Ultrastructural Abnormalities in Carcinogen-induced Hepatocellular Altered Foci Identified by Resistance to Iron Accumulation

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ABSTRACT

Hepatocellular altered foci were induced in rat liver by cycles of feeding of N-2-fluorenylacetamide and were distinguished by their resistance to iron accumulation following production of hepatic siderosis by dietary administration of 8-hydroxyquinoline and ferrous gluconate. The foci were readily identified by their iron exclusion in plastic-embedded sections stained for iron. Sections from iron-free regions processed for electron microscopy permitted ultrastructural study of cells in foci identified by reduced cytoplasmic ferritin.

Altered foci of the eosinophilic type produced by cyclic feeding of carcinogen for 16 weeks were composed of both normal-appearing hepatocytes and others with ultrastructural abnormalities, including increased agranular reticulum with associated glycogen particles, decreased rough endoplasmic reticulum with reduced length of cisternae, degranulated rough vesicles, altered and displaced Golgi complexes, and abnormal bile canaliculi. At 12 and 24 weeks after cessation of carcinogen exposure, cells in persistent eosinophilic foci continued to display ultrastructural abnormalities. They possessed increased rough endoplasmic reticulum with rather regular cisternal arrangement and relatively increased smooth endoplasmic reticulum. Golgi complexes were abnormal. Bile canaliculi were abnormal and occasionally increased in number. Nuclei displayed prominent nucleoli.

Cells in a basophilic focus were characterized by the presence of numerous free polyribosomes diffusely scattered throughout the cytoplasm, distended rough endoplasmic reticulum with loss of parallel-stack and hypertrophic dilated Golgi complexes, and prominent margined nucleoli.

The finding that persistent foci continued to display ultrastructural abnormalities, some of which changed or progressed in the absence of further carcinogen exposure, suggests that the persistent iron-excluding foci are a permanently altered population.

INTRODUCTION

A variety of focal hepatocellular lesions arise in the rat liver during induction of liver cancer by chemical carcinogens (2, 6–9, 13, 22, 31, 35, 36, 38, 40, 44, 55). The earliest of these, the altered (hyperplastic) focus, appears to give rise to the neoplastic (hyperplastic) nodule (9, 38, 39, 49, 50). Carcinomas have generally been considered to arise from nodules (7, 8, 30, 34, 35, 38) but have been suggested also to develop directly from the foci (1, 17, 49, 50).

The ultrastructure of cells in hyperplastic nodules has been extensively studied by Merkow et al. (27–29), who emphasized the uniformity of the cells in these lesions. Similar detailed studies on the ultrastructure of the antecedent foci have not been reported, probably because of the difficulty of establishing that sections for electron microscopy include cells from foci.

Foci, nodules, and carcinomas were shown by the work of Hirota and Williams (16) and Williams et al. (52–55) to be refractory to accumulation of stainable iron in the liver loaded with iron, either by administration of 8-hydroxyquinoline plus ferrous gluconate or by iron-dextran injection, and this phenomenon has been confirmed by others (25, 26, 46). Since ferritin aggregates can be visualized ultrastructurally, the lack of iron storage by altered foci provides a means of identifying them in electron micrographs (17, 46). In the present report, we document that cells derived from hepatocytes in iron-excluding altered foci induced by FAA2 possess to various degrees a number of ultrastructural abnormalities and that, in iron-excluding foci that persist following discontinuation of the carcinogen exposure, these abnormalities are constant or even progressive in nature. Thus, the cells of some foci display persistent phenotypic abnormalities and probably represent a stage in the development of liver neoplasms.

MATERIALS AND METHODS

Inbred male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 150 to 160 g were used. The basal diet was Purina laboratory chow (Ralston Purina Co., St. Louis, Mo.), to which all dietary additions were made (Bioserv, Inc., Frenchtown, N. J.). The diet contained 0.8% 8-hydroxyquinoline and 2.18% ferrous gluconate throughout the experiment to produce hepatic siderosis (55). FAA (0.02%) was administered in this diet for four 4-week cycles (total, 16 weeks on carcinogen diet) with a 1-week interval between cycles, effected by switching back to the basal iron-loading diet (17). After the treatments over a total course of 20 weeks, the carcinogen feeding was stopped, and the animals were continued on the iron-loading diet to maintain siderosis in the liver.

