
MEETING REPORT

16th Japanese Pathology for Carcinogenesis Meeting (Hatsugan-Byori Kenkyukai)

Shoji Fukushima

Starting from the time when Dr. Nobuyuki Ito became Professor at the Department of Pathology, Nagoya City University Medical School in 1974, the Department held a rehearsal every year to allow members of staff, both young and old, the opportunity to practise their presentations for the Japanese Cancer Association Meeting of that year before a friendly, if nonetheless critical, audience. The rehearsal meeting was soon expanded to include the groups of Dr. Michihito Takahashi, Dr. Yoichi Konishi, and Dr. Noboru Hiasa, also conducting research in the same area of toxicological pathology related to carcinogenesis and invited to participate because of their association with Dr Ito because of connections within Nara Medical University or Nagoya City University.

After they had joined the rehearsal in 1986 (The first official Hatsugan-Byori Kenkyukai, Carcinogenesis-pathology-Research Association) the same style was maintained until 1992. However, in 1993 it was felt that the general increase in interest in the fields covered demanded a remodeling of the Kenkyukai needed (for the 8th Hatsugan-Byori Kenkyukai), and the groups directly linked to Dr. Takashi Sugimura, Emeritus Director of the National Cancer Center, as well as those of Dr. Takatoshi Ishikawa, Dr. Katsuo Ogawa, Dr. Okio Hino and Dr. Shigeru Okada, among others joined in this opportunity to discuss advances in carcinogenesis research in the pathology area in a quiet and attractive setting conducive to developing research contacts. To allow coverage of the individual interests of the research groups it was decided to change the meeting structure to a symposium style (one speaker from each laboratory presenting their recent findings for open discussion). The success of the meeting, held in Hodaka View Hotel, Nagano guaranteed that interest would increase and now the vast majority of the groups active in Japan in toxicological pathology and carcinogenesis send representatives. The meeting has always been held in the end week of August. One group takes responsibility for organization and selection of the venue. At the 16th meeting, which was held on August 28-30, this year at Biwako Hotel, Ohtsu, it was the turn of with Dr Shoji Fukushima and his colleagues of Osaka City University, and a total of 126 researchers from 29 laboratories attended, .

A special attraction of the meeting is an invited lecture by a leading figure active at the cutting edge of Japanese research in the area of elucidation of mechanisms underlying biological processes. This year the research association was happy to be able to persuade Dr Takehiko Sasazuki to present the current state of knowledge regarding the 'Software' and 'Hardware' in the Organization of the Immune System.



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Special Invited Lecture: 'Software' and 'Hardware' in the Organization of the Immune System.

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Immune response is a crucial self-defense mechanism against invasion of microorganisms such as bacteria, viruses and parasites. This response which is mediated through the interaction of the β - β T cell receptors (TCRs) with antigenic peptides bound to major histocompatibility complex (MHC) molecules. To cope with the various pathogens, the diversity of TCRs theoretically reaches 10¹⁶ through rearrangement of five gene segments (V β , J β , V β , D β , J β) and at random nucleotide addition. However, mature T cells in the periphery express highly restricted TCRs, in that they exhibit tolerance to self-antigenic peptides and recognize foreign antigenic peptides in the context of self-MHC molecules. This mainly results from two reciprocal selection processes, positive and negative selection, acting during T cell development in the thymus.

Since the generation of TCR diversity is a stochastic process, the rearranged TCRs include 'harmful' TCRs that recognize MHC/self-peptide complexes with relatively high affinity. Negative selection is the process to eliminate CD4⁺CD8⁺ thymocytes expressing such TCRs, which plays a central role in establishment of self-tolerance. On the other hand, positive selection is the process that induces the differentiation of CD4⁺CD8⁺ thymocytes into CD4⁺CD8⁻ or CD4⁻CD8⁺ mature thymocytes only when their TCRs recognize self-MHC class I and class II molecules with sufficient, albeit weak, affinity and ensures that the specificity of mature TCR repertoire is directed against foreign antigenic peptides bound to self-MHC molecules.

How MHC/peptide complex determines the reciprocal fate of T cells in the thymus has been a major question in the field of immunology. Since thousands of self-peptides are expressed in association with a MHC molecule, it has been difficult to analyze the recognition by TCRs of MHC/self-peptide complexes in positive and negative selection at a molecular level. To overcome this problem, we developed transgenic-knockout mice where self MHC molecules are occupied with a single peptide. Based on the findings obtained from these mice, we will discuss about TCR-MHC-peptide interaction in the thymus as a 'software' that regulates organization of the immune system.

On the other hand, the immune system is distinguished from other complex systems, in that cells in the immune system are equipped with highly motile property. While macrophages and neutrophils rapidly migrate to the lesion to function in the initial defense, T cells and B cells differentiated in the primary lymphoid organs migrate to particular sites of the secondary lymphoid organs to achieve normal architecture of the immune system and provide the 'place' for proper immune response. Recent advance in chemokines and their receptors have revealed that different chemokines lead particular types of cells to the specific compartments of the immune system. However, the molecular basis underlying this polarized cell migration is poorly understood. We have recently identified a molecule indispensable for lymphocyte motility. Based on the findings of the knockout mice, we will discuss about the role of this molecule in the architecture of the immune system and refer to the 'hardware' in organization of the immune system.

Department of Experimental Pathology, JFCR Cancer Institute

Primal Force in Multistep Carcinogenesis “Eker Rat & Nihon Rat Models of Dominantly Inherited Renal Cancer”

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Cancer is a heritable disorder of somatic cells. Environment and heredity both operate in the origin of human cancer. Hereditary cancers in animals provide valuable experimental models for understanding the mechanisms of disease, and the development of the therapeutic treatments which can be translated into human patients as well as how environmental factors interact with cancer susceptibility genes. Animal models (I) are homologous to human genetic conditions and helpful toward understanding non-hereditary conditions, (II) provide opportunity to study earliest lesions, and (III) provide opportunity to study strain differences in phenotype which may result in finding the modifier genes.

