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SL-1

Toxicologic Pathology: Contribution to Drug Administration and Its Future

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“Pathology is the field of medicine which aims to elucidate the cause or pathogenesis of a disease, or to definitively diagnose a disease. Pathology involves the study of changes that take place in cells, tissues, and organs that underlie disease and involves the examination of specimens of cells, tissues, and organs macroscopically or microscopically.” (Japanese Wikipedia, November 3, 2011) The definition of toxicologic pathology has not been established in a general manner. However, it is a study that mainly involves the macroscopic and microscopic examinations of the following caused by drugs and agricultural chemicals: (1) side effects, (2) presence or absence of impairment, (3) site of impairment, (4) details, degree, and time course of impairment, and (5) pathogenesis. Toxicologic pathology can be used in conjunction with other sciences to elucidate toxicologic characteristics of drugs and other chemicals around us. When the resulting findings are reflected in governmental administrative decisions, toxicologic pathology can contribute to the maintenance of healthy living and the promotion of industries.

When the toxicity of chemicals is evaluated, it is unknown what toxicity will result. Thus, it is necessary to examine the toxic effects on all tissues and organs. A comprehensive examination is performed using various indices including general physical condition, morphological changes of organs and skeletal structures, blood profile, and serum and urine biochemical analysis. Toxicologic pathology is one component of a comprehensive examination. However, there are many impairments such as those involving the sensory organs and nervous system that are difficult to detect in animal experiments using other test methods. There are also diseases such as cancer for which the final diagnosis needs to be made histopathologically. Toxicologic pathology is a field that is considered the most important in the evaluation of toxicity. However, this field requires long training to attain technical skills and is highly specialized. Therefore, it is difficult for professionals from other fields to become skilled in toxicologic pathology.

There is a problem of subjective judgment that is used in pathological diagnosis. For example, pathological criteria differ greatly by institution. One institution can make various pathological diagnoses in the control group, but another institution might find no changes in the control group. Grading in pathological diagnosis can also differ by evaluator even if the evaluators are from the same institution. In many institutions, criteria and grading in pathological judgment have been standardized. . However, I have concerned of how adjustments are made in the final report. When carcinogenicity is evaluated, the same substance can be judged differently by different people. Thus, I have some papers that included diagnosis by multiple experts. . This type of ambiguity is disconcerting in a field where people outside the field have little choice but to believe the diagnosis of the toxicologic pathologists. There have been efforts to also use various new staining methods and chemical analyses to improve the accuracy of pathological diagnosis and to increase the objectivity of the decision. Dr. Michito Takahashi is the former director of the Pathological Department of the National Institute of Health Sciences, and he established the Pathology Peer Review Center. This act of establishing the center is very significant because he is a person close to an administration where on many occasions determinations need to be made with certainty by pathological diagnosis. The Japanese Society of Toxicologic Pathology certifies toxicologic pathologists, and I think such certifications are important in minimizing differences in diagnosis by individual and by institution.

The Toxicogenomics Project (TGP), in which I have been involved, has examined hepatotoxicity and nephrotoxicity of approximately 150 drugs and chemicals and has investigated changes of genes expression. When the second phase of the 10-year project ended, large amounts of tissue specimens and their images remained. We plan to complete electronic filing of photomicrographs in early 2012. We would like these slides and paraffin blocks to be used widely through the Japanese Society of Toxicologic Pathology. These materials will at least be useful in standardization of pathological diagnosis of the liver and in education. We also envision their future use in automated diagnosis using image analysis.

SL-2

Principles for The Risk Assessment of Chemicals in Food As Applied by FAO/WHO Expert Meetings

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The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) have served as scientific advisory bodies to the Codex Alimentarius Commission since its inception in the early 1960s, to member countries, and other interested parties. Previous general guidance for risk assessments applied by these expert committees were published in the late 1980s in two Environmental Health Criteria (EHC) monographs, EHC 70 (Principles for the safety assessment of food additives and contaminants in food, WHO 1987) and EHC 104 (Principles for the toxicological assessment of pesticide residues in food, WHO 1990).

Considerable changes have taken place in the procedures and complexity of assessments of chemicals in food since the preparation of these monographs. There have been significant advances in chemical analysis, toxicological assessment, and risk assessment procedures, and JECFA and JMPR have developed many new general principles. In light of this and the recognition of the importance of internationally harmonized risk assessment practices, as well as the recognition that the evaluations performed by JECFA and JMPR serve as the scientific foundation for international food standards that are of increasing importance within the Codex Alimentarius Commission and the World Trade Organization, FAO and WHO have undertaken a project to update and consolidate Principles and Methods for the Risk Assessment of Chemicals in Food, which has now been published as a single extensive EHC 240 monograph .

EHC240 contains the following nine chapters: Chapter 1: Introduction, Chapter 2: Risk Assessment and its Role in Risk Analysis, Chapter 3: Chemical Characterization, Analytical Methods and the Development of Specifications, Chapter 4: Hazard Identification and Characterization: Toxicological and Human Studies, Chapter 5: Dose-Response Assessment and Derivation of Health-Based Guidance Values, Chapter 6: Dietary Exposure Assessment of Chemicals in Food, Chapter 7: Risk Characterization, Chapter 8: Maximum Residue Limits for Pesticides and Veterinary Drugs, Chapter 9: Principles Related to Specific Groups of Substances. Included are also a number of annexes, most importantly an extensive glossary of terms, based on existing and agreed definitions. The principles outlined are applicable to food additives, contaminants, residues of pesticides and veterinary drugs. Special considerations are also given to substances consumed in small amounts, such as flavours or packaging migrants, and substances consumed in large amounts, such as ingredients and nutrients.

Details will be presented on the content of each chapter, illustrated by specific and practical examples. Focus will also be on special considerations, and an outlook given on further advances in chemical risk assessment.

S-1

Current Trends of Therapeutic Antibodies

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Therapeutic antibodies are developed as drugs that provide a swift solution to therapeutic targeting of disease-related molecules that have been discovered in genomic research because 1) a high level of efficacy and less adverse events are anticipated because of the high level of specificity and affinity to the target molecule (antigen), 2) the diversity of the target antigens and the mode of action allows the application to various drug targets, 3) modification and refinement by genetic engineering and the establishment of recombinant manufacturing technology has made industrial manufacturing possible.

The first therapeutic antibody was a mouse antibody that was launched in 1986 as an immunosuppressive agent used for organ transplantation. But because the antibody was a mouse antibody, adverse events such as antigenicity occurred and prevented the launch of therapeutic antibodies in the following 10 years. During this period, antibody engineering techniques such as the construction of chimeric or humanized antibodies from mouse antibodies or the production of human antibodies progressed, leading to the discovery and development of novel therapeutic antibodies that were serially launched in the mid 1990's.

Therapeutic antibodies exert their efficacy through the various natural functions of antibodies. Many therapeutic antibodies block the physiological function of their target antigens by neutralization. Therapeutic antibodies with antibody-dependent cell-mediated cytotoxic activity, complement-dependent cytotoxic activity or as drug delivery carriers (missile therapy) have been launched as anti-cancer agents. Recently, antibody engineering techniques have progressed and it is now possible to create antibodies with a diverse selection of functions such as antibodies with more efficient and long-lasting neutralizing effects, or agents that cause cytotoxicity at lower antigen expression levels, or bispecific antibodies that can recognize 2 different antigens with the same molecule to induce new biological responses. These recent advances along with the discovery of novel target antigens shed light on the possibility of new therapies. As the functions and target antigens of the antibodies become more and more diverse, the necessity to understand the biological function of the target antigens and the biological response to the modification of antibody functions is increasing. Evaluation and research of the toxicologic pathology associated with these issues will also require close attention.

S-2

MoA (Mode of Action) Analyses-A Toxicological Rationality in Pesticide Risk Assessment

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Safety evaluation of pesticide as a contaminant in food is done mainly through No Observed Adverse Effect Levels (NOAEL) obtained in toxicology testing using experimental animals. Essentially, a safety margin is taken into consideration for determination of Acceptable Daily Intake (ADI) using a Safety Factor (SF) or an Uncertainty Factor (UF) based on various toxicological observations in toxicology testing. Recent safety evaluation of pesticides involves toxicologically relevant analyses of mode of action that includes biological and molecular mechanisms existing behind the pathological observations to ultimately uncover cascade of molecular events to exert toxicity.

Studies on metabolism and disposition of pesticides, or, xenobiotics have revealed inter-species differences both on pharmacokinetics (PK)/toxicokinetics (TK) and pharmacodynamics (PD)/toxicodynamics (TD) between humans and experimental animals. In the course of safety evaluation of pesticides, quite careful consideration is needed on the possible inter-species differences in PK/TK and PD/TD between humans and experimental animals. In this symposium, a few examples which needs consideration on inter-species differences will be discussed: Avermectin required consideration on differences in genetics and age-development of P-glycoprotein; mesotrione on differences in incidence of higher blood concentration of tyrosine; and azoxystrobin on iron transporters.

S-3

The Juvenile Toxicity Study Guideline and Expectations for Toxicologic Pathology

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Pharmaceuticals have been used not only on adults but also children. The majority of prescriptions for children were "off-label use". Pharmaceuticals were investigated only through adult human clinical studies and adult animal nonclinical studies. Clinical studies in the pediatric population and juvenile animal nonclinical studies have not been conducted as frequently. No or little efficacy and/or concerns for safety were noted in some pharmaceuticals. Reflecting on this, clinical studies in the pediatric population were promoted after the release of the ICH E11 guidance "Clinical Investigation of Medical Products in the Pediatric Population" in 2000. In the nonclinical field, juvenile animal toxicity studies have become a general method to obtain the safety information for clinical studies in the pediatric population. The US FDA finalized the guidance of juvenile animal toxicity studies in 2006, and EMA was finalized in 2008. The timing of juvenile animal studies is also described in the ICH M3 (R2) guideline in 2009. The Ministry of Health, Labour and Welfare started to prepare the guideline for nonclinical safety studies in juvenile animals since October 2010. Public comments were collected on May 2011. The guideline will be finalized by March 2012.

One of the important endpoints in juvenile toxicity studies is toxicologic pathology in the developing organs. Reviews of the comparative organ system development (the bone, kidney, lung, male reproductive system, female reproductive system, heart, immune system, CNS, and gastrointestinal system) were reported in Birth Defect Research (Part B) from 2003 to 2005. The reviews were mainly based on older data, and described species base such as mice, rats, dogs or monkeys including various strains. As a first step, what is required is to clarify the normal histologic time course changes in the developing organs of the actual animals which are used for juvenile toxicity studies. The evaluation of toxicologic pathology based on that knowledge will increase the accuracy of juvenile toxicity studies.

S-4

The Role of Pathology in Alternative Methods for Toxicity Testing

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As we promote the reduction, refinement, and replacement (the 3Rs) of animals in laboratory studies worldwide, we hope to see both appreciation and implementation of these principles in toxicity testing. Along with reduction and refinement, we especially hope that advances in the development of alternative methods will produce results that obviate the use of laboratory animals altogether. Even though assessment based on relevant indices is highly likely to reduce the number of animals used in toxicity testing and so help to promote the 3Rs, many researchers still fail to recognize alternatives for tests involving pathology.

Nevertheless, when proof of mode of action (MoA) is sought, alternative methods that simply use comparison with the results of animal studies can also be employed to estimate localized toxicity from positive or negative results derived from the strength of the correlation. Many toxicity experts feel that there is not enough weight of evidence (WoE) to attribute MoA simply to the cytotoxicity of chemicals. Moreover, given current trends toward risk assessment from in vitro identification of toxicity, it is prudent to devise and employ models that utilize extracted tissues, three-dimensional cultures, and multiple mixed cultures in conjunction with the use of simple flat cell cultures. In such cases, pathological analysis is essential to clarify the MoA, increase the WoE, and ensure that continuous observation of changes for each chemical substance is reflected in ongoing risk assessment. Consequently, pathologists are also likely to play a major role in the development of alternative methods for toxicity testing.

WS-01

Evaluation of The Usefulness of MicroRNAs in Rat Hepatocarcinogenesis

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MicroRNAs (miRNAs), a class of small noncoding RNAs (~25 nucleotide) are known to be post-transcriptional regulators that bind to messenger RNAs (mRNAs), usually resulting in translational repression or target degradation. Recently, miRNAs are reported as promising biomarkers for several diseases, including cancer. MiRNAs is very stable as compare with mRNAs, because they have very few nucleotides. Therefore, miRNAs can be analyzed even in serum or formalin-fixed, paraffin-embedded (FFPE) tissue. In this study, we evaluate the usefulness of miRNA in rat hepatocarcinogenesis.

First, we extract RNAs from fresh frozen section, 6-month and 10-year old FFPE tissues and compared expression profiles of miRNA among them. The expression profiles of miRNA are closely resembled among these samples, so we could confirm that FFPE tissues were available for further miRNA analysis.

Circulating miRNAs in the serum are reported as good biomarkers for several cancers. Next, a study was conducted to provide concrete evidence using chemical-induced hepatocarcinogenesis in rat as a model. In this study, we use CYP inducer (Phenobarbital and DDT) and PPAR alpha agonist (Clofibrate). We thereby observed aberrant fluctuation of circulating miRNAs in the serum of rats not only with neoplastic lesions such as hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC), but also with preneoplastic lesions, such as foci of hepatocellular alteration (FHA). Additional qRTPCR analysis revealed gradual elevation of several circulating miRNAs with progress of hepatocarcinogenesis. Interestingly, increased levels of some miRNA were statistically significant even in the serum of rats at very early stages. Furthermore, some miRNA, whose levels were increased in serum, also increased in spontaneous HCA or HCC.

These findings provide the first evidences that circulating miRNAs have the potential to predict carcinogenesis at earlier stages, preneoplastic lesions than with previous biomarkers and that they might be utilized to monitor the progress of tumor development.

WS-02

The role of gap junctional intercellular communication in rat hepatocarcinogenesis and apoptosis

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Connexin 32 (Cx32) is a major gap junction protein in the liver with significant roles in the maintenance of tissue homeostasis. We previously reported that transgenic rats carrying a dominant negative mutant of Cx32 (Tg) demonstrated much reduced gap junctional intercellular communication in the liver and elevated susceptibility to diethylnitrosamine (DEN)-induced hepatocarcinogenesis. On the other hand, the Tg rats are less sensitive to hepatotoxicitic agents such as acetaminophen (APAP).

To understand the relationship between the effects of Cx32 on cancer prevention and cell damage in the liver, (i) 10 week-old Tg and littermate wild-type rats were given a single i.g. injection of 500 mg/kg APAP or 0.5% methylcellulose+0.1% Tween80, and hepatotoxicity was measured at 24 hours after dosing, (ii) 7 week-old Tg and wild-type rats were given a APAP (500 mg/kg, i.g.), or the vehicle alone, 10 times over 5 weeks. DEN (50 ppm) was then administered for 12 wks.

Under depletion of Cx32 expression, APAP-induced hepatotoxicity is much decreased as a result of reducing apoptosis. Pretreatment of APAP significantly accelerated development of DEN-induced GST-P positive foci only in Tg rats. The results suggest that Cx32 may play important roles in preventing carcinogenesis by inducing apoptosis in genetically damaged cells with cancer initiation.

WS-03

Role of the Canonical Wnt Signaling Pathway in Proliferation of the Gastric Epithelial Cells

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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 colon carcinogenesis. It has been reported that the Wnt signaling pathway is also activated in gastric cancers but it remains poorly understood how Wnt pathway contributes to the gastric carcinogenesis. In the present study, we generated the doxycycline-inducible ß-catenin mice and examined the effect of Wnt pathway activation on the proliferation of gastric epithelial cells. When mice were fed doxycycline, the increased expression of ß-catenin was confirmed in the gastric epithelial cells by immunohistochemistry and quantitative real-time PCR. The number of Ki-67 staining cells significantly increased in both fundic and pyloric glands in the doxycycline-treated mice as compared with those in the untreated control mice, indicating that the Wnt pathway activation led to the active proliferation of gastric epithelium. We are analyzing the expression of Wnt pathway tareget genes in the ß-catenin induced gastric mucosa, which would be useful to clarify the molecular mechanism underlying the gastric carcinogenesis.

WS-04

Inhibition of NADPH oxidase, an endogenous superoxide inducer, suppressed progression of prostate cancer.

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Recently, there are considerable evidences suggesting oxidative stress contributes to the etiology and pathogenesis of the prostate cancer. Therefore, we focused on NADPH oxidase, which generates intracellular superoxide, and utilized its inhibitor, apocynin for the suppression of prostate cancer progression.

In this study, we employed a xenograft model of prostate cancer with rat androgen-independent prostate cancer cell line, PLS10. The cells were inoculated subcutaneously into the back area of male nude mice. After the inoculation, mice were randomized into 3 groups and given drinking water with apocynin (0, 100 and 500 mg/L) for 4 weeks. The body weights and tumor volumes of each mouse were estimated every week. Mice were sacrificed at experimental week 4. At the sacrifice, implanted tumors and the liver, lung, kidneys, and lymph nodes were removed, and the size of primary tumors was measured. At least 1 section of each tissue was stained with HE, and implanted tumors were processed for Ki67 and CD31 immunostaining for analysis of cell proliferation and angiogenesis, respectively, and TUNEL assay for apoptotic activity.

During the experimentation, primary tumors were growing up with time, and which was suppressed by apocynin treatment. There were no differences of body weights and water consumption among the groups. At the sacrifice, the size of implanted tumors was found to be reduced in a dose dependent manner. The necrotic area, cell proliferation and vessel number were decreased by apocynin treatment. No increase of apoptosis in the tumor cells was noted. Incidence of metastatic tumors in the lungs and lymph nodes tended to be decreased in apocynin treated mice.

In conclusion, the present data suggest that apocynin possess a potential of anti-cancer property for castration resistant prostate cancer. We need further study to explore precise mechanisms underlining observed anti-cancer action of apocynin.

WS-05

Establishment of a new invasive urinary bladder cancer model Identification of invasion-associated protein using human c-Ha-ras proto-oncogene transgenic rats

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To establish animal models for identifying mechanisms of urinary bladder cancer (UC) invasion, male human c-Ha-ras proto-oncogene transgenic rats (Hras 128) were divided into 5 groups and treated with 0.05% *N*-butyl-*N*-(hydroxybutyl)nitrosameine (BBN) in drinking water and/or 0.1% phenylethyl isothiocyanate (PEITC) in diet, respectively, as follows: BBN (8 wks)→PEITC (8 wks); PEITC (8 wks)→BBN (8 wks); PEITC alone (16 wks); BBN alone (16 wks); non-treatment. At the end of week 16, higher incidence and number of invasive UC was observed in BBN→PEITC group compared to the BBN alone group. To identify the invasion-associated proteins of bladder cancer, proteome analysis was performed to compare the protein profiles of invasive and non-invasive UC of Hras 128 rats. We identified 32 proteins that overexpressed in invasive UC but not in non-invasive UC compared to normal bladder urothelium. Immunohistochemical analysis of carbonic anhydrase 2 (CA2), one of the above proteins, showed that incidence of CA2-positive UC was significantly higher in invasive UC compared to the non-invasive UC in rats. Furthermore, expression of CA2 was evaluated in 235 human UCs by immunohistochemistry. Incidences of CA2-positive UC were 0, 15%, 13%, 42%, and 65% in pTis, pTa, pT1, pT2 and pT3 UCs, respectively. Expressions of CA2 were significantly high in muscle invasive UC (pT2 and pT3) compared to pTis and pTa. These findings suggest that CA2 is an invasion-associated protein and could serve as a maker of aggressive and a potential therapeutic molecular target in urinary bladder cancer. In summary, treatment of Hras 128 rats with BBN followed by PEITC induced high incidences of invasive UC, and therefore provide a useful model for explore the mechanisms of UC invasion.

WS-06

Expression of Diagnostic Biomarkers in Central and Peripheral Squamous Cell Carcinoma of the Lung. Are They Different?

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BACKGROUND: Presence of specific biological characteristics and specific tareted molecule is critical to judge use of molecular target drugs. Also, examining high-throughput technique such as tissue microarray is important to predict positive and possible adverse effects for disease of interests. Lung adenocarcinoma has huge progress for current semi-personalized therapy. But squamous cell carcinoma (SCC) does not have effective molecular target drug. Referring to recent reports, pSCC may be biologically different from cSCC, but the evidence is weak. If they are biologically different pemetrexed and bevacizumab, currently prohibited drugs due to strong adverse effect, may be useful to pSCC.

DESIGN: 50 cases with SCC resected by lobectomy were collected. cSCC was defined as a tumor from trachea to segmental bronchi and pSCC as in more peripheral location.Tissue Microarray containing triplicated 0.6mm cores from each case were composed. Immunohistochemical staining for CK7,TTF1, p63, CK14, Napsin A, CK34βE12, CK5/6, and p53 were performed. Levels of entrapped pneumocytes inside the core were also scored by observing CK7.

RESULTS: Immunohistochemical patterns were identical between cSCC and pSCC, and none showed statistically significant difference. Observed single difference was a presence of entrapped pneumocytes highlighted by CK7 predominantly in pSCC (p=0.04). The-5-year survival showed no difference in the prognosis between cSCC and pSCC.

CONCLUSIONS: Immunohistochemical patterns and survival are not different between cSCC and pSCC. The central and peripheral SCC may not be biologically different except the ways of proliferation.

WS-07

Development of an analytical method for *in vivo* DNA adducts and its application to reporter gene transgenic rodents

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Liquid chromatography (LC) with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) using electron spray ionization (ESI) has been noted as a powerful tool to analyze chemical-specific DNA adducts for structural information and precise quantification. Development of transgenic rodents carrying reporter genes enables us to examine *in vivo* genotoxicity of chemicals while taking their biological behavior into consideration. In the present study, to understand molecular pathogenesis at an early stage of chemical carcinogenesis, we simultaneously examined DNA adduct formation and reporter gene mutations in *gpt* delta rodents. Lucidin-3-*O*-primeveroside (LuP)-, one of the components of madder color (MC), and estragole (ES)-specific DNA adduct formation were investigated by LC-ESI/MS after the reaction of each DNA base with the acetylated compounds produced by the mimic sulfotransferase metabolic pathway, and their precise chemical structures were determined by NMR analysis. Subsequently, quantitative analytical methods for the DNA adducts in *in vivo* samples were developed using LC-ESI-MS/MS. Modification by lucidin (Luc) at the N^2 and N^6-position of dG and dA, respectively, were found as the specific DNA adducts. In addition to the two reported adducts, ES-3'-N^2-dG and ES-3'-C8-dG, a new adduct ES-3'-N^6-dA was found. These newly developed quantitative methods for each adduct were able to detect at levels of several adducts per $10^{8\sim9}$ unmodified bases. Quantitative analysis of the specific DNA adducts and reporter gene mutation assays for each carcinogenic site was performed in *gpt* delta rodents administered LuP, MC or ES. Luc-N^2-dG and N^6-dA adducts were detected in the kidneys of rats treated with not only LuP but also MC, indicating that LuP might participate in MC-induced renal carcinogenesis. However, the fact that the mutation spectrum of *gpt* mutants induced by LuP was not identical to that by MC might imply the existence of other contributing components to MC carcinogenesis. Although ES-3'-N^2-dG, C8-dG and N^6-dA adducts were detected dose-dependently in the livers of ES-treated *gpt* delta mice, there was not a clear dose-dependent in increases of *gpt* mutant frequencies. The mechanisms underlying lack of a quantitative relationship between DNA adduct formation and subsequent gene mutation are to be determines hereafter.

WS-08

Involvement of Epigenetic Alteration in the Enhancing Effects of Chromated Copper Arsenate on Ultraviolet B-induced Skin Carcinogenesis

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Chromated copper arsenate (CCA) contains arsenic and chromium, and has generated public concerns over possible risk of skin cancer. CCA enhanced ultraviolet B (UVB)-induced skin carcinogenesis in a dose-dependent manner in SKH-1 hairless mice; particularly, CCA increased the incidences of moderately/poorly differentiated squamous cell carcinoma (SCC) and 8-OHdG levels of epidermis. To clarify the mechanism of the enhancing effects of CCA, gene expression profiling was performed in the tissues of epidermal hyperplasia and SCC in the UVB+CCA 0 and 90 ppm groups. Microarray analysis and quantitative RT-PCR demonstrated that CCA overrode genes involved in apoptosis, DNA repair, and antioxidant effect, all of which were enhanced by UVB. Next, to elucidate the involvement of epigenetic alteration in the gene expression, we quantitatively analyzed DNA methylation and histone acetylation in the CpG island nearby transcription starting site of p53 (apoptosis/DNA repair), Nrf2 and Msra (antioxidant), and Ogg1 (DNA repair/antioxidant). As a result, CCA hypermethylated CpG islands in Nrf2 and Ogg1, whereas CCA tended to increase histone H3 or H4 acethylation levels in CpG islands of p53, Nrf2, Msra, and Ogg1, suggesting that the regional DNA methylation, but not histone acetylation, were related to transcriptional silencing of these genes. Additionally, analysis of global DNA methylation status revealed that CCA enhanced global DNA hypomethylation even at the early stage of carcinogenesis. These results suggest that both regional hypermethylation and global hypomethylation may play important roles in the enhancing effects of CCA on the skin carcinogenesis.

WS-09

Possible Modes of Action Underlying Ochratoxin A-induced Renal Carcinogenesis

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Distribution of ochratoxin A (OTA), a mycotoxin, is very eccentrically located at the outer stripe of outer medulla (OSOM) possibly due to the kinetics of specific transporters. OTA can induce renal tumors that originate from the S3 segment of the proximal tubules, a main component of OSOM. However, the results of conventional mutagenicity tests have caused controversy regarding the role of genotoxic mechanisms in the carcinogenesis. In the present study, we have noted the site specificity of OTA. We conducted the reporter gene mutation assay and global gene expression in the cortex (COR) and the outer medulla (OM) in the kidney of *gpt* delta rats carrying *gpt* and *red/gam* (Spi^-) genes, given OTA at a carcinogenic dose. As a result, Spi^- mutant frequencies (MFs), indicating an increase in deletion mutations, but not *gpt* MFs in the OM were significantly higher than in controls despite the absence of changes in the COR. In addition, to identify genes relating to OTA-induced carcinogenesis, comparison of gene expression changes between the COR and OM in response to OTA was performed. Subsequently, changes in the levels of some mRNAs were confirmed by real time RT-PCR methods. In the comprehensive analysis, 1796 genes in the COR and 1726 genes in the OM were up- or down-regulated with $P < 0.05$ and fold change ≥ 1.5. Up-regulated genes observed in only the OM were as follows: genes associated with DNA double strand break repair (*Chek1*, *Rad18*, *Brip1*, and *Brcc3*, etc.), cell cycle progression (*Cyclins E1*, *A2*, and *B1*, etc.), G2/M arrest in response to DNA damage (*Chek1* and *Wee1*), Bcl-2 family (*Bak1* and *Bik*), and regulation by p53 or control of p53 function (*Phlda3* and *Chd8*, etc.). Significant changes ($P < 0.05$, fold change ≥ 1.5) in mRNA levels for many of these genes were confirmed by real time RT-PCR methods. In the group treated with OTA, significant changes of *Chek1* and *Rad 18* mRNA levels were characteristically observed in the OM, suggesting that OTA-induced deletion mutations might occur in the process of DNA double strand break repair. The observation of changes in several genes associated with DNA damage might imply that genotoxic mechanisms are involved in OTA-induced renal carcinogenesis.

WS-10

Functional Analysis of GPC3 as A Target Molecule for Noble Antibody Therapeutic

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Because antibody therapeutics are one of the major molecular target agent, it is crucial to know the molecular function of the target in the process of drug development. Currently, we are in the process of development for noble antibody therapeutics against one of membrane bound heparan sulphate proteoglycan, glipican 3 (GPC3) which is an onco-fetal protein expressed in more than 70% of hepatocellural carcinoma (HCC). On the other hand, since the biological functions of GPC3 were only scarcely known, we have been investigating its function on the HCC biology histopathologically.

First of all, we observed the detail GPC3 expression pattern in clinical HCC samples, and found that the specimens with fine membrane expression and with ambiguous membrane expression could be distinguishable. Further observation with this distinction revealed that the specimens with fine membrane expression pattern have large number of macrophage infiltration. Based on these observations, we established GPC3 transfectant cell line from GPC3 negative parent HCC cell line, and compared the relationship between its GPC3 expression pattern and level of macrophage infiltration. Since large number of macrophage were infiltrated in GPC3 expressing xenograft tissues same as in human HCC, we used this mice as a model to assess the characteristics of tumor infiltrating macrophage, and found the characters of the macrophage within the GPC3 expressing xenograft tissue were similar to that of tumor associated macrophage (TAM) which currently assumed to be “tumor promotive”.

Based on these results, we concluded that biology of the membrane expressed GPC3 in HCC, at least partially, were induction of TAM, and because of this biology, the molecule is considered to be very attractive target for antibody therapeutics to treat HCC.

WS-11

Expression profiling of iron-regulatory factors in thioacetamide-induced rat acute hepatic injury

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Attention has recently been focused on hepatic iron overload as a progression factor for chronic liver disease; however, the pathogenesis of iron dysregulation in hepatic disorders is still unclear. Here we analyze expression profiles of iron-regulatory factors in thioacetamide-induced rat acute hepatic injury, and investigate the role of the factors in transient iron dysregulation. Seven-week-old male F344 rats received a single intraperitoneal injection of thioacetamide at a dose of 300 mg/kg BW. Liver and serum samples were collected at 10 hours, 1, 2, 3, 5, 7 and 10 days after injection. Tissue sections were made from left lateral lobe of the liver for HE, iron histochemistry, and immunohistochemistry. Liver and serum iron levels were quantified from right lateral lobe and serum samples, respectively. Quantitative PCR and Western blot were conducted from right medial lobe to analyze expression profiles of iron-regulatory factors. In HE stain there were centrilobular necrosis of hepatocytes from 1 day postinjection (1DPI), and marked inflammatory infiltrates in the centrilobular area from 2DPI. These lesions were reduced from 5DPI and were recovered at 10DPI. Serum iron levels were elevated at 1DPI and returned to normal from 2DPI, while liver iron content gradually increased until 5DPI and returned to normal from 7DPI. Hepcidin expression levels peaked at 2DPI, which was accompanied by increased expression of transferrin receptor 1 and ferritin subunits. Ferroportin expression was increased at 3DPI. Consistent with acute hepatic injury, transient hepatic iron overload was observed. It suggests that the recovery from iron overload is achieved by upregulation of hepcidin, the key regulator of iron metabolism, and increases in cellular iron uptake and storage, and subsequent export of excess iron.

