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FORUM

Meeting Overview

Mechanisms of Susceptibility to Mouse Liver Carcinogenesis

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Mechanisms of Susceptibility to Mouse Liver Carcinogenesis, the first Society of Toxicology (SOT)-sponsored "Current Concepts in Toxicology" meeting, was held September 8–10, 1997, at the Carolina Inn in Chapel Hill, North Carolina. It was cosponsored by the Carcinogenesis Specialty Section in conjunction with the North Carolina Regional Chapter of SOT. The mouse liver carcinogenesis meeting was a success, with 140 registrants and a substantial amount of lively discussion. Opening remarks by SOT President Michael McClain set the tone for the conference by indicating the prevalence of rodent liver neoplasms in cancer bioassays and specifically the sensitivity of the mouse liver as an indicator of cancer risk for ostensibly nongenotoxic compounds. Thus, the need for understanding the mode of action for the induction of tumors at this site has continued relevance in delineating the pathogenesis of cancer development and in assessing whether there should be concern for human cancer risk from chemicals that increase the incidence of mouse liver tumors. The chairperson for the symposium, Thomas Goldsworthy, indicated that the main primary focus areas for the conference were the biology and regulation of liver tumor formation, the molecular genetics of liver cancer susceptibility genes, and risk assessment issues associated with the induction of mouse liver tumors.

On the first day of the conference, Carl Barrett made an interesting presentation on the primary issues associated with cancer susceptibility of populations and individuals, including the influence of gene interactions, gene–environment interactions, and the importance of understanding the context of chemical exposure on the background of cancer susceptibility, sensitivity, and risk-modifier genes. In this context, the development of mouse models for assessing cancer risk and the pathogenesis of hepatocarcinogenesis must emphasize both the similarities and the differences of the individual models for the purpose intended, as well as the age, disease status, gender, genetics, nutrition and diet, and coexposures on these endpoints.

Joe Grisham provided an overview of the similarities and differences in the molecular pathogenesis of hepatocarcinogenesis between rodents and humans; he emphasized the similarities in the histopathology, but the differences in the etiology and, to some extent, the pathogenesis of liver cancer. His talk stressed the early contribution of alterations in growth factor pathways to hepatic neoplasia in both mice and humans. Additionally, the genes involved in growth control, including oncogenes, suppressor genes, genes in the apoptosis pathways, cyclins, and their regulatory partners may be important in the cellular dysregulation that results in carcinogenesis. Specifically, increased mitogenic signaling, abrogation of cell-cycle checkpoints, decreased apoptosis, a lessening of differentiation, telomere shortening and telomerase activation, and progressive karyotypic instability may all contribute to the induction and progression of hepatocarcinogenesis. These processes may be modulated by chemical exposure, with each individual neoplasm the result of distinct cell and molecular changes. In his talk, Joe Grisham emphasized the role of alterations of proliferative control, apoptosis control, and differentiation status as potential contributors to mouse liver tumor development.

Bob Maronpot presented an excellent discussion of the history of the current bioassay and the choice of the B6C3F1 mouse as an important model for the NTP assessment of carcinogenic potential. He emphasized the use of models as surrogates for predicting human response. Alternative models for carcinogenicity testing are under consideration, and we need to be clear as to the purpose for selecting an alternative and whether the alternative is as a substitute, an adjunct, or a screen. In defining requirements for alternative models for hazard identification, we need to decide which types of carcinogens we wish to identify (and not identify), what the gold standard is for such decisions, and how the data generated will be used. New models range from the neonatal mouse model and initiation–promotion models to several transgenic models, including the TG.AC, the TgHras2, and p53+/− mice. The

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evaluation of new models should potentially include known human carcinogens, carcinogens from the 2-year bioassay, and some site-specific carcinogens, assuming that transspecies, multisite, high-dose, 6-month carcinogens will be detected. It is hoped that the findings will be tissue specific and mechanistically relevant. Several prevailing myths were dispelled in light of current database evaluations. For example, analysis of the database demonstrated that mouse liver tumors in females are the most prevalent single site in the bioassay. Strain differences are clearly observable in the database reevaluations, but were reflected primarily in a difference in the latency and magnitude of the response. If one increases the dose, the multiplicity of the observed tumor response is enhanced and the latency reduced. Mouse liver tumors are a reproducible response, but the biological relevance of the induction of mouse liver tumors to human risk is not known.