We present the unique Tsc genes mutants models for the study of problems in carcinogenesis; eg., cell stage and tissue/cell-type specific tumorigenesis, multistep carcinogenesis, species-specific difference in tumorigenesis, modifier gene(s) in renal carcinogenesis and cancer prevention.

Very recently, we also discovered a new hereditary renal carcinoma in the rat, and the rat was named the "Nihon" rat and its predisposing gene could be a novel.

Carcinogenesis looks like an opened Japanese fan, because initiated cells grow in several directions and clinical tumors suggest the edge of the fan having many gene abnormalities.

Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute

Possible Involvement of Fas (CD95) in Inhibition of Azoxymethane-Induced Colon Carcinogenesis by Bovine Lactoferrin in the Rat

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Lactoferrin, a multifunctional iron-binding glycoprotein present in various amounts in mammalian milk has physiological importance related to anti-bacterial, anti-virus and immunomodulating effects. Recently, bovine lactoferrin was shown to strongly inhibit development of colon tumors induced by azoxymethane (AOM) in rats. The results indicate that bovine lactoferrin has potential as a natural chemopreventive agent for colon carcinogenesis. To investigate the underlying mechanisms, we studied the influence of bovine lactoferrin on statement of tumor necrosis factor (TNF) receptor family members, Fas (CD95) and TNF receptor type 1 (TNFR1), involved in induction of cell death. F344 rats were administered AOM (15 mg/kg of body weight, s.c.) once a week for three weeks and starting one week after the last injection were fed diet containing 2% bovine lactoferrin or basal diet alone for 27 weeks. As expected, bovine lactoferrin significantly reduced the incidence of tumor in the proximal segment of colon. RT-PCR demonstrated bovine lactoferrin to increase mRNA statement of Fas, more than 2-fold, in the colon mucosa of rats, in good agreement with results for Fas protein statement, evaluated by western blot analysis. In contrast, mRNA and protein statement of TNFR1, as well as their ligands, FasL and TNF α , respectively, was not markedly altered. To further examine the involvement of Fas, effects of bovine lactoferrin on caspase-8 and caspase-3 activation, which contribute to Fas-mediated apoptosis signaling pathways, were analyzed. The active forms of both enzymes were significantly increased in the colon mucosa. Poly(ADP-ribose) polymerase, which is a substrate of caspase-3, was also shown to be reduced. Furthermore, immunohistochemical localization of Fas protein was only demonstrated within the proximal region of the colon mucosal epithelium, where TUNEL positive cells were detected. The results closely correlated with the observed inhibition of tumor lesions and suggest that the mechanism of the observed inhibition of colon carcinogenesis, especially in the proximal segment, may be explained by enhanced Fas statement, with activation of Fas-mediated apoptosis signaling and consequently reduced tumor growth.

Comprehensive Isolation of Genes Differentially Expressed in the Pylorus of Rat Strains with Different Proliferative Responses to Mucosal Damage

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ACI/N (ACI) rats show persistent and strong cell proliferation in response to gastric mucosal damage by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), while BUF/Nac (BUF) rats show transient and limited cell proliferation. This difference is implicated as one of the mechanisms for the high susceptibility of ACI rats to MNNG-induced stomach carcinogenesis. To identify genes involved in the differential cell proliferation, we performed comprehensive scanning for genes differentially expressed in the pylorus of ACI and BUF rats after MNNG exposure. Eight-week-old ACI and BUF rats were supplied ad libitum with 83 g/ml MNNG in drinking water for 2 weeks, and RNA was isolated from the pyloric mucosa of each pool of three rats. Comprehensive scanning was performed with cDNA-representational difference analysis (cDNA-RDA) and cDNA-RDA with elimination of excessive clones (RDA-WEEC). In cDNA-RDA using ACI as the tester and BUF as the driver, 88 clones were analyzed, 15 were considered to be differential in the initial screening, and 6 clones were confirmed to be overexpressed in ACI rats more than two fold by Northern blot analysis or by real-time RT-PCR. By cDNA-RDA-WEEC, one clone was additionally isolated. In cDNA-RDA using BUF as the tester and ACI as the driver, 4 clones were found to be overexpressed in BUF rats. By cDNA-RDA-WEEC, three additional clones were isolated. These clones included some MHC class II genes (highly expressed in ACI), and two interferon-inducible GTPase genes (ACI), MHC class Ia A1b antigen (BUF), and the cellular retinoic acid binding protein II gene (CRABPII, BUF). The overstatement of MHC class I gene in BUF was previously reported [Oka et al., *Cancer Res.*, 58:4107, 1998]. Retinoic acid inhibits the proliferation and invasion of many tumor types, and CRABPII mediates retinoic acid signal transduction. The genes identified in this study are potential candidates, alteration of whose statement could be responsible for the different cell proliferation.

Suppression of H. Pylori-induced Gastritis by Urease Inhibitors in Mongolian Gerbils

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Recently, the acquisition by *H. pylori* of resistance to antibiotics, including clarithromycin and metronidazole, has become a serious problem that may disturb treatment efficacy. Therefore, non-antibiotic agents, which are both highly effective and safe, are required to reduce *H. pylori*-induced gastric lesions. Since, urease has been suggested to be essential for colonization and pathogenesis of *H. pylori* infection, it is expected that urease inhibition might be effective in *H. pylori* eradication or suppression of *H. pylori*-induced gastritis. Therefore, we examined the effects of acetohydroxamic acid (AHA) and flurofamide (FFA), both are urease inhibitors, on *H. pylori*-induced gastritis in Mongolian gerbils. Animals

were orally inoculated with *H. pylori*, and given urease inhibitors in their diet throughout the experimental period of six weeks or for four weeks, starting from two weeks after *H. pylori* inoculation. Animals were sacrificed six weeks after inoculation and macroscopic gastric lesions including edema and hemorrhage were recorded. The wet weights of the whole stomach were also recorded to estimate the degree of gastritis. Viable *H. pylori* in the gastric mucosa was detected with segregating agar plates by loading the mucosal homogenate. With the administration of AHA at doses of 100, 500, and 2500 ppm throughout the experimental period, *H. pylori*-induced gastritis in animals was decreased in a dose-dependent manner, significantly at 2500 ppm. Bacterial infection rates were also significantly reduced to 40-50% of the control value of 100%, by the highest dose of AHA. The potent urease inhibitor, FFA, also caused marked reduction of *H. pylori*-associated gastritis on administration at 100 ppm throughout the six week experimental period or for four weeks after *H. pylori* infection. Animals treated with FFA had few visible gastric lesions, and the proportion infected with *H. pylori* was completely reduced. Thus urease inhibitors may have an ability to suppress *H. pylori*-induced gastritis or eradicate *H. pylori* in humans.

