Elevation of Serum Alpha-fetoprotein and Proliferation of Oval Cells in the Livers of LEC Rats

Hidetoshi Takahashi,[*1] Masahito Oyamada,[*1] Yoshinori Fujimoto,[*1] Mutsumi I. Satoh,[*1] Atsuo Hattori,[*1] Kimimaro Dempo,[*1, *4] Michio Mori,[*1] Toshiyuki Tanaka,[*2] Hiroyuki Watabe,[*2] Ryuichi Masuda[*3] and Michihiro C. Yoshida[*3]

[*1]Department of Pathology, Sapporo Medical College, S.1, W.17, Chuo-ku, Sapporo 060, [*2]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University, Kanazawa 1757, Tobetsu, Ishikari, Hokkaido 061-02 and [*3]Chromosome Research Unit, Faculty of Science, Hokkaido University, N.10, W.8, Kita-ku, Sapporo 060

Alpha-fetoprotein (AFP) in the sera of 35 LEC (Long-Evans with a cinnamon-like coat color) rats between 7 and 25 weeks of age was evaluated by enzyme-linked immunosorbent assay (ELISA). Elevation of serum AFP and proliferation of oval cells in the liver were observed in most LEC rats, which suffered from acute hepatitis. On the other hand, the serum AFP level was within the normal range before the onset of hepatitis. Immunohistochemical staining for AFP revealed that some of the proliferating oval cells produced AFP. Morphometric analysis of AFP-positive cells and ELISA for serum AFP demonstrated that there was a statistically significant correlation between the number of AFP-positive cells in the liver and the concentration of AFP in the serum. Histological examination revealed the transition and differentiation of the oval cells to small hepatocytes. These results suggested that the phenomena which occurred in LEC rats suffering from acute hepatitis were similar to those that occurred during the early stage of azo dye hepatocarcinogenesis, although the extent of the oval cell proliferation and the elevation of serum AFP in LEC rats were not as great as those in rats treated with azo dye. This is the first report on a rat strain with proliferation of AFP-producing oval cells during its natural history.

Key words: Alpha-fetoprotein — Oval cell — Hepatitis — Hepatocarcinogenesis — Animal model

The LEC[*5] rat is a new mutant that suffers from hereditary hepatitis associated with severe jaundice around 4 months after birth.[1, 2] About half of the rats died of submassive necrosis of hepatocytes within a week after the onset of jaundice. Genetic analysis revealed that a single autosomal recessive gene is responsible for the hereditary hepatitis.[3] The remaining rats survived more than one year. Enzyme-altered preneoplastic foci, similar to those in chemical hepatocarcino-

genesis, appeared in young (5-month-old) LEC rats after symptomatic or asymptomatic hepatitis, and their number and size increased with age.[4] Thereafter, hepatocellular carcinomas were observed at a high frequency (46/ 55; 84%) in the rats that survived more than 12 months.[5] From these findings, it is clear that the LEC rat strain provides a valuable animal model for studying the pathogenesis of hepatitis and hepatocellular carcinoma.

Alpha-fetoprotein (AFP) is a dominant plasma protein produced by both the yolk sac and the liver during the ontogenesis of mammals. Its concentration in the serum of neonates diminishes rapidly after birth and it is barely detectable in adults. Since the reexpression of AFP occurs in adults with hepatocellular carcinoma and yolk sac tumors, AFP is known as one of the oncodevelopmental proteins.[6] However, AFP appears in

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[*4] To whom all correspondence should be addressed.

[*5] Abbreviations used in this paper: LEC, Long-Evans with a cinnamon-like coat color; AFP, alpha-fetoprotein; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; ABC, avidin-biotin-peroxidase complex; 2-AAF, 2-acetylaminofluorene; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.

adult rats without any malignancies; that is, significant elevation of serum AFP occurs in the rats during the early stage of chemical hepatocarcinogenesis.[7-9] There are no reports on the reexpression of a large amount of AFP in adult rats without the use of any chemical.

We report here that AFP spontaneously increased in the serum of the LEC rats that survived after suffering from hepatitis. Histological study revealed that oval cell proliferation was prominent in the liver, and immunohistochemical studies using anti-AFP antibody suggested that AFP was produced by the proliferating oval cells.