Norman Drinkwater, the keynote speaker, described a genetic approach to understanding strain differences in mouse liver tumor susceptibility after chemical exposure. These studies examined either spontaneous tumors or those arising after perinatal exposure to carcinogens such as diethylnitrosamine. A concordance in the relative rank of a series of recombinant inbred strains with respect to the multiplicity of chemically induced and spontaneously arising tumors was observed, with one exception, the BxH-4 strain, which has a high incidence of spontaneous tumors and a low incidence of chemically induced liver tumors. Of the mouse strains in common use, the C3H is a more sensitive strain than the C57Bl/6 mouse with respect to both spontaneous and chemically induced tumors. The net growth rate of the preneoplastic lesions is greater in the C3H than in the C57Bl/6 mouse. The liver lesions in the DBA and the C3H mouse strains have similar growth rates, while the number of lesions as a function of age is greater in the C3H mouse. Analogous to the scheme proposed for mouse skin, it was suggested that the conversion step in early promotion is enhanced in the C3H mouse and that the differences in the response of the C3H and DBA strains to partial hepatectomy were supportive of this contention. Under certain conditions, an increase in Hras1 mutation is indicative of carcinogen exposure, while in other cases selection of preexisting Hras1 can occur as a result of chemical exposure. The number of genes underlying mouse liver tumor susceptibility is unknown. Mouse chromosome 1 contains the hepatocarcinogenesis susceptibility locus, which can account for 76% of the variance in susceptibility of the C3H and more than 90% in the CBA mouse strains. These studies indicate the potential for modifier loci to influence the carcinogenic process. Genetic approaches in combination with molecular pathology analyses are being used to elucidate the biological pathways of liver tumor development in mice. In these studies, the wide strain variation in susceptibility is used to map the genes that contribute to the susceptibility phenotype, under the assumption that the alleles surrounding the marker loci of interest will be inherited along with the marker and the phenotype of interest. A susceptibility locus for the induction of mouse liver tumors has been located in the distal part of mouse chromosome 1 with this genetic approach. While specific candidate genes have not been determined, genetic approaches can be used to determine the identity of these genes and their contributions to the development of liver carcinogenesis in the mouse and the implication of an induction of such neoplasms in the human.

Both Bill Kaufmann and Tony Fox discussed the potential role of altered cell cycle checkpoint controls on the development of chemically induced hepatocarcinogenesis. As discussed by Bill Kaufmann, the G1 checkpoint converges on the retinoblastoma protein, including its phosphorylation status and ability to bind and inactivate the E2F family of transcription factors that regulate many cell cycle-dependent genes. During G1, the genetic material is prepared for replication and the integrity of the DNA assessed. While loss of the G1 checkpoint did not correlate with tumorigenicity in several cell lines, loss of this control point was observed early in liver tumor induction and maintained during its progression. During G2, cells prepare for mitosis and the DNA is assessed for DNA damage and any resident damage is repaired. The G2 checkpoint pathways appear to converge on the MPF factor, and this checkpoint is intimately involved in the maintenance of chromosomal stability.

Tony Fox discussed the possibility that nongenotoxic compounds abrogate the G1 checkpoint. For example, phenobarbital administration results in a decrease in p53 levels and consequently a diminished G1 checkpoint control response, which may lead to an increase in mutations. The regulation of the cell cycle is complex, with a combinatorial interaction of the various components of these pathways. These observations implicate many potential targets for the disruption of cell cycle progression and ultimately for the induction of unregulated cell proliferation and support the contention that nongenotoxic agents can affect all stages in cancer development.

