

## UNIT V: Models of Liver Cancer, Non Genotoxic Carcinogens

### Assigned Reading:

Pitot et al. (1996). Quantitation of multistage carcinogenesis in rat liver. *Toxicologic Pathology*. 24:119.

- 1) Not all carcinogens are genotoxic, (i.e., directly alkylate DNA and cause mutations).
- 2) Chemicals that enhance carcinogenic responses to genotoxic carcinogens are known as promoters.
- 3) Modeling HCC in rodents can be performed by monitoring the evolution of carcinogen induced enzyme altered foci.

### Non Genotoxic Carcinogens:

Until now, we have been talking about carcinogens that act via direct/covalent modification of DNA ("alkylation"). We have talked about the early proposal that all carcinogens are mutagens. From the initial DNA alkylation studies and mutagenicity studies came the classification of mutagenic/alkylating carcinogens as "genotoxic carcinogens". If you read this early literature you will note that it is often stated that 90% of all carcinogens are mutagens. It is also implied that the remaining 10% would soon be discovered to be mutagenic once the appropriate bioactivation protocol was developed. Over the last ten years data has been generated to support the concept that a large fraction of carcinogens are not mutagenic and do not directly damage DNA. Current estimates are that about half of all carcinogens are mutagens and that the other half are not mutagenic. The mechanism to explain the carcinogenic activity of this latter group of compounds is an area of active investigation. Those carcinogens that are not mutagenic are often called "nongenotoxic carcinogens". In most of the literature you may also find them referred to as epigenetic carcinogens, implying that they do not act by directly damaging DNA. In this Unit, we will begin to discuss potential mechanisms to explain nongenotoxic carcinogens. It should be appreciated that many hypotheses are presented, yet none of them have overwhelming support. It is likely that there will be more than one mechanism to explain how these carcinogens work. The biology that underlies epigenetic carcinogenesis is a newer area of oncology and not nearly as well understood as the mechanism of genotoxicity.

### Here is my understanding of how scientists use the following terms (you will find a lot of differences):

**Genotoxic:** Refers to compounds that damage DNA. Most often used to refer to chemicals that alkylate DNA directly. Can also be used in its most general form to refer to compounds that lead to DNA strand breaks etc, but these compounds are more commonly referred to as clastogens. Genotoxic agents are often defined operationally. 1) By determining if they directly alkylate DNA in vitro or in vivo. This usually requires radiolabeled chemical or an antibody that recognizes the DNA adduct. 2) By determining if the compound can induce mutations in any of a number of simple system (e.g., Ames Assay, mammalian cell culture systems, mutamouse etc).

**Nongenotoxic (epigenetic):** Typically refers to chemicals that are carcinogenic in rodent models (or via human epidemiology), yet cannot be shown to alkylate DNA or lead to mutations.

**Given these widely held definitions, provide a mechanism by which a chemical can lead to DNA damage without the tested chemical forming a direct covalent bond with DNA (I am not asking about cell proliferation). Would you classify such a compound as a genotoxic or nongenotoxic compound?**

### Modeling Hepatocellular Carcinogenesis.

As a model of carcinogenesis, the rodent liver has both strengths and shortcomings. The shortcomings are significant. First is the observation that many chemicals cause rodent liver tumors, even though they cause cancer at other sites in humans and other animal models. This difference in sensitivity has prompted many scientists to speculate that many rodent hepatocarcinogens may not be human carcinogens at all. The rodent liver (especially the mouse liver) seems to be extremely sensitive to the carcinogenic actions of chemicals. One advantage of rats over mice is that many mouse strains seem to yield high rates of spontaneous liver tumors. Spontaneous liver tumors in rats are more rare. Finally, it is difficult to observe the progression of liver carcinogenesis in any system. Skin models can have significant advantages over liver because; 1) you can visually inspect tumors at various stages of skin carcinogenesis, whereas in liver, the animal must be killed and surgical sections taken; 2) spontaneous tumor rates in skin are quite low compared to rodent liver where spontaneous tumor rates can be quite high. The strengths of the rodent liver system include the fact that many chemicals induce this type of cancer in rodents. The rodent liver is a very sensitive indicator of chemical carcinogens. Thus, a single experimental protocol can be used to understand the mechanisms of a number of carcinogens. The low cost of rodents and its potential for genetic studies and manipulation (especially in the

mouse) are also attributes. These characteristics, coupled with a fairly extensive understanding of liver biology have made rodent hepatocarcinogenesis a popular model. To counter the inability to monitor hepatocarcinogenesis in real-time (like in skin), sensitive histological protocols have been developed as surrogates for early stages of neoplasia (see below). In addition, the liver model is a more appropriate model than skin for carcinogenic processes with etiologies related to dietary or parenteral exposures.