WS-12

Effects on the liver induced by the phase II drug-metabolizing enzyme inducers

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Drug metabolizing reaction is cosisted of two phases; phase I and phase II drug-metabolizing enzymes, and is important in toxicological evaluation. Many reports on the induction of phase I drug-metabolizing enzymes have been published so far. However, relatively little research has focused on the induction of phase II drug-metabolizing enzymes compared with phase I drug-metabolizing enzymes. In the present study, phase II drug-metabolizing enzyme inducers such as butylated hydroxyanisole (BHA) and 1, 2-bis(2-pyridyl)ethylene (2PY-e) was administered to male F344 rats (11 weeks-old) for up to 14 days, and the effects on the liver was compared to those treated with phenobarbital which induces both phase I and phase II enzymes. BHA and 2PY-e caused an increase in liver weight without apparent morphological changes including electron microscopic examination. Immunohistochemical examinations revealed that that BHA and 2PY-e induced GST Yp, not expressed in the normal hepatocytes, in the hepatocytes of the periportal and centrilobular area, respectively. Significant increase in the BrdU labeling indices were transiently found in the hepatocytes on Day 3 and double immunostaining confirmed that higher labeling indices were observed in the GST Yp-positive hepatocytes. In the GeneChip and 2D-DIGE analyses in the liver treated with BHA, transient increase in the cell proliferation related genes or proteins such as cell cycle, signal transduction and transcription was observed from Day 1 to Day 2, and formyltetrahydrofolate dehydrogenase involved in G1 cell cycle arrest was highly expressed on Day 4 and later. In conclusion, these results suggested that increased liver weight observed in the liver treated with phase II drug-metabolizing enzyme inducers mainly caused by hepatocyte proliferation and GST Yp induction might have some kind of relationship with hepatocyte proliferation.

WS-13

Hematopoietic toxicity of T-2 toxin in mice

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T-2 toxin is a kind of trichothecene mycotoxins produced by species of the genus Fusarium, and affects proliferating cells. Oral, parenteral and cutaneous exposures of trichothecene mycotoxin produced lesions in hematopoietic, lymphoid and gastrointestinal tissues and functional suppression of reproductive organs. Five-week-old female ICR:CD-1 mice were inoculated orally with 10 mg/kg b.w. of T-2 toxin. Hematological, blood biochemical examinations, bone marrow analysis and histopathological examination of the thymus, spleen, bone marrow and liver were done up to 48 hours after treatment (HAT). In addition, microarray analysis was done on the gene expression profile of the liver at 0.5, 3 and 24 HAT. The numbers of WBCs and platelets in T-2 toxin-treated animals were significantly depressed at and after 6 HAI. The numbers of RBC showed no significant changes. The coagulation test revealed the prolongation of both prothrombin time (PT) and activated partial thromboplastin time (APTT). In the T-2 toxin-treated group, the levels of AST and ALT increased while those of total cholesterol, total protein, blood glucose and fibrinogen decreased.
Histopathologically, T-2 toxin-induced lesions in the thymus, spleen, bone marrow and liver of mice were shown to be brought about by apoptosis of component cells. In the bone marrow and splenic red pulp showed a significant hypocellularity. In the bone marrow, the number of myelocytes significantly decreased due to loss of immature granulocytes, erythroblasts and lymphocytes. Microarray analysis on the liver revealed the up-regulated expression of oxidative stress-, cell cycle- and apoptosis-related genes and the down-regulated expression of lipid metabolism-, glycogen metabolism-, drug metabolism- and blood coagulation-related genes.

WS-14

Studies on Eosinophilic Substance in the Mouse Nasal Septum

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An eosinophilic substance is remarkably observed in the mature mouse nasal septum. Although it has been described as amyloid in some textbooks, there are no reports demonstrating it by Congo red stain or an ultrastructural examination. Meanwhile, some reports described it as a non-amyloid substance because there was a negative reaction with Congo red stain. In these reports, however, detailed investigations concerning the eosinophilic substance were not done. In this study, characteristics of the eosinophilic substance in the mouse nasal septa were investigated histologically and ultrastructurally.

Our studies clarified the following characteristics concerning the eosinophilic substance.

(1) The eosinophilic substance was not amyloid. It consisted of not only collagen but also a complex carbohydrate that was amorphous material ultrastructurally. In addition, the eosinophilic substance deposition may be a physiologic phenomenon.

(2) It was suggested that the complex carbohydrate was produced by the vomeronasal gland epithelial cells, accumulated at the basal portion, and migrated to the interstitium through a partial opening of the cell membrane and the basement membrane.

(3) The grades of the eosinophilic substance deposition were more enhanced in males than in females of the same age, indicating the grades have a sex difference. The grades increased with aging but reached a plateau by 58 weeks in males and 34 weeks in females, indicating the increase has a limit and did not show in seniles. In addition, the eosinophilic substance deposition may be a mouse specific phenomenon as it has been reported in mice only. Although the nasal septum of rats, guinea pigs, dogs, marmosets, and cynomolgus monkeys was examined, no eosinophilic substance was observed in these animals.

It is suggested that the eosinophilic substance has no effects on the function of the vomeronasal organ, a chemoreceptor for pheromone-mediated behavior, because the eosinophilic substance did not secrete into the lumen but into the interstitium of the vomeronasal gland. However, it was possible that the eosinophilic substance might deposit in response to certain action from and/or through the vomeronasal organ.

WS-15

Pathological Studies on Glomerulonephropathy Induced by Bolus Injection with Dibasic Sodium Phosphate Solution in Rats

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Phosphate has been widely used for drug substances as an electrolyte replenisher and as a buffer vehicle for injectable medicines. However, there is little toxicity data for dibasic sodium phosphate (Na_2HPO_4, CAS number: 7558-79-4), which is a form of phosphate. The objective of this study was to elucidate the nephrotoxicity of dibasic sodium phosphate in Sprague-Dawley rats.

Phosphate solution was given to Jcl:SD rats by daily bolus intravenous administration at concentrations of 0, 1, 25, 250 or 360 mM (0, 1, 28, 284 or 408 mg/kg Na_2HPO_4) at 8 mL/kg for 2 weeks. Urinalysis revealed mild to moderate proteinuria and histopathology showed panglomerular calcification, accompanied by degeneration of the glomerular epithelium and parietal epithelium in the 250 and 360 mM groups.
To investigate the early changes involved in glomerular calcification, 360 mM phosphate solution administered to Jcl:SD rats singly or repeatedly. Proteinuria was detected from day 3. Following single dosing of phosphate solution, electron microscopy revealed a number of vacuoles scattered within the Bowman's space. On day 4, minimal and focal mineralization was observed within the podocytes and parietal epithelial cells. On day 8 and 9, mineralization was minimal to mild and localized within the parietal epithelial cells and glomerular basement membrane. Increased urinary protein excretion correlated well with the glomerular changes. Up-regulation of *Kim1*, *Spp1* and *A2m* on day 8 is considered to be closely related to early onset of renal tubular injury by phosphate solution although no histopathological lesions were detected in the renal tubules.
Jcl:SD rats received tail-vein injections of 360 mM phosphate solution for 2 or 4 weeks. Persistent proteinuria developed without remission even after 2-week withdrawal in the phosphate-treated groups. Phosphate-treated animals developed lipemia and anemia on day 29. Histopathologically, glomerular changes consisted of mineralization in whole glomeruli, glomerular capillary dilatation, partial adhesion of glomerular tufts to Bowman’s capsule, and mesangiolysis. Marked tubulointerstitial lesions were tubular regeneration and dilatation, protein casts, mineralization in the basement membrane, focal interstitial inflammation, and fibrosis in the cortex.

High-dose phosphate transiently overloads the glomerular epithelium after filtration through glomerular capillaries and may produce insoluble calcium salt and regressive lesions, resulting in glomerular proteinuria. Clinical and morphological changes by phosphate treatment for 4 weeks were similar to features of human nephrotic syndrome.

WS-16

Morphological Characteristics of Luteal Toxicity in Rats Treated with Ethylene Glycol Monomethyl Ether, Atrazine, or Bromocriptine

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[Introduction] Ethylene glycol monomethyl ether (EGME), atrazine, and bromocriptine (BRC) are known as ovarian toxicants that target the corpora lutea (CL) in rats after repeated administration. In the 27th Annual Meeting of JSTP, we had reported that EGME and atrazine might have a direct stimulatory effect on new CL. Our findings suggested the possibility that EGME plays an indirect role in the luteal stimulatory pathway by increasing prolactin (PRL) secretion. The aim of this study was to morphologically characterize the luteal effects of EGME, atrazine, and BRC in detail with light and electron microscopic examinations in rats. This study also examined the direct effect of EGME on luteal cells *in vivo* under a PRL inhibitory state induced by co-administration of EGME and BRC.

[Materials and Methods] EGME (300 mg/kg), BRC (2 mg/kg), EGME+BRC, or atrazine (300 mg/kg) was daily administered to normal-cycling SD rats for 7 days. The ovaries were microscopically and ultrastructurally analyzed.

[Results and Discussion] Microscopically, EGME, EGME+BRC, and atrazine induced luteal hypertrophy in both new and old CL; the hypertrophied CL were classified into 2 types. In type I hypertrophied CL (CL I), the luteal cells contained prominent fine vacuoles, and in type II hypertrophied CL (CL II), the cells contained eosinophilic cytoplasm without vacuoles. In the EGME group, CL II were mainly observed, whereas CL I were predominant in the atrazine group. BRC increased the number of old CL. Ultrastructurally, the luteal cells uniformly contained lipid droplets, smooth endoplasmic reticulum (SER), and mitochondria in the control group at diestrus; however, CL I in the EGME, EGME+BRC, and atrazine groups contained abundant lipid droplets. CL II in the EGME and EGME+BRC groups were characterized by uniform and well-developed SER. No clear difference was observed between control CL and atrazine-treated CL II. These results indicate that EGME, atrazine, and BRC have different effects on luteal morphology. The luteal hypertrophy caused by co-administration of EGME and BRC supported the possibility that EGME directly affects luteal cells *in vivo*.

WS-17

Transitional Gene Expression Profiling of Ovarian Follicle in Rats Treated with Indomethacin and RU486

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Background: Single dosage on the proestrus day of indomethacin (IM, dual inhibitor of cyclooxygenase 1 and 2) or RU486 (RU, progesterone receptor antagonist) can induce unruptured follicle (UF) in rats. However, the morphology of follicular epithelium is different, and those in IM-treated rats are close to normal corpus lutea, and those in RU-treated rats look like graafian follicle, i.e., theca cells and granulosa cells in IM-treated rats are larger than those in RU-treated rats, and the borderline between theca cell layer and granulosa cell layer is blurry in IM-treated rats, whereas it's distinct in RU-treated rats. In the previous study, we determined transitional gene expression profile of ovarian follicles in normal rats during ovulation. In the present study, we characterized the difference of gene expression profile between RU-treated and IM-treated rats.

Materials and Methods: 21 female Crl:CD(SD) rats (8 weeks old) were used. Estrous phases of each animal was determined by vaginal smears for five consecutive days. RU (100 mg/kg) and IM (4 mg/kg) were orally administered at 10:00 and 15:00 on the proestrus day respectively, and ovaries were removed at 22:00 on the proestrus day and 10:00 on the estrus day, embedded in OCT compound and snapped frozen. Ovaries were also collected from untreated rats at 22:00 on the proestrus day, and 10:00 on the estrus day as a control. Each group consisted of three animals. Granulosa cell and theca cells of graafian follicle and peri-ovulatory follicle on the proestrus day, and post-ovulatory follicle were collected by Laser Micro Dissection technique, and total RNA was isolated from these follicles to be subjected to GeneChip（Affymetrix Rat 230.2.0 Array) analysis.

Results and Discussion: Comparing transitional gene expression profiles of untreated rats with those of RU-treated and IM-treated rats, statistically significant changes of genes were identified.

In IM-treated rats, genes for hyaluronan and proteoglycan link protein were down regulated, which may be related to insufficient follicle fluid production and seemed critical for rupture for follicles.

In RU-treated rats, many genes involved in lipid metabolism and steroid synthesis were affected, which is thought to correspond to immaturity of follicular cells. In addition, down-regulation of endothelin-2 and a disintegrin and metalloproteinase with thrombospondin motif 1 and 9 are observed, which were known to be indispensable for ovulation.

To analyze the difference of these profiles further, would make it possible to find genes or pathway which play important roles in the ovulatory impairment.

P-001

Calponin Expression in Cisplatin-Induced Rat Renal Tubulointerstitial Fibrosis, in Correlation with Myofibroblasts

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Myofibroblasts play an important role in fibrosis by producing excessive amounts of extracellular matrix. In renal tubulointerstitial fibrosis, besides the pre-existing interstitial fibroblasts and perivascular undifferentiated mesenchymal cells, renal tubular cells may be a possible origin of myofibroblasts via the epithelial-mesenchymal transition (EMT). However, the detailed mechanism remains to be investigated. Calponin is a smooth muscle-specific, actin-, tropomyosin- and calmodulin-binding protein, and the protein is implicated in the regulation and modulation of smooth muscle contraction. Calponin may be involved in myofibroblastic differentiation in fibrotic tissues. Little is known about the detailed expression of calponin in renal tubulointerstitial fibrosis. In this study, we immunohistologically investigated calponin expression in rat renal interstitial fibrosis, in correlation with myofibroblastic marker expressions as vimentin, desmin and α-smooth muscle actin (α-SMA). Renal interstitial fibrosis model was induced by a single dose of cisplatin (CDDP; 6 mg/kg BW) in 6-week-old male F344/DuCrj rats. Renal samples were corrected on 1-60 days after the injection; the samples were processed in periodate-lysine-paraformaldehyde (PLP) fixative, paraffin embedded by the AMeX method (PLP-AMeX method), or cryopreserved. In CDDP-induced lesions, calponin expressing cells appeared around injured and regenerating renal tubules in the cortico-medullary junction on day 9. Additionally, the injured epithelial cells expressed calponin. Calponin expressing cells increased with time. Calponin expressing interstitial cells also showed vimentin, desmin or α-SMA expression. Although injured epithelial cells express both vimentin and calponin, they did not react to desmin nor α-SMA, simultaneously. Along with intermediate skeletons, this study demonstrates that calponin is expressed in myofibroblasts, and that the protein may play a role in myofibroblastic differentiation through EMT.

P-002

Expression patterns of Biomarkers of renal injury in Cisplatin induced Rat Acute Renal Failure Model

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Osteopontin (OPN) or neutrophil gelatinase-associated lipocalin (NGAL) is one of the biomarkers of acute renal injury. The purpose of this study was to examine the expression patterns of OPN and NGAL in the cisplatin (CDDP)-induced rat acute renal failure model.
F344 rats were injected a single dose of CDDP (6 mg/kg body weight) intraperitoneally. On days 1, 3, 5, 7, 9, 12, 15, 20, 25 and 35 after the CDDP dosing, kidneys were removed. Histopathological changes were mainly observed in the proximal tubules in the cortico-medullary junction. Necrosis and apoptosis begun to be seen on day 1, and these changes were most apparent on day 5. Regeneration was observed from day 5. The expression of OPN was strongly observed from day 5, and mainly observed in the regenerating and dilated epithelial cells. mRNA expression of OPN was also increased from day 5 to 35. On the other hand, immunohistochemical and mRNA expression of NGAL were already increased from day 1. The NGAL immuno-expression was observed in the affected tubules and epithelial cells around the affected tubules.
These results indicated that the expression of NGAL was immediately increased after CDDP injection, but increase of OPN was later (on day 5 when necrosis and regeneration were clearly observed). OPN might be associated with the regeneration and differentiation of affected renal tubule; NGAL might play a protective role against CDDP induced renal injury. Further studies are in progress to clarify the role of OPN and NGAL.

P-003

Pathobiological Study of Nephrotoxicity due to Polymyxin B

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[Objective] Multidrug-resistant *Pseudomonas aeruginosa* (MDRP) is defined as *P. aeruginosa* showing resistance to carbapenems, aminoglycosides and fluoroquinolones. Although nosocomial MDRP infection has become a clinical problem, only a few antibiotics are effective against MDRP. Among these antibiotics, polymyxins are thought to be the most effective. However, polymyxins are reportedly associated with nephrotoxicity in patients with a background of renal impairent. Since polymyxins were not subjected to the drug development processes for compliance with contemporary regulatory requirements, there is little information about their toxicities, including nephrotoxicity, in laboratory animals. In the present experiment, we focused on nephrotoxicity due to polymyxins using rodents given a high dose of polymyxin B intravenously or subcutaneously.
[Methods] To clarify nephrotoxicity due to polymyxin B, a single intravenous dose and 14-day repeated intravenous dose toxicity studies were conducted in mice and rats, and a 14-day repeated subcutaneous dose toxicity study was carried out in rats. The dosage levels employed were 0, 5 or 10 mg/kg as the single intravenous dose, 0, 0.1, 0.3, 0.6, 1.2 or 2.5 mg/kg/day as the repeated intravenous doses, or 0, 2.5, 5, 10 or 20 mg/kg/day for the repeated subcutaneous dose study. Clinical observations, hematological and blood biochemical analyses, necropsy, body and organ weight measurements and pathological examinations were performed.
[Results] No nephrotoxicity was seen in the single intravenous dose or repeated intravenous dose toxicity studies. In contrast, animals treated with repeated subcutaneous doses of 20 mg/kg/day for 14 days showed decreased locomotor activity, cyanosis and deep respiration, as well as elevated plasma creatinine and blood urea nitrogen. On histopathological examination, pyknosis, basophilic change (regeneration) and sloughing (necrosis) of the proximal tubular epithelium, cellular/protein casts and tubular dilatation were noted.
[Conclusion] Polymyxin-associated nephrotoxicity was not observed with a single large dose or repeated intravenous doses, but did occur with repeated subcutaneous doses of polymyxin B. These results suggest repeated subcutaneous polymyxin B injection of rats to be a useful model for analyzing nephrotoxicity due to polymyxins in humans.

P-004

Characterization of the Cultured Podocytes derived from Osborne–Mendel Rats and the Response of their Actin Cytoskeleton to Angiotensin II

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[Introduction] According to the classical concept of the rennin–angiotensin system (RAS), anigiotensin II (Ang II), which is the final bioactive product of the blood-borne cascade, plays an important role in the maintenance of systemic blood pressure and fluid balance. Recent studies have revealed that the local angiotensin-generating system (so-called tissue RAS) can act locally in the glomerulus, and its action are independent of the circulating RAS. Osborne–Mendel (OM) rats develop mild hypertension and progressive glomerular injury with early-onset proteinuria. Our previous study suggested that podocyte injury in these rats may be a key determinant for progressive glomerulopathy; furthermore, our experimental study using RAS inhibitors indicated that activation of tissue RAS in the glomeruli of these rats may directly induce podocyte damage. In this study, we examined the characteristics of the cultured podocytes of OM rats and their response to AngII.

[Materials and Methods] Primary cultured podocytes derived from seven-week-old male OM rats and age-matched normal F344 rats were used for this study. Immunohistochemical examination and real-time polymerase chain reaction were used to analyze the expression of podocyte-associated proteins (nephrin, podocin, and synaptopodin) and Ang II type 1 receptor (AT1R) in the cultured podocytes. To evaluate the effects of Ang II on the expression of nephrin and on the actin cytoskeleton of the cultured podocytes, the cells were treated with $1 \times 10^{-8} – 10^{-6}$ mol/L of Ang II. The actin cytoskeleton was visualized by phalloidin staining.

[Results] The expression of podocyte-associated molecules was comparable between cultured podocytes derived from OM and those derived from F344 rats. However, AT1R mRNA expression was significantly higher in OM rat-derived podocytes than in F344 rat-derived podocytes. Although Ang II lowered mRNA expression of nephrin in cultured podocytes derived from both strains of rats, the downregulation was more significant in OM rat-derived podocytes. In addition, reorganization of the actin cytoskeleton by Ang II was observed in cultured podocytes derived from both strains of rats; however this phenomenon was more marked in OM rat-derived podocytes than in F344 rat-derived podocytes.

[Conclusion] The results of our study suggest that local activation of RAS in the glomeruli of OM rats may induce reorganization of the actin cytoskeleton in podocytes, leading to podocyte injury and progressive glomerulopathy in these rats.

P-005

Morphometrical Study for Podocytes on Gender Difference in SD Rats Kidney Glomeruli

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Up today, in Japanese human beings, the overall frequency of primary glomerulonephritis (GN) was 77.8%, and the ratio of renal function decline and outcome favored women over men in GN. These differences were not dependent of blood pressure and proteinuria, and then the gender difference concerning in GN of human beings has been unclear. In the present study, to clarify the gender differences the normal morphology of adult rat kidney glomeruli, we investigated the morpholometrical characteristics of the kidney glomeruli of adult SD rats by light microscopically observations with serial sections using three-dimensional morphostatistical analyses systems (Solution Systems, Inc., Japan). The present study was performed by the Register 2001 (Vey Tek, Inc., USA), the OZ/ 3D Reconstruction System V.3.01 (Rise, Inc., USA), and the Vox Blast 3.1 (Vey Tek, Inc., USA) on Macintosh and Windows computers. The relative weight of each rat kidney was similar between males and females. The number of the renal glomeruli of female rats was significantly higher than that of male rats, although the diameter of the renal glomeruli of female rats was significantly smaller than that of male rats. Moreover, the relative appearance ratio of podocytes in renal glomeruli of female rats was significantly higher that of male rats. The podocytes have been important cells for maintaining the normal structures and functions of the glomeruli. Recently, podocyte injury plays important roles in the pathogenesis of nephropathy, and podocyte number has conducted the expression of podocyte-associated molecules, suggesting podocyte number may play an important role on the pathogenesis in nepherosclerosis and diabetic nephropathy. Moreover, podocyte injury is a feature of GN has been used as prognostic factor of glomerular disease. The present study showed that there were gender differences in kidney glomeruli on adult rats, and these differences might be involved in the pathogenesis of glomerulonephritis.

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P-006

Intranuclear and Cytoplasmic Inclusions in Proximal Tubular Epithelium of the Kidney in Aged Wistar Hannover Rats

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[Introduction] We report cases presenting intranuclear and cytoplasmic inclusions in renal tubular epithelium in untreated 110-week-old Wistar Hannover rats.

[Materials and methods] The cases were 2 male and 1 female RccHan^{TM}: WIST rats (Japan Laboratory Animals, Inc.) from a long term study for collection of background data. The tissues were fixed with neutral formalin and examined histopathologically. Small pieces of tissue from the kidneys were also subjected to electron microscopic examination.

[Results] Intranuclear and cytoplasmic inclusions occurred diffusely in proximal convoluted tubules and proximal straight tubules and were characterized by eosinophilic and homogenous round corpuscles of a size comparable to that of a nucleus or less. Cytoplasmic inclusions appeared in both basal and luminal sides. Both intranuclear and cytoplasmic inclusions were negative for PAS staining. Ultrastructurally, both intranuclear and cytoplasmic inclusions consisted of homogenous and amorphous materials with a low-electron density, without a limiting membrane. There were no associated degenerative changes in the kidneys, and no similar changes were observed in the other organs. Furthermore, no clinical abnormality was recorded in any case.

[Discussion] Recently, RccHan^{TM}: WIST rats have been introduced into our country. To our best knowledge, no similar case has been reported in Europe where has been done many studies using Wistar Hannover rats. Therefore, it is not clear whether the change observed is one of the background lesions in this strain. Morphological characteristics indicate that the inclusions observed in proximal tubular epithelium might be biological materials but not virus particles or heavy metals. Further studies are needed for clarification.

P-007

Urinary Cystatin C as a Biomarker for Diabetic Nephropathy and its Immunohistochemical Localization in Kidney in Zucker Diabetic Fatty (ZDF) rats

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Cystatin C, a kind of cysteine protease inhibitor, is a novel biomarker of renal damage. In the present study, the urinary and plasma levels of cystatin C were measured to evaluate the usefulness for the detection of diabetic nephropathy in Zucker diabetic fatty (ZDF) rats, and were compared with those of other biomarkers (β2-microgrobulin, calbindin, clusterin, EGF, GST-α, GST-μ, KIM-1, NGAL, osteopontin, TIMP-1, and VEGF).

The urinary cystatin C level in ZFD rats was higher than that in control lean rats at diabetic early stage, and increased further according to the progression of diabetic nephropathy, though the plasma cystatin C level hardly changed. On the other hand, the plasma urine nitrogen and creatinine levels did not changed during the expansion of renal damage in ZFD rats. In addition, urinary levels of β2-microgrobulin, calbindin, clusterin, GST-μ, KIM-1, osteopontin, and TIMP-1 were also higher at any diabetic stage.

We also investigated the localization of cystatin C in ZFD rats. Cystatin C was predominantly localized in the proximal tubules, and its immunohistochemical expression was not affected by the progression of diabetic nephropathy. In addition, cystatin C was also observed in the lumen of renal tubules after the progression.

In conclusion, urinary cystatin C measurements can detect early diabetic nephropathy as well as β2-microgrobulin, calbindin, clusterin, GST-μ, KIM-1, osteopontin, and TIMP-1. Immunohistochemical cystatin C expression in the proximal tubule was hardly changed according to the diabetic stage, but it was observed in the tubular lumen after the progression of diabetic nephropathy.

P-008

Identification of novel biomarkers of rat renal carcinogenesis

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A useful preneoprastic lesion biomarker for rat renal carcinogenesis has not yet been established. The aim of the present study was to identify novel biomarkers for predicting rat renal carcinogenesis. Thirty male Wistar rats at 6 weeks of age were administered 500 ppm N-ethyl-N-hydroxyethylnitrosamine (EHEN) in the drinking water for the first 2 weeks, and were sacrificed at 26 weeks. Renal tumors and paired normal surrounding tissues were collected from formalin-fixed paraffin-embedded kidney tissues by needle microdissection. Comparative proteome analysis of renal tumor and normal tissue was carried out with QSTAR Elite LC-MS/MS. A total of 319 proteins were differentially expressed in EHEN-induced renal tumors consisting 135 overexpression and 184 downexpression proteins compared to surrounding tissues. Immunohistochemical analyses of top 10 overexpression proteins in the proteome analysis revealed that S100A11 was overexpressed in atypical hyperplasia and tumor compared to normal tubular. These findings suggest that S100A11 might be a novel biomarker for rat renal carcinogenesis. Expression analyses of other proteins in renal carcinogenesis models are ongoing.

P-009

Inhibitory mechanisms of HDAC inhibitor on prostate cancer cell proliferation

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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 gene expression. HDAC inhibitors have been reported to suppress growth of several solid malignancies including lymphoma. We previously showed inhibitory effect of HDAC inhibitor on cell proliferation in human prostate cancer cell lines. In this study, we examined inhibitory mechanisms of HDAC inhibitor on prostate cancer cell proliferation. We employed LNCaP (androgen-dependent prostate cancer cell line), and Trichostatin A (TSA) as HDAC inhibitor.
In the results, cell proliferation of LNCaP was significantly reduced by treatment of TSA at every concentration of 0.1, 1 and 10 μM in a dose dependent manner. Western blot analysis revealed that expression of androgen receptor, Cyclin D1, and cdc2 protein were decreased by 0.1, 1 and 10 microM TSA in LNCaP. On the other hand, that of cleaved caspase 3 protein was increased at a dose of 1 and 10 microM. High expression of HDAC1, one of HDAC family which has been reported to associate with cancer cell proliferation, was immunohistochemically detected in surgically resected human prostate cancer tissue (8 HDAC1 positive cases in 10 cases).
Thus, the present data suggested that inhibitory effect of HDAC inhibitor on prostate cancer cell proliferation is associated with regulation of androgen receptor, cell cycle, and apoptosis-related protein expression. The present data also suggested that inhibitory effect may be different among individual cases.

P-010

Chemopreventive Effects of Purple Corn Color on Prostate Cancer

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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 color extracted from these products is widely used as a food colorant. Previous studies reported that purple corn color (PCC) has anti-cancer effects on colon and breast cancer. This study is an initial investigation on its effect on PCa.

Two PCa cell lines, LNCaP (androgen-dependent) and VCaP (androgen-independent), were treated with PCC in vitro. In both of the cell lines, PCC dose-dependently inhibited cell proliferation, and increased the proportion of cells in the G1 stage of the cell cycle, but did not induce apoptosis or necrosis. It was also found that the expression of PSA was dramatically decreased by PCC, while there was no change in AR expression. ChIP analysis showed that a high concentration of PCC inhibited AR binding to AREs (Androgen Response Elements) on target genes.

The TRAP (Transgenic Rat for Adenocarcinoma of Prostate) model was employed to study the anti-PCa effects of PCC in vivo. Six-week old TRAP rats were divided into 3 groups, and fed with control diet, 0.1% PCC in the diet and 1% PCC in the diet for 8 weeks. Rats consuming the 1% PCC diet showed a significant decrease of incidence of prostate adenocarcinoma. The mechanism of PCC-mediated inhibition of PCa in vivo is being analyzed and we will report progress in our presentation.