Michael Schwarz indicated that a percentage of mouse liver tumors contain a mutated ras gene, while p53 mutation is not typically observed in mouse liver tumors. In culture, p53 mutations arise in hepatocytes derived from tumors in the mouse. Interestingly, the second allele is generally functional, but with increased time in culture there is a selection for those cells that contain inactivated p53. In mouse hepatocytes, p53 is not required for the induction of apoptosis, although it can regulate numerous other components of the cell cycle. Numerous genes are important for mouse liver carcinogenesis. Studies by Michael Schwarz on connexin 32 knockout mice indicated that no liver phenotype was associated with these mice, yet the perinatal administration of DEN resulted in an increased relative liver weight, an increased hepatic labeling index that was greater in female than in male mice, and a dramatic increase in the size of the tumors induced. Thus, connexin 32 negatively regulates the growth of mouse liver tumors in both male and female mice.

Rolf Schulte-Hermann discussed data which indicated that

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the strain differences are at the level of promotion and not initiation. In addition, he indicated that the nongenotoxic agent phenobarbital does not alter hepatic DNA synthesis and that the incidence of mitoses is low. In the mouse, the increased content of hepatic DNA that occurs with treatment persists upon removal of phenobarbital or nafenopin. There was not a strain difference in response to TGFβ nor in apoptosis between the sensitive and the resistant strain. Within lesions, an increase in both the mitotic and the apoptotic rate was observed and was correlated with tumor progression.

Snorri Thorgeirsson examined the hypothesis that continuous cell proliferation would result in a high probability of tumor formation through the use of albumin-myc transgenic mice, metallothionein-TGFα transgenic mice, and double transgenic mice. In the double transgenics the incidence of mitosis was high while that of apoptosis was low, which correlated with a more rapid focal growth. While the tumors in the double transgenics express TGFβ1, there is a decrease in TGFβ receptor 2 and bax, resulting in a diminished apoptosis rate. These tumors also overexpressed TGFα, cyclin D1, c-myc, cyclin B, phosphorylated RB, and free E2F family members.

Robert Isfort emphasized a different aspect of liver tumor formation, namely that of altered differentiation status. Inactivation of Tg737 was observed in 40% of the liver cell lines tested from both human and rodents. This gene contains a tetratricopeptide repeat motif. The function of Tg737 is largely unknown except that overexpression results in multinucleated cells and deletion results in the proliferation of oval cells, putative liver stem cells, without differentiation. This gene may be involved in differentiation of liver, kidney, and pancreatic ductular cells, and alterations of Tg737 are observed in tumors arising in these tissues. Interestingly, this gene is expressed in the early hepatic primordium and is lethal at embryonic day 10.5, when all four transcripts are lost. Focal areas of oval cells are observed in the Tg737+/− cells. In addition, this gene may function in a pathway to block differentiation induced with an EGF family member in combination with TGFβ. While alone these growth factor families may modulate proliferation and apoptosis in the liver, temporal and spatial overlap of expression of these two growth factor families may contribute to an independent pathway involved in control of differentiation and marked by expression of Tg737. The loss of Tg737 expression can then contribute to maintenance of the hepatocytes in an undifferentiated stage with expression of oval cell markers and formation of foci of altered hepatocytes. Apparently at least one modifier gene exists which may be different in different mouse strains.

Herve Lerat discussed work toward development of a transgenic mouse model that recapitulates the pathogenesis of hepatitis C virus-induced hepatocarcinogenesis in humans. Because hepatitis C is a major etiological agent for chronic viral hepatitis and frequently (>50%) results in a persistent infection, a model is necessary to understand the biology of the virus, the mode of pathogenesis, and the carcinogenesis mechanism(s). At present no *in vitro* model is available, and the only *in vivo* model is in the chimp. In an attempt to create transgenic mice expressing the RNA for this virus, several founders were developed. One line of animals contained fatty livers, while a second line contained well-differentiated hepatocellular carcinomas. The full-length RNA transcript of the transgene was not found in the tumors, indicating that further refinement of this valuable model will be required.