Six animals were killed just before discontinuance of carcinogen feeding; then 7 animals were terminated at Weeks 12 and 24 after carcinogen withdrawal. One animal from the 24-week group was fasted 24 hr prior to killing to deplete the liver of glycogen. For light microscopy, tissue slices obtained from each liver lobe were fixed in 10% buffered formalin and embedded in paraffin. Two serial sections from each block were prepared: one for iron reaction by the Prussian blue technique as described (16); and the other for hematoxylin-eosin staining.

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3 The abbreviations used are: FAA, N-2-Fluorenylacetamide; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum.

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For electron microscopy, liver tissues were minced into small cubes (1 to 2 cm) and fixed for 2 hr with 2% glutaraldehyde in 0.067 M phosphate buffer, pH 7.4, followed by postfixation in 2% osmium tetroxide for 1 hr. Blocks were embedded in Durcupan ACM, and sections were cut on either a Cambridge-Huxley or a Porter-Blum MT-1 ultramicrotome. One- to 2-μm-thick sections were reacted for phosphate buffer, pH 7.4, followed by postfixation in 2% osmium tetroxide containing the foci were stained with uranyl acetate and lead citrate and were studied with either a Siemens Elmiskop IA or a JEM 100B electron microscope.

Quantitative analysis for SER and RER was carried out on electron micrographs using standard stereological methods (48). The sample at each stage consisted of approximately 300 micrographs obtained from 10 to 15 tissue blocks containing the foci. Estimates were made for surface density by counting the number of intersections between the test lines and the membrane traces at about ×20,000 (primary magnification, about 6,600). To minimize the errors, a number of test lines were arranged in radial directions at uniform angular intervals. The surface density of membranes, S, per sq μm of cytoplasm was determined from twice the number of intersections, i, counted per μm of test line overlying cytoplasm:

\[ S = 2i \]

RESULTS

Light Microscopic Study

The histological features of the hepatocellular iron-excluding foci in this study and their behavior at intervals following the limited FAA cyclic feeding were the subject of a previous detailed report (17). Briefly, the foci induced by 4 cycles of feeding of FAA over 20 weeks were not necessarily visible grossly, being only about 1.0 mm in diameter without compression of the surrounding parenchyma. The plates of hepatocytes within the lesions were architecturally little altered and merged with those of the surrounding normal parenchyma (Fig. 1). Foci were classified on the basis of the predominance of cellular staining by hematoxylin and eosin of formalin-fixed tissues. Small foci of cells with dense eosinophilic cytoplasm (type I) were usually seen in the perportal region. Some cells had enlarged cytoplasms and enlarged nuclei. Another type of focus of acidophilic cells contained cells with a loose, pale cytoplasm (type II). Other foci were composed of clear cells (type III) or basophilic cells (type IV). Some type II acidophilic foci contained cells intermediate between acidophilic and clear cells, or even a minor fraction of basophilic cells. The 4 types of altered foci were readily identified by their resistance to iron accumulation in the siderotic liver.

At the end of FAA exposure, more than 90% of foci were type II; no type IV foci were present. After cessation of FAA exposure, the fraction of type II foci decreased at 12 weeks and then increased again at 24 and 48 weeks to represent about 80 to 90% foci. The incidence of type IV foci increased progressively, reaching up to 10% at 24 weeks after termination of treatment (17).

In plastic sections stained with toluidine blue, basophilic foci could be distinguished (Fig. 2), but not iron-excluding foci. The iron-excluding foci were recognizable in osmicated deplasti-
area of SER in the foci was about 200% that of the surrounding iron-loaded parenchymal cells; the RER in cells of the foci was decreased in density to approximately 70% of that of the surrounding parenchyma (Table 1).

