Kinetics of Phenotypic Maturation of Remodeling of Hyperplastic Nodules during Liver Carcinogenesis

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ABSTRACT

Hyperplastic nodules appearing during the preneoplastic phase of liver carcinogenesis were divided into two types, persistent and remodeling, according to the pattern of staining for γ-glutamyltransferase. In the resistant cell model of liver carcinogenesis used in this study, hyperplastic nodules, uniformly staining for γ-glutamyltransferase, rapidly emerge by 4 weeks after a single injection of diethylnitrosamine and brief selection by dietary 2-acetylaminofluorene plus partial hepatectomy. By 6 weeks, a majority of nodules (about 75%) show an obvious irregularity and loss of uniformity in staining for γ-glutamyltransferase while the remaining nodules continue to be uniformly stained. The number of irregularly stained nodules increases over the next 18 weeks until over 95% of nodules show the nonuniform loss of enzyme activity. The progressive loss of enzyme activity is accompanied by architectural remodeling. The uniformly stained nodules show the persistence of hepatocyte arrangements in plates two or more cells thick and in acini and of cytoplasmic hypertrophy characteristic of persistent hyperplastic nodules. Labeling indices are much higher in hepatocytes of the persistent uniformly stained nodules than in the remodeling ones. The possibility of exploiting this phase of the model further for in-depth analysis of the nodule-to-carcinoma sequence is discussed.

INTRODUCTION

Since the original discovery of experimental liver cancer induction with chemicals by Sasaki and Yoshida (30), it has been a common observation of most investigators that nodular hyperplastic lesions, composed predominantly of hepatocytes, precede the appearance of hepatocellular carcinoma in rats with many different carcinogens (7, 8, 10, 21, 23, 28, 31, 35–39). Also, with at least 5 carcinogens, Aramite (26), ethionine (10, 11), 3'-methyl-4-dimethylaminoazobenzene (16), 2-AAF4 (27), and DENA (35), hyperplastic nodules have been demonstrated to be one site of origin for liver cell cancer. These observations point to the obvious importance of hyperplastic nodules in one sequence of changes leading to hepatocellular carcinoma and to the need for a much closer examination of these lesions in the sequential analysis of liver cancer development.

At least 2 types of hyperplastic nodules have been described [persistent and "regressing," "maturing," or "remodeling" (10–13, 17–20, 22, 26, 30, 35, 38)]. Regressing nodules were observed by routine histological examination in several studies on liver carcinogenesis (10–13, 26, 30, 35, 38). Kitagawa (18, 19) and Kitagawa and Pitot (20) first reported the progressive change in some histochemical markers in some nodules, a phenomenon which he designated as "phenotypic maturation." This has been confirmed by using other histochemical indices (17, 22). The probable correspondence between regression and maturation and the phenomenon of remodeling was suggested (12). The persistent nodules show the continuation of the characteristic architectural pattern of hyperplastic nodules (plates 2 or more cells thick and acini and of several negative and positive biochemical markers). New cell populations, "nodules within nodules" (26), including metastasizing hepatocellular carcinoma (35), have been found confined entirely to a hyperplastic nodule with several carcinogens (10, 11, 26, 27, 35). The regressing or maturing nodules characteristically show a progressive remodeling to normal-appearing liver, with single-cell plates, and a concomitant gain or loss of negative or positive markers, respectively.

During previous studies by Ogawa et al. (22) and by Enomoto et al. (9), it appeared that it may be possible to distinguish fairly early and quite easily persistent from remodeling hyperplastic nodules with the use of histochemical staining procedures using a developing new model for the sequential analysis of liver carcinogenesis (13, 14). In this model, altered hepatocytes resistant to some inhibitory effects of carcinogens are induced by a single dose of DENA or some other carcinogen (34, 39). The resistant cells are rapidly selected by a brief exposure to dietary 2-AAF plus PH. Under these conditions, hyperplastic nodules rapidly appear in a reasonably synchronized manner, and these can be identified grossly and followed quantitatively and qualitatively as a function of time. Since metastasizing hepatocellular carcinoma has been found later in such nodules (35) in the absence of any further exposure to a carcinogen, the close relationship between hyperplastic nodules and cancer development in this model is indicated.

The present communication describes the early identification of at least 2 populations of nodules, persistent and remodeling after discontinuation of the selection procedure, using staining for γ-GT, (5) labeling with radioactive thymidine, and growth as functions of time and the possible utility of this phase of the model for the study of the nodule-to-carcinoma sequence.

MATERIALS AND METHODS

Animals and Treatment. Male Fischer rats (F-344) (Charles River Breeding Laboratories, Wilmington, Mass.) initially weighing 150 to

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180 g were used. The rats were maintained on a semisynthetic moderately high protein (24%) basal diet (Bio-Serv, Inc., Frenchtown, N. J.) and a 12-hr-light, 12-hr-dark daily cycle. They were given food and water ad libitum and were acclimatized to their environment for at least 1 week before the start of the experiment.

