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SL-1

Toxicologic Pathology Aspects of Chemically Induced Cardiovascular Toxicity, as Seen in National Toxicology Program (NTP) Studies

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Potential cardiovascular toxicity of environmental agents and pharmaceuticals poses a major concern to health and regulatory authorities. Epidemiological studies have associated cardiovascular and respiratory morbidity and mortality with particulate matter (PM) air pollution, particularly in susceptible humans with concurrent cardiovascular and pulmonary diseases. Between 1961 and 1992, 131 drug products were withdrawn from the markets in Europe and the US. Ten of the 131 were withdrawn as the result of cardiovascular toxicity.

Studies in laboratory rodents are used to investigate the potential toxicity of various agents, identification and characterization of lesions suggesting cardiotoxicity are vital. Morphologic criteria have been described for degenerative myocardial lesions in rodents, but even with these criteria, differentiation of spontaneous from toxicity-induced lesions may be difficult. The histopathological pattern of lesion development may help determine whether the myocardial damage is due to injury of the coronary vasculature (in which case lesions tend to be multifocal) or due to direct myocardial cell toxicity (in which case lesions tend to affect much or all of the myocardium diffusely). In view of this observation, a retrospective light microscopic evaluation was performed on the hearts of F344 rats and $B6C3F_1$ mice from National Toxicology Program (NTP) studies on six chemicals that produced myocardial toxicity in order to provide a detailed morphologic characterization of spontaneous versus treatment-induced myocardial lesions (Jokinen et al, *Cardiovasc. Toxicol.* 5:227-244, 2005). The findings at light microscopic evaluation, particularly when taken in conjunction with the results of other techniques, such as ultrastructural examination and special staining, may give a general indication of the potential mechanism of cardiotoxicity, and suggest possible areas for mechanistic studies to define more clearly the actual mechanism of toxicity. The lecture will present an overview of the morphologic aspects associated with chemically and drug-induced cardiovascular toxicity, as seen in the NTP studies.

SL-2

Carcinogenicity of PPARγ and Dual α/γ Agonists: Mode of Action and Human Relevance

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PPARs are a class of nuclear receptors involved in adipose tissue growth and differentiation, lipid metabolism, and epithelial cell functions. PPARγ and dual PPARα/γ agonists have been developed as therapeutic agents for diabetes mellitus and hyperlipidemias. Two year rodent bioassays have frequently shown a carcinogenic effect, most commonly rat urinary bladder carcinomas and subcutaneous fibrosarcomas and mouse hemangiosarcomas. The Health and Environmental Sciences Institute (HESI) formed a PPAR Agonist Project Committee, including academic, governmental, and pharmaceutical company scientists from Japan, Europe and the United States, to address their modes of action and relevance to human risk. This committee sponsored two Pathology Working Groups (PWG) (classification of sarcomas and related lesions and evaluation of bladder changes in monkeys). The mode of action for urinary bladder carcinogenesis in rats has been most extensively investigated with the dual agonist muraglitazar (Toxicol. Pathol., 34: 903-920, 2006), and includes a direct effect of the PPAR agonist inhibiting citrate synthesis, leading to hypocitratemia and hypocitraturia. When citrate, a chelator of calcium, decreases sufficiently in combination with other parameters in urine composition, calcium-containing urinary crystals form leading to cytotoxicity, regeneration, and malignancy. Demonstration of urinary solids has not always been shown for PPAR agonists, but this is likely related to methodology issues such as fasting of the animals, time of collection and analytical methods (Toxicol. Pathol., 35: 337-347, 2007). Abnormal crystalluria or calculus formation does not occur in humans in response to these PPAR agonists. Three purported lesions were identified in monkey urothelium after PPAR agonist administration, including intracytoplasmic eosinophilic granules, intercellular vacuoles, and hyperplasia. The PWG (Toxicol. Pathol., 36: 769-776, 2008) ascertained that the granules were a unique component of the normal monkey urothelium. Vacuoles occurred in untreated monkeys but were larger and more numerous in high dose monkeys. No mechanism could be ascertained conclusively. The PWG concluded that there was no evidence of hyperplasia, but rather, the differences in the number of layers of epithelial cells reflected the normal variation of the monkey urothelium similar to what is seen in the human urothelium, but in contrast to the rodent epithelium. The PWG that addressed sarcomas developed a specific classification system (Toxicol. Pathol., 35: 928-941, 2007). A specific mode of action was not developed for the sarcomas in rats. For the hemangiosarcomas in mice, it was noted that mice have a high background incidence of hemangiosarcomas. Possible modes of action that were hypothesized include a direct mitogenic effect on endothelial cells, an effect on bone marrow endothelial precursor cells, or an indirect paracrine effect of the agonist on adipocytes leading to secretion of endothelial cell growth factors, such as VEGF. Progress in the evaluation of these modes of action were presented and discussed at a December 4-5, 2008, workshop on hemangiosarcomas co-sponsored by the Society of Toxicology and HESI. The modes of action involving the induction of the various tumors by PPAR agonists could also be applicable to other classes of drugs and chemicals.

EL-1

Epigenetics and Epigenome for Toxicology

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Epigenetics is defined as “the study of mitotically and/or miotically heritable changes in gene function that cannot be explained by changes in DNA sequence”. Recently, epigenetics is more broadly defined as “the study of processes that produce a heritable phenotype that does not strictly depend on DNA sequence”. Based on a variety of physiological and morphological criteria, there are at least 200 differentiated cell types in mammals. Cell phenotypes may be maintained even after mitosis when the cell is equipped with epigenetic mechanisms that enable the inheritance of gene function. Thus, epigenetics is fundamental to development and maintain the cellular phenotypes.

DNA methylation is a major epigenetic event and causes gene-silencing in association with histone modifications and chromatin condensation. Every cell type or tissue has a unique DNA methylation profiles comprising at least thousands of tissue-dependent and differentially methylated regions (T-DMRs), suggested that the epigenetic change at T-DMRs underlies the differentiation of cells, because the epigenetic status is heritable to next cell generation and is changeable in the process of differentiation of cells.

Genome-wide epigenetic information is known as epigenome. Disruption of normal epigenome produces aberrant cells with prolonged abnormal phenotypes. Epimutation may be more common than our previous thought considering that there are numerous T-DMRs in mammalian genome. Mutation and epimutation is heritable to the next cell generation and both of them continue for whole life, while epimutation is reversible and drug treatment could potentially reverse the epigenetic status. Analysis of epigenome will provide a cue for a novel development of diagnosis, drug development for the complex chronic diseases and expanding toxicology.

In this meeting, I will introduce the recent study from “Epigenetics” to “Epigenome” as fundamentals of biology and as application to the Toxicology.

PD-1

A Panel Discussion

Regulatory Perspective for Pathological Data

Chair persons:
Yuji OISHI[1)], and Kazutoshi Tamura[2)]

Panelists:
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The Society of Toxicologic Pathology (STP) has released a position paper (Toxicol Pathol 35, 450-455, 2007) which provides recommendations on when pathology images should be considered raw data in nonclinical safety studies for the U.S. and the associated GLP requirements to handle this data. The paper defines the images used for data generation (e.g., the basis of a diagnosis or morphometric analysis) as raw data, and in contrast, indicates that illustrative images to convey information about a particular diagnosis or finding are not raw data. In addition, the paper discusses the status of images used during both formal and informal peer review which, in the U.S., are commonly performed before the pathology report is completed. In Japan, the Japanese Society of Toxicologic Pathology supports the STP position paper in principle, but at this moment, the routine work requires that the original image data of a particular finding attached to the report are treated as raw data and the study pathologist completes the pathology report before the pathology peer review. These differences between the three principal regions, Japan, U.S. and Europe and between current Japanese theory and practice were of concern to the 25th JSTP annual meeting program committee. They assembled a “questionnaire on pathology raw data and peer review” in cooperation with the International Federation of Societies of Toxicologic Pathologists / Regulatory Interaction Committee (IFSTP/RIC) and the Japanese Society of Toxicologic Pathology in order to obtain more information. The questionnaire was circulated and completed thanks to the cooperation of Japanese pharmaceutical companies and CROs. They also planned this panel discussion welcoming the participation of all representatives from domestic pharmaceutical companies, CROs, Japan Society of Quality Assurance (JSQA), American toxicologists/pathologists and IFSTP/RIC. This panel discussion will be the opportunity to build harmonization among these three regions, by introducing the outline of the STP position paper and the results of the above questionnaire as well as clarifying the differences in the pathology raw data and the pathology peer review processes between Japan, U.S. and Europe.

WS1-1

Evaluation of new blood and urinary biomarkers in rat hepatotoxicity models

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Optimal biomarkers for hepatotoxic evaluation are characterized as follows: 1) specificity of assessing severity and tissue distribution of the injury, 2) sensitivity of detecting early events in the injury, 3) a transportable test object among in vitro, experimental animals, and human depending on a phase of drug development, and 4) an easily measurable method. Defined biomarkers by histopathology reduce the background variation and provide clearer signal with increased specificity and sensitivity. The purpose of this study was to evaluate urine-based and blood-based biomarkers in different rat hepatotoxicity models, hepatocellular injury, cholestasis, and steatosis. Some chemicals were used in this study: concanavalin A (ConA), D-galactosamine, monocrotaline (MCT), dexamethazone (DEX) for hepatocellular injury, α-naphthylsothiocyanate (ANIT) and ethinyl estradiol for cholestasis, and Lieber-ethanol diet model and methionine-choline deficient model for steatosis. In these models, some time courses and doses were arranged for blood, urine, and tissue sampling. In blood-based biomarkers, candidates of serum biochemical parameters were examined: glutamate dehydrogenase (GLDH), ornithine carbamoyltransferase (OCT), glutathione-*S*-transferase (α-GST), and arginase-I as enzyme leakage markers, in addition to ALT activity, and serum osteopontin (OPN) level as an additional marker. All of the leakage marker candidates increased at the same time observed necrotic findings in the hepatocytes of the hepatocellular injury model. However, its measurement offered no additional information in either specificity or sensitivity, compared with ALT activity. The serum OPN level also significantly increased in the rats treated with ConA, MCT, DEX, and ANIT. Recently, it is suggested that OPN is implicated in infiltrating inflammatory cells to the liver. Thus, if the detailed role of OPN in liver inflammation is demonstrated, OPN will become a useful biomarker. In urine-based biomarkers, urinary metabolites were measured by UPLC-MS and GC-MS platforms. Metabolite profiling was clearly separated in each chemical-treated group by principle component analysis. Of them, some metabolites were commonly changed in each model. Annotation of metabolites and identification of a toxicological mode of action are necessary for application as valid urinary biomarkers. For example, the level of orotate was increased in rats treated with ConA, MCT, DEX, and ANIT in this study. The increase of orotate observed here could be an indication that urea cycle was inefficient as a result of liver malfunction. While a large database of metabolites is needed, the combination of histopathology and metabonomics will give well-qualified injury-related biomarkers.

WS1-2

ALT isozymes in Canine Tissues

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Serum/plasma alanine aminotransferase (ALT, also known as glutamate pyruvate transaminase, GPT) is widely used as a marker for hepatic damage in clinical and non-clinical practice. However, in non-clinical toxicity studies, increased serum/plasma ALT happens on occasion while no histopathological changes are observed in the liver. Therefore it is of concern that the cause of high ALT levels in serum/plasma might not be only hepatic damage. In recent years, it has been shown in humans, mice and rats that ALT has two isozymes (ALT1 and ALT2) coded on different chromosomes and each expression level is different between the organs. To comprehend more precisely increases in serum/plasma ALT, in the present study we investigated the ALT isozymes in dogs commonly used for pharmaceutical toxicity studies.

Sequence analysis revealed the full-length ORF sequences of canine ALT1 and ALT2, which are highly preserved in human and mouse genes. Based on the obtained sequences, specific polyclonal antibodies for each isozyme were developed. Using the antibodies, the expression level of each enzyme was analyzed in various canine organs by western blotting. The expressions in the organs were also analyzed at the gene level by quantitative real-time PCR. These analyses revealed that the isozymes were expressed differently in an organ-dependent manner; ALT2 was dominantly expressed in muscle, adipose tissue and kidney cortex while ALT1 was dominant in liver and heart. Immunohistochemistry using the specific antibodies showed that the immunoreactivity for each antibody was differently localized between the different cell types even in the organs.

In general, it is assumed that there would be some alterations in the liver with increases in serum/plasma ALT. However, as this study indicated, ALT1 and 2 are expressed at different levels in various canine organs other than liver, suggesting that serum/plasma ALT could possibly be increased by factors other than liver damage. In addition to the distribution data, an analysis method to distinguish serum/plasma ALT1 and 2 should be established. This would allow better determination of drug-toxicity targeted organs.

WS1-3

Echocardiographic Evaluation of Pulmonary Hypertension in Rats

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Alterations of the pulmonary artery flow velocity waveforms and right ventricle geometry, which can be determined by echocardiography, have been used as noninvasive biomarkers for the diagnosis of drug-induced pulmonary hypertension or cardiac valvular disease. However, the utility of these biomarkers in laboratory animals and the relationship to histopathological findings have not been sufficiently studied.

In the present study, monocrotaline (dosage level: 60 mg/kg), which is known to cause pulmonary hypertension ascribed to pulmonary vascular injury, was administered subcutaneously once to male Crl:CD(SD) rats (7-14 weeks old) and echocardiography, respiratory function measurements and histopathological examination were performed at 3 or 6 weeks after dosing.

At 3 weeks after dosing, marked interstitial pneumonia and endothelial swelling of the pulmonary arterial trunk suggesting an early phase of pulmonary vascular injury was observed on histopathology. At this phase, echocardiography revealed mild reduction of the acceleration time in the pulmonary arterial blood flow, without any change in cardiac morphology. A slight decrease in tidal volume accompanied by a slight increase in the respiratory rate was also detected by whole-body plethysmography.

At 6 weeks after dosing, in addition to the above-mentioned histopathological lesions, medial hypertrophy and neointimal formation in the pulmonary arteries and myocardial hypertrophy and interstitial fibrosis in the heart were observed. Cardiac changes in the right heart were more pronounced than those in the left heart. At this advanced phase of pulmonary vascular injury, marked reduction of the pulmonary acceleration time, cardiac morphological changes (enlargement of the right atrium and right ventricular cavity, thickening of the right ventricular wall and interventricular septum, shift of interventricular septum to the left ventricle, narrowing of the left ventricular cavity, mitral and tricuspid regurgitation, etc.) and cardiac functional changes (decrease in cardiac output and left ventricular dysfunction) were observed in the echocardiographic examination. A marked increase in respiratory rate and minute volume were also seen on the respiratory function measurements.

In conclusion, morphological and functional changes in the heart detected by echocardiography and respiratory functional changes detected by whole-body plethysmography correlated well with the histopathological pulmonary and cardiac changes induced by monocrotaline in rats. It was considered that these biomarkers could be useful for the evaluation of drug-induced pulmonary and cardiac toxicity in rats.

WS1-4

Detection of potential biomarkers for hepatotoxicity by urinary metabolic fingerprinting

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There has been a focus on omics research including transcriptomics, proteomics and metabolomics, as screening tools for assessing the toxicity of new therapeutic compounds in the early stage of drug development.

Metabolomics is defined as an attempt to measure the metabolome which is an integrated information of all the low molecular metabolites present within a cell, tissue or organism during a genetic modification or physiological stimulus. The metabolome consists of extremely diverse chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and complex natural products. That complexity makes it virtually impossible to simultaneously determine the complete metabolome. In practice, 'metabolic profile', a part of metabolome, is obtained. Metabolic fingerprinting has also been used to classify samples according to the origin or its biological relevance without aiming to determine the individual level of every metabolite. It is applicable to detecting toxicological effects and to search biomarkers obtained following dosing of compounds.

Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS), is possible to obtain an ultra-high resolution (> 100,000) mass spectrum in ~1s. By using FT-ICR MS, separation of the metabolites can be achieved solely by ultra-high mass resolution, eliminating the need for time consuming chromatography and derivatization. Identification of the putative metabolites, or class of metabolites to which they belong, can be achieved by determining the elemental composition of the metabolite based upon the ultra-high mass accuracy.

We examined toxicological assessment using urinary metabolic fingerprinting with FT-ICR MS in the toxic compound-dosing rat model to investigate its potential.

In this workshop, we would like to present a study of urinary metabolic fingerprinting using FT-ICR MS to detect the urinary metabolic alteration and the possible biomarkers involved in the acute hepatic toxicity.

WS1-5

Identification of Early Marker of Urinary Bladder Carcinogenesis in Rats

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The purpose of the present studies is to identify early markers of urinary bladder carcinogenesis in F344 rats. Microarray analyses of 12 *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder cancers and 11 dimethylarsinic acid (DMA)-induced bladder cancers revealed that 85 genes commonly overexpressed in all cancers compared to respective control bladder urothelium. Then, mRNA expression levels of 20 of above genes that selected based on the overexpression levels and the results of pathway analysis were evaluated in bladder urothelium of rats given BBN for 2, 4 and 8 weeks, respectively. Twelve of the 20 genes were shown to be consistently overexpressed, and therefore considered as candidate marker genes. Furthermore, their mRNA expression levels were evaluated in bladder urothelium of rats treated with 7 bladder carcinogens (DMA, 2-acetylaminofluorene, Sodium o-phenylphenol, Phenethyl isothiocyanate, Benzyl isothiocyanate, Uracil and BBN) and 3 nonbladder carcinogens (liver carcinogen: diethylnitrosamine; kidney carcinogen: N-ethyl-N-hydroxyethylnitrosamine; colon carcinogen: 1.2-dimethyhydrazine), respectively, for 4 weeks. Oncomodulin mRNA was found to be consistently significantly increased in all urothelium treated with any of bladder carcinogens regardless of existence of histopathologic changes or not, but not in any of urothelium treated with nonbladder carcinogens. These findings indicate that oncomodulin is a potential early marker of bladder carcinogenesis in rats.

WS1-6

Investigation of Toxicological Biomarker Discovery by Imaging Mass Spectolometry

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Many of the toxicity biomarker candidates obtained via omics technology are molecules that, based on our current knowledge, are unexpected. In addition, the biological relationships between the changes that occur in these candidates and the observed toxicological findings are not clear. To qualify a biomarker, first, we attempt to link its expression and the changes in its mRNA and protein in the target organ extract. Next, biomarker candidates can be histologically localized using histochemical methods like immunochemical staining. Protein localization is dependent, however, on the conservation of antigen and selectivity of the antibody. For lipid localization, there are few histochemical tools available; therefore, their relationships with molecular candidates and specific localization cannot be clarified. In such cases, it is difficult to estimate the biological significance of the biomarker. Recently, site-specific analysis has been possible using laser microdissection. However, although the transcriptional analysis with the minute amount of section is available, the analysis of protein including modification and lipids with that is not available because of low throughput and unamplification. Therefore, we focused on imaging mass spectrometry (IMS), which is able to directly analyze the section via mass spectrometry. However, few studies have used this method, especially for toxicity studies; therefore, we attempted to confirm the usefulness of IMS in this study.

We prepared fresh frozen sections of liver and lung from a rat with induced phospholipidosis, and applied the matrix to the affected area, which were then analyzed using IMS by AXIMA QIT (Shimadzu Corporation). Part of the spectra was analyzed using MS/MS and an opened database for identification. Frozen sections and paraffin embedded sections were also made from formalin-fixed organs and analyzed using the same IMS method.

As a result, we were able to successfully analyze and directly identify lipids and drugs, including the metabolites present on the IMS-analyzed section. These results demonstrate that IMS can contribute to drug discovery, like histopathology and ADME, and can also serve as an in situ comprehensive analysis method. However, since problems with analysis conditions are sometimes encountered with this technology, further investigation is necessary.

WS2-1

Histopathological Changes of Female Reproductive Organs in the Rat Treated with Medroxyprogesterone Acetate

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Medroxyprogesterone acetate (MPA) is a synthetic progestagen which has not only progestational effects but also anti-estrogenic, anti-androgenic and glucocolticoid-like effects. MPA has been reported to affect ovarian histology and female fertility after repeated dosing.

Methods: MPA was orally administrated to female Crl:CD(SD) rats at the dose levels of 0, 0.4, 2.0 and 10 mg/kg/day for 2 and 4 weeks. After the completion of dosing, the animals were euthanized and the histopathological examination of the female reproductive organs (ovary, uterus and vagina) was performed.

After 2- week treatment, decreased numbers of large follicle with increased atretic large follicles and decreased numbers of newly formed or old/large corpora lutea were observed at 2.0 mg/kg and greater. After 4- week treatment, atretic follicles are increased in number and all stages of corpora lutea including newly formed, old/large and old/small ones are decreased in number at 2.0 mg/kg and greater. Endometrial atrophy accompanying endometrial epithelial cell hyperplasia of the uterus and mucinous degeneration of vaginal epithelia were evident at 2 mg/kg and greater after 2- and 4-week treatment. These histopathological changes of the uterus and vagina were considered to be caused by progestational and anti-estrogenic effects of MPA. The ovarian histopathology suggests that MPA acts mainly on large follicles to induce atresia and consequent inhibition of ovulation and corpola lutea development. It was reported that MPA reduces the levels of estrogen- and progesterone receptors in the rat pituitary gland which results in decreased responsiveness to luteinizing hormone-releasing hormone. The present study suggested that impaired LH surge via decreased estrogen receptors in the pituitary gland resulted in decreased numbers of corpora lutea in the MPA-treated rats.

WS2-2

Ovarian Toxicity of 2- or 4-week Repeated Dose Study of 4-Vinylcyclohexene diepoxide in Female Rats

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4-Vinylcyclohexene diepoxide (VCD) is an occupational chemical that destroys small follicles in the ovaries of mice and rats. To determine the optimal administration period for evaluation of ovarian toxicity of VCD, VCD was intraperitoneally administered to female Sprague-Dawley rats at 0 (Control), 5, 20 and 80 mg/kg once a day for 2 or 4 weeks (2- or 4-week study). To identify small follicles, serial sections of the ovaries were stained with routine hematoxylin and eosin (HE) and proliferating cell nuclear antigen (PCNA) immunohistochemistry.

In the 4-week study, decrease in small follicles was observed in the ovaries at 20 and 80 mg/kg. In the 2-week study, the same change was also observed at 80 mg/kg. Identification of small follicles using PCNA-stained slides was easier than that using HE-stained slides.

In conclusion, histopathological findings in the ovaries are important for evaluation of female reproductive toxicity of VCD, and ovarian toxicity of VCD can be detected by administration for 2 weeks at an appropriate dose level. Furthermore, PCNA immunohistochemistry is effective for evaluation of small follicles destruction in chemical-induced ovarian toxicity.

WS2-3

Ovarian Toxicity of Cisplatin –Histopathological Evaluation of the Ovary in the Repeated Dose Toxicity Study in Rats.

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[Objective] Antitumor agents including cisplatin (CDDP) affect a female reproductive function, and cause amenorrhea during clinical use. In rats, the induction of ovarian histopathological changes by a single high dose of CDDP was reported; however, ovarian morphological changes by 2- and 4-week administrations of CDDP are not reported in general toxicity studies. Therefore, we conducted a detailed histopathological examination of the ovary in 2- and 4-week repeated dose toxicity studies of CDDP in rats, and evaluated the toxicity on female reproductive organs.
[Methods] CDDP was administered intraperitoneally to female Crl:CD(SD) rats at dose levels of 0.25, 0.5, 1.0 and 2.0 mg/kg from 8 weeks old for the 2-week study, and 0.125, 0.25 and 0.5 mg/kg from 6 weeks old for the 4-week study. The control animals received the same dose of physiological saline for the same period. During the dosing period, the estrous cycle was checked using vaginal smears. On the next day of the last dose, the ovaries were removed and weighed, and were fixed in 10% neutral-buffered formalin. Ovarian tissues were transversely halved to observe the maximum area. Paraffin sections were stained with H&E and applied to PCNA histochemistry. Ovarian follicles were classified into the following three groups with reference to the report by Pedersen and Peters: small follicles (type 1-3ab), medium follicle (type 4-5a) and large follicle (type 5b-8).
[Results and conclusion] In the 2-week study, the prolongation of the estrous cycle and the persistent diestrus were observed in some animals receiving 2.0 mg/kg. In the 1.0 and 2.0 mg/kg groups, a decrease in the absolute ovary weight was observed, and a decrease in large follicle, increases in medium and/or large atretic follicles, and/or a decrease in currently formed corpus luteum were observed. In the 4-week study, no abnormalities were observed in the estrous cycle and ovarian weight in the all treated groups; however, decreases in small and/or large follicles and an increase in large atretic follicle were observed in animals receiving 0.25 and 0.5 mg/kg. Small follicles and atretic follicles were easily detected by PCNA staining than H&E staining. In conclusion, histopathological changes attributable to CDDP dosing, increases in atretic follicles and decreases in follicles, were detected by the detailed observation of rat ovary in the 2- and 4-week studies, the histopathological examination is useful to detect the ovarian toxicity.

WS2-4

Ovarian Morphological Changes in Female Rats Treated Orally with Anastrozole

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Introduction: Anastrozole is an aromatase inhibitor and a decrease in fertility rate was reported in preclinical fertility studies as a result of its administration. As a part of a collaborative study with the National Institute of Health Science and the Japan Pharmaceutical Manufacturers Association, we performed a pathological examination of the ovaries and female reproductive organs of female rats treated with anastrozole. **Materials and Methods:** Anastrozole was administered to 7-week-old female F344/DuCrlCrlj rats at dose levels of 0, 0.01, 0.1, 1 and 50 mg/kg for 2 or 4 weeks. On the day of necropsy, the ovaries and vagina were sampled and histopathological examination was performed. **Results:** Increases in large atretic follicles and follicular cysts, a decrease in the corpus luteum and depletion of the developing corpus luteum were observed in the ovaries, and mucinous degeneration and single cell degeneration were observed in the vagina in the 50 mg/kg groups of the 2-week study. In the 4-week study, increase in large atretic follicle was observed in the 1 mg/kg group, and the histopathological findings and their incidence in the ovaries and vagina in the 50 mg/kg group were almost the same as those in the 2-week study. The estrus cycle stopped in the 50 mg/kg group in the 2- and 4-week studies. **Discussion:** After anastrozole administration for 2 weeks, morphological changes were observed in the ovaries and vagina. In the ovaries, large follicles were affected while small to medium follicles were intact. It was reported that aromatase expression became high in the large follicle, and therefore these stage-dependent morphological changes are likely associated with aromatase expression. Regarding the corpus luteum, there was no developing corpus luteum in the 50 mg/kg group. Accordingly, the late stage of follicular maturation and ovulation should be completely suppressed by anastrozole treatment. It was reported that vaginal changes observed in this study were also induced by tamoxifen treatment. Hence, it is considered that the vaginal changes were caused by the disturbance of sexual hormones along with the anastrozole treatment.

WS2-5

Ovarian toxicity of a novel PPAR α/γ dual agonist in rats

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As part of a collaborative study on toxicity related to female fertility, we performed experiments using a novel PPAR α/γ dual agonist. The test compound was administered to female rats by oral gavage at dose levels of 0, 4, 20, and 100 mg/kg/day for 2 and 4 weeks. Vaginal smears were taken daily to monitor the estrous cycle during the last week of administration. After exsanguination, the ovaries, uterus and vagina were fixed in 10% neutral buffered formalin. All of the organs were embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin and eosin for light microscopic examination. Consecutive sections of the ovary were subjected to immunohistochemical identification of PCNA antibody and stained for apoptosis using the in situ apoptosis detection kit.

The estrous cyclicity was not disturbed in both studies. A significant decrease in ovary weight was observed in the 100 mg/kg/day group in the 4-week study, and a similar tendency was seen in treated groups in the 2- and 4-week studies. On histopathological examination of the ovary, toxic changes were observed in the granulosa cells of large follicles. Most of those granulosa cells had undergone apoptosis, and atretic follicles increased in the dose dependent manner. These changes in the large follicles were considered to result in a decrease in the number of corpora lutea and an increase in stromal cells. On the other hand, small- and medium-sized follicles did not show any histological changes such as those seen in the large follicles. In addition, exfoliation of granulosa cells into the antrum in large follicles, especially Graafian follicles, was observed as a characteristic histopathological change in the ovaries of the animals at estrus in the groups administered the test compound. These exfoliated granulosa cells stained positively with anti-PCNA antibody in immunohistochemical staining, and they were negative for apoptosis. Furthermore, the corpora lutea with retained oocyte also reflected they have not been ruptured. Therefore, it was concluded that these unique findings indicate that the follicles with exfoliated granulosa cells were not becoming atretic, but were the cause of formation of unruptured corpora lutea. These findings indicate that PPAR α/γ dual agonists may cause functional changes in the granulosa cells.

WS2-6

Effects of Mifepristone on the Reproductive and Endocrine Organs in Female Rats

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Mifepristone is a synthetic steroid that possesses great affinity for progesterone receptors with no agonistic activity. Due to its antiprogesterone activity, it was proposed that mifepristone be used for the termination of early human pregnancy. In animal experiments in rats, it has been reported that post-coital administration of mifepristone induced post-implantation embryonic mortality or infertility and pre-coital administration also induced fertility impairment or decrease in the numbers of implantation. In the present study, the effects of mifepristone on the reproductive and endocrine organs were investigated in female rats treated by repeated oral administration.

Six-week-old Crl:CD(SD) female rats were allocated into 5 groups consisting of 20 animals each, and received daily administration of mifepristone by gavage at the doses of 0.8, 4, 20 and 100 mg/kg, respectively. Animals in control group were administered the vehicle only. During the administration period, measurement of body weight and food consumption, and investigation of estrous cycle by vaginal smears were performed. After completion of 14^{th} or 28^{th} day of treatment, ten rats each from all groups were sacrificed and examined, respectively.

The mifepristone treatment-related changes were observed in animals given 20 mg/kg and above in both the 2- and 4-week studies. Animals with irregular estrous cycles were sporadically observed in the 20 mg/kg groups and almost all of animals in the 100 mg/kg group had persistent estrus in the 4-week study. In histopathology, the number of luteinized cyst was increased in the ovary and large unruptured follicles with luteinized granulosa cell layer were observed in several animals showing estrus stage of the vaginal smear. Loss or decreases in number of newly or currently formed corpora and hypertrophy of previously formed corpora lutea were also observed. In addition, hypertrophy of the pars distalis in the pituitary and increased secretory activity of the mammary gland may be indicative of enhanced secretion of prolactin were observed. Bilateral cortical hypertrophy was observed in the adrenals whose weight was increased.

In conclusion, the treatment-related findings observed in the reproductive and endocrine organs were considered to be caused by the hormonal imbalance induced by progesterone- and corticosteroid antagonistic properties of mifepristone. The findings observed in the ovary such as increased number of luteinized cyst, unruptured luteinized follicle and loss or decreased newly and currently formed corpora indicate inhibition of ovulation and it may be one of causes to reduce the number of embryo and fertility ratio in the female fertility study in rats.

WS2-7

Collaborative Work on Evaluation of Ovarian Toxicity: Effects of Indomethacin in 2 and 4-week Repeated Dose Studies and Fertility Study in Female Rats.