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Reduced Expression of Apc Transcripts in Rat Colon Cancers Induced by PhIP

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The adenomatous polyposis coli (APC) gene plays a key role as a gatekeeper-type tumor suppressor gene in the development of colon cancers. Genetic alterations in *Apc* or β -catenin genes can cause accumulation of β -catenin protein in the cytoplasm and nucleus and as a consequence, Tcf/Lef transcription factors are activated. In turn this causes increased statement of cyclin D1, c-myc, peroxisome proliferator-activated receptor β (PPAR β) and other target genes. However, the situation with regard to epigenetic alterations in the *Apc* and/or β -catenin genes has yet to be fully elucidated. To clarify this point, a rat model of colon cancer induced by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), one of the most abundant carcinogenic heterocyclic amines contained in cooked meat and fish, was employed. After acclimatization to the housing environment for one week, six-week-old F344 and ACI male rats were fed an AIN-93G basal diet containing 400 ppm of PhIP for two weeks, and this two-week PhIP feeding was repeated three times with four-week intervals on only a high fat (HF) diet, followed by a continuous feeding of the HF diet up to the experimental week 60. A total of ten colon cancers, five from F344 and five from ACI animals, were subjected to genetic and immunohistochemical analyses. Accumulation of β -catenin protein in both cytoplasm and nucleus was observed in all the tumors analyzed, although only one half harbored mutations in either the *Apc* or the β -catenin genes. This result suggested that epigenetic molecular mechanisms, distinct from genetic alterations, could be responsible for accumulation of β -catenin in tumors. To further characterize alterations that might be responsible, statement levels of *Apc* transcripts were assessed quantitatively by means of real-time RT-PCR. Seven of ten tumors demonstrated a substantial decrease, by 60 % to more than 90 %. The remaining 3 tumors showed minimal decrease in *Apc* transcripts compared to non-cancerous counterpart tissue by 14, 20 and 32 %, respectively. Immunohistochemical analysis also revealed repressed statement of *Apc* protein in some of these tumors. No correlation was observed between the repressed statement of *Apc* and the presence or absence of mutations in either the *Apc* or the β -catenin genes. In order to clarify the molecular mechanisms underlying the suppressed statement of *Apc* transcripts, the 5'-RACE method was performed first to determine the 5'-end of the *Apc* transcript, and exon 1A, 59 bp in size, was identified, as in the human and mouse cases. The nucleotide sequence of the exon 1A and its upstream promoter region was then determined using two overlapping BAC clones, and found to show high homology to those of humans and mice, with 85.0 % and 92.5 % identity, respectively. Within the 240 bp promoter region, including exon 1A itself, eleven CpG sites were identified, giving a CpG score of 0.51. The methylation status of the exon 1A promoter region is currently being investigated using bisulfite DNA sequencing and methylation-specific PCR.

Molecular Profiling of Genes Up-regulated during Promotion by Phenobarbital-treatment in a Medium-term Rat Liver Bioassay

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In search of genes specific for the promotion stage in carcinogenesis, suppression PCR subtractive hybridization was performed using a phenobarbital (PB)-promotion model based on a medium-term liver bioassay. Two weeks after a single injection of diethylnitrosamine (DEN; 200 mg/kg body wt, i.p.), rats were given 600 ppm PB in the drinking water for up to 64 weeks. For comparison, animals fed 1 ppm ethinylestradiol (EE) or 3000 ppm butylated hydroxytoluene (BHT) in the diet at promotion stage were also included. Rats were subjected to partial hepatectomy (PH) at week 3. Fragments of a total of 67 different genes were isolated from the up-regulated gene population in the liver at day 10 of PB-treatment by subtracting from basal statement in the animal treated DEN+PH alone. To select genes steadily overexpressed during the promotion stage, Northern blot screening was employed for all genes obtained and up-regulation at week 8 of PB-promoted rat livers was found for twenty of signal-detectable 48 genes. The majority of these genes were also up-regulated at week 8 in the livers of rats treated with EE or BHT, and were also constitutively expressed in the DEN(-), PH(-)-untreated rat liver. Among them, 11 genes were found to be overexpressed in hepatocellular carcinomas, produced by PB-promotion at week 64, including *gstm2*, *umat*, apolipoprotein A4, nuclear receptor binding factor-2, CD81, hypothetical protein (HSPC014), *mlrq*-like protein, and one unidentified gene in particular. Considering constitutive statement in the normal rat liver, these genes might be potential biomarkers for the initial screening of non-genotoxic hepatocarcinogens by analysis in two-stage carcinogenesis models.

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Effect of Tamoxifen on Endometrial Adenocarcinoma Development in Mice Initiated with N-ethyl-N'-nitro-N-nitrosoguanidine.

