age under ether anesthesia, and necropsied and histopathological examination was performed.

Statistically significant increase in the hepatocellular carcinoma was observed in the males (wild type: 0/50, Cx32KO: 9/50, $p < 0.01$), and there was a tendency towards an increase in the hepatocellular adenoma (wild type: 0/50, Cx32KO: 4/50) in males, and hepatocellular adenoma (wild type: 1/50, Cx32KO: 5/50) and hepatocellular carcinoma (wild type: 0/50, Cx32KO: 2/50) in females. There was no apparent difference between Cx32KO mice and wild-type mice in the other organs. There was no increase in the incidence of spontaneous liver tumor in Cx32KO mice up to 18 months. However, the present study showed an increase in the incidence of spontaneous liver tumor in 24 months.

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Examination of *in vivo* mutagenicity and carcinogenicity in the *gpt* delta rat with Dammar resin

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Dammar resin is a food additive as a thickening agent. The purposes of this study are to evaluate carcinogenicity and *in vivo* mutagenicity of Dammar resin in *gpt* delta rats. 6 week-old male *gpt* delta rats were divided into 5 groups. Groups 1-3 were treated with five carcinogen (DMBDD) during the first 4 weeks of experiment as follows: diethylnitrosamine (100 mg/kg b.w. i.p.), N-methyl-N-nitrosourea (20mg/kg b.w. i.p.), 1,2-dimethylhydrazine (40 mg/kg b.w. s.c.), 0.05% N-butyl-N-(4-hydroxypropyl) nitrosamine in the drinking water from week 1 to 2, and 0.1% dihydroxybutyl-di-N-propylnitrosamine in the drinking water from week 3 to 4. Groups 4-5 were given vehicle. From experimental week 5, groups 1-3 were treated with Dammar resin in diet at doses of 0, 0.03, and 2% for 13 weeks. Groups 4-5 were treated with Dammar resin in diet at doses of 0, and 2%, respectively. At 18 weeks after starting the experiment, quantitative analysis of glutathione S-transferase placental form (GST-P) positive foci, which are preneoplastic lesions in the rat liver and mutation assays (*gpt* and *Spi*- mutation assays) were performed. Both numbers and area of GST-P positive foci were significantly increased in rats administered DMBDD → 2% Dammar resin (group 3) compared to rats administered DMBDD alone (group 1). 0.03% Dammar resin (group 2) had no effects on the development of GST-P positive foci. There were no significant differences in mutant frequencies of *gpt* and *red/gam* genes in the livers between rats administered basal diet (group 4) and 2% Dammar resin (group 5). These findings indicated that Dammar resin exerts promotion effect on liver carcinogenesis but lack of *in vivo* mutagenicity in the livers of *gpt* delta rats.

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Combined Effects of Food-Derived CYP1A2 Inducers on In Vivo Mutagenicity of Estragole

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Estragole (ES), a natural constituent of several herbs, has been used as a food additive for flavor. ES is metabolized to the active form by CYP1A2 and sulfotransferase to generate ES-specific DNA adducts. β -Naphthoflavone (β -NF) and thiabendazole (TBZ) known as food-derived chemicals are inducers of CYP1A2. It has been reported that the combined exposure to β -NF and TBZ at the highest dose each which does not induce CYP1A2 was able to elevate mRNA levels of the enzyme. In the present study, to clarify the combined effects of the three food-derived compounds, F344 *gpt* delta rats were given ES at a dose of 200 mg/kg bw by gavage, β -NF at a concentration of 200 ppm in the diet and TBZ at a concentration of 100 ppm in the diet for 4 weeks. Five groups were provided as control (basal diet), ES alone, ES + β -NF, ES + TBZ, ES + β -NF + TBZ. At necropsy, the livers were extirpated and were stored at -80 °C until examination of *in vivo* mutagenicity. The body weights in the combined groups of ES and the CYP inducers were significantly lower than the control or ES alone group, although exposure to ES alone did not affect the body weight. There were no changes in food consumption among the groups. The relative liver weight in ES alone, ES + β -NF or ES + β -NF + TBZ group was significantly higher as compared with the control. The combined exposure to ES and the two CYP inducers inhibited the body weight gain and caused an increase of the relative liver weight. *In vivo* mutation assay will be performed to be presented.

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In vivo genotoxicity and oxidative DNA damage in comprehensive toxicity studies using F344 *gpt* delta rats treated with safrole

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Safrole is the major component of sassafras oil, spices and essential oil, which is known to have carcinogenicity in the rodent liver. Safrole-specific DNA adduct formation and oxidative DNA damage have been reported to be found. However, it is unclear whether genotoxic mechanisms have been involved in its hepatocarcinogenesis along with lack of sufficient information on the general toxicities. In the present study, to investigate *in vivo* genotoxicity of safrole together with the toxicological profiles, F344 *gpt* delta rats were given safrole in the diet at doses of 0, 0.1 or 0.5% for 13 weeks. There was marked suppression of body weight gain in safrole-treated groups from week 2 to the end of the experiment. Absolute liver weights of males in 0.5% group and of females in the treated groups, and relative liver weights of females in 0.5% group were significantly increased as compared with the relevant controls. Histopathologically, centrilobular hypertrophy of hepatocytes was observed in safrole-treated groups of both sexes. In organ weights, hematological and serum biochemical analyses, there were no changes with toxicological significances. The number and area of glutathione *S*-transferase placental form (GST-P) positive foci, proliferating cell nuclear antigen (PCNA)-positive ratio and 8-hydroxydeoxyguanosine (8-OHdG) levels in the liver were significantly increased in safrole-treated groups of both sexes as compared with the controls. The *gpt* mutant frequencies (MFs) in males of the 0.5% group were significantly elevated despite the Spi MFs being unchanged. Serious toxic changes were not observed in rats treated with safrole at the carcinogenic dose for 13 weeks. The fact that *gpt* MFs and 8-OHdG levels were elevated in the livers suggested possible involvements of genotoxic mechanisms including oxidative DNA damage in safrole-induced hepatocarcinogenesis. Further data on analyses for *gpt* mutant frequencies in the females will be presented to discuss the modes of action underlying safrole-hepatocarcinogenesis

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Chemopreventive Effects of Silymarin in *gpt* Delta Transgenic Rat.

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Silymarin is the milk thistle (*Silybum marianum*, Family Asteraceae) extract contained three different flavonolignans (silybin, silychrystin, silydianin). *In vivo* genotoxicity study using rodent model is useful to assess mutagenicity and carcinogenicity in target tissues. To evaluate the chemopreventive mechanisms of silymarin against colon cancer, we examined suppressive effects of silymarin against carcinogenicity and genotoxicity induced by 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS) in the colon of F344 *gpt* delta transgenic rats. Male *gpt* delta rats were given a single s.c. injection of 40 mg/kg DMH, and followed by 1.5% DSS in drinking water for a week. They were fed diets containing silymarin for 4 weeks, starting one week before DMH injection. Silymarin at doses of 100 and 500 ppm suppressed the tumor formation in a dose-dependent manner and the reduction was statistically significant. In the mutation assays, DMH treatment enhanced the *gpt* mutant frequency (MF) in the colon about 120-fold over the value of untreated rats. Silymarin reduced the induced MFs by 20 %. To further characterize the suppressive effects, we conducted bacterial mutation assay with *Salmonella typhimurium* YG7108, a sensitive strain to alkylating agents, to examine whether silymarin inhibits genotoxicity of DMH. Silymarin reduced the genotoxicity of DMH by more than 80%. These results suggest that silymarin is chemopreventive against colon cancer induced by DMH plus DSS and also that the chemopreventive efficacy may be, at least in part, due to inhibition of genotoxicity induced by DMH.

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Development of colonic crypts from polyclonal to monoclonal: Analysis in chimeric mice

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The cancer-initiating cells or cancer stem cells played important roles for maintaining cancer population. In the normal counterpart, colonic stem cells are believed to reside bottom region of crypt throughout the normal mucosa and to be essential for the maintenance of the colonic epithelium. Thus, it should be important to reveal how colonic crypts would shape their unique form during development. To address this question, aggregation chimera mouse were produced using two strains, C57BL/6J Green mouse and C3H, and early developmental stage were analyzed for the crypt clonality. In results, in the neonatal period, quite a few crypts contained epithelial cells derived from both strains around the crypt patch borders, which indicated polyclonality of crypts at this stage of morphogenesis. Polyclonal crypts decreased exponentially and colonic crypts in adult stage around 56 days old became monoclonal in a crypt level and form patches consisted of several crypts from same strain. The results indicated that colonic crypts would obtain their monoclonality during several weeks after birth.

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Effects by baicalin on AOM/DSS-induced mouse colon carcinogenesis model.

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Huang Qin is used in traditional Chinese medicine, extracted from the dried roots of Baikal Skullcap (*Scutellaria baicalensis*). This plant, which contains a lot of chemical compounds, is distributed in the northeastern region of China. Huang Qin is known to have anti-inflammatory, anti-allergic, antimicrobial, antipyretic, sedative, diuretic, anti-hypertensive, anti-metabolic and anti-tumor effects. In this study, we examined the modifying effects of baicalin, a main compound of Huang Qin, in AOM/DSS-induced mouse colon carcinogenesis model, because it inhibited cell growth on colon cancer cultured cell lines in vitro.

Mice were divided into seven groups of 20 to 25 animals each, and fed CE-2 basal diet (groups 1 and 7) or 500 ppm of baicalin with diet (groups 2, 3, 5 & 6) and 100 ppm of bicalin with diet (group 4). Group 1, 4, 5, 6 and 7 were fed same diet till the end of experiment. Groups 2 and 3 were fed bicalin only for first 5 weeks and from two weeks after a cease of DSS treatment to the end, respectively. Colons in each group were dissected on 14 weeks after AOM injection. It was also observed and measured a body weight, kidney weight and liver weight and colonic lesions including neoplasms. It was examined the incidence and multiplicity of the colon tumors in each group, and survivin, one of inhibitors apoptosis proteins, by immunohistochemically, because the inhibition of the expression of survivin in previous in vitro assay with baicalin.

There were no significant differences on the tumor incidences among groups in spite of baicalin treatment. However, the positive cells in crypts reacted with anti-survivin antibody reduced in groups 4 and 5, treated with whole experimental duration (0.58 % and 0.59 %, respectively vs. 11.38 % in group 1). In addition, survivin positive cells of tumors in group 5 (22.0%) reduced to be compared with group 1 (40.3%).

It suggested that baicalin might be related to the inhibitory potential on survivin expression and inhibitory modefying effects on proliferative reaction and apoptosis in colon carcinogen-induced in vivo assay. Survivin protein is thought to inhibit caspase 3 and 7, and inhibiting activation of apoptosis. It is necessary to further study the effects of chemopreventive ingredients to either cancer cell or normal cell proliferation.

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Colorectal Tumors Induced by Benzo[a]pyrene and Dextran Sulfate Sodium in Mice

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[Introduction]

We previously reported the discrepancy between the frequency of mutations and carcinogenicity following benzo[a]pyrene (BP) treatment in transgenic mice (MutaMouse). BP induced tumors in target organs including the forestomach, lung and spleen; however, no tumors were developed in colon even though it showed the highest incidence of the mutation. In the present study, we examined the development of colon cancers by BP in a colon-colitis mouse model using dextran sulfate sodium (DSS), which was known to promote AOM-induced colon cancer in mice.