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[Introduction] Indomethacin is a well-known nonselective inhibitor of cyclooxygenase 1 & 2, and has been reported to inhibit ovulation by acting directly on the ovary. We report histopathological change found in 2 and 4-week repeated dose toxicity study, conducted as one of collaborative work on evaluation of ovarian toxicity. [Material and method] Six week old female Crl:CD(SD) rats were orally given indomethacin at doses of 0.3, 1.3 or 4 mg/kg (10 rats/group) for 2 or 4 weeks, and the ovary, the uterus and the vagina were examined histopathologically. [Results and Discussion] Unruptured follicle was observed in 3 and 2 animals at 4 mg/kg in the 2 and 4-week studies, respectively, and follicular cyst was noted in 1 animal at 4 mg/kg of the 4-week study. Unruptured follicles observed in estrus phase had large antral space and oocyte was occasionally seen in it. Theca cell and granulosa cell layer were luteinized and vascular genesis was observed. An unruptured follicle observed in metestrus phase was completely luteinized. It was thought to be unruptured since it was cystic and oocyte was seen in it. Unruptured follicle is identified easily in estrus phase as a large cystic follicle. It might be difficult to distinguish these unruputred follicles from cystic corpora lutea which were formed after normal ovulation in metestrus phase in the case that the oocyte was not seen on the specimen. There were no unruputred follicles in diestrus and proestrus phase. It was reported by several investigators that indomethacin could induce unruptured follicle. In those reports, animals in proestrus phase were selected and ovulation was stimulated by treatment of gonadtropin before a single dosage of indomethacin, and the ovary were removed on the estrus day. From the results of our present study, it was thought that animals in every estrous phase should be included in each group in order to evaluate ovarian toxicity of a compound which has potential of inhibitory effect on ovulation such as indomethacin in rats.

WS2-8

Female reproductive organ toxicity of ethylene glycol monomethyl ether in rats

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Ethylene glycol monomethyl ether (EGME) has been known to stimulate progesterone secretion from luteal cells. Detailed histopathological examination was performed on the toxicity of EGME in the rat female reproductive organs, with classification of the corpora lutea.

EGME was orally administered to female rats at doses of 0, 30, 100 and 300 mg/kg for 2 and 4 weeks. The reproductive organs were examined with hematoxylin and eosin (HE) staining, as well as PCNA immunohistochemistry of the ovary. In addition, BrdU was intraperitoneally administered to naive rats immediately after ovulation and autopsied after 1, 4, 8 and 12 days; the ovaries were examined with HE staining and immunostaining of BrdU.

Analysis with BrdU indicated corpora lutea of rats become largest at 1 cycle after ovulation, and regress over a 4–cycle period. They were classified into 4 types; newly-formed corpora lutea (Type I), mature corpora lutea (Type II), regressive corpora lutea (Type III) and residual corpora lutea (Type IV). The rats administered EGME showed irregular estrus cycle at 100 and 300 mg/kg. Histopathological examination showed luteal hypertrophy and increase in large atretic follicles in the ovaries at 100 and 300 mg/kg after both 2 and 4 weeks. The histological characteristic of these corpora lutea corresponded to Type II. These pathological changes were considered to be caused by the suppressive action of EGME on the regression of corpora lutea.

WS2-9

Effects of 2- or 4-week Repeated Administration with Sulpiride on the Reproductive Organs in Female Rats

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Sulpiride is a dopamine D2 receptor antagonist and is known to decrease pregnancy rate in a fertility study. As a part of the collaborative study of Japan Pharmaceutical Manufactures Association (JPMA) on ovarian toxicity, sulpiride was orally administered to female rats for 2 or 4 weeks to assess its effects on the reproductive organs by histopathological examination.

【Materials and Methods】Six-week-old female Crl:CD(SD) rats were treated with sulpiride at 1, 10 and 100 mg/kg/day or vehicle for 2 or 4 weeks. After completion of dosing, animals were euthanized and ovaries, uterus, vagina, mammary gland were removed. Hematoxylin and eosin (HE) specimens of these organs were prepared and examined microscopically. For ovaries, serial sections of HE specimen were stained immunohistochemically with anti-proliferating cell nuclear antigen (PCNA) antibody to detect developing follicles.

【Results and Discussion】

2-week study: In the ovary, increased atretic follicles were observed at 1 mg/kg or more, and increased follicular cysts were observed at 10 mg/kg or more. In the uterus and vagina, the incidence of diestrus increased at 1 mg/kg or more, and abnormal diestrus was noted at 10 mg/kg or more. In the vagina, increased epithelial mucification was noted at 10 mg/kg or more. Development of mammary gland and its hypersecretion were noted at 10 mg/kg or more.

4-week study: In addition to almost the same changes as seen in the 2-week study, increased abnormal proestrus and abnormal diestrus in the vagina, abnormal diestrus in the uterus were noted at 1 mg/kg or more. In the ovary, decreased large follicles were noted at 10 mg/kg or more and increased follicular cysts were seen at 1 mg/kg or more.

In this study, ovarian changes were observed in female rats treated with sulpiride at 1 mg/kg and above for 2-week. The main change induced by sulpiride in the ovary was arrest of development of types 7 to 8 large follicles, and there was no effect to small and medium follicles, or corpus luteum. In the uterus and vagina, an increased incidence of diestrus, abnormal diestrus and abnormal proestrus reflecting the arrest of estrus cycles were noted. The changes in ovary, uterus, vagina and mammary gland were considered to be related to dopamine D2 receptor antagonistic action and subsequent hyperprolactinemia by sulpiride.

O-1

Dietary tricin suppresses AOM/DSS-induced colon carcinogenesis in mice

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[Background and Aim] A flavone tricin, which occur in rice bran, possesses cancer chemopreventive properties in several preclinical rodent models and an *in vitro* system. The present study aimed to further investigate the potential chemopreventive efficacy of tricin in colitis-related colon carcinogenesis using an azoxymethane (AOM)/dextran sodium sulfate (DSS) mouse model.

[Methods] Male ICR mice were initiated with a single i.p. injection of azoxymethane (AOM, 10 mg/kg bw) and promoted by 1.5% DSS in drinking water for 7 days to induce colonic tumors. Starting 1 week after the DSS treatment, mice were fed with the diet containing 50 and 250 ppm tricin for 15 weeks. They were sacrificed to determine the incidence and multiplicity of tumors at wk 17.

[Results] Dietary feeding with tricin significantly inhibited tumor formation in the colon. Also, dietary tricin was able to inhibit the cell proliferation and to decrease the expression of Tnf-α in the colonic mucosa surrounding the tumors.

[Conclusions] Our findings suggest that dietary tricin effectively suppresses inflammation-related mouse colon carcinogenesis through multiple modulatory mechanisms of Tnf-α that are involved in carcinogenesis in these tissues. Our results also suggest that tricin should be further evaluated as a putative colorectal cancer chemopreventive agent.

O-2

Role of Canonical Wnt Signaling in Proliferation of Colonic Epithelial Cells: Analysis by ß-catenin Inducible Mouse

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In order to investigate the underling mechanisms on the control of cell proliferation in the colon epithelium, we first separated cycling cells from quiescent cells of colonic crypts using histone H2B–GFP inducible mice and compared the gene expression profiles of proliferating cells and quiescent cells. We found that proliferating cells express higher levels of ß-catenin/tcf target genes than quiescent cells, indicating the essential role of canonical Wnt pathway in active proliferation of colonic epithelium. Subsequently, we generated the ß-catenin inducible mice expressing stabilized the ß-catenin under the control of *tet-on* system to directly address the effect of the canonical Wnt pathway on the cell proliferation of the colon. Surprisingly, higher level of ß-catenin induction did not lead to rapid proliferation of colonic epithelium but they caused the frequent crypt fission/branching phenotype and upregulation of stem cell markers, musashi1, Lgr5 and Bmi1, indicating the stem call amplification. In contrast, lower level of ß-catenin induction increased cell proliferation activities, whereas they did not increase crypt fission rate. Taken together, these results indicate that canonical Wnt pathway plays distinct roles on the proliferation of colon epithelium and their expression regulation is important for the homeostatic proliferation of colon epithelium.

O-3

Crosstalk between PTEN/Akt2 and TGFβ signaling involving EGF receptor downregulation during the tumor promotion process from the early stage in a rat two-stage hepatocarcinogenesis model

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The present study investigated the involvement of signaling of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) /Akt and transforming growth factor-β (TGFβ) as well as receptor tyrosine kinases in the tumor promotion processes in a two-stage hepatocarcinogenesis model using male F344 rats. The cellular localization of related molecules was examined in liver cell foci expressing glutathione *S*-transferase placental form (GST-P) at the early stage of tumor promotion by fenbendazole (FB), piperonyl butoxide or thioacetamide. Distribution in the liver cell foci and neoplastic lesions positive for GST-P was also examined at the later stage of FB promotion. In contrast to the initiation-alone cases, subpopulations of GST-P-positive foci induced by promotion for 6 weeks, regardless of the promoting chemicals used, enhanced downregulation of PTEN and upregulation of phosphorylated (active) Akt2 and phosphorylated substrate(s) of Akt-kinase activity. Also, upregulation of TGFβ receptor I and downregulation of epidermal growth factor receptor (EGFR) were enhanced in the subpopulation of GST-P-positive foci in all promoted cases. A similar pattern of cellular distribution of these molecules was also observed in the neoplastic lesions at the late stage. These results suggest a crosstalk between Akt2 and TGFβ signaling that involves a mechanism requiring EGFR downregulation during the entire tumor promotion process starting from the early stage. In particular, a shift in subcellular localization of phosphorylated substrate(s) of Akt from the cell membrane in liver cell foci to the cytoplasm in carcinomas was observed, suggesting an alteration of the function or activity of the corresponding molecule(s).

O-4

Establishment of the New Two-stage Carcinogenesis Model for Lung Squamous Cell Carcinoma in Mice

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Recently, the development of the molecular-targeted anticancer drugs, which will be effective against different types of lung cancer is strong required. Although the lung adenocarcinoma animal models exist, there are no sophisticated model specifically induced lung squamous cell carcinoma (SCC) on mice lung carcinogenesis. Therefore, to establish the novel two-stage carcinogenesis model for lung SCC, we have performed the initiation by administration of N-nitroso-tris-chloroehylurea (NTCU), subsequently applied dimethylarsinic acid (DMA) and butylated hydroxytoluene (BHT) as the promoters, which are known to accelerate the lung adenocarcinoma. Female A/J mice, 6-week-old, were administered NTCU or acetone as vehicle, by topical application to the skin twice a week for 2 or 4weeks. After a week finishing NTCU treatment, mice were administered 0 or 200ppm DMA in drinking water and 0, 0.1, 0.25% BHT in diet. Sacrifices were performed at 18-week for the histopathological examination of the lungs. NTCU treatment induced lung squamous metaplasia almost in all mice. Incidences of lung SCCs were 54 and 53% in 4-week NTCU control and 4-week NTCU followed by 200ppm DMA treatment groups, respectively. Incidences of lung SCCs were 25, 16 and 22% in 2-week NTCU control, 2-week NTCU followed by 0.1 and 0.25% BHT treatment groups, respectively. Our results indicated that 4-week NTCU control were higher induction of lung SCCs compared to 2-week NTCU control. However, no evidence was obtained the promotional effects of DMA and BHT on lung squamous carcinogenesis in mice. We conclude these 2-week NTCU treatment suitable for two-stage carcinogenesis models of lung scc.

O-5

Establishment of a Medium-Term Lung Carcinogenesis Bioassay Model in A/J Mice by Employing Left Lung Collapse, and Possibility as a Bioassay Model for Mesothelioma of Pleura

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The risk of lung cancer development remains elevated and identification of potential chemopreventive or tumor promotive agents in the lung is also important. This present study was conducted in an attempt to reduce the experimental period, much shorter period than 12 weeks concluded by previous study, needed by incorporating procedures with strong tumor promotion effects. To induce strong promotion effect for lung tumor, in Experiment 1, there were examined the effects of thoracotomy to lung tumor initiated by 4-(methylnitrosamno)-1-(3-pyridyl)-1-butanone (NNK). In experiment 2, the polymer, a foreign material, was infused in left cavity of thorax to examine the effect of pulmonary collapse to lung tumor by NNK.

Female A/J mice (6 weeks of age) pretreated with NNK (2mg / 0.1ml saline / mouse i.p.) at weeks 0 and 1. At week 3, half of them underwent a left thoracotomy in Experiment 1 or infused 0.2 ml polymer gel in left cavity of thorax after left thoracotomy in Experiment 2. This polymer, sodium salt cross-linkage in the acrylic acid polymerization, material has absorption of moisture and brings about lung collapse by occupying the thoracic cavity for its expansion. In Experiment 1, the experiment was terminated after 8, 10, 12 and 16 weeks, and, in Experiment 2, was after 12 weeks.

The results of present experiments could not demonstrate the clear promotion effects by thoracotomy in Experiment 1 or pulmonary collapse by the foreign material in Experiment 2. It remains possible, however, that alternative approaches might have greater efficacy and these need more consideration.
In addition, histopathologically, there were observed the reactions of mesothelial cells with the infused polymer on the surface of the left lung with iron accumulation in Experiment 2. In addition, the observed reaction of mesothelial cells with the infused polymer, pointing to potential use in development of a suitable animal model for pleural mesothelioma. These findings might aid in development of a suitable animal model for pleural mesothelioma. To confirm this possibility, further experiment is now in progress.

O-6

New Strategy to Evaluate Brain Retardation Employing Neuronal Development Parameters: Availability of Reelin Immunolocalization Analysis and Monitoring of Neuron Distribution in the Hippocampal Dentate Gyrus

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To establish an appropriate assessment method that is available for detection of many types of brain retardation, we have focused on the developmental effects of neuronal and glial cells induced by developmental hypothyroidism, and searched marker molecules employing microarray analysis and immunohistochemistry in combination as well as morphometric methods in brain regions to detect irreversible effect. In the present study, we report a highly sensitive method for evaluation of abnormalities in neuronal development. Pregnant SD:IGS rats were administered 200 ppm methimazole, 3 propylthiouracil (PTU) or 12 ppm PTU in drinking water from gestation day 10 to postnatal day (PND) 21. Similarly, dams were given brominated flame retardants (BFRs) which have been shown to exert a weak anti-thyroidal activity, i.e., decabromodiphenyl ether at 10, 100 or 1000 ppm, hexabromocyclododecane or tetrabromobisphenol-A at 100, 1000 or 10,000 ppm in diet, respectively. Immunohistochemical distribution of Reelin-, EphA5- or NK3-positive neurons in the brain regions was examined in offspring at weeks 3 and 11. At week 11, NeuN-positive neuronal cells distribution in the hippocampal CA1 region and dentate gyrus were investigated. By measurement of serum thyroid-related hormone levels, an apparent hypothyroidism was confirmed in offspring exposed to anti-thyroidal agents, whereas a very slight hypothyroidism was observed with BFRs. At PND 21, increase of abnormally distributed EphA5- or NK3-positive cells was detected in hippocampal CA1 of PTU-exposed offspring. In contrast, Reelin-positive cells were increased in the area medial to granule cell layer in the dentate gyrus by anti-thyroidal agents and BFRs from middle doses, and this abnormality was also detected at the adult stage in the 12 ppm PTU group. At the adult stage, NeuN-positive neuronal cells were increased in the area medial to the granule cell layer of dentate gyrus at the highest dose of all chemicals examined. These results suggest that Reelin is a sensitive biomarker for detection of impairment of neuronal development. Moreover, changes in abnormally distributed neurons in the dentate gyrus at the adult stage were parallel to aberrant distribution of Reelin-positive cells at PND 21, suggesting the availability of both methods for detection of neuronal brain retardation.

O-7

6-Mercaptopurine Induces Apoptosis of Neural Progenitor Cell in the Developing Fetal Rodent Brain

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6-Mercaptopurine (6-MP), a DNA-damaging agent, is used for the therapy of lymphoblastic leukemia and causes fetal neurotoxicity. To clarify the mechanisms of 6-MP-induced fetal neurotoxicity, pregnant rats and mice were treated with 50 mg/kg 6-MP at embryonic day 13 or 12, and the fetal telencephalons and fetuses were collected 12 to 72 hours after treatment. The number of TUNEL- or cleaved caspase-3-positive cells remarkably increased in the rat telencephalon at 36 h. Electron microscopy showed typical ultrastructural characteristics of apoptosis. The expressions of p53, puma and cleaved caspase-9 proteins, intrinsic pathway factors, increased in the rat telencephalon from 24 to 72 h. 6-MP-induced apoptosis of neural progenitor cells was completely absent in *p53*-deficient mice. While, the expression of Fas protein, an extrinsic pathway factor, in the rat telencephalon did not change throughout the experimental period. The number of apoptotic neural progenitor cells was same between *Fas*-mutated and wild-type mice, suggesting that the Fas pathway is not involved in 6-MP-induced apoptosis of neural progenitor cells. These findings suggest that 6-MP induces p53-mediated intrinsic apoptotic pathway in neural progenitor cells in the rodent brain.

O-8

Epigenetic alteration of prostate enlargement induced by perinatal exposure to methoxychlor in rats

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In order to determine whether perinatal exposure to methoxychlor (MXC), an endocrine disrupting chemical, influences epigenetics in the rat prostate after maturation, we conducted microarray analysis, quantitative RT-PCR analysis and quantitative methylated DNA PCR (MeD-PCR) in addition to conventional histopathology.

For the present study, male pups were obtained from SD rat dams receiving MXC at a dietary level from 0 to 1000 ppm during the gestation/lactation period. The pups were fed a normal diet after weaning and kept up to 52 weeks of age. At 10 and 52 weeks of age, the prostates of 8 animals of each dose were sampled.

At 52 weeks of age, the prostate weights (absolute and relative) were significantly increased in 1000 ppm group when compared to the control, although no dose-dependent histopathological lesion was observed. From microarray analysis, 12 genes related to prostate enlargement were selected. In the quantitative RT-PCR analysis, prostate specific binding protein polypeptide C3 (Pbp C3) and 3 genes were significantly up-regulated, and testosterone repressed protein messenger (Trpm) and 5 genes were significantly down-regulated at 52 weeks of age. MeD-PCR analysis revealed that a CpG island in Pbp C3 promoter region was significantly hypomethylated and CpG islands in c-fos were significantly hypermethylated at 52 weeks of age. Furthermore, reductions of methylation status were observed in global DNA and several regions at 52 weeks of age in comparison with 10 weeks of age. The quantitative RT-PCR analysis of Dnmt-related genes revealed that Parp1 mRNA was down-regulated significantly in 1000 ppm group at 52 weeks of age and the transcriptional down-regulation of Np95 with the aging was observed in control rats.

In conclusion, these data suggest that the maintenance of DNA methylation system in rat prostate became fragile with aging, and that the epigenetic influence on this system by perinatal exposure to MXC became marked with aging.

O-9

Promotion of Nano-size Titanium Dioxide on Rat Lung and Mammary Gland Carcinogenesis – Involvement of Macrophage Inflammation Protein 1α

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Engineered nano-size titanium dioxide ($nTiO_2$) is used in a variety of commercial products such as toothpaste, sunscreen, and cosmetics. To examine the effects of $nTiO_2$ on carcinogenesis, Sprague-Dawley (SD)-derived female H*ras*128 transgenic rats were treated with DHPN (0.2% in the drinking water) for 2 weeks and then exposed to $nTiO_2$ from the end of week 4 through week 16: rutile type $nTiO_2$ 20 nm in average diameter without coating was suspended in saline and intra-tracheally sprayed into the lung at 250 ppm or 500 ppm once every two weeks. $nTiO_2$ treatment significantly increased the multiplicity of lung alveolar hyperplasia and adenoma and, surprisingly, mammary adenocarcinoma. Notably, aggregates of $nTiO_2$ were commonly observed in alveolar macrophages. Since macrophage activity and inflammation are strongly associated with fiber and particle toxicity in the lung, we treated nontransgenic female SD rats with 500 ppm $nTiO_2$ for 8 consecutive days and analyzed the lung tissue for inflammation associated factors. Treatment with $nTiO_2$ significantly increased SOD activity, 8-OHdG level, and expression of macrophage inflammatory protein1α (MIP1α). Importantly, MIP1α was detected only in macrophages with phagocytosed $nTiO_2$. MIP1α was also detected in the serum of $nTiO_2$ treated rats. Treatment of primary SD rat alveolar macrophages with $nTiO_2$ induced secretion of MIP1α. Finally, MIP1α enhanced proliferation of human lung cancer cells and H*ras*128 rat-derived mammary cancer cells *in vitro*. We conclude that $nTiO_2$ promoted lung and mammary carcinogenesis, and that MIP1α secreted by TiO_2 burdened alveolar macrophages caused proliferation of alveolar and mammary epithelial cells.

O-10

Induction of Mesothelioma by a Single Intrascrotal Administration of Multi-wall Carbon Nanotube in Intact Male Fischer 344 Rats

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Multi-wall carbon nanotube (MWCNT; 1 mg/kg bw, 7 animals), crocidolite (2 mg/kg bw, 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) was administered to male Fischer 344 rats (12 weeks old) by a single intrascrotal injection. Rats were autopsied immediately after the death, when becoming moribund or at the end of the maximal observation period scheduled to be 52 weeks. After 37-40 weeks, however, 6 MWCNT-treated animals died or became moribund due to intraperitoneally disseminated mesothelioma (6/7, 85.7%) with bloody ascites. Peritoneal mesothelium was generally hypertrophic, and numerous nodular or papillary lesions of mesothelioma and mesothelial hyperplasia were developed. While mesothelioid cells were predominant in relatively early stage tumors, advanced stage mesotheliomas were constituted by 2 portions occupied by mesothelioid cells in the surface and spindle-shaped sarcomatous cells in the depth. In the latter, the histological transition was apparently observed between these 2 portions. Mesotheliomas were invasive to adjacent organs and tissues, and frequently metastasized into the pleura. Only 1 rat survived for 52 weeks in the MWCNT-treated group, and similar findings except mesothelioma were observed. All 10 crocidolite-treated and 5 vehicle-treated rats survived for 52 weeks without any particular changes except deposition of asbestos in the former case. It is thus indicated that MWCNT possesses carcinogenicity to cause mesothelioma at a high rate in intact male rats under the present experimental conditions.

P-1

Single Terbutaline Infusion into the Internal Carotid Artery Induces Dark Cell Degeneration-like Change of Purkinje Cells in Adult Rats.

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INTRODUCTION: Dark cell degeneration (DCD) was induced by an increase in extracellular glutamate concentration (excitotoxitity) and differentiated from necrosis and apoptosis. The change is associated with the morphological adaptability against an increase in intracellular Ca++. We reported that single terbutaline (TB), β2-adrenoceptor agonist, infusion into the internal carotid artery after mannitol infusion induced DCD-like change of cerebellar Purkinje cells (PCs) in adult rats. (The 146th meeting of Japanese Society of Veterinary Science) In this study, we investigated the morphological structure of a DCD-like change in more detail and the alteration of calbindin, calcium binding protein, with immunohistochemistry.

MATERIALS AND METHODS: Nine Crl:CD(SD) male rats, 9 weeks of age, were treated with 30 mg/kg TB sulfate into the internal carotid artery after infusion of 25% mannitol solution. Two hours, 3 and 30 days after treatment, animals were deeply anesthetized and perfused with 10% neutral buffered formalin.

RESULTS: DCD-like change of PCs was detected in all of the TB treated animals. The change was prominent at 2 hrs and on Day 3. Whereas such change diminished on Day 30, the number of PCs did not decrease on Day 30. Vacuolation was observed around PCs at 2 hrs and on Days 3 and 30, and it was prominent on Day 30. Ultrastructually, fenestrated cisterns and large lamellar bodies were observed in the PC somas and dendrite on Day 30. PCs with a DCD-like change showed indentation of the nuclear membrane, many mitochondria and ribosome, large lamellar bodies and its deformed structure. Swelling of the Bergmann glias was observed around the PC somas and dendrites. Immohistochemically, granular reaction products were remarkable in the nucleus of PCs at 2 hrs and on Day 3, and they decreased on Day 30. Gross granular or laminar reaction products were observed in the cell edge region at 2 hrs and on Days 3 and 30, and they decreased on Day 30. In the controls, fine granular reaction products were observed in the nucleus and the cell edge region of PCs.

CONCLUSION: A DCD-like change was considered to be reversible, which differentiated from necrosis and apoptosis. The morphological changes were similar to DCD. Alteration of intracellular calcium kinetics in PCs seemed to be one of the causes of the DCD-like change. Our results suggest that a DCD-like change as well as DCD may be an excitotoxic neuronal degeneration elicited by glutamate receptor overstimulation.

P-2

Pathomorphological Examination on Neonatal Brain Development

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With regard to the evaluation of developmental neurotoxicity for pesticides, OECD finalized test guideline in 2007, following U.S.EPA. In Japan, recent establishment of the guideline is expected. In the study, the test substance is administered to maternal animals from the gestation day 6 to postnatal day (PND) 21, and their pups were examined histopathologically during immature (PND11-22) and young adult (PND60-70) periods. It is known that rodent brain continues to develop into the postnatal period, but there are few reports about detailed developmental process. To elucidate the neonatal brain development, we performed pathomorphological examination of pups at PNDs13, 22 and 70 using various staining methods. Paraffin-embedded brain sections including the striatum, hippocampus and cerebellum were routinely prepared. On PNDs13 and 22, high density of neuron distribution in the whole brain area was observed when compared to PND70. On PND13, PCNA-positive cells, which show proliferative activity, were observed in the area around the lateral ventricle, hippocampal dentate gyrus, cerebellar external granular layer and a portion of white matter. Although the number of PCNA-positive cells was low, same tendency was found on PND22. PCNA-positive cells were slightly observed in the area around the lateral ventricle on PND70. TUNEL-positive apoptotic cells were detected in the PCNA-positive area and the cerebral cortex on PNDs13 and 22. For the Bodian stain and neurofilament immunostain, the difference of the condition of dendrites and axons was observed in the cerebral cortex, hippocampus and cerebellum. Whereas thick dendrites and axons extended unidirectionally on PND70, reticulated thin dendrites and axons were observed disorderly on PND13. For synaptogenesis, whereas dot-like immunoreactive for synaptophysin were seen in the cerebral cortex, hippocampus and cerebellum on PND70, few positive reactions were observed on PNDs13 and 22. In the Kluver-Barrera's stain, myelination was almost completed on PND70. However, myelin was found in few or partial area on PNDs13 or 22, respectively. Based on these results, proliferation, differentiation and network formation of neurons continued into the postnatal period in the immature rat brain. Therefore, it is important to understand these development processes for evaluation of developmental neurotoxicity study.

P-3

Effects of Busulfan on Neonatal Rat Cerebellum

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[Purpose] Busulfan, an alkylating agent that is used as a chronic myelogenous leukemia drug and a chemotherapeutic agent before hematopoietic stem cell transplantation, is known to show neurotoxicity or bone marrow toxicity. In this study, cerebellar histopathological alteration of neonatal rats brought about by administering busulfan was observed chronologically.

[Materials and methods] Five male Crl:CD(SD) strain rats on day 6 of age were used at each dose and time point. Busulfan was suspended in olive oil and administered once subcutaneously to the dorsal area at dose levels of 0, 10, 30 or 50 mg/kg, and brain was obtained on day 1, 2, 4 or 7 or in week 5 after administration. At 30 and 50 mg/kg, some animals died or were sacrificed moribund due to sepsis with myelosuppression during the period from day 7 to day 16 after administration. After body weight was measured, neonates were bled from abdominal aorta under ether anesthesia, and brains were taken out and weighed. The brains were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin according to the conventional method, and examined histologically. In addition, they were subjected to immunohistochemical staining with anti caspase 3 (CAS), p53, p21 and Phospho-Histone H3 (HIS) antibodies and the TUNEL method.

[Results] In the Busulfan group, suppressed body weight gain and low value of brain weight were observed dose-dependently. In the cerebellum, pyknotic cells were strongly noted in granule cells in the external germinal layer, and showed positive reactions in the TUNEL method and to CAS, p53 and p21. Apoptosis appeared dose dependently from 1 day, peaked on day 2, and no longer observed on day 4 or 7 after administration. However, in the 50 mg/kg group, recovery was not observed, EGL completely disappeared on day 4, and cell density of the internal granular layer dropped. The CAS of EGL peaked on day 2 after administration in the 10mg/kg group and on day 1 after administration in the 30 and 50mg/kg groups, and decreased thereafter. HIS of EGL decreased dose- dependently in all groups on day 2 or day 4 after administration and was no longer observed in the 10 and 30 mg/kg groups, but disappearance was not observed in the 50 mg/kg group. In the animals in the 10 mg/kg group that survived for 5 weeks after administration, no significant findings were observed.

[Summary] It was revealed that single dose administration of Busulfan to neonatal rats at 6 days of age was associated with apoptosis mainly in granular cells of EGL in the cerebellum. The change was dose-dependent and reversibility was noted of 10 mg/kg where the damage was slight.

P-4

Study of Busulfan-Induced Central Nervous Toxicity in Apoptosis in Rat Fetuses — Sequential Changes of Apoptosis and Cell proliferation —

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Busulfan, an antineoplastic bifunctional-alkylating agent, is known to induce developmental anomalies; however, the mechanism of the fetotoxicity has not fully been understood. In the present study, we examined sequential changes of apoptosis and cell proliferation in the fetal central nervous system (CNS) exposed to busulfan.

Pregnant Crl:CD(SD) rats were equally divided into the control and busulfan groups. Animals of the busulfan group were administered intraperitoneally with 30 mg/kg of busulfan, and those of the control group with 10 mg/kg of olive oil on gestation day 13(GD13), and fetuses were collected at 6, 12, 24, 36, 48, 72 and 96 hours after treatment (HAT). All fetuses were weighed and fixed with 10% neutral buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE), and sections were subjected to immunohistochemical stainings for Cleaved caspase-3, p53, p21, phospho-histone H3, and BrdU, and the TUNEL method. Some telencephalic wall tissues were subjected to electron microscopic examination.

Fetal body weights of the busulfan group were significantly reduced at 72 and 96 HAT compared with those of the control group. The neuroepithelial cell death occurred from 24 to 72 HAT, with a peak at 48 HAT. Such cell death was confirmed to be apoptosis by the positive reaction for the TUNNEL method and Cleaved caspase-3 immunohistochemical staining and also by electron microscopic examination. In the fetal CNS, the number of Cleaved caspase-3 positive neuroepithelial cells began to increase at 24 HAT, peaked at 48 HAT, and returned to the control level at 96 HAT, while the number of p53 and p21-positive neuroepithelial cells appeared at 24 HAT, peaked at 36 HAT, and returned to the control level at 96 HAT. In contrast, the number of phospho-histone H3-positive cells and BrdU-positive neuroepithelial cells decreased from 24 HAT, reached the lowest level at 48 HAT, and returned to the control level at 96 HAT.

The present data clearly demonstrate that busulfan induces not only neuroepithelial cells apoptosis relating to p21 and p53 expression but also suppression of neuroepithelial cell proliferation, which may have a close relation to the subsequent occurrence of microencephaly.

P-5

The time-dependent changes in NTCU-induced A/J mice lung carcinogenesis

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Administration of *N*-nitroso-tris-chloroethylurea (NTCU) by topical application for forty weeks to female A/J mice has been reported to induce preneoplastic and neoplastic lesions, including lung squamous cell carcinoma (SCC). The present study was conducted to evaluate the time-dependent changes in NTCU-induced mouse lung carcinogenesis.

Female A/J mice, 6-week-old, were randomized into two groups. Mice were treated topically with NTCU (group 1), or acetone as vehicle (group 2) twice a week, with a 3-day interval. Sacrifices were performed at weeks 16, 20 and 24 after stopping treatment at week 20 for the histopathological examination of the lungs.

It was detected that lung lesions which include squamous metaplasia, dysplasia and SCC arise in the terminal bronchiole-alveolar sac and proceed through squamous metaplasia-dysplasia-carcinoma sequence. At weeks 16 and 20, incidences of lung dysplasia and SCC were time-dependently elevated. Furthermore, incidences of dysplasia and SCC was found to be increased at week 24. Development of SCC was found to be irreversible after discontinuation of treatment at week 20. Moreover overexpression of EGFR and p53 protein has been seen in investigated lesions similar to that in human.

Our results indicate that NTCU-induced SCC model in A/J mice might be applicable as a medium-term assay for mice lung carcinogenesis.