Linda Sargent examined the early cytogenetic breakpoints during carcinogenesis in transgenic mice. She then compared these early breakpoints with the cytogenetic alterations observed in neoplasms arising in the same model and demonstrated that the two sets of genetic changes are identical. In addition, the observed karyotypic changes correspond with the site of known susceptibility genes for mouse liver tumor development. For example, the primary chromosome involved in early breakpoints and in later cytogenetic rearrangements was mouse chromosome 1, near the primary locus defined by Norman Drinkwater as the site of the Hcs gene locus. Specifically, breakpoints in the mouse were observed on chromosomes 1, 4, 6, 7, 9, and 12. These karyotypic alterations are highly syngenic with chromosomal regions in the rat and human believed to be involved in liver cancer development and progression. Studies by Therese Poole indicated that both chromosomes 1 and 17 are involved in the sensitivity of the tested mouse strains to hepatocarcinogenesis and that the loci involved did not differ for male and female mice. The number of lesions in the mouse was greater in the Brown (BR) mouse than in the C57Bl/6 (B6) mouse. In this case, the BR and B6 males had a similar multiplicity, as did the male and female BR mice. However, the growth rate differed between the strains involved and in the duration of the experiment.

Jay Goodman discussed the hypothesis that altered DNA methylation is an epigenetic mechanism underlying abnormal gene expression, threshold effects, species specificity for carcinogenesis, tumor promotion, and heritable epigenetic phenomena. Studies of carcinogenesis need to move from simply investigating changes in the DNA sequence to the analysis of altered regulation of gene expression. Changes in methylation status can contribute to clonal tumor evolution, reversibility, and phenotypic multiplicity. There is a difference in the methylation status of Ha-ras in low- and high-sensitivity strains of mice. Following exposure to a promoting dose of phenobarbital, the sensitive B6C3F1 mouse has a lower hepatocyte proliferation rate and a decreased ability to maintain methylation status than the relatively resistant C57Bl/6 mouse. Methylation status can correlate with gene expression level, with hypomethylation generally indicative of expression as observed for ras and raf in the B6C3F1 mouse. Goodman implied that global hypomethylation occurs in the B6C3F1 mouse, resulting in increased sensitivity to hepatocarcinogens and lack of relevance for human risk because human cells appear to be better able to maintain normal methylation status.

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Randy Jirtle discussed the fact that the imprinting status of certain genes, especially when coupled with the presence of a polymorphism affecting gene expression, alters the number of targets necessary for inactivation by mutation or epigenetic changes. His research centered on the loss of expression of the mannose 6-phosphate/insulin-like growth factor 2 receptor, which is important in inactivating the hepatic apoptosis inducer, TGFβ. Loss of receptor function could occur by a point mutation or by loss of homozygosity, which invariably occurred on the nonimprinted allele. The species specificity of the imprinting-induced inactivation of one allele might provide an explanation why mice are so sensitive to hepatocarcinogens. The expression of IGF2 receptor is regulated by gene dosage, which is controlled by methylation status. The presence of a group of individuals with a polymorphism effectively inactivating one allele indicates that a sensitive human subpopulation exists for this important gene, wherein a single inactivating event is sufficient for loss of function of a tumor suppressor gene whose function is critical in liver.

James Klaunig addressed another interesting mechanism underlying species-specific responses to nongenotoxic carcinogens such as dieldrin, which is tumorigenic in mice but not in rats. The carcinogenic effect of dieldrin in mice is not strain specific and is secondary to increased cell proliferation, inhibition of apoptosis, modification of intracellular communication, and increased oxidative stress, including enhanced urinary excretion of 8-hydroxy deoxyguanosine. Measures of oxidative stress are elevated in mice compared to rats and are further elevated by administration of dieldrin to mice. Mice have a lower level of vitamin E than rats, and supplementation with vitamin E decreases markers of oxidative stress in mice. Hepatic labeling index in the presence of dieldrin is also suppressed, suggesting that the lower vitamin E levels in mice compared to rats may be responsible for an increased oxidative DNA damage concomitant with an increased hepatic labeling index. This may explain the greater sensitivity of mice to the carcinogenic potential of dieldrin, compared with rats.