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Tamoxifen (TAM) has been used as an anti-cancer drug for women with breast cancer, because TAM exerts anti-estrogenic effects by binding to the estrogen receptor. But it also acts as a weak estrogen agonist, especially in response to estrogen deficiency in postmenopausal women, and it has been pointed out that the risk of endometrial cancer is greater among women exposed to TAM therapy. In the present study, induction of preneoplastic and neoplastic lesions by TAM was studied in intact and ovariectomized (OVX) mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), using two-stage mouse uterine carcinogenesis method. In both intact and OVX mice experiments, animals were treated with cholesterol (ChL; control) or TAM (5 %) and/or 17 α -estradiol (E2)(0.5 %) pellets implant s.c. from 9 to 25 weeks, and all mice were given a single intra-uterine administration of ENNG (12.5 mg/kg) at 10 weeks of age, dissolved in polyethylene glycol. In both experiments, animals were divided into 4 groups: ENNG+ChL, ENNG+TAM, ENNG+E2 and ENNG+TAM+E2. At the termination of the experiment (week 15 after the ENNG treatment), all surviving mice were killed and the development of uterine proliferative lesions were assessed histopathologically. In intact mice experiment, endometrial proliferative lesions (hyperplasias and/or carcinomas) were observed in all groups, the incidences in TAM- and/or E2-treated groups being two times higher than ChL-treated animals. High induction (11/20, 55 %) of endometrial adenocarcinomas was observed in the E2 group, but this incidence was significantly decreased in animals combined with TAM (TAM+E2)(2/20, 10 %), and no carcinoma was found in the TAM group. Effects of TAM pellets (0, 0.5, 1.5, 3.0 and 5.0 %) on uterine carcinogenesis in mice given ENNG combined with or without E2 were examined. In groups without E2, incidences of all proliferative endometrial lesions in TAM-treated groups were significantly higher than that in ChL-treated animals, the increases being

dose-dependent (45, 75, 90, 95 and 100 %, respectively), although no endometrial adenocarcinomas were observed in all groups. On the contrary, incidences of adenocarcinomas in animals with E2 were disposed to decrease in groups of TAM-dose from 0 to 5 % (55, 40, 21, 15 and 10 %, respectively), although the incidence of all proliferative lesions was same in each group. Effect of TAM on uterine carcinogenesis was also examined in OVX mice. Endometrial proliferative lesions were observed in TAM- and/or E2-treated groups, and the incidences in these groups were significantly higher than that in ChL-treated animals ($P < 0.01$), because any proliferative endometrial lesions were not induced in ChL-treated animals. In the TAM group, however, no endometrial adenocarcinomas were found, and all proliferative lesions (hyperplasias) were only slight. In the E2 group, the incidence of adenocarcinoma was highest and significantly higher than that in the TAM group ($P < 0.01$), and the incidence was significantly decreased in the group combined with TAM (TAM+E2). These results indicate that TAM may have promotion effect, but anti-progression effect, on uterine endometrial carcinogenesis in adult mice initiated with ENNG.

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Mechanistic studies on the Hepatocarcinogenesis of Flumequine

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Flumequine (FL), a quinolone-antimicrobial used for veterinary treatment of infections, was found to elicit hepatocellular tumors in a conventional 18-month carcinogenicity study in mice and hepatocellular necrosis-regeneration cycle was considered to be a possible underlying mechanism. However, Yoshida et al. (Cancer Lett. 1999) reported that FL not only enhanced a development of altered foci and adenomas of the hepatocytes in CD-1 mice by dietary administration at 4,000 ppm for 30 weeks after an initiation of diethylnitrosamine, but also induced small numbers of hepatocellular foci at the same dose without any initiation, and concluded that direct or indirect genetic damage as well as liver tumor promoting effects may participate in the hepatocellular carcinogenicity of FL.

To examine whether FL has any modifying effects on the development of hepatocellular proliferation, groups of heterozygous p53 deficient CBA mice [p53 (+/-) mice], sensitive to genotoxic carcinogens, of both sexes and their wild-type littermates [p53 (+/+) mice] were fed diet containing 4,000 or 0 ppm FL for 26 weeks after an intraperitoneal injection of 5 or 0 mg/kg body weight of dimethylnitrosamine (DMN). Higher incidences of hepatocellular foci were observed in animals receiving FL, with or without DMN-initiation, than in the corresponding control groups in both p53 (+/-) and p53 (+/+) mice. Incidences and multiplicities of foci were generally similar in p53 (+/-) and p53 (+/+) mice, but, in the DMN+FL group, the multiplicity of foci and their PCNA labeling indices were greater in p53 (+/-) mice. There were also small numbers of hepatocellular adenomas and/or carcinomas in the DMN+FL group of p53 (+/-) mice, FL alone group of p53 (+/-) mice and DMN+FL group of p53 (+/+) mice. Induction of hepatocellular tumors in these mice within a relatively short period strongly suggests that mechanisms such as direct or indirect damage to DNA might be responsible for the hepatocarcinogenesis.

Since FL inhibits gyrase of bacterias, we determined the 50% inhibition concentration (IC50) of the activity of gyrase and topoisomerase II in FL and other quinolones to compare the degree of their inhibition in each chemical. The selectivity (IC50 of topoisomerase II/IC50 of gyrase) in FL was lower than that of other quinolones, suggesting the possibility that FL has an inhibiting effect of DNA synthesis to mammalian cells.

In order to clarify the mechanism of the hepatocarcinogenesis of FL, male C3H mice were fed diet containing 500 or 0 ppm phenobarbital for 13 weeks after the dietary administration of 4000 ppm FL for 2 weeks at an initiation stage. At 2 weeks of treatment of FL, the levels of mRNA of various enzymes, signal transduction-relating factors, and cell proliferating-relating factors in the liver were quantitatively analyzed using Atlas Mouse Cancer 1.2 microarray (Clontech Labs. Inc., USA). The microarray analysis showed that the mRNAs of glutathione S-transferase, MAP kinase 7, bcl-2, Cdk5, EphB2 receptor protein tyrosine kinase ligand, FGF7, frizzled homolog 3 and PKC-epsilon were up-regulated in the FL treated group. The results suggest that FL affects the signal transduction system indicating inhibition of apoptosis and enhancement of cell proliferation.

The results of our studies obtained so far strongly suggest that FL has an initiating effect as well as a promoting effect to the liver of mice. Further studies on the DNA damage of FL are now in progress.