[Material and methods]

CD2F₁ male mice (7-week old, 6-12 mice/group) were given either BP (125 mg/kg/day, for 5 days, p.o., BP/DSS group), AOM (10 mg/kg, single, i.p., AOM/DSS group) or no treatment (DSS group), followed by 2 cycles of DSS (4 %, 7 days, in drinking water) with a 2-week off-dose period. At 14 and 17 weeks after the start of experiment, the mice were subjected for pathological examination of the colon and rectum.

[Result and discussion]

In the BP/DSS group, multiple tumors including adenomas and adenocarcinomas were observed in all mice at 14 and 17 weeks. There was also a similar extent of tumor development in all AOM/DSS group mice at 14 and 17 weeks. In contrast, tumor development in the DSS alone group was limited to solitary colorectal tumors (adenoma, adenocarcinoma) in only 3 of 14 animals. These findings indicate that BP/DSS treatment induces tumors in the colon, even though BP alone is not carcinogenic to the colon. This finding further suggests a possible involvement of not only DNA damage but also epigenetic factors including inflammation in the colorectal carcinogenic process by BP/DSS.

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Time-Course Changes in a Murine Colon Carcinogenesis Model Induced by Benzo[a]pyrene and Dextran Sulfate Sodium

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[Introduction]

We previously reported the benzo[a]pyrene (BP) and dextran sulfate sodium (DSS)-induced colon carcinogenesis model, where BP/DSS treatment induced tumors in colon, even though BP alone induces mutations and yet not carcinogenic to the colon. The present experiment investigated the time-course changes in BP/DSS-induced colon carcinogenesis in CD2F₁ mice.

[Materials and Methods]

CD2F₁ mice were orally administered with BP at 125 mg/kg/day for 5 days, followed by 1 or 2 cycles (with an interval of 2 weeks) of 4% DSS in drinking water for 1 week from 10 days after the last BP treatment. In addition, negative control (no treatment), DSS alone, and BP alone groups were set. They were sequentially sacrificed at weeks 4, 7, 9 and 11 for histopathology of the colon and rectum.

[Results and Discussion]

In the BP/DSS treated group, tumors were observed in all animals at as early as 4 weeks. At 4 weeks there were animals with either adenoma, adenocarcinoma, or both (adenoma: 5.9/animal, adenocarcinoma: 1.5/animal), but at 7 week all animals developed adenocarcinoma (adenoma: 3.2/animal, adenocarcinoma: 16.0/animal). In contrast, tumor development was limited to few mice in the DSS alone group at Week 7 or later, and no proliferative lesion developed in mice exposed to BP alone, or mice in the negative control group. These results indicate that BP/DSS-induced colorectal adenocarcinoma developed within a short-time and also with a high incidence, and thus will be useful for investigating colorectal carcinogenesis.

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MicroRNA Changes Induced by Heterocyclic Amines and its Significance in the Early Stages of Colon Carcinogenesis

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Among various effects by PhIP, a carcinogenic heterocyclic amine (HCA) in the diet, our lab has been studying with regard to non-genotoxic aspects of HCAs; modulation of miRNA profile, for instance. Here we investigated whether or not miRNAs induced by HCAs could contribute to rat colon carcinogenesis. Specifically, we compared across the miRNA profiles in the colon epithelia of male F344 rats shortly after the 3-day treatment of a total of 6 HCAs (4 carcinogenic and 2 non-carcinogenic). Notably, carcinogenic and non-carcinogenic HCAs were successfully separated from each other by simple clustering analysis. Besides, a scoring system by weighting 5 miRNAs selected by discriminate analysis among differentially expressed miRNAs, was able to distinguish the two groups. It strongly suggests that a subset of miRNAs specifically induced by carcinogenic HCAs might play a role in rat carcinogenesis and that the system might be able to predict the carcinogenicity of yet uncharacterized HCAs in a short period of time. In vitro functional analysis of the 5 miRNAs is currently ongoing and hopefully the results are to be presented.

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Molecular Mechanisms of the Combination Treatment of Cetuximab and Dasatinib in Kras Mutant Colorectal Tumors

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Kras mutation is a predictive biomarker for resistance to cetuximab (Erbix[®]) in metastatic colorectal cancer (mCRC). This study sought to determine if Kras mutant CRC lines could be sensitized to cetuximab *in vivo* using the FDA approved Src family kinase (SFK) inhibitor, dasatinib (BMS-354825, sprycel[®]).

We analyzed 16 CRC lines for: 1) Kras mutation status, 2) dependence on mutant Kras signaling, 3) expression level of EGFR and SFKs. From these analyses, we selected three Kras mutant (LS180, LoVo, and HCT116) cell lines, and two Kras wild type cell lines (SW48 and CaCo2). *In vitro*, using PDL/laminin plates, Kras mutant cell lines were resistant to cetuximab whereas parental controls showed sensitive to cetuximab. Treatment with cetuximab and dasatinib showed a greater anti-proliferative effect on Kras mutant line as compared to either agent alone. To investigate a mechanism for this increased response in the combinatorial therapy we performed Human Phospho-kinase Antibody Array analysis (ARY003, R&D systems) measuring the relative phosphorylation levels of phosphorylation of 46 intracellular serine/threonine/tyrosine kinases in untreated, cetuximab, dasatinib or the combinatorial treatment in LS180, LoVo and HCT116 cells. The results of this experiment showed a compelling decrease in a broad spectrum of kinases when compared to the untreated or monotherapy treated controls.

To strengthen our *in vitro* findings we analyzed tumor growth delay with cetuximab, dasatinib or the combination *in vivo*. Kras mutant xenografts showed resistance to cetuximab therapy, whereas Kras wild type demonstrated an anti-tumor response when treated with cetuximab. Kras mutant tumors exhibited minimal response to dasatinib in monotherapy. However, *as in vitro*, Kras mutant lines exhibited a response to the combination of cetuximab and dasatinib as compared to controls. Combinatorial treatment of Kras mutant xenografts resulted in decreased cell proliferation as measured by Ki67 and higher rates of apoptosis as measured by TUNEL compared to controls.

The data presented herein indicate that dasatinib can sensitize Kras mutant CRC tumors to cetuximab and may do so by altering the activity of several key kinases. Further, these results suggest that signaling via the EGFR and SFKs may be necessary for cell proliferation and survival of Kras mutant CRC tumors. This data strengthen the rationale for clinical trials in this genetic setting combining cetuximab and dasatinib.

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Inhibitory Effects of Major Component of Spices on Chronic Gastritis in *Helicobacter pylori*-Infected Mongolian Gerbils

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Helicobacter pylori (*H. pylori*)-induced gastritis is known as an important risk factor for stomach cancer in humans. Although most Asian countries have high infection rate of *H. pylori*, there is a large difference in the incidence of gastric cancer, particularly between eastern Asia and other regions. In the present study, we examined inhibitory effects of major component of spices (turmeric, chili pepper, and black pepper), which are frequently-consumed in southern to south-eastern Asia, on *H. pylori*-induced chronic gastritis in Mongolian gerbils. All three compounds (curcumin, capsaicin, and piperine) inhibited *in vitro* proliferation of *H. pylori* in dose-dependent manner, and the suppressive effects of each component were significant at the dose of 100 μ M. Curcumin showed the highest inhibitory effect of all three components, and proliferation was also decreased at the dose of 10 μ M. To evaluate anti-inflammatory effects of the three components on *H. pylori*-associated gastritis, specific pathogen-free 6-week-old male Mongolian gerbils were intra-gastrically inoculated with *H. pylori*. Then, the animals were fed diet containing 5000 ppm curcumin, 100 ppm capsaicin, or 100 ppm piperine for 11 weeks, and sacrificed at experimental week 13. Capsaicin and piperine significantly alleviated infiltration of neutrophils and mononuclear cells in the gastric mucosa, while curcumin did not show any significant suppression of gastritis in contrast with the inhibitory effect on *in vitro* proliferation. In the pyloric mucosa, mRNA expression of inflammatory mediators including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , inducible nitric oxide synthase, IL-6, IL-10, KC (IL-8 homologue), and cyclooxygenase-2 was significantly reduced by piperine treatment. Similarly, capsaicin markedly decreased mRNA expression of TNF- α and KC in the antrum. These results suggest that capsaicin and piperine have suppressive effects on *H. pylori*-induced gastritis in Mongolian gerbils and anti-inflammatory effects may be more important for chemoprevention of *H. pylori*-associated gastric disorders than direct inhibition of bacterial proliferation.

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The Effect of *Raphanobrassica* for *Helicobacter Pylori*-induced Gastritis in Mongolian gerbils

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Helicobacter pylori infection is associated with chronic gastritis, as well as gastric cancer development. *Raphanobrassica*, an intergeneric hybrid of the genera *Raphanus* (radish) and *Brassica* (cabbages), includes Glucoraphanin which is the precursor of Sulforaphane exerting the antimicrobials, anti-inflammatory, antioxidative and antitumorigenic activity in the stomach. The purpose of the present study was to investigate the effect of *Raphanobrassica* on *H.pylori*-induced gastritis in Mongolian gerbils. Six-week-old male Mongolian gerbils were inoculated orally with *H. pylori* (ATCC 43504), fed diet containing the three kinds of freeze-dried *raphanobrassica* (2%): RB1; containing GR and GRe, RB2; containing GR, RB3; unanalyzed, after 2 weeks and sacrificed after 12 weeks. The grade of mononuclear infiltration in histopathological examination of gastritis, mRNA expression of IL-6, inflammatory mediator, and cell proliferation were significantly suppressed in RB1 treatment group. Oxidative DNA damage was suppressed in RB2 and RB3 treatment groups. In conclusion, these results indicate that *raphanobrassica* suppresses *H. pylori*-induced gastritis via the suppression of chronic gastritis and inflammation-associated gene in Mongolian gerbils, and might furthermore have a potential for prevention of gastric carcinogenesis due to the suppression of inflammatory, oxidative DNA damage and cell proliferation as the carcinogenesis-associated factors.

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Effects of Kuguacin J, Triterpenoid from *Momordica charantia* Leaf on Androgen-independent Human Prostate Cancer Cell Line, PC-3

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In this study, we examined the effects of kuguacin J (KuJ), one of the triterpenoids in that bitter melon (*Momordica charantia*) leaf extract (BMLE), against the androgen-independent human prostate cancer, PC-3, *in vitro*. KuJ treatment resulted in growth inhibition together with G-1 arrest in the cells. KuJ markedly decreased the levels of cyclins (D1 and E), cyclin-dependent kinases (Cdk2 and Cdk4) and proliferating cell nuclear antigen (PCNA). Moreover, treatment of KuJ with non-toxic dose significantly reduced PC-3 migration and invasion. Gelatin and plasminogen-casein zymography demonstrated that matrix metalloproteinase (MMP)-2, MMP-9 and urokinase-type plasminogen activator (uPA) secretions were significantly decreased by KuJ. These results suggest that KuJ exerts anti-invasion effects on PC-3 cells through the inhibitions of cancer cell motility and extracellular-matrix degradation enzyme secretion. Together, KuJ could be a candidate promising agent, which has potential against androgen-independent prostate cancer. **Acknowledgement:** This work was supported by grants from the Royal Golden Jubilee Ph.D. Program of Thailand and the Society for Promotion of Pathology of Nagoya, Japan.

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Chemopreventive Effect of Purple Corn Color and Purple Sweet Potato Color on Prostate Cancer Cell Lines

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Development of clinically manifested prostate cancer (PCa) usually requires an extremely long time. Consequently, PCa is an ideal target for chemoprevention. Purple corn and purple sweet potato have long histories as food products. Nowadays the purple colors extracted from these products are widely used as food colorants. Previous studies reported that purple corn color (PCC) and purple sweet potato color (PSPC) have anti-cancer effects on colon and breast cancer. This study is an initial investigation on their effects on PCa.

