Sequential Phenotypic Changes in Hyperplastic Areas during Hepatocarcinogenesis in the Rat

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Summary
Sequential phenotypic changes in hyperplastic areas of rat liver during N-2-fluorenylacetamide feeding were studied by enzyme and immunohistochemical methods combined with radioautography.

Hyperplastic areas showed a marked deficiency of β-glucuronidase and serine dehydratase during their developing phase, the 6th through the 9th experimental weeks, and were fairly specifically labeled by injections of tritiated thymidine after partial hepatectomy performed at the 9th week. A sequential observation on these labeled hyperplastic areas revealed a considerable elevation of the levels of these marker enzymes in the majority of the labeled areas in 3 to 18 weeks after labeling. On the other hand, there was a small group of hyperplastic areas in which the enzyme deficiency persisted during the observation period. This type of lesion was generally larger than those showing enzymic maturation. Labeled cells were not detectable either in distinct hyperplastic nodules at late phase or in carcinomas.

The metabolic regulation in the cells comprising hyperplastic areas was studied by checking the induction and repression of serine dehydratase after dietary stimuli. Serine dehydratase was not inducible in hyperplastic areas during the developing phase or in areas with persistent enzyme deficiency, but it was clearly induced and repressed in areas where there was an elevation of the endogenous enzyme level.

The areas of hyperplasia with persistent enzyme deficiency and growth appeared to be more important than the ones of phenotypic maturation in relation to the later development of carcinoma. The phenotypic maturation in hyperplastic areas might represent reversion of altered cells towards normalcy from the condition related with neoplastic transformation.

Introduction
The liver during carcinogen feeding has been extensively utilized for biochemical analysis of carcinogenesis. This is chiefly because the liver parenchymal cell has various marker enzymes and because the liver was considered to be a mass of relatively homogenous parenchymal cells, convenient for quantitative biochemical analysis. However, as histochemical observations (5, 11, 16) have shown, the "preneoplastic" liver is a mixture of degenerating cells from the original parenchyma and various kinds of hyperplastic lesions exhibiting different enzyme levels. Even the normal liver parenchymal cells have a considerable phenotypic heterogeneity within a lobule (12). Biochemical studies of preneoplastic liver should be carried out in close correlation with morphological observations.

Among various types or morphological changes in the liver during carcinogenesis, hyperplastic areas and nodules have been described as very important preneoplastic elements in the neoplastic transformation of liver parenchymal cells (3, 4, 15, 21). These are focal or nodular proliferations of somewhat altered liver cells appearing during the preneoplastic phase. With relatively low doses of carcinogen, most lesions grow slowly with minimal compression of the surrounding parenchyma and have been designated as hyperplastic areas, foci, or islands (5, 20, 21, 24). Distinctly nodular, generally larger lesions developing in a later phase of carcinogenesis were separately classified as nodules of hyperplasia or adenomas (21, 23). However, in conditions where degeneration of parenchymal cells proceeds rapidly, as is seen in experiments with high doses of carcinogen, or in conditions where the proliferation of the cells of hyperplastic areas is specifically accelerated, as is seen after partial hepatectomy during carcinogen feeding, hyperplastic areas grow more rapidly with apparent nodularity and grow large. In these cases, the clear-cut distinction between areas and nodules is difficult. Therefore, and probably for the sake of simplification, all the proliferative lesions of parenchymal cells have often been described simply as hyperplastic nodules (1-4, 13, 25, 26).

The technique of inducing large hyperplastic nodules by applying a high dose of FAA\(^1\) in the rat (3) offered a new system to obtain more uniform materials than ever for biochemical and morphological analysis of preneoplastic changes. Working with large hyperplastic nodules, Epstein et al. (3) reported a progressive decrease of glucose-6-phosphatase and phosphorylase levels in nodules with time. In their experiment, large hyperplastic nodules at a given phase were regarded basically as a homogeneous population and also as the representative of nodules of various sizes. Later, however, Kitagawa (11) demonstrated histochemically the variability of enzymic levels including glucose-6-phosphatase and phosphorylase in (large) hyper-

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2 The abbreviation used is: FAA, N-2-fluorenylacetamide.
plastic nodules. Teebor and Becker (25) reported heterogeneity among hyperplastic nodules in terms of their “persistence” after discontinuation of the carcinogen. Furthermore, Becker and Klein (1) pointed out the individual heterogeneity among nodules with respect to the pattern of protein synthesis, together with the difference in the level of protein production between “early” and “late” nodules. All these findings suggest the need for more careful consideration on the heterogeneity among hyperplastic nodules in analyzing progression of carcinogenesis.