Structural and Functional Characteristics of Blood Vessels in Mouse Hepatic Tumors

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Tumor growth requires development of new vessels for blood supply. However, it is poorly understood whether functions and structure of tumor vessels are different from those in normal tissues. We then compared blood vessels in dissected tissue samples of normal hepatic tissues and chemically-induced hepatic neoplasia in B6C3F1 mice by electromicroscopy and immunohistochemistry. Male B6C3F1 mice were intraperitoneally administered diethylnitrosamine (DEN) at a dose of 5 mg/g body weight at the age of 2 weeks. The mice were maintained on normal diet and water ad libitum and sacrificed after 8-12 months. The livers were fixed by perfusion through the portal vein or vena cava with 4 % formaldehyde solution and embedded in paraffin. H&E staining revealed that sinusoids in foci and adenomas were dilated, and hepatic plates were thickened due to layers of multiple cells. Electromicroscopy revealed that composition of sinusoidal wall cells in tumors is largely different from that in normal tissues, and the sinusoidal fenestration was lacking in tumors. In addition, blood vessels seem to be highly sensitive to LPS. When LPS was intraperitoneally administered at a dose of 10-100mg to tumor bearing animals, tumors became necrosis within 8-24 hours. As this change was not observed in the tumors of C3H/HeJ mice, that are resistant to LPS, the necrosis is clearly LPS-dependent. Statement of anti-coagulation factor, thrombomodulin, was less prominent in tumors than the normal tissues after LPS treatment, suggesting that altered statement of the factors relating to intravascular coagulation may be responsible for high sensitivity of hepatic tumors to LPS.

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Progression of Human Colonic Adenoma Cells to Adenocarcinoma Cells Through Chronic Inflammation Induced by Co-implantation with a Foreign Body in Nude mice

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The roles of inflammation in the malignant progression of tumors during the multistep carcinogenesis have been much discussed but remain to be elucidated. To determine the direct contribution of inflammation to the colon carcinogenesis, we established in a new model of progression of human colonic adenoma cells using a nude mouse, in which progression is accelerated by co-implantation of a foreign body.

FPCK-1-1 cell line, derived from a colonic polyp in a patient with familial adenomatous polyposis, is non-tumorigenic when injected s.c. into nude mice in cell suspension by the numbers up to 5 x 10⁶ cells per mouse. However, implantation of 1 x 10⁵ FPCK-1-1 cells attached to plastic plate induced first acute and then chronic inflammation, and formed progressively growing tumors, which were histologically determined as moderately differentiated adenocarcinoma in 65% of mice. On the other hand, gelatin sponge induced acute but not chronic inflammation due to its spontaneous absorbency and formed tumors in 8% mice after co-implantation with 5 x 10⁶ FPCK-1-1 cells. Cell lines established from the growing tumors were found to be tumorigenic when injected into mice even without foreign body. The arising tumor from the adenoma cells implanted being attached to plastic plate was surrounded by highly proliferating fibrous stroma. This fibrous tissue, rather than attachment to the plastic plate substrate, was considered essential for the malignant progression because the tumors were formed after injection of FPCK-1-1 cells into the fibrous tissue from which the plastic plate had been removed prior to the cell injection.

The present results demonstrated that inflammation-associated stroma promoted the conversion of colonic adenoma cells to adenocarcinoma cells and the model will be useful to detect possible chemopreventive agents against inflammation-associated carcinogenesis.

Department of Cancer, RIRBM, Hiroshima University

Regulation of Estrogen Receptors α and β in the Rat Prostate - The Involvement in Prostate Carcinogenesis

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It has been demonstrated that estrogen as well as testosterone plays a role in prostate carcinogenesis. Recent investigations have revealed that a new subtype of estrogen receptor (ER), β type, is expressed preferentially in the prostate gland in rats, suggesting that estrogen may act directly on the gland. We, then, examined mRNA levels of ER α and β in the gland in rats treated estrogen and/or testosterone at different ages.

1, 2, 4 and 9 week-old male F344 rats were castrated and/or treated with estrogen and testosterone for a week. Animals were sacrificed under ether anesthesia and the prostate tissues were removed and immediately frozen. Total RNA was prepared by a modified acid guanidium thiocyanate-phenol-chloroform extraction method. ER α , ER β and VEGF mRNAs were measured with a competitive RT-PCR technique. Rat prostate cancer cell lines, AT3 and PLS10, maintained in culture medium with charcoal stripped sera and treated with hormones, were also examined.

ER α was the major type of ER expressed in 2 week-old rats, while dominant statement of ER β mRNA was apparent at older age groups. ER α mRNA statement decreased by castration along with decreases in the VEGF statement as well as the prostate weight. In the castrated animals, testosterone administration induced ER α β mRNA levels within 24 hours. Estrogen administration combined with testosterone did not change the expression of ER α β . Although the higher ER β statement was detected also in rat prostate cancer cell lines, it was not regulated by testosterone. Our results indicated that the statement manner of ER α and β in the prostate gland changed dramatically during development from newborn to adulthood. This may be explained in part by the regulation of ERs with testosterone, whose production increases after puberty. The discovery of ER β has given a rise to new possibilities regarding estrogen's roles in the prostate gland. Our findings of developmental and/or hormonal regulation of ERs may suggest certain roles of ER α and β and its involvement in carcinogenesis in the prostate gland.

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A Processed Grain Food (Antioxidant Biofactor, AOB) Inhibits Renal Injury Induced by Ferric Nitrilotriacetate I in Wistar Rat.

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We have developed an experimental model of ferric nitrilotriacetate (Fe-NTA)-induced oxidative nephrotoxicity and renal cancer. This model has been proved excellent to see an antioxidant effect of food factors in vivo, and has been used widely now. In the present report, we tested the effect of a processed grain food (AOB) on acute renal damage in rats induced by Fe-NTA. AOB was provided by AOA Japan (Kobe, Japan). Other chemicals were of analytical grade from Wako Chemicals (Osaka, Japan). Male Wistar rats of six-week-old were purchased from SLC (Shizuoka), and were given an AOB-containing diet (AOB to basal diet :1:12 ratio) ad lib. Ten days after feeding, Fe-NTA (7.5 mg Fe/kg b.w.) was given intraperitoneally. All rats were sacrificed under ether anesthesia 3 hours, 5 hours and 24 hours after Fe-NTA. Histological observation by H & E staining, and an observation of apoptosis by 8 OH-dG immunohistological staining were performed. The lipid peroxidation was observed histochemically by 3-hydroxynonenal immunohistochemistry and chemically by thiobarbituric-acid-reactive-substance formation. The results showed the marked inhibition of the proximal tubular damage and lipid peroxidation in AOB-fed rats. It is shown that AOB is an effective antioxidant in vivo.