The PCa cell line LNCaP was treated with PCC and PSPC in vitro. After treatment, Guava Flow Cytometry was employed to count cell number, analyse cell cycle status and detect apoptosis; and Western blotting analysis was conducted to determine the expression of key proteins. Both PCC and PSPC dose-dependently inhibited the growth of LNCaP cells. PCC-treated cells were arrested in the G1 stage of the cell cycle, and PSPC arrested cells in G2/M. Neither apoptosis nor necrosis was induced by PCC or PSPC. In PCC-treated cells, expression of PSA and cyclin D1 decreased, suggesting that PCC treatment inhibited the growth of LNCaP cells by decreasing expression of cyclin D1. In PSPC-treated cells, AR expression increased slightly while CDC25c expression decreased, suggesting that PSPC treatment inhibited the growth of LNCaP cell by decreasing expression of CDC25c.

In our next study, the TRAP (Transgenic Rat for Adenocarcinoma of Prostate) model will be employed to study the anti-PCa effects of PCC and PSPC.

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Effects of HDAC Inhibitors on Prostate Cancer Proliferation and Differentiation.

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Histone acetylation plays an important role in chromatin remodeling and gene expression. Histone deacetylase inhibitor (HDAC inhibitor) promotes histone acetylation and regulates many cellular functions, including cell proliferation and differentiation. HDAC inhibitors have been reported to suppress growth of several solid malignancies including lymphoma. We examined the effect of HDAC inhibitor on cell proliferation and differentiation in human prostate cancer (PCa) cell lines, LNCaP (androgen-dependent) and PC3 (androgen-independent). Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA) and MS275 were employed as HDAC inhibitor.

In the results, cell proliferation of both LNCaP and PC3 were significantly reduced by treatments of TSA, SAHA and MS275 at a concentration of 0.1, 1 and 1 μ M, respectively. From this result, TSA was chosen for further study. In Western blot analysis, acetylated histone H3 protein was induced by 5 μ M TSA in both LNCaP and PC3 cells. High expression of keratin 8, as a marker of prostate luminal epithelium, was detected in PC3 cells after treatment with TSA. Morphologically, cytoplasmic enlargement was observed in PC3 but not LNCaP cells after treatment with TSA.

Thus, the present data demonstrated that HDAC inhibitor possesses cell growth suppression and differentiation potential for prostate cancer. Elucidation of mechanisms of HDAC underlying these effects is needed.

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Short Communication: Morphometrical Analysis on Pre-Noplastic Cellular Nuclei in BBN Induced Rat Bladder Carcinogenesis

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Many morphometric analysis systems have been developed to evaluate the prognosis of established carcinomas. Although the promotion stages in chemical induced carcinogenesis that are morphologically evaluated as epithelial hyperplasia with genomic instability, the cellular pleomorphism is difficult to distinguish by routine light microscopical observation. However, little image morphometric studies on cellular nuclei in promoting stage of carcinogenesis have been considered. Vascular endothelial growth factor (VEGF) has been observed in *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) induced rat bladder transitional cell carcinomas. We have previously described the up-regulation of VEGF in preneoplastic hyperplastic bladder epithelial cells of the promotion stage in BBN-induced rat bladder carcinogenesis (Vet Pathol 36: 111, 1999). We investigated the nuclear morphological features of VEGF-positive (V+) and -negative (V-) cells in hyperplastic lesions in BBN induced rat bladder carcinogenesis by the image morphometric nuclear analytic system. Sections were immunohistochemically stained by the anti-VEGF antibody and by the Foelugen method (Bacus Lab.Inc.IL), analyzed with the CAS200 Quantitative Cell Analysis System (Bacus,Lab.Inc.) in a minimum of 300 cells, and was determined by comparison with the control diploid cells of the normal rat lymphocytes. The measurements were transformed into QDA v3.0 Image List Mode file (Bacus,Lab.Inc.), and were analyzed by the Cell Sheet v2.0 software program (Bacus,Lab. Inc.). Moreover, nuclear images were transformed into Z-score scale in SD units. Details of the mathematical-method were previously described by Bacus JW (Bacus JW *et al.*, J Cell Biochem 28. 21, 1997). DNA ploidy analysis revealed the almost all V+ and V- cells showed diploid types. However, the nuclear grade Z-score of V+ cells was significantly higher than that of V-cells: the parameters on Area and Valley were significantly higher in V+ cells than V- cells, and the parameters on pg.DNA and Entropy were similar in both groups. General morphometric analysis revealed that the parameter of V+ cells showed significantly higher on Perimeter and lower on Elongation. General feature analysis revealed that the parameter of V+ cells showed significantly higher on Cfg.Run Length and Valley, and lower on Slope. The present study demonstrated the nuclear pleomorphism in BBN induced bladder hyperplasia was discriminated by cellular VEGF expression. This was supported by a research project grant awarded by the Azabu Univ. Res. Serv. Div.; and Grant-in-Aid (C) of The Minist.Ed.Cu.Sp.Tech.,Japan.

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Expression Analysis of Cell Cycle-related Molecules in Renal Tubules of Rats Treated for 28 Days with Karyomegaly-inducing Renal Carcinogens

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By exposure to renal carcinogens in experimental animals, bizarre nuclear enlargement, named as karyomegaly, often appears in the proximal tubular epithelial cells from the early stage. Recent studies have shown aberrant expression of cell cycle-related molecules in the karyomegalic cell area, indicating a chromosomal instability linked to carcinogenicity. To gain further insight into the molecular mechanism of karyomegaly, we performed immunohistochemical analyses of cell proliferation / cell cycle-related molecules in the kidneys of male F344 rats treated either with karyomegaly-inducing renal carcinogens, ferric nitrilotriacetate, ochratoxin A (OTA) or monuron, a karyomegaly-inducing non-renal carcinogen, *p*-nitrobenzoic acid, or a non-carcinogenic renal toxicant without inducing karyomegaly, acetaminophen, for 28 days at doses to induce karyomegaly or renal toxicity. Number of proximal tubular epithelial cells was quantitatively measured in the outer stripe of the outer medulla, a representative target site for carcinogenesis of most renal carcinogens. Ki-67-positive cells significantly increased in the renal carcinogen-treated animals as compared with the control animals. With regard to cell cycle-related molecules exerting function mainly at M phase, aurora B and phospho-histone H3 increased the number of positive cells in karyomegaly-inducing agents irrespective of carcinogenic potentials. HP1 α also showed a similar tendency, but without statistically significant difference. Separase and MKLP-1 did not show any specificity with karyomegaly-inducing agents. On the other hand, most carcinogens showed a tendency to increase the number of positive cells of topo II α , that functions during the late S and G2/M phases, as compared with the control group; however, OTA-alone significantly increased the positive cells. These results may suggest that renal carcinogens can be separated from other agents in terms of cell proliferation activity; however, there was no apparent relationship between the cell proliferation activity and cellular distribution of cell cycle-related molecules examined. On the other hand, positive responses of M phase-related molecules against karyomegaly-inducing agents irrespective of carcinogenic potentials suggest an outcome of the disruption of G2/M phase checkpoint. Because topo II α that functions from the late S phase showed an increase or increasing tendency of positive cells with carcinogens, it may be important to focus on molecules that function before G2/M phase checkpoint for searching carcinogenesis-related molecules.

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α -Mangostin Isolated from Pericarp of Mangosteen Suppresses Tumor Growth and Lymph Node Metastasis in a Xenograft Model of Metastatic Mammary Cancer

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The antitumor growth and antimetastatic activities of α -mangostin were studied in a mouse metastatic mammary cancer model having a p53 mutation. Mammary tumors, induced by inoculation of syngeneic BALB/c mice with BJMC3879luc2 cells, were subsequently treated with α -mangostin at 0, 10 and 20 mg/kg/day using mini-osmotic pumps.

Survival rates were significantly higher in the 20 mg/kg group than in the control group. Tumor volumes were significantly suppressed in the 20 mg/kg group. The multiplicity of lymph node metastasis was significantly decreased in the 20 mg/kg group. Levels of apoptosis were significantly increased in the 20 mg/kg groups. Microvessel density was significantly decreased in α -mangostin-treated groups. The numbers of dilated lymphatic vessels containing intraluminal tumor cells were significantly reduced in mammary tumors in the α -mangostin-treated groups. Western blotting and immunohistochemistry showed decreased phospho-Akt-Thr308 levels in the α -mangostin-treated cells and mammary carcinoma tissues.

These results suggest that α -mangostin may be useful as a complementary alternative medicine, adjuvant therapy and for the chemoprevention of breast cancer development.

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Effects of DAG edible oil and glycidol fatty acid ester on rat mammary carcinogenesis

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Regarding risk assessment of diacylglycerol (DAG) edible oil, a major concern contains issues that 1,2-DAG is a promoter of protein kinase C (PKC) and carcinogenicity of glycidol fatty acid ester (GLE). In human body, GLE may be converted to glycidol (GL) that is classified into Group 2A (probably carcinogenic to humans). To assess potential carcinogenic risk of DAG edible oil, it is necessary to examine the ADME analysis and also to investigate binding affinity to a target molecule. We examined the effects of DAG oil on the expression levels of PKC in Hras128 mammary carcinogenesis model in which tumors were induced by oral drops of DAG oil. Furthermore, we examined binding affinity of GLE and GL to the PKC protein using in silico toxicogenomics analysis. The rats were treated (oral drops) as follows: G1, 0.5 mL TAG x2/wk; G2, 0.5 mL DAG x2/wk; G3, 0.5 mL DAG x1/wk + 0.5 mL TAG x1/wk; G4, 0.5 mL DAG x1/2wks + 0.5 mL TAG x3/2wks. Experiment was terminated at 15 weeks after the start. Mammary tumors were histologically adenocarcinoma. Tumor incidence of G2 (77%) was significantly higher than G1 (22%). Tumor multiplicity of G2 (1.3) was also significantly higher than the other 3 groups (0.4-0.9). In tumor tissues, the mRNA expression levels of 6 PKC isoforms increased compared to the adjacent normal tissues. Also, DAG-treated mammary tissues exhibited increased expressions of 5 PKC isoforms compared to TAG-treated mammary tissues. Treatment of Hras128 rats with DAG enhances mammary carcinogenesis by inducing expression of several PKC isoforms. In silico analysis exhibited affinity of GLE and GL to the PKC protein. Further investigations are required to confirm the results.

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Involvement of Bronchiolar Alveolar Stem Cell for Early Stage of Carcinogenic Process in Mice Lung SCC

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We have previously shown that lung squamous cell carcinoma (SCC) is induced by 20 weeks treatment with N-nitroso-tris-chloroethylurea (NTCU). Histopathologically, it was suggested that NTCU-induced squamous metaplasia, dysplasia and finally SCC are arising in the terminal bronchiole. The aim of the present study was to identify the specific cell surface markers of dysplastic bronchiole epithelium which is the early lesion in mice lung SCC model.

Six A/J female mice were treated topically with NTCU in acetone (0.014M 75 µl/mouse) twice a week for 4 weeks and other 6 mice were used as a control and treated topically with acetone. All mice were sacrificed after 8 weeks and proteome analysis was carried out with QSTAR Elite LC-MS/MS in the frozen mice lung samples.