Kitagawa and Sugano (14) introduced a combination technique of enzyme histochemistry and radiomicrography that enabled them a more direct and sequential follow up of phenotypic changes in hyperplastic areas in close correlation between morphological and biochemical changes. The results of experiments with this technique compose a part of the present paper.

The impaired metabolic adaptation of hepatomas to substrate or hormonal stimuli has been documented (10, 18). Studies have also been carried out on enzyme regulation in liver during carcinogen feeding. Most of these studies revealed an inhibition of the adaptive formation of some enzymes of amino acid, carbohydrate, and hexobarbital metabolism (19, 22) or impaired cholesterol feedback (7). However, almost all the experiments thus far were carried out by “mass biochemistry” of the preneoplastic liver. In contrast with these results, Teebor and Seidman (26) reported retention of metabolic regulation in large hyperplastic nodules. In order to investigate the enzyme regulation in hyperplastic areas and nodules, Kitagawa and Pilot (12, 13) developed an immunohistochemical method for demonstration of serine dehydratase. Serine dehydratase had been extensively studied by quantitative analysis in enzyme regulation experiments but had never been visualized histochemically. This paper will describe the results obtained by sequential observations of the metabolic regulation in hyperplastic areas during FAA feeding utilizing the serine dehydratase immunohistochemistry.

Materials and Methods

Male Donryu rats (Nihon Rat Co., Urawa, Japan), weighing 150 to 170 g; or Sprague-Dawley rats (Sprague-Dawley Co., Madison, Wis.) were used. The rats were given chow containing 0.03% FAA either for the initial 12 weeks or continuously throughout the experimental period. The technique of the combined method of enzyme histochemistry and radiomicrography was described in detail previously (14). Briefly, rats were partially hepatectomized at the 9th week of carcinogen feeding, and a total of 400 μCi tritiated thymidine were injected every 3 hr from 18 to 27 hr after the operation. Liver tissues were obtained by biopsy or autopsy from treated rats 3 to 18 weeks after labeling.

Small pieces of the liver tissue were frozen on Dry Ice. Serial sections were made in a cryostat and stained for β-glucuronidase (6), serine dehydratase, and histological observation (hematoxylin and eosin). The sections stained for β-glucuronidase were covered with radioautographic films exposed for 8 weeks or more, and developed.

Serine dehydratase was stained by an indirect immunofluorescent technique. The preparation of specific antiserum to liver serine dehydratase rabbit serum was described previously (9). The immunohistochemical staining method was described elsewhere (12). The dietary induction of serine dehydratase was observed in the liver of rats fed a 90% protein diet containing 0.03% FAA for 5 days during 4, 6, 9, 12, and 15 weeks of carcinogen feeding. The effect of dietary glucose repression was observed in the liver of rats fed a 0% protein diet for 5 days during carcinogenesis. By feeding 90% or 0% protein diet for 5 days, a 10-fold difference in the level of serine dehydratase was seen in the normal rat liver (8). The presence or absence of induction or repression was judged by a comparative study of staining patterns and their intensities between tissues from treated (induced or repressed) and nontreated (control) rats.

Results

The morphological and histochemical changes occurring in the liver of rats fed a diet containing 0.03% FAA were described in detail previously (11, 15). Areas of hyperplasia develop in the midzones or periportal portions of hepatic lobuli around the 6th week, at the earliest during the 4th week. They rapidly increase in size and number, while original hepatocytes degenerate almost completely by the 12th week of carcinogen feeding. Usually, overt carcinomas develop later than the 24th week, with positive serum α-fetoprotein in 25% of rats at the 30th week (16).

During their developing phase, from the 6th through 9th weeks, hyperplastic areas showed marked deficiency of β-glucuronidase and serine dehydratase (Figs. 1, 3, and 5). At the 9th week of carcinogen feeding, the areas of hyperplasia were fairly specifically labeled by tritiated thymidine injected after partial hepatectomy (Fig. 3). The original hepatocytes were barely labeled, probably because their proliferative activity was inhibited by the toxic action of carcinogen.