The experimental design is based on the model described by Solt and Farber (34) and is outlined in Chart 1. The rats were given i.p. injections of DENA (Eastman Kodak Co. Rochester, N. Y.) dissolved in 0.9% NaCl solution at a dose of 200 mg/kg body weight. After a 2-week recovery period, they were subjected to a selection procedure consisting of 2 weeks of 2-AAF feeding (0.02% in the basal diet) with two-thirds PH performed at 1 week. Then the rats were kept on the basal diet until the termination of the experiment. Groups of several rats each (Chart 1) were sacrificed by decapitation at 4, 5, 6, and 8 weeks after the initial DENA injection and subsequently every 4 weeks for up to 24 weeks. All animals were fasted for 18 hr before sacrifice. The experiment was repeated at least twice for every time point.

Liver slices were always taken from the same portion of each liver lobe and were fixed in cold acetone for histochemical demonstration of γ-GT activity. γ-GT histochemistry was performed according to the method of Rutenberg et al. (29) as modified by Ogawa et al. (22). Contiguous sections were stained with routine H & E. The number of γ-GT-positive lesions were counted by low-power microscopy and expressed as foci per sq cm. The long and short axes of the foci were measured at intervals from 4 weeks to 6 months after the initial DENA administration. Nodules staining uniformly for γ-GT were designated as the persistent type, and those showing patchy staining were designated as the remodeling type. Several γ-GT-stained slides at various time periods were randomly photographed. γ-GT-positive lesions in the photographs were examined separately by 5 individuals using the above criteria, and the agreement with regard to uniform versus patchy staining was very close.

Identification of the 2 Types of Liver Lesions. All γ-GT-positive hepatocyte nodules were divided into 2 types according to their γ-GT staining pattern, i.e., completely uniform γ-GT staining or irregular patchy γ-GT staining. The former refers to those lesions which showed partial disappearance of γ-GT. The total number of γ-GT-positive nodules and the number and size of the uniform γ-GT-staining lesions were measured at intervals from 4 weeks to 6 months after the initial DENA administration. Nodules staining uniformly for γ-GT were designated as the persistent type, and those showing patchy staining were designated as the remodeling type. Several γ-GT-stained slides at various time periods were randomly photographed. γ-GT-positive lesions in the photographs were examined separately by 5 individuals using the above criteria, and the agreement with regard to uniform versus patchy staining was very close.

 Autoradiography and Labeling Index. At 3, 4, and 6 months after DENA injection, some animals were given [methyl-3H]thymidine i.p. at a dose of 0.5 μCi/g body weight (64 Ci/mol, New England Nuclear, Boston, Mass.). The rats were given 4 injections at 6-hr intervals and were sacrificed 24 hr after the first injection. The liver slices were fixed in cold acetone and then processed for γ-GT histochemistry. The γ-GT-stained sections were coated with Kodak NTB3 emulsion and stored in a desiccator for 3 weeks at 4°. After developing, the slides were counterstained with hematoxylin for microscopic examination. The number of total and labeled parenchymal cells per unit area were counted randomly in the persistent-type nodules, remodeling-type nodules, and surrounding tissues. About 1500 to 2000 cells in each lesion were examined in every liver. Contiguous sections were stained with H & E.

RESULTS

Appearance of Persistent and Remodeling Nodules. As indicated in previous reports (22, 35), the hyperplastic nodules appear rapidly and enlarge fairly synchronously in this model until 4 to 5 weeks after the time of the injection of DENA. In this study, at 4 weeks, the nodules were very similar in appearance to each other both with conventional H & E staining and with staining for γ-GT. However, the subsequent distinction between remodeling and persistent nodules is very difficult to make by ordinary light microscopy with H & E until many weeks after their initial appearance (12, 35). This is in contrast to the ease of distinction between uniform and nonuniform staining for γ-GT (Fig. 1). The irregular regional loss of staining for this enzyme activity in nodules was clearly evident very early, within 1 to 2 weeks, after their initial period of uniformity up to 5 weeks. The uniformly stained nodules were sharply demarcated from the surrounding liver in the whole of their circumference. The nonuniform nodules showed the beginning of loss of enzyme activity very quickly, and this often involved a segment of the peripheral region of the nodule. In the later stages, the multicell plates and acinar arrangement of hepatocytes in the uniformly stained nodules were readily distinguished from the areas of single cell plates in the nonuniform lesions.

In confirmation of the results in a previous study (22), over 90% of the early hyperplastic foci and nodules stain positively for γ-GT. This makes it possible to equate number of nodules to number of nodules staining for γ-GT with only a small degree of uncertainty. The correspondence between remodeling as seen with H & E slides and the nonuniform staining of nodules for γ-GT was clearly evident.