Rolf Schulte-Hermann examined the nongenotoxic carcinogens phenobarbital and nafenopin in sensitive and resistant mouse strains. Phenobarbital increased polyploidization and DNA synthesis in mouse liver. In contrast to rats, cessation of administration of either phenobarbital or nafenopin did not reverse the observed increase in DNA synthesis and did not result in an increase in apoptosis. Interestingly, mouse hepatocytes are less sensitive to TGFβ-induced apoptosis than are those of rats.

David Malarkey discussed the state of knowledge with respect to ras mutation and mouse liver carcinogenesis. The question as to whether carcinogens induce ras mutations or select for preexisting ones is still unsettled. Two pathways for tumor induction appear to exist in the mouse liver: one pathway that results in tumors containing Ha-ras mutations and a separate pathway that does not contain Ha-ras mutations. On one hand, a carcinogen and dose specificity is observed for the ras mutation obtained, while on the other hand an inverse relationship exists between the dose and the number of tumors with ras mutations. The mechanism for the induction of neoplasms that do not contain Ha-ras mutations is yet to be elucidated.

Jerry Ward discussed a problem that exists in some mouse colonies due to their infection with *Helicobacter*. The *Helicobacter* infection is passed from darn to pup and is associated with acute and chronic active hepatitis with hepatocellular carcinoma induction. Male mice are more susceptible than female mice to *Helicobacter*. The strains most susceptible to spontaneously and chemically induced neoplasms are also those most susceptible to tumor induction by *Helicobacter*. Because the background rate of liver tumors can be markedly elevated in control mouse populations, all mice should be tested for the presence of these infective agents through monitoring of bacterial 16S ribosomal subunit, bacterial heat shock protein 70, or PCR of the suspected organism. Angelo Turturro underscored the importance of diet, including adequate nutrition and appropriate caloric intake on assessment of tumor outcome. As body weight increases, cell proliferation increases and the apoptosis rate decreases. These two factors, increased proliferation and decreased apoptosis, are tied to an increased sensitivity to carcinogenesis. Harold Enzmann discussed several interesting findings from his analyses of an agrochemical database of 138 carcinogenicity studies. In these studies, hepatomegaly at 12 months was highly correlated with hepatocarcinogenesis. In these studies, preneoplastic lesions preceded the onset of tumors. Of the mouse liver positives, 31 of 37 chemicals were not positive in genotoxicity or mutagenicity tests. Tumor incidence in the control was not correlated with the strain of mouse used or the duration of the study in positive outcomes.

Numerous posters were presented that underscored the importance of the balances between cell proliferation, apoptosis, and differentiation in mouse liver cancer development. Corrie Dunn indicated that a differential focal to nonfocal hepatocyte labeling index could be observed after phenobarbital administration to the three mouse strains that had been treated with DEN. These studies implied that cell proliferation was an important component of mouse liver carcinogenesis but was not the sole factor underlying its progression. James Christensen demonstrated that distinct pathways for apoptosis exist in mouse hepatocytes and that nongenotoxic agents can act at several steps in these pathways. Administration of TGFβ resulted in a decreased level of inhibitors of apoptosis, including a phosphorylation of bcl2, a decrease in bcl-x1, and an increase in the apoptosis inducers bcl-xs and bax. Phenobarbital induced an increase in bcl-2, and bcl-2 knockout mice were more sensitive to TGFβ-induced apoptosis. Studies by Lisa Kamendulis indicated that dieldrin-induced DNA synthesis in mice is associated with an increase in polyploidization and is not observed in rats. An increase in apoptosis was not observed with dieldrin administration to mice.

