Cell Proliferation and Chemical Carcinogenesis: Conference Summary and Future Directions

by James A. Swenberg

Although it has been acknowledged for many years that cell proliferation is essential for carcinogenesis, this is the first meeting to directly focus on its role in carcinogenesis in a comprehensive manner. Many of the critical issues were outlined by Dr. Melnick in his opening remarks. Of particular interest is what data can be brought to bear on the recent controversies regarding the role of cell proliferation in studies of nongenotoxic carcinogens tested at maximum tolerated doses (MTD), and can cell proliferation alone be a cause of cancer? The symposium talks and poster presentations have been comprehensive and highly illuminating. It is impossible to truly summarize such a meeting, but I will try to place some of the major issues in perspective.

Speakers at this meeting have frequently referred to the multistep process of carcinogenesis. Many chemical carcinogens require metabolic activation to produce reactive electrophiles. Genotoxic agents directly damage DNA, whereas other chemicals may generate free radicals and indirectly damage DNA. In addition, spontaneous or background damage to DNA is always present. None of this DNA damage becomes a mutation unless cell replication takes place. This cell replication can be the *de novo* replication of a particular tissue or cell type, it can be modulated either up or down by toxicity, or it may be influenced by exposure to exogenous agents such as promoters. After fixation of such damage in the form of mutations at critical sites in the genome, we have the very first step in carcinogenesis, the initiated cell. In the past, we have focused primarily on point mutations; however, it is now known that chromosomal changes, such as insertions,

deletions, rearrangements, and gene amplification play important roles. One of the areas for future direction is to increase our ability to look at such end points. Initiated cells, however, are not cancer. They must undergo selection pressures and clonal expansion.

Cancer is a probabilistic disease, and it requires multiple mutational events. The probability of this happening is greatly enhanced through clonal expansion. If we look more closely at the events occurring in a cell or tissue after chemical exposure, it is clear that changes in the balance between metabolic activation and detoxication will occur at different places in the dose–response curve (*1*). In some cases metabolic activation will be saturated at high doses, leading to a supralinear dose response (Fig. 1, curve *b*). Two well-known examples of this type of response are vinyl chloride and (4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). If only high-dose data were used in assessing risk to this type of response, risk would be underestimated. With other compounds, detoxication or DNA repair saturates at high doses (Fig. 1, curve *c*), in which case the risk associated with low doses would be overestimated. These generalizations are further affected by dose-related changes in cell proliferation. Whether the promutagenic lesion in DNA is the biologically effective dose of the genotoxic chemical under test or background DNA damage, it does not become a mutation unless cell proliferation occurs. Cell proliferation is also a dose-related phenomenon that will affect extrapolation of risk. If we are to advance the scientific underpinning of risk assessment, it will be important to avoid making statements that are all-encompassing and instead refer to data on specific compounds and specific relationships.

To better understand the role of cell proliferation in carcinogenesis, we need to consider the sources of mutation. Figure 2 is a modification of Loeb's scheme for the sources of human mutation (*2*). Endogenous forms of DNA damage, free radicals, and polymerase errors are constantly present in all cells of the body. Regardless of whether these arise from endogenous or

Departments of Environmental Sciences and Engineering and Pathology, Campus Box 7400, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.
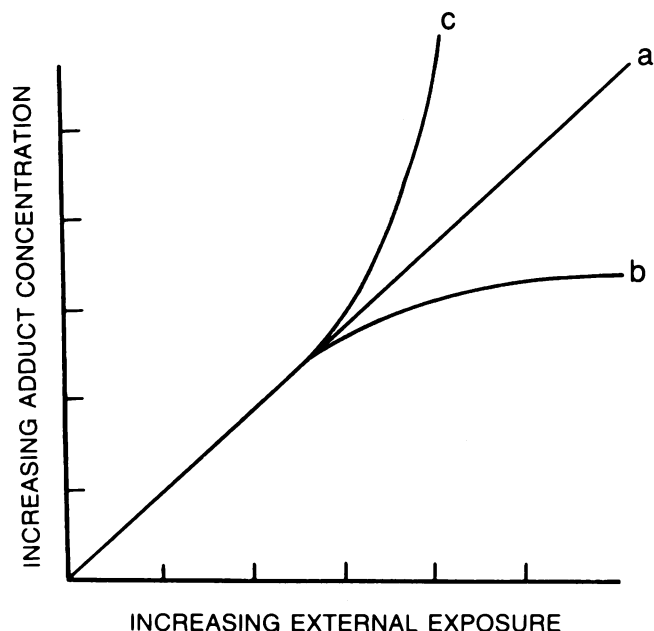
FIGURE 1. Schematic of theoretical dose–response curves. Curve *a* denotes a linear response, when biotransformation and DNA repair enzymes are not saturated. Curve *b* represents a supralinear response that results from saturation of metabolic activation. Curve *c* depicts a sublinear response that can arise when detoxication or DNA repair is saturated.
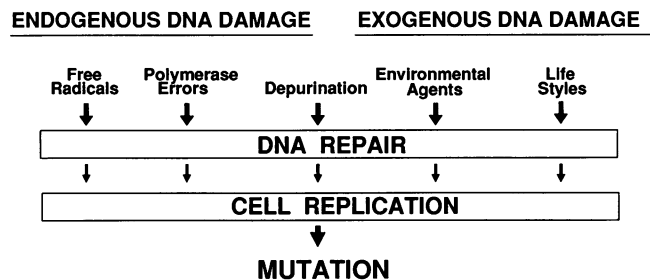