P-6

Rats Prenatally 3,3',4,4',5-Pentachlorobiphenyl (PCB126) Modulate ERK and ERα Expression in N-nitrobis(2-hydroxypropyl)amine (BHP)- Induced Lung Carcinogenesis

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PCB126 had been considered to transfer from mother to offspring via placenta and milk. Moreover PCB126 have been proposed that influence for carcinogenesis in laboratory animal and human. We investigated that BHP induced rats which prenatal exposed to PCB126, lung carcinogenesis by offspring rats.【Material and Method】SD rats were injected (i.g.) PCB126 (7.5ug, 250ng,and 2.5ng /kg BW) or the vehicle during 13-19th days. At 5-week-old offspring rats were treated 1,000ppm BHP for 8weeks, and were dissected at 25-week-old.【Result】Histological analysis showed the lung adnocarcinomas increased with PCB126 dose dependently. Immunohistchemical and westernblotting analyses showed in lung adenocarcinoas that the expression of AhR and CYP1A1 decreased with PCB126 dose dependently but the expression ERK and ERα increased with PCB126 dose dependently.【Discussion】The present study revealed that the enhance of lung carcinogenesis was unlikely by direct PCB126 cause. We consider that the lung carcinomas with prenatal PCB126 exposed offspring showed important role in upstream of expressions of ERK and ERα.

P-7

Gene alterations in rat lung tumors induced by intratracheal administration of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

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[Introduction] Previously, we reported that NNK, a tobacco-specific nitrosamine, induced neoplastic lesions of the lung by intratracheal administration in rats. In this study, we investigated gene abnormalities in lung carcinogenesis induced by intratracheal administration of NNK and compared them with the gene abnormalities in other experimental lung carcinogenesis models, such as *N*-nitrosobis-(2-hydroxypropyl)amine (BHP)-induced rat model. [Materials and Methods] The test article was administered intratracheally to male Wistar rats at 7 weeks of age once a week for 8 weeks. NNK was administered under 3 different conditions: 0.1 mL of the solution containing 140 mg/mL of NNK and 28 mg/mL of iron oxide; 0.2 mL of the solution containing 28 mg/mL of NNK and 14 mg/mL of iron oxide with 0.5 mL of air; and 0.2 mL of the solution containing 28 mg/mL NNK with 0.5 mL of air. The iron oxide was added to promote cancer while the air was given at the same time to allow the solution spread throughout the lungs. In week 69 after the final administration, the animals were necopsied and the lungs were removed. 10% phosphate buffered formalin was injected to fix the lungs, and paraffin thin sections were prepared by an ordinary method. DNA was extracted from proliferative lesions using microdissection method. Abnormalities of K-ras and EGFR genes were examined by PCR-SSCP and direct sequencing. [Results and Discussion] Intratracheal administration of NNK induced alveolar hyperplasias (AHs), adenomas (Ads) and adenocarcinomas (ACs). Mutations of K-ras or EGFR genes were not detected in 8 AHs, 4 ADs and 1 ACs. Gene mutation of K-ras has been reported in BHP-induced rat lung cancer and human lung adenocarcinoma, and gene mutations in EGFR genes in human lung adenocarcinoma. These results suggest that K-ras and EGFR gene alterations may not play an important role in the rat lung carcinogenesis induced by NNK. Further investigation is needed to clear the genetic pathway in the lung carcinogenesis induced by NNK.

P-8

Pulmonary effect in rat by inhalation and instillation of fullerene (C60).

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We operated two animal studies to evaluate the pulmonary toxicity of fullerene (C60) nanoparticles.

1) Instillation study: Wistar rats were single-instilled intratracheally of well-characterized fullerene (C60) with the dose of 0.1mg, 0.2 mg and 1mg (in 0.1% Tween 80) respectively. The 50% mean diameter of sample was 33 nm. The lung of the rats were examined at the time point of 3 days, 1 week, 1 month, 3 months and 6 months after instillation. The 1 mg instilled group in early phase after instillation showed a slight increase of total cell counts and PMN counts in bronchioalveolar lavage fluid (BALF). The pulmonary inflammation determined by point counting method showed that significant inflammation was observer in rats with 1mg instilled group within 1 week after instillation. However, no significant difference was observed after 1 month.

2) Inhalation study: Fullerene nanoparticles were generated in the gas phase by spraying suspension. Wistar rats were exposed fullerene nanoparticles for 4 weeks, and during the exposure, the consistent concentration and size distribution in the chamber were monitored and maintained. Rats were randomly sacrificed at 4 days, 1 mo, and 3 mo after the 4 wk of exposure. Total cell counts and PMN cell counts in BALF showed no significant difference between exposed rats and control. In the alveoli, the macrophage with pigment components were slightly observed, however, the infiltration of neutrophil or other inflammatory cells were not obvious during the time course after exposure. Point counting method for pulmonary tissue also showed that no significant difference were observed comparing with control rats.

In summary, the severe inflammation was not observed in rat lung by the instillation and the inhalation of fullerene nanoparticles.

P-9

Globule Leukocyte Infiltration in Rat Stomach Induced by Repeated Oral Dosing of Polyethylene Glycol 400

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Polyethylene glycol 400 (PEG 400) is a viscous liquid (mean M.W.: 400) and is considered to be a no- or extremely-low toxicity chemical. Taking advantage of these characteristics, PEG 400 is generally added to the dosing solution in preclinical studies as dispers medium, solvent and so on. We report here the increased globule leukocyte infiltration in the stomach observed histopathologically in rats administered PEG 400 repeatedly for 15 days.

Five, 50 and 100 v/v% PEG 400 aqueous solution (volume: 5 mL/kg B.W.) were orally administered daily for 15 days to Crl: CD (SD) [SPF] rats (5 males and females each at 7 weeks old for each dose-group). These rats were monitored their general conditions, body weights and food consumptions. After the completion of the dosing period, hematological examination and necropsy were conducted, followed by the histopathological examination for the digestive tracts. As the result, most animals administered 100 v/v% PEG 400 showed soft feces during the treatment period. While the values of body weight, food consumption, hematological parameters and gross findings in the PEG 400-treated groups were comparable to those of the control-group, globule leukocyte infiltration in males and increased epithelial spongiosis in both males and females were observed dose-dependently at the limiting ridge in the stomach. In the glandular stomach, proliferation of mucous neck cells and increased globule leukocyte infiltration at mucosa, and eosinophil infiltration at lamina propria and submucosa were observed dose-dependently at near the limiting ridge. Intracytoplasmic eosinophilic granules of the globule leukocytes in rats treated PEG 400 were larger than those in the control animals, and showed weak metachromasia with toluidine blue staining and positive reaction for anti-rat mast cell protease 2 (RMCP2) antibody. Although similar phenomenon on globule leukocytes has been reported on several chemicals, its biological meanings and pathogenesis are still unclear. Our approaches to clear them is now ongoing.

P-10

Roles of β-catenin Alternation and COX-2/mPGES-1 Expression in *N*-methyl-*N*-nitrosourea-induced Mouse Gastric Cancers.

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Helicobacter pylori (*Hp*) infection is closely linked with gastric cancer and induces cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in gastric mucosa. K19-C2mE transgenic (Tg) mice, simultaneously expressing COX-2 and mPGES-1 in the gastric mucosa under the influence of the cytokeratin 19 gene promoter, shows the promotion effect for *N*-methyl-*N*-nitrosourea (MNU)-induced gastric cancers. In present study, we investigated the involvment of Wnt/β-catenin signal pathway and COX-2/mPGES-1 expression in gastric carcinogenesis.

Tg and wild type (WT) mice were divided into 4 groups [*Hp*-infected, MNU treated, *Hp*+MNU treated, and none-treated control groups]. The mice in the MNU treated groups were given drinking water containing 120 ppm MNU on alternate weeks (total exposure was 5 weeks) and sacrificed at the 52nd experimental week. β-catenin accumulation and gene mutations were analyzed in observed gastric cancers.

As a result, immunohistochemical analysis demonstrated significantly greater β-catenin accumulation in pyloric tumors, compared with those in the fundus [MNU group: pyloric (15/15) vs. fundic (0/4), $P<0.01$; *Hp*+MNU group: pyloric (19/19) vs. fundic (1/4), $P<0.01$]. Mutations of exon 3 of the *β-catenin* gene in pyloric tumors frequently identified in β-catenin accumulating regions. Mutation frequency of β-catenin accumulating regions in WT and Tg mice with MNU alone were 18.2 and 21.4%. The corresponding figures in the mice with *Hp*+MNU treatment were 31.6 and 62.5%, respectively. Mutations were frequently observed in Tg mice with *Hp*+MNU treatment compared with Tg mice with MNU alone ($P<0.05$). In contrast, no mutations were detected in surrounding normal mucosa.

These results may suggest that Wnt/β-catenin signal pathway play important roles in mouse gasrtric carcinogenesis and *Hp* infection and COX-2/mPGES-1 expression affect the activation and proliferation of gastric cancer cells harboring mutations of exon 3 of the *β-catenin* gene, giving rise to promotion of gastric carconigenesis.

P-11

Influences of Pitavastatin on Stomach Carcinogenesis and Serum Lipid Profile in *Helicobacter pylori*-Infected Rodent Models

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Statins, HMG-CoA reductase inhibitors, are commonly-used drugs for the treatment of hypercholesterolemia, with beneficial effects on cardiovascular disease. Recent studies have shown multi-functionality of statins, and epidemiological research has also suggested chemopreventive effects for various types of cancers, including colorectal tumors. However, the true importance is still controversial, and no large epidemiological research into inhibitory effects of statin on gastric carcinogenesis has so far been conducted. Here we examined effect of pitavastatin, one of lipophilic statins, on *Helicobacter pylori* (*H. pylori*)-associated gastric cancer. Six-week-old Mongolian gerbils or mice were inoculated with *H. pylori*, fed diet containing pitavastatin (1, 3, or 10 ppm) and sacrificed at week 12 or 52. Gerbils were administered *N*-methyl-*N*-nitrosourea for long-term experiment. There was no significant difference of the incidence of gastric cancers between pitavastatin-treated and non-treated gerbils. In the former, serum total-cholesterol (T-Chol), triglyceride (TG) and low-density lipoprotein (LDL) levels were significantly increased in dose-dependent manner. In the pyloric mucosa, mRNA expressions of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were significantly up-regulated in pitavastatin-treated gerbils. In the short-term study, *H. pylori*-infected gerbils and mice also showed up-regulation of TG with pitavastatin treatment. These findings indicate pitavastatin to be ineffective for suppressing gastritis and chemoprevention of gastric cancer in *H. pylori*-infected Mongolian gerbils. Our results also suggest that pitavastatin failed to lower the serum lipid levels in rodent models, which might be related to *H. pylori* infection and consequent severe gastric inflammation.

P-12

Ephrin-A1 Promotes the Malignant Progression of Intestinal Tumors in *Apc*$^{min/+}$ Mice.

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The ephrin-A1 and EphA receptors are frequently highly expressed in different human cancers, suggesting that they may promote tumor development and progression. We generated transgenic mice carrying *Fabpl*$^{4xat\text{-}132}$ ephrin-A1, which express ephrin-A1 in the intestinal epithelial cells. Those mice were then mated with *Apc*$^{min/+}$ mice to produce the compound mice, which overexpress ephrin-A1 in the intestinal tumors of *Apc*$^{min/+}$ mice. We compared the number, size and histopathological features of the intestinal tumors in the *Fabpl*$^{4xat\text{-}132}$ ephrin-A1/ *Apc*$^{min/+}$ compound mice with those of the *Apc*$^{min/+}$ mice. The compound mice showed an increased number of intestinal tumors, significantly in the large intestine, and developed more invasive tumors. Among the 20 mice of each type examined, 5 *Apc*$^{min/+}$ mice developed 5 invasive tumors, 1 invasive tumor in each mouse, in the proximal or middle portions of the small intestine. On the other hand, 14 out of 20 compound mice developed 29 invasive tumors and 16 of them were in the distal small intestine and the large intestine, where transgenic ephrin-A1 was highly expressed. These results suggested that the increased expression of ephrin-A1 accelerated the malignant progression of the intestinal adenoma to invasive tumors.

P-13

Mechanistic study on hepatocarcinogenesis of piperonyl butoxide in mice

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Our previous studies demonstrated that several reactive oxygen species (ROS)-related genes were significantly up-regulated in the liver of male mice given 0.6% piperonyl butoxide (PBO) for 6 weeks after DEN-initiation. In order to clarify the possible mechanism of non-genotoxic hepatocarcinogenesis induced by PBO, male ICR mice were subjected to a two-third partial hepatectomy. Twenty four hours later, the mice received an i.p. injection of N-diethylnitrosamine (DEN) to initiate hepatocarcinogenesis. One week later, they received 0.6 or 0 % PBO-containing diet for 25 weeks. After sacrifice at the termination of this experiment, the livers were histopathologically examined and subjected to gene expression analyses using cDNA microarray and real-time RT-PCR. The incidences of preneoplastic foci, hepatocellular adenomas and hepatocellular carcinomas were significantly increased in mice given DEN + PBO. The formation of microsomal ROS in the liver was increased in the DEN + PBO group in comparison with the DEN-alone group. In the real-time RT-PCR, Cyp2a5, Por, Nqo-1 and Cyclin D1 were increased in the DEN + PBO group as compared with the DEN-alone group. Moreover, mRNA levels of c-jun, c-myc and ATF3 were significantly increased in the DEN + PBO group as compared with the DEN-alone group. Positive immunohistochemical staining for ATF3 was diffusely observed in non-proliferating hepatocytes of the DEN + PBO group, but a lot of altered foci, hepatocellular adenomas and carcinomas were negatively or weakly positive for ATF3. Thus, the results of the present study demonstrate that PBO can induce hepatocellular adenomas and carcinomas by PBO treatment for 25 weeks after DEN initiation, and suggest the possibility that the decreased expression of ATF3 in these proliferative lesions probably results in dysregulation of the cell-cycle during the hepatocarcinogenesis.

P-14

Possible participation of oxidative stress and consequent *in vivo* mutagenicity in tumorigenesis of non-genotoxic carcinogen, piperonyl butoxide

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Piperonyl butoxide (PBO) has been shown to induce hepatocellular tumors in rodents with higher incidences in spite of the classification as a non-genotoxic carcinogen. In this study, together with the role of p53, we investigated possible participation of oxidative DNA damage resulted from the induction of P450 isozymes in *in vivo* mutagenicity using *p53*-deficient *gpt* delta mice. Male *p53*-proficient and -deficient *gpt* delta mice were treated with PBO at a concentration of 6000 ppm, a reported carcinogenic dose, in the diet for 13 weeks. In an additional group, phenobarbital (PB), a potent tumor promoter as well as CYP inducer in the rodent liver, was fed at a concentration of 500 ppm in the diet. Severe suppression of body weight was observed in PBO treated mice, the relative liver weights being increased with statistically significance in both genotypes. Histopathologically, centrilobular hepatocytes hypertrophy was observed in all treated groups, and the lesion was accompanied with ground glass appearance in PB treated mice without any inter-genetic differences. 8-hydroxydeoxguanosine (8-OHdG) levels in liver DNA of PBO treated *p53*-proficient mice had a tendency to increase, but there were no changes in the *p53*-deficient mice. PB did not affect 8-OHdG levels in both genotypes. There were no statistically significant increments in *gpt* and Spi$^-$ mutation frequencies among the treated animals independently of p53. Those data are in line with the fact that CAR-mediated cell proliferation might be the major contributing factor to PB carcinogenesis. On the other hand, it is highly probable that PBO has no potential for damaging genomic DNA in the target site, which seems not to support the high incidence carcinogenicity. However, remarkable decrease of body weights in PBO treated mice allows us to speculate that hepatotoxicity induced by PBO exposure for longer period of time might participate in its hepatocarcinogenesis.

P-15

The Effects of Co-treatment with Tocotrienol on *in vivo* Mutagenicity and Preneoplastic Lesion Development in The Livers of F344 *gpt* delta rats Exposed to Diethylnitrosamine

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Tocotrienol (TTE) is an antioxidant agent like tocopherols and has been reported to show anticancer properties. In the present study, to clarify the chemopreventive effects of TTE against diethylnitrosamine (DEN)-induced *in vivo* mutagenicity and preneoplastic lesions in the livers, 7-week-old male F344 *gpt* delta rats were given TTE at a dose of 1% in the diet and intraperitoneally injected with 20 mg/kg body weight DEN once a week for 13 weeks. The other groups were fed only basal diet with or without DEN treatment during the experimental periods. At necropsy, the livers were removed for measurement of 8-hydroxydeoxyguanosine (8-OHdG) levels in liver DNA, *in vivo* mutation assay and quantitative analyses on immunohistochemical staining for GST-P and PCNA. Significant increases in 8-OHdG levels were observed in DEN treatment group, but not affected by TTE-treatment. *gpt* mutant frequencies were significantly increased in both DEN- and TTE-treated rats, but there were no significant inter-group differences. On the other hand, significant increases in number and area of GST-P positive lesions and labeling index for PCNA-positive cells were found in DEN treatment group, which were significantly enhanced by TTE treatment. In conclusion, TTE did not exert effectively preventive action against DEN-induced oxidative stress and *in vivo* mutagenicity. Alternatively, TTE did enhance liver preneoplastic lesion development induced by DEN.

P-16

Subchronic Toxicity study on synthesized planar catechin and its possible prevention against 2-nitropropane-induced liver injuries of rats

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We synthesized planar catechin (P-Cat), in which the catechol and chroman structure in (+)-catechin (Cat) are constrained to be planar. In the present study, in order to apply P-Cat to in vivo system, 28-day subchronic study using male F344 rats was performed, in which they were given P-Cat at doses of 0, 0.03, 0.1, 0.3 and 1.0% in the diet for 4 weeks. As a result, significant decreases of body and liver weights, and serum total protein, albumin and glucose levels were observed at the highest dose. In addition, a slight atrophy of hepatocytes occurred at the same dose. Therefore, we concluded that the second highest dose, 0.3% was a no-adverse-effect level. Based on the result, we examined whether P-Cat at the safety dose exerts preventive effects on 2-nitropropane (2-NP)-induced hepatotoxicity and oxidative stress in the liver DNA. Injections of 2-NP at a dose of 90 mg/kg three times per week for two weeks induced significant decreases of total cholesterol level and increases of ALT and AST levels without statistical significance. Co-treatment with P-Cat decreased slightly the raises of ALT and AST, albeit without statistical significance. Also, 2-NP caused significantly elevation of 8-OHdG levels in liver DNA, simultaneous administration of P-Cat failing to prevent the increment. Since P-Cat had certain toxicological effects at 1.0%, higher doses of P-Cat than in the present study are not applied. Therefore, in the further study, we make an attempt to synthesize P-Cat analogues showing less toxicity and/or lipophilic property.

P-17

Acetaminophen-induced hepatotoxicity in Connexin32 dominant negative transgenic rat.

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Connexin are components of gap junction channels, which allow direct transfer of ions, secondary messenger molecules, and other metabolites between contacting cells. Cx32 is a major gap junction protein in the liver and it is known that Cx32 expression is gradually decreased as chronic liver disease progression. On the other hand, acetaminophen (APAP) is a commonly used antipyretic and analgesic agent and acute overdoses and sometimes a normal dose of APAP can cause potentially fatal liver damage.

In the present study, to examine whether inhibition of GJIC effects APAP-induced hepatotoxicity, 10 weeks-old Cx32 dominant negative transgenic rats (Cx32ΔTg) and littermate wild rats were given a single i.g. injection of 250, 500, 1000 mg/kg APAP or 0.5% methylcellulose + 0.1% Tween80, and hepatotoxicity was compared between Cx32ΔTg and wild rats at 24 hours after dosing. As results, centrilobular cell damage and elevated serum AST, ALT levels were observed at all dose of APAP in wild rats, whereas Cx32ΔTg rats were less sensitive to APAP-induced these changes compare to wild rats. The injured liver cells by APAP were TUNEL positive and had induction of cleaved caspase-3 and Cx43 expressions.

These results suggest that apoptosis is a part of the mechanism of acute liver cell death induced by APAP, and induced Cx43 expression may play important roles for that.

Resistance to APAP-induced liver injury as a result of GJIC inhibition can be related to blocking apoptotic signal transmission through gap junction.

P-18

Expression and Localization of Heat Shock Protein 25 on Drug-induced Liver Injury in Rat

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Heat shock protein 25 (HSP25) was pathologically investigated in liver injury models in rats to elucidate the relationship between the induction of HSP 25 and macrophage. Carbon tetrachloride (CCl_4) or thioacetamide (TAA) was administered at a dose of 2 mL/kg or 300 mg/kg in male Crl:CD(SD) rats aged 6 weeks, respectively. Rats were sacrificed on 1, 2, 3, 5, 7 and 10 days after a single administration, and were examined by blood chemistry. Liver was removed, and examined by real time RT-PCR used a primer of Hsbp1 (HSP25), histopathology and immunohistochemistry for HSP25, ED1 and ED2 antibody. Blood chemistry revealed increased level of ALT, AST and total bilirubin, lactate dehydrogenase and GGT within Day 3 in both treated groups, and the changes recovered on Day 5. Histopathology and immunohistochemistry revealed degeneration/necrosis of centrilobular hepatocytes in both treated groups and HSP25-positive hepatocytes were in accord with the degeneration/necrosis only in CCl_4 group on Day 1. On Day 2, degeneration/necrosis of hepatocyte and infiltration of ED1-positive cells were observed in centrilobular area and necrosis/degeneration hepatocytes were replaced by ED1 -positive cells in the centrilobular area in both treated groups on Day 3. The hepatocytes around the centrilobular area displayed HSP25 positive in TAA group on Day 3. The pattern of HSP25 was immunohistochemically similar in CCl_4 group to in TAA group, but it was intermittent. ED1-positive cells gradually decreased in centrilobular zone on Day 5 thereafter and the number of HSP 25 positive hepatocytes also gradually decreased around the area of ED1-positive cells infiltration in both treated groups. Then there were no findings in both treated groups on Day 10. Real time RT-PCR showed that expression of Hsbp1 was markedly increased in CCl_4 group on Day 1, and the increase level was less than Day 1 but continued by Day 2. On the other hand it was increased on Day 2 in TAA group, and was gradually declined after Day 3. The appearance of the level and the change of the pattern corresponded with the results of immunohistochemistry in CCl_4 group. The expression of HSP25 was induced by only CCl_4 on Day 1 in the present study. The difference of the expression of HSP25 on Day 1 may reflect the toxicity by the formation of their metabolites resulting from CCl_4 and TAA because of free radicals from CCl_4, and electrophiles from TAA. The difference on Day 3 suggests the effect of some kind of cytokines and the level from ED1-positive cells or the number of ED1-positive cells.

P-19

Development of experimental nonalcoholic steatohepatitis (NASH) model using KK-A^y mice fed a high fatty choline-deficient L-amino acid-defined diet

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We reported previously that KK-A^y mouse is more useful than db/db and AKITA mouse as a NASH model animal caused by feeding with a 4-week methionine-choline-deficient diet (MCDD) but there was no obvious fibrosis, an important finding of NASH. On the other hand a considerable body weight decrease was recorded, concluding a more long-term the MCDD treatment is too severe for animals. Therefore, in the present study we tried to develop a NASH model using KK-A^y mouse fed a choline-deficient L-amino acid-defined diet, causing steatosis and fibrosis without body weight loss in rat as reported.

Male KK-A^y mice (4-week-old) were purchased from CLEA Japan, Inc. and were used at 7-week-old in the study. Mice were given a high fatty choline-deficient L-amino acid-defined diet (CDAAD [HF], 60%cal fat) for 2, 4 or 6 weeks following a 2-week high fatty diet treatment, and liver pathology was evaluated.

Macrovesicular steatosis and inflammation were observed after the CDAAD (HF) treatment, and these changes after the 6-week CDAAD (HF) treatment were more intense than the 2- and 4-week CDAAD (HF) treatment. Fibrosis was observed after the 4- and 6-week CDAAD (HF) treatment, and was more obvious after the 6-week CDAAD (HF) treatment.

NASH shows macrovesicular steatosis, inflammation, hepatocellular ballooning and fibrosis as major findings. In this study, macrovesicular steatosis, inflammation and fibrosis were observed after feeding with a CDAAD (HF). From these results, we considered that KK-A^y mouse fed a CDAAD (HF) became a useful NASH model.

P-20

Quantitative Assessment of Adipophilin Immunohistochemistry in Rodent Model of Steatohepatitis.

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During the development of small molecule therapeutic candidates, as well as investigative studies involving animal models of steatosis, intracellular lipid accumulation is commonly encountered in the liver. Standard histochemical lipid detection techniques require frozen tissue which is seldom obtained prospectively in GLP toxicology studies. A stain that can be used to define hepatic steatosis on formalin-fixed paraffin-embedded samples (FFPE) would be quite useful. Immunohistochemical staining for adipophilin is an infrequently reported method to define lipid vacuolation in tissues and immunopositivity can be used to quantify lipid accumulation in standard histologic sections.

In this study, we quantified the area of adipophilin immunopositivity in liver sections obtained from a rat model of diet-induced steatohepatitis and compared this to the total liver area. Adipophilin staining defined by diaminobenzidine was color deconvolved from hematoxylin counter-stained sections, and the area of adipophilin staining measured on a color score from 0 to 255. A threshold was chosen for identifying adipophilin positive areas and the same threshold was applied across whole tissue sections for each slide examined. The percent area was calculated for adipophilin positive tissue. The percent area of adipophilin positive tissue in liver sections ranged from 3 to 67 percent, and had high correlation with manual scoring of the slides.

These findings confirm the usefulness of adipophilin IHC for detecting lipid accumulation in FFPE liver sections. Using computer aided image analysis, adipophilin staining can be quantified in individual specimens, and this process is amenable to full automation. We recommend that this approach be considered when more definitive measurement of lipid accumulation is required in investigative and GLP toxicology studies in liver and other tissues.

P-21

Effect of Unilateral Nephrectomy or High-Fat And High-Sugar Diet on Glomerular Lesion in Diabetic WBN/Kob Rats.

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To investigate the effects of unilateral nephrectomy and feeding of high-fat or high-sugar diet on diabetic nephropathy, alloxan induced diabetic WBN/Kob rats were subjected to various examinations.

Materials and Methods: 10-week-old male WBN/Kob rats were dosed 50 mg/kg of alloxan (AL) once intravenously. Rats were allocated to each group fed normal diets after AL treatment (AL), fed high-fat and high-sugar diets after AL treatment (AL+HF group), fed normal diets after unilateral (AL+NX group), non-treated group (control), unilateral nephrectomized (NX group) fed with normal diet. Results: Blood and urinary glucose levels in all AL-treated groups were higher than control and NX groups. Control and NX rats showed high glucose levels in blood and urine just before the end point. Blood pressure was increased with age in all groups except AL+NX group, but not significantly different from others. Urinary albumin and protein increased sharply in AL+NX and NX group from 33 weeks. Triglyceride increased from 10 weeks after AL-dosing in AL+ HF group. Kidney weight was heavier in AL+NX and NX group. Glomerular size increased with mild diffuse increase in mesangial matrix and thickening of glomerular basement membrane, those were slightly high in AL+NX group. Furthermore hypertrophy and vacuolation of podocytes, fibrin cap, deposition of hyaline droplets in podocytes were most severe in NX group. Accumulation of glycogen in distal tubular epithelium was detected in all AL-treated groups. Hyaline droplets and hyaline casts in proximal tubules were often observed in AL+NX and NX group.

Discussion: Induction of mild diabetic nephropathy was confirmed in AL-induced diabetic rats under unilateral nephrectomy. It is evident that unilateral nephrectomy in addition to hyperglycemia deteriorated the glomerular lesion but high-fat diets show no apparent effects on glomerular changes in diabetic WBN/Kob rats.

P-22

Immunohistochemical study of crescentic glomerulinephritis in SCG/kj mice.

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Spontaneous crescentic glomerulonephritis (SCG)/kinjoh mice is the animal model developed rapidly progressive glomerulonephritis (RPGN)-like symptoms spontaneously. It is the characteristic to be observed crescentic glomerulonephritis and systemic small vessel vasculitis, attributed to the presence of aniti-neutrophil cytoplasmic antibodies(ANCA). In this study, we examined the histopathological change of the different stage of crescentic glomerulonephritis in SCG/kj mice with immunohistochemical staining.

【Method】We compared the histopathological change of slight SCG with the one of severe SCG. These grades were grouped by urinalysis and histopathological findings as follows: slight ; proteinuiria 1+, hematuria -, crescent formation <50%, and severe ; proteinuria 3+, hematuria ≧ 2+, crescent formation ≧50%.

To observe angioarchitecture in renal glomerulus, we analyzed kidney under a confocal microscope and fluorescence microscope after a single administration of FITC-conjugated tomato-lectin. Frozen or paraffin sections were stained with hematoxylin-eosin(H&E), periodic acid Shiff(PAS), colloid iron, congo red, and immunostained for vimentin, α-smooth muscle actin(α-SMA), neural glial cell 2(NG2), desmin, Masson-trichrome.

【 Result and discussion 】 In angiography using FITC-conjugated tomato-lectin, the constriction of capillary of renal glomerulus was observed in severe cases as vasucular disorder.

In immunohistochemical examination, a few NG2-positive cells were barely observed only around capillary in normal and slight SCG. On the other hand, in severe SCG, a lot of those cells were clearly observed on fibroblasts forming crescent. NG2 is a kind of the chondroitin sulfate proteoglycan. That is generally found on the surface of oligodendrocyte precursor cells, chondroblasts, proliferating capillary endothelial cells and so on. In this study, because a lot of NG2-positive cells were obviously found on the fibroblasts forming crescent, it was suggested to be a one of the marker for renal glomerulus disorder such as crescentic glomerulonephritis.

P-23

Nephrotic Syndrome induced by Dibasic Sodium Phosphate Injections for 28 days in Rats

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Nephrotic syndrome was successfully induced in Sprague-Dawley rats received once daily tail-vein injections of 360 mM dibasic sodium phosphate solution at 8 mL/kg for 28 days. Clinical examination revealed persistent proteinuria from 3 days after the first dosing and thereafter severe proteinuria from 8 days or later in the phosphate-treated groups. Proteinuria developed without remission even after 14-day withdrawal in the 14-day dosed group. Phosphate-treated animals developed lipemia, hypercholesterolemia, anemia, higher serum fibrinogen levels and lower serum albumin/globulin ratios on day 29. Renal weight increased significantly compared with control animals, and the kidneys appeared pale and enlarged with a rough surface. Histopathologically, glomerular changes consisted of mineralization in whole glomeruli, glomerular capillary dilatation, partial adhesion of glomerular tufts to Bowman's capsule and mesangiolysis. Ultrastructural lesions such as an increased number of microvilli, effacement of foot processes and thickening of glomerular basement membrane, and immunocytochemical changes in podocytes, mainly decreased podoplanin-positive cells and increased desmin expression, were also conspicuous in the phosphate-treated rats for 28 days. Marked tubulointerstitial lesions were tubular regeneration and dilatation, protein casts, mineralization in the basement membrane, focal interstitial inflammation and fibrosis in the cortex. These clinical and morphological changes were similar to features of the human nephrotic syndrome.

P-24

Modification of Renal Lesions in Hereditary Nephrotic Mice Treated with Nivalenol for 4 weeks

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Nivalenol (NIV) is a trichothecene mycotoxin produced by *Fusarium* fungi, which is known as a contaminant in wheat, rye and other cereals. It has been reported that short-term NIV treatment could induce slight mesangial expansion and IgA deposition in renal glomeruli and increase serum IgA concentration in mature mice, suggesting a possibility that NIV contamination might induce IgA nephropathy in human. However, the risk of NIV to high-risk population including children, especially ones with renal dysfunction, remains undetermined yet. Therefore, the present study was performed to investigate the modifying effect of NIV on renal toxicity in infant ICGN (ICR-derived glomerulonephritis) mice, a hereditary nephrotic model.

Male 3-week-old ICGN mice were treated with 0 (control), 6, 12 and 24 ppm NIV for 4 weeks. Final body and renal weights, levels of proteinurea and data of serum biochemistry except serum IgA did not differ between control and treated groups. Concentration of serum IgA was slightly increased in the NIV-treated groups, while localization and level of IgA deposition in the renal glomeruli were not different between groups. Moreover, histopathologically, the number of glomeruli affected with focal or diffuse mesangial expansion increased in the NIV groups. The number of glomeruli with α-smooth muscle actin (a marker of mesangial cell activation)-positive mesangial cells was also increased in 24 ppm NIV group.