Difference and Interaction between Endogenous and Exogenous Hepatocarcinogenesis in Rats

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Carcinogenesis is a process consisting of qualitatively different, multiple steps. These steps are processed as an accumulation of genetic and epigenetic alterations mediated by not only exogenous factors, like environmental chemicals and microorganisms, but also a variety of endogenous factors. It is thus apparently demanded for the control of cancers to investigate the details of both exogenous and endogenous carcinogenic mechanisms. Furthermore, differences and interactions between these two mechanisms should then be assessed. For such studies, appropriate animal models are required, but models for endogenous carcinogenesis are not sufficiently available, unlike exogenous cases. In this context, we got interested in the dietary choline deficiency model, because hepatocellular carcinomas (HCCs) are induced in rats without administration of any exogenous carcinogens. We developed a choline-deficient, l-amino acid-defined (CDAA) diet by replacing proteins of a widely-used, semipurified, choline-deficient diet by pure amino acids in order to establish an endogenous rat hepatocarcinogenesis model capable of inducing HCCs at a high incidence. Using this model, we have been investigating the endogenous hepatocarcinogenic mechanisms, comparatively with an exogenous rat hepatocarcinogenesis model featuring the initiation with diethylnitrosamine (DEN).

In the livers of rats, chronic feeding of the CDAA diet induced glutathione S-transferase-positive, putatively preneoplastic lesions that were then progressed into hepatocellular adenomas and eventually HCCs. Oxidative stress and the accumulation of a variety of its mediated hepatocyte injuries and signaling alterations have been revealed to participate in the endogenous hepatocarcinogenic mechanisms. Point mutations of the β -catenin gene were rare and lacked amino acid substitution in the endogenously induced HCCs by the CDAA diet, whereas such mutations with amino acid substitutions were detected in almost a half of the exogenously induced HCCs by DEN. In contrast, the endogenously induced HCCs frequently bore hypomethylation and thereby over-statement of the c-myc gene. One third of such HCCs was associated with altered TGF- β signaling due to either mutation of the Smad2 gene or reduced statement of the TGF- β receptor type II gene. The alteration on the TGF- β signaling pathway was not detected in the exogenously induced HCCs. It is, therefore, suggested that the endogenous hepatocarcinogenic mechanisms are different from the exogenous hepatocarcinogenic mechanisms.

N-(4-Hydroxyphenyl)retinamide and all-trans-retinoic acid inhibited both the CDAA diet-induced and DEN-induced hepatocarcinogenesis, whereas their inhibitory potencies were much superior in the endogenous case to those in the exogenous case. 1'-Acetoxychavicol acetate, a constituent of a Southeast Asian ginger-like plant, inhibited the endogenous, but not exogenous, hepatocarcinogenesis. Conversely, green tea extract inhibited DEN-induced, but not the CDAA diet-induced, hepatocarcinogenesis. Furthermore, nobiletin, a constituent of Citrus fruit, enhanced the endogenous and inhibited exogenous hepatocarcinogenesis. It is thus indicated that the CDAA diet-induced endogenous and the DEN-induced exogenous hepatocarcinogenesis models behave differently towards the modifying effects of chemicals, possibly due to their different underlying mechanisms. This is important information for the chemoprevention against cancers.

These results suggest the qualitative difference present between the CDAA diet-induced endogenous and the DEN-induced exogenous hepatocarcinogenic mechanisms in rats. The further studies are being conducted to elucidate the details of such a difference especially in the early phase and to obtain information about the interaction between the endogenous and exogenous carcinogenic mechanisms.

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Carcinogenicity of an Arsenic, Dimethylarsenic Acid in Animals

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Although numerous epidemiological findings have indicated that arsenics are associated with increased incidences of lung, skin, liver, and bladder cancers, experimental data from animal models to support the hypothesis of carcinogenic effects are limited. Dimethylarsinic acid (DMA) is a major form of organic arsenic in the environment. In addition, it is a major metabolite of ingested inorganic arsenics in most animals. We have recently shown that DMA has carcinogenic effects in F344 male rats with a various experimental protocols. Inorganic arsenics (arsenite and arsenate) are metabolized to monomethylarsonic acid (MMA), DMA, and trimethylarsine oxide (TMAO) in most mammals. Ingested DMA is also metabolized and excreted in urine as MMA, DMA, and TMAO. Promoting effects of sodium arsenite and these related organic arsenics (MMA, DMA, TMAO) in a rat submultiorgan-carcinogenesis test were therefore investigated.

To establish semi-widespectrum initiation of organs and tissue, male F344/DuCrj rats in groups 1-6 were treated sequentially with BBN (0.05 % in drinking water) for 4 weeks and EHEN (0.05 % in drinking water) for 2 weeks. The target organ of BBN is the urinary bladder and those of EHEN are liver and kidney in rats. After a 2-week interval, groups 2-6 were given 100mg As/L as MMA, DMA, TMAO, AsBe or 10mg As/L as NaAsIII in the drinking water; the dose of AsIII was one-tenth that of the other arsenicals because of the much higher acute toxicity of this agent. With regard to bladder carcinogenesis, DMA most-strongly enhanced the tumor induction, followed in decreasing order by MMA and TMAO, whereas AsBe and NaAsIII did not. The enhancement of bladder carcinogenesis was correlated with production of one unknown urinary metabolite of arsenics. These results revealed that MMA and TMAO have carcinogenic potential, as well as DMA.

