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α-Fetnprotein Containing Cells in Early Stages during Hepatocarcinogenesis in Rats Fed 2-Acetylaminofluorene

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Abstract The localization of AFP in certain cells in early stages during hepatocarcinogenesis in rats fed 2-AAF was revealed using indirect immunofluorescent technique. AFP containing cells appeared only around the portal triads in the 5th week after the commencement of a 2-AAF diet. With the lapse of time, AFP containing cells, which were shown to be hyperbasophilic hepatocytes by H & E stain, formed foci to nodules.

Key Words: α-fetoprotein (AFP), hyperplastic nodule, oval cell, 2-acetylaminofluorene (2AAF)

Introduction

It is now clearly established that AFP levels become elevated in the serum early in the hepatocarcinogenic process in experimental animals[1,2,11-15,25]. Several attempts to clarify the relationship between serum and its synthesizing cells have yielded two kinds of answers to the question of which cell population in the liver is associated with early appearance of AFP before the induction of hepatocellular carcinoma in rats subjected by 2-AAF[17,22,24], 3′-methyl-4-dimethylaminoazobenzene[3,8,18,19,24] or other carcinogenes[9,15,23]. One solution suggests that AFP is synthesized in the hyperplastic liver nodules[9,17]. Another possibility is the origination of AFP in the oval cell[3,8,15,18,19,22-24] and atypical hyperplastic zone[22]. It seems to be important to clarify the site of AFP synthesis in the carcinogenic process, thus identifying the new cell population that can evolve into hepatocellular carcinoma.

The results presented here strongly suggest that AFP production takes place in the small hyperbasophilic foci of a few hepatocytes risen from the portal triad and also in the larger foci of cells that ultimately form the hyperplastic nodules that are believed to be pre-cancerous lesion.

Materials and Methods

Treatment of Animals

Fifty male Wistar rats (Kuroda Farm, Kumamoto, Japan) weighing from 150 to 170 gms were used. These animals were fed a basal diet for 1 week before treatment with 2-AAF (Nakarai

The abbreviations used are: AFP, α-fetoprotein; 2-AAF, 2-acetylaminofluorene; FITC. fluorescein isothiocyanate; H & E, hematoxyand eosin.

Chemicals Ltd., Nagoya, Japan). Then, fourty animals were fed a basal diet (Oriental Food Co., Nagoya, Japan) containing 0.05% 2-AAF intermittently with intervals of the basal diet alone in order to induce large hyperplastic liver nodules as previously described by Epstein et al.[4]. Control animals (10 rats) were continuously fed the basal diet alone for 13 weeks. The animals were sacrificed during the 5th, 8th, and 13th week after ingestion of the carcinogen and the localization of AFP in the livers was studied using an indirect immunofluorescence.

Immunofluorescence

Monospecific antiserum to AFP was prepared by immunization of rabbits with purified AFP from rat amniotic fluid as described elsewher[17]. Purchased antiserum to rat AFP (Hokuiken, Sapporo, Japan) was also applied to the present study for the sake of impartiality. An IgG fraction of goat anti rabbit IgG serum was purchased from Miles-Yeda Ltd. (Rehovot, Israel) and labeled with FITC (Baltimore Biological Laboratories, Boltimore, Md.) by the method of Kawamura[10]. The specific fluorescent labeled antibody which was used for immunofluorescent study showed a molar fluorescence to protein (F/P) ratio of 1.2. The tissue blocks of the livers were fixed in pre-cooled 95% ethanol for paraffin embedding using the method of Sainte-Marrie[21]. Microtome sections, 4 μm thick, on clean glass slides were deparaffinized, hydrated, and washed in cold 0.01M phosphate buffered saline. The sections were incubated with rabbit anti rat AFP serum at room temperature for 2 hours. Following incubation, the sections were washed with cold phosphate buffered saline for 30 min, exposed to the labeled antibody in a moist chamber at room temperature for 45 min, and then washed again in the same buffer for 30 min. The stained sections were mounted in pH 9.5 buffered glycerol and examined under a vertical fluorescent microscope (AH-RFL, Olympus, Tokyo, Japan). Controls for specificity were carried out in the same ways as described previously[16]. After observation, the sections were washed in the buffer for over 30 min, and stained with H & E.