These findings suggest that NIV might worsen glomerular alterations observed in infant ICGN mice.

P-25

Morphological characteristics of normal cycling ovary in rats and their viewpoints for ovarian toxicity detection

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Detection of ovarian toxicities is very important for safety assessment of chemicals including drugs. The detection, however, is very hard without underlying normal ovarian morphology based

on reproductive physiology. This study was focused on practical analysis of ovarian morphology in each estrous cycle stage and guides of morphological viewpoints to detect the toxicities. Single ovary sections transversely dissected in maximum area in 143 rats with normal cycling were examined microscopically. The classification of follicles was referred to Pedersen and Peters (1968). The growing follicles are functionally divided into follicular stimulating hormone (FSH)-independent and dependent ones. The former small and medium follicles relevant to primordial/primary and preantral ones, respectively, were constantly distributed in all sections and easy to detect with immunohistochemical staining for proliferating cell nuclear antigen (PCNA). The latter large follicles relevant to antral or Graafian ones, and atresia showed synchronous histological changes depending on estrous cycle stage. Regarding to corpora lutea (CL), currently formed CL underwent remarkable changes in their appearance by each cycle, reflecting ovulation and progesterone production. Combined observation of follicle and CL alterations enhanced reliability of the analysis. These results indicate that qualified morphological analysis in the ovary enables us to classify each estrous cycle. Notification of morphological deviation in follicles and CL from normal estrous cycle is the first tier to detect ovarian toxicities. PCNA immunohistochemical staining is useful to detect follicular loss. Vaginal cytology is also informative to predict the ovarian toxicity.

P-26

Morphological Changes of the Ovary during the Estrus Cycle in GALAS Rat –Comparison with SD Rat-

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[Aim] BrlHan:WIST@Jcl(GALAS)rat (GALAS rat) is an outbred stock standardized internationally for non-clinical safety study. GALAS rat has been commonly used for toxicity study in Europe and USA, but uncommon in Japan thus far. As grow in use of GALAS rat is anticipated in Japan, data gathering about physiological and anatomical features of GALAS rat is promptly needed. For the purpose of data acquisition about GALAS rat, we conducted a comparative study on ovarian morphology during estrus cycle in GALAS rat and in Crl:CD(SD) rat (SD rat) based on the evaluating method adopted at "Collaborative work to evaluate toxicity on ovary".
[Methods] The ovary specimens obtained from one hundred and ten female SD rats (10-week-old) served as control group in several 4-week repeated toxicity studies were re-examined. The estrus cycle of each rat was determined based on uterine and vaginal histology, followed by detailed histopathological examination of ovary. Eighty eight GALAS rats (10-week-old) showing regular estrus cycle, no clinical sign and no abnormality at autopsy were also examined in the same manner.
[Results and Discussion] The number of early corpus luteum in estrus (GALAS: n=27, SD: n=28), of mature corpus luteum and cystic corpus luteum in metestrus (GALAS: n=26, SD: n=31) and of mature corpus luteum in diestrus (GALAS: n=14, SD: n=26) were not different between GALAS rats and SD rat. However, vacuolar change/necrosis in corpus luteum was more frequently observed in GALAS rat than in SD rat in all phases of estrus cycles. The severity of necrosis in corpus luteum was enhanced in proestrus, and gradually weakened. In addition, the number of large vesicular follicle in proestrus (GALAS: n=21, SD: n=25) was smaller in GALAS rat than in SD rat.
Present study shows that there are several morphological differences of ovary between GALAS rat and SD rat in physiological condition, suggesting strain-based difference should be taken into account at estimating ovarian toxicity.

P-27

Collaborative work to evaluate ovarian toxicity - effect of 2 or 4 week repeated and fertility studies of bromocriptine in rats –

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OBJECTIVE: As part of a collaborative study on toxicity related to female fertility, we performed experiments to determine the optimal administration period concerning toxic effects on ovarian morphological changes in a general toxicity study using bromocriptine, a dopamine agonist. **METHODS:** In the general toxicity study, bromocriptine was administrated subcutaneously to 6-week-old female Crl:CD(SD) rats at doses of 0, 0.08, 0.4 and 2 mg/kg for 2- or 4-week. Necropsy was performed on the day following the end of the administration period, and ovary was removed, weighed and fixed in 10% buffered formalin. After fixation, histopathological examination was performed. In the female fertility study, bromocriptine was administrated subcutaneously to 10-week-old females at the same doses for 2-week prior to mating, during the mating period and up to day 7 of gestation. Necropsy was performed on the day 14 of gestation, and the effects on fertility were investigated. **RESULTS:** In the 2-week study, increase of ovarian weights was observed at 2 mg/kg. In the 4-week study, ovarian weights were increased at 0.4 and 2 mg/kg. Although the number of corpora luteum was increased and apoptosis of luteal cells was inhibited in the 0.4 and 2 mg/kg groups of the 2- and 4-week studies by the histopathological examination of the ovaries, no effect was observed on estrous cycle. In the female fertility study, although animals in any groups mated successfully, no females in 0.4 and 2 mg/kg groups were pregnant. There were no adverse effects on the reproductive performance in the 0.08 mg/kg group. **CONCLUSION:** The histopathological changes in the ovary are considered important parameters for evaluation of drugs including ovarian damage. We conclude that a 2-week administration period is sufficient to detect the ovarian toxicity of bromocriptine in a general toxicity study.

P-28

Pathological Changes of Ovaries in Female Rats Treated with Atrazine for Two or Four Weeks

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To investigate whether it is possible for evaluating ovarian toxicity that reflects abnormal female fertility in the repeated dose toxicity study, atrazine, a potent herbicide with endocrine-disrupting activity, was administered to female Sprague-Dawley rats for two or four weeks at doses of 3, 30 or 300 mg/kg/day.

In the two- and four-week toxicity studies, small-sized ovaries and decrease in ovarian weights were observed in the 300 mg/kg group. In the estrous cycle observation, prolongation of diestrus was observed in the 300 mg/kg group of the two-week toxicity study and in the 30 and 300 mg/kg groups of the four-week toxicity study. In the ovarian histopathological examination, loss of currently formed corpus luteum, decrease of previously formed corpus luteum, increase of large-sized atresia and hypertrophy of previously formed luteal cell were observed in the 300 mg/kg group of the two-week toxicity study. And increase of large-sized atresia and hypertrophy of previously formed luteal cell were observed in the 300 mg/kg group of the four-week toxicity study.

In the female fertility study of atrazine, copulation failure caused by prolongation of diestrus was observed in the 100 mg/kg group.

These results indicate that the effect of atrazine on female fertility, which could be due to the anovulatory effect, can be assessed by histopathological examination of ovaries in a two-week repeated dose toxicity study.

P-29

Histopathological changes in rat ovary of short term repeated oral administration of Di (2-ethylhexyl) adipate

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As part of collaborative work on evaluation of ovarian toxicity in Japan Pharmaceutical Manufacturers Association, the present study was designed to confirm whether or not the ovarian toxicity of di(2-ethylhexyl)adipate (DEHA), which is known to have effects on female fertility, could be evaluated by the new method of histopathological examination of the ovaries in repeated dose toxicity. DEHA was orally administered to Crl:CD(SD) female rats at the doses of 0, 200, 1000 and 2000 mg/kg for 2 or 4 weeks in repeated dose toxicity study. Estrous cycles were determined everyday by vaginal smear for 2 weeks before the necropsy. Ovaries were transversally halved at dissection to observe maximum area of the ovary, embedded in paraffin, and stained with hematoxylin-eosin (HE) and anti-Proliferation Cell Nuclear Antigen (PCNA) antibody for immunohistochemistry. In the ovary, increase in atresia of large follicle, decrease in currently formed corpus luteum and follicular cyst were detected as DEHA treatment related changes, and the incidence and severity of these changes increased or intensified with time in the 4 weeks study. Small and medium follicles were normal, suggesting that there was no influence on the maturation from primordial follicle to large follicle. Pre-ovulation/Graafian follicles were observed in some females treated with DEHA that were judged estrus at estrous cycle observation, suggesting that DEHA disturbed ovulation, to trigger the onset of follicular cyst and a marked decrease in currently formed corpus luteum. In the estrous cycle observations, some females with abnormal estrous cycle and continuation of estrus were observed. In conclusion, a 2-week administration period is sufficient for detection of the ovarian toxicities following treatment with DEHA by new histopathological examination of the ovaries. Furthermore, performing the estrous cycle observation in the repeated dose toxicity study made it possible to evaluate ovarian toxicities taking into account estrus cyclicity.

P-30

Ovarian toxicity of a novel PPAR α/γ dual agonist in rats

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As part of a collaborative study on toxicity related to female fertility, we performed experiments using a novel PPAR α/γ dual agonist. The test compound, a PPAR α/γ dual agonist, was administered to female rats by oral gavage at dose levels of 0, 4, 20, and 100 mg/kg/day for 2 and 4 weeks. After exsanguination, the ovaries, uterus and vagina were weighed and fixed in 10% neutral buffered formalin. All of the organs were embedded in paraffin and stained with hematoxylin and eosin (HE). Consecutive sections of the ovary were subjected to immunohistochemical identification of proliferation cell nuclear antigen (PCNA) antibody and stained for apoptosis using the in situ apoptosis detection kit.

In 2-weeks and 4-weeks studies, the estrous cyclicity showed normal cycling, but decreased weight of ovary was observed in the dose dependent manner. On histopathological study of ovary, increased number of atretic large follicles with shrunken granulosa cells and apoptotic bodies was observed. In addition, decreased number of newly formed corpora lutea and increased number of stromal cells were observed. However, unlike large follicle, the small- and medium-follicles were not affected. On the other hand, exfoliation of granulosa cells into the antrum was observed in large follicles, especially Graafian sized ones, in a few treated animals. In some follicles with exfoliation of granulosa cells, new blood vessels were detected into cell layer. These exfoliated granulosa cells stained positively with anti-PCNA antibody in immunohistochemical staining, and they were negative for apoptosis. This was a characteristic histopathological change in the treated groups and only seen in animals at estrus. Furthermore, there were also several corpora lutea with retained oocyte in treated animals. Therefore, it was concluded that these unique findings indicate that the follicles with exfoliated granulosa cells were not becoming atretic, but were the cause of formation of unruptured corpora lutea. The ovarian histopathology suggested that a novel PPAR α/γ dual agonist acts mainly on granulosa cells of large follicles to induce atresia and unruptured corpora lutea.

P-31

Collaborative Work on Evaluation of Ovarian Toxicity. Effects of 2- or 4- Week Repeated Dose Toxicity and Fertility Studies with Tamoxifen in Female Rats.

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To assess whether ovarian histopathological examination in the repeated dose rodent toxicity study could reliably anticipate toxic effects on female reproductive function and to assess whether the ovarian change could be detected in a 2-week repeated dose toxicity study, tamoxifen was administrated orally to female rats at 0.005, 0.03, or 0.2 mg/kg/day for 2 and 4 weeks in the repeated dose toxicity studies, and for 2 weeks prior to cohabitation, during cohabitation, and through Gestation Day 7 in a female fertility study. The relationship between ovarian histopathological findings and fertility results was investigated.
Findings at 0.03 and 0.2 mg/kg/day included decreases in body weight gains associated with decreases in the food consumption, in 2- and 4-week repeated dose toxicity studies and fertility study. The ovarian histopathological findings included increases in the large atretic follicles, increases in the interstitium cells and absence of newly formed corpus lutea at 0.2 mg/kg/day in the 2-week study and at 0.03 and 0.2 mg/kg/day in the 4-week study. The treatment induced estrogenic and antiestrogenic reactions in the uterus, while mucinous degeneration was detected in the vagina. The effects on female fertility consisted primarily of disturbance of estrus cycle and decreases in numbers of pregnant rats which were considered to be related the ovarian histopathological changes.
Based on these findings, the ovarian histopathological evaluation in the repeated dose toxicity study could anticipate the effects of tamoxifen on female fertility via ovarian dysfunction at slightly toxic doses, and 2-week treatment of tamoxifen at appropriate dose could be sufficient to detect ovarian toxicity by microscopic examination.

P-32

Histopathological Examination of The Rat Ovary Treated with Di-(2-Ethylhexyl) Phthalate

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The effects of di-(2-ethylhexyl) phthalate (DEHP) on the ovaries were examined as a part of the collaborative project for the detection of drug-induced ovarian toxicity by the Japan Pharmaceutical Manufacturers Association. We report the histopathological findings of DEHP induced ovarian toxicity.

Female rats [Crl:CD(SD)] were orally administered daily with 0, 300, 1000 or 3000 mg/kg of DEHP for 2 or 4 weeks (n=10). The estrous cycle and pathological findings were examined.

In our study, at the estrous cycle, increased irregular estrous cycles and prolongation of mean estrus cycles were observed in the 3000 mg/kg group of the 4-week study. At necropsy, no change was observed in the ovaries. Ovary weight was decreased in the 3000 mg/kg group of the 4-week study. Histopathological examination of the ovaries revealed vacuolation of stromal cells in all treated groups. Increase of large atretic follicles was observed in the groups of 1000 mg/kg and higher. Furthermore, currently formed corpora lutea were decreased in the 3000 mg/kg group of the 4-week study. The changes seen in the follicles and corpora lutea were considered to be related to suppression of follicular growth and ovulation. In the liver, hypertrophy of hepatocytes with eosinophilic cytoplasm was observed in the groups of 300 mg/kg and higher in both the 2- and 4-week studies. In conclusion, histopathological changes of the ovaries were detected in the 2- and 4-week daily administration DEHP studies. DEHP is considered to affect the development of ovarian follicles and differentiation of granulosa cells, and also inhibits aromatase in granulosa cells. This causes reduction of estradiol and of the LH surge, resulting in ovulation disorders.

P-33

Distribution of Hormone-secreting Adenohypophysial Cells in Cynomolgus Monkeys -Immunohistochemical Analysis-

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In cynomolgus monkeys, distribution of adenohypophyseal cells, acidophil, basophil and chromophobe, is irregular. Moreover, these cells secrete different hormones respectively. Due to this irregular distribution, it is difficult to prepare uniform specimens for routine pathological examinations. As most of the drug-induced effect is specific to each hormone-secreting cell, it is important to prepare specimens that have a constant distribution of every hormone-secreting cell, and to understand the normal distribution and population of these cells. In this study, we investigated the distribution of hormone-secreting cell in the adenohypophysis of cynomolgus monkeys histologically and immunohistochemically. The entire pituitaries of 3 males and 2 females (about 3-year) were fixed in 10% neutral buffered formaline and routinely processed by embedding them in paraffin wax. Serial sagittal sections were cut and divided into 15 slides respectively (10~18 groups). In each group, sections were stained H.E and immunohistochemically using antiserum against prolactin (PRL), growth hormone(GH), thyroid-stimulating hormone(TSH), lutenizing hormone(LH) and adrenocorticotropic hormone(ACTH). In the H.E. stain, three distinct area, pale area, eosinophilic area (E. area) and peripheral area (Peri. area) appeared. The pale area surrounds the pituitary stalks and was mainly composed of chromophobe. The E area. takes the majority of pars distalis and was composed mainly of acidophil. The Peri.area contains basophil and chromophobe at various rates. Immunohistochemically, distribution of PRL cell and GH cell is consistent with the E. area. Moreover, these cells tended to cluster together and were more abundant in the males than in females. Distribution of TSH cell, LH cell and ACTH cell was inconstant, and tended to be scattered. Furthermore, two types of ACTH cell were observed. One was large but stained weakly and the other was small but stained strongly. No sex difference was seen in these cells. All cells tend to decrease toward the edge. In conclusion, the central sagittal section based on the stalk is the best to investigate all type adenohypophysial cells.

P-34

Preventive effects of Calcitriol on thyroid capsular carcinomas induced by promotion with sulfadimethoxine in a rat two-stage thyroid carcinogenesis model

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Recently we have shown activation of PI3K/Akt signaling in rat follicular cell carcinomas invading to the thyroid capsule that are highly produced by promotion with sulfadimethoxine (SDM) in a rat two-stage thyroid carcinogenesis model. To clarify the potential therapeutic target molecule for cancer invasion, we treated rats with Calcitriol (1α,25-dihydroxyvitamin D3) in this model, and analyzed the number of neoplastic lesions as well as immunolocalization of Akt/PTEN signaling molecules in association with cell proliferation activity. Experiment 1: One week after initiation with DHPN, male F344/NSIc rats were injected intraperitoneally with Calcitriol (0.1 μg/kg), three times a week, during the promotion with SDM for 15 weeks. Experiment 2: Using the same SDM-promotion model, Calcitriol (0.1 μg/kg) was injected to rats for 2 weeks before sacrifice at week 15. In Experiment 1, Calcitriol inhibited not only the incidence of capsular carcinomas but also the development of thyroid parenchymal carcinomas. Staining intensity of Phospho-PTEN, Phospho-Akt, and Phospho-Akt substrate in capsular carcinomas of Calcitrol-treated animals was similar to the vehicle controls. Also in Experiment 2, staining intensity of Akt/PTEN signaling molecules was unchanged by treatment. On the other hand, total numbers of $p27^{Kip1}$-positive cells as well as those showing nuclear immunoreactivity were significantly increased in capsular carcinomas by Calcitriol-treatment, associated with a tendency to decrease in the proliferation activity as estimated by Ki-67-immunoreactivity. Thus, while the inhibitory effect of Calcitriol on cell proliferation activity was mild during the stage of tumor invasion, the effects may be through activation of $p27^{Kip1}$ as a downstream target of PI3K/Akt signaling.

P-35

Serum Biochemistry and Histological Characteristics of Mammary Glands in Homozygous (*fa*/*fa*) and Heterozygous (+/*fa*) Zucker Rats

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Obesity is considered to be associated with the development of several serious health conditions, type 2 diabetes mellitus, and certain types of cancer including breast cancer. The etiology of increasing in breast cancer among obese women, however, has not yet been identified. Leptin is an adipocyte-secreted hormone that regulates energy homeostasis by binding to its receptor in the ventral medial nucleus of the hypothalamus, known as the "satiety center." Leptin is a serum growth factor that is positively associated with body weight or body fat storage as well as proliferation in mammary epithelium. The fatty Zucker rats with homozygous mutation of leptin receptor (*fa*/*fa*) have been reported to lead to overt obesity, hyperlipidaemia and insulin resistance. Because heterozygous mutant (+/*fa*) and wild type (+/+) Zucker rats show almost similar lean phenotypes, they have been used as negative controls of fatty Zucker rats (*fa*/*fa*). In the present study, potentials of Zucker rats with each genotype were comparatively investigated for establishment of mammary carcinogenesis model related to obesity. Seven-week-old female Zucker rats (+/+, n=8; +/*fa*, n=16; *fa*/*fa*, n=6) and 12-week-old female Zucker rats fed high-fat (10% corn oil) diet (+/+, n=6; +/*fa*, n=15) or basal diet (+/+, n=5; +/*fa*, n=14) for 5 weeks were investigated for serum biochemistry and histopathology of adipose and mammary tissues. *fa*/*fa* showed high levels of serum triglyceride, total cholesterol and insulin as compared to +/*fa* and/or +/+ rats ($p<0.05$, 0.01 or 0.001), and hypoplasia of mammary tissues along with small numbers of terminal end bud ($p<0.01$). Serum leptin levels not only in *fa*/*fa* but also +/*fa* rats were higher than +/+ rats ($p<0.001$). In addition, leptin receptors were expressed in mammary gland epithelia of each genotype. After feeding of high-fat resume for 5 weeks, serum leptin levels were increased ($p<0.05$) in +/*fa* and +/+ rats compared to respective basal diet groups and leptin mRNA expression in adipose tissue showed similar tendency. Increasing pattern of TNFα, but not of VEGF mRNA expression was shown in adipose tissue of +/*fa* rats and respective high-fat diet groups. These results suggest that +/+ fed 10 % corn oil diet or +/*fa* rats are useful for investigating the participation of leptin and leptin receptor in mammary carcinogenesis with some carcinogen or radiation treatments.

P-36

Susceptibility of Heterozygous (+/*fa*) Lean Zucker Rats to DMBA-Induced Mammary Carcinogenesis

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Susceptibility to 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis was investigated in heterozygous (+/*fa*) lean Zucker rats carrying mutated leptin receptor gene and the wild type (+/+). +/*fa* and +/+ rats at 7-week of age were given single administration of DMBA and divided into two groups each. Each one was freely accessed to basal diet containing 10% corn oil and the other was given basal diet alone. The minimum latent period of palpable tumors in both corn oil and basal diet groups of +/*fa* was 7 weeks after DMBA treatment whereas 10-11 weeks in +/+. The incidence and multiplicity of palpable tumors were higher ($p < 0.05$) in both corn oil and basal diet groups of +/*fa* than their counterparts of +/+. In our preliminary study, serum leptin levels in +/*fa* were higher ($p < 0.05$) than those in +/+, but no other serum biochemical parameters were changed. These results indicated that +/*fa* rats were more susceptible to DMBA-induced mammary carcinogenesis, and this might be at least partly associated with the higher serum leptin levels at the initiation stage.

P-37

Investigation on Local Reaction and Administration of the Local Irritation Study (repeated dose study)

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【 Introduction 】 Inactivated vaccines are inoculated repeatedly to increase the production of antibodies in clinical use. In the local irritation study of such repeated dose vaccines, local irritation including the stimulated immune response is evaluated using rabbit which received repeated administration of vaccine. The object of present study is appropriate evaluation of the local irritation induced by repeated administration of vaccine. We compared local reaction induced by repeated administration in same limb with that of repeated administration in different limb.

【Materials and Methods】Aluminum hydroxide gel was added to ovalbumin(test substance). In the first group, 6 male rabbits were injected 0.5mL/site of test substance in muscle of the left limb (1^{st} injection). After 14 days of 1^{st} injection, same test substance was injected to the right limb. In the second group, 6 male rabbits were injected 0.5mL/site of test substance in muscle of the right limb (1^{st} injection), and 2^{nd} injection were performed in the another site of the right limb. After 2 and 7 days of 2^{nd} injection, 3 rabbits each in the treated group were sacrificed under the pentobarbital sodium anesthetizing, necropsy and histopathological examination were performed.

【Result】＜The first group＞ 2 days after 2^{nd} injection, substance which was seemed to be caused by the injection of test substance was observed, and cell infiltration and degeneration/necrosis of muscle fibers also were seen. 7 days after 2^{nd} injection, severity of cell infiltration increased, and regeneration of muscle fibers were seen. ＜The second group＞ 2 days after 2^{nd} injection, substance which was seemed to be caused by the injection of test substance was observed, and cell infiltration and degeneration/necrosis of muscle fibers also were seen. 7 days after 2^{nd} injection, severity of cell infiltration increased, and regeneration of muscle fibers were seen. The kind of changes and degree of changes were similar to those of the first group.

【Discussion】As mentioned above, there were no obvious difference in comparison with the first group changes and the second group changes. Therefore, it was suggested that local reaction would not be greatly affected by the difference of injection site (same limb or different limb) in the local irritation study (repeated dose study).

P-38

Basic Investigation for 28-day Intratracheal Repeated Dose Toxicity of Physiological Saline without Anesthesia in Rats

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We have concentrated attention on accomplishing an intratracheal infusion method without anesthesia for a 4-week-repeated dose toxicity study in rats.

Groups of 10 male slc:Wistar-Hannover/RCC rats, 8 weeks of age, were treated intratracheally with saline at volumes of 0 (sham-treated), 0.1 or 0.2mL/rat for five days a week for 4 weeks, and compared with non-treated rats. Body weights, food consumption and water intake were determined weekly. At the end of experiment, total and differential cellular counts and biochemistry of the BALF and blood biochemistry and histopathology of the larynx, trachea and lungs were individually examined. During the experiment period, breathing/crepitant whistling was observed in all of the treated animals. In the histopathology, inflammation and squamous metaplasia of the larynx, and inflammation and fibrosis of the trachea due to manipulation of the infusion instrument were found in all the treated-groups, including the 0 mL group, with no significant differences due to the amount of saline infused.

We conclude that an intratracheal injection volume of 0.2mL/rat of physiological saline can be administered repeatedly without anesthesia.

P-39

Applying F344 *gpt* Delta Transgenic Rat for *in vivo* Genotoxicity Study of Structural Isomer 2,4-diaminotoluene and 2,6-diaminotoluene.

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In vivo genotoxicity study using rodent model is useful to assess mutagenicity and carcinogenicity in target organs. To evaluate sensitivity and specificity of *in vivo* mutagenicity test, it is important to perform validation studies using carcinogenic and noncarcinogenic substances. The aromatic amines 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT) are structural isomers that have been extensively studied for their mutagenic and carcinogenic characteristics. Both 2,4-DAT and 2,6-DAT are genotoxic in the Ames/*Salmonella* assay. It is reported 2,4-DAT induced liver tumors in rats, whereas 2,6-DAT was found not to be carcinogenic. In this study, to determine whether the results of *in vivo* mutagenicity test correspond to the results of rodent carcinogenicity test, we investigated the mutation frequency of liver and kidney DNA from male F344 *gpt* delta rats fed 125 ppm, 250 ppm and 500 ppm 2,4-DAT and 500 ppm 2,6-DAT for 13 weeks. The highest dose of 2,4-DAT induced body weight reduction. Hypertrophy and vacuolar degeneration of hepatocytes were observed in 2,4-DAT treatment groups, and cell proliferation was increased in 250 ppm 2,4-DAT treatment group. This transgenic rat has transgene lambda EG10 DNA with the *gpt* gene of *E. coli* used as a reporter for detection of point mutations. The mutant frequency in the liver was significantly increased in 2,4-DAT treatment group, whereas that was background level in 2,6-DAT group. In the kidney, however, it was not induced in any group. The results showed that *in vivo* mutagenicity was observed in the targeted organ of carcinogenicity. Thus, it is suggested that transgenic rodent assay with *gpt* delta rat may contribute to prediction of carcinogenicity.

P-40

Analysis of the Metastatic Mechanisms in the Lungs and Liver Using the Rat Hepatoma Cell Lines with Different Metastatic Ability

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Metastasis is one of the biggest causes of tumor death. It has been reported that cancer cells are often found in blood flow of cancer patient since early stage. Moreover, after intra-venous inoculation of $5x10^6$ cells in nude mice, only a few cells are selected then make metastatic focus. To find the mechanisms of tumor cell selection at *in vivo* delivered site, we analyzed histological changes of the lung or liver, shortly after intra-tail venous or intra-splenic inoculation of rat hepatocellular carcinoma cell lines with different metastatic ability.

[Methods] High metastatic cell lines (HMCs) or low metastatic cell lines (LMCs) were injected from either tail vein or spleen of nude mice. One hour or 1 day after cell-injection, lung, liver, spleen and kidneys were removed and histological specimens were made. By immunohistochemical staining with glutathion S-transferase placental form (GST-P) antibody, rat hepatoma cells were distinguishable. Thus, the numbers and areas of attached cell were analyzed. Apoptotic cells, positive for cleaved-caspase 3 antibody were also evaluated.

[Result] 1) One hour after injection from tail-vein, many cells ($11.3\sim15.8/mm^2$) were equally observed in the lungs of mice, inoculated either 2 kinds of HMCs or one LMCs. One day after injection, number of cells was decreased regardless metastatic potential; HMCs was seen in $0.5\sim0.6/mm^2$ and LMC was completely eliminated. One of LMC were seen only in $0.7/mm^2$ at 1 hour after injection, and eliminated at 1 day. The numbers of cleaved-caspase 3 positive cells in the lung were significantly high in LMCs-inoculated mice than HMCs-inoculated mice. Thus, it was suggested that selection of metastatic cells were related to either cell adhesion ability or escaping from apoptosis process in the lung.

2) One hour after intra-splenic inoculation, a little of LMCs ($15.2\sim23.3/mm^2$), and many HMCs ($48.7\sim77.1/mm^2$) were seen in the portal vein and sinusoidal space. At day one time point, almost none of the LMC cells, but appreciable numbers of HMCs ($13.3\sim15.0/mm^2$) were remained in the liver. Comparing with the cells in the lung of intra-tail vein inoculation, a few cells were positive for cleaved-caspase 3. Severe or focal necrotic changes with neutrophil infiltration were also observed in all mice unrelated to metastatic potential.

[Conclusion] These results suggested that most of cells reached to the metastatic site were eliminated within 24 hours, but its elimination process might differ between the liver and lung.

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Evaluation of New Protein Biomarkers in Mice Hepatocarcinogenesis

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To evaluate novel protein biomarkers and their role in mice hepatocarcinogenesis, the proteome of mouse hepatomas was investigated using microdissection in combination with mass spectrometry using iTRAQ labeling and QSTAR LC-MS/MS. To initiate hepatocarcinogenesis, 42 C57Bl/6J mice, 14-day-old, underwent intraperitoneal injection with diethylnitrosamine (DEN; 10 mg/kg b.w.) and were sacrificed at experimental weeks 27 and 38. Due to the results of mass spectrometry and ProteinPilot 2.0 Software analysis 109 proteins were identified in microdissected mouse hepatomas and expression of 67 of them was found to be altered. Ingenuity Pathway analysis showed that 31 proteins (e.g. intermediate filament member cytokeratin 18 (CK18), transcriptional regulator calreticulin (CALR) and acute phase response protein apolipoprotein A1 (APOA1)) might become potential biomarkers in mice hepatocarcinogenesis. Furthermore, significant up-regulation of CK8/18, CALR and APOA1 was immunohistochemically detected in mouse adenomas, hepatomas and preneoplastic lesions (basophilic foci) in concordance with the results of mass spectrometry. On the other hand, analysis of the proteome of mouse hepatomas have demonstrated suppression of protein expression of liver metabolizing enzymes, including the enzymes involved in urea cycle metabolism. Our results indicated that CK8/18, CALR and APOA1 might become novel biomarkers in mice hepatocarcinogenesis.

P-42

Metabolome Analysis of Serum from Rats Developing Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma is one of the most debilitating malignancies in humans, and the tumor incidence has been currently increasing. For importance of early diagnosis, a lot of studies for finding superior biomarkers in humans were carried out, and several candidates were identified. On the other hand, experiments of suitable animal model are needed along with research of human, because of complication and heterogenecity in human.

Transgenic rats in which express Ha-ras oncogene induced by the Cre/loxP system were used as the tumor model. Serum from tumor bearing rats and normal rats were deproteinized using MeOH/H_2O:8/1, freeze dried, and then transferred into pyridine. After derivatized by methyloxime and trimethylsilyl, samples were analyzed using GS-MS. Principal component analysis was conducted to survey the metabolites mainly contributed to distinguish the two groups.

The data showed clear difference between tumor bearing animals and control animals. Elevated levels of proline and hydroxyproline, which suggesting the high metastasis activity, were observed. In adition, the following changes were shown as the indicating markers of deteriorated systemic condition and impaired energy metabolism; decreased lipids and/or amino acids, altered metabolites in glycolysis and TCA cycle.

These data obtained in this study demonstrated that metabolomic profiling using blood could provide profitable information regarding tumor progression.

P-43

Using ToxWiz to explore the effects of WY14643, RU486, and Phenobarbital on nuclear receptors

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About 2 billion drug prescriptions were issued to patients during physician office visits in the U.S.A. in 2005, with the number of drugs prescribed per visit averaging 2.11. Co-administration of two or more drugs may result in complex drug interactions, increasing the risk of side-effects. For the safety of patients, it is important to consider these interactions and understand their mechanisms. In this report, ToxWiz software was used to find common molecular targets for WY14643, RU486, and Phenobarbital, and to predict toxicity mechanisms of combined exposure to these drugs.