FIGURE 2. Mutations arise from endogenous and exogenous factors that are modulated by DNA repair and replication. Modified from Loeb (2).

Table 1. Approximate rates of endogenous DNA damage per cell per day (3).

Type of damage	Amount
Depurination	24,000
Depyrimidination	1,300
Cytosine deamination	400
Single-strand breaks	120,000
N-7-methylguanine	84,000
O^6-methylguanine	3,000
Oxidation products	3,000

exogenous processes, they are subject to DNA repair. Only those forms of DNA damage that pass through this filter of DNA repair and are present during cell replication will lead to mutations. This is true whether it is background DNA damage or chemically induced DNA damage.

If one examines the amount of background DNA damage present in cells, one finds background damage is enormous. Table 1 provides estimates from a recent report of the National Academy of Science (*3*). One type of damage that may be an important mechanism for mutations in *p53* is the deamination of cytosine. Likewise, apurinic sites and oxidative damage can lead to the types of mutations being found in *p53*. If we look at the amounts of these types of DNA damage, the estimates range from 50,000 to 250,000 events per cell

per day. Normally, the cell has very good DNA repair systems available to correct these background lesions. It should be recognized that when cell proliferation increases, the time available for repairing DNA damage decreases, whether the damage is endogenous or exogenous.

In addition to its role in initiation, cell replication is also important in clonal expansion. A cell that has a single hit will have to be clonally expanded to the point that the probability of a second genetic event becomes likely. Cell replication is a key factor in this expansion of the initiated cell population. If we are dealing with a genotoxic agent, the probability of that second event occurring happens with a much smaller population of initiated cells than if we are dealing with DNA adducts that are much rarer or that have a much lower efficiency for causing mutations. Therefore, we should not expect equal effects for the role of cell replication across the broad range of chemicals examined for carcinogenic potential. However, there can be little doubt that the more we know about the type and amount of DNA damage, coupled with the extent of cell proliferation, the more likely we can incorporate science into the risk assessment process and improve its accuracy.