Cells within the altered foci at this stage and later, although refractory to accumulation of iron stainable at the light microscopic level, occasionally displayed the presence of ferritin aggregates and siderosomes (Fig. 8). Peroxisomes, most with nucleoids containing a polytubular crystallloid, were present in normal numbers, or perhaps even slightly increased in number (Fig. 8). Lysosomes apparently converging on the cytoplasmic zones bordering on the bile canaliculi were sometimes increased in number. Most of these peribiliary lysosomes had small osmiophilic droplets, but the presence of ferritin was usually equivocal or absent (Fig. 10). Mitochondria were not abnormal.

Nuclear abnormalities were not noted in these altered cells.

At 12 Weeks off FAA Feeding. Ultrastructural changes were still present in the cells of persistent iron-excluding foci. The cisternae of RER were usually lengthened but regular in parallel-stack arrangement (Fig. 11). The areas of membranes of both RER and SER were significantly greater than those in the surrounding iron-loaded hepatocytes; on the average, the surface area of the RER at this stage was increased to 154% of that of the surrounding normal parenchyma, while the SER was increased 208% (Table 1). Compared to the areas of membranes present in altered cells at the end of FAA exposure, the RER was increased approximately 2.5 times in density and the SER was slightly decreased.

Glycogen particles in the form of fine rosettes were distributed evenly throughout the cytoplasm or located at the periphery of most of the altered cells (Fig. 11), as in normal hepatocytes. Peroxisomes were almost as abundant in foci as at the end of FAA feeding. Cells with cytoplasmic fat (Ito cells) were often encountered among the altered hepatocytes (Fig. 11) and in several foci; erythrobasts were present in the sinusoids or in Disse’s space (Fig. 12), representing the occurrence of extramedullary hematopoiesis. Polyribosomal particles lying free in the cytoplasmic matrix were occasionally present and most prominent in cells from foci in the periportal region; these perportal altered cells were also characterized by the presence of large elongated Golgi complexes, some of which were displaced to a perinuclear location, and scantiness of RER and glycogen storage (Fig. 13). Mitochondria were not abnormal.

Nuclei occasionally displayed increased interchromatinic granules and enlarged nucleoli.

At 24 Weeks off FAA Feeding. In some cells within persistent iron-excluding foci, the RER was increased in amount, displaying a lessening of parallel-arrayed arrangement or increased irregular contours (widening or irregularity of the space between each cisterna) (Fig. 14). A well-developed SER network was often diffusely distributed. Continuities between the irregularly contoured RER and proliferated SER were also frequent (Fig. 14). As shown in Table 1, the surface area of RER and SER was increased to 141 and 174%, respectively, relative to the surrounding normal parenchyma (Table 1). Compared to cells in foci at the end of the carcinogen exposure, the RER was increased approximately 2.4 times, while the SER was slightly decreased.

A few foci included altered cells containing abundant polyribosomes lying free in the cytoplasm. The distribution of the free polyribosomes varied from focal (Fig. 14) to diffuse throughout the cytoplasm in a basophilic focus (Fig. 15).

Cells in the basophilic focus were also characterized by an abundance of RER with moderately distended or somewhat vesicular cisternae and a paucity of SER and glycogen areas (Fig. 15). The RER found in these cells assumed no parallel-stack appearance and was ribosome studded, unlike that in the other foci. The Golgi complexes in these cells were hypertrophied, dilated, and moderately distorted, occasionally displaced to a perinuclear location (Fig. 16). Some dilated Golgi cisternae contained granular material of medium density. The nuclei of basophilic cells occasionally displayed increased interchromatinic granules (Fig. 15), and nucleoli were prominent and margined (Figs. 15 and 16).

In altered cells of other foci, peroxisomes were well represented but not as abundant as at earlier stages (Fig. 17). Mitochondria showed no particular morphological abnormalities.