Results

Localization of AFP in the Livers from the Animals in the 5th Week

Histologically, the disorganization of the hepatocytes and bile duct cells (so-called "oval cell" [5, 20]) proliferation were particullary evident around the portal triads (Fig. 1, 5). Immunofluorescent study revealed that AFP containing cells were localized around the portal area. These cells were shown not to be proliferated bile duct cells, but rather hyperbasophilic hepatocytes according to the H & E stain of the sections for immunofluorescence (Fig. 3-4, 5-6). No AFP positive cells were seen in the sinusoid.

Localization of AFP in the Livers from the Animals in the 8th Week

As shown in Figs. 8, 9, and 13, hyperbasophilic hepatocytes around the portal area had a tendency to proliferate towards the middle zone of the lobule. Also, the cytoplasm of some nodules in this stage appeared to be somewhat like ground-glass. The localization of AFP was seen evenly in the cytoplasm of the cells forming hyperplastic liver nodules (Fig. 8, 10, 13).

Localization of AFP in the Livers from the Animals in the 13th Week

The rats sacrificed in the 13th week showed the many large nodules on and in the liver. AFP containing cells appeared mainly in the nodules and occasionally in hepatocytes close to the nodules. Comparative studies of H & E stain and immunofluorescence of the same sections revealed the localization of AFP in the hepatocytes of hyperplastic liver nodules. However, AFP positive cells were not uniformly distributed in the nodules (Fig. 16, 18).

AFP localized cells were not found in the livers of any of control animals.

Discussion

Previously, we reported that AFP might be synthesized not only in the fetal liver and in hepatocellular carcinoma, but also in hyperplastic liver nodules that appeared through 2-AAF induced hepatocarcinogenes-

is in the rat[17]. On the other hand, recently Sell[22] mentioned that" 1% of the oval cells and about half of the zones of atypical hyperplasia included cells that contain AFP, but none of the neoplastic nodules or normal hepatocytes have any AFP-containing cells, in the livers of Fischer rats fed four cycles of 2-AAF". Therefore, in the present studies we carefully followed AFP containing cells in the rats fed a 0.05% 2-AAF diet from the 5th week until the 13th week when the large hyperplastic nodules could be recognized macroscopically on and in the livers, by means of indirect immunofluorescence. We were unable to detect any AFP positive cells among the bile duct cells in this experiment. However, in the livers of the rats killed in the 5th week, hyperbasophilic hepatocytes proliferated around the portal triads, which look like the zone of atypical hyperplasia described by Sell, showed the localization of AFP. The small hyperplastic nodules which were seen in the livers on the 8th week were uniformly positive for AFP in the cytoplasm. This observation is not pointed out by Sell.

In the comparison of our study and those of Sell and several other investigators using 3′-methyl-4-dimethylaminoazobenzene, the following questions remain unresolved; (a) What are the histological criteria of the oval cell ?, (b) What kind of function does the oval cell have ?, and (c) What are the differences between the hyperbasophilic foci and the atypical hyperplastic zone?

Kuhlman reported that areas of hyperplastic appearance developed from the oval cells in the livers of BDX rats fed high dose of n-nitrosomorphine[15]. This new observation may be important and should be further researched in order to resolve the 2 oppositing opinions about possible AFP synthesizing cells in very early stages of hepatocarcinogenesis in rats.

Morphologic evidence suggesting the remodelling or maturation of hyperplastic nodules has been published[6,7]. It seems that this hypothesis is also parallel to the conception of reversibility of the hyperplastic nodules in the whole process of hepatocarcinogenesis. Immunofluorescent study on the localization of AFP revealed various forms of AFP containing cells in the nodules. This observation may also support the remodelling of the hyperplastic nodules through the induction of hepatocellular carcinoma.

In the present study, we reconfirmed that the distribution of AFP containing cells over time is consistent with the hypothesis that hyperplastic liver nodules might be an important premalignant population.