MATERIALS AND METHODS

Animals The LEC strain was established from non-inbred Long-Evans rats maintained under normal conditions at the Center for Experimental Plants and Animals of Hokkaido University, as described by Sasaki et al.[1] and Yoshida et al.[2] Thirty-five LEC rats between 7 and 25 weeks of age were examined.

Detection of AFP in the Serum of LEC Rats For the measurement of AFP concentration in the blood, blood samples were collected when rats were sacrificed. The serum was separated by centrifugation and stored at $-70°$ until measurement. AFP concentration was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using horse anti-rat AFP antibody (IgG), which was purified on an affinity column of rat AFP coupled to Sepharose 4B. The secondary antibody was coupled to horseradish peroxidase by using periodate. Substrate reaction using o-phenylenediamine was determined at 492 nm in a Microelisa Autoreader (Dynatec, VA).

Histological Study The liver was excised and cut into 2 to 3 mm slices with a razor blade. Some sections were fixed in Bouin's solution or Carnoy's solution for staining with hematoxylin and eosin (HE) and others were fixed in ice-cold acetone.

Immunohistological Study Immunohistochemical staining of AFP was performed by the avidin-biotin-peroxidase complex (ABC) method. Sections 5 μm thick were deparaffinized with benzene and an alcohol series. After inactivation of endogenous peroxidase with a methanol solution containing 0.6% H_2O_2, the sections were sequentially incubated in the following: rabbit anti-AFP (1:2000), biotin-labeled goat anti-rabbit IgG (1:50, Vector Laboratories Inc., Burlingame, CA) and ABC (Vector Laboratories Inc.). Diaminobenzidine was used to localize the bound peroxidase. The sections were counterstained with hematoxylin.

Morphometric Study Morphometric analysis of AFP-positive cells in the livers of LEC rats whose concentration of AFP in the blood was greater than 500 ng/ml was performed on their left lateral, left medial and middle lobes. After immunostaining, AFP-positive cells were counted in 100 randomly selected fields, in which a portal triad was placed in the center of the field and the central area in the hepatic lobules was in the periphery at $\times 400$ magnification under a Nikon Microphoto FX microscope. The number of AFP-positive cells in each LEC rat liver was expressed as the total number of AFP-positive cells in 100 fields.

RESULTS

AFP Concentration in the Blood of LEC Rats
Figure 1 shows the concentration of AFP in the blood of LEC rats between 7 and 25 weeks of age, measured by sandwich ELISA. In more than half of the rats (12/23) from 15 to 19 weeks of age, AFP concentration was greater than 500 ng/ml. In the young LEC rats between 7 and 8 weeks of age, before the onset of hepatitis, no rat showed an AFP concentration greater than 150 ng/ml.
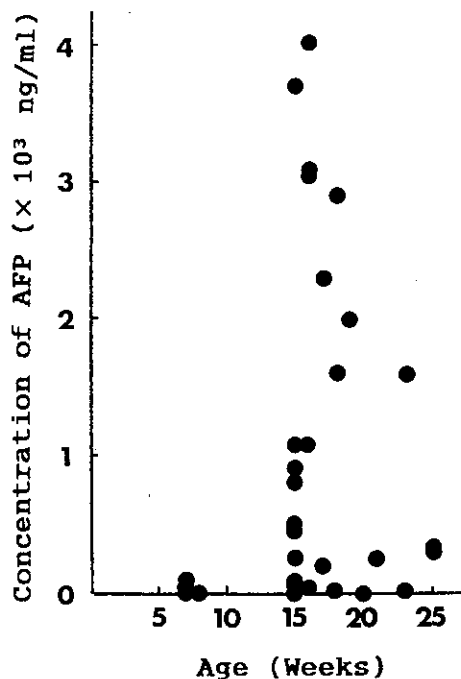