Some bile canaliculi were dilated or distorted in shape, with a tendency toward either smaller or decreased numbers of microvilli. An unusual finding was multiple bile canaliculi between adjacent cells (Fig. 17). Nucleoli in general were prominent with central location (Figs. 17 and 18).

In the liver of an animal fasted for 24 hr prior to killing, the normal iron-containing hepatocytes were almost devoid of glycogen particles, whereas glycogen retention was observed in some iron-excluding altered cells (Fig. 18).

DISCUSSION

Numerous electron microscopic studies have been performed on carcinogen-exposed liver (2-4, 10, 11), “hyperplastic” nodules (7, 27-29, 33), and hepatocellular carcinomas (5, 12). Although the earliest lesion in hepatocarcinogenesis, the hepatocellular altered focus, has been suggested to be an essential precursor to neoplastic development (2, 9, 17, 22, 34, 38, 49, 50), the ultrastructure of the focus has received limited study (20, 33), probably because of the difficulty of identifying foci with certainty in electron micrographs. The utilization of the sensitive marker of resistance to iron accumulation in altered foci (49, 53-55) enables the investigation of the ultrastructure of the carcinogen-induced altered focus (16, 46).

In the iron-excluding foci, some hepatocytes did possess ferritin in the form of small solitary siderosomes or free clusters in the cytoplasm. Nevertheless, the presence of ferruginous material was sparse in the peribiliary lysosomes of cells in the foci, as Timme (46) also noted. Thus, the resistance of cells in foci to storage of cellular iron (49, 55) is confirmed at the ultrastructural level. Additionally, the ultrastructural characterization of iron-excluding cells provides some further insight into the basis for this abnormality. Hennigar et al. (14) recently suggested, from the finding of beaded iron particles on the inner aspect of the SER, that proliferation of this membranous system was involved in iron uptake in hepatocytes affected by secondary hemochromatosis. Within the proliferated SER present in the persistent foci, which seems to contribute to eosinophilia of most cells within the type II foci, no iron particles were observed. Thus, this alteration appears not to be capable of mediating iron uptake in the iron-excluding foci and may indicate that the proliferated SER is not normal. In a study by...
Hultercrantz et al. (18) of rat liver following iron overload, X-ray microanalysis revealed 2 types of lysosomal bodies, one characterized by the presence of reaction product containing iron and lead and the other displaying iron deficiency. In the present study, the peribiliary lysosomes occasionally found in the liver cells of iron-excluding foci may be of the second type. This could reflect the loss of the type competent to store iron. The precise mechanism of the resistance to iron accumulation in the carcinogen-induced altered foci remains to be established, but increasingly appears to be related to an abnormality in cellular storage rather than to deficient uptake (56).

A variety of ultrastructural abnormalities were found in foci at the termination of carcinogen exposure and at later stages. Generally, cytoplasmic alterations were more conspicuous than were nuclear ones. The variation between cells in the type and extent of abnormalities differs from the report by Bryant et al. (4) of constant abnormalities of both SER and RER in the cells of premalignant lesions induced by diethylnitrosamine or 3'-methyl-4-dimethylaminobenzene in the livers of guinea pigs and rats. However, the precise identification of premalignant lesions by Bryant et al. (4) does not seem certain. The variety of ultrastructural changes in foci in the present study is considered to reflect the heterogeneity of cell types in foci induced by FAA (17).

Vesiculation and degranulation of hepatocyte RER in rats after administration of noxious agents such as carbon tetrachloride, ethionine, α-naphthylisothiocyanate, puromycin, and various other chemicals has been described by a number of investigators (37, 41, 43, 47). This RER change was considered to indicate disturbance of protein synthesis by the toxins. Therefore, the impaired RER of this type found in the foci at the end of FAA feeding is probably nonspecific. Cells with this impairment produced by a hepatocarcinogen, however, may be different from those affected by a non-hepatocarcinogen, in that the RER increased in amount following the termination of carcinogen exposure, and other alterations were persistent or progressive.