Histopathologically, dysplastic epithelium in the terminal bronchioles were detected in NTCU-treated group, and bronchiolar alveolar stem cell (BASC) which is double positively with CCSP and SPC were significantly increased in NTCU-treated group compared with control group. Tunel and Ki-67 positive cell index were not significantly different with both groups. 1185 proteins were identified with 66% confidence or higher and quantified with ProteinPilot 2.0 Software. 319 proteins were significantly elevated in NTCU-treated mice lungs compared with control. Furthermore, 25 up-regulated proteins (e.g. GAP43, nephronectin, Ly6C1) located in the cellular membrane are likely to become potential biomarkers of mouse dysplastic bronchiole epithelium.

The present study suggested that proliferation of BASCs is involved in the development of NTCU-induced lung carcinogenesis at early stage of carcinogenic process. Furthermore, identification of dysregulated membrane proteins will facilitate to identify the cancer-initiating BASC.

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Study on modification of tumorigenesis in the central nervous system by early-life exposure to manganese

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Early-life exposure to chemical carcinogens is known to increase susceptibility to cancer when compared with adult exposures. However, the information and number of evaluated chemicals have been limited. Medium-term protocols like *N*-ethyl-*N*-nitrosourea (ENU)-induced transplacental carcinogenesis are considered to be useful for the detection of the chemicals susceptible in the central nervous system (CNS) carcinogenesis by early-life exposure. An essential trace element, manganese (Mn) causes neurotoxicity and mutagenicity in mammalian cells, but not carcinogenicity in long-term animal studies. In the present study, we investigated that the modification activity of early-life exposure to Mn on rat CNS tumorigenesis induced by transplacental ENU treatment. Dam rats were treated with ENU (20mg/kg b.w., i.v.) at 17th pregnant day. Dams during the lactation period and their offspring after weaning until postnatal week 34 were given diet containing manganese chloride tetrahydrate at 0, 0.002%, 0.01% or 0.05%. Dams were autopsied after weaning and no significant differences in delivery data, body weight and clinical findings were observed among the groups of dams. Offspring were autopsied when the treatment was finished. Intergroup differences of body weight, food consumption, survival rate and incidence of clinical findings were not evident. Relative liver weight of 0.05% female offspring was significantly decreased, compared with the control without obvious histopathological changes. In histopathological assessment, astrocytomas, malignant astrocytomas, oligodendrogliomas and glioblastomas were observed in CNS, and Schwannomas in trigeminal nerve. The incidences of CNS tumor were totally 62% and 74% in male and female control, significantly. In the nervous tissues, none of the treated groups showed significant differences in the incidence, multiplicity and volume of tumor over the control group. Also, proliferative lesions were noted in lung, kidney and thyroid. These results indicate that offspring transplacentally exposed to ENU developed neoplasia properly within 34 weeks. Thus the present model was suggested to be useful for screening the modifier of CNS carcinogenesis and Mn does not exert promote CNS carcinogenesis in the present model.

P-98

Localization of Matrix Metalloprotease Activity in Macrophages Aggregated Adjacent to Thyroid Capsular Invasive Carcinomas Induced by Promotion with Sulfadimethoxine in Rats

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Matrix metalloproteinases (MMPs) are secreted from host-derived stromal cells as well as neoplastic cells to aid invasive growth and distant metastasis of neoplastic cells. In the present study, to clarify the role of stromal cells on neoplastic cell invasion in relation with activation of MMPs, we examined the activity distribution of MMPs and their cellular source in the rat capsular invasive thyroid carcinomas in a two-stage thyroid carcinogenesis model, male F344 rats were treated with sulfadimethoxine (SDM) at 1500 ppm in the drinking water for 11-23 weeks beginning one week after initiation with *N*-bis(2-hydroxypropyl)nitrosamine. One day prior to sacrifice, MMPsense 680 that can produce fluorescence signal through cleavage by MMPs was intravenously injected to rats. Thyroidal MMP activity visualized by bioimaging method at autopsy was later compared with the distribution of zymographical activity and immunohistochemical distribution of MMPs. As a result, MMPsense activity was detected specifically at the location nearby invasive carcinomas. Gelatinase (MMP-2 and/or -9) activity as detected by *in situ* zymography was similarly distributed at the location of capsular invasive carcinomas. Immunohistochemically, expression of MMP-9 was observed in both neoplastic cells and adjacent stromal cells distributed to the invasion front of neoplastic cells; however, MMP-2 expression was much less specific to these lesions. Moreover, these MMP-9-positive stromal cells were found to be CD68-positive macrophages from an early stage of the development of capsular invasive carcinomas. Myofibroblasts immunoreactive for alpha-smooth muscle actin lacked MMP-9-expression. These results suggest that MMP-9 secreted from both neoplastic cells and exudate macrophages cooperatively act to local invasion of neoplastic cells into the capsule in a SDM-promotion model of rat two-stage thyroid carcinogenesis.

P-99

Capsaicin propagate cancer cells and cancer stem like cells

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Capsaicin (N-vanillyl-8-methyl-1-nonamide), the main pungent component of red pepper, have demonstrated anti-proliferative and cell death-stimulating effects in various cancer cell lines. We confirmed that capsaicin treatment induces necrotic cell death in HepG2 human hepatoblastoma cells. However after capsaicin treatment, small populations of cells were always survived. There is accumulating evidence that many type of cancers are initiated and maintained by a small populations of cancer stem cells. In this study we identified various differentiated cells such as nerve or fat-like cells, together with epithelial cells in the capsaicin resistant HepG2 cancer cells. Western blotting analysis revealed overexpression of CD133, vimentin, connexin 32 and connexin 43 in cells with resistance to capsaicin treatment. These results indicated that capsaicin resistant cells may possess stem cell or cancer stem cell characteristics with morphological features of both epithelial and mesenchymal differentiation. Furthermore, our simple method to enrich cancer stem-like cells may be useful for chemoprevention or toxicologic studies.

P-100

Two-step Ultra-short-term Carcinogenicity Test of Carcinogen and Non-carcinogen Using rasH2 Mice

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[Purpose] We demonstrated possibility of evaluation skin tumor promoting effect in 8 weeks using rasH2 mice with TPA (as a positive control) and DMBA (as an initiator) in last annual meeting of JSTP. In this study, we investigated the promoting effects of 4-vinyl-1-cyclohexane diepoxide (4VCD), a skin carcinogen, and three non-carcinogens: diisopropylcarbodiimide (DC), benzethonium chloride (BC) and oleic acid diethanolamine condensate (OADC).

[Methods] DMBA (50 µg/100 µL acetone) was applied once to shaved dorsal skin of female rasH2 mice (7 weeks old, 10 mice/group). One week after the treatment, 4VCD (2.5 or 0.5 mg/200 µL), DC (40 mg/kg b.w.), BC (1.5 mg/kg b.w.) or anhydrous ethanol were applied 5 times a week, respectively. OADC (30 mg/kg b.w.) and 99.5 % ethanol were applied everyday. Moreover, non-initiated mice (5 mice/group) were also similarly treated with 4VCD (2.5 mg/200 µL), DC or BC. All animals were sacrificed at 12 weeks after DMBA initiation.

[Results] At 4 weeks after DMBA treatment, skin nodules were observed in all DMBA-treated groups except 99.5% ethanol group, but the nodules increased very little during the treatment period. The incidence and average number of nodules at the sacrifice were within 20~30 % and 0.2~0.4, respectively. In histopathological examination, the nodules were specified as squamous cell hyperplasia, squamous cell papilloma or squamous cell carcinoma. In contrast, no skin tumor was induced in 99.5% ethanol or non-initiated groups.

[Conclusion] 4VCD, a skin carcinogen, induced very little skin tumors in rasH2 mice after DMBA initiation. The low incidence might be caused by low dosage. Non-carcinogens or solvent induced very little skin tumors similarly to 4VCD. Further investigation using solvent or non-carcinogens will be necessary to establish a two-step ultra-short-term carcinogenicity test. Aftertime, we aim to examine the detection for 4VCD skin tumor at dose levels according to the two-year carcinogenicity studies.

P-101

Scanning Electron Microscopic Observations of Cultured Mammalian Cells Treated with Multi-wall Carbon Nanotubes

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A variety of nano-materials useful in wide areas has been developed recently. A cell culture system for screening toxicity of multi-wall carbon nanotubes (MWCNT), made of nano-materials, has been exploited in our Center. Our system has elucidated that needle-like (straight) MWCNT induced ploidy changes and double or multinucleated cells in CHL cells and an epigenetic transformation in BALB cells. The present report describes findings of the intake of MWCNT in both cells by SEM. [Methods] Both CHL/IU and BALB/c3T3 cells were treated with straight MWCNT (MWNT-7, supplied by Mitsui-bussan Co. Ltd; 88±5 nm in diameter, 5.0±4.5 µm in length, containing those of more than 5.0 µm in length in 39.8%) , suspended in culture media, for two days. Culture cells were fixed with PBS containing 1% glutar aldehyde, replaced with *t*-butanol after dehydration with ethanol, frozen-dried in vacuum and evaporated with platinum for observation by SEM (FE-SEM Hitachi:SU-8000) . [Results, Discussion] (1) Intake of straight MWCNT was observed in both culture cells. (2) Straight MWCNT were found connecting two cells like as a bridge. Observed findings suggested that straight MWCNT give an effect on cell division after the intake in both culture cells, inducing ploidy changes and double or multi-nuclei in CHL/IU cells and an epigenetic transformation in BALB/c3T3 cells. (supported by the Scientific Fund from the Ministry of Health, Labor and Welfare, Japan)

P-102

Sequential Distribution of FITC Conjugated PLGA Nanoparticles after Intratracheal Administration

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(Purpose) PLGA nanoparticle has been developed as a drug delivery carriers for pulmonary administration. However, its behavior has not been fully examined so far. In this study, we examined sequential distribution of PLGA nanoparticles after intratracheal administration to investigate its behavior in body. **(Method)** Emulsion of FITC-PLGA nanoparticles was administered intratracheally to rats. At 5, 30 or 60 minutes after single intratracheal administration, the rats were sacrificed and the lungs and the kidneys were collected for further analyses. The rats in the control group were untreated. The lungs and kidneys were collected immunohistochemically. A part of the lungs collected were examined for PLGA nanoparticles using a transmission electron microscope. **(Result and Discussion)** In the electron microscopic study, many pinocytotic vesicles were observed in vascular endothelial cells and type 1 alveolar epithelial cells after administration. The vesicles were electron-lucent dense and had average size of 60 nm in diameter. These vesicles were observed also in the control groups. There was little morphological difference between the two groups. However, the number of vesicles observed after administration was more than that of the control group. These vesicles were observed also in alveolar macrophages after administration. The vesicles had the same features as vesicles observed in type 1 alveolar epithelial cells and vascular endothelial cells. However, the vesicles in alveolar macrophages varied in size and those of 150nm in length were present sporadically. In immunohistochemical study, FITC was found in alveoli, alveolar macrophages and proximal tubular epithelia. It was suggested that FITC-PLGA nanoparticles existed in pinocytotic vesicles in the kidney. FITC-PLGA nanoparticles were detected from 30 minutes after administration and its staining intensity reached the highest level at 60 minutes after administration. The results of this study suggested that nanoparticles administered intratracheally were absorbed in alveoli immediately and were carried to the kidney. In addition, it was indicated that agglomerated nanoparticles were uptaken by alveolar macrophages.