Fig. 1. The concentration of AFP (ng/ml) in the sera of 35 LEC rats between 7 and 25 weeks of age, measured by sandwich ELISA.
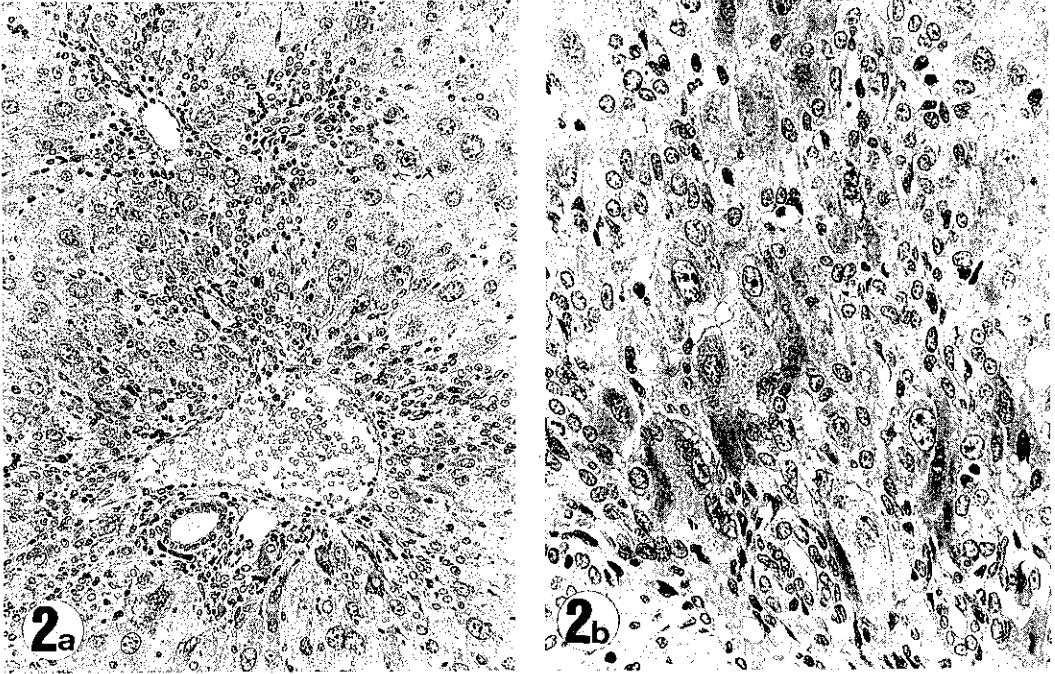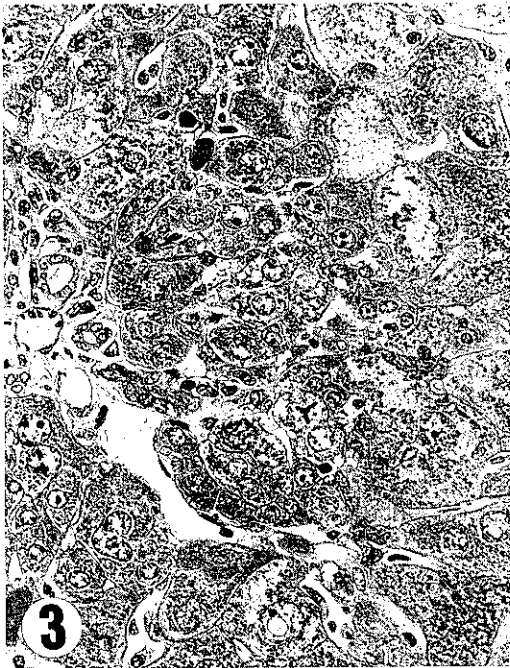
Fig. 2. Light microphotographs of the liver of a LEC rat of 16 weeks of age (HE staining). a) Oval cells are observed in the periportal area ($\times$170). b) Higher magnification of part of Fig. 2a ($\times$340).

Histological Study Histological changes in the livers of LEC rats during their natural history were reported in our previous paper.[2] Briefly, no histological abnormality was found in the livers of young LEC rats between 7 and 8 weeks of age before the onset of hepatitis. After 15 weeks there were many enlarged hepatocytes with large nuclei, which often had pseudo-inclusions, and there was eosinophilic degeneration of hepatocytes. Bile pigments in activated Kupffer cells and foci of extramedullary hematopoiesis were often observed. While few inflammatory cells, such as lymphocytes and neutrophils, were seen, mononuclear phagocytes containing erythrocytes were often found in the sinusoid. The histological changes described above were seen in all rats examined after 15 weeks of

Fig. 3. Proliferation of small hepatocytes in the periportal area of the liver in a LEC rat that survived after recovery from jaundice (HE staining, $\times$340).