The proliferation of SER observed in altered cells at the end of FAA feeding has been noted in hepatocytes during carcinogen administration (3, 10, 32, 45) and in the cells of foci (33). Such proliferation may be a result of induction of carcinogen-metabolizing enzymes. However, since the cells in foci are resistant to the cytotoxic and antimitotic effects of carcinogens requiring metabolism (19, 24, 53), such proliferated SER, as suggested earlier, may be altered in function. Stereological measurement of the surface area of endoplasmic reticulum indicates a persistent abnormality of both SER and RER, which would result in alterations in metabolism after cessation of carcinogen exposure.

Other abnormalities in cellular membranes were found in foci after discontinuation of carcinogen exposure. Golgi were distorted and occasionally displaced from their normal pericanalicular location to perinuclear sites. Another significant membrane abnormality in the persistent foci was aberrations in the structure of bile canaliculi, which corresponds with a recent report by Ogawa et al. (33) of this feature in preneoplastic liver lesions. This, and the unusual development of multiple bile canaliculi between the adjacent altered cells present at 24 weeks after cessation of FAA feeding, may reflect a permanent alteration of the cell membrane in these persistent foci. Williams (51) has suggested that an abnormal cell membrane may be involved in rendering altered liver cells resistant to regulation by tissue growth and differentiation factors that are transferred between cells to maintain homeostasis. Impermeability of the altered cell to these regulatory factors may be essential to its persistence in a phenotypically altered state.

Some investigators have previously suggested that basophilic foci may play an essential role in neoplastic transformation (6, 17, 20, 21, 30, 31). In the present study, a higher fraction of persistent foci were basophilic in comparison to the types present at the end of carcinogen administration (17). The increase of these lesions with prolonged maintenance is consistent with their postulated role in neoplastic development. The cells in a basophilic focus possessed distinctive features, consisting of numerous free polyribosomes diffusely scattered in the cytoplasmic matrix, prominent distended RER throughout the cytoplasm, with both decreased length of cisternae and loss of parallel-stack arrangement, abnormal Golgi complexes, and paucity of SER component. These characteristics are similar to those of the basophilic foci induced by N-dimethylaminobenzene described by Timme (46). Abundance of free polyribosomes in the cytoplasm is one of the striking characteristics of immature or undifferentiated cells in general, such as stem cells, blast cells, embryonic cells, and cells in culture. The free polyribosome-rich cells composing basophilic iron-excluding focus presumably reflect a state of active synthesis of endogenous proteins needed for cell growth or some other abnormal function. Such alterations in ribosomal distribution, including those seen at earlier stages, may possibly be related to the synthesis of different types of proteins.

The persistent phenotypic abnormalities in the cells of foci presumably result from a genotypic change. However, nuclear alterations were not observed, except for the tendency toward a single enlarged nucleolus previously documented at the light microscopic level (54). The minimal nuclear changes may indicate that the genetic alterations in foci are not as severe as in more advanced lesions and carcinomas.

In conclusion, the finding of diverse ultrastructural changes of various degrees in the iron-excluding foci that persisted following cessation of FAA exposure supports the concept that some altered foci are composed of permanently altered populations which may be the precursors of neoplastic nodules or carcinomas.

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Fig. 1. Iron-excluding foci (arrows), at 12 weeks after FAA feeding, in formalin-fixed, paraffin-embedded tissue. Prussian blue reaction. × 68.

Fig. 2. Glutaraldehyde-fixed, osmicated epoxy section stained with toluidine blue, at 24 weeks after FAA feeding. Center, a group of hepatocytes with prominent nuclei stained more strongly (basophilic) with toluidine blue (arrows) than the surrounding parenchyma. × 133.

Fig. 3. Iron-excluding focus (arrows) from the same area as Fig. 2. Deplasticized section reacted with Prussian blue, × 133.

Fig. 4. Hepatocytes at the end of FAA feeding in the siderotic liver. Pilemorphic electron-dense iron-containing bodies are distributed throughout the cytoplasm. × 4,400.