Promoting potential of DMA and related organic arsenics (MMA and TMAO) on rat liver carcinogenesis was further investigated using a rat medium-term bioassay for carcinogens. The three compounds were administered to male F344/DuCrj rats at a dose of 100ppm in their drinking water. Increases in the numbers and areas of GST-P-positive foci in the rat livers treated with all three compounds were found as compared with controls. The potency of promoting activity was almost the same with MMA, DMA, and TMAO. Formation of 8-hydroxydeoxyguanosine was increased in the livers along with induction of CYP2B1 and hydroxy radicals. These results suggest that DMA and related organic arsenics (MMA and TMAO) have promoting potential in rat liver and its mechanisms could be related to generation of hydroxy radicals and DNA damage.

Male F344/DuCrj rats were administered 12.5, 50, or 200 ppm of DMA in the drinking water for 104 weeks. From weeks 97 to 104, urinary bladder tumors were observed in 0 of 33, 8 of 31, and 12 of 31 animals, respectively. No bladder tumors were observed in the controls. PCR-SSCP analysis of the bladder tumors revealed mutations to be few in H-ras (exon1: 10%, 2/20) and lacking in p53, Ki-ras, and -catenin genes. Also no alterations were observed in 18 microsatellite loci in 16 carcinomas. Immunohistochemical analysis of the bladder tumors revealed high statement of COX-2 and cyclin D1 and decreased statement of p27. These results indicate that DMA induced urinary bladder cancer has characters different from those induced by genotoxic carcinogen such as BBN.

Susceptibility to Lung Carcinogenesis in F344, Long-Evans Cinnamon and Long-Evans Agouti Rats

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Individual susceptibility to lung cancer may depend on genetic capacity to metabolize environmental carcinogens. In the present study, susceptibilities to N-diethylnitrosamine (DEN), N-methyl-N-nitrosourea (MNU) and N-nitrosobis(2-hydroxypropyl) amine (BHP)-induced carcinogenicity in F344, Long-Evans Cinnamon (LEC) and Long-Evans Agouti (LEA) rats was examined.

Experiment 1: Six-week-old male F344 and LEC rats (n=21) were given s.c. injections of 100 mg/kg DEN in weeks 0, 2 and 4, and killed in week 50. Experiment 2: Seven-week-old male F344 and LEC rats (n=21) were given weekly i.p. injections of 50 mg/kg MNU (pH 6.0) in weeks 0 to 5, and killed in week 24. Experiment 3: Six-week-old male F344, LEC and LEA rats (n=21) were given 0.1% BHP in their drinking water for 12 weeks, and killed in week 24.

Experiment 1: Incidences of lung adenomas in F344 and LEC rats were 20/21 (95%) and 0/21 (0%), respectively. Incidences of hepatocellular carcinomas in both strains were 100%. Experiment 2: Incidences of lung adenomas in F344 and LEC rats were 12/14 (86%) and 0/13 (0%), respectively (animals died by the end of the experiment were excluded). Incidences of colon cancer in F344 and LEC rats were 3/14 (21%) and 12/13 (92%), respectively. Experiment 3: Incidences of lung tumors (adenomas plus carcinomas) in F344, LEC and LEA rats were 14/14 (100%), 2/10 (20%) and 7/7 (100%), respectively (the experiment was not terminated, yet). Histologically, multiple adenomas developed in F344 rats, and the incidence of squamous cell carcinomas was higher in LEA rats than those in other two strains. Incidences of liver tumors (adenomas plus carcinomas) in these strains were 0/14 (0%), 10/10 (100%) and 5/7 (71%), respectively.

LEC rats were the most resistant strain to DEN, MNU and BHP-induced lung carcinogenesis, and it was thought that this might be a useful model strain to investigate the susceptibility to lung carcinogenesis. Causes of strain difference must be elucidated.

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Genetic Steps of Rat Hepatocarcinogenesis

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Carcinogenesis consists of multiple biochemical steps. These steps involved a number of host genes, which can be polymorphic in a species. Genetic susceptibility or resistance to cancer is a sum of effects of such polymorphic genes. Studying difference in cancer susceptibility among inbred strains of mice or rats, we can expect to elucidate such a biochemical step critical to carcinogenesis, and on the other hand, to pursue the mechanism of genetic differences in cancer susceptibility among individuals. These are our general purposes to study various carcinogenesis models in experimental animals.

The DRH strain rats, an inbred strain selected out by continuous feeding of liver carcinogen 3'Me-DAB to parental closed colony Donryu rats, were highly resistant to hepatocarcinogenesis by 3'Me-DAB and other hepatocarcinogens. We studied genetic resistance on precancerous and on progression stages by feeding 3'Me-DAB to F2 rats between DRH and susceptible F344 either for 7 weeks or 20 weeks. Quantitative trait locus (QTL) analysis on parameters for GST-P enzyme altered foci, such as number and size of foci, GST-P mRNA levels in the liver after 7 weeks of feeding revealed 2 clusters of QTL on RNO1 and 4. Both QTL affected all parameters. On the other hand, analysis of quantitative parameters for liver cancers after 20 weeks feeding revealed that the QTL on RNO1 affected on GST-P mRNA level and the QTL on RNO4, size and number of tumors moderately. DNA adduct formation by the carcinogen is equivalent between DRH and F344. DRH rats were resistant despite of much weaker induction of a detoxifying enzyme GST-P. Therefore, we assume that the primary defect in DRH rats is failure to develop enzyme altered foci, in which secondary genetic events leading to hepatocellular carcinoma take place as a rare event.

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Screening of a Putative Connexin 26-associated Protein

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It is widely known that connexins form a family of tumor suppressor genes. Our laboratory has previously shown that transfection of connexin 26 (Cx26) suppresses growth of HeLa cells, while the other connexins cannot in spite of their ability to restore gap junctional intercellular communication efficiently. Based on a series of results obtained from our own and other laboratories, we have hypothesized that Cx26-mediated cell growth control should involve protein-protein interactions between Cx26 and some other proteins, that may function downstream of Cx26 signal transduction.