Fig. 4. Immunohistochemical staining of AFP of the liver in a LEC rat of 16 weeks of age. AFP is detected exclusively in oval cells ($\times 340$).
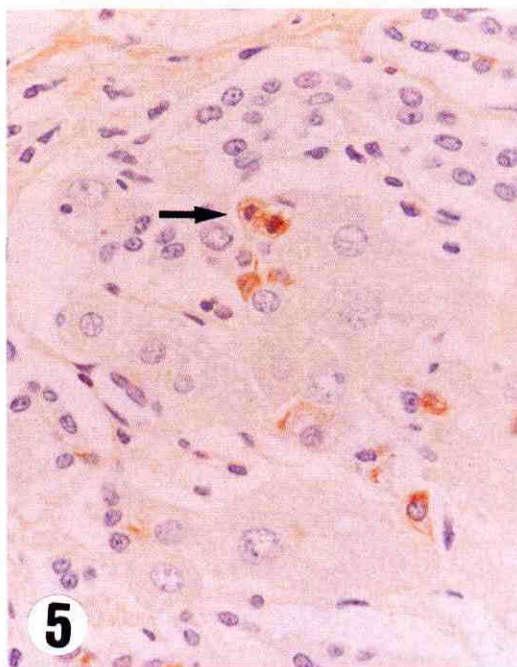

Fig. 5. Immunohistochemical staining of AFP of the liver in the same LEC rat as in Fig. 4. An arrow shows a mitotic oval cell ($\times 470$).

age. In the rats in which AFP in the serum was greater than 500 ng/ml, a large number of small cells with oval vesicular nuclei and scant cytoplasm, which correspond to "oval cells," was observed in the periportal area of the hepatic lobules (Fig. 2). The proliferation extended further toward the midzone of the hepatic lobule and mitotic figures were frequently found in these oval cells. On the other hand, though, few mitotic figures were observed in mature hepatocytes. Some of them formed glandular structures and others had more cytoplasm and round nuclei, corresponding to intermediate cells between cholangiolar cells and small hepatocytes.

In the rats surviving after recovery from jaundice, small hepatocytes with basophilic cytoplasm and small round nuclei were discerned in the periportal area adjacent to oval cells (Fig. 3). Mitotic figures were seen not only in oval cells but also in the small hepatocytes. Small foci, composed of the small hepatocytes with vacuolar or basophilic

cytoplasm and the samll round nuclei, were detected in the livers of some rats, as reported in our previous paper.[4]

Immunohistochemical Staining of AFP The anti-AFP antibody revealed fine granular reaction products in the cytoplasm of some oval cells and intermediate cells, which had a little more cytoplasm and round nuclei (Fig. 4). Some oval cells in mitosis were also stained for AFP (Fig. 5). It was also shown that some oval cells stained by this antibody formed a lined arrangement. Neither enlarged hepatocytes nor bile duct cells in the portal triad were stained, nor was AFP detectable in hepatocytes within preneoplastic foci.

Morphometric Analysis of AFP-positive Cells in the Livers of LEC Rats We performed morphometric analysis of AFP-positive cells in the livers of 11 LEC rats in which the AFP concentration was more than 500 ng/ml. Figure 6 demonstrates that there was a statistically significant correlation between the number of AFP-positive cells in the liver and
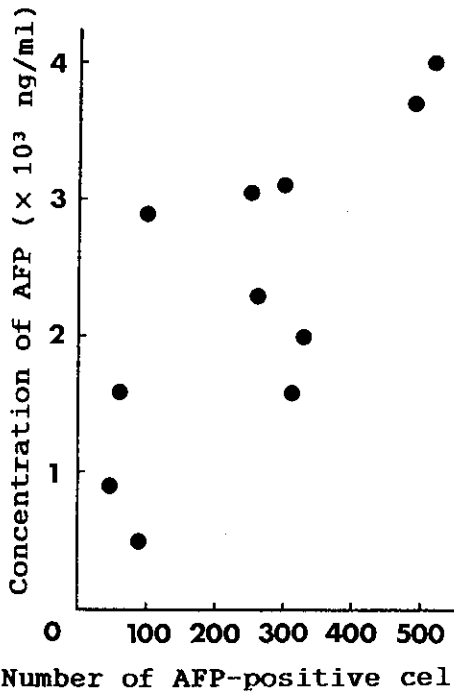
Fig. 6. Correlation between the number of AFP-positive cells in the liver counted in 100 randomly selected fields and the concentration of serum AFP (ng/ml).

the concentration of AFP in the serum ($P <$ 0.01); the correlation coefficient was 0.77. There was, however, no correlation of this number with age (data not shown).