P-011

Assessment of Biomarkers in Gentamicin-induced Renal Lesions of Cynomolgus Monkeys

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Seven urinal biomarkers were claimed as acute renal injury biomarkers by Predictive Safety Testing Consortium, FDA and EMEA, but assessment of renal biomarkers in non-rodent has not been conducted due to limitations on the assay system. To assess renal biomarkers in monkeys, gentamicin was subcutaneously injected into 1 male or female monkey per group at 10, 30, 50 mg/kg and clinical biochemistry, urinalysis and histopathology were performed. For urine, 15 markers were examined using Human Kidney MAP v1.0 (Rules-Based Medicine Inc.). In addition, the western blot of urine and immunohistochemistry of the kidney were performed using anti-human NGAL antibody. Degeneration of proximal epithelium and tubular basophilia was observed at 30 mg/kg and more, and UN and CRE values increased for 2 to 3-fold in comparison to the pre-value. One male given at 30 mg/kg, additionally showed necrosis of proximal epithelium and hyaline cast. In urinalysis, ALP, LDH, NAG, GLU, PRO, IP, β2MG, clusterin, KIM-1 and microalbumin increased on Day 8 and calbindin and VEGF elevated on Day 14 for more than 10-fold, in comparison to the pre-value in the male showing tubular necrosis. On Day 14, β2MG, clusterin, KIM-1, microalbumin and TIMP1 increased from 16 to 965-fold at 30 mg/kg and more. The NGAL signal in the western bolt of urine increased on Day 8 and later. Degenerative proximal epithelium was stained strongly for NGAL, however, the intensity was reduced in the regenerative tubular epithelium. In conclusion, β2MG, clusterin, KIM-1 and microalbumin measured by Human Kidney MAP v1.0 were considered to be candidates for sensitive renal injury biomarkers and NGAL increased early as well as the parameters in urine of monkeys treated with getamicin.

P-012

Immunohistochemical Examination of Progressive Glomerulonephropathy in Common Marmosets (*Callithrix jacchus*)

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Progressive glomerulonephropathy occurs frequently in common marmosets (Callithrix jacchus). In this study, we investigated the renal glomeruli of 11 common marmosets (age range of 0 to 10 years old; 7 males and 4 females) using HE, PAS, PAM, and Masson's trichrome stains and transmission electron microscopy (TEM). Moreover, we investigated the involvement of IgM, IgA and IgG with this nephropathy by immunofluorescence technique using the fresh frozen sections. **Results:** The renal lesions of 11 common marmosets were classified into no light microscopic lesion (2 animals; 1 and 2 years old), minimal nephropathy (3 animals; 0, 3 and 8 years old), mild nephropathy (3 animals; 2, 4 and 7 years old) and moderate nephropathy (3 animals; 6, 7 and 10 years old) by the light microscopic observation. In cases classified into no light microscopic lesion, focal detachment of the loose inner layer of the glomerular basement membrane (GBM), flattening of the podocyte foot process and microvilli were observed by the TEM examination. The focal detachment of the loose inner layer was found mainly in the GBM adjacent to the paramesangium area, and occasionally in the GBM on the peripheral side. In immunostaining, IgM deposits were observed as fine granular or liner pattern at the GBM and mesangium area. IgA deposits were less frequent than IgM in the same region, but IgG deposits were not observed. In the minimal and mild nephropathy cases, the mesangial cells and matrix proliferated in the glomerular hilum. Ultrastructurally, irregularity of the GBM and flattening of the podocyte foot process were observed more extensively than in the cases classified into no light microscopic lesion. In immunostaining, IgM and IgA deposits were more remarkable. Furthermore, IgM and IgA deposited to the glomerular hilum in which proliferation of the mesangial cell and matrix was occurring. IgG deposits were slightly observed. In the moderate nephropathy cases, proliferation of the mesangial matrix expanded globally. Ultrastructurally, electron dense deposits were found in the GBM. In immunostaining, IgM and IgA deposits were extensively observed as fine granular, coarse granular and linear patterns at the glomerular hilum, peripheral mesangium area and GBM. Although the linear deposition of IgG was also observed, the degree was much lower than those of IgM and IgA. **Conclusion:** The results in the present study suggested that the initial lesion of the progressive glomerulonephropathy in common marmosets occurs as abnormalities of the GBM and paramesangial area, and IgM deposits are probably related to the development of the glomerular lesions.

P-013

A study of partial nephrectomized model using common marmoset (*Callithrix jacchus*) monkeys

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In order to establish a chronic renal failure (CRF) animal model of non-rodent animal species, we observed the pathophysiological condition of renal failure at 13 weeks after about 5/6 nephrectomy (Nx) in female marmoset monkeys (aged 42 to 65 months old). At same time, we also observed the condition of 5/6 nephrectomized female rats (aged 9 weeks old), and identified similarities and differences of pathophysiology between the marmoset monkey and the rat. Each animal underwent about 5/6 surgical nephrectomy or sham operations (rat only) in two steps.

Nx marmoset monkeys showed a remarkable decrease of body weight promptly after nephrectomy, and then sustained slightly. In Nx marmoset monkeys, urine volume, and total urine elimination amounts of creatinine, NAG and cystatin C (Cys-C) and serum levels of BUN, creatinine and Cys-C were increased after nephrectomy. These parameter changes continued till Week 5, then recovered gradually. Nx marmoset monkeys, however, did not show a significant proteinurea observed in Nx rats. The Nx marmoset monkeys with high levels of the above renal disorder parameters showed a decrease level of serum calcium and lipid metabolism parameters, likely total cholesterol, phospholipids and triglycerides. While Nx rats showed an increase of levels of lipid metabolism parameters, the species difference was observed on lipid metabolism parameters between marmoset monkeys and rats after nephrectomy. In hematology, Nx marmoset monkeys showed a decrease of red blood cell parameters at Week 13. These animals showed an increase of fatty marrows in bone marrow, suggesting anemia related to renal failure. The residual renal tissue in Nx marmoset monkeys showed degeneration/ necrosis, atrophy and regeneration of renal tubules, and interstitial fibrosis with cellular infiltration. An enlargement of Bowman's capsule was also observed, but the severity of glomerular damages was weaker than that in Nx rats. In addition, Nx marmoset monkeys showed hyperplasia of parathyroid chief cells and cortical bone changes showing high bone turnover, but these changes were not clear in Nx rats.

In this study, we can observe some different pathological changes including renal changes in Nx female marmoset monkeys in comparison of Nx female rats. The 5/6 Nx marmoset monkey model might have a potential for useful method to evaluate the unique mechanism of renal failure development which is difficult to evaluate by Nx rat model.

P-014

Effects of Pentachlorophenol and *N*-acetylcysteine on Safrole-induced Hepatocarcinogenesis in F344 *gpt* delta rats

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At the last annual meeting, we reported that genotoxic mechanisms might contribute to safrole (SF)-induced hepatocarcinogenesis. In the present study, to clarify the underlying mechanisms, the effects of pentachlorophenol (PCP), a known inhibitor of sulfotransferases, and N-acetylcysteine (NAC), an antioxidant, on several carcinogenic parameters were examined in F344 *gpt* delta rats treated with SF for 4 or 8 weeks. Animals were divided into 6 groups (Group 1: control, Group 2: 0.5% SF alone, Group 3: 1% NAC alone, Group 4: 0.02% PCP alone, Group 5: 0.5% SF+1% NAC, Group 6: 0.5% SF+0.02% PCP). SF and PCP were administered in the diet and NAC was administered in the drinking water. Absolute liver weights of rats in the SF-treated groups were significantly increased compared with the relevant controls at 4 and 8 weeks. 8-hydroxydeoxyguanosine (8-OHdG) levels in liver DNA were significantly increased in SF-treated groups as compared with controls at 8 weeks. Combined administration of PCP further increased 8-OHdG levels with statistical significance, but the effect was not observed with the administration of NAC. Conversely, the number and area of glutathione *S*-transferase placental form (GST-P)-positive foci in the liver increased due to SF exposure, but this was significantly inhibited by combined administration of PCP. Likewise, *gpt* mutant frequencies (MFs) were significantly increased by SF-treatment alone, but MFs showed a tendency to decrease by combined administration with PCP at 4 weeks. Given that oxidative stress is considered to occur in the process of glutathione conjugation of SF, the enhancing effects of PCP on 8-OHdG formation due to SF exposure might result from a compensatory increase of glutathione conjugation in response to a decrease of sulfate conjugation. The inhibitory effects of PCP on *gpt* MF and GST-P formation due to SF exposure indicate that SF-specific DNA modifications might play a crucial role in SF-induced hepatocarcinogenesis. Further data on analyses of *gpt* MFs at 8 weeks will be presented to discuss the modes of action underlying SF-induced hepatocarcinogenesis.

P-015

Enhancing Effects of Flumequine on *in vivo* Mutagenicity of MeIQx in the Mouse Liver

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Purpose: A variety of carcinogens are produced or contaminated in foods accidentally. Many studies on the toxicities of individual substances have been reported; however, there are few reports on their combined effects. MeIQx, one of the heterocyclic amines produced in heated foods, is a genotoxic hepatocarcinogen. Flumequine (FL), which is a veterinary drug and of concern as a pesticide residue, promotes hepatocellular tumor formation following hepatotoxic injury. In the present study, the effect of FL on the *in vivo* mutagenicity of MeIQx in the mouse liver was investigated. The effect of phenobarbital (PB), a non-hepatotoxic tumor promoter for hepatocarcinogenesis, on MeIQx mutagenicity was also examined.

Method: Groups of 5 male B6C3F$_1$ *gpt* delta mice (6-week-old) were given a basal diet (Control) or a diet containing 0.03%MeIQx, 0.4%FL, 0.05%PB, 0.03%MeIQx+0.4%FL, or 0.03%MeIQx+0.05%PB for 13 weeks. At necropsy, the livers were sampled for histopathological examination, BrdU immunostaining, analysis of reporter gene mutations (*gpt* and Spi$^-$ assays), and comprehensive gene expression analysis by cDNA microarray.

Result: There was suppression of body weight gain in the MeIQx+FL combined group. Increases in liver weight and centrilobular hypertrophy of hepatocytes were observed in all of the PB and FL groups. In addition, hepatocytes with vacuolation and inflammatory cell infiltration were evident in the FL groups. The addition of FL further elevated *gpt* mutant frequencies (MFs) increased by MeIQx alone more than 10 times, with statistical significance. On the other hand, there was no effect of PB on MeIQx-induced *gpt* MFs. In the *gpt* mutant spectrum, GC:TA transversion mutations, the predominant mutation observed in the MeIQx alone group, also appeared in the FL combination group, with a 10-fold increased frequency. In comparison of gene expression changes (more than 3-fold) between the MeIQx alone and MeIQx+FL groups, 433 genes corresponded.

Discussion: FL enhanced the *in vivo* mutagenicity of MeIQx in the mouse liver. Given that PB had no effect on *gpt* MFs, various hepatotoxicity-related factors might contribute to these enhancing effects. Further data from analysis of BrdU incorporation, Spi$^-$ assay, and functional analysis of candidate genes are presented to clarify the molecular basis of the effects.

P-016

Examination of in vivo mutagenicity and carcinogenicity in the rat with Kojic acid

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Kojic acid (KA) is known to suppress the biosynthesis of melanin at melanocytes in epidermal skin by inhibiting the activity of tyrosinase. KA reported that KA has carcinogenicity in liver of rats. The purpose of this study is to simultaneous detection for carcinogenicity and mutagenicity of KA and IQ in rats. IQ used as an appropriate standard mutagen for mutational analysis.6 week-old male F344 rats were divided into 3 groups (Group 1-3) and gpt delta rats were divided into 3 groups (Group 4-6). Groups 1-3 were treated with five carcinogens (DMBDD) during the first 4 weeks of experiment. Groups 4-6 were given vehicle. From experimental week 5, groups 1-6 were treated with KA in diet at doses of 0, and 2%, and 0.01% IQ for 13 weeks. At 18 weeks after starting the experiment, quantitative analysis of GST-P, which are preneoplastic lesions in the rat liver and mutation assays were performed. Both numbers and area of GST-P positive foci were significantly increased in rats administered DMBDD→ 2% KA and administered DMBDD→0.01% IQ compared to rats administered DMBDD alone. These findings indicated that KA and IQ exerts promotion effect on liver carcinogenesis. There was no significant difference in mutation frequency in liver between Kojic acid and control group, whereas mutation frequency was significantly increased in IQ group compared to control group.

P-017

Possible Involvement of Genotoxic Mechanisms in 5-(hydroxymethyl)-2-furfural-Induced Hepatocarcinogenesis in Mice.

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One of the major products formed in the process of the Maillard reaction is 5-(hydroxymethyl)-2-furfural (HMF), present in various foods and beverages such as honey and fruit juice. HMF has been shown to be a hepatocarcinogen in female mice using the long-term bioassay. Although HMF is not a mutagen in the conventional *in vitro* mutation assays, 5-sulfoxymethylfurfural (SMF), which is a reactive metabolite of HMF following sulfotransferase conjugation, shows mutagenicity. Thus, it is probable that hepatocarcinogenesis of HMF involves genotoxic mechanisms. In the present study, to clarify the modes of action underlying HMF-induced hepatocarcinogenesis, female B6C3F$_1$ *gpt* delta mice were given HMF at a carcinogenic dose (188 or 375 mg/kg b.w.) by gavage 5 days per week for 4 weeks. There were no changes in general condition or body weight during the experimental period. Liver weights in HMF-treated groups showed no significant differences compared with the control group and there were no obvious macroscopic lesions in the treatment groups. There were no significant differences among the groups in mutant frequencies (MFs) of *gpt* mutations primarily indicating point mutations, and Spi$^-$ mutations showing chiefly deletion mutations. Positive control sample (rat liver treated with tamoxifen) showed significant increases of MFs in both *gpt* and Spi$^-$ assays. These results suggest that genotoxicity does not contribute to HMF-induced hepatocarcinogenesis. It has been reported that HMF treatment significantly induced hepatocellular adenoma in female mice, but not in male mice or rats of both sexes. Overall, HMF might be considered to be a tumor-promoter in the livers of female mice. Further analyses of histopathology and the proliferating cell nuclear antigen (PCNA)-positive ratio in the liver will be performed in future studies.

P-018

IQ Promotes Mouse Hepatocarcinogenesis by Activating Transforming Growth Factor-β and Wnt/β-catenin Signaling Pathways

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The purposes of the present study were to investigate modifying effects of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a genotoxic carcinogen produced during cooking of protein-rich foods, and elucidate underlying mechanisms in a two-stage hepatocarcinogenesis mice model. Six-week-old B6C3F1 mice were subjected to two-thirds partial hepatectomy at the beginning of the study, followed by an intraperitoneal injection of diethylnitrosamine (DEN) on day 1. Starting one week later, they were fed diets containing IQ at doses of 30, 100 or 300 parts per million (ppm), for 39 weeks. A dose-dependent trend for increase in eosinophilic altered foci as well as eosinophilic hepatocellular adenomas was observed, along with significant elevation in the incidence of hepatocellular carcinomas in the 100 and 300 ppm IQ groups as compared with initiation control group. Furthermore, IQ elevated protein expression levels of Wnt1, transforming growth factor-β (TGF-β), TGF-β receptor 1 and 2 (TβR1 and TβR2), and phosphorylated c-Jun (p-c-Jun), while suppressing those of E-cadherin and $p21^{WAF1/Cip1}$. Moreover, translocation of β-catenin to the nuclei as well as up-regulated nuclear expression of c-Myc and cyclin D1, which are downstream targets of β-catenin and p-c-Jun, were detected at 100 and 300 ppm. These findings suggest that IQ exerts dose-dependent promoting effects on mice hepatocarcinogenesis by activating TGF-β and Wnt/β-catenin signaling pathways and inhibiting cell adhesion.

P-019

Analysis of gene expression in rat liver treated non-genotoxic carcinogens for 28 days

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In general repeated dose study in rat, many of non-genotoxic carcinogens take for long time, for 6 months or more, to be observed the precancerous lesion or tumor in the liver. Some alternative studies such as short-term carcinogenecity study are able to effectively investigate the potential of non-genotoxic carcinogenesis with more short time. But these studies are limited to do due to be required the special technique. It makes possible to extremely reduce the cost and time that the screening system that can detect the potential of non-genotoxic carcinogenesis in general repeated dose and more short time would be established.

In 2011, Open TG-GATES (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation system , http://toxico.nibio.go.jp) was disclosed. We can download the gene chip data of 131 compounds from rat liver via this database and analyze under the license agreements. Some of non-genotoxic carcinogen such as thioacetamide and methapyrilene etc. are included in that database.

In this study, we analyzed the gene chip data of some non-genotoxic carcinogens included Open TG-GATES. We focused our gene expression analysis on the mechanism of non-genotoxic carcinogenesis such as oxidative stress and cellular injury etc. and explored the differential expression genes which are associated with the above mechanism and regulated expression depended on dosage and dosing period.

In the results of gene expression analysis, we identified some genes which were regulated expression depended on dosage and dosing period. Some of these genes were associated with the known mechanism of non-genotoxic carcinogenesis. We will report that whether we can predict the potential of non-genotoxic carcinogenesis with the identified genes.

P-020

Threshold Levels for Tumor Promoting Effects of Ethyl *tertiary*-Butyl Ether (ETBE) on Hepatic and Renal Carcinogenesis in Rats

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As one goal of achieving the "Kyoto protocol", fuel derived from renewable sources (biomass or biofuel) might be introduced onto the marked in the near future, and there are plans to introduce ETBE, derived from biomass ethanol, in Japan. Evaluation of potential carcinogenicity of ETBE is very important in this context. The present study was conducted to detect any threshold level in the renal and hepatic tumor promotion activity previously found in a medium-term multi-organ carcinogenesis bioassay carried out in our laboratory. Male Wistar rats were given drinking water containing 500 ppm *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine (EHEN) as an initiator for 2 weeks. One week thereafter, the animals received ETBE by gavage at dose levels of 0 (control), 100, 300, 500 or 1000 mg/kg/day until experimental week 22. Necropsy of all rats was performed at week 23, and the livers and kidneys were examined histopathologically. Incidences of hepatocellular adenomas, and of hepatocellular adenomas or carcinomas were significantly elevated in rats given 1000 mg/kg/day ETBE, with significant increase in the average numbers of hepatocellular carcinomas and of hepatocellular adenomas or carcinomas. No significant differences in incidences and average numbers of renal tubule neoplasms were found in rats administered ETBE. However average numbers of atypical renal tubules, considered to be pre-neoplastic lesions, were significantly increased in the rats given 1000 mg/kg/day, but not 500 mg/kg/day or lower.
These results imply that 500 mg/kg/day may be a no-effect level for hepatic and renal tumor promotion of ETBE on EHEN-induced carcinogenesis in male rats. As accumulation of α2u-globulin in renal tubules, which is specific for male rats, was also found in ETBE treated animals, promoting effects of ETBE on a renal carcinogenesis could not be extrapolated to humans.

P-021

Modification of Liver Tumor Promotion Effects with Co-administration of β-Naphthoflavone and Piperonyl Butoxide in Rats

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Some chemicals which induce CYP1A and CYP2B are known to have a liver tumor promoting effect. β-Naphthoflavone (BNF), a CYP1A inducer, and piperonyl butoxide (PBO), a CYP1A and CYP2B inducer, have a liver tumor promoting effect. In this study, we investigated whether co-administration of BNF and PBO exerts modifying effect on tumor promotion in rats. Male rats were subjected to partial hepatectomy, and given 0.125% or 0.25% BNF, 0.125% or 0.25% PBO or 0.125% BNF+0.125% PBO in diet for 6 weeks after diethylnitrosamine initiation. In treated groups, liver weights and glutathione *S*-transferase placental form (GST-P)-positive foci significantly increased, but GST-P-positive foci in BNF+PBO group were not significantly higher than that in high dose of PBO or BNF group. Real-time RT-PCR revealed up-regulation of *Cyp1a1* and NRF2 gene batteries such as *Gpx2*, *Yc2* and *Akr7a3* in treated groups and up-regulation of *Cyp2b1/2* in PBO groups. Interestingly, the transcript level of *Cyp1a1* and *Cyp2b*1/2 in BNF+PBO group was much lower than that in 0.125% BNF or 0.125% PBO group. The results of our study indicate that the co-administration of BNF and PBO did not result in additive effects in the liver tumor promotion in rats, probably in relation with rather suppressed induction of *Cyp1a1* and *Cyp2b1/2* in this group.

P-022

Toxicopathological Impact of Co-exposure to Different Phthalate Esters on the Liver and Male Genital System by 90 Days Repeated Administration in Rats

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Many phthalate esters are identified as peroxisome proliferator activated receptor α (PPARα) agonists, but there are a variation in the subtype and affinity of PPAR to be activated and its toxicological profile depending on the type of alkyl group. We have recently shown that repeated oral administration of dihepthyl phthalate (DHP) to rats for 90 days through diet resulted in dose-dependent induction of liver cell foci positive for glutathione *S*-transferase placental form (GST-P) in a manner independent of PPARα-mediated mechanism, as well as a lack of testicular toxicity even at a dose showing 30% body weight reduction (20,000 ppm). The present study was performed to examine whether co-exposure to phthalate esters that are different in the mechanism of action or toxicological outcome exerts synergistic effect on the liver and male genital system. For this purpose, male F344 rats were administered with di(2-ethylhexyl)phthalate (DEHP; 12,000 ppm), di-n-butyl phthalate (DBP; 12,000 ppm), DHP (10,000 ppm), DEHP+DBP or DEHP+DHP for 90 days. Histologically, hepatocyte hypertrophy with increased cytoplasmic eosinophilia, a typical change reflecting peroxisome proliferation, was observed with DEHP-alone, resulting in enhancement by DBP co-exposure. In contrast, swelling and vacuolar degeneration of hepatocytes, that were observed in DHP-alone, disappeared with DEHP co-exposure. With regard to α2-macroglobulin (α2M), a molecule that is reported to be a marker of preneoplastic lesions induced by PPARα agonists, weakly positive liver cells were diffusely observed in groups treated with DEHP and/or DBP. On the other hand, similar to the induction of GST-P-positive liver cell foci, DHP also induced α2M-positive foci, and interestingly, both types of cellular foci disappeared with DEHP co-exposure. In the male genital system, treatment with one compound alone did not induce any changes; however, both of co-exposure groups induced seminiferous tubular atrophy characterized by shedding of germ cells as well as decreased testicular absolute weights. These results suggest that DBP exerts enhancing effect on PPARα activity of DEHP in the liver, while DEHP exerts antagonistic activity on PPARα-independent liver changes induced by DHP. With regard to testicular atrophy, phthalate esters in combination treatment might have exerted either additive or enhancing effect on PPARα activity.

P-023

The Role of Constitutive Androstane Receptor in Liver Hypertrophy by Triazole Fungicides.

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[Introduction] Constitutive Androstane Receptor (CAR), a nuclear receptor, plays an important role in induction of CYP2B, liver hypertrophy and hepatocarcinogenesis in mice. In the present study, we investigated the relationship between CAR and liver hypertrophy treated with triazole derivatives, which have been reported to induce liver hypertrophy and tumors in rodents.

[Materials and Methods] C3H derived male CARKO mice were administered cyproconazole (Cyp), tebuconazole (Teb) or fluconazole (Flu) in their diet for 4 weeks, and compared with wild-type (WT) mice (C3H/HeNCrlCrlj). After the treatment, mRNA expression of CYPs (2b10, 3a11) and pathological changes in the liver were evaluated.

[Results] Cyp group: Cyp induced an increase in liver weight and severe centrilobular/diffuse hepatocellular hypertrophy in WT mice, but not in CARKO mice. In mRNA analysis using real time PCR, Cyp2b10 expression in WT mice was markedly increased, although the increase was less in CARKO mice. Cyp3a11 expression levels in WT and CARKO mice were both markedly increased. Immunohistochemistry showed thathepatocytes in the centrilobular/panlobular area were strongly positive to CYP2B in WT mice, although the signal was weak and only in centrilobular hepatocytes in CARKO mice. Teb group: Teb induced an increase in liver weight and severe diffuse hepatocellular hypertrophy in both genotypes. Marked increases in Cyp2b10 and Cyp3a11 expression and a strong positive reaction to CYP2B in panlobular hepatocytes, were detected in both WT and CARKO mice. Flu group: Increases in liver weight were moderate in WT mice and mild in CARKO mice. Cyp2b10 expression in WT mice was markedly increased, although it was not increased in CARKO mice. Cyp3a11 expression in WT and CARKO mice was markedly increased. Hepatocytes in the centrilobular area were strongly positive to CYP2B in wild mice but were weakly positive in CARKO mice.

[Conclusion] Our results indicate that liver hypertrophy by Cyp and Flu is mainly CAR-mediated. Other pathways, with the exception of CAR, are crucial to liver hypertrophy by Teb. In addition to CAR, PXR might be involved in the process of liver hypertrophy by all three triazoles.

P-024

Expression of cell proliferation-related proteins and involvement of constitutive androstane receptor (CAR) in altered liver foci/adenomas induced by CYP2B inducers

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The present study was performed to evaluate the involvement of cell proliferation-related factors in the mechanism of CAR-mediated hepatocarcinogenesis induced by CYP2B inducers. 6-week-old male mice, C3H (wild type) and CAR knockout (CARKO) were fed a diet containing 500 ppm phenobarbital (PB), 50,000 ppm piperonyl butoxide (PBO) or 500 ppm decabromodiphenyl ether (DBDE) for 13 or 27 weeks after diethylnitrosamine (DEN) initiation. The protein expression levels of cyclin B1, forkhead box protein M1 (FoxM1), and growth arrest and DNA damage (GADD) 45γ in eosinophilic or basophilic altered liver foci/adenomas were evaluated by immunohistochemistry. At week 13, cyclin B1 was weakly positive in both types of small foci observed in wild mice. Cyclin B1 and FoxM1 were positive in the foci/adenomas in all treated groups at week 27. Their intensities were stronger in basophilic lesions but weaker generally in CARKO mice. GADD45γ was weakly positive in eosinophilic and basophilic foci observed in wild mice at week 13. At week 27, GADD45γ was strongly positive in both types of foci/adenomas in each genotype. In DEN alone controls, the positive level of each protein in the basophilic lesions of both genotypes was as low as in non-proliferative areas. In conclusion, these proteins might be related to hepatocarcinogenesis, especially induction of basophilic foci, by CYP2B inducers. Among these proteins, Cyclin B1 and FoxM1, but not GADD45 γ, might be partly involved in the CAR-mediated hepatocarcinogenesis process.

P-025

Transcriptional Down-regulation of ALT Gene and AST Gene in the Liver Induced by Phenobarbital Sodium

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Phenobarbital (PB) is a well-known non-genotoxic hepatocarcinogen in rodents, but the toxicological significance of reduction in plasma activity of deviation enzyme such as ALT and AST is still unclear. Thus, PB was administered by gavage to female rats at doses of 0, 8 and 80 mg/kg/day for a period of 4 weeks, and we performed molecular analyses using liver samples in order to clarify these mechanisms. Although LPO was significantly increased in the 80 mg/kg/day group, plasma activities and gene expression levels of ALT and AST were significantly decreased in the 80 mg/kg/day group at 4-week of treatment. As ALT and AST participate in glucometabolism, Creb and Hif1a were selected for ChIP analysis. ChIP-PCR revealed that Creb binding to its response element in the genomic AST region and Hif1a binding to its response element in the genomic ALT region were significantly decreased in the 80 mg/kg/day group. Western blot analysis revealed that Creb and Hif1a were significantly decreased in the 80 mg/kg/day group. These indicate that down-regulations of Creb and Hif1a were responsible to the down-regulations of ALT and AST, respectively. Down-regulation of Hif1a gene by siRNA in rat hepatocyte primary culture induced significantly low ALT and AST transcription levels. This result indicated Hif1a has an important role in the transcriptional regulation of ALT and AST. From the evidences that Foxo1 regulates Mtor pathway including Hif1a and Car cross-talks with Foxo1, we are now investigating the role of Foxo1 in the liver.

P-026

Interstrain comparison of pathophysiological condition in high-fat (70kcal%) diet fed rats

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The occurrence mechanism of non-alcoholic steatohepatitis (NASH) remains highly uncertain. In order to clarify the pathogenesis and develop new drugs, a number of animal models have been developed.

Above all, diet-induced NASH models are widely used for their simple method for producing and the high reproducibility. We previously reported that high-fat (HF) diet fed rat could be an appropriate NASH model based on a diet comparison study in male Wistar rats (The 26th annual meeting of the Japanese Society of Toxicologic Pathology). However, inflammation and fibrosis in the liver, considered to be progressive findings of NASH, were relatively mild and considerable individual variation was found in Wistar rats.

In the present study, we fed HF (70kcal%) diet to male Wistar rats, Crl:CD(SD) rats and Brown Norway (BN) rats for 16 weeks and compared the results of body weight, food consumption, blood chemistry and liver histopathology to conventional diet fed rats.

As a result, Wistar rats fed with HF diet increased body weight compared to those in the control group, SD rats showed no difference between HF and control group, on the other hand, body weight gain was suppressed in BN rats fed with HF diet compared to those given a conventional diet. Food consumption was decreased in rats fed with HF diet compared to control group in each strain. In blood chemical analysis, cholesterol level was increased, triglyceride was decreased and ALT and AST activities were higher in rats fed with HF diet than those in the control group in each strain. In interstrain comparison of HF fed rats, ALT and AST activities increased greatly in BN rats than Wistar or SD rats. Histopathological examination of the liver showed that inflammation and fibrosis were more severe in BN rats than in Wistar rats, but the severity of SD rats were comparable to that of Wistar rats.

These results suggest that BN rats could be the most desirable strain to use as HF feeding NASH model. However, suppressed body weight gain along with severely reduced food consumption in BN rats is different from human clinical symptom, and thus further improvement in study design is needed.