P-103

Translocation of Intratracheally Administrated Multiwall Carbon Nanotubes to Brain

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【Introduction】 Recent studies reported the translocation of Fullerene (C₆₀) and Titanium dioxide (TiO₂) nanoparticles to the fish and rodent brain, and therefore, serious concerns for the biological effects by nanomaterials have been arisen. This study reports the deposition of multiwall carbon nanotubes (MWCNTs) found in the brain of rats, which were intratracheally administered (a single dose).

【Materials and Methods】 MWNT-7 (Mitsui & Co., Ltd.), of which mean width and length were 88 nm and 5.0 μm respectively, was used as the test material. After suspension in PBS containing 0.1% Tween 80, MWCNTs (160 μg/rat) were intratracheally administered to 13-week-old male F344 rats. The rats were sacrificed and necropsied on day 1, 7, 28, and 91 after the treatment. For microscopical examination, the brains were fixed by perfusion with 10% neutral buffered formalin, and the sections of brain tissue were stained with Kernechtrot. The fibers in the brain were examined as follows, (1) distribution in the brain (2) time-dependent changes of the number of the fiber deposited (3) histopathological changes in the area of the fiber-deposition. Moreover, the fibrous materials observed microscopically in the Kernechtrot-stained sections were also examined with the Field Emission Scanning Electron Microscope (FE-SEM: HITACHI SU-8000). The FE-SEM samples were prepared by the following steps, (1) Targeted area containing the fibrous materials was chosen from the Kernechtrot-stained sections. (2) The area was fixed with 2% OsO₄. (3) The area was sputter-coated by Pt.

【Results】 The following results were obtained by the microscopical examination. (1) Fibers were observed in the whole area of the brain. (2) Fibers were found in the brain on day 1 after the treatment, and the deposition increased time-dependently. (3) No histopathological changes, such as an inflammation, were observed in the area of the fiber-deposition in the brain. Additionally, FE-SEM confirmed the fiber observed in the brain by microscopical examination to be 115 nm in width.

【Discussion】 Oberdörster *et al.* (2004) reported the deposition of ¹³C carbon black nanoparticles in the brain by inhalation exposure in rats. They proposed the translocation pathway of nanoparticles to the brain from the nasal cavity through the olfactory nerve. In the present study, the olfactory pathway of the deposition to the brain is excluded because MWCNTs were administered by the intratracheal instillation.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan.

P-104

Effects of Magnetite Nanoparticles on Lungs of Fischer 344 Rats after Repeated Intratracheal Spray Instillation.

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Ferric oxide nanoparticles are of considerable interest for the application in nanotechnology related fields. However, as iron being a highly redox-active transition metal, the safety of iron nanomaterials need to be further studied. In this study, ferric oxide nanoparticles (magnetite) were used to test the pulmonary responses in rats by an intratracheal spray instillation.

Ten-week-old male and female Fischer 344 rats (n=20/group) were exposed every four weeks intratracheal spray instillation of 0 (vehicle), 0.2, 1.0 or 5.0 mg/kg body weight magnetite. After 52 weeks, the rats were sacrificed and hematological, serum biochemical or biological consequences were investigated. There were no significant, treatment-related changes with regards to body weight, food intake, urinalysis, hematology or serum biochemistry. The lung weights of high-dose group male and female rats were significantly higher than those of the controls. The lungs of high-dose group rats showed enlargement and black patches originating from the color of magnetite, macroscopically. The histopathological changes in lungs of treated rats were infiltration of macrophages phagocytosing magnetite, inflammatory cell infiltration in the walls and spaces of the alveoli and alveolar type II cell proliferation. The hyperplasia of the bronchial/alveolar epithelium and perivascular edema were observed in high-dose group rats.

In the repeated toxicity test, magnetite caused a foreign body inflammation with the magnetite accumulation and hyperplasia of the bronchial/alveolar epithelium in the lung. The responses of pulmonary lesions were dose-dependently increased.

P-105

Risk Evaluation of Carbon Nanotube on Rat Lung Carcinogenesis

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Carbon nanotube (CNT) is expected to have significant beneficial impacts in fields such as medicine. However, there is an urgent need to determine potential human health hazards.

We have reported secreted MIP1 α from nanoscale TiO₂-laden alveolar macrophages can cause cell proliferation in lung alveoli. This suggests that factors from nano-particle-laden alveolar macrophages promote proliferation of lung epithelial cells and this increased proliferation could be the basis of the possible carcinogenic activity of CNT in the rat lung.

In this study, we investigated the capability of single walled CNT (SWCNT) and multi walled CNT (MWCNT) to promote proliferation of lung tumor cells via macrophages. To examine the effects of CNT on lung carcinogenesis, CNT was administered to F344 rats by intrapulmonary spraying five times over 9 days. We used asbestos (crocidolite) as a positive control. Microscopic observation showed scattered inflammatory lesions with infiltration of numerous macrophages in CNT-treated animals. CNT aggregates were found in macrophages, and the number of macrophages in the alveoli was significantly increased in the CNT-treated animals as well as the crocidolite-treated animals. Treatment with SWCNT and MWCNT (A Inc.) significantly increased 8-hydroxydeoxy guanosine (8-OHdG) levels, but crocidolite and MWCNT (B Inc.) did not. The culture medium collected from macrophages treated with CNT promoted proliferation of the human lung cancer cell line A549. We conclude that CNT promotes proliferation of lung cancer cells, and that factors secreted from alveolar macrophages phagocytosing CNT is a probable determinant.

P-106

Lack of promoting effect of titanium dioxide particles on skin carcinogenesis in rats and mice

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Titanium dioxide (TiO₂), nano- and larger scale, is used in sunscreens and cosmetics as an ultraviolet light screen. TiO₂ is known to have carcinogenic activity in the rat lung and is classified as a possible human carcinogen, Group 2B category by WHO/IARC. The carcinogenic effect on the skin, however, has not been reported. By using two-stage skin carcinogenesis models, we did 3 independent experiments to examine the promoting/carcinogenic effect of 2 types of nano-size TiO₂ particles in animal models known to be sensitive to skin carcinogenesis: c-Ha-*ras* proto-oncogene transgenic (*Hras*128) rats and Ha-*ras* transgenic (H2) mice. Type 1 TiO₂ particles (rutile, without surface coating, primary 20 nm in mean diameter) were suspended in Pentalan 408 and type 2 particles (rutile, surface coated with silicone, 35 nm in mean diameter) were suspended in silicone. Experiment 1: male and female *Hras*128 rats at the age of 10 weeks, were initially treated with ultraviolet B (UVB) radiation on shaved back skin 2 times weekly for 10 weeks (800 mJ/cm² 7 minutes, 20 cm at the target distance). UVB treatment was followed by painting with 100 mg/ml TiO₂ of type 1 suspension on the shaved area at 3 x 3 cm 2 times per week until sacrifice. Male *Hras*128 rats were treated with TiO₂ for 42 weeks and killed at week 52; female *Hras*128 rats were treated with TiO₂ for 6 weeks and killed at week 16. Experiment 2: male *Hras*128 rats at the age of 6 weeks were initially treated with a single dose of DMBA (2.5 mg in 0.5 ml acetone) on shaved back skin. A week later, the rats were painted with 50 or 100 mg/ml TiO₂ of type 1 suspension on the shaved area 2 times per week until sacrifice at week 28. Experiment 3: female H2 mice at the age of 6 weeks were treated with a single dose of DMBA (0.2 mg in 0.1 ml acetone) on shaved back skin. One week later, the mice were painted with 10 or 20 mg/ml TiO₂ of type 2 suspension on the shaved area 5 times per week until sacrifice at week 20. In all 3 experiments, the incidence and average number of skin tumors were not significantly increased compared with those of vehicle controls. TiO₂ particles were detected only in the upper stratum corneum and not in the underlying skin tissue layers or remote organs, indicating that TiO₂ particles did not penetrate the epidermis. Furthermore, TiO₂ particles did not penetrate the human epidermis model *in vitro*. Our data suggest that TiO₂ does not cause skin carcinogenesis, probably due to its inability to penetrate through the epidermis and reach the underlying skin structures.

P-107

Spontaneously Occurring Intracranial Lipomatous Hamartoma in a Young BALB/c Mouse

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Lipomatous hamartoma is a benign lesion characterized by the accumulation of mature adipose tissue within the ventricles or midline of the brain and considered to be one of the malformations. This type of lesion has been described in humans and several kinds of laboratory animals, e.g., rodents and canine, but the incidence is very low in mice; 15 out of 45,983 BALB/c mice¹⁾. We encountered lipomatous hamartoma in a young BALB/c mouse, and the histopathological features are summarized.

[Materials and methods] The case was from a 7-week-old female BALB/cAnNCrIj mouse in an acute chemical toxicity study. No abnormal changes were observed during the in-life phase. The mouse was euthanized by isoflurane and necropsied at the end of the study. The brain and other organs were fixed in 10% neutral formalin and coronal sections of cerebrum and cerebellum were processed into hematoxylin and eosin-stained slides, and sequential sections were immunostained with anti-PCNA and anti-GFAP antibodies.

[Results] A nodule composed of mature white adipose cells containing one large fat droplet was observed in the third ventricle. These cells revealed no cytological atypia and were negative for PCNA. Brain parenchyma at frontal cortex to hippocampus in cerebrum was slightly compressed and chorioid plexus was located downward. GFAP-positive glial cells did not proliferate in the surrounding brain parenchyma and capillary vessels were scattered penetrating into the nodule from the surrounding tissue. Other animals in the same study had no abnormal changes in the brain, that lipomatous hamartoma was considered to be occurred spontaneously, and not related to chemical exposure.

[Conclusion] Based on the above findings, the present case was diagnosed as lipomatous hamartoma. The localization of the nodule indicated that this lesion occurred from the roof of the third ventricle. To best of our knowledge, this strain of mice is not very popular to use in toxicity studies, and historical control data of brain have not been well reported. Our present case may provide valuable information to histopathology of BALB/c mice.

[References]

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P-108

A Malignant Mixed Tumor Suspected To Be Submandibular Gland Origin in a Wistar Hannover Rat

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Tumors in the salivary gland are rare in rats. Most of them reported were originated from epithelial cells. In this report, we describes pathological and immunohistochemical characteristics of a tumor with epithelial and myoepithelial components suspected to be submandibular gland origin in a rat.

At necropsy, a white mass approximately 2 cm in diameter involving the junction of the submandibular gland was observed in a male GALAS rat (Br/Han:WIST@Jcl) (110 weeks old of age) given a diet containing 0.3% catechin (middle dose group) at termination of two-year carcinogenicity study. Similar masses approximately 1~5 mm in diameter were observed in the lung, prostate and the abdominal cavity. All tissues were stained with HE and tumors were stained immunohistochemically with following antibodies: pan-keratin, cytokeratin 7 (CK7), vimentin and α smooth muscle actin (α SMA). Histopathologically, epithelial and/or spindle to polygonal cells with atypia proliferated surrounding the necrotic area in the central part of masses. This feature was common to the masses. The well-differentiated cuboidal epithelium arranged in single-layered ductal structures was observed at the boundary of the submandibular gland and in metastatic area in the lung. Spindle to polygonal neoplastic cells were arranged in bundles and wavy pattern. Immunohistochemically, the neoplastic ductal cells and a part of spindle to polygonal cells were positive for pan-keratin. In addition, a part of epithelial cells were positive for cytokeratin 7. Most of the striated ductal cells and excretory ductal cells in the normal part of the submandibular gland were positive for cytokeratin 7. The spindle to polygonal cells were positive for vimentin and α SMA.