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Tony DeAngelo examined the dose response for dichloroacetic acid and noted that while neither 0.05 nor 0.5 g DCA/L in the drinking water increased the incidence of hepatic neoplasms relative to the control, a dose of 1–3.5 g/L clearly did. In addition, all tested doses increased the multiplicity of tumors. Interestingly, 10 weeks of 3.5 g DCA/L was sufficient to induce hepatic neoplasms 90 weeks later. Further studies on DCA by Tonya Moore indicated that this high (3.5 g/L) dose increased serum corticosterone levels by 26 weeks at least in part through an inhibition of 11β hydroxysteroid dehydrogenase. Studies by Sharon Leavitt indicated that 1 or 3.5 g DCA/L provided evidence that DCA may have a genotoxic component based on an increased incidence of T:A–G:C mutations in lacI mice after 60 (but not 4 or 10) weeks of DCA administration. An elegant study by Anja Stauber indicated that, unlike hepatic neoplasms induced by TCA, DCA-induced tumors were c-jun and c-fos positive. Her development and application of a soft agar method for assessment of transformation of hepatocytes by these agents permitted the parallel assessment of the *in vivo* results. Colonies transformed by TCA were negative, while those transformed by DCA were positive for fos and jun immunoreactivity, indicating distinct pathways of selection by these agents. Transformation of Syrian hamster embryo (SHE) cells by peroxisome proliferating agents was examined by Tore Sanner. The SHE cells were effectively transformed by peroxisome proliferating agents without an alteration of the number or size of peroxisomes and without altering the observed level of oxidative stress. A transient depression of gap junctional communication was observed. These agents present a mechanism for transformation in this cell type different from oxidative stress or peroxisome proliferation. Kiersten Gressani indicated that Ki-ras was a common and early event in hepatic tumors induced by transplacental administration of 3-methylcholanthrene. An overexpression of Ha-ras, Cip1, and c-jun was observed in 30% of the tumors, while only a low incidence of mutations in INK4a or p53 was observed, indicating the early nature of oncogene activation and the late inactivation of tumor suppressor genes in this model. An exciting finding of a positive marker for focal changes in the mouse liver came from studies performed by Ute Wastl. In these studies, P450 2A5 overexpression was found in half of the foci detected in untreated mice, in more than 90% of those from DEN-treated mice, and in all adenomas and carcinomas on the basis of analyses by immunocytochemistry and *in situ* hybridization. The expression of P450 2A5 was enhanced focally in both sensitive and resistant mouse strains. Studies by Jason Isenberg indicated that cell proliferation induced by a partial hepatectomy is associated with reduced or altered expression of connexin 32 and a decrease in gap junction communication. David Malarkey demonstrated that Brca1 expression was enhanced in hepatic foci and adenomas, but not in carcinomas in B6C3F1 mice. These findings were suggested to imply that the early focal expression of Brca1 might be associated with loss of negative growth control, lack of differentiation, or genomic instability in the carcinomas that then arise.

Michael McClain concluded the conference by discussing what is known about the hepatic response in mice compared to humans. The nongenotoxic hepatic tumor promoters do not appear to play an important role in the etiology of human liver cancer, which is a conclusion also supported by negative human epidemiological data for phenobarbital and related compounds. In the assessment of potential human cancer risk, the modes of action should be considered. Genotoxic compounds should be viewed with considerable concern. However, for the nongenotoxic hepatic tumor promoters, the weight of evidence indicates that a mouse liver tumor response is of questionable relevance to humans.

The conference provided a great deal of data about the pathogenesis of mouse liver neoplasms, as pointed out by Linda Birnbaum, the rappoteur for the meeting. Specifically, the meeting resulted in a discussion of the genetic and epigenetic changes that accompany the process of mouse liver carcinogenesis, including the similarities to and the differences from other species. Alterations in the balance between proliferation, apoptosis, and differentiation signals appear to be critical factors in mouse hepatocarcinogenesis and the modulation of this process by chemicals.

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