### Altered Hepatic Foci.

Early studies of chemically induced hepatocellular carcinoma noted the early appearance of altered hepatic foci (AHF) in the livers of treated animals. AHF have been noted as early as the 1960s, with each laboratory using an independent histological protocol for their identification. For example, AHFs have been defined primarily by histochemical staining techniques; 1) deficiency in glucose-6-phosphatase (G6P) staining, 2) H&E staining, 3) accumulation of glycogen, 4) increased expression of gamma-glutamyl transpeptidase (GGT), 5) deficiency of iron staining, expression of placental GST, 7) expression of alpha fetoprotein, or 8) canalicular ATPase. In each case, the focal appearance or disappearance of a marker corresponds to a cellular lesion/proliferation (focus) with a phenotype that is different from surrounding cells. In any given protocol, more than one type of foci may be observed (although each lab seems to have its favorite and only looks at that one). In addition, these foci do not necessarily overlap (see figure below, left, taken from Fundamentals of Oncology, 3rd Ed, HC Pitot). In many cases, this change appears to be expected given the idea that cancer may often be thought of as a process of dedifferentiation (anaplasia).



Figure 6.5 Composite drawing of "islands" of hepatocytes or enzyme-altered foci in three serial sections of liver from a rat given a single dose of diethylnitrosamine 24 hours after partial hepatectomy followed by feeding of phenobarbital according to Pitot et al. (1978). Open circles = glucose-6-phosphatase-deficient areas; dashes = ATPase-deficient areas; dots =  $\gamma$ -glutamyl transpeptidase-positive areas. In foci exhibiting more than one enzyme alteration, combined and/or parallel symbols are used.

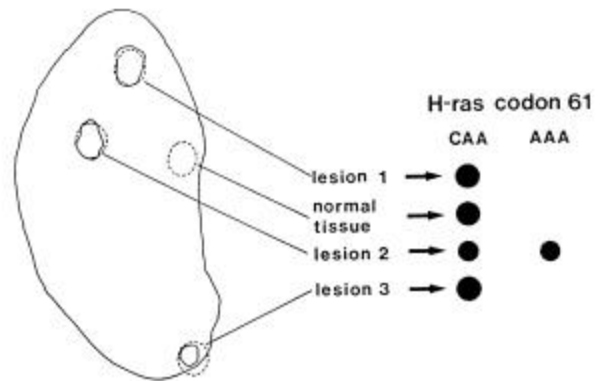


Figure 1. Representative example of H-ras mutations in early enzyme-altered liver lesions. A 10- $\mu$ m liver section from a DEN-treated mouse was prepared and stained for glucose-6-phosphatase activity. Small tissue samples of approximately 5  $\mu$ g were taken from three individual liver lesions and from normal liver

using punching cannuli with a diameter of 0.7 mm (indicated by dotted circles). These samples were used for in vitro amplification of DNA by the PCR method and analyzed for mutations at codon 61 of the H-ras gene by selective oligonucleotide hybridization.

A typical experiment with perinatal carcinogen treatment (usually day of birth) of sensitive mouse strains to DEN might yield 100-300 AHF of a given type per  $\text{cm}^3$  of liver tissue between 14 and 52 weeks. Remember that multiple types of AHFs can be detected if desired and that they don't always overlap, so the total number of AHFs in such a protocol is much larger. This same protocol might yield between 20-40 tumors after about 32 weeks. This is one piece of evidence that not all foci become neoplastic.

### What is the proof that AHF are a marker of carcinogenesis:

- 1) There is a close correlation between the number of AHF and tumors (both adenomas and HCC). In the data described at the right (from Cancer Research 43:4253), note that numbers are per mouse. Basophilic foci is the method of identifying AHF by this group. Hyperplastic nodules are foci that are beginning to compress the normal parenchyma around them
- 2) Tumors often display similar biochemical parameters used to identify foci (e.g., increased GGT etc.
- 3) The foci often contain high frequencies of altered Ras genes. Thus a fraction of the foci appear to have been the target of a mutational event. (see foci figure above right). A mutational event like that commonly observed in Ras is often thought of as the first step in chemically initiated carcinogenesis, to a certain degree based upon the mutational spectra data we have discussed previously.