P-027

M1/M2 Macrophage Polarization in Thioacetamide (TAA)-induced Acute Rat Liver Lesions

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Macrophages activated after tissue injury control either inflammation or remodeling. IFN-γ induces “classically activated macrophages (M1)” for inflammation, whereas IL-4 induces “alternatively activated macrophages (M2)” for remodeling. M1 and M2 cells also participate into Th1 and Th2 responses, respectively. To clarify the M1/M2 polarization, M1- and M2-related factors were analyzed in hepatic lesions induced in F344 rats by a single injection of TAA (300 mg/kg BW), and liver samples were collected on 10 hours and 1 to 10 days after injection. In contrast to controls, on 10 hours, mRNA expressions of IFN- γ, TNF- α, and IL-1 for M1, and IL-4 for M2 were significantly increased, followed by significantly increased expressions of IL-10 and TGF- β 1, both for M2, on days 1 and 2. Remodelling (including reparative fibrosis by myofibroblasts) by M2 cells was delayed as a matter of course. Th1 and Th2 responses are regulated by antigen-presenting cells under respective INF- γ and IL-4. In injured perivenular areas of hepatic lobules, macrophages reacting to MHC class II (OX6) appeared on days 1 to 3 with a peak on day 2, and CD3-positive T cells increased transiently on day 2. Double immunolabelling revealed that on days 1 to 3, there were macrophages reacting both to OX6 and CD68 (ED1; phagocytosis), or to OX6 and CD163 (ED2; inflammatory factor production). In connection with M1- or M2-related factors, collectively, antigen-presenting cells may show various functional properties in hepatic lesions, accompanied with T cells. M1/M2 cell paradigm would be useful for hepatotoxicity analysis.

P-028

Localization of Connexin32 and Connexin26 in Spontaneous Liver Lesions in Mice

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Connexin32 (Cx32) and Connexin26 (Cx26) are components of gap junction (GJ) and are expressed in various organs and tissues including the liver. Cx32 and Cx26 have 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 expression levels of Cx32 protein decreases in neoplastic cells. In the present study, spontaneous liver lesions in mice such as hepatocellular altered foci and hepatocellular neoplasm were examined, where immunohistochemistry and localization of Cx32 and Cx26 in spontaneous lesions were compared to those in normal hepatocytes. The spontaneous lesions such as clear cell foci, basophilic foci, eosinophili foci, hepatocellular adenoma and hepatocellular carcinoma observed in the mice carcinogenecity study (C57BL6J, 24 months, p.o.) were stained with anti-Cx32 or anti-Cx26 antibodies and image analysis was conducted using an image analyzer. There was no clear difference in localization of each lesion between Cx32 and Cx26. Decrease in Cx32 and Cx26 expression was observed in the clear cell foci and some of the eosinophilic foci. In the eosinophilic foci, and adenoma and carcinoma which have eosinophilic cytoplasm, the expression level decreased in the intercellular area, but increased in the cell membrane that faces the perisinusoidal space. In the basophilic foci, and adenoma and carcinoma which have basophilic cytoplasm, an increase in the number of positive spots and spot area were observed. In conclusion, localization of Cx32 and Cx26 in spontaneous lesions in mice has been classified into the above 3 patterns.

P-029

Antibodies useful for Macrophage Detection and Distribution in the Liver in Cynomolgus Monkeys

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The role and function of macrophages in the liverare important in terms of cytotoxicity, cytoplasmic or tissual repair, immune response. However, the mechanisms of toxic effects are still not enough to have led to the elucidation.To explore the usefulness of three different antibodies developed for human macrophages (EBM11, AM-3K, SRA-E5) and clarify the distibution, liver samples were obtained from cynomolgus monkeys (7 males and 5 females aged 3 to 5 years, and 12 females aged 18 to 19 years) (China, purpose-bred). The data were evaluated by counting positive cells in the pericentral or periportal areas in the terms of age and gender differences. The Wilcoxon rank sum test was employed for each antibody. All three antibodies showed cross reactions to monkey macrophages, and EBM11 and SRA-E5 could be more useful for macrophage detection, because the immunolabeling was available for formalin-fixed tissues. Although rodent macrophages are more predominat in periportal areas than perivenular areas, there were no significant differences in the present data. As compared with positive cell numbers to EBM11 and SRA-E5 in the periportal area in old females (18-19 years old), those of young females (3-5 years old) tended to increase, and those of young males (3-5 years old) significantly increased. The results obtained would be useful background data on liver macrophages in normal cynomolgus monkeys.

P-030

Identification of Novel Protein Biomarkers for Human Liver Cancer

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To search for new diagnostic and prognostic biomarkers and compare the molecular background of liver cancer occurred in non-alcoholic steatohepatitis (NASH) and HCV positive patients, the proteomes of dissected carcinomas and adjacent normal-appearing liver tissue from formaline-fixed and paraffin embedded (FFPE) sections were analysed using QSTAR Elite LC-MS/MS coupled with iTRAQ technology. Significant specific elevation of mitochondrial proteins and transcriptional regulators prohibitin 1 (PHB1), prohibitin 2 (PHB2) and YME1-like 1 (YME1L1), canopy 2 homolog (CNPY2), cache domain containing 1 (CACHD1), immediate early response 5-like (IER5L) and WD and tetratricopeptide repeats 1 (WDTC1) was detected in both NASH and HCV-positive hepatocellular carcinomas (HCCs). Specific up-regulation of cytochrome P450 isoenzymes CYP2A6, CYP3A5, CYP4A11, CYP51A1, and CYP8B1 was found in HCCs of NASH patients. Furthermore, significant elevation of glutathione S-transferase kappa 1, hypoxia up-regulated protein 1 associated with tumor invasiveness, ADP-ribosylation factor 5 and specific down-regulation of lactate dehydrogenase A (LDHA) and monoamine oxidase B was observed in NASH HCCs. On the other hand, specific overexpression of actinin alpha 4, LDHA and phosphogluconate dehydrogenase, was detected in HCV-positive HCCs. In conclusion, CNPY2, CACHD1, IER5L and WDTC1 might become potential biomarkers for human liver cancer.

P-031

Effects of carbon nanotubes on lung tissues, cell proliferation, and gene expression

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We examined the effects of carbon nanotubes on lung tissues, cell proliferation, and gene expression. Using multi wall carbon nanotubes (CNT), we examined which suspension is the optimal for dispersion, and also investigated whether each fraction of CNT fragment (remaining, R; flow through, FT; whole, W), which was separated with a sieve, affects the levels of expression of a specific gene *in vitro*. In addition, histological examination was done with the areas of parietal and visceral pleura after intratracheal instillation. The optimal suspension was the saline in a polymer solution. FT exhibited stable dispersion, but R and W showed aggregation in the solution. Electron microscopic examination revealed that FT consisted of short CNTs whereas R and W consisted of CNTs of various length and aggregates. By exposure of FT particles, an inflammatory change with CNT phagocytosis of macrophage was seen. Formation of granuloma and infiltration of macrophage were also seen with R and W particles. After treatment of macrophage with each CNT fraction, the growth of human lung carcinoma cells significantly increased in FT, R, and W. However, treatment of CNTs did not affect the growth of human mesothelioma and fibroblast cells. By treatment of macrophage with CNTs, common expression spectrum was found between proliferation and cytokine expression profiles. Collectively, physicochemical character of CNTs was different among the fractions. This difference affected histological changes of the lung and localization of macrophage.

P-032

Application of Spiral Array to Survival Analysis of Lung Cancer - Tissue Heterogeneity of Ki67 Matters

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The use of tissue microarray for biomarker validation is powerful. However, heterogeneity within certain tumors may complicate the interpretation of small core stainings. We recently developed new technique, Spiral Array, which observes the side of thick reeled sections that enable to find whole morphology included in the one axis of paraffin sections. Using Spiral Array, we investigated prognostic significance of Ki-67 staining from the view of staining heterogeneity.

DESIGN: 100 cases of lung cancer were collected. Spiral Array blocks were generated form sections cut at 100 um thick. 4 um thick sections of the Array block were stained for Ki-67. Staining results in the each reel were scored for areas with lowest (LS), highest (HS), and dominant (DS)expression frequencies in the cancer cells. The scores were divided into four grades (0, ＜ 1%; 1, 1-10%; 2, 11-30%; 3, ＞30%). Prognostic significance of Ki-67 was analyzed using Log rank test.

RESULTS: 78 cases had clinical data. The proportion of Ki-67 staining was 18 score 3, 28 score 2, 29 score 1, and 21 score 0. Cases with score 2 and 3 of HS showed significant poorerprog nosis (P＜.001), whereas LS or DS did not show any prognostic values.COX mu ltivariate analysis showed HS is an independent prognostic factor.

CONCLUSION: Ki-67 is a strong prognostic marker for lung cancer when highest staining frequency is considered. Considering tissue heterogeneity is important for the establishment of tissue-based biomarkers.

P-033

Ovarian toxicity potential of dibromoacetic acid in rats

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[Introduction] Dibromoacetic acid (DBA), one of a number of haloacetic acids generated during the water disinfection process, is reported to induce suppression of estradiol (E2) catabolism in liver resulting in serum E2 level and persistent estrus in rats in the previous studies. However there are no information of morphological changes in the female reproductive tract by DBA treatment. In the present study, we examined morphological changes in the female reproductive tracts, estrous cyclicity and hormone profiles in rats treated with DBA.

[Materials and Methods] Matured normal cycling female BrlHan:WIST@Jcl(GALAS) rats (8 to 10 wks of age) were treated with DBA at 250mg/kg bw/day for 4, 8, 16 and 28 days (1, 2, 4 and 7 estrous cycles, respectively), and examined morphological changes in the female reproductive tracts, vaginal cytology and serum hormone levels.

[Results] Estrous cyclicity was abnormal during the experimental period, but persistent estrus was not observed. In several rats on 8 and 16-day, morphological features in the ovary and uterus were still proestrus while vaginal cytology was estrus. Luteinized cysts were detected in a few rats on 16-day. On 28-day, many rats showed the similar asynchronized changes between the vaginal cytology and the morphology in the ovary and uterus. Large atretic follicles was increased on 28-day. In hormonal assay, higher FSH levels and lower levels of progesterone and E2 were detected at proestrus on 28-day.

[Conclusion] These results indicate that DBA disrupts ovarian follicle growth in female rats by short-term treatment.

P-034

Histological Characteristics of Ovaries in Wistar Hannover Rats: The Comparisons with SD Rats

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<Introduction>
As there have been only a few studies conducted using Wistar Hannover rats (WH) in Japan, we are acquiring their background data. In the 38th Annual Meeting of the Japanese Society of Toxicology, Kimura and Yoshida, et al. reported that a smaller numbers of corpus luteums, implantations and surviving fetuses observed macroscopically in WH compared with SD rats (SD) through the cesarean operation at 32-week old and 20 days of pregnancy. Moreover, we found heavier ovary weights in WH (97.3mg) than SD (82.3mg). In this study, we investigated the histological characteristics of ovaries in WH.
<Materials and methods>
Ovaries were collected from 44 WH (RccHan^{TM}:WIST) at 32–week old and 30 SD of same age as the comparative control. We investigated the ovarian size, numbers of corpus luteums and atretic follicles, and beginning and processes of luteal involution observed in the maximal area of the ambilateral ovary section.
<Result>
The size was larger in WH than SD. The average number of total corpus luteums (currently and previously formed corpus luteums) in ambilateral ovaries was larger in WH (29.4) than SD (19.7) in all cycles. There was no difference in the numbers of newly (WH3.1, SD3.0) and currently (WH 4.2, SD4.6) formed corpus luteums or atretic follicles (WH4.8, SD5.2). The beginning of luteal involution (characterized by degeneration and necrosis of luteal cells) was observed in the currently formed corpus luteum in the diestrus in both strains. However, as a trend, while degeneration and necrosis of the luteal cells occurred sporadically in SD, they occurred massively in the center of the corpus luteum in WH. The beginning of size reduction (characterized by atrophy in luteal cells and interstitial fibrosis) associated with luteal involution was observed in the previously formed corpus luteum in the estrus in SD. Meanwhile in WH, it occurred in the diestrus, 2 cycles later than SD.
<Conclusion>
The slower beginning of luteal size reduction in WH compared to the SD rats was thought to be associated with the larger number of the total corpus luteums observed histologically, and it could be one reason for the larger and heavier ovary in WH. There was no difference in the numbers of newly and currently formed corpus luteums or atretic follicles in these strains, which did not reflect the fewer corpus luteums observed macroscopically in WH through the cesarean operation. Further investigations are required to clarify the causes concerning the fewer implantations and surviving fetuses.

P-035

Histopathological Characteristics of Testicular Toxicities Induced by Topoisomerase Inhibitor I (TP300) in Rats: Morphological Changes of Nucleus in Spermatid

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Introduction: Topoisomerases (Topo) are nuclear enzymes that remove torsional stress in DNA. Their function is important for replication, transcription, chromosome condensation, and chromosome segregation during mitosis and meiosis. Topo I and II participate in single and double strand DNA breaks, respectively. Both Topo I and II are present and potentially functional in both spermatocytes and spermatids. In the testicular toxicities by a Topo II inhibitor in rat, in addition to the changes such as apoptosis, degeneration/necrosis and desquamation /decrease of spermatocyte and spermatogonia, the characteristic morphological change of nucleus in seminiferous tubules, such as chromatin condensation of peripheral nuclear membrane has been reported. However, histopathological features of testicular toxicities mainly focus on the characteristic change in the nucleus and have not been reported in detail in either Topo I or II inhibitors. In this study, we evaluated the histopathological characteristics of testicular toxicities induced by Topo I inhibitor (TP300) in rats. We have mainly focused on the morphological changes of nucleus.

Materials and methods: Intravenous intermittent administration of TP300 for 4 weeks was carried out (5 weekly dose) with male SD rats (6-week age at dosing) at dose levels of 0 (vehicle), 1, 6 and 30 mg/kg. Animals were necropsied on days 3 and 28 after the final injection, respectively (n=10 and 5 in each group). Histopathological examination and immuno-histochemistry (IHC) for anti-Cleaved Caspase-3 antibody (cCasp-3) of the testis and epididymis were conducted.

Results and discussion: Testicular toxicities were observed at 6 mg/kg and above. At necropsy, atrophy and low organ weight of the testes were noted. Histopathological examination showed decrease of the spermatogenic cells and multinucleated giant cell in seminiferous tubules in the testis, and decrease of sperm and desquamated spermatogenic cells in duct of the epididymis. Over the 4-week recovery period, all of the changes recovered at 6 mg/kg and showed a tendency to recover at 30 mg/kg. The characteristic nuclear change, as previously reported in a Topo II inhibitor, such as chromatin condensation of peripheral membrane, was noted in the spermatid and multinucleated giant cell in the testis and desquamated spermatogenic cell in duct of the epididymis, and these cells were negative for cCasp-3 in IHC. These characteristics suggest that the pathogenesis of the nuclear changes in spermatid induced by Topo inhibitors is related to the effects to spermatogenesis including spermiogenesis, and is though to be different mechanism from apoptosis.

P-036

Comparison of Age-related Male Reproductive Performance, Spermatozoa and Spermatogenesis in RccHan:WIST Rats

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【Objective】Sprague Dawley rats have been the major strain in preclinical toxicity studies in Japan, although, Wistar Hannover rats are considered as one of the major strain in Europe. Recently, the latter strain is regarded as more appropriate for the evaluation in preclinical toxicity studies in Japan, because they have the advantage of long life span. In developmental and reproductive toxicity (DART) studies, mating should be conducted with animals reached full sexual maturity. This study was performed, therefore, confirming the age of sexual maturity in Wistar Hannover male rats.

【Materials and Methods】Naïve male RccHan:WIST rats at ages of 8, 10 and 12 weeks (8-, 10-, or 12-week-old group, respectively) were mated with 12-week-old female rats. Reproductive performance, sperm counts, computer-assisted sperm motion analysis (CASA), sperm morphology and testicular histopathology were compared with each group.

【Results】No changes were noted in the sexual behavior. The 8-week-old group showed a low fertility index. No effects on fertility were observed in the 10- and 12-week-old groups. There was an age-related increase in both weights of epididymides and testes at ages of 8 to 12 weeks. CASA indicated that the percentage of motile sperm in the 8-week-old group was significantly lower than that of the other groups. Morphologically abnormal sperm showed high incidence except for the 12-week-old group. Significantly low epididymal sperm counts were observed in the 8-week-old group compared with the other groups. Daily sperm production in the 8- and 10-week-old groups was significantly lower than that of the 12-week-old group. The evaluation of spermatogenesis in the seminiferous tubules, which was conducted for four spermatogenic stages (II-III, IV, VII, and XII), indicated no differences between each age group. These results suggested that the impaired fertility in the 8-week-old group was probably related to low sperm counts, low sperm motility and high sperm morphological abnormalities. There were slight changes in sperm morphology and daily sperm production in the 10-week-old group, nevertheless, the impact of them on male fertility was not observed.

【Conclusion】RccHan:WIST male rats at age of 8 weeks have not yet reached sexual maturity completely. Reproductive performance attained maturity by age of 10 weeks although spermatogenesis has not yet been fully established. It is recommended that RccHan:WIST male rats at ages of 12 weeks or older should be used to acquire enough the number of pregnant animals in DART studies.

P-037

Suppression of Lymph Node Metastasis by New Endogenous Soluble VEGF Receptor-2 Isoform in a Mouse Mammary Cancer Model

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An endogenous soluble VEGF receptor-2 (esVEGFR-2) that we recently identified is a selective inhibitor of lymphangiogenesis and associated with normal alymphatic cornea. To evaluate the antimetastatic potential of esVEGFR-2, gene therapy with vector expressing esVEGFR-2 or endostatin (pEndo) as a positive control was conducted on murine metastatic mammary cancer. **Results:** Tumor volume was significantly lower in the pesVEGFR-2 and the pEndo groups as compared to the pVec group as a control throughout the study. Multiplicity of lymph node metastasis was significantly suppressed in these two groups. Moreover, total number of overall metastasis including the other organs was also decreased in these groups. In mammary tumor tissues, the number of blood microvessels was significantly decreased in the pEndo group, while the number of lymphatic vessels was significantly decreased in the pesVEGFR-2 and pEndo groups. In addition, a significant reduction in the number of dilated lymphatic vessels containing intraluminal cancer cells was observed in the pesVEGFR-2 and pEndo groups. **Conclusion:** Our data demonstrate that esVEGFR-2 can inhibit mainly lymph node metastasis. The antimetastatic activity of esVEGFR-2 may be of high clinical significance in the treatment of metastatic breast cancer.

P-038

Age related susceptibility of MNU-induced mammary carcinogenesis in female Lewis rats

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N-methyl-*N*-nitrosourea (MNU)-induced mammary cancer model is widely used in breast cancer research. Mammary susceptibility against MNU to induce mammary cancer is age dependent; MNU at 3 weeks of age is more susceptible compared with 8-week-old rats, and aged rat is in low susceptibility[1]. However, MNU susceptibility on immature rat (< 3 weeks of age) is inconsistent. Therefore, we compared three different studies performed in our laboratory using female Lewis rats with different age at MNU administration to assess the degree of susceptibility of MNU in relation to age at administration and the development of mammary cancers.

[Materials and methods] Newborn, 3 or 4-week-old female Lewis rats (Charles River Japan) received an intraperitoneal injection of 50mg/kg MNU. All rats were fed commercial pellet diet (CMF 30kGy) and fresh water *ad libitum* and sacrificed 16, 16 or 26 weeks after MNU, respectively. All palpable mammary tumors and bilateral cervical-inguinal mammary fat chains were processed to hematoxylin and eosin staining. Mammary cancer incidence (the number of rats with mammary cancer), multiplicity (number of mammary cancers per animal), and tumor histology were compared. Newborn female rats that received physiological saline instead of MNU, and sacrificed at 16 weeks of age served as controls.

[Results] In each group, palpable mammary tumor arose 8-9 weeks after MNU administration, and all tumors were histologically diagnosed as adenocarcinomas. Mammary cancer incidence and multiplicity in newborn, 3 or 4-week-old rats was 21.4% and 0.43, 60.0% and 1.20, and 88.2% and 1.82, respectively. Induced cancers showed similar morphology.

[Conclusion] Four-week-old rat was most susceptible to MNU followed by 3-week-old rats, and less susceptible in newborn rats. The results may reflect the hormone levels and the degree of mammary gland differentiation at the time of MNU administration[2]. Additional immunohistochemical results will also be presented.

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P-039

Expression of TWIST-1 in Stromal Cells of Rat Mammary Fibroadenoma .

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The most frequently encountered types of mammary gland neoplasms are fibroadenoma (FA) and phyllodes tumor (PT). However, differential diagnosis between these two types is difficult due to their similarity in histopathology. Currently, two types of human patients with theses tumors are on a rise due to external environment-dependent hormone changes. Moreover, PT tends to be more malignant and thus has a poorer prognosis than FA. An accurate differential diagnosis is therefore urgently needed. Here, we show that TWIST-1 is highly expressed in the stromal cells of FA, but not in PT and thus, we suggest that TWIST-1 is potentially useful as molecular markers for the differential diagnosis between FA and PT.

Eighteen Fischer 344/Brown Norway F1 hybrid rats were used. They were 14- to 16-week-old nonpregnant (n=3) , pregnant (n=2) or lactating (n=4) rats and 102- to 107-week-old rats (n=9). Breast and other organs from these rats were collected, fixed in formalin (10% neutral buffered solution), embedded in paraffin and sliced into 4 μm sections. These specimens were stained with hematoxylin-eosin or immunostained.

We found that TWIST-1, which has been reported to be up-regulated in cancer-associated fibroblasts, was expressed in stromal cells of FA, but not in stromal tumor cells of PT or normal tissue. As a further characterization of the TWIST-1-positive stromal cells of FA, we demonstrated by immunohistochemistry that these cells did not express any of myoepithelial and myofibroblastic markers such as α-SMA, S-100, p63, calponin, and CD10, which was consistent with the previous literature.

These findings suggest that we can use TWIST-1 expression not only to distinguish FA from PT but also as a novel molecular marker of FA. Further study is necessary to elucidate the mechanism underlying TWIST-1 expression in the stromal cells of FA.

P-040

Analysis of Cryoglobulin Reactivity Against Worm Antigens in Experimentally Cryoglobulinemia Model of ICR Mice Induced with *Capillaria Hepatica* Infection

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Experimental infection with *Capillaria hepatica* (*C. hepatica*) causes type II mixed cryoglobulinemia in ICR mice. This is the first experimental animal model of type II mixed cryoglobulinemia, which is characterized by formation of cryoglobulin contained a monoclonal immunoglobulin (Ig) M rheumatoid factor and IgA (Aihara et al, 2011). Using this animal model, we aimed to study the in vitro reactivity of cryoglobulin (CG) and Ig isolated from glomerular deposits with soluble *C. hepatica* worm antigens isolated from the serum.

ICR mice, experimentally infected with *C. hepatica* eggs, were euthanized at 10, 20, and 30 days post injection (DPI). Serum samples were tested for CG formation and *C. hepatica* antibody titer. Igs were extracted using acid buffer from isolated glomeruli samples of 30 DPI mice kidneys. The reactivity of IgMs derived from glomerular deposits with the soluble fraction of *C. hepatica* worms was analyzed using the Western blot analysis. Immunofluorescence demonstrated localization of the CG reactive site in the *C. hepatica* worm.

The formation of cryoprecipitate and increase in the antibody titer for *C. hepatica* were detected in all infected mice at 30 DPI. In the Western blot test, the serum of 30 DPI mice reacted with multiple worm antigens. CG solution and glomerular-isolated Ig from all 30 DPI mice reacted with 55 kDa worm antigens. However, the serum of the control mice (10 and 20 DPI) did not react with the worm antigens. In immunofluorescence, CG solution strongly reacted with the wall of the worm body.

Polyclonal Igs against *C. hepatica* significantly increased in the serum of 30 DPI mice. On the other hand, IgMs formed CG or glomerular deposits and reacted with a specific antigen of the *C. hepatica* worm. These results indicate that the reaction of monoclonal IgM with specific antigens of the *C. hepatica* worm plays a key role in the pathogenesis of type II cryoglobulinemia in this mouse model.

P-041

Influence of variable host immunity on the incidence of cryoglobulinemia in mice experimentally infected with *Capillaria hepatica*.

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Experimental infection with *Capillaria hepatica (C. hepatica)* causes type II mixed cryoglobulinemia in ICR mice (Aihara et al, 2011). The status of host immunity is an important factor in the pathogenesis of cryoglobulinemia. In this study, we aimed to elucidate the influence of host immunity on cryoglobulinemis in mice infected with *C. hepatica* by comparing pathological findings between three mouse strains, each exhibiting a different immune response. We used C57BL and BALB/c mice as strains having Th1-type and Th2-type dominant immune responses, respectively. In addition, ICR mice were used as the positive control group. Mice infected with *C. hepatica* were euthanized at 30 days post infection, and their livers, spleens, and kidneys were histopathologically examined. Using immunoflorescence, we detected mu heavy, kappa light, and lambda light chains of immunoglobulin (Ig) in the spleens and kidneys of these mice. Ultrastructure of the renal glomeruli of each group was studied by electron microscopy. Histological examination revealed marked eosinophilic infiltration in the livers of BALB/c and ICR mice injected with *C. hepatica* eggs. On the other hand, the livers of C57BL mice were characterized by the infiltration of macrophages with multinucleated giant cells. Eosinophile was also detected in the spleens and bone marrows of ICR and BALB/c mice. In the renal glomeruli of ICR and BALB/c mice, periodic acid-Schiff-reactive materials filled the capillary lumina. Electron microscopy revealed that these materials were composed of lamellar structures, which are the characteristic features of cryoglobulin deposits. Interestingly, no histological change was observed in the glomeruli of C57BL mice. Immunofluorecence staining revealed significant deposition of Ig mu heavy and kappa light chains in the glomeruli of ICR and BALB/c mice compared with that in the glomeruli of C57BL mice. The number of double-positive cells for mu heavy and kappa light chains of Ig significantly increased in the spleens of BALB/c mice and mildly increased in the spleens of ICR and C57BL mice. The deposition and characteristic structures of Ig observed in the glomeruli of ICR and BALB/c mice suggested that cryoglobulinemia occurred in these two strains but not in C57BL mice. In addition, marked eosinophilic infiltration in the livers, spleens, and bone marrows of ICR and BALB/c mice suggested that Th2-type immune responses were dominantly activated in *C. hepatica*-infected mice. These results strongly suggest that predominance of the Th2-type response in the immune status of the host promotes the production of cryoglobulin in *C. hepatica* infection.

P-042

Immunopathological Changes in Immunotoxicological Study of Mice Treated with Methoxychlor.

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Accumulating evidence suggests that several chemicals can potentially disrupt the immune system, thereby affecting human health. WHO proposed a new guidance for evaluating immunotoxicology in 2010, and immunotoxicolgical evaluation is one of the most important topics in toxicology assessment. We previously reported that methoxychlor (MXC) induced apoptosis of double positive T cells in Balb/c mice. Here we investigated the pathological changes on immune systems in Balb/c mice treated with MXC and also evaluated the strain difference among Balb/c, C3H/HeN, and ICR mice to MXC immunotoxicity. The three strains of female mice were fed MXC in diets at 0, 150, 500, and 1500 ppm for 2 or 4 weeks. At 4 days before necropsy, mice were immunized with sheep red blood cells (SRBC). Plasma IgM levels were measured by ELISA. In spleen, the specific IgM responses and numbers of lymphocyte cells (T and B cell) were detected by plaque forming cell (PFC) assay and flow cytometric analysis, respectively. Paraffin-embedded spleen, thymus, several lymph nodes, Peyer's patch, and bone marrow were processed for histopathology, TUNEL, and/or immunohistochemistry for CD3, CD45R, CD138, IgG κ-light chain, and Ki-67. As a result, decreases in plasma IgM levels, PFC responses, and the numbers of splenic T and B cells were showed in MXC treated Balb/c mice by immunological analysis. By the same token, decrease in the number of T cells in the splenic PALS, undevelopment of germinal center in the spleen, and apoptosis in the thymic cortex were detected by histopathologic analysis. Furthermore, morphometry supported the possibility of undevelopment of germinal center in the mesentery lymph node and Peyer's patch. No effect was detected in the cervical and axillary lymph nodes or bone marrow. As for strain difference, C3H/HeN mice had similar potential to MXC immunotoxicity to Balb/c mice; however, C3H/HeN mice showed prominent increase of plasmacyte-like cell (CD138+) expressing IgG κ-light chain in splenic PALS after injection of SRBC. Meanwhile, ICR mice were less sensitive to MXC and immune response to SRBC. Overall, these results suggested that MXC induced apoptosis in immature thymic T cells, followed by decrease in number of T cells in splenic PALS and thereby undevelopment of splenic and lymphatic germinal center. There was strain difference of sensitivity to MXC or immune response to SRBC.

P-043

Pathological Examination of Upper Respiratory Tract in Rats Administered Amiodarone Hydrochloride for 4-days

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[Objective] The upper respiratory tract has been recognized as a target of toxicity and gavage related reflux has been suggested as one of the toxicological mechanisms, namely reflux of dosing formulation from esophagus to nasal passage to induce nasal lesion. However, reports are limited on this toxicity using model compounds. To examine the toxic effect of a compound with cytotoxic, amphiphatic and soluble property on the upper respiratory tract, histopathological examinations were conducted in rats administered amiodarone hydrochloride solution. [Materials and methods] In experiment 1, amiodarone hydrochloride was administered to male F344 rats for 4-days at dose levels of 0, 150 and 500 mg/kg/day. Histopathological changes of the upper respiratory tract were examined. In experiment 2, the dose level was set as 150 mg/kg/day and 3 groups were made; a group of 10 mL/kg with feeding *ad libitum*, a group of 3 mL/kg with feeding *ad libitum*, and a group of 10 mL/kg with restricted feeding. Histopathological changes of the upper respiratory tract were examined as well. [Result] In experiment 1, upper respiratory tract lesions were observed in the nasopharynx and nasal cavity. The lesions were observed in 0/5, 3/5 and 2/4 animals at dose levels of 0, 150 mg/kg and 500 mg/kg, respectively. In each animal with nasal lesions, degeneration and/or necrosis of respiratory epithelium was observed in the nasopharynx and level 3 nasal cavity. Some animals had degeneration and/or necrosis of olfactory epithelium and some had those of respiratory epithelium of level 1 or 2 nasal cavities. In experiment 2, upper respiratory tract lesions were observed in the group of 10 mL/kg with feeding *ad libitum* and the group of 10 mL/kg with restricted feeding. The lesions were similar to those of experiment 1. In contrast, no lesions were observed in the upper respiratory tract in the group of 3 mL/kg with feeding *ad libitum*. [Discussion] This study demonstrated induction of upper respiratory tract lesion by 4-day repeated administrations of amiodarone hydrochloride (150 mg/kg, 10 mL/kg). The frequent site was a caudal part of the nasal passage consisting of nasopharynx and level 3 nasal cavity. The lesions were prevented by the lowering of dosing volume. The frequent site and effect of dosing volume was consistent with the gavage-related reflux theory. The effect of feeding conditions was not clear in this study. Amiodarone hydrochloride would be a useful model compound to elucidate a toxic mechanism of upper respiratory tract lesions in rats.