Two examples illustrate the role of cell proliferation in carcinogenesis. Formaldehyde is a chemical for which there has been great deal of research conducted to understand the mechanisms responsible for a highly nonlinear dose response. For instance, we know from the research of Heck et al. (*4*) that the extent of DNA protein cross-linking per ppm of formaldehyde is markedly decreased at concentrations below 6 ppm. Between 6 and 15 ppm, the dose to DNA is linear. Yet, this is the observable portion of the concentration-response curve for nasal cancer that is highly nonlinear, i.e., a 50-fold increase in cancer for a 2.5-fold increase in dose (Fig. 3). Data from the new mechanisms-based bioassay reported at this meeting (*5*) replicates the initial bioassay, but has an additional important exposure group of 10 ppm. This provides new information on the concentration response for tumor induction and clearly demonstrates that the nonlinear response is a straight line from 6 to 15 ppm. The other three lines in Figure 3 present cell proliferation data at various time points in this study. What is clear from these data is that sustained increases in cell proliferation exhibit virtually the same concentration
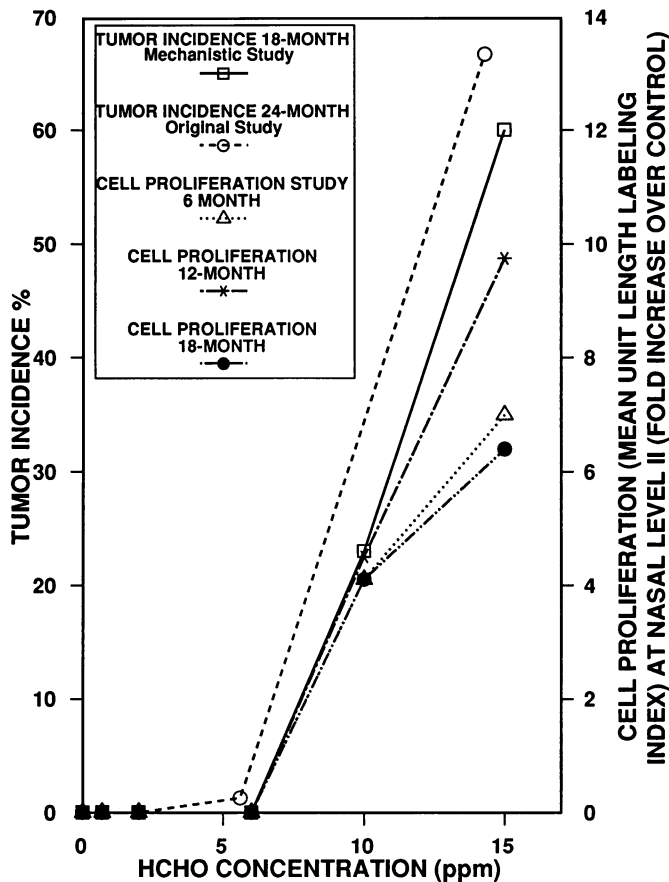
FIGURE 3. Tumor incidence and cell proliferation in rats exposed to formaldehyde. See Monticello et al. (5) for details.

es a 4- to 5-fold increase in cell proliferation in the epithelial cells of the proximal tubules in the male Fischer rat, a strain of rat that makes α_{2u}-globulin, but that no increase in cell proliferation occurs in the NBR rat, which does not synthesize α_{2u}-globulin (6). As pointed out in a recent review by the Environmental Protection Agency (7), this male-rat-specific protein selectively binds certain chemicals or their metabolites, is freely filtered by the glomerulus, and is resorbed by the proximal tubule of the nephron, where it accumulates in cellular lysosomes. The lysosomal digestion of the chemical–protein complex is decreased compared to α_{2u}-globulin alone, leading to a marked accumulation and subsequent cytotoxicity. In response to cell death, cell replication is specifically enhanced. What is important in the d-limonene study is the fact that the presence of initiated cells in control animals was clearly demonstrated. Control animals have large numbers of atypical tubules, the first recognizable focus of initiated cells. Atypical tubules are present in Fischer rats and NBR rats in similar numbers (Table 2). Thus, there is not a strain difference in spontaneous initiation. Identifying these early lesions requires perfusing the kidney and looking at six sections per kidney. It is not necessary to initiate these animals with a genotoxic chemical to demonstrate that d-limonene causes a statistically significant increase in the number of these initiated cells in F344 rats, but not in NBR rats. Note that promotion of these lesions only occurs under conditions where increased cell proliferation is present. Neither increased cell proliferation nor promotion of these spontaneously initiated cells occurs when α_{2u}-globulin is not synthesized.

The same phenomenon is evident in more advanced lesions. The number of atypical hyperplasias in all groups is less than the number of atypical tubules. These studies were conducted for 32 weeks; however, it is likely that the numbers of preneoplastic lesions would continue to increase throughout the life span of the animal. Only small numbers of these lesions can be demonstrated in control rats of either strain. When F344 rats are treated with d-limonene, a statistically significant increase in atypical hyperplasia occurs. Again, promotion only occurs when sustained increases in cell proliferation are present. This provides

response as the induction of squamous cell carcinomas. It is almost inconceivable that cell replication is not the driving factor for the induction of nasal cancer because we know that the molecular dose to DNA is linear over this portion of the concentration response curve. This provides strong evidence that sustained increases in cell proliferation play a major role in determining the dose response for tumor induction, and as such they should be factored into risk assessment.

d-Limonene is the other example I would like to highlight. Table 2 demonstrates that d-limonene caus-

Table 2. d-Limonene-induced cell proliferation and promotion in kidney (6).