A sequential study with the liver tissue obtained at the 12th, 15th, 18th, and 27th weeks of carcinogenesis, i.e., 3 to 18 weeks after labeling, showed a considerable elevation of enzyme activities in the majority of labeled hyperplastic areas in 3 to 6 weeks after labeling (Figs. 2 and 4). The elevated level of the enzymes was variable in individual areas but in many areas was comparable to that of normal adult liver. The staining pattern of β-glucuronidase also varied, including normal pericanalicular granular activity and atypical diffuse or large droplet cytoplasmic activities. Although each hyperplastic area appeared to have its own level of enzymes in comparison with those of other areas, a closer observation often revealed some differences of enzyme activity among cells within an area. Generally, more grains were found in the cells with higher enzyme level. Occasionally, foci of enzyme-deficient cells with very few silver grains were observed in a matured hyperplastic area, the composite cells other than the foci being rather thickly labeled. Probably because of this uneven proliferative activity of cells within an area of hyperplasia, many labeled, matured cells were observed fragmentally, surrounding other hyper-
plastic areas or nodules. It seemed there was a tendency for reorganization of matured hyperplastic areas or matured cells in the liver tissue.

On the other hand, there was a small group of hyperplastic areas in which enzyme deficiency persisted during the observation period. This type of area increased in size during the observation period up to 5 mm in diameter. Proliferative activity of the cells within these areas was also uneven, as judged by the distribution of silver grains.

Labeled parenchymal cells were hardly detected in distinct hyperplastic nodules or in carcinomas, even of microscopic sizes, at the 27th week.

The enzymic maturation of hyperplastic areas was seen both in the animals returned to chow without carcinogen after the 12th week of feeding and in animals fed the carcinogen continuously throughout the experiment. In the latter group, however, the liver has less hypertrophic and more abundant with enzyme-deficient hepatocytes and proliferated cholangiolar cells, and the disappearance of labeled hepatocytes was more rapid.

Serine dehydratase was not inducible in hyperplastic areas of developing phase where there was no original enzyme level. Later, there was a considerable elevation in the endogenous level of this enzyme in the majority of hyperplastic areas, and in these areas a distinct enzyme induction was observed (Fig. 6). The level of induction seemed to be proportional to the original endogenous enzyme level. In the hyperplastic areas with persistent enzyme deficiency, no induction was observed. Glucose repression was complete in every type of hyperplastic area.

The results are shown in Chart 1 and Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Sequential change of original hepatocytes and areas of hyperplasia during hepatocarcinogenesis</th>
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<tr>
<td><strong>Original hepatocytes</strong></td>
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**Chart 1.** Sequential changes of labeled areas of hyperplasia. Complete circles, areas of hyperplasia; oblique lines, sites of enzymatically mature hepatocytes; black dots, sites of labeled hepatocytes. At the 9th week, areas of hyperplasia are enzymatically deficient and are labeled selectively and intensely with [14C]thymidine after hepatectomy. At the 18th week, 9 weeks after labeling, most labeled hepatocytes are abundant with the enzyme. Many are arranged like normal liver tissue. Occasionally, foci of hepatocytes with enzyme deficiency are seen in areas of enzyme-rich cells (A). Some areas of hyperplasia show persistent enzyme deficiency (B). Unlabeled areas of hyperplasia are scattered (C).

**Discussion**

The phenotypic maturation of hyperplastic areas during FAA feeding has been described both in the endogenous level of enzymes and in their metabolic adaptation. The same phenomenon has also been observed during diethylene-nitrosamine or azo dye feeding (13). The phenotypic maturation of hyperplastic areas was basically independent of the presence or absence of carcinogen although, in the case of continuous carcinogen feeding, enzyme-deficient areas were more abundant and the disappearance of labeled cells was more rapid.

There appeared to be a tendency for matured hyperplastic areas or cells to be reorganized in the liver tissue, functionally compensating the original parenchymal cells. In this respect, this type of hyperplastic area has a "regenerating"
nature. However, in the precise meaning of "regeneration" as the proliferation of normal parenchymal cells to compensate the defect of the same type of cells or tissue, the cells of hyperplastic areas do not seem to be simple regenerating cells. The evidence suggesting the altered character of the cells of hyperplastic areas includes the following: (a) only carcinogenic substances can induce hyperplastic areas after a certain period of their administration; (b) no such enzyme-deficient cells are detectable in normal adult liver; (c) the cells and the tissue of hyperplastic areas are morphologically and histochemically different, if not very much, from original parenchyma even after a process of phenotypic maturation; (d) the changing process of their phenotype is different from those of fetal-neonatal or regenerating liver; (e) the cells of hyperplastic areas are resistant to the toxicity of the carcinogen; and (f) the hyperplastic areas are individually heterogeneous with respect to the above-mentioned features.