P-044

Ultrastructure of the Bronchial Cilia in Congenital Hydrocephalic Rats

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Hydrocephalic rats were found in the colony of Crl:CD(SD) rats in our laboratory. The results of a backcross-mating and breeding experiment showed that this congenital hydrocephalic brain anomaly could be by autosomal recessive inheritance. The pathogenesis of hydrocephalus is important for defining the scientific value of this rat model, therefore, we performed transmission electron microscopic examination on the bronchial cilia of the affected animals because ciliary abnormalities have been reported in some mouse models of hydrocephalus.

Bronchial ciliated epithelia from 5 hydrocephalic rats and 6 normal SD rats at 10 days of age were examined (300 cilia/animal). In the normal SD rats, all the cilia were arranged in parallel orientation of the ciliary axis and almost all axonemes which are microtubule cytoskeletons in the cilia had the normal 9 outer doublet microtubules and a central pair of single microtubules (9+2) structure. On the other hand, in the hydrocephalic rats, the arrangement of cilia was misaligned due to inconstant orientation of ciliary axis. Some axonemes showed abnormal structures such as 9+0, 8+2 and 8+0, abnormal position of the doublet microtubules or compound cilia. The incidence of the abnormal cilia accounted for 14.0-17.1% of the cilia examined.

In conclusion, the congenital hydrocephalic rats have bronchial cilia with cytoskeletal abnormalities in the ciliary axes and axonemes which were nearly identical to primary ciliary dyskinesia (PCD) in human patients with chronic respiratory disease, male sterility and situs inversus. There are no appropriate rat models for PCD, thus our hydrocephalic rats would be useful as disease models of PCD and PCD-related hydrocephalus.

P-045

Histopathological study of the mechanism of cardiac toxicity by microtubule-disassembling drugs

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Microtubule-disassembling drugs (MDDs), which include colchicine (COL) and vincristine (VCR), have been recognized as cardiotoxic. However, there are few histopathological reports on the cardiac toxicity of MDDs, the mechanism of which has still not been elucidated. In this study, we histopathologically and electron microscopically analyzed the heart of rats administered COL and VCR, to elucidate the mechanism of cardiac toxicity of MDDs.

Glucose (5%, for 2 days with single daily dosing), COL (1.00 or 1.25 mg/kg for 2 days with single daily dosing or 2.00 mg/kg single administration) and VCR (0.50 or 0.75 mg/kg for 2 days with single daily dosing or 1.00 mg/kg single administration) were administered intravenously to male Crl:CD (SD) rats aged 6 weeks. The day after administration, the hearts were excised and examined histopathologically and electron microscopically.

On histological examination, degeneration and necrosis of cardiac myocytes with vacuolation were observed in rats administered COL 1.25 mg/kg, COL 2.00 mg/kg and VCR 0.75 mg/kg. Furthermore, pyknosis and karyorrhexis of spindle or oval cells in the cardiac interstitial region were observed in all rats administered COL and VCR. These spindle or oval cells were positive for TUNEL staining. On electron microscopic examination, swelling of mitochondria was observed in cardiac myocytes. Furthermore, basement membrane was observed in the oval cells and spindle cells that showed pyknosis or karyorrhexis.

From electron microscopic study, it appeared that spindle or oval cells expressing apoptotic figures in the interstitial region were vascular endothelial cells. These findings suggest that apoptosis of vascular endothelial cells may play a role in the mechanism of degeneration and necrosis of cardiac myocytes induced by MDDS such as COL and VCR.

P-046

Signet ring cell type adenocarcinoma of unknown origin in a rat

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Signet ring cell type adenocarcinoma or mucinous carcinoma is rare tumor in the rats and only a few cases with this spontaneous tumor in the small intestine have been reported (Maekawa et al., 1990 & Chandra M & Frith CH., 1994). The tumor mass is characterized by the morphologic feature suggesting the mucous production of tumor cells, such as mucous-filling cytoplasmic cysts or extracellular accumulation of mucinous fluid (mucous lake) and suggesting the poorly differentiated tumor cells such as incomplete glandular structure or invasion into adjacent tissue.

We encountered a case of signet ring cell type adenocarcinoma (mucinous carcinoma) of unknown origin with prominent cytoplasmic eosinophilic granules in many tumor cells.

A male F344/DuCrl rat from a control group of a carcinogenicity study was sacrificed at 109 weeks of age. This rat had no clinical signs with good general conditions during the test period for 2 years (104 weeks). No significant changes were detected by gross observation at necropsy, but a brown nodule sized 10×8×3mm was detected in the surface of the duodenal wall at the tissue trimming. Tumor cells of signet ring cell type with round eccentric nuclei and mucous-filling cytoplasm proliferated and partially formed incomplete ductal structure in the duodenal serosal layer corresponding to the brown nodule at gross observation. Neoplastic proliferation was detected also in the pancreatic tissue but there were no significant lesion in the duodenal mucosa. The similar cells were observed in the adipose tissue adjacent to the mesenteric lymph node, and serosal, muscle layer and mucosal propria of the seminal vesicles. In some area, each tumor cells floated in the mucous substance (mucous lake) accumulate in the interstitial tissue. Abundant eosinophilic granules in tumor cells were similar to zymogen granules of the Paneth cell or pancreatic acinar cell.

To determine the tumor cell origin, the morphological and staining property of eosinophilic granules was investigated.

P-047

A Poorly Differentiated Salivary gland Adenocarcinoma with Prominent Squamous Metaplasia in a Pregnant Wistar Hannover Rat

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Salivary gland tumors often develop in carcinogen-treated or irradiated rats. Spontaneous tumors are less common; however, the cases reported were mostly diagnosed as undifferentiated form of tumor which was positive for epithelial (keratin) and/or mesenchymal (vimentin) cell markers in relatively young rats. In this report, we describe the histological characteristics of poorly differentiated tumor, which was suspected to be a salivary gland origin, in a pregnant 16-week-old Wistar GALAS rat (BrlHan: WIST@Jcl). Clinically, a subcutaneous mass was found in the ventral neck on day 8 of gestation and it enlarged up to approximately 30 mm in diameter on day 18. The rat was euthanized on day 20. At necropsy, a pale brown-colored mass was found adjacent to the salivary glands, and necrotic and hemorrhagic foci were evident on the cut surface. The tumor was fixed in 10%-buffered formalin and embedded in paraffin. The sections were stained with H&E and immunohistochemically with several antibodies including keratin, vimentin, p63, glial fibrillary acidic protein (GFAP), prominin-1 and synaptophysin. The tissue was also stained with Alcian blue, PAS and Masson-trichrome stain. Microscopically, the mass was well demarcated from the adjacent tissues such as the epidermis, with slight compressions of the residual submandibular and parotid glands. Partial invasion into connective tissues was noted; however, no evidence of metastasis to other organs including the regional lymph node was observed. The majority of the mass consisted of a diffuse sheet of poorly differentiated epithelial-like cells, but acinar or ductal structure was extremely limited. Some of the peripheral cells were arranged in a nest-like structure. In both areas, the neoplastic cells were comprised of irregularly-shaped nuclei with prominent nucleoli, basophilic cytoplasm and distinct cell membrane. Mitotic figures and apoptosis were observed frequently. Foci of squamous metaplasia, necrosis, hemorrhage and interstitial vascularization were present throughout the mass. Immunohistochemically, the neoplastic cells were positive for keratin (an epithelial marker), vimentin (a mesenchymal marker), GFAP and p63 (myoepithelial markers), prominin-1 (a marker for intercalated ducts and acinar cells), slightly positive for synaptophysin (a neuroendocrine marker). A part of neoplastic cells showed similar stainability of prominin-1 to the submandibular and parotid glands. Collectively, we diagnosed the tumor as a poorly differentiated adenocarcinoma derived from epithelial, myoepithelial and/or neuroendocrine lineages in the submandibular and/or parotid glands. To our knowledge, this case was the first report on a salivary gland tumor in a pregnant female animal.

P-048

A Paraganglioma at Posterior Wall of Left Atrium Originated from Aortic Body in a Wistar Hannover Rat

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The small cardiac tumor was detected at posterior wall of the left atrium of a 110-week-old female Wistar Hannover rat (Slc: Wistar Hannover/Rcc) in carcinogenicity background study. Tumor cells were polygonal to oval in shape which had slightly basophilic and granular cytoplasm. Also, these cells were arranged in distinctive cell nests, called Zellballen, which were separated by reticulin fibers. The nuclei were round to slightly oval. A few mitotic figures were found. Cytoplasmic granules of tumor cells were negative by Fontana-Masson and Periodic acid Schiff (PAS) staining. By immunohistochemical stainings, tumor cells were positive for neuroendocrine markers such as synaptophysin and chromogranin A, but were negative for S-100, vimentin, cytokeratin, and α smooth muscle actin. On the other hand, the surrounding sustentacular cells were positive for S-100. The immunohistochemical features of tumor cells were quite similar to those of aortic bodies. Tumor cells were infiltrated into the myocardium of the left atrium, and were also noted within vessels. Based on these findings, it is reasonable to consider that the present tumor is diagnosed as a paraganglioma originated from aortic body.

P-049

A case of intratubular seminoma in a young SD rat

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There are many reports of seminoma in humans and canines, however, the incidence of seminoma is extremely rare in rodents, and all of them reported were originated from carcinogenicity studies. In this case report, we describe histological and immunohistochemical characteristics of intratubular spermatocytic seminoma observed in a 10 weeks old SD rat.

A male Crl;CD(SD) rat from high-dose group in a 4-week toxicity study was sacrificed at 10 weeks old. In this animal, there were no gross lesions at autopsy.

Histopathologically, proliferative lesion was observed in a seminiferous tubule of the right testis. The proliferative cells were round to polyhedral, with, slight to moderate eosinophilic cytoplasm, distinct cell boundaries, and sheet-like proliferation. The nuclei were large, round to polyhedral, contained one to several nucleoli, and mitotic figures were numerous. Smaller round cells with dark condensed nuclei as well as larger mononucleated or binucleated giant cells were observed occasionally. Some spermatids were found in the seminiferous tubule. The proliferative cells showed PAS-negative staining. Immunohistochemistry was strong positive for PCNA and slight positive for c-kit, but negative for PLAP. Other animals in the same study had no proliferative lesions in the testis. Additionally, structurally analogous compound was used to conduct Ames test and 13-week toxicity study. The result of the former was negative, and no proliferative lesions were observed in the latter. Therefore, this case was considered to be occurred spontaneously.

In this case, the proliferative cells did not show the spireme-like distribution of chromatin, but had three cell populations (small, medium, and large size). Moreover, it is reported that the neoplastic cells of the spermatocytic seminoma in humans are negative for PAS staining, and immunohistochemically variable staining for c-kit and negative for PLAP. In conclusion, this case was diagnosed as intratubular spermatocytic seminoma.

P-050

Eosinophilic Granules Observed in the Mammary Gland of a Cynomolgus Monkey

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Background: In preclinical toxicology studies using cynomolgus monkeys, scattered cytoplasmic small eosinophilic granules in the mammary gland epithelium are sometimes observed, although the nature is not well understood. Recently, we experienced a case where the mammary gland had unusually numerous and large eosinophilic granules in the epithelium of many lobules. In the present report, we present the histopathological findings in comparison with non-lactating (normal) and lactating mammary glands to investigate the biological implication of the increased size and number of eosinophilic granules.

Animal: The animal was 4 year and 11 month-old nulliparous female imported from China. There were no noteworthy findings in the in-life observations or on necropsy of this animal.

Materials and Methods: Immunohistochemistry examinations for lactalbumin and casein, the major milk proteins, were performed for this case, normal animals with small eosinophilic granules in the mammary gland (N = 4), normal animals without granules (N = 2) and a lactating animal (N = 1). Electron microscopic examination was performed for this case and a normal animal without granules (N = 1).

Results: The eosinophilic granules were negative for lactalbumin and casein both in this case and in the normal animals with small granules while, the cytosol of the lactating mammary gland epithelium was diffusely positive for lactalbumin and casein, but eosinophilic granules did not exist. On electron microscopy, electron-dense spherical materials were observed both in this case and in a normal animal without granules. Though the size and number of materials were much larger in the case compared to that in the normal animal, there were no apparent differences in the ultrastructures.

Discussion: Eosinophilic granules in non-lactating mammary gland epithelium in monkeys seemed to be a form of secretory granules which were not derived from the major milk proteins. Because immunohistochemical and electron microscopic features of the eosinophilic granules in this case and in normal animals were similar, the increase in the size and number of the eosinophilic granules is considered to be a continuum of change in a physiological response. However, their incidence and severity should be examined further to elucidate the physiological roles of these granules.

P-051

Spontaneous Squamous Metaplasia in the Prostate of Rabbits

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[Background] Spontaneous epithelial squamous metaplasia and/or hyperplasia are known to occur in the prostate of rabbits. Recently, very few toxicity studies used rabbit are conducted, and thus, the chance encountering these lesions is very rare. Furthermore, to our knowledge, there has been no report concerning detailed morphological characteristic properties of these lesions. In the present study, we investigated the morphological properties of prostatic epithelial squamous metaplasia and/or hyperplasia in rabbits.

[Methods] Prostates removed from five month-old male rabbits (New Zealand White) were fixed by 10% phosphate buffered formalin. The prostate was cut in a cross-section to provide HE-stained sec lesions tions. Silver-stained, and cytokeratin 5 and Ki-67 immunohistochemical stained sections were also conducted.

[Results] The frequently occurred in the dorsal region in the prostate, and were located in the apical portion of papillary mucosal folds of glandular epithelium. The lesions showed two morphological patterns: One was nodular basal cell hyperplasia with squamous epithelium. Glandular epithelium of the prostate was replaced by squamous epithelium. The nodular basal cell hyperplasia was consisted of basophilic cytoplasmic cells that showed downgrowth toward the interstitium. The other was hyperplasia consisting only of the basal cells that showed intraepithelial growth. The lesion, nodular basal cell hyperplasia with squamous metaplasia, has eosinophilic cytoplasmic granules similar to trichohyalin granules, and ghost cell-like cells and eosinophilic laminate materials were also found in the squamous metaplasia. In both lesions, lymphocytic infiltration was observed around the lesions. Basal cells in both lesions revealed positive reaction for cytokeratin 5 and Ki-67, and there was no cell growth cross the basal membrane.

[Discussion] These prostatic lesions were considered to be essentially nodular hyperplasia of prostatic basal cells with squamous metaplasia similar to epithelium of hair follicle. The pathogenesis of these lesions is unclear. However, it is considered to be related to inflammatory change because that lymphocytic infiltration was present around the lesions. These lesions may be specific to rabbits as no similar lesion has been found in any other species.

P-052

Interstitial Lung Disease in Beagle Dogs

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Interstitial lung diseases (ILDs), more accurately known as diffuse parenchymal lung diseases, describe a heterogeneous group of parenchymal lung disorders in humans such as usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP), idiopathic interstitial pneumonias (IIPs), drug-induced ILDs and occupational and environmental lung diseases. In dogs, ILDs are reported in some breeds, especially in west highland white terriers which show UIP-like features. However, ILDs of dogs are poorly characterized. We report here two beagle dogs with severe interstitial fibrosis in their lungs. The dogs were 8-months-old, and showed no specific clinical signs. At necropsy, spontaneous focal emphysema-like lesions were found in the lungs. The lesions were fixed in 10% buffered formalin and routinely embedded in paraffin. The sections were stained with hematoxylin and eosin, Periodic acid-Schiff and Masson-trichrome stains. The lesions consisted of expanded alveolar ducts and spaces, and thickening of alveolar walls were prominent. Thickened alveolar walls were comprised of dense connective tissues, proliferation of fibroblastic cells, and focal accumulation of lymphocytes and macrophages, without any typical epithelial lining cells. The lesions are basically lacking in patchy fibrosis with remodeling of lung structure which is a feature of UIP. These histopathological changes resemble the human NSIP. Human NSIP takes two forms, cellular pattern and fibrosis pattern. The cellular pattern is characterized by mild to moderate interstitial chronic inflammation with hyperplastic pneumocytes, whereas the fibrosis pattern is characterized by diffuse thickening of alveolar walls by dense or loose connective tissues and interstitial chronic inflammation. Based on the histopathological changes, we diagnosed the lesions of the two dogs as ILD, which resemble fibrosis pattern of NSIP. To our knowledge, the cases were the first report of severe form of ILD in dogs.

P-053

Histopathological studies on the siderotic nodule of the spleen in beagle dogs.l

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Siderotic nodule, also called as siderotic plaque or siderofibrosis is the changes consisting of the deposition of basophilic substances or brownish pigment and old fibrosis located in the sub-capsular region of the spleen, which is often seen in untreated beagle dogs. The changes are often associated with localized hemorrhage nearby. The change is considered to be similar to Gamna-Gandy body which is observable in human patients of chronic splenic congestion such as Banti's splenomegaly or in sickle cell anemia. In these diseases, the basophilic substance deposition was demonstrated to be hemosiderin and calcium. In the beagle dogs with the change, however, no splenic congestion or hematological abnormalities were observed. In order to find out similarity or dissimilarity to the human change, the basophilic substance deposition was identified by Berlin-Blue staining and Perls' method for iron, and Kossa's method for calcium.

The basophilic substance deposition in the siderotic nodule was stained with Berlin-Blue and Perls' staining but not stained with Kossa's method for calcium. From the result, siderotic nodule in the spleen observable in dog is considered to be dissimilar to Gamna-Gandy body in human.

P-054

Inflammatory Fibroid Polyp in the Duodenum of Common Marmoset (*Callithrix Jacchus*)

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A 32-month-old male common marmoset (*Callithrix Jacchus*) had a mass in the duodenal wall. The lesion was white colored, and the cut surface showed grayish white and firm. Histologically, the mass consisted of a proliferation of spindle cells with oval to spindle-shaped nucleus in scant eosinophilic cytoplasm, and the spindle cell proliferated without specific proliferation pattern in loose myxoid or fibrotic background. Some of the cells formed concentric pattern around vessels like onion-bulb structure. Additionally, marked inflammatory cellular infiltration, mainly eosinophils, was observed throughout the mass. Immunohistochemically, the spindle cells were positive only for vimentin, alpha smooth muscle actin, and fascin, whereas negative for S-100, factorVIII related antigen, and c-kit. These histological and immunohistochemical features did not meet differetial diagnoses including gastrointestinal stromal tumor, hemangiosarcoma, hemangiopericytoma, schwannoma, smooth muscle tumor. Collectively, we diagnosed the mass as the lesion corresponding to an inflammatory fibroid polyp (IFP) in human. IFP is defined as mesenchymal proliferation composed of a mixture of stromal spindle cells, small blood vessels, and inflammatory cells, particulary eosinophilis, and currently classified into non-neoplastic lesion. To our best knowledge, this is the first report of spontaneous IFP in the animal.

P-055

nNOS or iNOS expression level is not involved in the different susceptibility to dopaminergic neurotoxicity induced by MPTP between C57BL/6 and BALB/c mice

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces severe degeneration of dopaminergic neurons when administered to C57BL/6 mice, but such lesions are not observed in BALB/c mice. To clarify the factors which influence such marked strain differences in the susceptibility to MPTP, the involvement of nNOS and iNOS was investigated. MPTP was ip administered to adult C57BL/6 (highly sensitive) and BALB/c (resistant) mice. Immunohistochemical analysis using an antibody to tyrosine hydroxylase (TH) showed a significant decrease in TH-immunopositive areas in the striatum and TH-positive cells in the substantia nigra pars compacta of MPTP-treated C57BL/6 mice at 1 and 7 days (d) after administration. On the other hand, MPTP-treated BALB/c mice showed no significant changes. By Western blot analysis, TH, MAO-B, DAT, nNOS and iNOS protein expression levels were examined in intact and MPTP-treated mice. Intact BALB/c mice showed higher DAT protein expression in the striatum and TH protein expression in the midbrain than intact C57BL/6 mice. In addition, MPTP-treated BALB/c mice showed a more significant increase of MAO-B expression than MPTP-treated C57BL/6 mice at 12 h. The increase of nNOS and iNOS protein expressions in MPTP-treated BALB/c mice was more pronounced in the striatum and midbrain than in MPTP-treated C57BL/6 mice at 12 h and 2 d. These results indicate that MAO-B, DAT, nNOS or iNOS expression levels are not involved in the different strain susceptibility to MPTP.

P-056

Changes of Nigrostriatal Tyrosine Hydroxylase (TH) and Dopamine Transporter (DAT) in the Hamster After a Single Intrastriatal Injection of 6-Hydroxidopamine

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One of the most important models for analyzing the pathomorphological aspects of Parkinson's disease (PD) is the 6-hydroxydopamnie (6-OHDA) model where lesions of the nigrostriatal axis are observed when 6-OHDA is intrastriatally injected. In the present study, we evaluated for the first time the effects of a single intrastriatal injection of 6-OHDA (20μg in 2μl of vehicle) in young-adult golden hamsters. A significant decrease in tyrosine hydroxylase (TH)-positive area and dopamine transporter (DAT)-positive area was found in the ipsilateral striatum 3 days after the injection. The decrease continued for 7 days and a recovery trend was found at 15 and 21 days post injection. On the other hand, no effect of the injection was found on the contralateral side. In the substantia nigra pars compacta (SNpc), a significant decrease in the number of TH-positive cells appeared one week after the injection with the peak-value in the loss of TH-positive cells being recorded two weeks post-injection.

On the basis of the present results, we believe that the golden hamster is a suitable model for studying axonal regeneration as well as the response of nigrostrialtal cells to neuroprotective agents involved in possible therapeutics for PD.

P-057

Light and scanning electron microscopic changes of rat brain treated with multiwalled carbon nanotubes by intratracheal instillation

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【Introduction】Oberdörster *et al*. (2004) proposed the translocation pathway to the brain from the nasal cavity through the olfactory nerve on the basis of their single inhalation study of graphite nano particles in rats. In the last year, we reported the following points on deposition of the multiwalled carbon nanotubes (MWCNTs) found in the brain of the rat treated with intratracheal instillation at the dose of 160 μg/rat : (1) MWCNTs were observed in whole area of the brain, (2) no histopathological changes were observed, (3) the incidence of MWCNT deposition and the number of deposited MWCNTs increased time-dependently, (4) MWCNTs were found in the cerebral blood vessels by scanning electron microscopy (SEM). In this study, the data of 40 μg group was added, and SEM examination of MWCNTs translocation to rat brain was conducted.

【Methods】An intratracheal instillation of MWCNT (MWNT-7, Mitsui & Co., Ltd) to 13wk-old male F344 rats was conducted at the dose of 0(vehicle control), 40 and 160 μg/rat. The rats were examined on days 1, 7, 28 and 91 after the single instillation of MWCNTs for light microscopy with Kernechtrot staining and for SEM.

【Results】The following results were obtained by light and SEM examinations : (1) No histopathological changes, such as an inflammation, were observed in surrounding area of the deposition in brain tissues of both 40 and 160 μg groups, (2) MWCNTs were observed in the brain from day 1, and the incidences of MWCNT deposition reached 100 % on day 28 in both groups, (3) the number of deposited MWCNTs increased linearly with time after instillation, and the deposition in the 40 μg group was approximately 1/10 of the deposition in the 160 μg group. (4) SEM image analysis indicated that MWCNTs were probably present in the brain parenchyma**.** Futher study is needed to clarify the actual situation about the MWCNTs in the brain, since MWCNTs have not been accurately identified. (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-058

Brain lesions by Ketamine Treatment in the Juvenile Rats

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[Introduction]

It has been reported that multiple injections of the toxic dose of ketamine (NMDA receptor antagonist) at 90-min intervals over 9-hr induced increases of neuronal apoptosis or neuronal degeneration in the developing rat brain (Pediatric Anesthesia 2002;12;770). During development of the brain, the window of vulnerability to the toxic effects of anesthetic including ketamine is restricted to the period of rapid synaptogenesis (Anesthesia & Analgesia 2008;106;1599). To investigate the vulnerability of different age of rats to ketamine toxicity we counted the number of TUNEL-positive cells in the brain after ketamine treatment to different age of juvenile rats. The toxic effect to brain by repeated daily treatment of ketamine was also examined.

[Materials and Methods]

Post-natal day (PND) 7 or PND 14 male and female rats (n=4-5/group) were administered 0 (saline control) and 25 mg/kg of ketamine diluted with saline (2.5 mL/kg) by i.p. multiple injections with 90-min intervals over 9-hr. Twenty-four hours after the initial injection, the rats were perfused with buffered 4% paraformaldehyde (PFA) under pentobarbital anesthesia (PND7_24h and PND14_24h necropsy groups). After fixation in 4% PFA, paraffin-embedded brain sections (levels 3, 5 and 7) recommended by STP (Toxicol Pathol 2006;34;296) were stained with HE and TUNEL method, and were histopathologically examined. Number of TUNEL-positive cells in the thalamus, hypothalamus, amygdala, striatum and retrosplenial cortex was also counted and was compared with each control. PND 7 rats were administered ketamine, and were necropsied after 2 weeks to confirm the reversibility (PND7_2w necropsy group). In addition, PND 7 rats were daily administered 0, 25 and 50 mg/kg of ketamine for 3 weeks, and the brain sections were subjected to histopathology in the same way (PND7_3w repeated group).

[Results and Discussion]

Increased number of TUNEL-positive cells was observed in the thalamus, hypothalamus, amygdala, striatum and retrosplenial cortex of PND7_24h group. However, no brain lesions were observed in PND14_24h, PND7_2w, and PND7_3w groups. These results indicated that developing brain (PND 7) was most vulnerable to ketamine toxicity, and that affected brain (increased apoptosis) was returned to normal level after 2-week recovery period. Repeated daily treatment of toxic doses of ketamine for 3 weeks did not induce brain lesions.

P-059

Effect of developmental exposure to nicotine on the neurogenesis of the hippocampal dentate gyrus in rats

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In order to establish an *in vivo* evaluation system for developmental neurotoxicity, we focused on the neurogenesis of the dentate gyrus in the hippocampus. In the present study, we examined developmental exposure effect of nicotine, a cholinergic neurotoxicant and a risk factor of the effect of *in utero* cigarette smoke exposure, on neurogenesis in rats. Pregnant SD rats were administered (−)-nicotine hydrogen tartrate salt at 2, 10 and 50 ppm in the drinking water supplemented with 1% saccharin from gestational day 6 to postnatal day (PND) 21. Control animals were administered L-(+)-tartaric acid at 50 ppm supplemented with 1% saccharin in the same manner. For the offspring on PND 21 and 77, immunohistochemical analysis in the hippocampal dentate gyrus was performed for proliferating cell nuclear antigen (PCNA), doublecortin (DCX) and Reelin, as well as TUNEL-assay. Viability index on day 4 after birth decreased at 50 ppm. Decreased body weight was observed at 50 ppm in both sexes from PND 7, 10 ppm in males from PND 28 and 2 ppm in males on PND 70 and 77. Decreased food consumption was observed in males in all treated groups and females at 10 and 50 ppm from PND 28 and females at 2 ppm on PND 70 and 77. Brain weight at 50 ppm in both sexes showed significant decreases on PND 21 and 77. Urine cotinine level on PND19 in nicotine treated groups increased with a dose-dependent manner. In the immunohistochemical analysis, DCX-positive cells in the subgranular zone (SGZ) of the hippocampal dentate gyrus increased significantly at 10 and 50 ppm although there were no differences on PND 77. There were no effects on any other immunohistochemical parameters or TUNEL-assay. DCX, a molecule expressed in the late stage progenitor cells (type-2b, type-3 cells) and postmitotic immature neurons, in the SGZ of the hippocampal dentate gyrus increased at 10 and 50 ppm at the end of the nicotine exposure on PND 21. Considering no changes in cell proliferation and apoptosis, the increase of DCX-positive cells may be due to facilitation of neuronal maturation rather than neurogenesis in these animals.

P-060

Effect of Developmental Exposure to Chlorpyrifos on the Neurogenesis in the Hippocampal Dentate Gyrus in Mice

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The organophosphorothionate, chlorpyrifos (CPF), has been extensively used as a pesticide, since it kills insects by irreversibly inhibiting cholinesterase (ChE) and disrupting cholinergic function in the nervous system. However, it remains unclear whether CPF affects neurogenesis during the development in mammals. In the present study, we examined the effect and reversibility of developmental exposure to CPF on neurogenesis of the hippocampal dentate gyrus in mice. Oral doses of CPF (0, 4, 20, or 100 ppm in diet) were given to maternal mice from gestational day 10 until weaning on postnatal day (PND) 21, and the hippocampal dentate gyrus of male offspring were immunohistochemically examined at the end of exposure and also at the adult stage of PND 77. There were no dose-related changes in the body weight in dams and male and female pups during the study, maternal food consumption, body and organ weights and serum thyroid-related hormone levels on necropsy at both PND 21 and PND 77, except for a decrease of serum T_4 at 100 ppm on PND 77. On the other hand, dose-related decreased concentrations of total blood and plasma ChE were observed from 4 ppm in both dams and pups on PND 21. At this time point, brain ChE concentrations (forebrain) decreased in offspring at 20 ppm or more and in dams at 100 ppm. At PND 77, the blood and brain ChE concentrations were recovered in offspring of all exposure groups, while the decreases of plasma ChE concentrations were sustained in these animals. Immunohistochemically, decreases in doublecortin-expressing cells were observed in the dentate subgranular zone of offspring at 20 and 100 ppm on weaning, and they were recovered at PND 77 at both concentrations. Decrease in NeuN-positive postmitotic neurons was also observed in the granular cell layer of offspring at 100 ppm on PND 21, while it was recovered on PND 77. The number of subgranular cell populations immunoreactive for PCNA, Tbr 2 or Pax6 and hilar interneurons expressing Reelin were unchanged at both PND 21 and PND 77. These results suggest that CPF at 20 ppm (3.3 mg/kg body weight/day) or more directly targets the immature granular cells to suppress maturation in the subgranular zone during the exposure. However, the effect itself was reversible.