Based on the histopathological findings of the tumor in the submandibular gland and immunoreactivity for cytokeratin 7, this tumor was strongly suspected to be submandibular gland origin. Active proliferation of both epithelial cells and myoepithelial cells which were positive for vimentin and α SMA led to a diagnosis of malignant mixed tumor in the submandibular gland. The reason why the tumor cells disseminated in the abdominal cavity was still unknown.

P-109

Cystic Cholangioma in the Thoracic Cavity of a Rat

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A female congenic rat which was produced by repeated backcrossing of Nihon rat, a model for hereditary renal cell carcinoma, to Brown Norway rat was necropsied at 24 months of age.

At necropsy, a white mass about 1 centimeter in size was observed in the thoracic cavity, and the mass partly adhered to the esophagus and the diaphragm. In the abdominal cavity, a part of the liver adhered to the diaphragm, and the membrane in the adhesion area was markedly thickened.

Histopathologically, the mass was clearly circumscribed by connective tissue, and consisted of neoplastic cuboidal epithelial cells which showed cystic-tubular proliferation. Some islands of well-differentiated hepatocytes and some vessels were observed in the mass. In the adhesion area of the liver, the component cells were very similar to the tumor cells in the mass. Immunohistochemically, the tumor cells were strongly positive for cytokeratin and partly for vimentin, but negative for mesothelin and Von willbrand Factor. The positive rate for Ki-67 was 2.4%. No significant histopathological findings were observed in the esophagus, the diaphragm and the other area of the liver.

Based on these histopathological evidences, we diagnosed this tumor as a cystic cholangioma. To our knowledge, this is the first case of cystic cholangioma appeared in the thoracic cavity of a rat.

P-110

Intracytoplasmic Eosinophilic Inclusion Bodies Spontaneously Occurred in a Rat Liver

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Detailed investigation of the intracytoplasmic inclusion bodies in rat hepatocytes has scarcely been reported. In this report, we describe the histological, ultrastructural and immunohistochemical characteristics of intracytoplasmic inclusion bodies spontaneously occurred in a rat liver.

A female Crl:CD (SD) rat from a 2-week toxicity study was sacrificed at 8 weeks of age. Though no abnormalities were observed in general conditions, body weight, food consumption, hematology or at necropsy, blood biochemical test revealed a slight elevation in AST (161 U/L).

Histologically, a number of eosinophilic inclusion bodies were found in the cytoplasm of the hepatocytes. These inclusion bodies were strongly positive for PAS staining and resistant to giastase digestion. Ultrastructurally, the inclusion bodies were surrounded by limiting membranes and composed of moderately electron dense, homogenous materials. Immunohistochemical examinations revealed that the inclusion bodies were positive for albumin and IgG; however, they were negative for lysosome markers.

From histological, ultrastructural and immunohistochemical analyses, these inclusion bodies were diagnosed as intracytoplasmic blood plasma inclusions.

P-111

Histopathological features of postmortem changes and artifacts in rat pancreas

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Introduction: Pancreas is susceptible to postmortem and artifactual autolysis due to the abundance of digestive enzymes. Autolysis is sometimes difficult to differentiate from lesion. Recognition of artifactual and postmortem effects is important in the toxicity evaluation. We report histopathological features of artifacts and postmortem changes in rat pancreas.

Methods: Pancreas was removed from rats (mainly SD) 1-24 hours after death and processed into hematoxylin-eosin specimens for microscopic examination. To investigate artifactual effects, pancreas that was removed after euthanasia, left for an hour and damaged by compression, was compared with non-treated pancreas. PCNA immunostaining and TUNEL method were also used for some pancreas.

Results and Discussion: An hour after death, there were no obvious changes in non-treated pancreas. On the other hand, sporadic pyknosis-like figures and focal lysis around interlobular pancreatic duct were found in the acinar cells of compressed pancreas. That indicated mechanical damage caused autolysis by leakage of pancreatic juice from the injured interlobular duct rather than by effect of intracellular zymogen granules. Several hours after moribund death, zymogen granules were decreased and the acinar structure was massively obscure due to progressed lysis. In the remaining acinar cells, binuclear acinar cells, which were oval and nonpolar, were often observed. These cells were also found among the necrotic acinar cells in a rat with acute pancreatic necrosis. Electron microscopic examination revealed that these binuclear cells were not syncytial cells but true binuclear cells existing adjacent to debris of the disrupted cells. In addition, these cells showed negative for PCNA and TUNEL, indicating they were not proliferating, degenerative or dying cells. Some researcher reported that binuclear cells somewhat exist in normal rat pancreas. It was considered that binuclear cells were often observed since they were more resistant to autolysis and damage than mononuclear cells and remained longer.

P-112

Two Cases of Parathyroid Carcinoma in C3H Mice

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Parathyroid tumor is extremely rare endocrine tumor in mice. Two cases of parathyroid carcinoma were seen in male C3H/HeNrs mice of our radiation carcinogenicity study. In this report, the histopathological natures were presented. In the radiation carcinogenicity study, mice were irradiated by fast neutron or gamma-rays at the age of 8 weeks and all mice were kept until natural death. One mouse was killed moribund 637 days after the irradiation of 1Gy of fast neutron (Case 1), and the other was killed moribund 798 days after the irradiation of 0.1Gy of fast neutrons (Case 2). Macroscopically, Case 1 showed white nodules of left thyroid, lungs, livers, kidneys and spleen, dark red nodules of livers, and small white nodules in adrenal glands, and Case 2 showed a white nodule in the right thyroid, dark red nodules in livers and a small white nodule in the adrenal. Histopathologically, in both cases, tumor developed in the region corresponding to the parathyroid gland, and showed solid tubular growth pattern. Necrosis, cell debris or pooling of blood were sometimes seen in center of the tubular structure. Tumor cells were small and uniform with scanty cytoplasm, cell margins were indistinct, and basal cells were aligned next to a vascular stroma. Mitotic figures were frequently observed. Metastasis to renal cortex, livers, spleen, lungs, endocardium, bone marrow, and stroma surrounding accessory reproductive gland was observed in Case 1, and metastasis to renal cortex were observed in Case 2. No abnormal changes were seen in digestive tract in both cases. Immunohistochemistry, in both cases, the tumors had numerous PCNA-positive cells and showed PTH-negative, while normal parathyroid epithelial cells showed PTH-positive. In conclusion, these cases were diagnosed as parathyroid carcinoma. Hyperparathyroidism might not be included in pathogenesis of these cases. Whether radiation induced parathyroid tumor or not is not clear at the present.

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A case of spontaneous adrenal medullary proliferative lesion in Wistar Hannover rat

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In this case report, we describe proliferative lesion diagnosed as ganglioneuroma in the adrenal medulla, which was observed in a male Wistar Hannover rat (RccHanTM: WIST) at 20 weeks of age. In this animal, there were no macroscopic abnormal changes at autopsy. In the microscopic examination, abnormal cell proliferative lesion was observed in the adrenal medulla suppressing slightly on the zona reticularis. The proliferating cells were predominantly composed of ganglion cells and schwann cells. Ganglion cells had round or oval nuclei, large nucleolus and abundant eosinophilic cytoplasm. Atypical adrenal medullary cells were also exhibited in the periphery of this lesion. In part of the lesion, eosinophilic serous fluid accumulated in the interstitium. The cellular atypia and nuclear atypia of the proliferating cells were slight and no necrosis or invasion to the adjacent tissue was observed. By PCNA immunostaining, positive to weak positive-stained nuclei were shown in stromal cells and stellate cells. By S-100 immunostaining, many stromal cells and stellate cells were positively stained. By Chromogranin A staining, the cytoplasm of the atypical adrenal medullary cells were positive. In these immunostaining, no ganglion cells were positively stained. According to the international classification of rodent tumors of the rat, adrenal medullary tumors were classified as Pheochromocytoma type, Complex pheochromocytoma type, Ganglioneuroma type and NOS. In this case, the lesion originated from adrenal medulla and was judged to be a benign tumor with no strong cellular proliferation, cellular atypia or invasion to the adjacent tissue. Finally we diagnosed this lesion as ganglioneuroma because this lesion was composed of ganglion cells and schwann cells, and the portion of atypical adrenal medullary cells was minor. The incidence of ganglioneuroma was very low even in the 2-year carcinogenicity study; therefore, the present case was an extremely rare case observed in a young rat at 20 weeks of age.

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Case Report: A Spontaneous Basal Cell Carcinoma in The Lower Abdominal Subcutis of A Young RccHanTM:WIST Rat

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In order to collect background data, a life-span study using a female RccHanTM:WIST rat was conducted. Clinically, at 18 weeks of age, the animal developed anemia induced by hemorrhage from a subcutaneous mass in the lower abdominal region; the animal was euthanized due to unfavorable prognosis. The subcutaneous mass was 1.5 cm in diameter, and its surface was ruptured and showed partial hemorrhage. The cut surface was whitish and dense. The mass could be easily removed from the normal skin, with no adhesion to the neighboring muscle tissue. Other macroscopic findings were pale appearance and slight splenomegaly. Histopathologically, proliferating oval-to-spindle-shaped tumor cells with pale eosinophilic cytoplasm showing small nodules were found below the epidermis. These cells showed diffuse proliferation from the middle to deep layers of the mass. The tumor cells had large nuclei and clear round-to-oval-shaped nuclear bodies. Mitotic cells were occasionally observed, and the borders of the tumor cells were unclear. Masson's trichrome staining revealed few collagen production. Watanabe's silver impregnation method revealed alveolar pattern surrounding the tumor cells, with fine reticular fibers. Periodic acid Schiff (PAS) reaction yielded negative results. Immunohistochemically, the tumor cells were strongly positive for pan-cytokeratin and positive for cytokeratin 14, p63, and proliferating cell nuclear antigen (PCNA), but negative for vimentin and estrogen receptor- α . On the basis of these findings, we diagnosed the tumor as basal cell carcinoma.

P-115

A Spontaneous Histiocytic Sarcoma of a Male Sprague-Dawley Rat

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In the present study, we report a case of spontaneous histiocytic sarcoma developed in the liver and metastasize other organs including regional lymph nodes, lung, heart, spleen, and skeletal muscle. A male Sprague-Dawley rat was found dead with rough hair coat and emaciation. Grossly, the animal had multiple gray nodules which were randomly scattered through the swollen liver. Also, nodular gray spots were randomly found in the pulmonary parenchyma and the pleural surface which was adhered to the heart and thymus. Histopathologically, the hepatic nodules were characterized by neoplastic foci composed of oval to spindle cells resembling macrophages with central area of necrosis. The neoplastic cells had foamy cytoplasm, indistinct cell boundaries, and often contained phagocytized cellular debris and erythrocytes. The multiple nodules around blood vessels were noted in the lung and the neoplastic cells infiltrated into the overlying serosal surface. Multinucleated giant cells were often found in the neoplastic foci. The morphology and growing patterns of neoplastic cells were similar to those in other organs such as heart, lymph node, spleen, and skeletal muscle. Immunohistochemically, the tumor cells were positive for vimentin and lysozyme but negative CD3 and CD79 α . Based on the histopathological and immunohistochemical features, this case was diagnosed as histiocytic sarcoma in the rat; it presumably developed in the liver and metastasized to other organs, although we absolutely could not rule out multicentric origin.