Fig. 5. Higher magnification of an iron-containing hepatocyte at the end of FAA feeding. Single membrane-bound lysosomal residual bodies (large arrow) and free ferritin clusters (small arrow) are occasionally present. × 29,500.

Fig. 6. Cell in an iron-excluding focus at the end of FAA feeding. Granular reticulum is sparse and somewhat fragmented in appearance, showing decreased length of rough cisternae. Rough vesicles sparsely covered with ribosomes are present (arrow). × 24,400.

Fig. 7. Cell in an iron-excluding focus at the end of FAA feeding. Rough vesicles are markedly degranulated, giving resemblance to SER. Numerous solitary ribosomes appear to be attached to tangentially cut membranes, and some may be derived from disaggregated polyribosomes (arrow). × 20,200.

Fig. 8. Cells in an iron-excluding focus at the end of FAA feeding. A considerable amount of SER is present near the canaliculus. Peroxisomes (mb, microbodies) are abundant. Ferritin aggregates (small arrow) and single membrane-bound siderosomes (large arrow) are present. BC, bile canaliculus. × 22,400.

Fig. 9. Cell in an iron-excluding focus at the end of FAA feeding. Profiles of SER with a meshwork of branching and associated glycogen particles are prominent. × 20,700.

Fig. 10. Cells in an iron-excluding focus at the end of FAA feeding. The bile canaliculus (BC) is dilated with reduced numbers of microvilli. Peribiliary lysosomes are increased in number and density. Granular ferritin is not evident. × 19,500.

Fig. 11. Cells in an iron-excluding focus at 12 weeks after FAA feeding. The altered cell in the upper portion of the field displays a well-developed RER. A fat-storing cell is located in the lower portion of field. × 8,200.

Fig. 12. An erythroblast located among the altered cells in an iron-excluding focus at 12 weeks after FAA feeding. Arrow, micropinocytosis at the coated surface of the erythroblast destined to the formation of vesicles (double arrows). × 13,800.

Fig. 13. Cells in an iron-excluding focus in the periportal area at 12 weeks after FAA feeding. Free polyribosomal particles (arrow) are diffusely scattered throughout the cytoplasm, and large elongated Golgi complexes (go) are present. The one marked go is displaced to a perinuclear location. Bile duct cells are seen in the lower portion of field with adjacent basal lamina (BL). × 13,400.

Fig. 14. Cell in an iron-excluding focus at 24 weeks after FAA feeding. Proliferated RER with reduction of parallel-arrayed arrangement is prominent. Continuities between irregular contoured RER and well-developed SER network are seen. Free polyribosomes are focally present (small arrows). A tangentially cut nucleus (large arrow) has prominent nuclear pores. × 14,000.

Fig. 15. Cells in the basophilic iron-excluding focus shown in Figs. 2 and 3, at 24 weeks after FAA feeding. RER is diffusely distributed instead of forming focal parallel stacks. Evenly spread polyribosomes lie free throughout the cytoplasm. Most RER cisternae, which are distended and vesicular, contain granular material. Interchromatinic granules are abundant and nucleioli are margined. × 11,000.

Fig. 16. Cells in the same focus as Fig. 15. Golgi complexes (go) show hypertrophy and dilation with moderate distortion. Some dilated Golgi cisternae contain granular material of medium electron density (arrows). The complex in the lower portion of the field (arrow) is displaced to a perinuclear location. The nucleus of the cell in the lower portion of the field has a margined nucleiolus. × 11,000.

Fig. 17. Cells in an iron-excluding focus at 24 weeks after FAA feeding. Multiple bile canaliculi between the adjacent cells are present. Nucleoli are prominent. × 6,000.

Fig. 18. Iron-excluding and iron-containing cells from a fasted rat at 24 weeks after FAA feeding. The altered iron-excluding cell (top) shows glycogen retention, but the iron-containing hepatocyte (bottom) does not. The single nucleiolus of the altered cell is enlarged. × 9,100.
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