P-061

Early Changes in Medulloblastoma and Cerebellar Development in Patch1 Heterozygous Knockout Mice

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Patched1 is one of the major genes related to medulloblastoma (MB) formation in humans. Heterozygous Ptch1 knockout mice (Ptch1 +/- mice) develop MBs that resemble human MBs. Human MBs are thought to derive from residual cells located in the external granular layer (EGL) of the cerebellum, although EGL cells migrate inward to form the internal granular layer (IGL) during normal cerebellar development. This study was conducted to clarify the early changes of MBs in Ptch1 +/- mice during the process of cerebellar development. Cerebellar development in Ptch1 +/- and wild-type mice was sequentially examined during postnatal days (PNDs) 0 to 21. In addition, a single intraperitoneal injection of 100 mg/kg of 5-Bromo-2 ' -deoxyuridine (BrdU) was given to mice at PND8 and 14 to investigate the migration of EGL cells. The cerebellums were stained with HE and the following antibodies for immunohistochemical analyses: BrdU, Ki-67, and neuronal differentiation markers such as NeuN, Nestin, and $p27^{kip1}$. Microscopically, EGL thickness peaked at PND7. The EGL gradually thinned and disappeared by PND21. The EGL cells that showed positive staining for BrdU immediately after administration sequentially migrated from the EGL to the IGL. A thickened, proliferative lesion that was continuous from the normal EGL and MBs were observed in Ptch1 +/- mice at PND10 and 12, respectively. This indicates that MBs developed within two weeks after birth, which is a dynamic developmental period for the cerebellum. Since these proliferating cells were weakly positive for BrdU, the cells were considered to be derived from the EGL. In Ptch1 +/- mice, EGL-like cell foci were detected in the outermost region of the molecular layer after PND16, when migration of EGL cells to the IGL is almost complete. These cells were also positive for BrdU, showing that the foci were derived from EGL cells. Furthermore, the foci were classified into two types: Ki-67-positive and Ki-67-negative foci. The latter, which were positive for NeuN and $p27^{kip1}$ and negative for nestin, suggests a differentiation into neural cells. In contrast, the Ki-67-positive foci were negative or weakly positive for NeuN and $p27^{kip1}$ and positive for nestin. These results indicate the possibility that the Ki-67-positive foci without apparent neuronal differentiation may progress to MBs.

P-062

Development of An Early Induction Model of Medulloblastoma in Ptch1 Heterozygous Knockout Mice Initiated with N-ethyl-N-nitrosourea

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[Background] The heterozygous ptch1 mouse (Ptch1 +/-) is known as an animal model of medulloblastoma (MB), a common brain tumor in children. Similar to human cases, MBs in Ptch1 +/- mice are thought to arise from granule cell precursors (GCPs) in the external granule layer of the developing cerebellum in a multi-step process. Although the Ptch +/- mouse is a valuable model for studying MBs, the relatively low tumor frequency and wait time for tumor development are disadvantages for evaluating the modulatory effects of chemicals on MBs. Therefore, we attempted to develop an early MB induction model in Ptch +/- mice initiated with N-ethyl-N-nitrosourea (ENU). **[Materials and methods]** Ptch +/- mice and their wild-type littermates received a single intraperitoneal injection of ENU (10, 50 or 100 mg/kg) on postnatal day 1 (d1) or 4 (d4). Intact mice were set as the control. Histopathological assessment of brains was conducted at 12 weeks of age. **[Results]** Although most mice were asymptomatic, a reduced cerebellar size was apparent in d1-treated groups at 50 and 100 mg and the d4-treated group at 100 mg regardless of genotype. Histopathologically, early lesion of MBs occurred with a high incidence in Ptch +/- mice receiving 10 mg on d1 or d4, or 50mg on d1. Comparatively, an induction of MBs was not found in Ptch +/- mice injected with 50 mg on d4, or 100 mg on d1 or d4. At 50 and 100 mg, reduced granular layer thickness, Purkinje cell disarrangement and reduced cerebellar size were observed dose-dependently. In wild-type mice, there was no MB occurrence in either group. **[Discussion]** These results demonstrate that ENU is available for the induction of MBs in Ptch +/- mice, and 10 mg is a sufficient dose. Early induction of MBs at a high rate may provide a useful model for the study of modifier effects on MB tumorigenesis. At the 100 mg dose, it is considered that MBs were not induced since the damage was too severe to allow for GCPs to generate MBs.

P-063

Histopathological study of lesion of the spinal cord in SJL/J mouse's EAE model

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[Introduction]

Experimental autoimmune encephalomyelitis (EAE) is an experimental model of multiple sclerosis. One of the EAE models is that in SJL/J mice (S-EAE). In this model, the mice have repeated relapse and remission after the first onset of neurological symptoms as the disease becomes chronic. Thus, this model is known to more closely mimic human multiple sclerosis than other EAE models. However, there have been no detailed reports on the relationship between neurological symptoms and spinal cord tissue injury in S-EAE. In this study, we conducted a detailed histopathological examination of spinal cord tissue injury in S-EAE. This report presents the results of analysis of the relationship between neurological symptoms and spinal cord tissue injury.

[Methods]

Six-week-old SJL/J mice were subcutaneously injected in the inguinal region with the $PLP_{139-151}$ antigen in complete Freund's adjuvant and with nonviable Mycobacterium tuberculosis. The mice were intraperitoneally injected with Bordetella pertussis toxin immediately after and two days after the aforementioned subcutaneous injection. The scores of neurological symptoms were examined from immediately after antigen injection until 50 days after the injection. SJL/J mice were sacrificed by exsanguination under anesthesia at 7, 12, 27, and 50 days after antigen injection. The lumbar spinal cords (L1-L6) were removed, and L1, L2, and L4-L6 were fixed in 10% neutral buffered formalin solution. Paraffin sections were prepared by standard procedures. The sections were stained with hematoxylin and eosin or a combination of Kluver-Barrera stain and Bodian.

[Results]

Demyelination was observed with inflammatory cell infiltration of varying degrees in various areas of examined lumbar spinal cords in S-EAE. Some mice had demyelination with inflammatory cell infiltration in the reticulospinal tract and vestibulospinal tract. They showed partial paralysis in the hind legs or tail.

[Conclusion]

Mice with paralysis of the hind legs or tail had tissue injuries in the reticulospinal tract and vestibulospinal tract which maintain equilibrium and control muscle flexion and extension. The results of this study suggested that tissue injuries in the reticulospinal tract and vestibulospinal tract affected the neurological symptoms in the S-EAE model.

P-064

Utility of Immunohistochemical Application for Glial Brain Tumors in Rats

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Purpose: We report that glial brain tumors observed in one Wistar Hannover (RccHan^{TM}:WIST) rat and three Crl:CD (SD) rats were suggested malignant reticulosis by immnohistochemical approach.

Material and method: A male wistar hannover rat was necropsied at 84-week-old. In three male SD rats, one of them was moribund sacrificed at 84-week-old, and two of them were found dead and necropsied at 90- or 99-week-old. No abnormal findings in the brain of these animals were observed at the necropsy.

Result: In the H&E staining specimen of the wistar hannover rat, the tumor cells showed solid growth, and localized from piriform lobe to meninges in the cerebrum. The boundary of the tumor and normal tissue was not clear. The tumor cell has an oval or pleomorphic small nucleus with abundant eosinophilic cytoplasm. In H&E staining specimen of the 3 SD rats, the tumor cells showed solid growth, and localized mainly cerebral cortex or hypothalamus. No tumor cells were observed in the meninges. The tumor cell has a small oval nucleus with eosinophilic cytoplasm. In a silver staining, no fiber formation was observed in the stroma of the all 4 grial tumors. Although, GFAP showed negative, Iba-1 and ED-1, the markers of microglia/macrophage, were positive for immnohistochemical stainings of the all 4 glial tumors.

Conclusion: We could not distinguish astrocytoma from malignant reticulosis in these glial tumors in the H&E staining specimens. However, we diagnosed all these glial tumors as malignant reticulosis based on the result of immunohistochemical staining. We propose that glial tumor in rat might be difficult to diagnose by H&E staining, and the immnunohistochemical staining of microglia/macrophage makers such as Iba-1 and ED-1 are useful for a diagnosis of glial tumor in rats.

P-065

GFAP-Positive Neoplastic Cells in Spontaneous Oligodendroglioma and Mixed Glioma of Rats

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Introduction: Glial fibrillary acidic protein (GFAP), which is the principal intermediate filament of mature astrocytes, is used to diagnose astrocytoma in animals including humans. In rats, neoplastic astrocytes are reported to be negative for GFAP except for some induced astrocytomas, although normal or reactive astrocytes are positive for GFAP. In this study, GFAP-positive neoplastic cells in spontaneous oligodendroglioma and mixed glioma of rats are discussed.

Materials and methods: Immunohistochemical examination was performed on spontaneous oligodendroglioma (25 cases) and mixed glioma (5 cases) of F344 and SD rats from repeated dose toxicity studies and carcinogenicity studies.

Result: Neoplastic oligodendroglias, which mainly consisted of clear cells with smaller round dense nuclei and pale eosinophilic cells with round nuclei, were mostly positive for olig2, and some pale eosinophilic cells were positive for GFAP. In the mixed glioma, spindle neoplastic cells with oval nuclei and large round neoplastic cells with large round nuclei were observed in addition to the neoplastic oligodendroglia described above. The spindle and large round cells were negative for olig2 but some spindle cells were positive for GFAP. GFAP-positive and olig2-negative cells, which were observed around or inside of the tumor, were judged to be reactive astrocytes, since the cell was characterized by long process, eosinophilic cytoplasm or clear nuclei.

Discussion: Olig2-positive and GFAP-positive neoplastic cells in oligodendroglioma were suggested to be undifferentiated oligodendroglia, because GFAP reacts positively in immature oligodendroglia. Olig2-negative and GFAP-positive neoplastic cells in mixed glioma were thought to be neoplastic astrocytes, therefore it was suggested that GFAP-positive neoplastic astrocytes exist in rats as well as in other species.

P-066

Background Data of Retinal Function and Morphology in Wistar Hannover (RccHan^{TM}:WIST) Rats

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[Purpose] To collect the ophthalmological basic data based on electroretinography (ERG) and spectral-domain optical coherence tomography (OCT) in retina of Wistar Hannover (WH) rats.

[Methods] Four-week-old WH (RccHan^{TM}:WIST) rats (30 per sex) were quarantined/acclimated for a week, and randomly divided into native (10 per sex, 20 eyes/group) and ophthalmological examination (20 per sex, 40 eyes/group) groups. The rats were housed in stainless-steel cages for 26 weeks without treatment, and general condition and body weight were recorded once a week. In the ophthalmological examination group, scotopic ERG and Spectralis OCT were measured before (baseline) and at 4, 13 and 26 weeks after observation. At the end of the observation, histological examination was performed. SD (Crl:CD(SD)) rats (10 per sex, 20 eyes/group) were used to compare with the WH rats.

[Results] From 4 weeks after observation, ERG a-wave was reduced significantly in male WH and SD rats. Statistically significant decreases in female rats of both stains were observed at 13 weeks, and mean amplitude of ERG a-wave indicated the low value 60% from baseline at 26 weeks. However, the amplitude of ERG b-wave was increased from 4 weeks and recovered to baseline at 26 weeks. Compared with baseline value in male and female of WH and SD rats, mean retinal thickness from OCT image at 26 weeks was significantly decreased in 93%, 92%, 90%, and 89%, respectively. In histopathological examination, retinal dysplasia was observed in three eyes of male WH, and one eye of female SD rats. In one eye of retinal dysplasia in WH rats, OCT from baseline showed a clear low density area associated with spontaneous fluorescence in the outer plexiform layer of retina. Moreover, subretinal hemorrhages were observed in one eye of female WH rats. Compared with those in native group, a higher incidence of serious corneal injury was observed in the ophthalmological examination group, especially in female rats.

[Discussion] The corneal injury which originated in the ophthalmology inspection may be influence in the retinal function. One of WH rats had retinal alteration from the beginning of this study. Its incidence, however, might be within background data of any rat strain.

P-067

***N*-Ethyl-*N*-Nitrosourea Induces Retinal Photoreceptor Damage in Adult Rats**

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Seven-week-old male Lewis rats received a single intraperitoneal injection of *N*-ethyl-*N*-nitrosourea (ENU) (100, 200, 400, or 600 mg/kg), and retinal damage was evaluated 7 days after the treatment. Sequential morphological features of the retina and retinal DNA damage, as determined by the TUNEL assay and phospho-histone H2A.X (γ-H2AX), were analyzed 3, 6, 12, 24, and 72 hr, 7 days, and/or 30 days after 400 mg/kg ENU treatment. Activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) was analyzed immunohistochemically by poly (ADP-ribose) (PAR) expression in response to DNA damage of the retina. All rats that received ≥ 400 mg/kg of ENU developed retinal degeneration characterized by the loss of photoreceptor cells in both the central and peripheral retina within 7 days. In the 400 mg/kg ENU-treated rats, TUNEL-positive signals were only located in the photoreceptor cells and peaked 24 hr after ENU treatment. The γ-H2AX signals in inner retinal cells appeared at 24 hr and peaked at 72 hr after ENU treatment, and the PAR signals selectively located in the photoreceptor cell nuclei appeared at 12 hr and peaked at 24 hr after ENU treatment. However, degeneration was restricted to photoreceptor calls, and no degenerative changes in inner retinal cells were seen at any time points. Retinal thickness and the photoreceptor cell ratio in the central and peripheral retina were significantly decreased, and the retinal damage ratio was significantly increased 7 days after ENU treatment. In conclusion, ENU induced retinal degeneration in adult rats that was characterized by photoreceptor cell apoptosis through PARP activity.

P-068

Pathogenesis of Mice Ocular Coloboma: Relation between Basement Membrane Disintegration and Macrophage Recruitment at Optic Fissure Margin

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Purpose: Typical coloboma is a congenital ocular anomaly, and is caused by a failure in closure of the embryonic fissure of the optic cup. Previous studies have suggested that a failure of basement membrane disintegration at the optic fissure margins might cause the faulty closure of the optic fissure. We have reported that positive gelatinase activity was remarkably increased at the fusing optic fissures in normal fetuses, whereas its activity was weakly positive or indistinguishable at unfused optic fissures in FLS fetuses. This study was attempted to clarify the relationship between gelatinase activity and macrophage recruitment at optic fissure margins immunohistochemically.

Materials and Methods: Serial coronal sections of eyes from F1 fetuses between FLS and CBA mice and FLS fetuses were made, and gelatinase activity was examined there by *in situ* FITC-conjugated zymography. Then, the same sections were followed by immunohistochemical stain with anti- F4/80, anti-Type IV collagen, anti-MMP 2 and anti-MMP 9 antibodies.

Results: In normal fetuses, F4/80-positive macrophages aggregated, and they showed strong gelatinase activity *in situ* zymography at optic fissure margins, where type IV collagen–positive basement membrane was gradually disintegrated. Macrophage was positive for MMP 2 (gelatinase A), but negative for MMP 9 (gelatinase B). Meanwhile, in almost FLS fetuses, type IV collagen-positive basement membrane completely persisted and F4/80-positive macrophages recruitment is rather mild at fissure margins. In addition, MMP 2 and MMP 9 expression were weak.

Conclusion: Macrophage recruitment and intramacrophage MMP 2 expression may cause disintegration of basement membrane at optic fissure margins.

P-069

Oto-Neurotoxicity Induced by 6-Aminonicotinamide, A Potent Antimetabolite of Nicotinamide, in Mice

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[Introduction**]** 6-Aminonicotinamide (6-AN) is gliatoxic by inhibiting various nicotinamide dinucleotide phosphate (NADP)-dependent enzymes, particularly 6-phospho-gluconate dehydrogenase, via the pentose-phosphate pathway and can result in a reduction of ATP. We introduce the histopathological features of oto-neurotoxicity induced by a single dose of 6-AN in mice.

[Materials and Methods**]** Adult male CD-1 mice, 7 weeks of age, received a single intraperitoneal injection of 20 mg/kg of 6-AN. The mice were perfused transcardially with 10% buffered formalin 24, 48 or 72 hrs after administration of the drug. The adult male control CD-1 mice, 7 weeks of age, receiving equivalent volumes of 0.9% saline were transcardially perfused with the same fixative solution. The skulls with the brain and inner ear were decalcified with EDTA-4Na solution and embedded in paraffin wax. Frontal step-serial sections of the whole brain and the organ of Corti were sectioned at 6 μm, and stained with hematoxylin and eosin. A Galton's whistle was used to produce a low pitch sounds at 4,000 to 5,000 Hz and a high pitch at 13,000 to 15,000 Hz for the ototoxicity examination at pre-treatment and postadministration hours (PAH) 24, 48 and 72.

[Results**]** All animals showed auricle and head reactions in response to both the low and high pitches of the Galton's whistle before treatment and at PAH 24. Treated animals did not react to either pitch at PAH 48 and 72.

Microscopic findings in the auditory pathways: All lesions induced by 6-AN were observed bilaterally. At PAH 24, swelling of the oligodendroglia and astroglia was observed in the areas of the cochlear nucleus and superior olivary nucleus. At PAH 48, a spongy state which consisted of hydrophilic swelling of the oligodendroglia and astroglia, and vacuolation of neuropil was observed in the areas of the cochlear nucleus, nucleus of the trapezoid body, superior olivary complex, lateral lemniscus, inferior colliculus, medial geniculate body, and auditory radiation. In these lesions, oligodendroglia with crescent-shaped deformation of the nucleus or pyknotic nucleus were prominent. Astroglial processes around the blood vessels were frequently swollen. Microglia were scattered in the lesions. No changes were observed in the neurons. At PAH 72, the spongy state was more advanced in the areas of the cochlear nucleus, superior olivary complex and lateral lemniscus. Small hemorrhagic focus and thrombus were observed in the lateral lemniscus. Enlarged astroglia were observed first in the cortical layers Ⅲ to Ⅴ in the auditory cortex. Microscopic findings in the organ of Corti: Severe damage of Deiters' cells at PAH 24 and severe damage to inner hair cells at PAH 48 and to outer hair cells at PAH 72 were observed. Swelling of oligodendroglia in the central projection of the acoustic nerve was observed, however no changes were seen in the peripheral process of the acoustic nerve or the spiral ganglion at any point of the investigation.

[Conclusion**]** Our data suggest that the auditory disturbances in mice induced by 6-AN intoxication are produced by two distinct events, acute gliopathy in the brain stem and the cell degeneration of the organ of Corti, which occur independently of each other.

P-070

Feasibility Analysis of Whole Brain Fixation with Methacarn for Global Molecular Analysis in Specific Brain Areas of Rats

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For global analysis of genes and proteins in anatomically specific brain areas in the framework of conventional toxicology testings using rodent animals, it is necessary to establish a procedure for quick and accurate tissue sampling and processing to achieve high integrity and minimal inter-animal variability in the expression of extracted macromolecules. In the present study, we compared the integrity of extracted molecules and variability of expression data with regard to the total RNAs and polypeptides extracted from specific brain areas between methacarn and acetone-fixed tissues using brain-matrix cast after fixation of whole brains. Twenty one- and 63-day-old male non-treated Crl:CD (SD) rats were used. Whole brains were fixed in each fixative for 5 hours and dehydrated in ethanol overnight at 4 °C. Coronal slices were prepared using the brain-matrix cast, and each portion of the specific brain areas, i.e., hippocampal dentate gyrus, corpus callosum, cingulate cortex and cerebellar cortex, was cut out using punch-biopsy devices to extract total RNAs and polypeptides. To examine the integrity and variability in molecular expression between samples, quality check of extracted total RNAs and global polypeptide expression analysis were performed using 2100 bioanalyzer and 2D-DIGE, respectively. As a result, integrity of total RNAs at both age was maintained in the order of unfixed tissue, methacarn-fixed tissue, and acetone-fixed tissue. The integrity of total RNAs in both of the fixed tissue samples was high enough for conducting global expression analysis, such as microarray technique. 2D-DIGE analysis of polypeptides resulted in fluctuations in the spot volume ratio against that of total peptides in small population of high-molecular-weight molecules in both of the fixed-tissue samples as compared with the unfixed ones at both age. However, magnitude of fluctuations in methacarn-fixed tissues was mostly smaller than that of acetone. Moreover, inter-animal variability in the spot volume ratio with methacarn was better than with acetone. In conclusion, whole brain tissue fixation with methacarn revealed to provide availability for global analyses using microarrays and proteome approaches, judging from the quality of total RNAs and polypeptides close to that in unfixed tissue samples. Moreover, polypeptide expression analysis provided less variable data with methacarn compared with acetone. We are now investigating inter-animal variability of global RNA expressions in fixed-tissue samples using microarrays for comparison of availability of methacarn with acetone.

P-071

Novel Rapid Tissue Fixation by Using a Water Oven (Superheated Steam Oven)

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A Water Oven for home-use has a unique heating function by using superheated water to cook food and different from an ordinary reheat function of most microwave ovens, We report a novel rapid tissue fixation method by using this unique function.

We investigated a suitable mode for tissue fixation from three cooking modes of the Water Oven, “Mushimono (steam)”, “Nimono (stew)” and “water oven (roast & bake)”. Various organs from rats were fixed for about 5 min by using these three cooking modes and submitted to routine dehydration, paraffin embedding, and H&E staining. histological examination of these H&E stained sections revealed that the “Mushimono (steam)” mode is most suitable for tissue fixation. Preservation of tissue structures were compareble to formalin fixed tissues, except for melting of connective tissues.

To clarify the cause of melting of connective tissues, tissues were heated in water at 40, 60, 80 or 100 °C. In the tissues treated at 40 or 60 °C, the connective tissues were intact, but other tissue structures poorly preserved/fixed. On the other hand, treatment at 80 or 100 °C led to melting of connective tissues, but adequate preservation of other tissue structures. From these results, the melting of connective tissues was considered to be induced by high temperatures.

To avoid the heat-induced melting of connective tissues, organs were pre-fixed in neutral-buffered 10% formalin for 5 min and fixed by the Water Oven with “Mushimono (steam)” mode for 5 min. The heat-induced effects on the connective tissues were prevented and tissue structure preservation was comparable to formalin fixation. In addition, immunohistochemical staining was also comparable between Water Oven-fixed and formalin-fixed tissues.

In conclusion, we have established a novel rapid fixation method by using the Water Oven. In addition, our method has great advantages for immunohistochemistry by reducing the time of formalin fixation to minimize formalin induced cross-linkage and masking of antigens. No heat induced inactivation has been observed so far.

P-072

Muscle Injury Tests Using Erector Spinae in Minipigs

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A draft guideline for test methods on localized injury published in 1979 recommends the usage of rabbits in muscle injury tests, since (1) the rabbit muscle is a suitable size for injection and analysis and (2) macroscopical observation for injury was easy, since the color of the rabbit muscle is white. On the other hand, it is stated in the guideline that rabbits may be unsuitable for muscle injury tests, since some drugs cause specific responses in rabbits. Generally, dosage per administration is set at a half amount of the clinical dosage. However, the upper limit of dosage was set at 1 mL for the vastus lateralis in rabbits in a revised draft guideline. Therefore, we tried to use minipigs, in which dosage can be higher than in rabbits due to larger muscle size, in a muscle injury test to perform the test under dosing conditions close to the clinical conditions.

For the present study, 6 male minipigs (Göttingen) were used. Injection of 1 mL each of 0.425 and 1.7 w/v% acetic acid and saline, which are used as the control articles in muscle injury tests, was given into the erector spinae. The dosing sites were removed and subjected to macroscopical observation and histopathological examination for 3 animals each 2 and 14 days after administration.

The following findings were noted 2 days after administration: hyperemia, hemorrhage, and white in color in the macroscopical observation and degeneration and necrosis of the muscle fibers, cellular infiltration, and hemorrhage in the histopathological examination. Severity of these findings depended on the concentration of acetic acid. The following findings were noted at the dosing site of 1.7 w/v% acetic acid 14 days after administration: white in color in the macroscopical observation and regeneration of the muscle fibers, fibrosis, mineralization, and cellular infiltration in the histopathological examination. Only regeneration of the muscle fibers was noted in the histopathological examination at the dosing sites of 0.425 w/v% acetic acid and saline.

From the above results, it was confirmed that muscle injury was macroscopically observable in the erector spinae in minipigs, and muscle injury tests were practicable. A large amount of injection can be given to minipigs, and multiple substances can be administered into 1 side of the erector muscle of spine. Therefore, it is considered that muscle injury tests using erector spinae in minipigs are useful.

P-073

Histological Changes Associated with Long-Term Feeding in Clawn Miniature Pigs

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[Objective] In recent years, Clawn miniature pigs (minipigs) have been increasingly used as useful animals for studies on the development of medical devices and other purposes because these animals preserve physiological and anatomical similarities to humans. We have been performing histological examinations on minipigs during their aging process in order to obtain certain data as an useful experimental model animal; we reported more diverse and severe histological findings after 9 months of feeding compared to after 3 months of feeding at the 27th annual meeting of the JSTP. In the present study, we performed histological examinations on minipigs after 15 months of feeding to compare histological changes with those after 3 and 9 months of feeding. [Methods] Male and female minipigs (n = 3/gender) that bred for 15 months after stent implantation in the coronary arteries were used in this study. Animals were sacrificed, all organs and tissues except for the heart were removed and performed histological examination. At necropsy, animals were aged 30–42 months and weighed 45–53 kg. [Results] The most frequently observed findings were cellular infiltration mainly by mononuclear lymphoid cells including eosinophils in the gastrointestinal tract, lungs, spleens, eyelids, and mesenteric lymph nodes. The findings frequently observed were deposition of yellow-brown pigment in the proximal tubular epithelium of the kidney, tubular atrophy associated with hyperplasia of interstitial cells in the testis, and enlargement of follicular cysts and proliferation of granulosa cells in the ovary. Follicular dilation of the thyroid gland in one female, abscessive pneumonic lesion associated with neutrophil infiltration and sparsely mixed with multinucleated giant cells in the cranial lobe of the right lung (one male) were noted only in the animals fed for 15 months. There was no evidence of neoplasms. [Discussion] Inflammatory cell infiltration in the gastrointestinal tract and respiratory organs in the present study was frequently observed after 15 months of feeding, and also these findings had been detected in 3 months and 9 months of feeding. Therefore, it was considered that these histological changes frequently occurred during feeding of minipigs. Tubular atrophy and interstitial cells hyperplasia in the testis and follicular cyst enlargement and proliferation of granulosa cells in the ovary were considered to be aging and/or disuse of these organs. In this study, we used animals that were used for medical device implantation tests, so we need to perform histological examinations on non-treated animals in order to obtain background data. For all that, these results indicate that minipigs are useful animal model for long-term feeding studies when used in consideration of the histological changes observed in this study.

P-074

Development of Two-Stage Ultra-Short-Term Carcinogenicity Model Using rasH2 Mice – Evaluation of Skin Tumor Promoting Effects in rasH2 and Non-Tg Mice

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[Purpose] Short-term carcinogenicity tests using rasH2 mice are possible replacements for conventional two-year tests. To establish an ultra-short-term skin carcinogenicity model using rasH2 mice, we have investigated skin tumor promoting effect of various chemicals in rasH2 mice. In this study, we review results of tests using DMBA as an initiator and TPA as a positive control, comparing rasH2 mice and wild type (Non-Tg) mice.

[Methods] DMBA (50 μg/100 μL acetone) was applied to shaved dorsal skin of female rasH2 and Non-Tg mice (7 weeks old). One week after the treatment, 8 μg or 4 μg TPA was applied to shaved dorsal skin once or twice/week for 6 weeks. Acetone (200 μL) was applied twice a week.

[Results] Non-Tg mice: Skin nodules were observed in 8, 6, 5, and 7 weeks after DMBA treatment in 8 μg (twice/week), 8 μg (once/week), 4 μg (twice/week), and 4 μg (once/week), respectively. The incidence and multiplicity was 30% (0.4), 30% (9.7), 60% (26.8), and 10% (1.6).

RasH2 mice: At 4 weeks after DMBA treatment, skin nodules were observed in all groups, and tumor incidence reached 100% at 5 or 6 weeks in all groups. The multiplicity was 62.4, 47.1, 63.3, and 32.8 with 8 μg (twice/week), 8 μg (once/week), 4 μg (twice/week), and 4 μg (once/week), respectively. The skin nodules were diagnosed as squamous cell hyperplasia, squamous cell papilloma, squamous cell carcinoma, and keratoacanthoma, histopathologically.

[Conclusion] It was confirmed that TPA induced skin nodules in both rasH2 and Non-Tg mice after DMBA initiation. Skin tumors started to be induced at 4 and 5 to 8 weeks in rasH2 mice and Non-Tg mice, respectively.

P-075

Granulation Tissue in the Process of Regeneration of Rat Skin Injury – Case Report in a Wistar-Hanover Rat -

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[Introduction] There have been a few reports of toxicologic skin lesions in repeated dose toxicity studies in rats, although the skin of guinea pigs is usually evaluated in skin irritation and photosensitizing studies. On the other hand, the histopathological process and characteristics are rarely reported in the toxicity study reports. We have started to evaluate spontaneously-occurring skin lesions in rats or dogs used in toxicity studies to obtain higher quality evaluation of toxicity in the skin. In the presentation, we introduce a case report of the skin lesion of a Wistar Hanover rat. [Materials and methods] The sample was obtained from a male Wistar Hanover rat that had ulcerated lesion in the base of the tail. The rat was purchased from Charles River Laboratories (Kanagawa, Japan). The tail lesion was embedded in paraffin after fixation in 10% phosphate-buffered formalin solution. Approximately 4 μm-thick sections were cut, and stained with hematoxylin-eosin, Masson Trichrome and elastica van Gieson. An additional section was stained immunohistochemically with anti-α-SMA. These sections were examined under a light microscope. [Results and Conclusion] The Wistar-Hanover study was started when the rats were 6 weeks old. The tail lesion was observed in the rat at the age of 14 weeks, and was continuously confirmed through the age of 24 weeks. The rat was euthanized at the age of 24 weeks, and the lesion was sampled. The lesion was observed in the epidermis and dermis but did not extend to the tail bone. The ulcer was characterized by a large area of granulation tissue consisting of inflammatory cellular infiltration, proliferation of fibroblasts and fibrocytes, and neovascularization. However, the distribution of fibroblasts showed no uniformity, and there were multifocal areas of α-SMA positive fibroblasts. Many other fibroblasts showed negative α-SMA staining. In conclusion, there were many regeneration processes in the lesion which were observed continuously in the lesion for 10 weeks.