ToxWiz is a comprehensive software and database system that provides extensive information on chemicals and their biological effects on organisms. Using previously collected data on chemicals from the public domain and proprietary company datasets, this software generates networks of interacting chemicals, genes, proteins, pathways, and biological effects. ToxWiz may be used to quickly assess or predict the toxicity of combinations of drugs, or drugs in combination with other chemicals in the environment. Complications or even fatal outcomes in patients, and resulting costs of prolonged hospitalization may be avoided with the use of ToxWiz.

Using the ToxWiz software, common targets for WY14643, RU486, and Phenobarbital were identified, including peroxisome proliferative activated receptor alpha isoform 1 (PPARA) and pregnane X receptor isoform 3 (NR1I2). The software further revealed possible mechanisms of action for each drug, and allowed the user to infer possible drug interactions at the level of these two target molecules. Wide use of combinations of drugs may lead to unexpected and unwanted side-effects in patients, which may be predicted with the application of ToxWiz.

P-44

Systems Toxicology Investigation of Rat Hepatotoxicity Elicited by Lipopolysaccharide and Ranitidine

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[Introduction] Hepatotoxicity elicited by ranitidine (RA) is augmented by pretreatment of lipopolysaccharide (LPS). In the present study, comprehensive gene expression analysis was performed on rat livers to further investigate the molecular dynamics elicited by LPS/RA co-treatment.
[Methods] Ten-week old male F344 rats were treated intravenously with either LPS (0.06 mg/kg) or saline 2 h before RA treatment (30 mg/kg, i.v.). Livers were removed 2 or 6 h after the RA treatment, and microarray analysis was performed using Affymetrix Rat 230 2.0 GeneChip array.
[Results and Discussion] A total of 209 probe sets were identified whose signal levels were synergistically elevated by LPS/RA co-treatment. Gene Ontology (GO) analysis revealed that the GO terms associated with inflammation (*Irf1*、*Cxcl10*、*Tnfrsf14*), cell death (*Bcl2l2*、*Ripk2*), apoptosis (*Gadd45b*、*Casp1*、*Casp8ap2_predicted*), cell differentiation (*Atf3*、*Map2k1*、*Adm*) and hypoxia response (*Pdgfa*) were significantly enriched in the 209 probe sets. In the saline/RA-treated group, expression levels of the 209 genes showed high at 2 h after the RA treatment, but were lowered at 6 h after the RA treatment. On the other hand, expression levels of these genes were relatively high at 6 h after the RA treatment in the LPS-pretreated group, suggesting that the continuous high-level expression of the genes associated with inflammation, apoptosis and hypoxia plays crucial roles in augmented hepatotoxicty elicited by LPS/RA co-treatment. In addition, a network analysis using ToxWiz® software indicated that CD14, TLR4, IL-10 and TNF-α may play crucial roles in the LPS/RA-elicited molecular perturbation in the rat liver. Based on these results, we performed a literature search using PubMed database by keywords of “LPS”, “Ranitidine” and “TNF”, and found couple of literatures describing a protective effect of histamine on LPS-induced hepatotoxicity in the mouse through H_2 receptor.
[Conclusion] Since RA is a H_2 receptor antagonist, the LPS-induced hepatotoxicity may be augmented by inhibition of H2 receptor-mediated cellular protection effects. The systems toxicology approach successfully gave us clues to generate a new hypothesis to extend our understanding toward LPS/RA-elicited hepatotoxicity.

P-45

Spontaneous extraskeletal osteosarcoma in an aged rat

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Spontaneously extraskeletal osteosarcoma in rat is extremely rare tumor. In this study, we found a case of telangiectatic type of extraskeletal osteosarcoma in an aged rat, and then carried out macroscopic, histopathologic, immunohistochemical, and electromicroscopic examination.

Female F344/DuCrj rat was used as control animal of a carcinogenicity study, died at 90 weeks of age.

At necropsy, the body weight of the rat was 185 g. Dark reddish mass was 60 x 25 x 35 mm, and observed in right abdominal cavity. The mass involved in no abdominal organ and adjacent bone. The cut surface of the mass was lobular pattern with various size cysts.

Histopathologically, the tumor was consisted of osteoblastic-like tumor cell, multinucleated giant cell, calcified osteoid tissue and blood lake. Immunohistochemically, the tumor cell and giant cell were positive for vimentin and osteopontin. Some of tumor cell and osteoid tissue expressed for collagen type I and osteocalcin. The tumor cell was positive for PCNA, and the giant cell for ED1. In electromicroscopic examination, the tumor cell showed irregular nuclei and dilatation of rough endoplasmic reticulum with a little of cytoplasmic organization. The giant cell had numerous small mitochondria.

In conclusion, this case was diagnosed as telangiectatic type of extraskeletal osteosarcoma characterized with proliferation of osteoblastic-like cell and formation of multinucleated giant cell, osteoid tissue and blood lake.

P-46

Emphysematous Cystitis in A Chemical Induced Diabetic Dog

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Emphysematous cystitis is a rare disorder characterized by gas accumulation within the bladder wall with cyst formation. Although some cases of emphysematous cystitis have been reported in diabetic dogs, cats and humans, few reports describe histopathological details. This report describes histopathological characteristics of emphysematous cystitis found in a chemically induced diabetic dog.

A female CSK beagle dog received a single intravenous injection containing a mixture of streptozotocin and alloxan to induce diabetes mellitus at 1 year old. The dog was sacrificed by exsanguination from the common carotid artery under deep pentobarbital anaesthesia at 8 years old. Clinically, moderate to severe hyperglycemia (150~400 mg/dL) was observed. The dog occasionally revealed heamaturia during the period of half-year before sacrifice. At necropsy, the urinary bladder was fixed in formalin and embedded in paraffin. The sections were stained with HE and gram stain. Furthermore immunohistochemistry was performed against vimentin, α-smooth muscle actin, macrophage scavenger receptor class A, CD31, and cytokeratin.

Macroscopically, the mucosa of the urinary bladder was irregular and dark red areas were observed. On the cut surface, multiple cyst-like structures about 1mm in diameter were observed in the elevated mucosa. Small pieces cut from the mucosa floated in the fixative solution. Histopathologically, multiple cysts varying in size were observed in the area from the lamina propria to the muscle layer. Most of the cysts were lined by a single layer of flattened cells, but partially was not lined by any cells. Multinucleated giant cells and neutrophils were observed around the cysts. Gram-negative short bacilli were observed sporadically in the lumen of the urinary bladder. In the mucosa, degeneration, desquamation, erosion and hyperplasia of transitional epithelium were observed. Hemorrhage and inflammatory cell infiltration of neutrophils, lymhocytes and macrophages were observed in the mucosal epithelium and lamina propria. Immunohistochemistry revealed that the flattened cells covering the cysts were positive for vimentin, partially positive for α-smooth muscle actin or macrophage scavenger receptor class A and negative for cytokeratin and CD31.

The dog had prolonged diabetes with persistent hyperglycemia, and presented chronic cystitis thought to be caused by gram-negative bacilli. The cystitis was histopathologically characterized by cystic structures lined with flattened myofibroblasts, fibroblasts and macrophages with infiltration of multinucleated giant cells around the cysts in the mucosa. From these findings, this case was diagnosed as emphysematous cystitis.

P-47

Characteristics of mast cells in canine thymomas

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In the normal thymus of humans and animals, mast cells are located mainly in the non-parenchymal connective tissue, and rarely in the parenchyma. Although the physiological roles of mast cells in the thymus remain to be clarified, perivascular distribution of mast cells implies a role in angiogenesis. The presence of mast cells in canine thymomas has been reported, however, there is no detailed description of their characters. This study focuses on the characteristics of mast cells in canine thymomas.

Tissue samples from 2 canine thymomas (7- and 9-year-old Labrador retriever) and 1 normal thymus (2-month-old Miniature Schnauzer) were fixed by 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE), toluidine blue and alcian blue-safranin.

Compared to the normal thymus, the number of mast cells in thymomas was remarkably increased, and they were arranged perivascularly. The cytoplasm of these mast cells was eosinophilic and the cytoplasmic granules were unclear. Mast cells in both the normal thymus and thymomas stained positively with safranin and negatively with alcian blue, indicating a histological phenotype of mucosal-type mast cells. These findings are almost identical to those in normal thymus and thymoma in humans.

P-48

Femoral Myxoid Liposarcoma in a Dog

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Liposarcoma is one of the most common soft tissue sarcoma in humans and various histological variants, such as myxoid, pleomorphic, spindle and dedifferentiated types have been reported. Canine lipoma is commonly encountered, however, liposarcoma is relatively uncommon, and a few myxoid liposarcomas have been documented. We report pathological findings of the canine myxoid liposarcoma occurred in the femur.

A 9-year-old male Labrador retriever dog developed a mass, approximately measuring 5 x 4 cm, in the left femur. MRI examination revealed multiple cysts in the lesion. The mass was removed and subjected to the histopathologic examination.

Macrocopically, the mass was composed of variously-sized cysts containing a lot of mucin. The cysts separated by fibroconnective tissues were found and spindle to ovoid neoplastic cells were distributed sparsely in the cysts. The neoplastic cells have cytoplasmic vacuoles, which were positive for Oil red O and negative for alcian blue stains. The stroma was myxomatous and stained positively with alcian blue stain. Immuhohistochemistry using anti-adipophilin revealed many granular positive reactions in the neoplastic cytoplasm. Invasive growth of the tumor was found in the adipose tissue and the joint cavity of the affected femur.

Based on these macroscopic and pathological findings, we diagnosed this case as myxoid liposarcoma and malignant behavior was suggested. Immunohistochemistry using anti-adipophilin is considered to be useful for the differential diagnosis.

P-49

Spontaneous Arteritis in Cynomolgus Monkeys (*Macaca fascicularis*)

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Summary
In toxicity studies in cynomolgus monkeys, the incidence of spontaneous arteritis is low and only 2 cases of polyarteritis in cynomolgus monkeys have been reported. In this report, we introduce a case of a high incidence of spontaneous arteritis observed in a toxicity study in cynomolgus monkeys.

In a 13-week intravenous toxicity study in cynomolgus monkeys (*Macaca fascicularis*) with a 8-week recovery period using 19 males and 18 females (2–6 years old), 5 males and 4 females showed arteritis. These arteritides were observed randomly in each group including the control group and were considered to be spontaneous lesions and not treatment-related, because of the lack of dose-dependency. There were no abnormalities as clinical signs or in the gross pathology. The lesions were noted, variously, in the kidney, liver, gallbladder, urinary bladder, gastrointestinal tract (all segments except rectum), pancreas, testis, heart (intra- and extramural), and sciatic perineural connective tissue. Small to medium sized arteries were affected mostly singly or as multifocal lesions. The lesions were segmental and of variable severity and/or stage of development. The least developed lesions were characterized by an intimal proliferation, a fibrinoid appearance mainly in the tunica media and proliferation of the intimal and advential cells, sometimes accompanied by mononuclear cell infiltration in the adventitia. The more developed lesions were characterized by degeneration and/or necrosis of the tunica media along with proliferation of the intimal and adventitial connective tissue, sometimes with disruption of the internal elastic lamina. These findings resemble those of *polyarteritis nodosa* (PAN); however, inflammatory infiltrates were not generally prominent, regardless of the stage of development, and the appearance was not quite the same as in PAN as described in the literature for cynomolgus monkeys. Additionally, some characters that the lesions are specific in the arteries, the finding of necrosis in the tunica media and the lack of findings in the meningeal vessels were not typical for perivasculitis/vasculitis which is usually seen in cynomolgus monkeys and a lymphocyte infiltration is characteristic.

This case is considered to be useful as historical control data for toxicity studies in cynomolgus monkeys.

P-50

Focal Hyperplastic Lesion in the Liver in Cynomolgus Monkeys

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Although hepatic hyperplastic lesions are rare in cynomolgus monkeys, we report here on two cases observed in female cynomolgus monkeys (*Macaca fascicularis*, purpose bred).

(Materials and Methods) Case 1 was three years old, case 2 was four years old, and both were born in China. Their livers were fixed in 10% natural buffered formalin, routinely processed for paraffin section, and stained with hematoxylin and eosin (H.E.), periodic acid-Schiff stain (PAS), Masson's trichrome stain (MT), silver impregnation, and immunohistochemically with mouse anti-PCNA antibody.

(Results and Discussions) Case 1: Grossly, a single raised mass approximately 3 cm in diameter was observed in the left hepatic lobe. The lesion was pale compared with the surrounding normal tissue, and swelled from the cut surface. Histologically, the lesion was not encapusulated by fibrous tissue except a small portion, and it compressed adjacent normal tissue. Basic lobular structure was maintained; however, fibrosis characteristically showed a wagon-wheel-like pattern in the center of the lesion. The pseudolobule-like structures were observed in one part. Hepatic cords divided by the fibrous tissue, proliferated bile ducts and vascularization were presented. Hepatocytes are often arranged into 2 or 3 layers. The lesion showed many anti-PCNA-positive hepatic cells when compared with normal tissue, particularly in its center. Additionally, intracytoplasmic inclusions, that were acidophile with H.E. stain and positive for PAS, were often observed in hepatocytes. Corresponding to the inclusions, a highly electron dense, homogeneous, amorphous substance was observed in electron microscopy. Case 2: Grossly, a single raised nodule, approximately 0.5 cm in diameter, was observed in the left hepatic lobe. The nodule was pale compared with the surrounding normal tissue. Essentially, it was histopathologically the same as in case 1. However, the fibrous tissue was less than in case 1, and no pseudolobule-like structure was observed in case 2. More hepatic cells were anti-PCNA positive than in case 1.

Neither lesion was bordered with fibrous tissue, except at one point, and basic lobular structures were maintained. Additionally, atypical neoplastic hepatocytes were not observed. The two cases were concluded to be regenerative hepatocellular hyperplasia (as categorized by the WHO for domestic animals, 2003). However, in case 1 there was a resemblance to focal nodular hyperplasia in humans (as categorized by the WHO, 2000).

P-51

Goblet Cell Hyperplasia and Muscular Layer Thickening in the Small Intestine of a Cynomolgus Monkey

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We encountered an interesting case of cynomolgus monkey showing goblet cell hyperplasia and muscular layer thickening throughout the small intestine without any clinical symptoms, and presented it here.

[Case] The animal was a 5-year-old, male cynomolgus monkey, and it was allocated in the control group of a 1-week toxicity study and killed at the study termination. There were no noteworthy clinical findings including general condition, body weight, hematology and blood chemistry throughout the quarantine, acclimation and toxicity study periods. At necropsy, thickening of the intestinal wall (5 mm in thickness) was observed from the jejunum to the ileum with some conglomerated mucus on their mucosal surface. All of organs and tissues were fixed in 10% neutral buffered formalin, and paraffin-embedded sections were prepared and stained with hematoxylin and eosin.

[Results] Both mucosal and muscular layers remarkably thickened in the jejunum and ileum, and slightly in the duodenum. In the mucosa, goblet cell hyperplasia together with the extension of the circular folds and villi was prominent. The mucosal surface was widely and thickly covered with the mucus containing desquamated mucosal epithelial cells. The mucus was positive for PAS and stained blue with alcian blue(pH2.5), and the stainability was the same as the mucus in the normal intestine. No atypia was found in the epithelium of mucosa and crypt. In addition, the proliferative activity was normal. Minimal to slight inflammatory infiltration, mainly composed of lymphocytes and plasmacytes, was observed in the lamina propria of the duodenum and jejunum, but not prominent in the ileum. No organisms such as protozoas and parasites were observed in the intestine, and no pathogenic bacteria were identified by Gram, Giemsa and Wartin-Starry stains. Both inner and outer muscular layers were markedly thickened due to smooth muscle hypertrophy. No histologic abnormality was observed in the nerve plexuses of the intestinal wall. An increase in intracellular mucus of the superficial mucous cells and slight extension of gastric pit were also observed in the gastric mucosa. No significant findings were observed in other organs and tissues.

To our knowledge, no similar case has been reported previously in any animals including humans. Therefore, this case was considered to be exceedingly rare entity of unknown pathogenesis in cynomolgus monkey.

P-52

Pathophysiological study on vacuolar degeneration of thyroid follicular epithelial cells noted in a cynomolgus monkey

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We reported the histopathological aspects of vacuolar degeneration of thyroid follicular epithelial cells found in an untreated female cynomolgus monkey at the 23rd JSTP meeting. Similar change was observed in a female cynomolgus monkey obtained from the same farm as the previous case (Primate Quality Control Center, PQCC, INA RESEARCH PHILIPPINES, INC.). In the present study, in addition to morphological examination, thyroid function was examined by the analysis of plasma levels of thyroid related hormones.

Five female cynomolgus monkeys including one animal with the thyroid change from the control group in a 13 weeks toxicity study were used (2-3 yrs old, orally administered with vehicle of 0.2w/v% HCO-60 and 0.5w/v% CMC). Plasma levels of thyroid stimulating hormone (TSH), T3, and T4 were determined on the samples obtained at 7 and 13 weeks of the study. The thyroid glands were fixed in 10% formalin and processed routinely to histology sections. The sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) reaction, and immunohistochemistry for thyroglobulin (TG). In addition, ultra-thin sections prepared from the formalin-fixed thyroid were observed under a transmission electron microscope.

Microscopically, large vacuoles with PAS-negative and anti-TG negative were observed in the cytoplasm. Nuclei were located characteristically at the apical region adjacent to the follicular lumen. Ultrastructural examination revealed dilatation of rough endoplasmic reticulum (rER) corresponding to the vacuoles observed by light microscopy. No abnormalities were seen in thyroid gland weight or in the histology of other organs. The plasma levels of TSH, T3, and T4 of the monkey with the thyroid change were not obviously different from the control values [7th week : 2.1 μIU/mL, 187 ng/dL, and 4.0 μg/dL, respectively (mean values ± S.D. of 4 normal control animals; 1.4 ± 0.9, 195 ± 8, 4.7 ± 1.1, respectively) and 13th week : 1.6 μIU/mL, 203 ng/dL, and 3.3 μg/dL, respectively (mean values ± S.D. of 4 normal control animals; 1.2 ± 0.8, 188 ± 21, 4.2 ± 1.0, respectively)].

From the results showing no abnormalities in plasma levels of TSH, T3, or T4, it is suggested that the thyroid gland with vacuolar degeneration was functionally normal. Although vacuolar degeneration of thyroid follicular epithelial cells without functional alteration has been reported in rodent[1)], there are no report in non-human primate. The present case provides useful information on non-neoplastic spontaneous changes in the histopathology of cynomolgus monkeys.

1) Vacuolar change in the thyroid follicular cells in BrlHan: WIST@Jcl (GALAS) rats. Shimoi A., et al. Journal of Toxicologic Pathology, Vol.14 (2001)

P-53

A Case of Cynomolgus Monkey Suspected to Be Gaucher's Disease

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Gaucher's disease (Glucocerebrosidosis) is a rare, hereditary, lysosomal storage disease characterized by decreases or deficiencies of glucocerebrosidase, leading to the accumulation of glucocerebroside in the reticuloendothelial system, especially histocytes in the spleen, lymph nodes and bone marrow. It has been reported in dogs, sheep and pigs. We report a case suspected to be Gaucher's disease in a cynomolgus monkey.

The female cynomolgus monkey was five years and 7 months old and belonged to the vehicle control group in a 4-week repeated-dose toxicity study. The monkey was necropsied on the final day of the administration period and observed histopathologically in systemic organs.

No abnormalities were observed in clinical examination, urinalysis, hematology, blood chemistry or macroscopy. Histopathologically characteristic lesions were appearances of Gaucher cells in lymphoid organs (spleen, thymus, lymph node and lymphoid tissue of intestine) and bone marrow. The Gaucher-like cells were 20 to 50 μm in size and had a few pyknotic round nuclei and eosinophilic reticular materials in the cytoplasm. The reticular materials were positive to the PAS reaction, were stained blue with Masson stain and Azan stain, and were not stained with Oil Red O stain and Sudan black B stain. The Gaucher-like cells were observed in the cortex in the thymus, the germinal center in the spleen and lymphoid tissue and in bone marrow. The foamy macrophage proliferation was observed in the lamina propria of the small intestine and large intestine. The macrophage proliferation with eosinophilic cytoplasm was observed in the medullary sinus of the mesenteric lymph node. No remarkable changes were observed in the brain and liver.

This case was suspected to be a lysosomal storage disease because many macrophages (Gaucher-like cells) appeared in the lymphatic system and bone marrow. According to the cell specificity of macrophages and appearing organs, we suspect it is Gaucher's disease. We will make an electron microscopic observation.

P-54

Subchronic Toxicity of Semicarbazide Hydrochloride in B6C3F1 Mice

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Semicarbazide (SEM) has been known as a contaminant in different types of bottled foods, including baby food. One of the sources of SEM in foods is thermal degradation of azodicarbonamide, a blowing agent used to foam plastic gaskets of the metal lids for glass jars. Although some toxicological profiles including osteolathyrism of SEM hydrochloride (SEM·HCl) have been reported in rats, toxicity data in mice is limited. In the present study, subchronic toxicities of SEM·HCl was investigated in male and female mice. A total of 50 male and 50 female B6C3F1 mice were divided into five groups and fed diet containing SEM·HCl at concentrations of 0 (control), 125, 250, 500 and 1000 ppm for 90 days. As results, significant reduction of body weights was noted in 1000 ppm males and in 500 and 1000 ppm females. Food consumption showed a tendency for decrease in 500 and 1000 ppm males. No hematological change with toxicological significance was detected in any groups. In serum biochemistry, alkaline phosphatase was increased in 1000 ppm males. In females, relative spleen weights in the 1000 ppm group were increased. Macroscopically bowing deformity of the tibia was observed in 500 and 1000 ppm males. Histopathologically, thickening of epiphyseal cartilage in the tibia in 125 ppm and above males, thickening of articular cartilage in the femur in administered male groups and in 500 and 1000 ppm females, discontinuous/irregular array of elastic lamina in the thoracic aorta in 1000 ppm males, enhancement of extramedullary hematopoiesis in the spleen in 500 and 1000 ppm females and decrease of corpora lutea in the ovary in 1000 ppm females were observed. Based on above findings, the no observed adverse effect level (NOAEL) of SEM·HCl in B6C3F1 mice was estimated to be less than 125 ppm (19.3 mg/kg bw/day) for males and 250 ppm (41.1 mg/kg bw/day) for females.

P-55

Comparison of Toxicities of L-methionine and Homocysteine

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<Objective> Many studies have reported that dietary excess of L-methionine (Met) caused various toxic symptoms including growth retardation and hemolytic anemia. Met is metabolized to taurine via various intermediates, such as homocysteine (Hcys) and S-adenosylmethionine (SAM). These Met metabolites could be involved in toxic changes by Met excess. We have reported that Hcys is one of toxicity biomarkers for Met excess, whose concentrations in the plasma increased by excessive intake of Met. The other studies have shown that administration of Hcys caused growth retardation and behavioral abnormalities. Thus present study aimed to investigate possible involvement of Hcys in Met toxicities by comparing their toxicities in rats. <Methods> Thirty-four 6-week-old male F344 rats were randomly allocated to six groups (n=4 or 6). The rats were fed purified diet with AIN-93G composition containing additional Met (1.2% or 2.4%), homocystine ($Hcys_2$, 1.1% or 2.2%) or 2.2% $Hcys_2$+3.8% betaine ab libitum. Their body weights and food intakes were recorded daily. The animals were sacrificed on the 14th day of the experiment for hematological and histopathological examinations. Liver samples were taken for metabolite analysis. <Result & Discussion> 1) Feeding of 2.4% Met increased splenic non-hem iron content and hemosiderin deposition, indicators for splenic hemolysis. These changes were accompanied with congestion in the spleen and Heinz body formation in RBC. These toxic changes were not observed in rats fed additional $Hcys_2$, indicating no involvement of Hcys in hemolytic changes caused by Met excess. However, feeding of additional $Hcys_2$ in combination with betaine stimulated splenic hemolysis. Since betaine is known to promote Met synthesis from Hcys, the results suggested possible involvement of Met itself or SAM, both of which are upstream to Hcys, in hemolysis caused by excessive intake of Met. Indeed, metabolite analysis in the liver revealed marked accumulation of Met and SAM in rats fed either Met or $Hcys_2$+betaine, but not in rats fed $Hcys_2$ alone. 2) Fatty degeneration in centrilobular region of liver was observed in rats fed 1.2 or 2.4% Met. This was also evident and severer in rats fed $Hcys_2$, while betaine addition to $Hcys_2$ diet ameliorated the histopathological changes in the liver, indicating possible involvement of Hcys in fatty degeneration caused by excessive intake of Met.

P-56

Evaluation of the Toxicity of *Equisetum arvense* L. with 90 Days Dietary Administration to F344 Rats

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The *Equisetum arvense* L., a plant containing a lot of minerals such as silicon, has been used as diuretic and cough drug in the traditional Chinese medicine. However, little is known about the safety of *Equisetum arvense* L. in experimental animals. The present study was conducted to evaluate the toxicity of *Equisetum arvense* L. in male and female F344 rats administered test diet for 90 days. A total of 80 rats (6-week-old), were divided into eight groups 10 rats each. Groups 1~4 (males) and 5~8 (females) received *Equisetum arvense* L. at concentrations of 0, 0.3, 1 and 3% in CRF-1 diet. For all treatment groups, no differences of body weight, food, water intake or urinalysis data were found compared to the control. In hematological and blood biochemistry analyses, no changes demonstraning the toxicity of *Equisetum arvense* L. were detected. From the results of histopathological analysis, eosinophilic bodies in the kidney were detected in all male rats. However, no differences in the incidence has been found. Therefore, it was concluded that this histopathological characteristic is due to the natural disease or is an accidental event. Our results indicated the safety of administration of *Equisetum arvense* L. at doses of 0~3%. Therefore, the no-observed adverse effect level (NOAEL) of the *Equisetum arvense* L. in rats was considered to be 3% (male; 1.79 g/kg b.w./day, female; 1.85 g/kg b.w./day) under the conditions employed in this study.

P-57

A 90-day Repeat Dose Oral Toxicity Test of L-Serine in Fischer 344 Rats

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L-serine (L-Ser) is a nutritionally dispensable amino acid and is listed in the list of existing food additives in Japan. Recently, L-Ser has become widely used as an ingredient of supplements or health foods. There are only few reports available, however, regarding toxicity of L-Ser.

A subchronic oral toxicity study of L-Ser was conducted with groups of 10 male and 10 female Fischer 344 rats that were fed a powder diet containing 0, 0.06, 0.5, 1.5 and 5.0% of L-Ser for 90 days. There were no toxicologically significant, treatment-related changes in body weight, food consumption, water intake or hematology. The serum biochemistry showed dose-dependent decreases of total protein and albumin values of both sexes. Values of urea nitrogen or aspartate aminotransferase of male rats were dose-dependently decreased, while values of gamma glutamyltransferase of treated females tended increased in comparison with the controls. Relative kidney weights were significantly increased in the 5.0% male and female rats. These changes were within the range of the historic control values or lacked corresponding pathological findings. In the histological study, only sporadic spontaneous lesions were observed.

In conclusion, the no-observed-adverse-effect-level (NOAEL) for L-Ser is, therefore, determined to be a dietary dose of 5.0% (2765.0 mg/kg body weight/day for males and 2905.1 mg/kg body weight/day for females) under the present experimental conditions.

P-58

5-Fluorouracil-induced Apoptosis Death and Cell Proliferative Activity in The Brain of Rat Fetuses

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5-Fluorouracil (5-Fu), a well-known thymidylate synthesis inhibitor, induces developmental anomalies mainly in the craniofacial tissues and limb buds. Recently, it was reported that microencephaly was also induced in rat neonates after 5-Fu-treatment in late phase of pregnancy.

In this study, pregnant rats were treated with 5-Fu (50 mg/kg) on Day 13 of gestation, and their fetuses were examined for histopathological changes at 3, 6, 9, 12, 24, 48 and 72 hours after treatment (HAT). Two-μm paraffin sections of the fetus were stained with hematoxylin and eosin for histopathological examination. TUNEL, cleaved caspase 3 (caspase 3), p53, p21, phospho-histone H3 (histone 3) and bromodeoxyuridine (BrdU)–positive neuroepithelial cells in the ventricular zone of the telencephalic wall were counted in 2 randomly chosen fetuses from a dam.

No deaths occurred in dams or fetuses of any group. In the 5-Fu group, the weights of the fetuses were significantly reduced at 48 and 72 HAT.

In the 5-Fu group, the number of apoptotic cells increased at 6 HAT, peaked at 9 and 12 HAT, and then decreased gradually toward 72 HAT. The number of p53- positive cells was most numerous at 3 HAT, thereafter decreased gradually until 12 HAT, and then decreased suddenly toward 24 HAT. After 48 HAT, most of the p53- positive cells disappeared, and returned to the control level. The number of p21-positive cells was not observed. The number of BrdU-positive cells decreased from 12 HAT to 48 HAT, and then returned to the control level at 72 HAT. The number of histone 3-positive cells decreased slightly from 3 HAT to 48 HAT, and then returned to the control level at 72 HAT. Thus, increase of apoptosis and depression of cell proliferative activity in the fetal brain returned to the control level at 72 HAT.

It was evident that 5-Fu induced apoptosis and depressed cell proliferative activity in the neuroepithelial cells in the rat fetal brain, the expression of p53 increased prior to the induction of apoptosis and decreased cell proliferative activity. It is strongly suggested that excessive apoptosis and depression of cell proliferative activity in the fetal brain may have a close relation to the latter occurrence of microencephaly.

P-59

Observation of fetal brain in a rat valproate-induced autism model

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We have examined the direct effects of prenatal sodium valproate (VPA) exposure on the fetal brain (gestational days (GDs) 14 and 16). The GD14 fetal brain was too young to reveal VPA-specific neurotoxicity, even though developmental delay was observed. GD16 examination detected VPA-induced neurotoxicity in the cortex, midbrain and pons.

In this study, a more developed GD20 fetal brain, which is an endpoint in teratogenicity studies, was examined after VPA exposure (800 mg/kg) on GD9 (VPA9) or GD11 (VPA11). At Caesarean section on GD20, male fetuses were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA), embedded in 10% gelatin, and coronal serial sections were cut. The sections were observed after immunohistochemical staining with tyrosine hydroxylase (TH) and serotonin (5-HT).

In the cerebral cortex, cortex layers revealed no remarkable changes on GD20, whereas a decrease in TuJ1-positive cells (a marker of immature neurons), and hypoplasia of the cortical plate were observed on GD14 and 16, respectively, after VPA exposure.

In the pons, a conspicuous round structure, composed of a disorganized neural fasciculus with scattering TuJ1-positive cells observed in a GD16 sample, was also noted on GD20 materials in the VPA11 group. Immunohistochemical examination revealed that TH-positive cells were detected in the anterior portion of these structure and 5-HT-positive cells were distributed in the posterior part of this structure. The anatomical location of this abnormal area is compatible with the ventral tegmetum (A10 and VTA) and median raphe. From these results, migration of TH as well as 5-HT-positive cells was affected due to this rounded structure. No remarkable changes were observed in this area in the VPA9 group.

Thus, we have examined three stages of fetal brain after VPA exposure. We discuss the usefulness of fetal brain examination, and place special emphasis on the timing and areas of observation in a developmental neurotoxicity test.