An issue you should be aware of: How do you know that these foci actually become the neoplastic lesions that are observed. You cannot be absolutely certain that none of these foci ever become a true cancer. This is

Estimated frequencies of basophilic foci, hyperplastic nodules, adenomas, and carcinomas per liver 40 weeks following DEN treatment

DEN dose ( $\mu\text{g/g}$ )	Basophilic foci		Hyperplastic nodules	Adenomas	Carcinomas
	A	B <sup>a</sup>			
0.000	0	0	0	0	0
0.625	99	13	0	0	0
1.250	196	13	53	0	0
2.500	378	27	53	10	0
5.000	946	42	184	34.75	0.25

because you cannot follow them over time. You can only look at individual livers at various time points. Recent data suggest that this progression can be observed. A number of labs have been developing the use of TGF $\beta$  as a marker for cells that are progressing from a focus (altered marker expression, but morphologically normal) to a neoplasm (invasive, morphologically abnormal). In these early studies, AHF can be observed (very rarely, as expected) that have a new TGF $\beta$  focus within them. This focus within a focus is thought by many to represent an additional step in the progression to neoplasia.

Taken in sum, these data suggest that liver neoplasia can be segregated into at least three steps. 1) The initiation event whereby a mutation has occurred in the genome (commonly thought of as mutations in Ras). 2) the growth of that cell at a rate greater than its neighbors to form an AHF. 3) The progression of the AHF to a frank neoplasm, often displaying genomic instability and invasive and metastatic potential.

**Recently a group has published that single hepatocytes can be stained for increased Jun expression after DEN initiation (and these progress into AHF). Do you think Jun has a mutation from DEN exposure (like Ras)? Could it be the first hit, why or why not?**

## Early studies of carcinogen/chemical interactions in rodent HCC.

### Protocols

As noted in previous lectures, the protocol used to elicit liver carcinogenesis can have a significant impact on the mutational spectra and the metabolism of the carcinogen. **Why might you expect to get different outcomes if you gave an animal one 10 mg dose of benzo(a)pyrene, vs. ten 1 mg doses give 2 days apart. What mechanisms are at play?** The protocol used in an experiment is dictated by the experimental question. For example, in the Ames review you learned that protocols to detect carcinogens are performed at the maximal dose that does not induce notable toxicity (often called the maximal tolerated dose or MTD). In most experiments of this type, carcinogen exposure is often chronic.

In experiments designed to understand the mechanism of carcinogenesis, such MTD protocols may not be appropriate. Chronic exposure of a carcinogen presents a number of problems, not the least of which is the hassle involved in frequent handling and dosing of animals with a carcinogen. Therefore a number of model liver carcinogenesis protocols exist. Typically a dose of a genotoxic agent is used to initiate the carcinogenic process. This dose is preferably administered at a dose that does not induce toxicity, regeneration or necrosis (each of these can be a confounding variable). To enhance the sensitivity of rodent models it is often desirable to increase the probability that hepatocytes are more prone to mutations by attempting to “fix” them via replicative bypass. To ensure this, investigators often follow the mutagen with a partial hepatectomy. Alternatively, investigators dose perinatally when the liver cells are actively dividing. These protocols will increase tumor yields at least an order of magnitude over protocols not eliciting an acute phase of cell division.

In addition to fixing mutations (the “initiation step”), investigators have learned that exposure to other chemicals can have synergistic effects when they are chronically administered after the initiation step. Phenobarbital, dioxins, peroxisome proliferators and sex steroids are classic examples of such a compounds. Additionally, all but phenobarbital are classic examples of nongenotoxic carcinogens. Followup/chronic administration of these compounds can lead to increases in tumor yield of an order of magnitude or more. In addition, they can increase the models sensitivity such that doses of an initiator that were previously non carcinogenic, are now yielding tumors. Alternatively, they can shorten the time for appearance of tumors, thus increasing data acquisition and reducing costs. In the rat initiation-promotion model, an initiator (typically a genotoxicant like DEN) is followed by partial hepatectomy and after two weeks, the animals are put on a chronic Phenobarbital diet. More recent models have added a “progression” step, which hits the animal with a third stimulus later in the protocol (typically, another hepatectomy and exposure to ENU). Such a stimulus is believed to provoke genomic instability a postulated requirement of neoplasia. The progression step can increase tumor yields by 3-10 fold. Importantly, perinatal protocols seem to be more useful in the murine system (not as useful in the rat). This has been suggested to be because the mouse hepatic environment may already have “an endogenous promoter” or a “promoting environment” (to be discussed in a later unit).

### Additional Reading:

Chemical Carcinogenesis, in “Casarett and Doull's Toxicology, The basic Science of Poisons. Pages 201-267 (CD Klaassen ed.) 5th Edition, McGraw Hill, San Francisco, CA.)

Pitot, HC. (1990). Altered Hepatic Foci: Their Role in Murine Hepatocarcinogenesis. Annu. Rev. Pharmacol. Toxicol. 30: 465.