P-116

Histopathological, immunohistochemical and ultrastructural studies of congenital mesoblastic nephroma in a young Beagle dog

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A 15-month-old male beagle dog used in a toxicity study had a primary renal mesenchymal tumor. Macroscopically, the tumor was a gray-white mass which was found in the right kidney, and extended from the capsule to a position slightly compressing the medulla. Microscopically, most of the tumor cells showed a myxoid pattern, in which the matrix was positive for Alcian blue staining. In the other parts of the tumor, a fascicular and wavy pattern was observed, and the matrix was full of collagen fibrils. Immunohistochemically, tumor cells were positive for vimentin and fibronectin, and negative for cytokeratin, desmin, α -smooth muscle actin, Von Willebrand factor, cyclooxygenase-2, fibronectin and myelin basic protein. As a result, we diagnosed this case as a congenital mesoblastic nephroma based on the findings of abundant collagen fibrils and myxoid materials in the matrix, positivity for vimentin and fibronectin by immunohistochemistry, and negative staining for other immunohistochemical markers.

P-117

Pathological Features of Congenital Cyst in the Pelvic Cavity of A Dog.

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In humans, congenital cysts derived from various kinds of embryonic tissues occur in the retrorectal space. Here we report pathological features of congenital cyst in the pelvic cavity of a dog. An 8-year-old castrated male Welsh Corgi dog was presented to a veterinarian for perianal swelling. Perineal hernia was initially suspected. A perineal incision revealed a cyst with a calcified wall. The operation was then given up because of the difficulty of removal of the cyst. After that, the dog suffered from repeated accumulation of pus within the cyst and repeated rupture of the cyst. Administration of antibiotics and aspiration of the cyst fluid failed to improve the symptom. Seven months later, the dog was presented to the Veterinary Medical Center at Osaka Prefecture University. X-ray and CT tests revealed the cyst from perianal region to the pelvic cavity, displacing the rectum to the right dorsal position. The cyst was surgically removed. It had a bony consistency and was adhered to the rectum and urethra without direct communication. The anal sac, prostate and urinary bladder were normally located.

Histopathologically, there was a nodular proliferation of cuboidal to polyhedral epithelium with eosinophilic or pale granular cytoplasm in the luminal part of the cyst. The proliferating cells were arranged in nest or glandular pattern, and had prominent nuclear and cellular atypia. Mitotic figures were frequently observed. These cells were positive for uroplakin III, a transitional epithelium marker. Occasionally, the cyst was lined by a single to pseudomultilayer of epithelial cells without cellular atypia. The cyst wall consisted of fibrous connective tissue with abundant blood vessels, osteoid and mature bone tissue, small aggregates of lymphocytes and plasma cells, and occasional irregular bundles of smooth muscle. The present case was characterized by neoplastic proliferation of transitional epithelium and bone formation. The lack of direct communication between the cyst and urinary systems (bladder or urethra) suggests a congenital cyst derived from remnants of embryonic tissue such as the cloaca and urogenital sinus.

P-118

Examination of Histopathological Alterations in Organs and Tissues of Clawn miniature pigs Surely Associated with Their Aging in Rather Early Stage of The Life.

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【Background】

In recent years, Clawn miniature pigs have been adopted as the laboratory animals for testing the safety of medical devices due to their physioanatomical similarities with human's. However, so far not many results about histological alterations caused by aging process have been reported. The present study has been planned to clarify their histological alterations through possible aging process but not toxicological influences, though present study deals with rather earlier stage of life to Clawn miniature pigs.

【Method】

Male and female Clawn miniature pigs used in this study received implantation of coronary stent for a performance test. Sixteen animals, 12 males and 4 females, in total, were divided into 2 groups, Group A (6 males and 1 female) and Group B (6 males and 3 females). Group A animals were bred for 3 months and Group B were bred for 9 months just started after stent implantation. Before necropsy, all the animals received coronary angiography. Almost all organs and tissues except for heart of all the animals were examined histologically with H&E stained sections. Animal ages at necropsy were 16 to 24 months in Group A and 22 to 42 months in Group B animals.

【Results】

Findings frequently observed in Group A were cell infiltration mainly composed of lymphocytes and/or eosinophils in the lamina propria of gastrointestinal tract, interstitial tissues of trachea and bronchioalveoli of lungs, eyelids, and mesenteric lymph node, and fat tissue infiltration in pancreas. Findings observed in Group B were quite similar to those observed in Group A, but their grade of the findings were more significant and expanded compared with Group A. Infiltration of mononuclear cells were only observed in lamina propria of the urinary bladder of Group B and infiltration of mononuclear and eosinophils were widely and intensely observed in lamina propria of gastrointestinal mucosae of Group B. Testicular atrophy associated with interstitial cell hyperplasia were observed only one third of males in Group B. Neoplastic lesions were not observed in all the animals of both groups.

【Conclusion】

Histopathological findings observed in both groups, age 18.4±2.9 (Group A) and 27.0±6.2 (Group B), were rather severer in Group B and some findings were only observed in Group B. These results suggest that some of histological findings detected were aging associated alterations in Clawn miniature pigs and some of lesions may appeared in rather younger age of their life span.

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Spontaneous changes of the macula lutea and optic nerve in cynomolgus monkeys

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【Background】The cynomolgus monkey (*Macaca fascicularis*) is considered to have clear macula lutea structure among the laboratory animals and a detailed examination of the macula lutea is important for extrapolating the ophthalmologic toxicity in humans. In this study, the collected histopathological background data of spontaneous changes seen in the macula lutea and optic nerve in cynomolgus monkeys used in toxicity studies were reported.

【Materials and Method】Cynomolgus monkeys (2-6 years old, from the Philippines, Vietnam and China, 875 males and 803 females) from vehicle dosed control animals and test article administered animals that were used in toxicity studies between 2007 and 2010. The hematoxylin-eosin (HE) stained sections of the eyes and optic nerve were examined histopathologically particularly on the macular and optic nerve lesions.

【Results】A total of 17 animals from both males and females (incidence rate: males 1.94% and females 2.11%) showed slight to moderate atrophy of the ganglion cell layer of the macula lutea and the optic nerve. In addition, coincidentally, slight gliosis (neuroglia cells infiltration) of the median part of the optic nerve accompanied by foamy macrophage infiltration were also observed. These lesions of the macular region and the optic nerve were seen only in cynomolgus monkeys from Vietnam and were not observed in monkeys from the Philippines and China. Moreover, above-mentioned lesions were not detected at in-life ophthalmological examination.

【Conclusion】In conclusion, based on the morphological characteristic of the macular and optic nerve lesions, they are similar to idiopathic bilateral optic atrophy reported in rhesus monkeys (*Macaca mulatta*), Leber hereditary optic neuropathy (LHON) or dominant optic atrophy (DOA) in humans. The occurrence is possibly hereditary.

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Spontaneous cardiac changes seen in cynomolgus monkeys

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Introduction

Cynomolgus monkeys (*Macaca fascicularis*) are widely used in preclinical toxicology studies due to their phylogenetic relationship to humans. Since limited number of animals used in non-rodent toxicity studies, it is difficult to distinguish test-compound-related lesion from spontaneous occurrence changes. In the present study, the spontaneous cardiac changes seen in the cynomolgus monkeys which are thought to be important information in toxicity assessment were retrospectively examined.

Material and methods

Cynomolgus monkeys (117 males and 117 females) aged 2.5 to 4 years, from general toxicity studies conducted Ina research, Inc. between 2006 and 2008, were used for the present study. These monkeys received vehicle only by either the oral, intravenous or subcutaneous routes of administration. The animals were purpose-bred for laboratory use and originated from Philippines, Vietnam and China. Histopathological specimens of the heart from monkeys were retrospectively investigated.

Results and conclusion

Focal mononuclear cell and inflammatory cell infiltration, myocardial degeneration/necrosis, fibrosis, mineralization, karyomegaly and hemorrhage were noted. In those findings, focal mononuclear cell infiltration was often observed. Significant differences in the geographic origin of monkeys were not detected in the present study.

Among the spontaneous cardiac changes seen in

cynomolgus monkey, myocardial degeneration/necrosis was required more attention. When myocardial degeneration/necrosis is seen in test compound treated monkey of toxicity studies, distinction from background lesion is important to consider. Thorough understanding of the morphological characteristics and incidence of these spontaneous changes is necessary to prevent misidentifying test-article-related cardiac changes in toxicity studies. In the present study also reports on the results collected in 2009 monkey studies.

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Lymphatic Leukemia in a Japanese Macaque (*Macaca fuscata*)

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In the study on naturally-occurring lesions in the breeding colony of Japanese macaques maintained in the Institute of Primatology in Kyoto University, we found a case of lymphatic leukemia. The case was female adult, and was examined because of swelling of the left cheek and wobbling walk. The animal was humanly euthanized 5 days after submission because of suspected sepsis with a large abscess in the gum around left fore-molar and advancing anemia. At necropsy, marked splenomegaly and generalized lymphadenopathy were seen. Histologically, there was prominent infiltrative proliferation of the lymphoid neoplastic cells in the white pulp in the spleen, and splenic lymphoid follicles frequently showed hyalinization. The neoplastic cells possessed round or ovoid nuclei and fairly scant cytoplasm, and showed frequent mitotic figures. In the lymph nodes the neoplastic cells infiltrate in the paracortical area, compressing cortex and medulla with prominent central necrosis. In the liver, there was multi-focal infiltration of neoplastic cells around periportal areas. In the kidneys, the neoplastic cells were infiltrating in the interstitial tissues. The lungs had also infiltrative growth of the neoplastic cells in the alveolar septa. There was infiltration of the neoplastic cells in the lamina propria in the intestines. Immunohistochemically, the neoplastic cells were positive for CD3, CD56 and CD30, and negative for CD5, CD8, CD20, CD25, CD68 and CD79a. These finding indicated that the neoplastic cells might NK/T cell origin. Further study was needed to investigate correlation with Simian T-lymphotropic viruses (STLV).

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Bilateral Macular Degeneration in a Cynomolgus Monkey

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Macular degeneration, one of the leading causes of irreversible blindness in adult humans, is characterized by hyperpigmentation of the retinal pigment epithelium (RPE) cells and/or sub-RPE deposits. Ophthalmological findings similar to human macular degeneration have been reported in the cynomolgus monkey. We report on histopathological changes in a case of bilateral macular degeneration in a cynomolgus monkey (*Macaca fascicularis*).

The animal was a naïve four-year-old female imported from China, in which suspected bilateral macular degeneration had been observed in an ophthalmic examination one month before necropsy. The necropsy was performed following examination of the fundus, electroretinography (ERG) and optical coherence tomography (OCT). After euthanization by exsanguination, the left eye was fixed in a mixture of 2.5 % formaldehyde and 3% glutaraldehyde solution and examined microscopically (HE) and electron-microscopically (TEM). The right eye was fixed in 4% paraformaldehyde solution, then specially stained (periodic acid-methenamine-silver, masson trichrome) and immunostained (keratin, β - amyloid, CD34).

No abnormalities were observed by ERG; however, a severe bulge was observed bilaterally in the RPE layer in the fundus of the peripheral macula flava by OCT. Histopathological examination revealed a 40 μ m thick, oval granulation tissue focus (drusen) surrounded by RPE cells formed bilaterally within an area of 500 μ m of the macula flava fundus. Plentiful fiber (collagen fiber), isolated spindle cells, and 2 or 3 continuous cuboidal cells were observed in the drusen. These spindle or cuboidal cells were positive for keratin, and negative for CD34 in immunohistochemical examinations, and almost all contained melanin. Therefore, they were considered RPE cells. Amyloid deposition and choroidal neovascularization were not observed.