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P-60

Heart Lesions in F344 Rats with Long Term Breeding after MeIQx Treatment

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2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) is the one of the most important compounds in heterocyclic amines as same as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). PhIP has been reported to induce colon, beast, and prostate tumors. On the other hand, MeIQx has not been reported the carcinogenicity in those organs, but in liver, skin and connective tissue. Takahashi et al. found the cardiotoxicity of PhIP in Fischer rats. Watanabe et al. reported the cardiomyopathy in Donryu rats one year after treated with PhIP, but not in rats treated with MeIQx. In this study, we found the cardiac lesions of F344 rats in long term breeding (2 years) with high fat diets after the short-term intermittent treatment of MeIQx.
56 F344 rats were divided 4 groups. The animals in Groups 1 and 3 were given corn oil high fat (COHF) diet and those in Groups 2 and 4 were fed the mixed lipid high fat (MLHF) diet. Groups 1 and 2: 25 rats were given the short-term intermittent treatment (Nakagama model) with 400 ppm MeIQx and high-fat diets following long term breeding with high fats diets till natural death. Groups 3 and 4 were fed the each experimental diets throughout the termination. After necropsy, all of tumors and main organs in animals were examined histopathologically.
After 114 weeks, all of animals were died. Hearts were performed istopathological examination in 23 rats in Group 1 and 24 rats in Group 2. Incidence of cardiac lesions were 7 cardiomegaly, 1 dilation, 8 fibrosis, 4 local fibrosis (12 fibrosis), 6 inflammatory cells infiltration, 3 calcification in Group 1, and 4 cardiomegaly, 1 dilation, 7 fibrosis, 5 local fibrosis (12 fibrosis), 7 inflammatory cells infiltration, 4 calcification in Group 2. No particular findings could be found in Groups 3 and 4.
No significant differences could be found between Groups 1 and 2. In this study, No particular findings could be found in control groups. However, SLC reported fibrosis was found in 90 % in F344 rats after 2 years breeding, but not cardiomegaly, dilatation, and calcification. Watanabe et al. reported the cardiac lesions were observed in Donryu rats from 6 months (to the termination (1 year)). Takahashi et al. found the heart lesions in F344 rats fed 400 ppm PhIP diet for 52 weeks. In this study, the cardiac lesions were found after 18 months. Cardiotoxicity of MeIQx in F344 rats develops later than that of PhIP.

P-61

Usefulness of Immunohistochemistry with Anti-Troponin Antibodies for Investigation of Drug-Induced Myocardial Damage in Cynomolgus Monkeys

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Blood troponin concentration has been reported to be a useful biomarker for drug-induced myocardial injury; however, the investigation of changes in myocardial troponin with immunohistochemistry in cynomolgus monkeys has not been reported. We examined the relationship between immunohistochemical changes with mouse anti-myocardial troponin antibody, H.E.-stained histopathological changes, and blood troponin concentration.

(Materials and Methods) Myocardial injury was induced by concomitant administration of isoproterenol and vasopressin. Isoproterenol (0.03, 0.3, or 3 mg/kg, 2 ml/kg) was administered once subcutaneously, and then vasopressin (0.3 mg/kg) was administered once intravenously to 3 male cynomolgus monkeys (aged 3 to 5 years, purpose-bred in China). Physiological saline was administrated to a control group. Blood Troponin concentration (TnT, TnI) was measured from 2 hours after administration until the seventh day, on which the animals were necropsied. The heart was fixed in 10% neutral buffered formalin and processed for paraffin section. Specimens were H.E. stained and immunohistochemically stained with mouse anti-myocardial troponin antibodies (TnI, TnT).

(Results) Blood troponin concentrations (TnT, TnI) increased dose-dependently, peaking at 4 to 8 hours after administration, and could still be detected by the fifth or seventh day. Vacuolation and necrosis of the myocardium were observed in H.E.-stained specimens, mainly in the left ventricular wall. Peak TnT and TnI concentrations correlated highly with the necrosis score in histopathological examinations of H.E.-stained specimens. Decreased immunoreactivity of troponins (TnI, TnT) was observed in the myocardium, which showed vacuolation or necrosis in H.E.-stained sections. Furthermore, decreased immunoreactivity was also observed in some myocardial specimens that showed no abnormality in H.E.-stained sections.

(Conclusion) Decreased immunoreactivity of troponins (TnT, TnI) in morphologically normal H.E.-stained myocardial sections showed release of troponin. Therefore, it was suggested that immunohistochemistry with anti-troponin antibodies is a useful method for detection of myocardial injury without morphological change.

P-62

Induction of MCP-1, CCR2 and Oxidative Stress in Arsenate and Hexavalent Chromium-Induced Pulmonary Inflammation and Remodeling

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Chromated copper arsenate (CCA), which is used worldwide as a wood preservative, can adversely affect human health. Whereas accumulating evidence suggests that hexavalent chromium (Cr) and arsenate (As) can potentially disrupt the redox balance and cause respiratory diseases including cancer in humans, combined effects of these metals have not been fully elucidated. Previously we have reported oxidative stress could be involved in As and Cr-induced pulmonary injury in mice. Herein we determined sequential changes up to 14 days after intratracheal co-treatment of As with Cr. Those induced inflammation, followed by fibrosis in the lung with increased type I collagen mRNA level. By the bronchoalveolar lavage analysis, monocyte chemotactic protein-1 (MCP-1) level was markedly higher than other chemokines, along with increased level of its receptor, chemokine (C-C motif) receptor 2 (CCR2) mRNA in lung tissues. To clarify the further mechanism on oxidative stress, we examined the gene expression of the catalytic subunit of glutamylcysteine ligase (Gclc), the modified subunit of Gcl (Gclm), glutathione peroxidase 1 (Gpx1), Gpx2, glutathione reductase, thioredoxin 1 (Trx1), Trx2, Trx reductase 1 (Trxrd1), superoxide dismutase 3 (Sod3), methionine sulfoxide reductase A (MsrA), MsrB1, peroxiredoxin 1, heme oxygenase-1 (HO-1), 8-oxoguanine DNA-glycosylase 1, and Nrf2 on Day 2. Of these genes, co-treatment enhanced mRNA levels of Gclc, Trx1, HO-1, and Gpx2, while As alone reduced Trx2, SOD3, MsrA, and MsrB1 levels. These findings suggest that pulmonary injury by co-treatment with As and Cr could be partially mediated by MCP-1, signaling via CCR2, and the effect may be exerted through a disruption in the balance among several antioxidant genes.

P-63

Acute Alteration of Lung by Intratracheal Instillation of CuO Nano- and micro-sized particles in F344 Male Rats

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Toxicity and carcinogenesis of nanomaterials remain uncertain, despite nano-sized materials are widely used. In this study, acute alteration in Fischer 344 male rats has been examined after intratracheal instillation (i.t.) of CuO nano- and micro-sized particles.

A total of 76 rats were divided into three groups, with 28 rats in groups 1and 2, and 20 in group 3. The rats in group 1 were administered by i.t. of nano-sized particles (0.5mg/0.2ml 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine/rat). In group 2 were administered micro-sized particles. Group 3 was no treatment (control) group. All rats were sacrificed on 1, 3, 7 and 14 days after i.t. administration. Histopathological examination and Cu contents analysis by atomic absorption spectrometry (AAS) were performed on blood serum and the liver.

In histopathological findings, there were severer acute inflammatory changes of the lung on day 1 and 3 in nano-sized particle group than in micro-sized particle. The Cu content of blood serum on day 3 was increased in nano-sized particle group as compared with in micro-sized particle. The Cu content of the liver on day1 was higher in nano-sized particle group than in micro-sized particle.

These results indicated that the induced inflammatory change was different from the size of the particles, even if the components of the particles were the same, and the inflammatory changes appeared earlier and severer in nano-sized particle than in micro-sized particle.

P-64

Promotion Effects of Fullerene (C60) on Rat Lung Carcinogenesis

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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.

To examine the effects of fullerene (C60) on carcinogenesis, 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 44. C60 was suspended in rock-candy solution and intra-tracheally sprayed into the lung at 250 ppm or 500 ppm once every two weeks. C60 treatment significantly increased the multiplicity of lung alveolar hyperplasia, adenoma and carcinoma. Notably, aggregates of C60 were commonly observed in alveolar macrophages. C60 was not detected in other organs. Since previous reports have shown that C60 induces oxidative stress, we treated F344 rats with 500 ppm C60 for 8 consecutive days and analyzed the lung tissue for oxidative stress. Treatment with C60 significantly increased 8-OHdG levels. Although it was not significant, an increase in the activity of superoxide dismutase (SOD) in the lung was observed. We conclude that C60 promoted lung carcinogenesis, and that oxidative stress resulting from alveolar macrophage phagocytosis of C60 is a probable factor.

P-65

Lactoferrin, Surfactant Protein, and β-Defensin in Mice Following Intratracheal Instillation of Didecyldimethylammonium chlorides (DDAC).

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As their location of direct contact to exogenous environment, the respiratory organs are always exposed to risk of invading pathogenic microorganisms. The pulmonary innate immune system mainly constructed by airway mucus containing many primary materials. Lactofferin (LF), an inclusion of exocrine secretions such as milk, tear, and saliva, or mucosal secretion, exists in airway secretions. It has been reported that LF possess antimicrobial activity and is lymphocyte proliferator. Surfactant protein A and D (SP-A and SP-D) of lung collectins and β-defensins (BD) produced from epithelial cell also have antimicrobial activities. There play integral roles in pulmonary defense system. However, there were few reports about the relationship between these defense system and inhalation of chemicals. DDAC is widely used chemical as a detergent of germicides, antiseptics, and wood preservatives and a series of evaluations on its respiratory toxicity are on going. In the present study, DDAC was applied by intratracheal instillation to C57BL/6J mice to assess the effects on pulmonary defense system. On 1, 3, and 7 days after intratracheal instillation of 0.01 % DDAC, three or four mice in each group were sacrificed and lung tissue were collected. The levels of pulmonary LF, SP-A, B, C, D, and BD-1, 2, 3 mRNA expressions were measured by real-time PCR. LF mRNA level in treated groups showed a trend toward a decrease with time dependently. BD-2 and 3 mRNA levels in the treated groups were significantly decreased when compared with those in the controls. Clear changes could not be obtained with BD-1 because there was no detectable level of its mRNA. There were no statistically significant differences in SP-A, B, and C mRNA levels between the control and treated groups, while SP-D mRNA level was significantly increased when compared with that in the controls. These results suggested that DDAC instillation to mouse lung caused an imbalance of pulmonary antimicrobial peptides or proteins and these changes possibly affected the defense system of the lung.

P-66

Effects of Chronic Inflammation on *in vivo* Mutagenicity and Carcinogenicity in the Colon of *gpt* delta Mice Treated with MeIQx

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Chronic inflammation is regarded as a risk factor of cancer development. To investigate whether chronic inflammation affects mutagenicity and carcinogenicity induced by 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a genotoxic carcinogen derived from cooked foods, we examined *in vivo* mutation assay and measurement of aberrant crypt foci (ACF), as precancerous lesion of colon cancer, in the colon of *gpt* delta transgenic mice. Male B6C3F1 *gpt* delta mice were given a diet containing 300 ppm of MeIQx for 7 weeks, and were given water containing 2% dextran sulphate sodium (DSS) at week 2 and 5 of experimental period. At week 7, a part of mice were sacrificed, the colonic mucosa being collected for *in vivo* mutation assay. Some mice of each group were housed for 19 weeks to investigate carcinogenicity in murine colon. The end of examination, mice were sacrificed and were measured the number of ACF. In the *gpt* mutation assay for detection of point mutations on the *gpt* locus, the mutant frequency (MF) was increased in MeIQx treatment mice as compared to the control mice albeit without statistical significance. In the MF in the combined treatment group, the increase in MF was not observed in comparison to any groups. At necropsy on 26-week, the incidence of ACF was significantly increased in distal colon of DSS-treated mice. Likewise, number per mouse of ACF had a tendency to increase. However, the combined treatment with MeIQx did not affect these changes. The effects of chronic inflammation on mutagenicity and carcinogenicity of MeIQx in the colon of mice were not clear under the present experimental condition. The administration period of MeIQx in the present study might be too short to clarify the influence of inflammation because the increase of *in vivo* mutagenicity of MeIQx was not observed in the MeIQx alone group. Alternatively, the fact that DSS-induced colitis at the early stage is characterized by inflammatory cell infiltration with erosion of mucosal epithelium allows us to speculate that the initiated cells by MeIQx did not fully receive the effects of the inflammatory factors due to enhancement of cell turnover.

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Cancer Risk in Diabetic Rat Models

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Recent epidemiological data suggest that type 2 diabetes is a risk factor of colorectal cancer in humans. We examined the cancer susceptibilities in diabetes-prone Long-Evans Agouti (LEA) and Long-Evans Tokushima Fatty (OLETF) rats. For controls, F344, Long-Evans Tokushima Otsuka (LETO) and LEA.F-*Xdm1*, a congenic strain introgressing D20Rat47-D20Mgh4 QTL region of F344 onto LEA background, rats were used. In experiment 1, six-week-old male F344 and LEA rats (n=15) were whole body X-irradiated at a dose of 2 Gy and killed at 72 weeks. The incidences of small intestine tumors in F344 and LEA rats were 0% and 7%, respectively, and those of colon tumors were 0% and 14%, respectively. In experiment 2, LETO and OLETF rats (n=15) were whole body X-irradiated at a dose of 4 Gy and killed at 72 weeks. The incidences of small intestine tumors in LETO and OLETF rats were 0% and 30%, respectively, and those of colon tumors were 0% in both strains. In experiment 3, F344 and LEA rats (n=21) were administered 50 mg/kg *N*-methyl-*N*-nitrosourea, i.p. (x4) and killed at 32 weeks. The incidences of small intestine tumors in F344 and LEA rats were 19% and 38%, respectively, and those of colon tumors were 10% and 24%, respectively. In experiment 4, LEA.F-*Xdm1* and LEA rats (n=24) were administered 15mg/kg azoxymethane, s.c. (x3) and killed at 30 weeks. The incidences of small intestine tumors in LEA.F-*Xdm1* and LEA rats were 0% and 4%, respectively, and those of colon tumors were 70% and 83%, respectively. In these experiments, diabetic rats were more susceptible to small intestine/colon carcinogenesis than respective controls, although the differences were not significant.

P-68

Analysis of the Lesion Development in Serine Palmitoyl Transferase (SPT) Conditional Knock Out Mice

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[Background] Sphingolipids are considered to have important function in biological process not only as components of cell membrane but also as signal transmitters. Although Serine Palmitoyl Transferase (SPT) is known as an enzyme which catalyzes the first step of the biosynthesis of sphingolipids, the precise role of SPT in vivo is not well understood since knockout (KO) of SPT results in fetal lethal phenotype. Thus, a conditional KO (cKO) mouse was developed. In this cKO mouse, knockout of SPT can be induced by administration of tamoxifen after their berth. Time course analysis was performed for the development of the lesions observed in SPT cKO mice.
[Materials and Methods] 16-week-old Sptlc2lox/lox: CreERT2 mice were administered 225 mg/kg/day of tamoxifen (TAM). Blood and tissues were sampled from 3 rats/sex/group at 24, 48 and 72hours (24, 48, 72H) after the first administration of TAM. Hematological, serum biochemical and histomorphological analyses were performed. Sptlc2lox/lox: CreERT2 mice administrated vehicle and Sptlc2lox/lox: wt mice administrated TAM were also examined as controls.
[Results and Discussion] 24H: There were no noteworthy changes in body weight, organ weight and results of hematological and plasma biochemical analyses of cKO mice. In the small and large intestine, small number of single cell necrosis was observed in and near the crypt. 48H: Decreasing trend of body weight, decrease in spleen and thymus weights and decrement in the number of white blood cells and reticulocytes were observed in cKO mice. Histologically, prominent single cell necrosis was observed at the base of villi in the small intestine and crypts in the large intestine. At this point, mucosal epithelium of the small and large intestine and the length of villi in the small intestine were relatively normal. Lymphoid necrosis in the thymus and depletion in the bone marrow were also observed. 72H: Decreases in body weight, spleen and thymus weights and the number of white blood cells and reticulocytes became obvious in cKO mice. Changes considered to be related to the dehydration and poor physical condition were also observed. Histologically, atrophy of villi in the small intestine and atrophy of mucosa of the large intestine were observed. These changes were accompanied by necrotic debris in the intestinal lumen and crypts. Lymphoid necrosis and depletion become severe in lymphoid and hematopoietic tissues.
The results suggest that defect of SPT have some effects on the actively proliferating cells such as intestinal epithelial cells, and induce necrotic lesion followed by atrophic change of the tissue in short term.

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Giant Mitochondria in Pancreatic Acinar Cells of Alloxan-induced Diabetic Rats.

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Giant cytoplasmic eosinophilic granules had been often observed in various cells under some pathologic condition, however, they were rarely derived from mitochondria. In this study, we investigated the morphological features of eosinophilic granules derived from mitochondria encountered in pancreatic acinar cells of alloxan-induced diabetic rats.
Diabetic conditions were induced in total 5 male F344 rats by single intravenous dosing with 35 mg/kg of alloxan at 6 weeks of age. Rats were sacrificed after 25 weeks of treatment for histopathological and ultrastructural examination in the pancreas.
Histologically, pancreatic tissues were diffusely atrophied along with severe atrophy and loss of islets due to direct effect of alloxan treatment. Eosinophilic granules in varying size and small vacuoles were observed in the basal area of acinar cell. These eosinophilic granules showed negative reaction for PAS, Alcian blue, Oil red O and Sudan III, but small vacuoles were positive for Alcian blue, Oil red O and Sudan III. Ultrastructually, giant granules corresponded to eosinophilic granule were surrounded by double membrane, and had irregular cristae-like structure. Small zymogen granules with low density accumulated near the lumen, and a large amount of small lipid droplets were shown at the basal area of acinar cells. Immunohistochemical analysis of prohibitin, a kind of protein located in the mitochondrial inner membrane, was partially positive in marginal area of some eosinophilic granules, but negative for central area. The positive reaction for succinic dehydrogenase (SDH), one of the mitochondrial enzymes, showed similar localizing pattern to prohibitin by enzyme histochemistry. Almost all of acinar cells were negative for amylase.
Electron microscopic and immunohistochemical findings confirmed that the eosinophilic granules in exocrine pancreas of alloxan-induced diabetic rats were giant mitochondria. In addition, location of prohibitin and SDH suggested inadequate function of giant mitochondria.

P-70

Modifying effects of enzymatically modified isoquercitrin or melatonin on oxfendazole-induced liver tumor promotion in rats

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We previously reported that administration of oxfendazole (OX), a benzimidazole anthelmintic, in rats enhanced liver tumor-promoting activity resulting from the oxidative stress such as DNA damage and lipid peroxidation due to reactive oxygen species (ROS) generated. To clarify more whether oxidative stress is involved in the liver tumor promoting effect of OX, male rats were administered a single intraperitoneal injection of *N*-diethylnitrosamine (DEN) and were fed a diet containing 500 ppm of OX for 10 weeks from 2 weeks with or without 2,000 ppm of enzymatically modified isoquercitrin (EMIQ) or 100 ppm of melatonin (MLT) in the drinking water after DEN initiation. One week after the commencement of the administration of OX, all rats were subjected to two-thirds partial hepatectomy. Immunohistochemical examinations of glutathione S-transferase placental form (GST-P) showed that the number of GST-P-positive foci promoted by OX was significantly inhibited by the combined administration of antioxidant EMIQ or MLT, and the area of GST-P-positive foci was inhibited by the administration of MLT. Quantitative real-time RT-PCR analysis revealed decreases in mRNA expression levels of *Cyp2b1/2* and *Me1* in the DEN-OX-EMIQ and DEN-OX-MLT groups and decreases in mRNA expression levels of *Cyp1a1* and *Akr7a3* in the DEN-OX-MLT group, as compared with the DEN-OX group. In addition, in *in vitro* reactive oxygen species (ROS) production assay using hepatic microsomes in rats of the DEN-OX group, inhibited nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ROS production was observed when treated with 3 μM of EMIQ or more or 3 nM of MLT or more. These results suggest that coadministration of EMIQ or MLT suppresses the hepatocellular tumor-promoting activity of OX in rats through the decrease in ROS production by hepatic microsomal activation.

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The effects of liver tumor promoters on mice initiated by PhIP

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2-Amino-1-methyl-6-phenylimidaso[4,5-*b*]pyridine (PhIP) is one of the most abundant heterocyclic amine contained in cooked meat and fish. In mice, PhIP has been shown to induce lymphomas and weakly induce small intestinal tumors. I*n vivo* mutagenicity study using *gpt* delta transgenic mice, increased mutant frequencies in the colon, spleen and liver were reported. In the colon, recent studies have revealed that adnocarcinomas are induced by PhIP followed by the treatment of dextran sulfate sodium, a tumor promoter associated with colitis. In the present study, to ascertain tumor initiating potential of PhIP in the mouse liver, the 2-stage liver carcinogenicity study was performed using the two different categories of liver tumor promoters following PhIP treatment.

Male C57BL/6J mice at age of 7 weeks were given 400 ppm PhIP in the diet for 13 weeks as in the case of the previous *in vivo* mutagenicity study. After the 2 weeks recovery period, they were received phenobarbital (PB, 500 ppm), a nuclear receptor-mediated hepatocyte proliferator, or acetaminophen (APAP, 10000 ppm for 37 weeks, and then 7500 ppm), a compensatory hepatocyte proliferator, in the diet for 63 weeks (total experimental period was 78 weeks). Histopathological examination was conducted in the liver, spleen, mesenteric lymph nodes and small intestine.

PhIP induced lymphoma and small intestinal tumors with high incidence in male mice. However, there were no effects on the liver tumors by the treatment of any tumor promoter. Thus, the present study demonstrated the lack of liver tumor initiating activity of PhIP. *In vivo* mutagenicity in the colon of mice treated with PhIP was more than 10 times higher than that in the control group. However, in the liver, it was only about 3 times higher than that in the control group. The result in the present study suggests that there might be a threshold between the increased mutation frequency and the induction of tumors as an endpoint.

P-72

CYP1A Inducers-Induced Hepatocellular Tumor Promotion in Rats, and the Expression of Histone Deacetylases during their Tumor Promotion.

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Some cytochrome P450 IA (CYP1A) inducers are known to have hepatocellular tumor-promoting activities in rodents, such as the strong CYP1A inducer β-naphthoflavone (BNF); however, their molecular mechanisms are still unclear. By using a rat two-stage hepatocarcinogenesis model, we confirmed the potential of tumor-promoting activities of an anti-androgen fultamide (FUL) and a dietary compound indole-3-carbinol (I3C) as compared with that of BNF, and the molecular expression changes in altered foci induced by these CYP1A inducers were examined immunohistochemically as compared with their surrounding tissues. Male 6-week-old F344/N rats were treated with CYP1A inducers (1% BNF, 0.5% I3C or 0.1% FUL) in the diet for 6 weeks after DEN initiation (200 mg/kg, i.p.). Two-third partial hepatectomy was applied one week after the start of promoter treatments. The number and area of GST-P positive foci were significantly increased in the livers of rats treated with promoters (BNF>>FUL>I3C>DEN) and paralleled with the induction of mRNA expression of *Cyp1a1*, a representative AhR-regulated gene. In GST-P positive foci, altered foci comprising of eosinophilic or clear cell type were induced (BNF>I3C>>FUL=DEN). Immunohistochemically, the staining intensity of CYP1A1 in these foci was decreased compared to the surrounding tissue. In addition, PCNA-positive hepatocytes in these foci were significantly increased along with the decreased intensities of p21 and C/EBPα, negative regulators of cell cycle progression. Furthermore, the nuclear intensity of histone deacetylases 1 (HDAC1) and HDAC5 in these foci was increased or decreased, respectively, compared to the surrounding tissue. These results confirm that CYP1A inducers FUL, I3C and BNF have the hepatocellular tumor-promoting activities in rats. Furthermore, differential cellular localizations of HDAC is found in altered foci appeared during the process of tumor promotion, suggesting that the epigenetic mechanisms in the regulation of gene expressions may be involved in the induction of altered foci by CYP1A inducers.

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Comparison of Inhalation Exposure and Oral Administration in *N*,*N*-Dimethylformamide-induced Liver Lesions

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N,*N*-Dimethylformamide (DMF) has been widely used in industry as an organic solvent, and it occurs in the environment, including the atmosphere and water. In long-term inhalation studies, DMF induced liver tumors in rats. In the present study, we examined the difference in liver toxicity induced by inhalation exposure compared to oral administration of DMF.

[Materials and Methods] Groups of 5 male rats (F344 /DuCrj) were exposed to DMF by inhalation or oral administration for 4 weeks. The inhalation groups were exposed to DMF for 6 hours/day, 5 days/week by whole body inhalation at 200 or 400 ppm (v/v). The oral groups were given DMF-formulated drinking water at 800, 1,600 or 3,200 ppm (w/w) *ad libitum*. A control group was given clean air and clean untreated water. All organs of the rats were examined histopathologically.

[Results and Discussion] Terminal body weight significantly decreased in the 400 ppm inhalation group and 3,200 ppm oral group compared with the control. DMF-induced effects were observed in the liver. Liver weight significantly increased in all DMF-treated groups by both inhalation and oral routes, the oral groups more significantly than the inhalation groups. Incidences of single cell necrosis and centrilobular hypertrophy of hepatocytes were increased in all DMF-treated groups. Although increased liver weight was higher in the oral groups, these DMF-induced liver lesions were more severe in the inhalation groups than oral groups by histopathological evaluation. The percentages of PCNA-positive hepatocytes were increased in a dose-related manner in both the inhalation and oral groups. These results suggest that DMF-induced cell proliferation of hepatocytes is due to not only regenerative proliferation but also DMF itself.

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In vivo Liver Genotoxicity and Twenty-eight Day Repeated Oral Dose Toxicity Study of Diheptyl Phthalate (DHP) in Rats

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Diheptyl phthalate (DHP), one of the phthalate esters (PAEs), have been widely used as solvents, plastics softeners, and additives in paper coatings. Since DHP has also been categorized as a PPAR alpha agonist, it induces not only testicular toxicities but also swelling and necrosis of hepatocytes in the liver of rats. Accordingly, DHP, like other PPAR alpha agonists, probably has a hepatocarcinogenic potential in rats, but it is unclear as for the genotoxicity and carcinogenicity of DHP. On the other hand, recently, there are a lot of reports demonstrating that PPAR alpha agonists induce DNA damage secondary to oxidative stress resulting from the generation of reactive oxygen species (ROS). Based on these historical backgrounds, in order to clarify the *in vivo* genotoxicity and carcinogenicity targeting the liver of DHP, male F344 rats were subjected to repeated oral administration of DHP at the dose levels of 0, 2.5 or 5 g/kg for 28days. In addition, F344 rats were subjected to once or 14 times oral administration of 5 g/kg/day of DHP, and their livers were evaluated in an alkaline single-cell gel electrophoresis (comet) assay. Furthermore, based on the results of these studies, we perform *in vivo* liver initiation assays by once, three times, or 14 times oral administration of DHP in rats. In the 28-day toxicity study, there was a marked suppression of body weight gain in DHP-treated groups from the 14th day. Absolute and relative liver weights were significantly increased in DHP-treated groups with a dose-dependent manner. On immunohistochemical examinations, the mean area and number of GST-P positive foci significantly increased in DHP-treated groups compared with those in the control group. In comet assay, cell migration, a marker of DNA damage, was significantly induced in the liver of rats subjected to 14 times oral administration of DHP at 24 hr after administration. In *in vivo* initiation assay, an increase in the number and area of GST-P positive foci was observed in rats of DHP-treated groups subjected to 14 times oral administration of DHP as compared to the control group. The results of these studies suggest the possibility that DHP is a genotoxic carcinogen in the liver of rats.

P-75

Steroid receptor expression in hepatocellular hypertrophy

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Although hepatocellular hypertrophy is a common aspect in toxicity tests, it is not always clear whether this is an adverse effect or adaptive response. The mechanistic understanding is important to make reliable risk characterizations and assessments from this hepatic change. The liver is a target organ for steroid hormones and it has been known that development of hepatic tumors was related to the sex hormones status. In this study, steroid hormone receptor expressions were investigated during the development of hepatocellular hypertrophy to understand the possible relationships. 6 week-old F344 rats were fed with phenobarbital (PB), chlofibrate (CF), and insecticide chemicals X and Y. The animals were killed at day 3, weeks 4 and 13. The recovery groups were also set feeding with standard diet for another 4 weeks. Liver tissues were dissected and subjected to the total RNA extraction. The mRNAs including steroid hormone receptors were measured by the real-time RT-PCR method. All of the four chemicals were confirmed to induce hepatic hypertrophy. Decrease in estrogen receptor α expression was evident in all the groups at week 4 and it was consistent through the experiment in the CF group. Androgen receptor mRNA significantly decreased in the male PB group. The further investigations were needed to clarify the relationship between change in sex hormone receptor expressions and hepatocellular hypertrophy.

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Utility of An *in vivo* Medium-term Initiation Assay Model using 4 Week-old Rats

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An *in vivo* medium-term initiation assay model using 7 week-old rats was required 5 week-long study and cell proliferation stimulus (such as the two-thirds partial hepatectomy (PH)). PH is laborious and causes loss of condition of experimental animals in addition to the response individual variation that may result in inaccurate interpretation. Therefore, improvements of cell proliferation stimulus in this model have been tested in past studies. This study was to investigate the utility of an *in vivo* 4-week initiation assay model using 4 week-old rats without cell proliferation stimulus.

In experiment I, using 4, 4.5 and 8 week-old Fscher 344 male rat livers, cell proliferation of hepatocyte was analysed by the bromodeoxyuridine (BrdU) labeling method, and the total Cytochrome P450 (CYP) contents and enzyme activities of CYP isozymes (CYP1A, 2A, 2B, 2C, 2E, 3A) were measurerd. In experiment II, 4 week-old Fischer 344 male rats were administrated orally single (4 or 16 mg/kg) or 4-day repeat (1 or 4 mg/kg) of 1,2-dimethylhydrazine (DMH), which is well-known mutagenetic carcinogen. In the control group, saline was administrated orally. All rats were fed basal diet for 1 week, and then a diet containing 0.015% of 2-acetylaminofluorene for the following 2 weeks. Two weeks after first DMH dosing, all animals received a single dose of Carbon tetrachloride (0.8 ml/kg, p.o.). At the end of 4 weeks after first DMH dosing, the induction of glutathione S-transferase placental form (GST-P) positive foci by DMH in the liver was evaluated immunohistochemically using image analysis software.

Experiment I, it was found that 4 and 4.5 week-old rats had approximately 3 times of hepatic BrdU index compared with 8 week-old rats, and similar total CYP content and enzyme activities except for CYP2C. In experiment II, the inductions of GST-P positive foci in all DMH-treat groups were dose-dependent and significantly greater than that of the control group. GST-P positive foci in the DMH 1 mg/kg 4-day dosing group and the DMH 4 mg/kg 4-day dosing group were induced in a similar way of the DMH 4 mg/kg single dosing group and DMH 16 mg/kg single dosing group, respectively. From these results, an *in vivo* 4-week initiation models using 4 week-old rats without cell proliferation stimulus was detected initiation activity of DMH and addivitiy of initiation activity, so that this medium-term initiation assay model is considerd to be useful to detect iniation activity of chemicals.

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The Glomerular Lesions of Japanese Quails by Dietary Administration of 17β-estradiol

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[Introduction] To investigate additional endpoints for screening of endocrine-disrupting effects in birds, one-generation reproduction test of 17β-estradiol (E2) in Japanese quails was assessed according to the OECD Testing Guideline 206. As a result of this test, distinctive renal lesions were observed in the parental quails.

[Material and methods] 10-week-old WE strain of Japanese quails in both sexes, were given E2 mixed diet at concentration of 0, 0.3, 3 and 30 ppm for 6 weeks. We measured serum E2 and Vitellogenin (VTG) concentrations and performed macro- or microscopical examinations (HE stain, special stains and immunostain for anti-VTG antibody), and electron microscopical examinations.

[Results] Serum E2 concentration was significantly higher in the 3 ppm or higher groups in males or 30 ppm group in females than that in the controls. Serum VTG concentration of males increased dose-dependently, although that was not detected in the control group. Serum VTG concentration was almost same level in female 0, 0.3 and 3 ppm groups, but that in the 30 ppm group was significantly higher. Grossly, discoloration and swelling of the kidney in both sexes were observed in the 30 ppm groups.

Histopathologically, swelling and eosinophilic deposition of the glomerulus and eosinophilic cast and degeneration of the renal tubules were observed. Those eosinophilic materials of the glomerulus and renal tubule were positive for PAS reaction and anti-VTG antibody.