To examine the above hypothesis, we screened a cDNA library from HeLa cells with the use of the Cx26 internal loop domain as the bait by yeast two-hybrid system. Only one candidate gene, (named AP26) was isolated and identified. The sequence analysis revealed that the AP26 gene was identical to the reported UXT gene, which is located closely to the ELK1 locus in Xp11.23-p11.22, and that the nucleotide sequence was well conserved between human and mouse. AP26 statement was detected ubiquitously in every examined organ by Northern blotting or RT-PCR as well as in many cancer cell lines. Results from characterization of AP26 in cultured cells by immunoprecipitation and immunostaining analysis will be presented.

Analysis of Activation Mechanism of Gelatinase A and B and Their Predictive Values for Metastasis

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Venous permeation leading metastasis is one of the critical matters when considering prognosis of patients with malignant tumor. The secretion and activation of matrix metalloproteinases (MMPs) play an important role in degrading tissue around tumor including wall of blood vessel. In the inoperable cases, clinicians select the way of chemotherapy binding other methods such as irradiation, but sometimes there are no effective kinds of treatment for prevention of metastasis through venous permeation. We investigated gelatinase A and B in the urine of patients (73 cases, Noto General Hospital 2000) and tried to individually correlate between gelatinolytic activity and result of urinary cytology. Also, the study of autopsied patients (4 cases, KMU 2000-2001) who received operation to resect their primary malignancy with observation of venous permeation indicated that primary tumors of systemic organ except urinary tract have activation mechanism of MMP-9 (92kDa gelatinase/gelatinase B) via plasmin system. In order to apply our data of comprehensive gelatinolytic activity in tissue levels to clinical use, we developed new double stain technique using non-RI, film in situ zymography (FIZ). The results on venous permeation of cancer cells using this method were similar to those occurred in rupture of atheromatous plaque. We suggest that the unique system of estimation of comprehensive gelatinolytic activity with morphological reports works well as the novel way of tissue-orientated evidence based medicine (TOEBM). TOEBM helps clinicians deciding the most optimal kind of drug and its dose individually, and makes the prognosis of patient with malignancy better.

Examination of β -Catenin-accumulated Crypts, Putative Premalignant Lesions for Rat Colon Cancer

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Our previous studies clarified that β -catenin-accumulated crypts, independent of aberrant crypt foci (ACF), are probably direct precursors for colon cancers in rats. Presently, we investigated effects of chemopreventive agent or tumor promoter on the statement of β -catenin-accumulated crypts, and the results were compared with those on ACF. Male F344 rats were administered azoxymethane (AOM) s.c. at a dose of 15 mg/kg body weight, once weekly for 3 weeks to induce the premalignant lesions. As the chemopreventive agent or tumor promoter, was used COX-2 inhibitor, celecoxib or cholic acid. The dietary administration of celecoxib (150 and 1500 ppm) caused significant inhibition in the frequency and growth of β -catenin-accumulated crypts. The efficacy of celecoxib on β -catenin-accumulated crypts was more evident than of ACF. In contrast, the cholic acid (0.5%) increased the statement of β -catenin-accumulated crypts. These findings may represent additional evidences that β -catenin-accumulated crypts are direct precursor lesions of colon cancer. The results also suggest that β -catenin-accumulated crypts could be a novel biomarker for chemopreventive agents for colorectal cancers.

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In Vitro Analysis of Lymphnode Metastasis by Human Colon Cancer Cells Using Immortalized Human Thoracic Duct-derived Lymphatic Endothelial-like Cells

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Although various evidence has been accumulated regarding hematogenous metastasis, the mechanism of lymphnode metastasis still remains unclear. This is partly because of the lack of lymphatic endothelial cell lines of human origin. In this study, we established a lymphatic endothelial-like cell line (HuTLEC) and a human colonic carcinoma cell line (COLM-5) with high lymphnode metastatic potential and investigated their interaction in vitro. An immortalized lymphatic endothelial-like cell was established from a primary endothelial cell culture from the human thoracic duct by the infection of a retroviral vector containing human papillomavirus HPV16 E6 and E7 gene and human telomerase reverse transcriptase subunit (hTERT). This cell line grows stably with more than 100 population doubling and showed bFGF, VEGF and heparin-dependent growth. The line also revealed statement of Flt-4, a receptor for VEGF-C, at the mRNA and protein level. Immortalization of HuTLEC cells was found to result from activation of TERT and inactivation of Rb (retinoblastoma) pathway. Tube formation by HuTLEC cells was significantly stimulated by the addition of the conditioned medium prepared from COLM-5, but not from other COLM cell lines without lymphnode metastatic potential. These results suggest that lymphangiogenesis induced by tumor cells may play a role in lymphnode metastasis of colon cancer. Thus, it may well be a useful model for understanding the mechanism of lymphnode metastasis.

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Radio Frequency Electromagnetic Fields and Cancer Risk

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Epidemiological studies have indicated that exposure to magnetic fields may be associated with an increased cancer risk in the general population, although the data for specific groups at high risk do not necessarily lend any support for this. The situation is complicated by differences between industrial high exposure to electric and/or magnetic fields, derived not only from 50 or 60 Hz power frequency sources, but also from high frequency (radio frequency) transmitters. Since the use of portable cellular phones is rapidly expanding in many countries, the potential health hazards to humans of the radio frequency electromagnetic fields that they use is therefore of great concern. We have conducted a series of animal experiments to assess effects on diethylnitrosamine-initiated rat liver carcinogenesis by 900MHz and 1.5GHz electromagnetic near fields and 1,2-dimethylbenz[a]anthracene-initiated mouse skin carcinogenesis by 1.5GHz electromagnetic near fields, but the results were all negative. The present review of the literature suggests that long-term exposure to electromagnetic fields is unlikely to result in carcinogenesis in rats or mice. Furthermore, findings with promotion assays after carcinogen initiation provide little support for any enhancing effects although weak influence cannot be ruled out. Attention should therefore be concentrated on feasible mechanisms of action so that firm conclusions can be drawn. Mutation assay of mouse brain has also been conducted using Big Blue Mouse, which had been exposed by 1.5GHz electromagnetic near fields. Furthermore, long-term studies of 1.5GHz electromagnetic near fields on rat ethylnitrosourea-initiated brain carcinogenesis have been now undergoing in our laboratory.