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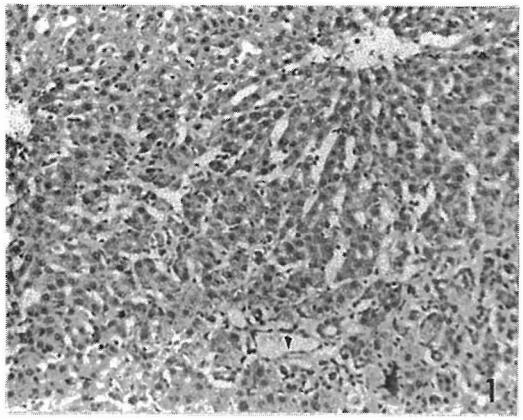
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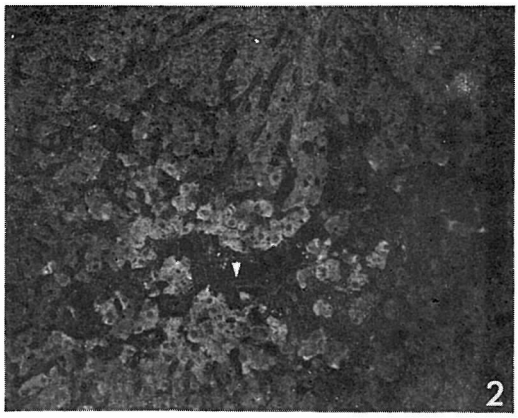
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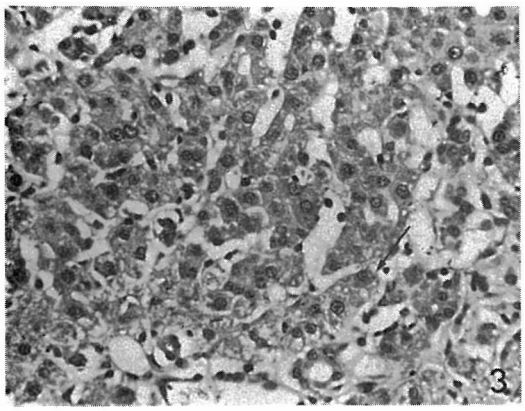
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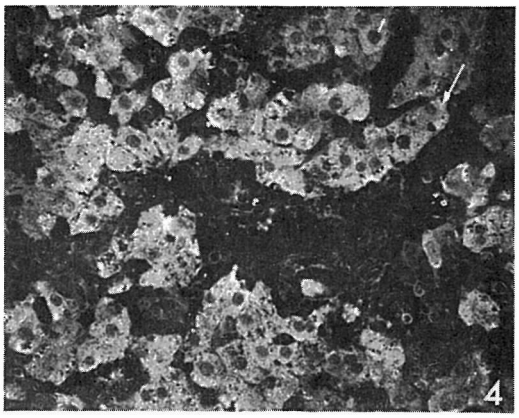
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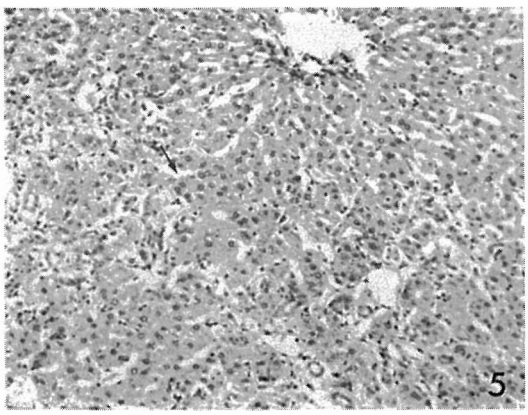
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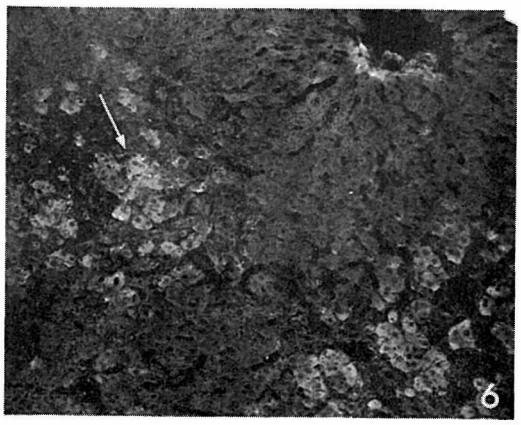
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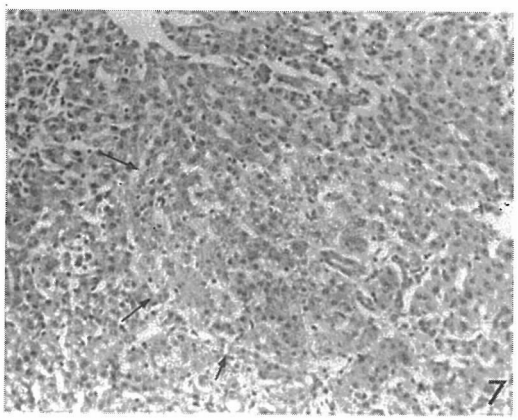

