Functional Markers and Growth Behavior of Preneoplastic Hepatocytes

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Summary

Functional markers and growth behavior of abnormal hepatocytes at several stages of liver carcinogenesis were studied. Early lesions, i.e., hyperplastic foci and areas, did not accumulate iron in siderotic livers, had persistent glycogen stores, were not more agglutinable by concanavalin A, and were associated with α-fetoprotein secretion, but were not independent secretors of high amounts. The cells in the early lesions had an increased mitotic index, but cells from livers with early lesions did not have an increased survival in cell culture or the ability to grow in soft agar. The more developed lesions, hyperplastic nodules, also did not store iron, had persistent glycogen, did not display increased concanavalin A agglutinability, and were not independent secretors of high levels of α-fetoprotein. Similarly, nodule cells were proliferative but did not display an increase in survival in cell culture. In addition, both iso- and autotransplantation of nodules into mammary fat pads resulted in persistence but not growth of nodule cells. On the other hand, hepatocellular carcinomas regularly grew upon transplantation. Thus, early lesions and hyperplastic nodules were proliferative lesions but did not possess autonomous growth capability comparable to that of hepatocellular carcinomas.

Introduction

During the administration of hepatocarcinogens to rats, several distinct focal lesions of hepatocytes develop sequentially (15, 16). The understanding of the pathogenesis of hepatocellular carcinoma will depend upon establishing the origin of the populations in these lesions, their relationship to one another and to carcinoma, and their biological behavior. The critical biological property to be assessed in these lesions is growth behavior, since all neoplasms are characterized by an overgrowth of their tissue of origin, and therefore, this loss of growth control must be acquired at some stage. This review discusses studies performed in collaboration with various colleagues in which certain functional properties of focal lesions were used as markers for studies of their origin and relationship to one another. Studies underway on the growth behavior of these lesions are also described.

Early Lesions. Although the hyperplastic nodule is easily recognized because of its compression of the surrounding parenchyma, the early lesions that precede nodule formation are not so well characterized and have been given a variety of names. Reuben (14) describes the hyperplastic focus as a small collection of abnormal cells less than 1 mm in diameter in which there is architectural disorganization, but which is not clearly demarcated from the surrounding parenchyma. The hyperplastic area is larger and is well demarcated, but does not compress the surrounding parenchyma. Just as the hyperplastic nodule is induced with a variety of hepatocarcinogens (5, 11, 15, 27), so too are these hyperplastic foci and areas (11, 15, 27). Also, in our experience, both types of lesions are induced only by carcinogens. Our study of early lesions has been greatly facilitated by the discovery that hepatocellular carcinomas and hyperplastic nodules that developed in livers with 8-hydroxyquinoline-induced siderosis did not contain stainable iron (28). Subsequently, it was shown that hyperplastic areas and, apparently, foci were also unable to store iron (27). This feature has recently been examined in greater detail by inducing siderosis through the feeding of 8-hydroxyquinoline and ferrous gluconate to rats for 3 months before commencing carcinogen treatment. In this study (25), very early lesions of iron-negative cells were detected that were not as well-developed as any previously described (15). These, as well as hyperplastic foci and areas, are referred to hereafter as "early lesions." At 3 weeks of 0.05% FAA feeding, small periportal foci of abnormal iron-free hepatocytes that did not encroach on the surrounding parenchyma were present (25). The cells in these foci had occasionally enlarged nuclei and hyperbasophilic cytoplasms. They were the only proliferative cells (MI, 1.03) in the carcinogen-treated liver, even after a mitotic stimulus, indicating that all newly formed cells may be identified by the absence of iron. By 4 weeks, the lesions were larger and had lost architectural arrangement, but they still did not compress the surrounding parenchyma (Fig. 1). At this time the cells retained glycogen when it was depleted from surrounding iron-containing cells (Fig. 2). The cells in these iron-free lesions also had an MI of 0.99, while the surrounding iron-containing hepatocytes were nonmitotic. The absence of mitoses in the normal iron-containing hepatocytes was shown to be due to inhibition by the carcinogen and not by the iron. Thus,
these early lesions displayed a resistance to the inducing carcinogen and arose at least in part by cell division. Furthermore, they were also frequently spared from dimethylaminonitrosamine-induced necrosis (25), which probably indicates a reduced metabolic capability, as has been found for hyperplastic nodules. The basis for the lack of iron accumulation by these cells has not been determined, but it appears to be a consequence of decreased cellular uptake or storage. AFP is produced by hepatocellular carcinomas induced with a variety of carcinogens (1, 9, 20). Watabe (22) first showed for 4-dimethylaminoazobenzene, and we (10, 11), for 3'-methyl-4-dimethylaminoazobenzene, FAA, N-hydroxy-N-2-fluorenylacetamide, and aflatoxin B₁, that high levels [calculated from previous studies (9) to be >80 μg/ml] of serum AFP were induced by carcinogen treatment within 2 weeks, long before the appearance of carcinomas. Early lesions of hepatocytes and hyperplastic nodules were present at the time AFP secretion was induced. However, none of these lesions was capable of secreting amounts of AFP comparable to that of carcinomas in the absence of the carcinogen, as proven by 2 findings: (a) when carcinogen was withdrawn, AFP production fell below detectable levels (10), and (b) low levels of carcinogens induced only a temporary appearance of high levels of AFP, although administration was continued (11). Thus, early lesions were not independent producers of high levels (>80 μg/ml) of AFP in the absence of a carcinogen. Con A has been reported to agglutinate hepatocellular carcinomas but not normal liver (2). Using collagenase perfusion of adult rat liver to dissociate liver cells (12, 24), the Con A agglutinability of viable cells capable of initiating cultures (12, 24) was studied after intervals of FAA treatment. Normal liver plasma membrane has been shown to have Con A binding sites (4, 19), and normal cells were agglutinable (Chart 1), confirming the findings of other investigators (8). This agglutination occurred even if perfusion was performed with only a chelating solution (12), and was specific, as shown by inhibition with methyl-α-D-mannopyranoside. Even after 10 weeks of 0.02% FAA feeding, at which point early lesions were present, cells from treated liver were not more agglutinable than normal cells (Chart 1). Agglutinability should, of course, be studied on isolated altered cells, but these results gave no indication of an enhancement.