[Conclusion] Although the glomerular lesions were observed in all groups including the control group for females, they were observed only in the treatment groups for males. The severity and incidence of the lesions increased as serum VTG concentration increased in both sexes. VTG is the yolk precursor phosphoprotein synthesized in the liver induced by the E2 in the female oviparous vertebrates. VTG is not synthesized in the normal male quails, while mature female quails keep constantly high serum VTG level. Therefore, females may have glomerular lesions as a background lesion. The result of this study suggested that the excessive serum VTG resulted in the eosinophilic deposition in the glomerulus and might cause the renal lesions.

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Lucidin-3-O-primeveroside might be a promising candidate for a responsible constituent for Maddar color-induced rat renal carcinogenesis

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Madder color (MC) has been demonstrated to induce kidney and liver tumors in F344 rats. To explore the major cause in MC carcinogenesis, among many constituents, we have noted lucidin-3-O-primeveroside (LP) and its metabolites, lucidin (Luc) and Rubiadin (Rub) because of their genotoxicity. In fact, Luc-specific DNA adducs in the rodent target sites were detected by ^{32}P-postlaebeling analysis, but the precise chemical structure remains uncertain. Additionally, since alizalin (Alz) has potential of oxidation, it was also considered to be a candidate agent despite lack of genotoxicity. In the present study, we characterized Luc-specific DNA adduct and examined reporter gene mutations in the kidneys of *gpt* delta rats given MC (5.0%), LP (0.08%), Rub (0.3%) or Alz (0.04%) in the diet for 8 weeks along with 8-hydroxydeoxyguanosine (8-OHdG) measurement.

The Luc-specific DNA adduct formation in the reaction mixture of dG or dA with acetylated-Luc involving carbocation was searched by LC-ESI/MS analysis. Subsequent ^{1}H NMR analysis provided Luc-N^2-dG and Luc-N^6-dA as the precise chemical structures for each adduct. Along with these adducts formation in calf thymus DNA being confirmed, the present data suggest that Rub might be partly metabolized to Luc, leading to the Luc-specific adducts formation.

Significant increases in 8-OHdG levels were observed in all the treated rats except Rub treated group. *Gpt* mutant frequencies (MFs) in rats treated with MC, LP and Rub were significantly elevated. Analysis of the mutation spectra revealed that AT:TA transversion was a common mutation in those groups, especially being prominent in Rub treated rats. In view of the fact that 8-OHdG adduct gives rise to GC:TA transversion mutations, the present data showing the observation of this mutation in all treated groups imply participation of oxidative DNA damage in the MC carcinogenesis. However, since the MFs were remarkably increased in rats treated with LP and Rub as compared with that in Alz treated rats, the bulky DNA adducts induced by LP and its metabolites may be a major cause in MC carcinogenesis. Furthermore, the observation of AT-TA transversion mutation in common in MC, LP and Rub groups might result from Luc- and Rub-induced dA modifications.

P-79

Age dependence of radiation-induced renal tumorigenesis

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Since the amount of radiation exposure to children during medical procedures has been increasing, it is important to evaluate the radiation risk to children and fetuses. However, there is only limited information on the kidney in relation to age-dependent radiation risk. In this study, we attempted to assess the risk of cancer in developing kidneys.

F1 rats were born to male Eker rats (Tsc2 mutant) and female F344 rats. In one group, F1 fetuses were irradiated with 2 Gy γ-rays on gestation days 15 and 19, and in another group, F1 pups were irradiated with the same doses on postnatal days 5, 20, and 49. Both groups were euthanized at 27 weeks, and their kidneys were removed. After removing the weights of the kidneys, they were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and then stained with HE. Prepared sections were examined for proliferative lesions in the renal tubular epithelium.

In both male and female F1 rats with the wild-type Tsc2 gene, proliferative lesions were not observed in the renal tubular epithelium regardless of when they were irradiated. But hyperplastic lesions were observed in the renal tubular epithelium in both male and female rats with Tsc2 heterozygous mutation regardless of irradiation. Compared with the non-irradiated control group, the number of developing tumorigenic lesions of the renal tubular epithelium was higher in both male and female rats irradiated with 2 Gy at gestation day 19. This suggests that the period when kidney cells are actively growing is a window for tumorigenic transformation of the renal tubular epithelium.

P-80

Influence of cholinesterase inhibitor on smooth muscle of urinary bladder

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Cholinesterase inhibitor inhibits the enzyme cholinesterase which breaks down acetylcholine and increases the level of acetylcholine and duration of its action as a neurotransmitter and, subsequently, the muscarinic and nicotine receptors are situmulated excessively. Salivation, miosis, incontinence and twitching are well known clinically observed adverse effects of cholinesterase inhibitors; however, organic changes in the urinary bladder are less known. Degeneration of the vesicular smooth muscle was reported in a 1-year oral dog toxicity study of galantamine which inhibits cholinesterase although the mechanism is unclear. Therefore, we investigated the effect of anti-cholinesterase activity on the vesicular smooth muscle using fenitrothion, which is a potent cholinesterase inhibitor, with ganglionectomy (GN) of the pelvic ganglia.

GN was performed on the left pelvic ganglia, using metal probe cooled with liquid nitrogen, on 16 of 32 female Crl:CD(SD) rats. One week after GN, corn oil as control or 300 mg/kg fenitrothion was administered to operated or non-operated rats for 2 days. On the day after the last administration, their urinary bladders were collected and whole-mount specimens for acetylcholinesterase activity to identify the nerve distribution and HE sections were prepared and evaluated.

In the whole-mount slides stained for acetylcholinesterase, a decrease in nerve fibers was found in the operated side in 3 out of 4 control rats with GN. On histopathological evaluation, degeneration of the vesicular smooth muscle was found in all animals treated with fenitrothion. The grade of the degeneration was less on the operated side compared with the non-operated side in 3 of 4 GN animals treated with fenitrothion. Accordingly, it is concluded that cholinesterase inhibition could lead to degeneration of the smooth muscle in the urinary bladder in rats.

P-81

In a Sprague-Dawley Rat:

Sertoli-Leydig Cell Tumor of the Testis

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A rare intratubular gonadal stromal tumor was observed in the testis of a 7-week-old male Sprague-Dawley rat. The tumor comprised an intratubular mixture of two different types of tumor cells with intercellular junctions: the predominant tumor cells were consistent with a Sertoli cell origin, and the others were a relatively small number of tumor cells situated on basolateral side of the tubuli consistent with a Leydig cell origin. The neoplastic Sertoli cells had large pleomorphic nuclei and clear cytoplasm with many tubulovesicular cristae and free ribosomes, while the neoplastic Leydig cells showed relatively small pleomorphic nuclei, dark cytoplasm with rich smooth endoplasmic reticulum, numerous mitochondria, and lipid droplets. Occasionally, a few transitional type neoplastic cells were observed. The presence of a thick and/or multilayered basement membrane was confirmed except in tumor-infiltrative lesions. The present case was considered to be a testicular mixed tubular Sertoli-Leydig cell tumor in a Sprague-Dawley rat.

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Tranilast Suppressed Prostate Cancer Growth *in Vitro* and *in Vivo*.

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[INTRODUCTION] Development of a new therapeutic approach to improve the prognosis of prostate cancer is needed. Tranilast is generally used for several allergic disorders such as atopic dermatitis and bronchial asthma. Tranilast is also reported inhibitory effect against tumor cell proliferation or invasion such as oral squamous cell carcinoma and gastric carcinoma. Therefore, we investigated whether Tranilast would exhibit inhibitory effects on prostate cancer growth *in vitro* as well as *in vivo*. [Materials & Methods] Four prostate cancer cell lines (LNCaP, PC3, DU145, and PLS10) were used. Each cell line was treated with Tranilast, generous gift from KISSEI Pharmaceutical Co., Ltd, at 4 different doses (0.001mM, 0.01mM, 0.1mM, and 1mM). Cell growth was measured by WST-1 assay. Cell apoptosis was investigated with Guava Nexin kit. For detection of molecular mechanisms of Tranilast against prostate cancer cell lines, western blot analysis was performed LNCaP treated 1mM Tranilast by using several antibodies of signal transduction. As an *in vivo* study, Tranilast (200 and 400mg/kg/day) was given intragastrically to prostate cancer transplanted rats at cranial region for 3 weeks. Sequentially tumor volume was measured during 3 weeks experimentation. At sacrificed, cranial transplanted tumor was removed and histologically examined. Tumor cell proliferation was investigated with Ki67 staining. Tumor cell apoptosis and necrosis was evaluated with TUNEL staining and image analyzer. [Results] *In vitro* study, cell proliferation of LNCaP, PC3, DU145 and PLS10 was significantly suppressed at 0.1mM and 1mM doses of Tranilast as compared with the control. Tranilast also increased apoptotic rate in LNCaP. Using LNCaP cells, western blot analysis suggested that phospho-GSK3β was up-regulated and phospho-Akt was down-regulated after the treatment with Tranilast. *In vivo* study, the tumor volume of cranial transplanted PLS-P, rat prostate cancer, was significantly decreased by the highest dose of Tranilast. Cell proliferation of transplanted prostate cancer was not significantly different between control group and Tranilast-treated group. TUNEL positive area was significantly increased by the highest dose of Tranilast. [Conclusion] Tranilast suppressed prostate cancer cell proliferation *in vitro*, and promoted prostate cancer cell apoptosis *in vitro* as well as *in vivo*. Based on our results, Tranilast may be a candidate for conventional therapy of prostate cancer. Further study is needed for application of Tranilast for prostate cancer.

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Prostate Cancer Regrowth in TRAP Rats by Restoration of Testosterone after Bilateral Orchiectomy

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We established transgenic rat for adenocarcinoma of prostate (TRAP) model using rat probasin promoter/SV40 T antigen gene construct. TRAP rats develop high-grade prostatic intraepithelial neoplasia (PIN) from 4 weeks of age and well-moderately differentiated adenocarcinomas with high incidences by 15 weeks of age. These adenocarcinomas were androgen dependent and almost all were noninvasive phenotype. Therefore we tried to establish an animal model that mimic advanced prostate cancer in human cases by restoration of testosterone after bilateral orchiectomy in TRAP rats.

Twenty-four male TRAP rats were divided into 2 groups. Rats were underwent bilateral orchiectomy at 20 weeks of age in group 1 and 10 weeks of age in group 2. All rats in both groups were implanted testosterone propionate (TP)(40mg)-containing Silastic tubes into subcutis 5 weeks after castration, and 3 or 4 rats each were sequentially killed at 0, 2, 5 and 15 weeks after TP implantation.

Microinvasive adenocarcinomas were found in 3/4, 2/4 rats in ventral prostate and 2/4, 0/4 rats in lateral lobe in groups 1 and 2, respectively, at week 15. No invasive adenocarcinoma larger than 2mm was observed. Sequential immunohistochemical analysis demonstrated that kinetics of androgen receptor, SV40 T antigen and Ki-67 expression did not differ in both groups.

It was difficult to establish an animal model for invasive adenocarcinoma of prostate by castration-TP restoration in expectation that induce mechanical disruption of basement membrane of prostatic acini.

P-84

Establishment and characterization of rat prostate cancer cell lines derived from the transgenic rats with SV40T antigen expression under probasin promoter control.

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We generated a transgenic rat with the SV40 T antigen under probasin promoter control, allowing prostate-specific gene expressions. Males demonstrate atypical epithelial cell proliferation in the prostate from 4 weeks of age and develop prostate carcinomas at 100% incidence before they are 15 weeks old. From the transgenic rat of 55 week-old, we established a transplanted tumor in nude mice with castration treatments. And then, we have tried to established cell lines from the nude mice tumor. Finally we established 3 cell lines. One cell line designated PCai-1 shows charcoal treated FBS insensitive growth, and two other cell lines PCai-2 and PCai-3 are sensitive for the charcoal treatment FBS with stopping growth. Characteristic gene expression profiles have been obtained for each 3 cell lines. From the nude mice tumors without castration, we also established another new line A1-1F which may have cancer stem cell characters. These cell lines may be useful to study mechanisms of progression of prostate cancers, and to study chemo-prevention of prostate cancers.

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Morphological Analysis of Blood- and Lymphatic-Vessel distributions in the Ovary and Uterus

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To examine the morphological distribution of blood vessels and lymphatic vessels in some organs may be useful on toxicity evaluation. As preliminary technical approach, we performed morphological analysis of blood- and lymphatic-vessel distributions in the ovary and uterus.

The ovary of rat, which contains abundant blood vessels or lymphatic vessels, undergoes morphological and functional changes according to the estrus cycle. Involvement of regulators, such as vascular endothelial growth factor (VEGF), on the morphological changes caused by estrus cycle has been reported in the uterus. So we immunohistochemically tried to identify the morphological changes of blood vessels and lymphatic vessels caused by estrus cycle in 10 intact Slc:WitarHannover/Rcc female rats aged 9, 18 and 31 weeks, respectively, after evaluating estrus cycle-stages with hematoxylin-eosin staining.

Five proestrus, 2 estrus and 3 diestrus females aged 9 weeks, 2 proestrus, 1 estrus and 7 diestrus females aged 18 weeks and 4 proestrus, 2 estrus and 4 diestrus females aged 31 weeks were observed. Lymphatic vessels showed positive reaction on anti-podoplanin staining in serial sections, and were mingled with arteries and veins at medulla in the ovary. Their size and number were smaller than those in blood vessels. Lymphatic vessels were also observed at around follicle, corpus luteum and at inside of corpus luteum. Lymphatic endothelium was identified along blood vessels in the uterine endometrium or muscle layer. In addition, the size of lymphatic vessels was smaller than that of blood vessels at endometrium. Our approach for this morphological analysis is still ongoing.

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Effects of Sulpiride on Reproductive Organs and Pituitary in Female Rats

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Sulpiride is a dopamine D2 receptor (D2DR) antagonist and is well known to induce the hyperprolactinemia in animals and human. In this study, we investigated the histopathological changes induced by sulpiride in reproductive organs and pituitary in female rats.

Six-week-old female Crl:CD(SD) rats were treated with sulpiride at 1, 10 and 100 mg/kg/day or vehicle for 2 or 4 weeks. After completion of dosing, animals were euthanized and ovaries, uterus, vagina, mammary gland and pituitary were removed. Hematoxylin and eosin specimens of these organs were prepared and examined microscopically. In addition, the pituitary of the representative animals in the control and 100 mg/kg/day groups for 2 weeks was stained immunohistochemically with D2DR antibody.

In the rat treated with sulpiride for 2 weeks, the following changes were observed; an increased number of atretic follicles (type 7 and 8 large follicle) in the ovary and an increased incidence of diestrus in the uterus and vagina at 1, 10 and 100 mg/kg/day; an increased incidence of follicular cyst in the ovary, abnormal diestrus in the uterus and vagina, an increased incidence of epithelial mucification in the vagina and development/hypersecretion in the mammary gland at 10 and 100 mg/kg/day. Additionally, eosinophilic materials in the intermediate cells of the pituitary were observed at 10 and 100 mg/kg, and these materials were positive for antibody against D2DR. In the rat for 4 weeks, abnormal proestrus in the vagina at 1, 10 and 100 mg/kg/day and a decreased number of large follicle in the ovary at 10 and 100 mg/kg/day were observed in addition to almost the same changes as those noted in the 2-week study.

In this study, the main change induced by sulpiride in the ovary was arrest of development of types 7 to 8 large follicles, and there was no effect to small and medium follicles, or corpus luteum. In the intermediate cells of the pituitary, the expression of D2DR protein was increased by sulpiride administration. It is suggested that D2 receptor in the pars intermedia responded more strongly to D2 antagonistic action than that in any other pituitary cells.

P-87

Modifying Effects of Isoflavone Aglycon on Mammary Gland and Uterine Carcinogenesis

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The soybean isoflavone have been shown to possess estrogenic activity in human and laboratory animals, therefore the maximum of its intake for human has been established by Japan Food Safety Commission. To investigate the modifying effects of isoflavone aglycon (IA) on mammary gland and uterine carcinogenesis, Donryu rats were administered the dose of IA equal to the established maximum intake for human.

5 week-old female Donryu rats were treated by a single 7,12-dimethylbenz[a]anthracene (DMBA) injection to initiate mammary carcinogenesis, and thereafter intrauterine treatment with double injection of N-ethyl-N□-nitro-N-nitrosoguanidine (ENNG) at a dose of 10 mg/kg body weight on days 7 and 11 was performed via the vagina to initiate uterine carcinogenesis. IA-rich extract was administered to rats at a dose of 0.6% in Phytoestrogen Low Diet (NIH-07PLD) for 36 weeks.

From the results of histopathological analysis, incidence of mammary gland adenocarcinoma was significantly elevated in the IA-treated as compared to the DMBA and ENNG initiation control group. Furthermore, in the uteri of IA-treated animals, incidences of total proliferative lesions (adenocarcinoma and hyperplasia) and polyps were significantly increased, as compared to the initiation control group.

Our results indicated that IA administered to Donryu rats at a dose of 150 mg/kg b.w. /day, which was shown to possess estrogenic activity, exerts promoting effects on mammary and uterine endometrial carcinogenesis initiated by DMBA and ENNG.

P-88

Histological Findings of Aberrant corpus luteum in Ovaries of Cynomolgus Monkeys

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Abstract: The menstrual cycle of monkeys is similar to that of humans, for instance the average days in a cycle (about 28-day), number of ovum at ovulation (single ovulation), and histological structure of the ovary. There are some active lutein-like cell foci remaining in the degenerated corpus luteum (DCL) of monkeys, which has been reported as "aberrant corpus luteum" (ACL) in a previous report of rhesus monkeys. Though the ACL is usually observed in the ovaries of cynomolgus monkeys, the histopathological feature and function of the ACL has not been detailed. We investigated the histological, immunohistochemical, and ultrastructural features of ACL of cynomolgus monkeys in this study. Incidence of ACL: The histological examination was performed for both sides of the ovaries of 3 years and older cynomolgus monkeys, which were observed with cyclical changes in their ovaries. The ACL was observed in 26/104 monkeys. The number of ACL is between one and three in each monkey. Histological findings: The ACL was observed as an island in DCL, and occasionally occupied the entire area of a previously formed DCL. The majority of the ACL was distributed to the inner part of DCL except for the outer layer, which was composed of the former theca lutein cells. The size of the ACL cells was smaller than the functional corpus luteum (FCL) cells, and the ACL cell had eosinophilic to basophilic and solid cytoplasm and round to oval nucleus. There were abundant blood capillaries in ACL. Immunohistochemical findings: The ACL cells were positive for inhibin α. In addition, the FCL cells were positive, and the DCL cells were negative for inhibin α. Ultrastructural findings: The ACL cells had some rER, mitochondria, lysosome, but no lipid droplets in cytoplasm. A typical feature of an ACL cell, "open nucleoli" and "ring-shaped/chondriospheres mitochondria" were observed. Discussion: Although ACL has been known for a long time, there are no details about ACL, because it was considered that the ACL cells had no ovarian hormone production such as progesterone. We revealed that the ACL could secrete inhibin in this study. Inhibin can inhibit the secretion of FSH by negative feedback mechanisms of pituitary FSH. This negative control of FSH is very important for prevention of follicular grows in the luteal phase, induction of follicular atresia except for a dominant follicle in follicular phase. The ACL may have a role in controlling the number of follicles by inhibin secretion for a single ovulation in monkeys.

P-89

A Histopathological Evaluation for Progression of Dermatitis Induced by Repeated Topical Application with Dermatophagoides Farinae Extract in NC/Nga mice.

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[Background] For the study of atopic dermatitis, we had used a spontaneous dermatitis model in NC/Nga mice. However, this model has some inadequacies for histopathological evaluation (e.g large individual and regional variabilities in the severity of the lesions) due to spontaneous onset. To seek a suitable atopic dermatitis model for histopathological evaluation, we evaluated another dermatitis model[1] induced by topical application of ointment containing dermatophagoides farinae extract on back skin of NC/Nga mice.
[Material and method] Thirty-eight female NC/Nga mice at 6 to 9 weeks of age were allocated to 3 groups and topically applied of ointments containing dermatophagoides farinae (0, 30 and 70 mg/animal, respectively) on their back (6 times with 3 or 4 days interval). The first day of experiment was defined as Day 0 and the experiment period was 22 days. The back skin regions were removed from each mouse, routinely processed and stained with hematoxylin and eosin.
[Result and discussion] In macroscopic observation, severe dermatitis was occurred on back skin in ointments treated groups. Histopathologically, the dermatitis was composed of crust, hyperkeratosis and hyperplasia of epidermal squamous cells in epidermis, ulcer, hyperplasia of external roof sheath cells, and fibrosis, inflammatory cell infiltration and pigmentation in dermis. There were no differences in histopathological feature and severity of dermatitis between 30 and 70 mg/animal groups. In both applied groups, the lesions was apparently observed on Day 4 and later, and aggravated until on Day 14. And then, the severity/condition of dermatitis continued. On the same sampling day, no obvious individual variability was observed in each group. No obvious regional variability of dermatitis was also observed in each animal. In conclusion, this model has a certain advantage for histopathological examination, specially, less individual and regional variabilities and less secondary influences, as well as that macroscopic and histopathological examinations could perform using same skin region, in comparison with the spontaneous dermatitis model in NC/Nga mice. In addition, the time-course change of macroscopic dermatitis score measured by our criteria mostly coincided with that of histopathological semi-quantitative dermatitis score. Therefore, this dermatitis model was suggested to be suitable for histopathological studies for atopic dermatitis.
[Reference] 1.Yamamoto M. et al., Allergol Int 56: 139-48. 2007.

P-90

Distribution and Pathogenesis of Skeletal Muscle Lesions in Dysferlin-deficient SJL and A/J Mice

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[Object] The pathogenesis of limb girdle muscular dystrophy 2B (LGMD2B) in humans remains incompletely understood. We investigated the distribution and pathogenesis of skeletal muscle lesions in two different LGMD2B mice models, SJL and A/J, and compared the differences between these strains.

[Method] The femoral, crural, brachial, forearm, abdominal, pectoral, masseter, lumber and bulbocavernosus muscles, as well as the diaphragms and tongues were collected from 6 males each of SJL and A/J mice and 3 males of BALB/c mice (normal controls) at the age of 10 and 35 weeks, and were examined histopathologically and immunohistochemically (an anti-slow-twitch fiber antibody applied to confirm the specificity of skeletal muscle lesion for muscle fiber types).

[Result] At the age of 10 weeks, minimal to slight hyaline degeneration and necrosis of muscle fibers were observed in the femoral, brachial, abdominal, masseter and lumber muscles in 5/6 and 6/6 SJL mice, and in the crural, forearm and pectoral muscles and the diaphragms in 1/6 to 3/6 SJL mice. On the other hand, neither A/J nor BALB/c mice showed the abnormal findings in the skeletal muscles examined. At the age of 35 weeks, skeletal muscle lesions in SJL mice were increased in the frequent and degrees, and atrophy of muscle fibers accompanied with fatty infiltration was observed in the longissimus and lateral vastus muscles, indicating exacerbation with age. A/J mice developed minimal hyaline degeneration and necrosis in the femoral, crural, brachial, abdominal and lumber muscles. There were no abnormal findings in the skeletal muscles of BALB/c mice. The specificity for muscle fiber types was not seen in these muscle lesions in both SJL and A/J mice.

[Conclusion] The skeletal muscle lesions in SJL mice developed earlier than did A/J mice. In SJL mice, the femoral and lumber muscles showed the most significant progress with age, and the longissimus muscle is a common site of skeletal muscle lesions on muscular dystrophy. Additional examinations are under way to investigate the relationship between the endoplasmic reticulum stress and the skeletal muscle lesions.

P-91

Different Macrophage Populations, Myofibroblasts, and Galectin 3-expressing Cells in the Rat Excisional Wound Healing

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Wound healing is a basic process of body restoration. Mechanisms remain to be clarified. Appearance of macrophages, myofibroblasts, and galectin 3 cells was ivestigated in rat wound healing. Samples were examined on post-wounding days 1-26. Macrophages reacting to ED1 (CD68), ED2 (CD163), and OX6 (MHC class II) appeared on day 1, and peaked on days 3, 5, and 15, respectively. OX6 cells were present beneath regenerating epithelia. These findings indicated that ED1 and ED2 cells are responsible for inflammatory and subsequent granulation tissues, whereas OX6 cells might relate to epithelial regeneration. Myofibroblasts reacting to alpha-smooth muscle actin began to be seen on day 5 in periphery of wounds, forming granulation tissues; the cells reached maximum on day 9 following peaked number of ED1 and ED2 cells. Galectin 3 cells peaked on day 1 and many galectin-3 cells also reacted to ED1. This study showed that diverse macrophages participated into wound healing, with myofibroblasts.

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P-92

Histopathological investigation of local reations induced by various adjuvants

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【Introduction】There are some inactivated vaccines which contain adjuvant. It is considered that the grade and distribution of the local reaction are different depending on the kind of adjuvant. In the present study, we investigated local reactions induced by some adjuvants which were added to human or animal inactivated vaccines.

【 Methods 】 Male Japanese White rabbits received intramuscularly a single injection of 0.5mL/site of aluminum hydroxide gel, liquid paraffine and squalene, respectively. After 2 and 7 days of injection, 3 rabbits each in the treated group were sacrificed under the pentobarbital sodium anesthetizing, histopathological examination was performed. In the histopathological examination, paraffine-embedded sections of injection sites were routinely prepared and stained with hematoxyline and eosin for the light microscopic observation.

【Results】<Aluminum hydroxide gel> At 2 days after injection, substance which was seemed to be caused by the injection of aluminum hydroxide gel was localized. Although degeneration/necrosis of muscle fiber was observed, inflammatory reaction was weak. At Day 7, infiltration of the mononuclear cell was stronger than that of Day 2, and the substance like aluminum hydroxide gel was still localized. <Liquid paraffin> At Day 2, big vacuoles which were seemed to be caused by the injection of liquid paraffin were observed in muscle, and slight mononuclear cell infiltration was seen. At Day 7, same changes were seen and fasciitis also was observed. < Squalene > At Day 2, vacuoles which were seemed to be caused by the injection of squalene were observed in muscle, and slight mononuclear cell infiltration was seen. The vacuoles were smaller and more diffuse in comparison with those of liquid paraffin. At Day 7, size of vacuoles was bigger than that of Day 2, and fasciitis also was observed.

【Conclusion】In the aluminum hydroxide gel, substance which was seemed to be caused by an injection of aluminum hydroxide gel was localized and cellular infiltration was mild. On the other hand, although vacuoles were seen in the liquid paraffin and squalene, cell reaction was weak. Fasciitis also was observed. Although the histopathological changes in liquid paraffin and squalene were similar, the vacuoles of squalene were smaller and more diffuse in comparison with those of liquid paraffin. As mentioned above, the distribution of adjuvant and the grade of the cellular infiltration differed depending on the kind of the adjuvant, therefore it was suggested that adjuvant was a major factor in evaluating the local reaction of vaccines.

P-93

Evaluation of DMBDD Carcinogenesity in ZDF Type 2 Diabetes Model Rat

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DMBDD model is a well-known rat multi-organ carcinogenesis test. In the present study, we investigated the carcinogenic effect of DMBDD treatment in Zucker Diabetic Fatty (ZDF) rat, used for model of type 2 diabetes and control Lean rat.

6 week-old male ZDF rats (26) and Lean rats (27) received a single i.p. injection of diethylnitrosamine (100 mg/kg b.w.) at the commencement, four i.p. injections of N-methyl-N-nitrosourea (20 mg/kg b.w.) during two weeks, four s.c. injections of 1,2-dimethylhydrazine (40 mg/kg b.w.) from week 3 to 4, 0.05% N-butyl-N-(4-hydroxybuthyl) nitrosamine in the drinking water for 2 weeks from the commencement, and 0.1% dihydroxybutyl-di-N-propylnitrosamine in the drinking water for the following 2 weeks (DMBDD treatment). Histopathological examination of target organs was performed 30 weeks after starting the experiment.

In the urinary bladder, incidences of transitional cell carcinoma (67%) and total bladder tumors (87%) were increased significantly in DMBDD-treated ZDF rats. Furthermore, incidences of colon adenocarcinoma (63%) and total colon tumors (75%) in DMBDD-administered ZDF rats were significantly elevated as compared to the control Lean rats.

Our results demonstrated that carcinogenesis in DMBDD target organs is enhanced in ZDF rats indicating their high sensitivity to carcinogens exposure.

P-94

Histological Expression of Metallothionein in the Developing Rat Placenta

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In order to clarify the metallothionein (MT) localization in the developing placenta, we histologically investigated the sequential MT expression in placentas and fetal livers using pregnant rats during gestation days (GDs) 9 - 21. The placentas were sampled and weighed on GDs 9, 11, 13, 15, 17, 19 and 21.

In the early post implantation period, the expression of MT was slightly detected in the yolk sac and the primary decidual zone around the embryo. MT was then mainly present in the deciduas parietalis and yolk sac. After the deciduas parietalis ruptured, MT was subsequently detected in the yolk sac and deciduas basalis. MT continued to be detected in the yolk sac until GD 21, but it was reduced in the deciduas basalis in accordance with development of the fetal liver with elevated MT expression.

In conclusion, the main expression site of MT changes from the maternal placenta to the fetal placenta, and then to the fetal liver in accordance with the fetal development. However, we speculate that the MT-positive cells in the placenta are positioned between the maternal and embryonic environments throughout the gestation period and always surround the embryo/fetus.

P-95

Histopathological Investigation of Sequential Changes in Skeletal Muscle in a Collagen-induced Arthritis Monkey.

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We have established a collagen-induced arthritis (CIA) model in cynomolgus monkeys to assess the effectiveness of anti-rheumatic drugs in pre-clinical studies. In this model, in addition to joint swelling, muscle wasting and body weight loss are also observed. Along with joint disorders, muscle wasting is a complication that negatively affects the quality of life of rheumatoid arthritis sufferers. In this CIA model study, we examined sequential histopathological changes in skeletal muscle.

CIA was induced in twenty-two female cynomolgus monkeys by two immunization with bovine type Ⅱ collagen in the presence of Friend's complete adjuvant. Symptoms of CIA were monitored to evaluate the swelling level, and food intake, body weight, and serum biochemical parameters including CPK and creatinine were measured sequentially. Tissue samples were obtained from five or six animals at 11, 21, 35, and 63 days after the first immunization. Quadriceps femoris specimens from these animals were stained with H.E., NADH-TR, ATPase, and Gomori's trichrome, and examined histopathologically, and also by electron microscopy.

At 11 days after the first immunization, slight degeneration and necrosis of muscle fibers, hemorrhage, and neutrophil infiltration were observed. At 21 days, these histopathological changes were severer, with increased areas of collagen fiber. At 35 days, histopathological changes were severest, and included regeneration of muscle fibers. At 63 days, histopathological changes were less severe than at 35 days. The infiltrating cells were almost all macrophages, except for neutrophils at an early stage. CPK level transiently increased in the early phase. Creatinine level sequentially decreased, accompanying decrease in body weight or suppression of body weight gain. Special staining showed that type Ⅱ muscle fibers were more damaged than type Ⅰ muscle fibers. Electron microscopy revealed irregularity of myofibril alignment and increased mitochondria and microvesicles possibly derived from sarcoplasmic reticulum in muscle fibers.

The histopathological changes observed in the CIA model represented myositis consising of necrosis, degeneration and atrophy of muscle fibers, mainly type Ⅱ fibers. These histopathological changes were correlated with arthritis severity. This suggests the possibility that myositis occurs due to inflammation factors.