DISCUSSION

The present results have shown that serum AFP was significantly elevated in LEC rats after the symptomatic or asymptomatic onset of acute hepatitis around 4 months of age, that AFP-producing oval cells proliferated in their livers, and that there is a significant correlation between the amount of serum AFP and the number of oval cells containing AFP. We consider that these findings in LEC rats are analogous to those in the early stage of chemical carcinogenesis. This is the first report on rats with a proliferation of AFP-producing oval cells during their natural history.

Concerning the proliferation of oval cells and the appearance of AFP, proliferation of oval cells has been observed in livers of rats

treated with many carcinogens,[8-21] some non-carcinogenic hepatotoxins such as alpha-naphthylisothiocyanate,[22-24] or common bile duct ligation.[21, 25] It is said that serum AFP does not elevate and that oval cells possessing AFP do not proliferate in the liver under the latter two conditions.[21, 24] Some investigators reported that AFP was transiently elevated in adult rats following partial hepatectomy or treatment with CCl_4 or galactosamine, but they noticed that the amount of AFP in the serum was low and proliferation of oval cells was not observed in these situations.[26, 27]

There seems to be a general agreement that oval cells are responsible for the early elevation of serum AFP during hepatocarcinogenesis with various chemical carcinogens such as azo dyes,[12-14, 16, 17, 20] 2-acetylaminofluorene (2 - AAF) and choline - deficient diet containing ethionine.[24, 28] Some investigators suggested that oval cells in the early stage and their transformed atypical cells ("atypical hyperplasia") in the later stage are possible precursor cells for hepatocellular carcinomas, and that the initial elevation of AFP represents one phase of a specific sequence of gene alteration related to carcinogenic evolution.[19] Since we could detect neither atypical hyperplasia nor AFP-positive hyperplastic foci, the latter of which are generally accepted as precancerous lesions, in LEC rat livers, we do not have any evidence that AFP-positive oval cells transform into carcinoma cells. However, we observed that oval cells, most of which show a positive reaction for placental glutathione S-transferase, proliferate in the early phase and that hepatocellular carcinomas develop with a fairly high frequency later in life. In LEC rats which had hepatocellular carcinomas, an elevation of serum AFP (6500 ng/ml) was observed (data not shown). From these findings, it appears that proliferation of AFP-positive oval cells may play a role in the development of precancerous lesions and hepatocellular carcinomas in LEC rats, even though oval cells are not precursors for hepatocellular carcinomas themselves.

Transition and differentiation to small hepatocytes is another interesting finding in LEC rats. Although the fate of the oval cells during chemical carcinogenesis is still a subject of dispute, some reports have clearly

demonstrated that the oval cells have the capability to transform into hepatocytes through various transitional phases in 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) hepatocarcinogenesis.[10, 11, 13–17, 20] As far as we know, there are few reports that histologically demonstrate the apparent transition of oval cells to small hepatocytes during hepatocarcinogenesis induced by various chemicals, including 2-AAF, or choline-devoid diet, except for azo dyes. Dempo *et al.*[16] proposed in the previous paper that this phenomenon is a kind of regenerative process that occurs when hepatocytes are selectively damaged by carcinogens and cannot repair the damaged liver through their own division. We suppose that the impairment of regeneration occurs in enlarged hepatocytes of LEC rats in a manner similar to that in 3'-Me-DAB hepatocarcinogenesis. In a preliminary study, we found that the enlarged hepatocytes did not respond to epidermal growth factor (EGF), while the oval cells proliferated with EGF, in primary culture with the addition of EGF to the culture medium, when liver cells were isolated from the livers of LEC rats with jaundice (unpublished observation).

In the previous paper, we reported that placental glutathione *S*-transferase-positive foci appeared in 5-month-old LEC rats after the onset of hepatitis and that their number and size increased with age.[4] Since a remarkably high incidence of hepatocellular carcinomas was observed in LEC rats surviving more than 1 year,[5] we suppose that hepatocytes of LEC rats possess a genetic predisposition to the development of hepatocellular carcinoma. Necrosis of hepatocytes and the following regenerating process, represented by oval cell proliferation and successive transition to hepatocytes, seems to play an important role in causing the early appearance of enzyme-altered foci. We are undertaking further experiments to clarify whether "genetically initiated cells" exist in LEC rats.

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