P-076

Histopathological Changes of the Dorsal Skin by Preparative Procedures in Dermal Administration to Beagle Dogs

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[Background] We reported the back ground data of skin lesions (treated sites) found in the repeated dose percutaneous toxicity studies in beagle dogs in the 27th annual meeting of this society. As the result, very slight acanthosis was observed in the sham treatment control groups which had not been administered with the test or base substances, and it was suspected that this change was associated with the administration procedures. In the present study, we investigated the influence of the administration procedures, fitting of a jacket or hair removal (hair clipping and shaving), routinely performed in the repeated dose percutaneous toxicity studies.
[Methods] Three male and three female beagle dogs, 9 to 12 months old, were used in the present study and the fitting of a jacket and the hair clipping and the shaving on the dorsal skin were carried out as parts of the administration procedures. Two males and two females were fitted with a jacket throughout the experiment period (7 days). The hair of the dorsal skin was clipped with an electric clipper on day 1 and was shaved with an electric shaver once (day 7 only), 3 times (day 1, day 4 and day 7) or 7 times (every day). The hair removal areas were 6 sites of about 8 cm × 8 cm on the back (the right and left sides of the neck, middle back, and buttock), and thereby the influence of shaving times was compared in same animal. The sites with no hair removal were prepared as a control. The dorsal skins were sampled on day 8, fixed in 10% phosphate buffered formalin and stained with hematoxylin and eosin. In addition, immunohistochemical staining for Ki-67 was performed.
[Results] In the animals fitted with a jacket, the influences of hair removal on the dorsal skin were observed in all shaving times. That is, the degree of acanthosis, crust and inflammatory cell infiltration increased. The degree of histological changes were largest for 7 times, followed by once and 3 times in this order. The same tendency was observed in the ratio of Ki-67 positive cells in keratinocytes. The influence of hair removal on the dorsal skin was also observed in the animals without a jacket. However, the degree of these changes was less in the animals without a jacket including those with no hair removal.
[Conclusion] Fitting of a jacket or even one-time hair removal induced the histological changes on the dorsal skin. We should therefore consider the additional influences of the preparative procedures and the setting of a sham treatment control group to evaluate accurately the influence of the test substance on the skin in the percutaneous toxicity studies.

P-077

Antimicrobial agent, Tetracycline, enhanced upper alimentary tract *Candida albicans* infection and its-related mucosal proliferation in alloxan-induced diabetic rats

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Previously, we reported that proliferative changes of the forestomach accompanied by chronic inflammation due to *Candida albicans* (*C. albicans*) infection, some of which progress into squamous cell carcinoma, in alloxan-induced diabetic rats. In clinical practice, antimicrobial therapy, particularly tetracycline treatment, is frequently mentioned as a cause of candidiasis. The role of tetracycline treatment is explained that alteration of the bacterial flora is thought to allow an overt fungal infection in the upper alimentary tract. The objective of this study is to ascertain whether or not tetracycline treatment can accelerate early-onset of *C. albicans* infection and the proliferative changes in this diabetes model. Alloxan-induced diabetic rats were given chlorinated water (AL group) or tetracycline solution (0.1% during week 1 and 0.01% thereafter) as drinking water (AT group). They were sacrificed after 25 weeks of drinking. Infection rate of *C. albicans* was 93.3% (14/15) in AT group, and was significantly higher than AL group (28.6%, 4/14). Incidence and severity of squamous cell hyperplasia were enhanced in AT group compared to AL group. Squamous cell carcinoma was detected in one animal in AT group. These findings demonstrate that tetracycline rapidly induces *C. albicans* infection and enhances forestomach proliferative lesion in alloxan-induced diabetic rats. Thus, alloxan-induced diabetic rat model treated with tetracycline is useful for identifying the risk between *C. albicans* infection and its-related carcinogenesis under the diabetic condition.

P-078

Experimental *Candida albicans* infection induces chronic inflammation and mucosal proliferation of upper alimentary tract in alloxan-induced diabetic rats

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Alloxan-induced diabetic rats exhibit mucosal proliferative lesions accompanied by chronic inflammation, and some lesions progress to squamous cell carcinoma (SCC) after 50 weeks of diabetic condition. Diabetic condition and *Candida albicans* (*C. albicans*) infection is profoundly involved in the pathogenesis of lesions. The objective of this study is to induce early-onset inflammation and proliferative changes by experimental *C. albicans* infection in alloxan-induced diabetic rats.

Female 10-week-old WBN/Kob rats were divided into alloxan-induced diabetic rats (AC group) and non-diabetic rats in (C group). They were experimentally infected with *C. albicans* by oral gavage, and sacrificed at 35 weeks old for histopathological examination of upper alimentary tract.

Moderate to severe mucosal proliferative lesions developed in 93.3% (14/15) in AC group. These lesions were consistently accompanied by *C. albicans* infections with suppurative inflammation of mucosal epithelium and lymphoplasmacytic infiltration of submucosa. Squamous cell carcinoma was detected in one rat. Mucosal proliferative and inflammatory lesions with *C. albicans* were not seen in C group

These findings strongly suggest that *C.albicans* infection can induce early-onset and proliferative changes accompanied by chronic inflammation of the forestomach in alloxan-induced diabetic rats.

P-079

Gene Expression Analysis of a *Helicobacter pylori*-Infected and High-Salt Diet-Treated Mouse Gastric Tumor Model

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Although *Helicobacter pylori* (*H. pylori*) infection and excessive salt intake have been known as important risk factors of gastric cancer, interactions of these two factors with expression profiles during gastric carcinogenesis remain unclear. In the present study, we investigated the global gene expression profile in a mouse gastric tumor model combined with chemical carcinogen (*N*-methyl-*N*-nitrosourea; MNU), *H. pylori* infection, and high-salt diet. Tumor multiplicity in the glandular stomach of MNU-treated mice was significantly increased by combination of *H. pylori* and high-salt diet. Gene expression profiles in the gastric mucosa were examined by oligonucleotide microarray, and 36 up-regulated and 31 down-regulated genes with more than 2-fold difference were detected in the combination group compared with *H. pylori*- or high-salt-alone groups. Quantitative RT-PCR confirmed significant over-expression of several candidate genes including *Cd177*, *Muc13*, and *Reg3g*. In immunohistochemical analysis for CD177 using human gastric cancer specimens, over-expression of CD177 was found in 33 of 55 cases (60.0%), significantly correlated with favorable prognosis. Multivariate analysis including clinicopathological factors as covariates revealed high expression of CD177 to be an independent prognostic factor for overall survival. These results suggest that our mouse model combined with *H. pylori* and high-salt diet is available for genetic analysis in gastric carcinogenesis, and CD177 may be a novel prognostic factor for stomach cancer.

P-080

Mouse colon cancer model using benzo[*a*]pyrene and dextran sulfate sodium: histological analysis in the process of an early stage of neoplastic lesion

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[Introduction] Benzo[*a*]pyrene (BP) is highly mutagenic but not carcinogenic in the colon by oral exposure in mice. Our previous study showed that oral treatment of BP followed by dextran sulfate sodium (DSS) in drinking water, which induces colitis in the mouse, induces neoplasms in the colon after 4 weeks (the 27th annual meeting of JSTP, 2011). In the present study, early stages leading to neoplastic lesion were studied histologically in this BP/DSS model.

[Materials and Methods] 9-Week old male $CD2F_1$ mice (6-8 mice/group) were treated with BP orally at 125mg/kg for 5 days, and subsequently with 4% DSS in drinking water for 7 days (BP/DSS-group). Mice were necropsied either immediately, 1, 3, or 5 days after the final treatment with DSS, and the colon was examined histologically. Vehicle-, BP-, and 4% DSS-groups were set as reference groups.

[Results and Discussion] In the BP/DSS-group, there was desquamation of almost all of colonic epithelial cells immediately after DSS treatment. At 3 days following DSS treatment, dysplastic crypts with a branching structure appeared among regenerating colonic crypts; these crypts were considered to originate from stem cells that survived the treatment and had been mutated by BP. At 5 days following treatment, adenomas were present in the colonic mucosa that had been mostly covered with colonic epithelial cells. There was no adenoma in the DSS-group, even though the histological pictures followed a similar time-course to those in the BP/DSS-group. Mice in the BP-group showed normal histology. Immunohistochemical analysis with β-catenin (protein closely associated with both differentiation/proliferation of colonic crypts and initiation/progression of colon tumors) and Ki-67 (indicator of cell division) is underway, and will be discussed together.

P-081

Effect of Caloric Restriction and High Fat Diet on Azoxymethane-induced Carcinogenesis in LETO and OLETF Rats

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We showed recently that diabetic and mildly obese Otsuka Long-Evans Tokushima Fatty (OLETF) rats are more susceptible to azoxymethane (AOM)-induced Zymbal gland and intestinal carcinogenesis than control Long-Evans Tokushima Otsuka (LETO) rats. In the present study, we examined the effect of caloric restriction and high fat diet on AOM-induced carcinogenesis in these strains.

Experiment 1: Six-week-old male LETO and OLETF rats (n=21) were given s.c. injections of 15 mg/kg AOM once weekly for 3 weeks, then fed, 1) control diet, 2) 20% caloric restriction (CR) diet, or 3) high fat diet (HFD, control diet with 10% safflower oil), and killed at 36 weeks of age. The incidences of colon tumors (adenoma plus adenocarcinoma) in control, 20% CR and HFD groups of LETO rats were 45%, 33% and 55%, respectively. Average number of tumors/rat in CR group was fewer than that in HFD group ($P<0.05$). The incidences of colon tumors in control, 20% CR and HFD group of OLETF rats was 76%, 48% and 62%, respectively. Zymbal gland tumors in CR group developed later than those in control and HFD groups in OLETF rats. Experiment 2: Male LETO and OLETF rats (n=5) were given control diet, 20% CR diet or HFD for 5 weeks, and killed at 24 weeks of age. In LETO rats, serum triglyceride, total cholesterol, insulin and leptin levels decreased by CR, and increased by HFD. In OLETF rats, those levels decreased by CR, and increased by HFD except leptin. Expression of hepatic IGF-1 and Sirt-1 mRNA elevated in 20% CR groups of both strains. The liver of OLETF rat HFD group showed severe fatty change histologically.

In conclusion, CR inhibited, and HFD promoted colon carcinogenesis in LETO rats. CR inhibited, but HFD did not promoted colon carcinogenesis in OLETF rats because of early development of Zymbal gland tumors. Hyperinsulinemia and/or hyperlipidemia may exert promoting effect in rat carcinogenesis.

P-082

Modifying effect of castration in PhIP-induced colon carcinogenesis

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Human colonic carcinogenesis is thought to be influenced by androgen. Epidemiological studies suggest that androgens influence colonic carcinogenesis. Animal studies were confused. In the experiment of azoxymethane（AOM）-induced F344 rat carcinogenensis, castration before carcinogen treatment resulted in a decrease of incidence of colon tumors. In 1,2-dimethylhydrazine-induced CBA mice carcinogenensis, castration did not affect the appearance and the incidence of tumours of any site. In AOM-induced SD rat colon carcinogenesis, Chemical castration, but not surgical castration, resulted in increased colonic tumorigenesis. By contrast, rats given testosterone after surgical castration showed decreased colonic tumorigenesis. In this study, we examined the effect of castration in PhIP-induced colon carcinogenesis using F344 rats. Rat were castrated, and then given 400ppm PhIP containing diet for 2 weeks and then given control diet for 4 weeks. This treatment was repeated 3 times, and then all rats were sacrificed and performed autopsy. Each colon was segmented 3 portions, and counted ACF to analyze the effect of castration for PhIP-induced colon carcinogenesis. ACF induced by PhIP was not found a lot. The average number of ACF and the average number of crypts per ACF in PhIP alone group were Total: 9±3、2±1.5, Proximal：2.3±1.2, 2.1±1.7, Middle：2±0.8, 2±1.1, Distal：4.7±1.2、1.8±1.6. Those in PhIP + castration group were Total: 11±5.6, 2.4±1.7, Proximal : 3.7±4.7, Middle : 2±1.6, 2.7±1.4, Distal：5.3±2.9, 2.6±2.4. No statistical significances were found between them, although those in PhIP + castration group were slightly higher than in PhIP alone group. Castration could not be found the prominent modifying effect in PhIP-induced colon carcinogenesis using F344 rats. These results were similar to the results in the study of SD rats.

P-083

Effects of an Angiotensin II Receptor Blocker on Obesity Associated Colon Carcinogenesis

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Obesity is a major cause of metabolic syndrome including hypertension, and is a risk of colon cancer. Moreover, dysregulated expression of adipocytokines is suggested to play important roles in cancer developments. Angiotensinogen, which is the component of renin angiotensin system, is one of the adipocytokines possessing cell growth function.
In the present study, we examined the effects of an angiotensin II receptor blocker on obesity associated colon carcinogenesis using obese KK-A^y mice.
Six week-old KK-A^y mice were treated with azoxymethane (200 μg/mouse) once a week for 3 weeks and given 10, 20 and 50 ppm angiotensin II receptor blocker, 2 days after last azoxymethane treatment. All mice were sacrificed at 13 week-old and the number of colon aberrant crypt foci was evaluated. The number of aberrant crypt foci was decreased around 20% in KK-A^y mice treated with 50 ppm angiotensin II receptor blocker. The number of dysplastic aberrant crypt foci was also decreased in these mice. The expression levels of Pai-1 mRNA in adipose tissue and of c-myc protein in colon mucosa were decreased in the mice treated with 50 ppm angiotensin II receptor blocker.
These results indicated that angiotensin II receptor blocker could suppress obesity-associated colon carcinogenesis partly through the decrease of Pai-1 and c-myc expression.

P-084

Congenic *db/db-Apc*$^{Min/+}$ Mice Increase Intestinal Tumors

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<Bachgroud> Obesity and diabetes are high-risk conditions for chronic diseases, including certain types of cancer, such as colorectal cancer (CRC).

<Aim> The aim of this study was to develop a novel animal model in order to clarify the pathobiology of CRC development in obese and diabetic patients.

<Methods> We developed an animal model of obesity and colorectal cancer by breeding the C57BL/KsJ-*db/db* (db/db) mouse, an animal model of obesity and type II diabetes, and the C57BL/6J-*Apc*$^{Min/+}$ (Min/+) mouse, a model of familial adenomatous polyposis.

<Results> At 15 weeks of age, the N9 backcross generation of C57BL/KsJ-*db/db-Apc*$^{Min/+}$ (db/db-Min/+) mice developed an increased multiplicity of intestinal adenomas (males, 61±15, p<0.001; females, 58±13, p<0.001) when compared to the db/m-Min/+ (males, 32±8; females, 33±6) and m/m-Min/+ mice (males, 31±8; females, 31±6). Blood biochemical profile showed a significant increase (8.3-fold to 11.7-fold) in insulin and 1.4-fold to 2.6-fold increases in RNA levels of insulin-like growth factor (IGF)-1, IRF-1R, and IGF-2 in the db/db-Min/+ mice, when compared to those of the db/m-Min/+ and m/m-Min/+ mice.

<Conclusion> Our findings suggested that the IGFs promoted adenoma formation in the db/db-Min/+ mice and the db/db-Min/+ mice should be invaluable for studies on the pathogenesis of CRC in obese and diabetes patients and the therapy and prevention of CRC in obese people and diabetic patients. (Supported in part by a Grant-in-Aid from the Ministry of Health, Labour, and Welfare of Japan)

P-085

Histopathological Characteristics of Adenomatous Hyperplasia of Glandular Gastric Mucosa in Untreated and Vehicle Treated ICR(CD1) Mice

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Adenomatous hyperplasia (AH) is well-known spontaneous lesion of the glandular stomach in ICR(CD1) mice used in long-term studies. However, the detailed morphology and pathogenesis of AH is still unclear. In the present study, AH of glandular gastric mucosa were investigated histopathologically and immunohistochemically to clarify the histopathogenesis in ICR(CD1) mice untreated or treated with vehicle for two years. 50 male and female ICR(CD1) mice were allocated to each of the two groups, non-treated and gum arabic (GA) group. Mice of GA group were dosed 5% GA in water by gavage daily for 2 years from 6 weeks of age. At the end of experimental period, all survived mice were sacrificed. Stomach tissues from all dead, moribund and sacrificed animals were examined histopathologically and immunohistochemically using Ki-67 antibody. As results, survival rates in non-treated and GA group were 42 and 44% in males and 36 and 28% in females, respectively. Incidences of AH in non-treated and GA group were 48 and 26% in males and 26 and 24% in females, respectively. The AH lesions protruded into the lumen expanding from limiting ridge to fundic region and detected as irregular thickening of the mucosal layer due to proliferation of the mucous neck cells and/or surface mucous cells. The severe lesions of AH were consisted of only glandular structures with branching or dilated glandular lumen, and papillary proliferation at the surface was also frequent. Invasive growth of proliferative epithelium into submucosal layer was also detected, however, the invaded tissue differentiated into normal fundic gland or formed cyst in about half of AH. Mucosal height was markedly reduced in some area and resulted in prominent loss of flatness of the mucosa. Erosions/ulcers with/without proliferative change were noticed in over half of AH. Expansion of proliferative zone of the mucosa was evident and Ki-67 positive cells increased in proliferating areas of AH, severe lesions showed very high Ki-67 positive indices and several surface epithelial cells were also Ki-67 positive. In conclusion, high incidence of spontaneous AH of glandular gastric mucosa in the glandular stomach was confirmed in aged ICR(CD1) males and females. Mucosal epithelium in AH often showed invasive growth penetrating into the submucosal layer, however, it was considered to be non-neoplastic because of differentiation into normal fundic gland even in the submucosal layer with retaining a polarity of each cell. Enhancement of proliferative activity caused by repeated erosions/ulcers and inflammatory reactions seemed to play a pivotal role in the pathogenesis of AH.

P-086

Induction of p21 at Intestinal Crypt Bottom in X-Irradiated Mice

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Different part of the crypt may have varying sensitivity to radiation exposure. Thus, in vivo reaction to X-irradiation in murine intestinal crypts were analyzed in region specific manner. Three Gy was X-irradiated to adult (7 weeks old) and baby (12 days old) C57BL/6J mice. Colonic and small intestinal crypts were isolated, further divided into crypt bottom, middle, and top regions and villi, and analyzed for expression of p21, p53, cyclin D1, and β-catenin. In an adult normal colonic crypt, p21 is 15-fold more transcribed in the crypt top compared to the bottom part. Upon X-irradiation, p21 mRNA was induced more than 10 fold in the bottom, but not significantly changed in the top. p53 and cyclin D1 transcription was high in the bottom but low in the top cells, which were not increased with X-irradiation. β-Catenin level was relatively homogeneous along the crypt with little effect of X-irradiation. In babies, crypt bottoms also showed upregulation of p21 mRNA with X-irradiation irrespective of the bottom bifurcation. p53 and p21 protein levels were increased after X-irradiation in baby colon epithelium. These data imply that X-irradiation induces p21 specifically in the crypt bottom region in order to protect intestinal stem cells.

P-087

Brunner's Gland Lesions in Rats Induced by A Vascular Endothelial Growth Factor Receptor Inhibitor

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Vascular endothelial growth factor (VEGF) receptor inhibitors for cancer have been reported to cause proliferative changes in the duodenal mucosa in rats. A diagnosis of adenosis, an unusual proliferative duodenal lesion characterized by epithelial hyperplasia with transmural invasion, was recently reported in a rat chronic toxicity study of a VEGF receptor inhibitor and affected the clinical trials (ESTP, Hannover, CD-ROM, 2009).
We have also experienced similar duodenal changes in rat 4-, 13-, and 26-week toxicity studies of E7080, a VEGF receptor-2 tyrosine kinase inhibitor. At 4-weeks, the change was first noted and characterized by neutrophil infiltration around Brunner's glands, which in severe cases extended into the duodenal mucosa and/or muscular layers. At 13-weeks, the Brunner's gland inflammation was still present with secondary regenerative hyperplasia of duodenal and pyloric epithelium. The Brunner's glands were atrophic with flattened epithelial cells, and occasionally replaced by regenerative duodenal crypt epithelium. At 26-weeks, the inflammatory change was more advanced and chronic. Almost all Brunner's glands were replaced with crypt epithelial cells forming cystic dilatation in the submucosa/muscular layer, as if down-growth of duodenal epithelium had occurred.
Considering the pathogenesis of the duodenal lesion by VEGF receptor inhibitors, the results of our longitudinal histologic examination of the duodenum revealed that the duodenal lesion is reactive hyperplasia subsequent to inflammation in the Brunner's gland. This notion differs from that of adenosis in previous publications, which was interpreted as a proliferative change with down-growth, with potential of malignancy. Full evaluation of the Brunner's gland is needed in preclinical toxicity studies of VEGF receptor inhibitors for understanding the precise pathogenesis of the duodenal lesions.

P-088

Particokinetics and Extrapulmonary Translocation of Intratracheally Instilled Triiron Tetraoxide Nanoparticles (Magnetite)

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【**Introduction**】Iron nanomaterials are of considerable interest for the application in nanotechnology-related fields including environmental catalysis, biomedical imaging, drug delivering and hyperthermia, because of their super-paramagnetic characteristics and high catalytic abilities. However, information about potential risks of iron nanomaterials is limited. The present study assessed particokinetics and extrapulmonary translocation of intratracheally instilled magnetite nanoparticles in rats.

【**Methods**】Ten-week-old male Fischer 344 rats (n=15/group) were exposed an intratracheal spray instillation of 0 (vehicle) or 15.0 mg/kg body weight of magnetite. After 1, 3, 7, 21 and 50 days, three rats each from every group were housed individually in metabolism cages for 24 hours, and feces and urine were collected. The blood samples were then collected via the abdominal aorta. The rats were sacrificed, and the lung, liver, kidney and brain were excised and weighed. Frozen blood, urine, feces and organs were homogenized and, pretreated for analysis, and iron was detected by the inductively coupled plasma mass spectrometry (ICP-MS).

【**Results and Discussion**】One day after the instillation of magnetite, a high concentration of iron was detected in the lung, while only minimal concentrations of iron were detected in the liver, kidney and brain. The concentration of iron in the lung promptly decreased to day 7, and gradually decreased thereafter. It is estiamated that the biological half-time of magnetite in the lung was about 7 days. The iron concentration in the feces was higher than that in the urine, and the excretion of magnetite to the urine was very slight. On the other hand, the excretion to the feces was continued from 1 to 50 days after the dosing. It is thus thought that instilled magnetite is mainly excreted in feces.

In the histopathological findings of magnetite-treated rats, the infiltration of dark-brownishly pigmented macrophages, phagocytosing magnetite, were seen in the alveolar walls and spaces of the lung. Also in the lymph nodes, the infiltration of magnetite phagocytosing macrophages and the deposits of magnetite were seen in the dosed rats. It is thus suggested that a part of magnetite was translocated to such lymph nodes.

P-089

Promotion Effects of Rat Lung Carcinogenesis of Fullerene (C60) administered by intra-pulmonary spraying

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Fullerene (C60) is expected to have significant beneficial impacts on fields such as medicine. However, there is an urgent need to determine potential human health hazards. We showed that treatment with C60 significantly increased 8-OHdG levels in the lung, and cytokines resulting from alveolar macrophage phagocytosis of C60 promoted proliferation of lung cancer cells. In this study, we examined the effects of fullerene (C60) on carcinogenesis. Male and female F344 rats were treated with DHPN (0.2% in the drinking water) for 2 weeks and then exposed to C60 from the end of week 4 through week 24. C60 was suspended in rock-candy solution and intra-tracheally sprayed into the lung at 250 μg/ml or 500 μg/ml once every week. Notably, aggregates of C60 were commonly observed in alveolar macrophages. C60 treatment significantly increased the multiplicity of lung alveolar hyperplasia. No significant increase of adenoma and carcinoma was observed. These results suggest that C60 might promote lung carcinogenesis.

P-090

Alveolar hyperplasia like lesions induced by intra tracheal spray of carbon black particles.

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Carbone black particles (CB), which are employed in rubber products and in laser toner, may produce discomfort to the upper respiratory tract. The current IARC evaluation is Group 2B. The present study was conducted to detect carcinogenic activity of carbon black administered by a novel intrapulmonary spraying (IPS). Male and female F344 rats were treated first with DHPN in the drinking water for 2 weeks. The CB suspended in rock candy solution was administered by IPS once every week from week 4 to week 24. Microscopic observation in both male and female showed scattered inflammatory lesions with infiltration of numerous macrophages mixed with a few neutrophils and lymphocytes. Clusters of alveolar macrophages with CB were observed throughout the lung alveoli. Alveolar epithelium cells surrounding the clusters were swollen. This alveolar hyperplasia like lesions (AHLL) were observed in the rats treated with CB regardless of DHPN treatment and this AHLL were located independently of DHPN-induced lesions such as alveolar hyperplasias, adenomas and adeno- carcinomas. The average numbers of AHLL in the lung were higher than that of DHPN-induced lesions. Although IPS of CB did not increased the multiplicity of DHPN- induced lesions, this IPS treatment increased the average number of the combined lesions (AHLL + DHPN-induced). These results suggest that it is important for the evaluation of the carcinogenic activity of carbon black to determine whether the alveolar hyperplasia like lesions are preneoplastic or not.

P-091

The reactions to potassium octatitanate fibers (TISMO) administrated to left thoracic cavity in A/J female mice.

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The present study was performed to assess effects of potassium octatitanate fibers, trade name TISMO, with the chemical formula $K_2O \cdot nTiO_2$, on mouse lung and thoracic cavity.

Thoracotomy was performed to infuse test particles directly into the left thoracic cavity of A/J mice. Fiber-shaped particles of TISMO, supplied by Otsuka Chemical Co., Ltd. (Osaka, Japan) with dimensions mostly <50μm in length and <2μm and granular-shaped micro- and nano-size order particles of titanium dioxide (TiO_2) were employed (1.5 mg in 0.2 ml saline/mouse). The experiment was terminated after 21 weeks to assess mesothelial reactions. Only the fiber-shaped TISMO, morphologically similar to asbestos, induced a severe reaction of the pleura. Following Berlin blue staining, positive spots were observed around the TISMO, indicative of iron accumulation. These positive spots corresponded with mesothelial cells. The results indicate that the risk of mesothelial cell reaction may depend on the shape as well as the particle size.

A second 52 weeks experiment was employed to examine long-term effects and possible tumor induction by fiber-shaped TISMO infused into the thoracic cavity of A/J mice. This experiment showed numbers of TISMO fibers in the alveoli, indicating penetration through the visceral pleura. Atypical mesothelial cells were also observed with severe pleural proliferation, but no malignant mesothelioma was induced. The additional detection of TISMO fibers in the liver and kidneys histopathologically and under scanning electron microscope (SEM) indicated their movement outside of the thoracic cavity.

In conclusion, the experiments demonstrated fiber shaped TISMO infused into the thoracic cavity of A/J mice induced severe pleural proliferation but not malignant mesothelioma, within the one year period studied. Hazard risk should also take into account that asbestos-like fiber spread to the whole body from the thoracic cavity.

P-092

Multi-walled Carbon Nanotubes Translocate into the Pleural Cavity and Cause Mesothelial Proliferation

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Multi-walled carbon nanotubes (MWCNT) induce mesothelioma when directly applied to the abdominal cavity. However, whether inhaled MWCNT migrate into the pleural cavity is still obscure. In this study, rats were treated by intra-pulmonary spraying with 500 ppm MWCNT or crocidolite (CRO) suspension for 5 times over a 9 day period, and translocation of the fibers into the pleural cavity and pleural lesions were examined. The results indicated that 1) MWCNT and CRO were found in the macrophages of the pleural cavity; 2) MWCNT and CRO applied to the lung caused hyperplasic proliferation of the visceral mesothelium; 3) MWCNT and CRO treatment also caused infiltration of inflammatory cells into pleural cavity, and a significant percentage of these infiltrating cells were macrophages; 4) MWCNT and CRO fibers were found in the mediastinal lymph nodes and 5) penetration of the dosed fibers was not found in the proliferated mesothelium lesions. These observations indicate that a major route of the translocation into the pleural cavity is probably through the lymphatic flow. Mesothelial proliferation lesions were associated with inflammation inflammatory reaction in the alveoli and pleural cavity, mainly mediated by macrophages.

P-093

Influence of the product level physicochemical property on the carcinogenicity of multi-wall carbon nanotube in rats

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We previously reported that mesothelioma is induced in F344 rats by the intrascrotal or intraperitoneal injection of multi-wall carbon nanotube (MWCNT). There have been no reports showing carcinogenicity, except one using p53 gene deficient mice, which may be due to the influence of MWCNT characteristics like fiber size. In this context, we performed a comparative study for the mesothelioma induction in rats by five MWCNTs with different charceristics. MWCNTs from M-company (M-CNT; 1-9 μm 74.3%, >10 μm 25% in length, 50-80 nm 97.2% in diameter , Fe content in 3500 ppm), N-company (N-CNT; 1-9 μm 79.1%, >10 μm 21% in length, 50-80 nm 94.8% in diameter , Fe content in 20-40 ppm), W-company (2 types; WL-CNT, 0.5-10 μm in length, 85-200 nm in diameter and WS-CNT, 0.5-2 μm in length,40-70 nm in diameter) and T-company (T-CNT, 10-100μm in length, 20-100 nm in diameter) were injected intraperitoneally to male F344 rats (12 rats per group) with a single dose of 1mg/kg BW. Rats were kept for up to 55 weeks after the injection.