P-96

Histopathological Study of Bone Toxicity in Rats

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There are many cases where femur and sternum are recommended as organs of standard tissue lists of bone in repeat-dose toxicity studies. It is known that the time of epiphyseal closure differs in some regions. Concerning about distal femur, proximal humerus and tibia, the time of fusion of the secondary ossification centers are various in X-ray radiography on rats aged about 50 weeks. The aim of this study is to investigate histopathologically the different effects of bone from some regions having the different time of epiphyseal closure, therefore rats aged about 50 weeks were dosed with doxorubicin (DOX) which is known to affect epiphyseal growth plate in youg animal. Male Crl:CD (SD) rats aged 46 weeks were used, then DOX was administered every other week by injection in the tail vein, at 15 and 0 (control) mg/m^2 body surface area in all 5 times, that is known to be effective in epiphyseal growth plate of young rats. Aged 55 weeks, all rats were sacrificed and histopathologically examined epiphyseal growth plates of distal femur, 4th sternebra, proximal humerus and tibia. In proximal humerus and tibia, thinning of epiphyseal growth plate was remarkably observed same as young rat tibia, but the others were not. These data suggest that there are some differences in the regions of bone and it was related to the time of epiphyseal closure, therefore it is important for analysis of bone in toxicologic pathology to consider selection of the regions and the age of animal.

P-97

GLP /ERES Compliance Strategies in Whole Slide Imaging

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Whole slide digital images can be produced from glass slides at resolutions of 20x, 40x, and 100x, and assembled without producing image artifacts or seams. This technology offers exciting opportunities in toxicology, for recording results, viewing images remotely, and applying quantitative morphology or IHC algorithms. However, the implications of working in a GLP environment in compliance with 21 CFR 11 and Japanese ERES guidelines must be addressed. We discuss what we consider to be vendor responsibilities, and offer some suggestions in the adoption digital slide technology in GLP studies.

P-98

***In Vivo* Fluorescent Imaging of MMP Activity in the Thyroid in Relation with Neoplastic Lesions Induced using a Rat Two-stage Thyroid Carcinogenesis Model**

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Molecular imaging has now become a powerful tool for visualization of the biological process in living animals. In cancer research area, this technique has been mainly applied using transplanted tumor models. In this study, we employed an induction model of invasive thyroid carcinomas with high frequency using a two-stage carcinogenesis model that is predicted to mimic the development of clinical cancers, to visualize the process of *in vivo* carcinogenesis. Matrix metalloprotease (MMP) activity was visualized by fluorescent imaging. Male F344 rats were treated with sulfadimethoxine (SDM) at 0.1 % in the drinking water for 11-15 weeks beginning 1 week after initiation with DHPN. To observe MMP activity, MMPSense 680 (VisEn Medical) was intravenously injected to rats one day prior to analysis. *In vivo* imaging was performed after surgical incision under inhalation anesthesia. MMPSense signal was detected at the thyroidal surface sterically enhanced at the periphery, reflecting the signal in the thickened capsule in association with infiltrated inflammatory cells nearby invasive carcinomas. In parallel with enhanced MMPSense signal, high protease activity was detected at the capsule by *in situ* zymography. The results suggest that the *in vivo* imaging of MMPSense signal could be a powerful tool for rapid capture of protease activity linked to invasive growth of carcinomas, and thus this technique could be applied to rapid screening of anti-cancer drugs.

P-99

Histology Pattern Recognition on Common Toxicological Problems

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Whole slide imaging offers the ability to observe and quantitate areas of interest on much larger sample sizes, including an entire tissue sample. Quantitation is typically either morphologic (e.g. cellular hypertrophy or tissue infiltrates) or biomarker expression with special stains (e.g. in-situ hybridization or immunohistochemistry). Quantitation requires that a pathologist either draw a region of interest or provide highly specific tissue staining prior to the computer calculating the results. This step can be particularly challenging in cases with large and morphologically intricate areas of tissue, or when tissue staining is nonspecific. The pathologist needs to be able to identify regions to include or exclude, and then ask the computer to find all similar regions and classify the tissue. Once this histology pattern recognition step has been completed, the pathologist can then run quantitative image analysis on the regions found by the computer across the entire slide.

The histology pattern recognition algorithm Genie (GENetic Imagery Exploitation) from Los Alamos National Laboratory was used in conjunction with Aperio quantitation algorithms to access the ability of a pathologist to train the computer to find different tissue types or histopathology common in toxicology and other studies. The following four examples are demonstrated: human bronchoalveolar carcinoma, mouse spleen, rat liver with necrosis and rat liver with biliary hyperplasia and peribiliary lymphoid infiltrates. It was found that lesions and tissues of interest could be consistently distinguished by histology pattern recognition in the above examples evaluated.

P-100

Malignant Hibernoma in a Crl:CD(SD)IGS BR Rat

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Hibernoma in rats is an extremely rare tumor of brown fat origin, although it is easy to diagnose with its histological and cytological findings. We encountered a case of malignant hibernoma occurred spontaneously in a male Crl:CD(SD)IGS BR rat. In the present report, we describe its histopathological features. 【Case】The rat was applied to the control group in a carcinogenicity study and found dead at 53 weeks old. Before the animal died, labored respiration, decreased food consumption and weight loss were observed over 3 weeks. No palpable mass was detected by the external observation. At necropsy, thoracic cavity contained a firm, tan and solid mass that measured 2.5×3.0×3.5 cm. The mass had comparatively smooth surface, whereas it was partially adherent to the thoracic aorta, esophagus and lung. 【Methods】The mass and other organs were fixed in neutral buffered formalin. Hematoxylin and eosin and oil red O staining were performed in accordance with standard procedures. Formalin-fixed, paraffin-embedded sections were immunostained using an anti-uncoupling protein 1 (UCP-1) antibody. Ultrastructural examination was also performed with the tumor tissue fixed in formalin. 【Results】Histologically, the mass was almost completely encapsulated by fibrous connective tissue. The tumor mass appeared incompletely lobulated with fibrovascular septa. Tumor cells were arranged compactly and most of them were oval to polygonal in shape with multivacuolated cytoplasm and centrally located one nucleus. Vacuoles in cytoplasm were positive for oil red O. The architectural and cytological characters described above revealed this tumor was of brown fat tissue origin. In addition, the areas composed of tumor cells with marked nuclear pleomorphism including karyomegaly and multinucleation, higher nuclear-cytoplasmic ratio and anisocytosis were present. In those areas, tumor cells showed frequent mitoses. Several thrombi, hemorrhagic or necrotic foci were scattered within the tumor mass. Vascular invasion in the tumor capsule was observed, although metastasis or invasion toward the thoracic aorta, esophagus or lung, or distant metastasis to other organs was not observed. Ultrastructurally, tumor cells were characterized by abundant, round to oval mitochondria with transverse closely-packed cristae which is a characteristic finding for brown adipocytes. Various numbers of non-coalescing vacuoles in cytoplasm were also observed. Immunohistochemically, the cytoplasm of the tumor cells was positive for UCP-1 which is known as a specific antigen for brown adipocytes. With the findings mentioned above, this case was diagnosed as a malignant hibernoma.

P-101

One case of synovial sarcoma in the thigh of a male F344/DuCrj rat

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Introduction: Spontaneous synovial sarcoma of rodents is extremely rare. No case of synovial sarcoma was found in 2249 male and 2097 female rats used as control groups of 2-years carcinogenic studies (45 studies in males and 42 studies in females) carried out from 1987 to 2008 at the Japan Bioassay Research Center. The histological characteristics of this synovial sarcoma developing in the thigh are presented.

Materials and methods: A SPF F344/DuCrj rat was housed individually in stainless-steel wire-mesh hanging cages a barrier system animal facility. Water and γ-irradiation-sterilized diet (Oriental Yeast) were available ad libitum. The rat was euthanized under ether anesthesia and necropsied at 110 weeks of age. The specimen was fixed in 10% buffered formalin and was embedded in paraffin. The sections were stained routinely with hematoxylin and eosin (HE), and were also stained with silver and alcian blue (pH 2.5) after hyaluronidase treatment. In addition, immunohistochemical staining was performed for vimentin, cytokeratin and fibronectin. The reactivity was detected using EnVision+ (EV+, Dako, Copenhagen, Denmark) of the two-layer dextran polymer visualization system.

Results: A red mass, approximately 45 mm in maximum diameter, was observed in the left thigh. The mass enclosed the knee joint and was cystic. Microscopically, the cystic mass was composed of two kinds of lining cells, epithelioid cells and fibroblast-like cells. The epithelioid cells had round nuclei and relatively abundant cytoplasm. The fibroblast-like cells had elongated or spindle nuclei and scanty cytoplasm, and were surrounded by the interstitial material. The interstitium was lightly stained with alcian blue and was degraded by hyaluronidase digestion. The rich reticular fiber surrounding the fibroblast-like cells was indicated by silver stain. Immunohistochemically, the fibroblast-like cells stained positive for vimentin and fibronectin. Neither the epithelioid-like cells nor the fibroblast-like cells stained with cytokeratin.

Discussion: The synovial lining layer in the rats consists of two kinds of lining cells: type A and B cells. The type A cell is regarded as a macrophage-like cell, and the type B as a secretory fibroblast-like cell. The present tumor was morphologically characterized by biphasic features composed of epithelioid cells and fibroblast-like cells. The tumor invaded the surrounding skeletal muscle tissue. Therefore, the present case was diagnosed as synovial sarcoma.

The present case had been in the medium dose group for two-year carcinogenicity study. It was considered that this case represented a spontaneous tumor, since was a single case and there were no cases of the synovial sarcoma in the high dose group.

P-102

Case Report: Spontaneous Extra-skeletal Osteosarcoma in Femoral Subcutis of a Female F344 Slc/N Rat.

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A subcutaneous mass of 4x3x3 cm was noted on the right femoral region of a control female F344 Slc/N rat at 83 weeks of age. The rat was killed *in extremis* by unfavorable prognosis due to emaciation, anemia and nasal bleeding.

At necropsy, the mass invaded surrounding skeletal muscle and skin, but there was no relation with the bones. The cut surface of the mass revealed whitish solid area, many cysts containing bloody fluid, and necrotic area. Multiple nodules with hemorrhagic areas and bloody-foamy fluid were observed in the lung and trachea, respectively.

Histopathology revealed sheet-like growth pattern of tumor cells resembling round or comma-shape nucleus in pale eosinophilic cytoplasm. Osteoid tissue was observed at a few areas. Vascular structure lined by normal endothelial cells was noted. The lung nodules were metastasis of the tumor. Hyaline droplets were seen in the renal proximal tubular cells.

Masson-trichrome staining indicated poor collagen. For Watanabe silver staining, each tumor cell was randomly surrounded by argentophil fibers. There was no positive component for Oil Red O or PAS.

Immunohistochemically, tumor cells revealed strongly positive for PCNA and vimentin, partially positive for CD-68(ED-1), Osterix and Osteocalcin (osseous area), but negative for keratin, S-100, von Willebrand factor (factor IIIV related antigen), CD-31, CD-34, desmin, α-smooth muscle actin, lysozyme, α1-antitrypsin and rat MFH antigen. Double-staining for CD-68 and PCNA indicated that most of CD-68 positive cells were negative for PCNA.

These findings indicated that the present case was diagnosed as “extraskeletal osteosarcoma”.

P-103

A case of extraskeletal osteosarcoma in the stomach of an F344 rat

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[Introduction] Extraskeletal osteosarcoma is occuurs in tissues unrelated to the bone or the periosteum. It is such a rare tumor that there are few reports. In some reports, extraskeletal osteosarcoma was found in the subcutaneous tissue and the thoracic cavity (Yoshizawa et al., 2005. Minato et al., 1988). In this report, we describe extraskeletal osteosarcoma in the stomach of an F344 rat.

[Material and method] The animal was a female F344 rat in a long term examination which was sacrificed at the age of 110 weeks. The tumor was fixed in 10% phosphate buffered formalin (pH7.2) and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (HE) stain, Masson’s trichrome stain and immunohistochemical stain for histopathological examination.

[Result] At necropsy, the mass, 18x18x18 mm sized, was observed at the greater curvature of the glandular stomach and the mucosal surface was depressed. At histopathology, tumor cells showed solid growth pattern, and osteoid or bone tissue formation that appeared mesh-shaped was observed in the interstitium. Tumor cells were round to polygonal, but spindle cells were observed partially too. Tumor cells had pale eosinophilic cytoplasm and round nuclei with prominent nucleoli and anisokaryosis, and showed strong atypia. But mitotic figures were few. At immunohistochemistry, the tumor cells were positive for osteocalcin (osteoblast-like cells and osteoid matrix), PCNA and vimentin, but negative for keratin, S-100, ED-1 and α-smooth muscle actin.

[Conclusion] In this case, histopathological and immunohistochemical findings showed that this tumor was osteosarcoma. In addition, since there were no tumors from the bone in other tissues except the stomach, this case was diagnosed as extraskeletal osteosarcoma originating from the stomach. Osteosarcoma in the rat is classified into osteoplastic, fibroblastic, osteoblastic, telangiectatic and compound type by the main components (International classification of rodent tumors, IARC (WHO), 1993). In the present case, it was suggested that this tumor was osteoblastic type because this tumor consisted of osteoblast-like cells and osteoid or bone tissue was scattered.

P-104

Highly differentiated teratoma of the uterus in a mouse

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Teratoma most commonly occurs in the testis and ovary, whereas teratoma of the uterus is rare. We present the histopathological features of uterine teratoma, characterized by highly differentiated tissues, in a mouse.

A mass, 5×5×10 mm in size, was detected in the lumen of the right uterine horn in a 26-weeks-old C57/BL female mouse. This mouse has an experience of pregnancy and parturition three months before sacrifice, but no clinical abnormalities were observed during pregnancy and postpartum periods.

Microscopically the lesion consisted almost of highly differentiated bone including growth plate and myeloid cells and cartilage. In addition, striated muscle, exocrine gland, eye-like structure and melanocytes were observed. These tissues showed little cell-proliferative activity.

These findings indicated that the lesion was composed of mesodermal, ectodermal and/or endodermal components with mature tissues and was regarded as a mature teratoma. Some previous studies have reported that highly differentiated teratomas could be induced from visceral yolk sac in rodent uterine, and they were characterized by highly differentiated components with little cell-proliferative acivity. Our case closely resembled these experimentally induced teratomas.

P-105

Spontaneous Thymic Lymphomas in The NOD/SCID/γ_c^{null} Mouse

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The NOD/SCID/γ_c^{null} mouse is a severely immunodeficient mouse derived from the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse. In addition to lacking T and B lymphocytes, this mouse has multifunctional defects in NK cell activity, macrophage function, complement activity, and dendritic cell function. This strain is known to be an excellent recipient for engraftment of human hematopoietic cells and solid tumors. In this study 2406 mice (8-62 weeks old, 503 males and 1903 females) were subcutaneously engrafted with human tissues. Masses in the thorax were seen in 1 out of 503 male and 15 out of 1903 female animals. The total count was 16 out of 2406 mice (0.7 %). They occurred mainly in the younger mice of 12 to 26 weeks old. Grossly, the masses were located in the anteroventralis of the thorax. They were 0.4 to 2 cm in diameter and whitish with a lobular structure. In 6 animals, large spleens were noted. No significant changes were found in the other organs. Histopathologically, sheets of lymphoblastic cells were observed with a “starry sky” pattern in the thoracic mass. Lymphoblastic cells were small to medium in size and round to slightly irregular in shape with scant cytoplasm. The nuclei were rounded to oval with condensed chromatin. Mitotic figures were numerous. Immunohistochemically the lymphoblastic cells were positive for Thy 1. The lymphoblastic cells were also seen in the spleen, lung, liver, kidney and heart. The gross and histopathological findings lead to the diagnosis of spontaneous thymic lymphoma in NOD/SCID/γ_c^{null} mice.

P-106

A case of suspected malignant mesothelioma in the thoracic cavity of a mouse

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[Introduction] Malignant mesothelioma is known to occur spontaneously in the thoracic or abdominal cavity in male F344 rats, but its occurrence in mice is rare. We report a B6C3F1 mouse in which malignant mesothelioma in the thoracic cavity was suspected.
[Materials and method] This was an untreated male B6C3F1 mouse which died at 100 weeks of age. After necropsy, the thoracic nodules (including the rib), liver, spleen, heart, lung and kidney were fixed in phosphate buffered 10% formalin, embedded in paraffin, and H&E stained specimens prepared. In addition, the nodules were subjected to Masson trichrome staining, PAS staining, Alcian blue staining and anti-WT-1, TTF-1, Keratin and Vientin antibody immunohistochemical stainings, and examined microscopically. Using the part of the nodule fixed in formalin, electron microscopic examination was performed.
[Results] Macroscopically, retention of approximately 1 mL of bloody pleural fluid and sporadic white nodules (3 to 5 mm in diameter) in the dorsal surface of costal pleura were observed. Histopathologically, the tumor consisted mainly of glandular and papillary glandular proliferation associated with slight fibrillar connective tissues, partially showing solid and nest proliferation, and infiltrated into the muscle layer and in the rib. The part of glandular and papillary glandular proliferation consisted of comparatively large and round or roughly round nucleus and tall columnar or cubic cells containing acidophilic cytoplasm. In the solid and nest part, spindle-shaped cells were also observed. Tumor cells showed slight atypia, but mitosis was rare. In a part of tumor cells, Alcian blue- or PAS-positive mucous substance was observed in the cell or glandular cavity. In immuno-histochemical staining, keratin was strongly positive in almost all glandular and papillary glandular structure parts, while vimentin was positive in spindle-shaped cells. WT-1 was positive only in a small number of cells. Other antibodies were all negative. Electron microscopic examination showed microvillus and developed desmosomes.
[Conclusion] Based on the results described above, it was likely that the tumor observed in this mouse could be malignant mesothelioma from its morphological feature.

P-107

Spontaneous Hemangiosarcoma in a 9-week-old Male SD Rat

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The incidence of spontaneous hemangiosarcoma in SD rats was very low. We report a spontaneous hemangiosarcoma in a 9-week-old male SD rat. [Materials and methods] The animal was a 9-week-old male Crj:CD(SD)IGS rat that was applied to the mid dose group of a 1-week toxicity study. During the study period, no abnormalities had been observed in the clinical signs, hematology and blood chemistry. At necropsy, there were multiple and white nodules which varied in size were seen in the enlarged spleen. The cut-surface of the nodules showed solid and grayish white in color with red spots. In addition, there were multiple, small and white nodules in the liver. No gross lesions were seen in any other organs and tissues. All of the organs and tissues, including the nodules, were fixed in 10% neutral-buffered formalin. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin. The nodules in the spleen and the liver were subjected to immunohistochemical staining and electron microscopic examination. [Result] Histopathologically, the spleen was almost displaced by the tumor nodules, which showed invasive proliferation without a capsule. The tumor nodules were composed of spindle shaped cells, with basophilic cytoplasm, indistinct cell borders and round to spindle nuclei showing many mitotic figures. These cells were usually arranged in sheets and solid, but partially in whorls and/or faint alveolar patterns with irregular capillary-like structures containing blood within the lumen. The grossly red spots corresponded to hematomas. The nodules of the liver showed the same histopathological features as those observed in the spleen and the tumor cells in the liver mostly adherent with a sinusoid. There were no particular lesions in other organs and tissues, excluding the proliferation of the same tumor cells in the adrenal medulla (unilateral) and the pancreatic lymph nodes.
Immunohistochemically, the tumor cells were positive for vimentin (mesenchymal cells marker), factor VIII-related antigen, and CD34 (vascular endothelial cells marker), while negative for podoplanin (lymphatic endothelial cell marker). Under electron microscopy, Weibel-Palade like bodies, specific organelles for vascular endothelium, were detected in the tumor cell cytoplasm.
[Conclusion] From these findings, the diagnosis was confirmed as hemangiosarcoma. Spontaneous vascular neoplasms of any structure are uncommon (0-2.8%) in rats, much less the hemangiosarcoma (0.1-0.9%), and the average age for rats with hemangiosarcoma is about 90 to 107 weeks. Hence, occurrence of hemangiosarcoma in a 9-week-old rat is extremely rare.

P-108

One-Year Chronic Toxicity Study of Ozokerite in F344 Rats.

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[PURPOSE] Ozokerite which is refined from wax has been used for a gum base of chewing gum and solidification agents of an oiliness ingredient of cosmetics such as lipstick and lip cream. However, there were few reports regarding the comprehensive toxicological study. To investigate the effects of ozokerite for longer period of time, 1-year chronic toxicity study was performed following 90-day toxicity study recently reported. [Method] Male and female F344 rats were given diet containing 0(control), 0.05, 0.1 and 0.2% ozokerite for 52 weeks. During the treatment period, body weights and food consumptions were measured. At necropsy, rats were anesthetized with ether, and blood samples were collected from the abdominal aorta for hematology and the serum biochemistry. After the blood collection, main organs were weighed and all tissue were routinely processed to carry out the histopathological examination. [Result] In the male, body weights were significantly decreased at doses of more than 0.1%. In the hematology, several parameters such as RGB and MCHC at doses of 0.05% and above, PLT at doses of 0.1% and above were significantly reduced, significant increase of WBC being observed at the highest dose. In the female, changes in almost the same parameters were found at dose of more than 0.1%. In the serum biochemistry, several parameters related to hepatotoxicity were changed in both sexes of all treated rats. Significant elevations of the weights occurred in the liver and spleen at doses of 0.1% and above, and in the lungs at all treated rats of both sexes. In all treated rats, granulomas partly included histiocytosis were found in the liver, spleen, pancreatic, mesenteric, submandibular and Hilar lymph nodes, and bronchus- and Gut-associated lymphoid tissue (BALT and GALT) dose-dependently. [Discussion] In the hematology, the changes of parameters indicating anemia were observed in all treated groups, the lack of decrease of RBC suggesting that the degree of anemia was not serious. It is likely that increases of AST and ALT, and weights of the liver, spleen and lungs result from the formation of granulomas. Considering that Ozokerite is refractory macromolecular substance, it is highly probable that intake of a large amount of ozokerite caused histiocytosis, subsequently systemic granuloma formation.

P-109

Evaluation of the Toxicity of Kombu Extract with 90 Days Dietary Administration to F344 Rats

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Kombu, a seaweed which contains a lot of minerals and vitamins, is used widely in Japanese cuisine. Recently, kombu extract has been consumed as a functional food. However, little is known about the toxicity of kombu extract. The present study was conducted to evaluate the toxicity of kombu extract in male and female F344 rats administered test diet for 90 days.

A total of 80 rats, 6-week-old, were divided into eight groups of 10 rats each. Groups 1~4 (male) and 5~8 (female) received kombu extract at concentrations of 0, 1, 2 and 4% in CRF-1 diet.

No significance in body weight, food consumption, water intake and urine were found in any treatment group compared to the control. Histopathologically, no changes indicating the toxicity of kombu extract were noted in 4% groups of male and female.

In male rats, significant increases in ALP and T-Cho were observed in 4% group compared to the control. In addition, significant increases in absolute weight of thymus were observed in 2% and 4% group compared to the control. In female rats, significant increases in Ht, MCV, and a significant decrease in MCHC were observed in 2% and 4% group compared to the control. Furthermore, significant increases in T-Cho, absolute weights of heart, spleen and liver were observed in 4% group compared to the control.

Therefore, the no observed adverse effect level (NOAEL) of the kombu extract in rats was considered to be 1% (male rats; 0.57 g/kg b.w./day, female rats; 0.58 g/kg b.w./day) in the present study.

P-110

Histopathological findings of SHR/NDmcr-cp rats, a model of the metabolic syndrome

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The aim of the present study: SHR/NDmcr-cp (SHRcp) rats carry nonsense mutation of the leptin receptor gene and are known to develop hypertension, obesity and hyperlipidemia spontaneously. SHRcp rats are known to be an animal model of the metabolic syndrome and diabetic nephropathy, but there have been few detailed reports on histopathological changes other than kidneys so far. In the present study, clinical and pathological examinations were conducted after feeding normal and diabetes-inducing diet for 20 weeks to evaluate detailed characteristics of the model.

Materials and methods: SHRcp rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and randomly divided into two groups at 7 weeks of age, and were fed either a standard diet (MR-Stock; Nosan Corporation, Yokohama, Japan) or diabetes-inducing diet (MR-DBT; Nosan Corporation, Yokohama, Japan) for 20 weeks. Body weight, blood pressure, blood glucose, HbA1c, and urinalysis were examined every 4 weeks. At the end of the study, necropsy was performed after blood sampling for hematology and blood chemistry. Liver, kidney, heart, lung, spleen, pancreas, aorta, and mesenteric artery and vein were subjected to histopathology.

Results: Systolic blood pressure (SBP) of SHRcp rats elevated time-dependently from the starting average of 125 mmHg to the average of 160 to 180 mmHg for 8 weeks, and maintained the range for the rest of the study. Blood glucose levels increased in MR-DBT-fed SHRcp rats from 4 to 12 weeks after the beginning of the experiment, whose average values were 170 to 230 mg/dL, but it declined afterwards. In MR-Stock-fed SHRcp rats, elevations of blood glucose levels were very slight. Blood chemistry performed at sacrifice showed higher values in total cholesterol, triglyceride and phospholipid. Histopathologically, vacuolation of hepatocytes, mesangial expansion and segmental sclerosis of glomeruli, hyaline cast, increase in basophilic renal tubule, inflammatory cell infiltration in renal cortex, hyperplasia of pancreatic islets were observed as disease-related changes. Vacuolation of hepatocytes, hyaline cast and increase in basophilic renal tubule were more severely noted in MR-DBT-fed SHRcp rats. No disease-related changes were observed in the other organs examined.

Conclusion: SHRcp rats developed clinical conditions which resemble human metabolic syndrome and renal histopathological changes which are similar to other animal models of diabetes. In the present study, however, elevations of blood glucose values were transient. Hyperplasia of pancreatic islets was a pronounced character compared to other diabetic animal models.

P-111

Spontaneous neoplastic and non-neoplastic lesion development in aged Slc:WistarHannover/RCC rats

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Recently, NTP made a decision to switch to the WistarHan strain (initially the F344 rat was used) for toxicity and carcinogenicity studies, because of the lower spontaneous tumor incidence and other health related concerns. Spontaneous neoplastic and non-neoplastic lesions in Slc:WistarHannover/RCC rats, expected to undergo increased use for toxicity and carcinogenicity studies in Japan, were therefore investigated to compile background data in our laboratory.

Six-week old 55 male and 55 female rats were housed in polycarbonate cage with hard wood chips, and maintained on MF pellet diet and city water *ad libitum* for 104 weeks, and underwent urinalysis, hematology, blood biochemistry, and pathology examinations.

At 104 weeks, survival rates were 62% for males, 55% for females, and average body weights were 602 g for males, 411 g for females. As spontaneous neoplastic lesions, the most frequent tumors in males and females were pituitary tumors, followed by tumors of the thyroid, testes and mammary glands. However, the incidence of testicular tumors, a problem inherent in F344 rats, were quite low, and LGL leukemia was not found in the present study. A number of non-neoplastic lesions were also evident in heart, liver, kidneys and other organs.

Thus, overall tumor incidences of the Slc:WistarHannover/RCC rats were low, when compared to the spontaneous background tumors in F344 rats. These results clearly indicated that this strain of rats might be appropriate in models for toxicological and carcinogenic studies.

P-112

Age-Related Histopathological Changes in Female Cynomolgus Monkeys and the Effects of Ovariectomy

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We have previously reported on age-related changes in the reproductive organs and mammary glands of female cynomolgus and rhesus monkeys. In this study, we examined age-related changes in the organs and systemic tissues of cynomolgus monkeys originating from China, and the effects of ovariectomy. Formalin-fixed, paraffin-embedded organs and tissues from twelve 10 to 16-year-old non-ovariectomized (NOVX) monkeys and twelve 15 to 17-year-old monkeys which had been ovariectomized (OVX) at the age of about 10 years were examined histopathologically. Hematological and blood chemistry samples, and estradiol-17β and progesterone in serum were examined at necropsy.

As age-related changes, proliferation of mesangial cells and the matrix, thickening of glomerular capillary walls, mononuclear cell infiltration and interstitial fibrosis in the kidney, yellow-brown pigment deposition in the cerebrum and cerebellum, and vacuolation in the pancreatic islet cells were observed in the NOVX monkeys. Amyloid deposition in the pancreatic islets was observed in the three of the OVX monkeys and the incidence of proliferation, swelling, and vacuolation of basophilic cells in the anterior lobe of the pituitary, and intimal thickening of the aorta was greater in the OVX monkeys than in the NOVX animals. The incidence and degree of age-related changes in the OVX and NOVX monkeys were almost identical. As proliferative lesions, ovarian teratoma and mammary hyperplasia in the NOVX monkeys, and bronchioalveolar hyperplasia and papillary hyperplasia of Brunner's gland in the duodenum in the OVX monkeys were observed. Glucose levels in the OVX monkeys were higher than in the NOVX animals, particularly in those with amyloid deposition in the pancreatic islets. Plasma values of gonadal steroids in the OVX monkeys were below the lower limit of quantification, except for progesterone in one animal.

Glomerular lesions in the kidney were a prominent age-related change, but there was no difference in the incidence between the NOVX and OVX monkeys. Amyloid deposition was observed in the OVX monkeys, and proliferation, swelling, and vacuolation of the basophilic cells in the pituitary and intimal thickening of the aorta were greater in the OVX monkeys than in the NOVX animals. It was suggested that ovarian hormones influenced the occurrence of these changes.

P-113

Epidermal Growth Factor Receptor (EGFR) Cooperates with Src Family Kinases (SFKs) in Acquired Resistance to Cetuximab

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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that plays a major role in oncogenesis. Cetuximab is an EGFR-blocking antibody that is FDA approved for use in patients with metastatic colorectal cancer (mCRC) and head and neck squamous cell carcinoma (HNSCC). Although cetuximab has shown strong clinical benefit for a subset of cancer patients, most become refractory to cetuximab therapy. We reported that cetuximab-resistant NSCLC line NCI-H226 cells have increased steady-state expression and activity of EGFR secondary to altered trafficking/degradation and this increase in EGFR expression and activity lead to hyper-activation of HER3 and down stream signals to survival. We now present data that Src family kinases (SFKs) are highly activated in cetuximab-resistant cells and enhance EGFR activation of HER3 and PI(3)K/Akt. Studies using the Src kinase inhibitor dasatinib decreased HER3 and PI(3)K/Akt activity. In addition, cetuximab-resistant cells were resensitized to cetuximab when treated with dasatinib. These results indicate that SFKs and EGFR cooperate in acquired resistance to cetuximab and suggest a rationale for clinical strategies that investigate combinatorial therapy directed at both the EGFR and SFKs in patients with acquired resistance to cetuximab.

P-114

The Role of Phosphoinositide 3-kinases/Akt Signaling Pathway in Maintenance of Cancer Stem-like Cells of Human Malignant Mesothelioma Cell Lines

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Side population (SP) cells determined by Hoechst 33342 dye staining have been identified in various human cancers and are known to be enriched for tumor initiating cells, so called cancer stem cells (CSCs). Here, we report the existence of SP cells in human malignant mesothelioma (HMM) cell lines and their biological properties. A SP assay comprised of staining with Hoechst 33342 dye and flow cytometric analysis revealed SP cells in HMM cell lines, including NCI-H513 (H513), MS-1, LRK1A, NCI-H2373 and MeT-5A cells. The SP cells accounted for 0.05 to 1.32% in the HMM cell lines. The biological properties of SP cells from H513 and MS-1 were further characterized. And the cells were significantly decreased by treatment with verapamil hydrochloride, a blocker of ABC transporter, and survived treatment with cisplatin, a DNA-damaging anticancer drug. Sorted SP cells exhibited higher clonogenicity than non-SP (NSP) cells. Repopulation of SP cells generated both SP and NSP cells, indicating self-renewability. Stemcell properties of SP cells were further supported by up-regulated stemness genes such as ABCG2, Bmi-1, Oct-4 and Notch1. Moreover, phosphoinositide 3-kinases (PI3K)/AKT inhibition by LY294002 decreased SP fraction in conjunction with down-regulation of OCT4.

To the best of our knowledge, this is the first report demonstrating that mesothelial SP cells are enriched for stem-like cells, which warrants further studies to provide new insights on HMM carcinogenesis.