The growth behavior of these early lesions is presently

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being studied in cell culture and in soft agar. Dissociation of the liver following collagenase perfusion produces a high yield of cells (12) which can be reproducibly cultured (12, 24). Using procedures that gave a recovery in culture of 14% of the liver (12), there was a considerable pleomorphism of cultured treated cells, but no increase in survival compared to controls. Cells from normal adult rat liver seeded directly in soft agar have not formed colonies and, at 3 weeks of FAA treatment when early lesions were present, there was still no colony formation in agar. This property is currently being studied after longer intervals of treatment.

Thus, these early lesions are abnormal in a variety of ways; the cells have abnormal nuclei, they do not store iron, they retain glycogen under conditions in which normal hepatocytes are depleted, and they are resistant to the mitotic inhibition of carcinogens and to necrogenic effects of agents requiring metabolism. Haddow (7) and others (5) have favored the concept that carcinogens might initiate their effects by selective toxicity, allowing a resistant population to emerge. The principal question concerning the origin of such a resistant population is whether it starts out by proliferation of preexisting cells or whether it is a newly induced population from the beginning. Considering the abnormalities in these lesions which have not been identified in normal hepatocytes, it seems more likely that such a population would arise from cells with alterations induced by the carcinogen. This could occur by 2 mechanisms: (a) mutation or (b) induction of a new stable state of differentiation. Isolation of the cells in the early lesions could permit studies to examine these possibilities. Fortunately, the lack of iron storage by these cells undoubtedly results in a difference in density between them and normal hepatocytes and, thus, may make physical separation and isolation possible.

These foci can persist long after the removal of the carcinogen (27), which raises the question whether they are autonomous growths. This is not necessarily the case, however, since the lifetime of hepatocytes is of the order of 200 days (13) and, thus, any induced population could persist for such long periods until diminished by cell death or differentiation.

With increasing intervals of carcinogen exposure, iron-free lesions became larger and were eventually associated with hyperplastic nodules composed of cells morphologically identical to those in the hyperplastic areas (25).