The present experiments also have shown the presence of a small group of hyperplastic areas in which enzyme deficiency and unresponsiveness to the induction stimuli persisted as long as the observation period. This type of lesion attained a larger size than those showing phenotypic maturation and appeared to be more important as "preneoplastic" change. These lesions may consist in part of hyperplastic nodules or "persistent nodules" (25). In the present experiment, however, labeled cells were hardly detectable in distinct hyperplastic nodules or microcarcinomas developing in later phase. It should be noted also that not all the hyperplastic nodules or carcinomas are deficient of the marker enzymes used in the present experiment (10, 11). These facts suggest that there should be further steps of single cell alterations within areas or nodules of hyperplasia after the 9th week of carcinogenesis before the achievement of cancerous transformation. In this context, the irregularity of the proliferative activity within a hyperplastic area and the finding suggesting a de novo development of enzyme-deficient foci within a matured area are also important. The presence of heterogenous cells in hyperplastic nodules resistant to L-asparaginase was described (1).

The enzyme deficiency has often been described as "persistent" in hyperplastic areas or foci induced by diethylnitrosamine (20, 22). On discussing the discrepancy between such statements and the present results, the differences in the criterion of "enzyme deficiency," the marker enzymes used, and the type, dose, and method of application of carcinogen should be taken into consideration. The present author noticed that hyperplastic lesions with persistent enzyme deficiency were much more numerous during diethylnitrosamine feeding compared with those induced by FAA or azo dyes (13). The elevation of canalicular ATPase activity is much less common or less remarkable than that of β-glucuronidase, glucose-6-phosphatase, and serine dehydratase. Furthermore, the present author has also observed that hyperplastic areas induced by short-term application of carcinogen, such as by a single injection of diethylnitrosamine after partial hepatectomy (24) or by feeding weaning rats a low dose of FAA for only 2 weeks (17), are likely to belong to the type that has persistent enzyme deficiency (unpublished data). Presumably, in conditions where degeneration or loss of parenchymal cells are not remarkable and the growth of hyperplastic areas is very slow, the proliferative activity of the altered cells may stop when there is a phenotypic maturation before detectable foci of cells are formed. Supporting evidence for this assumption is the presence of very small foci of cells with abnormally high activity of glucose-6-phosphatase.

The real nature of areas of hyperplasia with or without phenotypic maturation in relation to the development of carcinoma is still obscure. However, I would like to put the emphasis on the possibility of reversion of altered cells from a state related to the neoplastic transformation towards normalcy, phenotypically expressed as the elevation of enzyme levels and normal responses to the induction stimuli.

References


Fig. 1. The liver at the 9th week of FAA feeding showing many enzyme-deficient hyperplastic areas. β-Glucuronidase, x 5.

Fig. 2. The liver at the 12th week of FAA feeding showing considerable elevation of the enzyme activity in some of the hyperplastic areas. β-Glucuronidase, x 10.

Fig. 3. The liver at the 9th week of FAA feeding showing the specifically labeled cells in hyperplastic areas. The original parenchymal cells rich in enzyme activity (dark) are barely labeled. β-Glucuronidase and radioautography, x 60.

Fig. 4. The liver at the 15th week of FAA feeding, 6 weeks after labeling, shows marked elevation of the enzyme activity in the labeled cells of hyperplastic areas. β-Glucuronidase and radioautography, x 120.

Fig. 5. The liver at the 9th week of carcinogenesis after feeding 90% protein diet for 5 days. Serial sections stained for glucose-6-phosphatase (A) and serine dehydratase (B). Serine dehydratase was not induced in the hyperplastic areas. x 30.

Fig. 6. Induction of serine dehydratase in hyperplastic areas at the 12th week of carcinogenesis. x 80.
Sequential Phenotypic Changes in Hyperplastic Areas

1

2

3

4

5A

5B

6
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