A difference in the gross appearance of the 2 types of nodules was obvious. The persistent nodules remained grayish-white and sharply demarcated from the surrounding reddish-brown liver while the remodeling ones showed a grayish-brown mottling with increasing blending into the surrounding liver. Equally important is the observation that the remodeling nodules, unlike the persistent ones, show a progressive merging with the surrounding liver. Thus, by 12 to 16 weeks, the liver becomes smooth and assumes a marbled grayish-brown color, and scattered throughout this are the discrete grayish-white persistent nodules.

Quantitation. One of the most striking aspects of this study is the rapid loss of γ-GT staining in a majority of the nodules by the sixth week (Table 1). The total number of nodules showed...
an apparent increase from 4 to 5 weeks and then began to show a slow decline. One week after the cessation of the 2-AAF diet, i.e., at 5 weeks, the total number of γ-GT-positive nodules per sq cm showed an apparent 148% increase as compared to the base-line values at 4 weeks. By 24 weeks, the total number of γ-GT-positive nodules decreased to 42% of the value at 4 weeks.

The slow rate of loss of the total number of nodules is in very sharp contrast to the decrease in the number of uniformly stained lesions relative to the total. As is easily seen in Table 1, the vast majority of γ-GT-positive nodules showed evident areas of loss of staining by Week 6, and this increased only very slowly over the next 18 weeks. The ratio of uniformly stained nodules to the total γ-GT-positive nodules dropped dramatically from 97% at the fifth week to 24% at the sixth week and 15% at the eighth week and thereafter showed only a very slow decline to 7% at 24 weeks. Thus, within 2 weeks of their uniform appearance at 4 weeks, the majority of nodules already show evidence of nonuniformity of staining, no doubt as an accompaniment of the remodeling process. However, despite this relatively rapid appearance of the earliest evidence of phenotypic maturation or remodeling, the degree of change among the many nodules is quite variable. Some nodules show advanced degrees of loss within three weeks, while others showed far less loss even after many weeks.

**Proliferation Activity.** In addition to differences in appearance in architecture and staining with H & E and γ-GT between the 2 nodule populations, a clear-cut difference in proliferative activity was seen. As shown in Table 2, the labeling indices of the hepatocytes in the uniformly stained nodules were considerably and significantly larger at 12, 16, and 24 weeks than were those in the hepatocytes in the nonuniformly stained nodules and in the surrounding liver. In the uniformly stained nodules, the labeled hepatocytes tended to be localized in the peripheral areas of the nodules and were sometimes concentrated in one or more areas (Fig. 2). In the nonuniformly stained nodules, the infrequently labeled hepatocytes were seen not only in the γ-GT-positive areas but also in the γ-GT-negative regions (Fig. 3) with no apparent differences in the labeling indices between these areas. However, it is interesting to note that some areas in a few nonuniformly stained (remodeling) nodules at 24 weeks had as high a labeling index as was seen in some of the uniformly stained (persistent) nodules.

Consistent with the labeling data are the results of measurement of the mean diameter of the uniform γ-GT-positive nodules. As can be seen in Chart 2, these nodules, the persistent ones, showed a progressive increase in mean diameter as a function of time. At 4 weeks, the mean diameter of all the nodules was 0.45 mm, and this value increased to 2.1 mm at 24 weeks. Comparable measurements of the nonuniform γ-GT-positive nodules, the remodeling ones, could not be accurately done because of their irregular shape and the irregular patterns of loss of γ-GT activity.

**DISCUSSION**

The most important facet of this study is the clear-cut demonstration that the 2 recognized options for hyperplastic nodules, persistence or remodeling, are expressed early in the life history of the nodule in this model of carcinogenesis and that the majority of nodules take the second option under these circumstances. Equally important is the progressive loss of the nodular character of the liver except for the few scattered persistent nodules. Such a liver should be readily amenable to isolation of intact persistent nodules separate from the surrounding tissue (6).

A phenomenon similar to that seen with γ-GT is also seen with epoxide hydrolase (9). With the use of staining for this enzyme activity, a rapid onset of a patchy loss of activity in nodules that were also losing γ-GT activity was found. Interestingly, the areas that lose γ-GT retain the hydrolase and vice versa (9). Perhaps this is a reflection of an early zonal differentiation since the hydrolase is highest in activity in Zone 3 and lowest in Zone 1 (9), while γ-GT is seen most frequently to appear in hepatocytes in Zone 1 and least often in Zone 3.

Not only does the ratio of uniform (persistent) to nonuniform (remodeling) and total nodules change slowly with time, but the total number of nodules also decreases as a function of time. The most likely explanation for this appears to be the blending of the remodeling nodules with the surrounding liver as the architecture changes to that of normal liver. This phenomenon is not special to this model, since it has been described as "reversibility" and "regression" of nodules in different models with either prolonged or intermittent exposure to one of several carcinogens (10, 12, 15–20, 30, 38).