**Hyperplastic Nodules.** The nodule is larger than the early lesions and clearly represents an overgrowth, since it compresses the surrounding parenchyma (5, 15, 17). Like the early lesions, nodule cells did not store iron (25, 27, 28). Although they are a site of AFP production (14), they did not independently secrete high levels of AFP (10, 11). Becker (2, 3) has reported that hyperplastic nodule cells are not agglutinable by Con A, while hepatocellular carcinoma cells are. We found that nodule cells, including isolated ones, were agglutinable, but not significantly more so than were normal liver cells (Chart 2). Thus, nodule cells did not differ from the early lesions in the properties we have studied. This is consistent with the possibility that nodules develop simply by enlargement of early lesions, rather than by selection of a special subpopulation. This process was also suggested by the study of the development of iron-free lesions (25). After 6 weeks of FAA treatment, there were only iron-free hyperplastic areas. FAA was withdrawn for 2 weeks and then was fed again for an additional 2 weeks, after which iron-free nodules were present. For the nodules to have developed from iron-containing cells in so short a time would have required a massive synchronous loss of iron from several hundred associated cells or an explosive onset of proliferation. With regard to the latter, the MI of the nodules was 0.98, comparable to that of the early lesions at 3, 4, and 6 weeks. That is, nodules had about the same rate of proliferation as the early lesions. Therefore, it was concluded that the nodules developed by enlargement of hyperplastic areas (25). Thus, the process up to this stage seems to be simply the proliferation of a fairly uniform abnormal population.

The biological significance of hyperplastic nodules is being examined through a study of the growth behavior of nodule cells in cell culture, in soft agar, and in transplants. Although cultures from nodule cells displayed considerable pleomorphism, there was no obvious increase in the survival, compared with normal cells. The transplant studies have used both isologous and autologous transplantation into the 4th or 5th mammary fat pad. Both normal liver cells and nodule cells persisted for months at this site. While nodule cells remained histologically recognizable, they showed no autonomous growth. On the other hand, hepatocellular carcinoma cells grew regularly in this location. Thus, these studies confirmed the observations of Reuber and Firminger (17) and Reuber and Odashima (18) on the lack of transplantability of nodules (17, 18). By demonstrating for the first time the persistence, but lack of growth, of autotransplanted nodules, these studies also eliminated immune rejection as the mechanism for this absence of autonomous growth. Thus, these various studies on the growth behavior of nodule cells have not revealed any autonomous growth ability of the cells outside the liver. However, this does not preclude the possibility that nodules are progressively growing in situ.

Reuber (16) suggests that hyperplastic nodules are at the stage at which they are no longer dependent upon exposure to the carcinogen and will continue to progress. Other studies suggest that nodules may be regresible (21) or

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**Chart 2.** Con A agglutinability of collagenase-dissociated control liver (X) and FAA-induced hyperplastic nodule (HN) (·) cells.

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4 G. M. Williams, M. Klaiber, and E. Farber. The Difference in Transplantation between Hyperplastic Nodules and Hepatocellular Carcinomas, manuscript in preparation.
reversible (5). There are several issues to be clarified here, but, in general, it has been my experience that not all nodules progress to carcinoma. In a current experiment,\(^3\) nodules induced by the intermittent feeding of ethionine for 20 weeks were followed up to 11 months and, while they persisted, they did not progress to form carcinomas.

**Hepatocellular Carcinomas.** It is not yet definitely established from which cells carcinomas originate. Farber and Ichinose (6) first identified within nodules the development of regions of atypicality that were morphologically identical to malignant cells. Reuber (15) also suggested that small carcinomas arise in nodules. We have confirmed the development of such changes inside nodules (23), but have also observed them outside of nodules (23). In addition, in a current experiment,\(^3\) carcinomas were induced following development of hyperplasias, but without formation of distinct nodules. Thus, it seems that the nodule is not an obligate precursor to carcinoma. It remains to be determined what percentage of carcinomas do arise in nodules and what percentage of nodules do progress to form carcinomas.

**Conclusions**

The experiments reviewed suggest that the earliest focal lesions arising during hepatocarcinogenesis are, at least in part, proliferative. An important aspect of their development is their resistance to the inducing carcinogen. These lesions appear to enlarge to form hyperplastic nodules. The early lesions and the nodules are similar in several ways and lack certain features that characterize hepatocellular carcinomas. In particular, nodules do not secrete high levels of AFP, do not have increased Con A agglutinability, and do not grow upon autologous or isologous transplantation. Thus, the nodule should not be considered equivalent to the carcinoma.

The absence of stainable iron from the preneoplastic and neoplastic cells is the easiest and perhaps the most reliable marker for these altered cells. The early induction of iron-free focal lesions could serve as a rapid bioassay for carcinogens, just as does the induction of high levels of AFP (10, 11). In addition, because of the almost certain difference in density between cells loaded with iron and those free of it, the absence of iron from early lesions is the only marker that might make possible the physical isolation of these cells. The techniques for recovery and culture of hepatocytes (12, 24, 26) have reached the level where critical questions concerning the growth potential in vitro of altered cells can be answered. Such studies will significantly contribute to an understanding of the biological behavior of preneoplastic cells.

**References**


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