Exposure group	Strain	Labeling index	Atypical tubules	Atypical hyperplasia	Adenomas
H$_2$O–corn oil	F344	4.6 ± 0.3	3.3 ± 0.7	0.0 ± 0.0	0/31
H$_2$O–d-limonene	F344	24.2 ± 1.5	10.7 ± 1.3	0.4 ± 0.1	0/31
EHEN–corn oil	F344	5.2 ± 0.4	15.6 ± 1.5	1.2 ± 0.2	1/31
EHEN–d-limonene	F344	20.5 ± 1.3	65.0 ± 3.4	15.5 ± 1.5	9/31
H$_2$O–corn oil	NBR	4.5 ± 0.3	4.7 ± 0.8	0.1 ± 0.1	0/30
H$_2$O–d-limonene	NBR	4.7 ± 0.2	4.6 ± 0.8	0.1 ± 0.0	0/31
EHEN–corn oil	NBR	5.2 ± 0.4	9.6 ± 1.2	0.2 ± 0.1	0/30
EHEN–d-limonene	NBR	5.6 ± 0.4	8.0 ± 1.0	0.2 ± 0.1	0/31

EHEN: N-ethyl-N-hydroxyethylnitrosamine.

strong support for the concept that cell proliferation is causal for the induction of neoplasms for some chemical carcinogens. Exposure to genotoxic agents, such as in the EHEN-initiated groups in this study (6) further magnifies the response.

We can summarize the role of cell proliferation in carcinogenesis in the following way: a) Increased cell proliferation decreases the time that is available for DNA repair. This is a first principle that is not up for debate. b) DNA replication converts repairable DNA damage into nonrepairable mutations. Support for this comes from numerous experiments in cell culture, as well as from in vivo systems. c) Cell replication and DNA synthesis are also necessary for chromosomal aberrations, insertions, deletions, and gene amplification, which are important mechanisms in chemical carcinogenesis. d) Cell proliferation is required to clonally expand initiated cell populations. An important point is that a mutated Rb or mutated p53 gene product increases cell proliferation, decreasing time available for repair. This eliminates one of the cell's important check points and contributes to genomic instability. The mechanisms that I have discussed for sustained increases in cell proliferation are therefore similar to what happens when the p53 or Rb gene is inactivated, i.e., increased cell proliferation. Why is it acceptable that aflatoxin's induction of point mutations at codon 249 of p53 is a mechanism that is causally related to carcinogenesis and results in increased cell proliferation, but when cell proliferation is induced by other means it is deemed an unlikely mechanism for carcinogenesis? Such a rationale lacks scientific objectivity. Likewise, to accept that cell proliferation alone is a primary mechanism for some nongenotoxic agents is totally different from making generalizations that cell proliferation is the mechanism for all MTD carcinogens. There are no data that support such a generalization.

Not all agents that increase cell proliferation are carcinogens. There are many reasons why all chemicals that cause increases in cell proliferation are not carcinogens: The first of these and no doubt the most common is related to the quality of the data being cited. Transient increases in cell proliferation are common responses to the administration of many chemicals, but these do not provide sufficient evidence for or against a causal link between cell proliferation and cancer. Evidence of sustained increases in cell proliferation that are not associated with increases in neoplasia is, on the other hand, much less available, but must be seriously considered. The exact temporal association between increased cell proliferation and the induction of neoplasia is unknown at this time. It is clear from the data presented at this meeting on formaldehyde that a minimum of 3 months of sustained cell proliferation is needed to sharply increase the slope for nasal cancer incidence (5). Likewise, it took 12 months for the nongenotoxic agent ethyl acrylate to induce forestomach tumors in rats (8). Studies on a series of jet fuels that induce α_{2u}-globulin nephropathy demon-

strated that none of the fuels induced renal tumors when male rats were exposed for 90 days and then held for an additional 21 months, although several did induce kidney tumors when the exposures were for 12 months and the rats were held for an additional 12 months (7). Thus, it is likely that sustained increases in cell proliferation are required to induce carcinogenesis. If examples are found where sustained increases are present and no increase in neoplasia occurs, these will be excellent examples to pursue to better understand the critical mechanisms that are involved. Possible examples include the α_{2u}-nephropathy-inducing agent gabapentin (9).

The third factor that could explain the lack of neoplasia in the presence of increased cell proliferation is selective cytotoxicity of initiated cells. Before this meeting, this mechanism seemed plausible, in that it represented the reverse of the Solt-Farber model for haptocarcinogenesis. However, initiated cells are far more susceptible to the cytotoxicity and apoptosis induced by some agents (10). Likewise, terminal differentiation of proliferating cells would remove this population of initiated cells from progressing to cancer. Two additional mechanisms were presented at this meeting. Different responses to cell–cell communication could alter the carcinogenic response (11), and maturation arrest could decrease the likelihood of these cells progressing to neoplasms (12). Thus, a clear area for future research is to gain a better understanding of why some agents that enhance cell proliferation are carcinogenic, while others are not. The fact that not all agents that enhance cell proliferation are carcinogens does not negate enhanced cell proliferation as a causal mechanism for other agents. As is clear from the arguments presented above, the two situations are not mutually exclusive.