In conclusion, drusen observed in this young cynomolgus monkey contained RPE cells separated by collagen fibers. The histopathological features of the present case differ from human, early age-related macular degeneration (basal laminar-deposit, basal linear deposit).

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Bilateral Glaucoma in a Cynomolgus Monkey

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No cases of suspected spontaneous glaucoma in the eyes of cynomolgus monkeys have been reported on in relevant histopathological examinations. We report on histopathological changes in the bilateral eyes of a cynomolgus monkey, characterized by cupped optic disc and degeneration of the retina.

The animal was a naïve female (aged 4 years) imported from China, in which abnormal ocular fundi, mainly pale discoloration of the retina around the optic disc, had been observed 1 month before necropsy. Necropsy was performed following fundus, intraocular pressure, electroretinography (ERG) and optical coherence tomography (OCT) examinations. For histopathological examination, the right eye was fixed in a mixture of 2.5 % formaldehyde and 3% glutaraldehyde solution and specimens were prepared from it with Hematoxylin-Eosin (HE) staining. For immunohistochemical examination, the left eye was fixed in 4% paraformaldehyde solution and specimens were prepared from it with Glial Fibrillary Acid Protein (GFAP) staining.

In ophthalmic examinations, intraocular pressure and ERG findings within the normal range were exhibited. Bilateral cupped optic discs and disarranged retinal layers around the optic disc were confirmed by OCT images. Histopathological examination revealed an abnormal, cupped optic disc accompanied with the dilatation of retinal central artery/vein. In the retina around the optic disc, disarrangement of the laminar structure caused by decrease in or loss of cells of the inner/outer nuclear layer and ganglion cell layer was observed. An increase in glial cell number was observed in the optic nerve. No abnormal changes were observed in the sclera, cornea, angle or iris. The retina and optic nerve exhibited markedly positive reactions in GFAP staining.

In conclusion, the glaucoma in the present case was similar to primary open-angle glaucoma (including normal-tension glaucoma) in humans and that in an experimental glaucoma model in animals.

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Nasal-Associated Lymphoid Tissue (NALT) of the Cynomolgus Monkey

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Mucosa-associated lymphoid tissue plays an important role in immune response to exogenous antigens. Although lymphoid tissues are found in the nasal mucosa of humans and non-human primates, they have not been investigated in detail. We examined the distribution of nasal-associated lymphoid tissue (NALT), and the morphologic and immunohistochemical features of NALT and the tonsils in cynomolgus monkeys.

Nasal mucosa, pharyngeal tonsils, tubar tonsils, palatine tonsils and lingual tonsils of male and female cynomolgus monkeys (aged 3 to 5 years) were fixed in 10% neutral buffered formalin. The number and distribution of NALT were examined macroscopically with Harris hematoxylin staining. The nasal mucosa and tonsils were embedded in paraffin, sectioned and stained with Hematoxylin-Eosin. For immunohistochemical examination, specimens were stained with CD3 (T cell), CD20 (B cell), CD68 (macrophage) and CD83 (dendritic cell).

NALT was diffusely observed in the nasal mucosa in the macroscopical and histological examinations, and was mainly present in the respiratory region of the nasal mucosa, particularly at the bottom of the ventral nasal meatus. There was a marked variation in the density of NALT between individuals. NALT was composed of various sized lymphoid follicles and mononuclear cells infiltrating the overlying epithelium. Immunohistochemical examinations revealed that the lymphoid follicles of NALT and tonsils were mainly composed of B cells, and lymphocytes around the follicles were mostly T cells. There were scattered macrophages and a small number of dendritic cells in the follicles. In the epithelium of NALT, the majority of infiltrated mononuclear cells were T cells, although small numbers of B cells, macrophages and dendritic cells were observed. Conversely, mononuclear cells infiltrating the epithelium of tonsillar crypt were mainly composed of B cells.

The present data clarified the distribution of NALT, and morphologic and immunohistochemical features of NALT and the tonsils in cynomolgus monkeys. It was suggested that there was a marked difference in T/B cell ratio infiltrating the epithelium between NALT and the tonsillar crypt.

P-125

Cortical nodule in the adrenal of cynomolgus monkeys

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It is widely known that the spontaneous occurrence of atypical cell populations (cortical nodule: CN) has been observed in the adrenals of cynomolgus monkeys in laboratories. These entities were reported by Fuji *et al.* (JSTP, 2002). In this study, we investigated the difference in occurrence between ages and distribution of CN in the adrenal cortex.

The adrenals of 7,248 cynomolgus monkeys (*Macaca fascicularis*, 3,912 males and 3,336 females) were stained with Hematoxylin and Eosin (H.E.) and immunohistochemically [for six cytochrome P450 (CYP) enzymes (CYP1A1, CYP2B1, CYP2C11, CYP2E1, CYP3A2 and CYP4A1), proliferating cell nuclear antigen (PCNA), Ki-67, c-erbB-2, IGF-2, and p53], and examined microscopically.

The incidence of CN increased markedly with age. CN was classified morphologically into three types: eosinophilic, clear cell, and mixed. Clear cell type CN was observed only in the zona fasciculate and eosinophilic type CN was observed in the zona reticularis and deep in the zona fasciculate. Mixed type CN and clear cell type CN, and eosinophilic type CN, were distributed on the side closer to the adrenal surface and on the side closer to the medulla, respectively. In immunohistochemical examinations, there was a difference in stainability of CYPs between CN and the normal cortex around CN. No positive reaction was noted for PCNA, Ki-67, c-erbB-2, p53, or IGF-2 with immunohistochemical staining.

From the stainability and localization of cells, it was concluded that clear cell type CN and eosinophilic type CN were derived from zona fasciculate cells and zona reticularis cells, respectively. From the difference in CYP staining for CN, it was considered that each CN was a cell population with altered enzyme manifestation. The findings of CN in cynomolgus monkeys in this study were suggested unlikely to have been precancerous lesions since CN showed little proliferation in immunohistochemical examinations and was negative for the product of oncogenes and tumor-suppressor genes, and tumors in the adrenal cortex are extremely rare in cynomolgus monkeys.

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Spontaneous Glomerular and Tubulointerstitial Lesions in Common Marmosets (*Callithrix jacchus*)

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Although spontaneous progressive nephropathy dominated by glomerular lesions in common marmosets has been described (Brack M 1988, 1995), their histopathological characteristics, including the relationship between glomerular and tubulointerstitial lesions, have not been reported in detail. In the present study, we examined the histopathological characteristics of the background lesions of the kidney in common marmosets.

Materials and methods: Twelve common marmosets (3 to 8-year-olds; 3 males and 9 females) were necropsied in this study. The kidneys were fixed in formalin and embedded in paraffin for histopathologic examination. Hematoxylin and eosin staining, periodic acid-Schiff (PAS) reaction and immunohistochemical staining for Proliferating Cell Nuclear Antigen (PCNA) were performed. The severity of glomerular lesions was graded into 3 classes: Grade I; no alteration, Grade II; segmental/focal increase of mesangial cells and matrix, Grade III; global/diffuse increase of mesangial cells and matrix. The area of mesangial matrix in each glomerulus was measured by using imaging analysis software. Tubulointerstitial lesions (interstitial inflammation and fibrosis, tubular regeneration and tubules with high nuclear density) were scored according to the area of each lesion: Score 0; 0%, Score 1; 1□20%, Score 2; 21□40%, Score 3; 40% and above).

Results and Discussion: Histopathologic changes were characterized by enlargement of glomeruli, expanded mesangial area (increase of PAS-positive mesangial matrix) with mesangial cell proliferation, interstitial inflammation and fibrosis, tubular regeneration and tubules with high nuclear density. Glomerular lesions progressed with increasing mesangial matrix and with aging. Additionally, the tubulointerstitial lesions became exacerbated with progressing glomerular lesions. Tubules with high nuclear density were divided into 4 types according to the structure of the cell layer (simple or stratified-like), the area of high nuclear density (partial or entire), cytoplasmic staining (eosinophilic or basophilic) with or without brush border and thickened basement membrane, and the activity of cell proliferation (PCNA-positive or -negative). In conclusion, the background lesions of the kidney in common marmosets were characterized by mesangial proliferative glomerulonephritis which progressed with aging, and secondary tubulointerstitial lesions, including tubules with high nuclear density.

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Spontaneous Renal Lesion in Common Marmosets: Details of Glomerular Change

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Introduction: Spontaneous renal lesions often occur in common marmosets (*Callithrix jacchus*). However, detailed reports about the progressive process of glomerular changes related to aging are very few. We investigated 21 common marmosets' kidneys (2 to 11 years old; 9 males and 12 females) using HE, PAS, PAM, and Masson's trichrome (MT) stains and transmission electron microscope.

Results: The grade of renal lesion became stronger in accordance with aging. The early lesions were interstitial inflammatory cell infiltration, basophilic tubule and hyaline cast. At this stage, no distinct glomerular lesions were recognized. The glomerular lesion was firstly recognized in the HE stain sections and increases in the mesangial cell and matrix at the glomerular hilum. In the mildly progressed lesion, the increased mesangial cell and matrix extended along the tuft. In the MT stain sections of the moderately progressed lesion, large red deposits were observed at the paramesangial area. These findings were similar to the feature of human IgA-nephropathy, which was classified to the mesangial proliferative glomerulonephritis. On the other hand, slight ruggedness of basement membranes was seen in a part of the epithelial side of the capillary loop. In the most progressed lesion, capillary dilatation and deposits at the rugged capillary loop were observed in MT stain sections. Part of the deposits was occasionally washed-out like a moth-eaten capillary wall. Ultrastructurally, adhesion of the podocyte processes was observed in the early stage. These findings suggested that a protein leakage occurred. In the progressed lesions, the high electron-dense area was observed in the increasing mesangial matrix. The capillary basement membrane thickened remarkably and was rugged on the epithelial side. In the most progressed lesions, many humps were washed-out and many organelles were observed at these areas.

Conclusion: The renal lesions in this study have been reported as Callitrichid IgM-nephropathy in previous studies. In our investigation, it was obvious that the early lesions of this progressive nephropathy were adhesion of the podocyte process. This glomerulonephropathy extended accompanied by mesangial proliferation and rugged capillary basement membrane along with aging.

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Rat Chronic Nephropathy-like Lesion with Tubular Hyperplasia and Adenomatous Proliferation in the Ageing Common Marmoset (*Callithrix jacchus*)

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Rat chronic nephropathy-like lesion with tubular hyperplasia and adenomatous proliferation was found in the kidneys of three ageing female marmosets maintained to breed. Macroscopically, the kidneys were pale slightly to severely in all cases. The surfaces of the kidney were granular in two cases, and dark red cysts were found in one case. Histopathologically, tubular dilatation, atrophy, degeneration and regeneration, hyaline cast, interstitial fibrosis and cell infiltration, and glomerular sclerosis were found in the kidneys of all cases. These lesions were similar to chronic nephropathy known as age-related disease in rats. Additionally, fibrosis in the left ventricular wall was found in two cases, and calcification of arterial wall and hyperplasia of the parathyroid gland in one respectively. Furthermore, tubular hyperplasia (eosinophilic, basophilic or vacuolar) was found in the kidneys of all cases, and one case had eosinophilic adenomatous proliferation.

Though glomerulonephritis with tubular degeneration and interstitial cell infiltration has been reported before, rat chronic nephropathy-like lesion with tubular hyperplasia and adenomatous proliferation was not mentioned. These cases will be valuable information for background data on ageing marmosets.