An impressive aspect of this study is the contrast between the fairly clear-cut rapid rate of onset of remodeling, within 1 to 2 weeks after the maximum number of nodules appear, and the large spread in the rates at which advanced remodeling is seen in different nodules. Thus, the individual focal proliferations, the hyperplastic nodules, show a diversity in rates of

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**Table 2**

<table>
<thead>
<tr>
<th>Time after initial DENA injection (wk)</th>
<th>Uniformly stained (persistent)</th>
<th>Nonuniformly stained (remodeling)</th>
<th>Surrounding liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (2)</td>
<td>2.0 ± 0.3*</td>
<td>0.7 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>16 (3)</td>
<td>2.5 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>24 (4)</td>
<td>3.1 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

*Significantly different from b and c at each time period (p < 0.001).

Numbers in parentheses, number of effective animals.

Mean ± S.E.
remodeling even though the onset of the process is reasonably synchronous. A similar diversity is seen in other biochemical markers in both early and advanced focal proliferative lesions in several other models (8, 19, 22, 24, 25, 27, 31).

With respect to the number of nodules in the liver as a function of time, the apparent rapid increase between the fourth and fifth week deserves some comment. We interpret this as a reflection of the end of the selection pressure created by the dietary 2-AAF plus PH. It is possible that the proliferation of some foci of initiated cells may be inhibited by the dietary 2-AAF and that these rapidly enlarge to become "countable" as the degree of inhibition wanes. It must be emphasized that the apparent increase in number between the fourth and fifth week may be largely if not entirely an artifact, due to an increase in the mean diameter of the nodules. The larger the diameter of a nodule, the greater is the probability of seeing it in a random section (32). This may also be the case with the persistent nodules at later times. Obviously, this complication could decrease the absolute number of persistent nodules per unit area at the later time points.

The phenomenon of "maturation" or "phenotypic maturation" of hyperplastic areas or nodules was described first by Kitagawa (18, 19) and was studied further by Kitagawa and Pitot (20), and by us (22) using various markers. We consider this to be similar to or the same process as architectural remodeling of nodules described a few years ago (12). Thus, even though the focal cell proliferations designated as hyperplastic nodules begin as a basically irreversible phenomenon, initiation (8, 33), their contained hepatocytes as a population have as a major option the ability to undergo a complex series of biological and biochemical changes leading to the ultimate differentiation to normal-appearing liver.

The availability of biological options for the carcinogen-induced focal proliferative lesions selectively stimulated to grow by promoting environments is not unique to the liver but is seen in a very analogous manner in the skin as papillomas (1-4) and also elsewhere (14).

An interesting and potentially important aspect of this study is the observation correlating persistence with increased labeling index. Virtually all persistent or uniform nodules contained many more proliferating hepatocytes than were found in the remodeling nodules or in the surrounding liver. However, the 2 to 3% labeling indices of these nodules after the intense selection pressure has subsided are to be compared with the 80 to 90% labeling indices seen during the formative growth of the nodules during Weeks 3 to 5 when the selection pressure is intensive and maximal (22, 35). Although an occasional "hot spot" was found in "nonuniform nodules," this was quite uncommon. Whether the persistence of enzyme markers such as γ-GT and the increased proportion of proliferating hepatocytes in the persistent nodules have any close causal relationship is not known.

The basis for the elevated labeling index is not evident as yet. Whether this represents a true change in the control of cell proliferation in the involved hepatocytes toward autonomy or is a reflection of a persistence of a mitogenic stimulus in the liver generally remains to be established. Since the hepatocytes in the surrounding liver also show an elevated level of labeling above adult resting liver, it is conceivable that a mitogenic environment is being exerted on the whole liver. Clearly, carefully controlled comparisons between the proliferative capacity of these different hepatocyte populations in vitro and in vivo seem indicated.

Finally, it should be emphasized that the results of the present study show that carcinogen-induced new cell populations still have the potential to differentiate to normal-appearing tissue. Thus, the manifestation of a complex differentiation phenomenon is not proof that a population of cells has not sustained some damage by a carcinogen.

REFERENCES

K. Enomoto and E. Farber


Fig. 1. Persistent nodule (P) and remodeling nodule (R) observed at 12 weeks after DENA injection. γ-GT, × 70.

Fig. 2. Autoradiograph showing a focal aggregation of labeled hepatocyte in a persistent nodule. Surrounding liver (S) shows no labeling. γ-GT and hematoxylin, × 175.

Fig. 3. Autoradiograph of a remodeling nodule. A labeled hepatocyte is seen in γ-GT negative area. γ-GT and hematoxylin, × 175.
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