How then can we begin to factor new data into the risk assessment process? Data were presented at this meeting where increased incidences of cancer were only seen after animals were exposed to high doses of a nongenotoxic agent under conditions that result in increased cell proliferation, while humans are exposed to much lower doses. Likewise, the genotoxic agent formaldehyde induced a markedly increased incidence of nasal cancer under conditions that caused a similar increase in cell proliferation, even though the molecular dose was linear over that portion of the exposure-response curve and humans are only exposed to concentrations not associated with increased cell proliferation. This meeting did not discuss nonlinearities in molecular dose; however, I mentioned vinyl chloride and formaldehyde as two examples. It should be clear that incorporating mechanistic data such as cell proliferation and molecular dose does not automatically reduce the risk assessment. When a chemical saturates metabolic activation or exposure is to young children whose tissues are rapidly proliferating, the risk is likely to be greater.

At the IARC meeting on mechanisms held in June

1991, it was clearly pointed out that mechanistic data could increase or decrease the classification of a chemical carcinogen. This is only appropriate. Risk assessments should be modified if an agent exhibits large species differences in response. An example of this is thyroid-stimulating hormone (TSH)-mediated thyroid tumors. This is a well-characterized example of the role of cell proliferation in chemical carcinogenesis. The normal means of maintaining hormonal homeostasis involves a feedback system between the hypothalamus-pituitary gland and the thyroid. When circulating thyroxin levels become low, the pituitary releases TSH, which in turn stimulates hypertrophy and hyperplasia of the thyroid follicular cells to synthesize more thyroxin. Exposure to some chemicals interferes with the production or metabolism of thyroxin, leading to sustained increases in TSH release and subsequent cell proliferation. This increase in cell proliferation leads to the induction of thyroid neoplasia (*13*). The induction of thyroid tumors could be completely ablated by feeding the animals thyroxin. To show that such thyroid tumors were due to chronic TSH stimulation of thyroid follicular cell replication, experiments done more than a quarter-century ago used transplantable pituitary tumors that secrete TSH to induce thyroid neoplasms (*14*).

Perhaps the most important outcome of this meeting is the identification of future research needs to clarify the role of cell proliferation in carcinogenesis. It is clear that we need to expand our database in several directions. Future bioasssays should be designed in a manner to provide much more and better data on cell proliferation. Beginning with the subchronic studies, cell proliferation can be used to help establish the MTD. The data will be useful in understanding nonlinearities in dose response and will establish the correlations for increased cell proliferation and the induction or noninduction of neoplasia. These studies will provide test compounds for elucidating critical mechanisms in carcinogenesis. Understanding interactions between species-, sex- and tissue-specific susceptibility factors and cell proliferation in chemical carcinogenesis is another major need. Why are some tissues susceptible to cancer induction while others are not? Why is the rat bladder susceptible to calculi-induced bladder cancer and the mouse bladder not? Once a series of chemicals that have an adequate database on cell proliferation is available, we may be able to identify elements of the mutational spectra that are associated with background DNA damage and cell proliferation. If one can identify the cell proliferation equivalent of the aflatoxin-induced *p53* mutation at codon 249, it may provide a marker for proliferation-induced neoplasia.

Let me end by addressing one of the organizing committee's opening questions: Should cell proliferation be used in risk assessment? This could also be restated as: Can science replace the emperor's new clothes? We have discussed the different degrees of rigor that are present in experiments and experimental data sets on cell proliferation. We should give equal thought to the degree of rigor that is present in the mathematical extrapolations of risk that are currently practiced. What is their basis? Have they ever been validated, and where they are taking us? Regulation is important to human health, but it also does not come without costs. These costs are many fold. If we do a poor job of communicating the causes of human cancer to society, we are not promoting the prevention and control of cancer. If society equates cigarette smoking with the "carcinogen of the week" because the risk assessments all look alike, we as scientists involved in cancer control have failed. We must begin to incorporate knowledge of mechanism into the risk assessment process to improve its accuracy. In this regard, cell proliferation is one piece of mechanistic data that should be evaluated for its impact on the dose response for carcinogenesis. It is clear that accurate risk assessments will be required to identify and prioritize those health issues of greatest importance.

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