All WL-CNT rats were autopsied due to death or moribund after 26-43 weeks. In M-CNT group, 4 rats died, and 7 rats became moribund after 32-50 weeks. In N-CNT group, 5 rats died, and 2 rats became moribund after 39-53 weeks. At 55 weeks after injection, each group's survivors, 1 rat in M-CNT, 3 rats in N-CNT, all rats of WS-CNT and T-CNT were autopsied. In M-, N-, WL-CNT-treated groups, intraperitoneal tumor nodules were detected in all animals, and most of rats were accompanied with bleeding ascites fluid. Gross findings related to mesothelioma were not found in rats of WS-CNT or T-CNT groups. These results show the possibility that the size of MWCNT fiber was one of the most critical factors for its carcinogenicity. It is necessary to examine further influence of the product level physicochemical property on the carcinogenicity of MWCNT.

P-094

The strain difference for the effect of chronic inflammation to lung carcinogenesis in F344 and Wistar-Hannover rats

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It is well known that intratracheal instillation (i.t) of quartz fine particles induces severe chronic inflammation in the lung. And the chronic inflammation is also cause of some pulmonary disease.

In this study, the effects of chronic inflammation on N-bis (2-hydroxypropyl) nitrosamine (DHPN) lung carcinogenesis, focusing attention on strain difference in F344 and Wistar-Hannover male rats, were examined.

Male F344 rats and Wistar-Hannover rats were maintained in the Kagawa University Animal Facility according to the Institutional Regulations for Animal Experiments. On week 0, lung tumorigenesis was initiated by DHPN in drinking water for 2 weeks and 2 mg quartz was administrated by i.t. on week 4. On week 25, rats were sacrificed and their lungs were examined histopathologically after sampling their blood and bronchoalveolar lavage fluid (BALF). Carcinogenic potential was analyzed by comparing the numbers, incidences and areas of their tumors for each histopathological lung proliferative lesions. The area of lung tumors was assessed using an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice Corporation, Osaka, Japan). The percentage of tumor's area was calculated as (total area of lesions / total area of lung per a slide) × 100. And lung inflammation was evaluated by hematological data, Interleukin-6 (IL-6) in the serum, BALF, cellular fraction in BALF and histopathological paramaters.

In hematological data and IL-6 in serum and BALF, there was no inter-group difference. However, the cellular components in BALF, the number of white blood cells were increased significantly in F344 rats. And in both F344 and Wistar-Hannover rat strains, the percentage of lymphocytes and neutrophils were increased significantly. Histopathologically, severe infiltration of inflammatory cells in the groups of quartz i.t. and F344 rats showed stronger than Wistar-Hannover rats. Furthermore, both of incidence and areas of lung tumors (hyperplasia and adenoma) with quartz i.t. showed a trend to increase in both strains. In addition,

This experiment indicated that chronic inflammation by quartz i.t. trends to have a potential of promoting effect on lung carcinogenesis in F344 rats and Wistar-Hannover rats.

The novel experiment is going on with longer experiment period and larger number of rats.

P-095

4-Nitroquinoline 1-Oxide Induced Pulmonary Tumorigenesis in TSOD Mice

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[Objective] Obesity, hyperlipidemia and diabetes increase the risk of carcinogenesis in several tissues. However, the relationship between these metabolic abnormalities and pulmonary carcinogenesis is unclear. ddy mouse is susceptible to 4-nitroquinoline 1-oxide (4-NQO), and pulmonary tumor occurs frequently by subcutaneous injection of 4-NQO. It was reported that these pulmonary tumors are promoted by glycerol in drinking water (Jpn J Cancer Res, 1986). Tsumura Suzuki Obese Diabetes (TSOD) mice, which generated from ddy mice, represent obesity, hyperlipidemia, and diabetes. In the present study, we investigated the lung tumor tumorigenesis of the TSOD mice initiated with 4-NQO.

[Methods] Six weeks of age male TSOD (group 1) and their littermate TSNO mice (group 2), which don't develop metabolic impairment were given a single subcutaneous injection of 4-NQO (10 mg/kg bw). TSOD and TSNO mice injected saline served as control groups that belonged to groups 3 and 4, respectively. At week 30, their lung was histopathologically examined.

[Results] The mean weight of whole body, liver, kidney, perigonadal fat, and the average levels of serum triglyceride, total cholesterol and free fatty acid of TSOD mice were significantly increased than those of TSNO mice. The mean free fatty acid value was decreased by 4-NQO exposure in TSOD mice. Both the incidence and multiplicity of the pulmonary tumor of mice in group 2 (11/16, 69%, $p<0.003$; 0.88 ± 0.72, $p<0.002$) were significantly larger than those of mice in group 1 (3/20, 15%, 0.15 ± 0.37). The values of groups 3 and 4 were the same, and were 8 % (1/13) and 0.08 ± 0.28.

[Conclusion] TSNO mice exhibited higher sensitivity to 4-NQO in pulmonary tumorigenesis than TSOD mice. Our findings indicate that metabolic disorders may not be a risk factor of pulmonary tumorigenesis.

P-096

Laccaic Acid in Lac Color Targets Plasma Hyaluronan-binding Protein (PHBP) and Inhibits Induction of Thyroid Capsular Invasive Carcinomas in Rats

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At the 27th meeting, we have reported that dietary administration of lac color (LC), a coccid-derived natural food colorant potentiating inhibition of plasma hyaluronan-binding protein (PHBP), suppressed the formation of capsular invasive carcinomas (CICs) in the initial phase of cancer invasion in a rat two-stage thyroid carcinogenesis model using sulfadimethoxine (SDM) as a promoter. We also found that this suppression was mediated through inhibition of inflammatory responses and angiogenesis by inhibition of tissue proteolysis resulting from activation of PHBP through the pharmacological action of the major constituent of LC, laccaic acid (LA). The present study was aimed at demonstrating the direct evidence of the inhibitory effect of LA on CICs formation, and we examined the modifying effect of the isoforms of LA, LA-A (90% of LA in LC) and LA-B (less than 10 % of LA in LC), on the development of CICs in the CICs-induction model. One week after initiation with DHPN, male F344/NSIc rats were treated either with LA-A or LA-B (3 mg/kg body weight) by intraperitoneal injections three times a week or fed a diet containing 200 ppm 3,3'-diindolylmethane (DIM), a potent inhibitor of fibrinolysis via the mechanism independent of PHBP-inhibition, during the promotion with SDM for 8 weeks. Increased body weights and decreased thyroid weights were observed by DIM-treatment, while no changes on these parameters were found by treatment with LA-A and LA-B. DIM inhibited the multiplicity and total area of intracapsular foci immunoreactive for Tenascin-C (TN-C), representing an early stage of CICs, and the ratio of TN-C-positive interstitial area in the whole area forming TN-C-positive foci. Both LA-A and LA-B exhibited insignificant reductions in the multiplicity and area of TN-C-positive foci. LA-B further significantly reduced TN-C-positive interstitial area in the whole area of TN-C-positive foci accompanied with transcript downregulation of genes involved in PHBP-related tissue proteolysis in CICs. Serum TSH level mildly reduced with DIM, but no difference with LA-A and LA-B. Thus, we could judge that the main inhibitory constituent of LC on CICs formation is LA-B. Reduction of CICs by DIM accompanied with weak reduction of serum TSH level suggests that inhibition of fibrinolysis independent of PHBP-inhibition may also be effective for suppression of CICs formation.

P-097

A 28-day repeated dose study of glycidol, 3-MCPD and associated esters in rats.

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Glycidol is classified as a probable human carcinogen and 3-monochloropropane-1,2-diol (3-MCPD) is regarded as a rodent carcinogen. Furthermore, esters of these compounds have recently found to be generated in many foods and food ingredients as a result of food processing. But there are few reports about toxicity of their esters.

In this study, we examined toxicity of glycidol, 3-MCPD and its various esters to rat by genotoxicity test and histopathological analyses. We administered glycidol (male and female, 800 ppm in drinking water), 3-MCPD (male, i.g., 40 mg/kg, 5 times a week) and their esters (the same molar concentration of glycidol oleate ester, glycidol linoleate ester, 3-MCPD palmitate diester, 3-MCPD palmitate monoester, 3-MCPD oleate diester) to 6 week old F344 *gpt* delta rats for 28 days. We examined *in vivo* genotoxicity by micronucleus (MN) and *Pig*-A assays. For *Pig*-A assays, peripheral blood was collected from each animal before and 2 and 4 weeks after start of administration, then *Pig* mutant RBCs were detected as REC^{CD59-} cells. For micronucleus (MN) assays, bone marrow samples were collected from each animal at scheduled sacrifice. Furthermore we examined histopathological analysis. (kidneys, liver, spleen and testis (for 3-MCPD))

In result, the relative its organ weight of kidney in the 3-MCPD and its various esters, glycidol groups were significantly increased to those of control. The frequency of *Pig*-A mutant RBCs was not different among groups in *Pig*-A assay. In MN assay, the frequency of micronucleated reticulocytes (MNRET) did not differ significantly among groups. In histopathological analysis, there are no significant observations to those of control.

In our present experiment, glycidol, 3-MCPD and its various esters induced increased relative kidney weight without apparent histological change, however no-genotoxic effect was observed in their hematopoietic cells.

P-098

Application of Renal Carcinogen-responding Markers to Other Carcinogenic Target Organs in Rats Treated with Carcinogens Targeting Other Organs for 28 Days

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Bizarre nuclear enlargement, known as karyomegaly, often appears in the renal epithelial cells reflecting cell cycle aberration by exposure to renal carcinogens in experimental animals and is suggested to be involved in the early carcinogenic mechanism. Until now, we found increases in TUNEL-positive apoptotic cells and immunoreactive cells for MCM3 and Ki-67 (cell proliferation markers), Topoisomerase IIα and Ubiquitin D in the renal tubules of rats treated with renal carcinogens irrespective of karyomegaly-inducing potential for 28 days. Topoisomerase IIα acts for DNA decatenation reflecting cell proliferation, and Ubiquitin D is a proteasome proteolysis-related molecule involved in multiple cellular functions. In the present study, we extended our strategy to examine cellular responses of other carcinogenic targets against these renal carcinogen-responding markers in rats treated with carcinogens targeting other organs for short term. Either carcinogenic or tumor-promoting dose was orally treated for 28 days with thioacetamide (TAA), fenbendazole (FB), piperonyl butoxide (PBO) and methyleugenol (MEG) targeting the liver; sulfadimethoxine (SDM) targeting the thyroid; and phenylethyl isothiocyanate (PEITC) targeting the urinary bladder. As non-carcinogenic control compounds, we selected acetaminophen and α-naphthyl isothiocyanate with hepatotoxic dose for the liver; and caprolactam, a genotoxic non-carcinogen, with maximal dose lacking induction of carcinogenicity in any organs for the thyroid and bladder. As a result, both MCM3 and Ki-67-immunoreactive cells significantly increased with TAA, FB and MEG as compared with untreated controls or non-carcinogens, and with SDM and PEITC as compared with both untreated controls and non-carcinogens. These carcinogens also significantly increased immunoreactive cells for Topoisomerase II α and Ubiquitin D, and the apoptotic cell index except for the non-significant increase of apoptosis with SDM. PBO did not increase cell proliferation, apoptosis, or immunoreactive cells for Topoisomerase II α and Ubiquitin D. These results suggest that carcinogens showing high proliferative activity after the 28-day treatment simultaneously facilitated apoptosis and proteasome proteolysis, the latter probably linking to cell cycle acceleration in response to cell proliferation. Increased apoptosis may be the reflection of facilitation of cell proliferation irrespective of the simultaneous disruption of cell cycle regulation.

P-099

Expression Characteristics of Cell Cycle-related Molecules in The Liver of Rats Treated with Hepatocarcinogens for 28 Days

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Some hepatocarcinogens have a potential to induce karyomegaly in hepatocytes after repeated exposure to experimental animals, and this cellular change reflects aberration in cell cycle. Recent studies have shown that aberrant expression of cell cycle-related molecules in the area exhibiting cellular karyomegaly may undergo a chromosomal instability linked to carcinogenicity. In the present study, to search for rapid screening markers of hepatocarcinogens, we examined expression characteristics of cell cycle-related molecules in rats treated with hepatocarcinogens inducing karyomegaly. We first examined global gene expression changes using microarray followed by real-time RT-PCR in the liver of male F344 rats administered with hepatocarcinogenic doses of thioacetamide, a typical hepatocarcinogen inducing karyomegaly, to obtain cell cycle-related genes upregulated. We, next, examined immunohistochemical cellular distribution of candidate molecules selected based on the gene expression profile in the liver of rats treated either with thioacetamide or other karyomegaly-inducing hepatocarcinogens, fenbendazole, piperonyl butoxide or methyleugenol, or non-carcinogenic hepatic toxicants without inducing karyomegaly, acetaminophen or α-naphthyl isothiocyanate, at doses to induce carcinogenicity or toxicity. Immunoreactive cells for phospho-Wee1, a G_2/M checkpoint molecule, did not fluctuate specifically to all hepatocarcinogens, as with those for Ki-67, a proliferation marker; however, cells positive for Cdc2, driving G_2/M transition, increased with most hepatocarcinogens as compared with untreated controls or non-carcinogens. With regard to molecules exerting function mainly at M phase, i.e., Aurora B, phospho-Histone H3, Incenp and HP1α increased the number of positive cells with most hepatocarcinogens. Among CDK inhibitors that play a role for G_1/S checkpoint, $p21^{Cip1}$ specifically increased the positive cells in response to hepatocarcinogens. These results suggest that cell proliferation markers and G_2/M checkpoint molecules except for Cdc2 could not separate all hepatocarcinogens from non-carcinogens. On the other hand, increases of cells immunoreactive for M-phase molecules as with those for $p21^{Cip1}$ in response to hepatocarcinogens suggest an outcome of an increase of cells stayed at M phase due to M phase disruption that may eventually result in cell cycle arrest by postmitotic checkpoint. Thus, the present study indicates that Cdc2, M-phase molecules and $p21^{Cip1}$ may be the candidate of early detection markers of hepatocarcinogens.

P-100

Rat 90-Day Repeated Oral Toxicity Study of Peach Gum

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[Objective] Peach gum, obtained by separating peach tree resin, is an “Existing food additive” primarily applied as a stabilizer in foods. Here, a 90-day repeated oral toxicity study using rats was conducted to investigate safety of peach gum.

[Method] Five-week-old male and female F344 rats were fed 0, 0.5, 1.5 and 5.0% of peach gum (each 10 rats) in their diet for 90 days. Over the treatment period, general conditions were observed daily, and body weight, food consumption and water consumption were measured once a week. In the final week of the treatment period, urinalysis and ophthalmoscopic examination were performed. At the termination of the treatment, blood samples were drawn from the abdominal aorta, and then hematological and blood biochemical assessments were performed. All animals were subjected to a full, detailed gross necropsy and organ weights were measured. Those in the control and 5.0% groups were examined histopathologically.

[Results] No deaths and no signs of abnormality were observed in either sex of the control and peach gum groups during the course of the study. There were no treatment-related adverse effects on body weights, food consumption, water consumption, and findings of urinalysis, ophthalmology, hematology, gross pathology or histopathology. In blood biochemistry, blood urea nitrogen was significantly increased in the male 5.0% group. However, it was considered that this change was not toxicologically significant, since it was very slight and no lesions were noted histopathologically. The significantly increased relative testes weights found in 5.0% group were also not considered related to toxicity since histopathological examination showed no changes.

[Conclusion] Thus, the no-observed-adverse-effect level (NOAEL) of the peach gum was concluded to be 5.0% (2,920 mg/kg/day for males and 3,465 mg/kg/day for females).

P-101

A 90 day Feeding Toxicity Study of Magnesium Hydrogen Phosphate ($MgHPO_4.3H_2O$) in Sprague-Dawley rats.

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As magnesium hydrogen phosphate ($MgHPO_4.3H_2O$) is affirmed as GRAS, it has been used as food additive (a nutritional supplement and a pH control agent) with no limitation in U.S. However the safety evaluation studies using experimental animals has not been done.

This study was designed to evaluate and characterize any subchronic toxicity of $MgHPO_4.3H_2O$, when administered to both sexes of Sprague-Dawley rats (10 males and 10 females in each group) at dietary levels of 0% (as control), 0.5%, 1.5%, 5.0% for 90 days. There were no deaths, and the treatment had no toxicologically siginificant effects on general conditions, body weights, food consumption, water consumption, ophthalmology, urinalysis, hematology, blood biochemistory, gross pathology, organ weight, or histopathology. Therefore, the no-observed-adverse-effect level (NOAEL) for magnesium hydrogen phosphate was concluded to be a dietary level of 5.0% (3045 mg/kg/day for males and 3702 mg/kg/day for females).

(MHLW Food, Food Additives Test Program)

P-102

Can a carcinogenicity of a pharmaceutical be predicted without a rat 2-year carcinogenicity study? – The analysis of data collected from package inserts of pharmaceuticals in Japan

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According to Frank D.Sistare *et al*., data collected from 182 marketed and nonmarketed pharmaceuticals demonstrate little value in conducting rat 2-year carcinogenicity studies for compounds that lack (1) histopathologic risk factors for rat neoplasia in chronic toxicology studies, (2) evidence of hormonal perturbation or (3) positive genetic toxicology results. When all of these 3 criteria were negative, 62 out of 76 pharmaceuticals (82 %) were correctly predicted to be rat non-carcinogens. In this context, they have proposed a possibility to refine a regulatory criteria for conducting a 2-year rat carcinogenicity study, based on the assessment of histopathologic findings from rat 6-month studies, evidences of the hormonal perturbation, the genetic toxicology results and the findings of 6-month transgenic mouse carcinogenicity studies (*Toxicol. Pathol*. 39, 716~744, 2011).

We collected data of rat 2-year carcinogenicity studies from package inserts of marketed pharmaceuticals in Japan, and researched into correlation between data of rat-carcinogenicity study and those for 3 criteria of (1) histopathologic risk factors for rat neoplasia in chronic toxicology studies, (2) evidence of hormonal perturbation and (3) positive genetic toxicology results. Furthermore, we also researched into non-neoplastic changes that could not be detected in rat 6- or 12-month chronic toxicology studies but were detected in rat 2-year carcinogenicity studies.

As a result, there were pharmaceuticals demonstrating positive results for rat 2-year carcinogenicity studies, but not satisfying the 3 criteria. On the other hand, it was demonstrated in several cases that the retinal denaturation or atrophy, heart hypertrophy or dilation, and kidney infarct were observed as non-neoplastic changes not in rat 6- or 12-month chronic toxicology studies but were found only in rat 2-year carcinogenicity studies.

These results suggest that it may be difficult to predict a carcinogenicity of a pharmaceutical without a rat 2-year carcinogenicity study, and that the skip of such a study may cause oversights for some non-neoplastic changes even after the enforcement of appropriate chronic toxicology studies. Further assessments are, therefore, demanded to decide whether the proposed refinement of the regulatory criteria for conducting a rat 2-year carcinogenicity study can be accepted.

P-103

Nasal Lesion in Rats Exposed to 2,4-pentanedione for 13-weeks and 104-weeks

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2,4-pentanedione is used as a catalyst (metal chelate), intermediate material for synthesis solvent, adhesive, plating and fuel additive. It was reported that 2,4-pentanedione induced inflammation and necrosis in the nasal mucosa of the rats by short-term inhalation exposure. In the present study, the nasal influence of 2,4-pentanedione were examined by inhalation exposure of 2,4-pentanedione to male and female F344 rats for either 13 weeks or 104 weeks.

【Methods】The 13-week study consisted of 5 exposed groups and one control group, each comprising of 10 rats (starting at an age of 6 weeks) of both sexes, and the 2,4-pentanedione concentrations used were 25, 50, 100, 200 or 400 ppm (v/v). The 104-week study consisted of 3 exposed groups and one control group, each comprising 50 rats of both sexes, and the 2,4-pentanedione concentrations used were 100, 200 or 400 ppm (v/v). The nasal cavity was fixed in 10% buffered formalin and was decalcified in formic acid-formalin solution The nasal cavity was trimmed at three levels, and was embedded in paraffin. The sections were stained routinely with hematoxylin and eosin.

【Results】In the 13-week study, squamous cell metaplasia and transitional cell like change of the ciliated columnar epithelium in the nasal cavity of male rats were occurred by exposure to 2,4-pentanedione at 400 ppm. In the 104-week study, no increased in tumors was indicated. As non- neoplastic lesion, transitional cell hyperplasia, squamous metaplasia and inflammation in the respiratory epithelium and atrophy in the olfactory epithelium were observed in both sexes exposed to 200 and 400 ppm.

【Conclusion】Thirteen weeks inhalation study of 2,4-pentanedione demonstrated the lesions in the respiratory epithelium such as squamous metaplasia, and the transitional cell hyperplasia were shown in 104-week inhalation study. The squamous cell metaplasia in the respiratory epithelium, which was found by 13-week inhalation exposure, did not progressed to the tumor by the prolonged exposure period.

P-104

A Chronic Toxicity Study of Acrylamide in Hamsters

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Acrylamide (AA) induces tumors in various organs/tissues in rats and mice. Epidemiological studies for the oral exposure to AA have revealed controversial results, and mortality studies of AA workers showed increased rates of pancreatic cancer. Among rodents, Syrian golden hamsters are sensitive to pancreatic ductal carcinogenesis. In the present study, effects of chronic exposure of AA on various organs including pancreas were evaluated in hamsters. A total of 90 females and 90 males were divided into three groups each, treated with AA at 0, 10 or 20 mg/kg body weight in drinking water for 78 weeks. Organs/tissues were routinely processed for embedding in paraffin, and stained with H&E for histopathological examination. Animals that died or were killed in a moribund condition during experimental period were also examined similarly. The final survival rate was 90, 83 and 67% for males and 77, 63 and 30% for females in 0, 10 and 20 mg/kg groups, respectively. Papillomas/squamous cell carcinomas in the forestomach were increased in 20 mg/kg males and females ($p<0.05$ and 0.001, respectively). In the cecum, adenocarcinomas were found in one animal each in 10 and 20 mg/kg female groups. Also in the lung, adenomas/carcinomas showed a tendency for increase in AA-treated groups. In conclusion, carcinogenicity of AA in the forestomach of hamsters was found. Further studies are needed to confirm whether the induction of forestomach tumors was related to genotoxicities of AA or not.

P-105

A Carcinogenicity Study of Semicarbazide Hydrochloride Administered in The Diet to B6C3F$_1$ Mice

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Semicarbazide (SEM) has been used as a marker metabolite for abuse of the banned veterinary antibiotic nitrofurazone. However, it was recently found that the compound can also be generated by thermal breakdown of azodicarbonamide, used as a blowing agent in plastic gaskets. Other sources such as processed foods undergoing hypochlorite treatment have additionally been suggested. In view of possible exposure from various foods, independent of any nitrofurazone use, it has become important to assess the health risk of SEM. In the present study, carcinogenicity of SEM were assessed in male and female B6C3F$_1$ mice fed diet containing the compound at concentrations of 0, 10, 50 or 250 ppm for 78 weeks. Intergroup differences in body weights and food consumption were not evident, but a significantly high incidence of mortalities was observed in 250 ppm males, mostly featuring intrathoracic hemorrhage. Enlargement and deformation of knee joints were obvious in 250 ppm males and females from weeks 11 and 57, respectively, and absolute and relative heart weights in 250 ppm males were also significantly increased. In histopathology of the thoracic aorta, number of elastic lamina was significantly decreased, while focal interruption of elastic lamina, disappearance of internal elastic lamina, medial thinning and adventitial thickening were increase. In female 250 ppm group, focal interruption of elastic lamina also significantly increased. Enlargement and deformation of the knee joints and prominence of the thorax were found in male at 50 and 250 ppm and female at 250 ppm. In histopathology, the incidences of osteoarthritis, patellar enlargement and sternal cartilage degeneration were significantly increased in male at 250 ppm. SEM-HCl treatment did not induced any significant difference of the incidences of neoplastic lesions. In conclusion, SEM is not carcinogenic in B6C3F$_1$ mice of either sex and toxicological effects of chronic exposure to SEM-HCl were prominently observed in bone, cartilage and the aorta.

P-106

Background Data for Carcinogenicity Studies in RccHan™:WIST Rats

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[Introduction] Wistar Hannover (RccHan™:WIST) rats are newly produced Wistar Hannover rats which maintain the advantages but not the demerits of the established strains. In this meeting, we report the data on 104-week non-treated animals, which we collected to use as the background data of carcinogenicity studies using this strain. [Method] One hundred male and 100 female rats were received at 4 weeks of age, quarantined/acclimated for 2 weeks and divided into 2 groups — a pellet diet group and a powder diet group (CR-LPF, radiation-sterilized, Oriental Yeast Co., Ltd.). They were housed individually in stainless-steel cages which were placed in an animal room where temperature was kept at 23 ± 3°C, relative humidity at 50 ± 20%, air ventilation 10-15 times per hour and 12-hour lighting per day. They were allowed free access to feed and tap water during the 104-week period. During the experimental period, animals were subjected to clinical observation (every day), measurement of body weight and food consumption (once in 1 or 2 weeks) and ophthalmological examination (Weeks 4, 13, 26, 52, 78 and 104). At the end of the experimental period, after collecting blood samples for hematology, animals were subjected to histopathological examination. The data obtained were compared with the historical background data of SD (Crl:CD(SD)) rats and F344 (F344/DuCrlCrlj) rats of the test facility. [Results] For both the pellet diet and powder diet groups, the survival rate of this strain was between those of SD and F344 in males while it was higher than that of SD but comparable with that of F344 in females. Body weight and food consumption of this strain were lower than those of SD but higher than those of F344 for both males and females. In the ophthalmological examination, focal corneal opacity was observed at a higher incidence than in SD. In the hematological examination, leukocyte count was lower than that of SD but comparable to that of F344. In the necropsy, males and females showed nodule of pituitary, males showed granular surface, large and pale color of the kidney and females showed nodule of the thymus, nodule in the subcutis and polyp in the uterus at high frequency. In the histopathological examination, the incidence of hepatocellular adenoma was higher than that in SD and F344, pituitary anterior adenoma and mammary gland adenocarcinoma/fibroadenoma were lower than in SD but higher than in F344 and thymoma was higher than in SD and F344 in males and females. We will also report the data of non-tumor lesions in the meeting.

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Long Term Husbandry in House Background Data of Wistar-Hannover Rats

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Background and Purpose: In this study, Wistar-Hannover rats that have been considered as replacement for Sprague-Dawley (SD) rats were housed for 2 years without treatment to collect background data. In addition, results obtained were compared with SD rats.

Materials and Methods: Six-week-old RccHan™:WIST (Japan Laboratory Animals,Inc) rats, 40 animals per sex, were housed without any treatment for 2 years. General condition and body weight measurement were conducted. In the 2nd year, all animals of both sex were necropsied and organ weight measurements and pathological examination were conducted. For comparison, SD strain rats, Crl:CD (SD) (Charlesriver Laboratories Japan, Inc.), were similarly examined.

Results and Discussion: In the Wistar-Hannover rats, the survival rate showed the high value both 67.5% in males and 65.0% in females compare with SD rats, 32.7% in male and 45.5% in females. Compared to SD rats, body weight gain was slow, mass occurrence was low and mass generating age was also late at the Wistar-Hannover rats during the study period. In macroscopic observation, masses were observed in pituitary, subcutis, cerebellum, ovary and changes like a chronic nephrosis observed also by SD rats. Organ weight measurements showed that several organs tended to have lower values. Histopathological examination is still continued up to the present, presentation of the progress, and the analysis of additional characteristic are planned.

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Natural Occurrence of Neoplastic and Preneoplastic Lesions in Young Sprague-Dawley Rats

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[Introduction] We previously reported the historical control data on the occurrence of spontaneous neoplasms in young Sprague-Dawley rats[1)]. In the present study, we revised the data by including the recent data on the occurrence of neoplasms, and also surveyed the occurrence of preneoplastic lesions as well as neoplasms. [Materials and Methods] Data were obtained from Sprague-Dawley rats in control groups in 4- (10 weeks old, 1132 males, 1120 females), 13- (19 weeks old, 1317 males, 1349 females) and 26- (32 weeks old, 1015 males, 1018 females) week studies conducted at Bozo Research Center Inc. from 2000 to 2011. The rats were obtained from Charles River Laboratories (Kanagawa and Shiga, Japan). [Results and Conclusion] No neoplastic lesions were seen in any 10-week-old animals. The tumors which were observed in 19-week-old animals included malignant lymphoma, erythroid leukemia, nephroblastoma in the kidneys, adenocarcinoma in the mammary glands, adenoma of pars distalis in the pituitary, benign basal cell tumor of the skin, thymoma, follicular adenocarcinoma and C cell adenoma of the thyroids. Thereafter, oligodendroglioma in the brain, malignant schwannoma of the cranial cavity, histiocytic sarcoma, fibroadenoma in the mammary glands, malignant basal cell tumor of the skin, adenocarcinoma in the submandibular glands, hemangiosarcoma in the tongue and endometrial stromal polyp of the uterus were detected in animals before 32 weeks of age. Tumors observed at a relatively high incidence were adenocarcinoma in mammary gland and adenoma of pars distalis in the pituitary in females, and endometrial stromal polyp of the uterus. Preneoplastic lesions were limited to occurrence at very low incidence at 10 weeks old. At 19 and 32 weeks old, altered cell focus in the liver (0.3 and 4.4 %, respectively) and focal hyperplasia of pars distalis in the pituitary (0.2 and 3.8 %, respectively) were seen as relatively high incidence lesions. The present results showed the profile of the occurrence of spontaneous neoplastic and preneoplastic lesions in young Sprague-Dawley rats up to 32 weeks of age, and demonstrated that the tumors as reported in aged rats could also develop as small neoplasms or preneoplastic lesions in younger rats.

[1)]Natural occurrence of neoplastic lesions in young Sprague-Dawley rats. J Toxicol Pathol 2011;24:37-40.