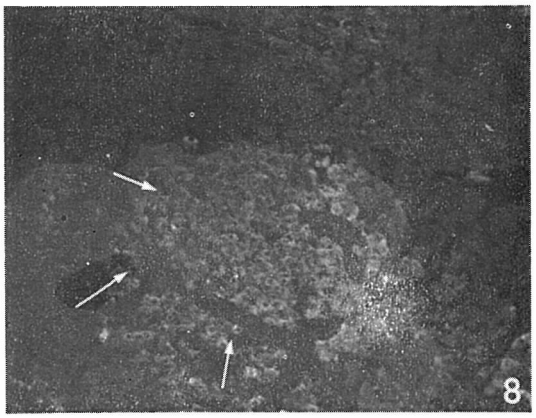

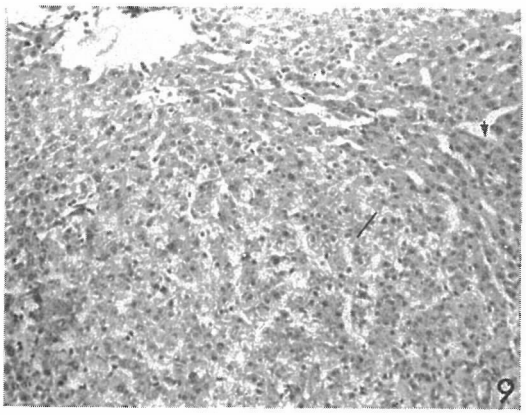

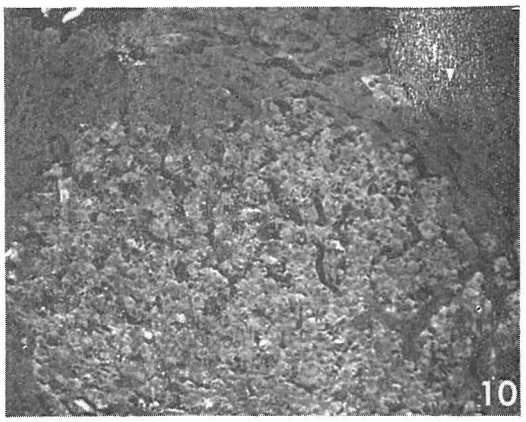

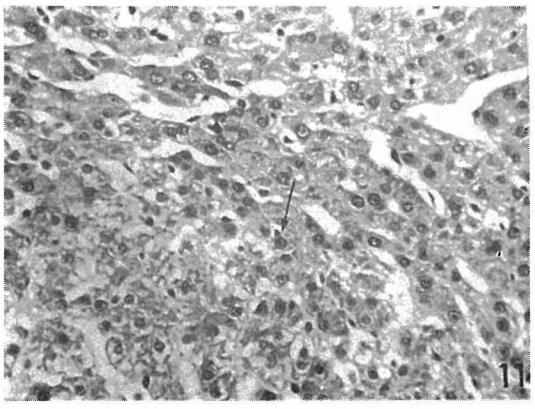

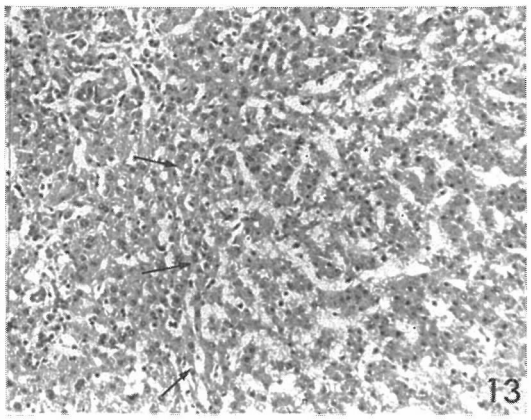
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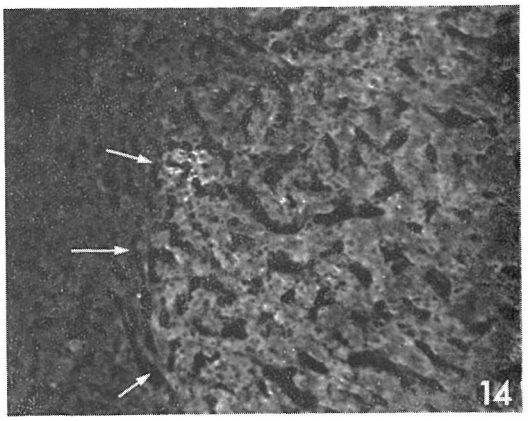
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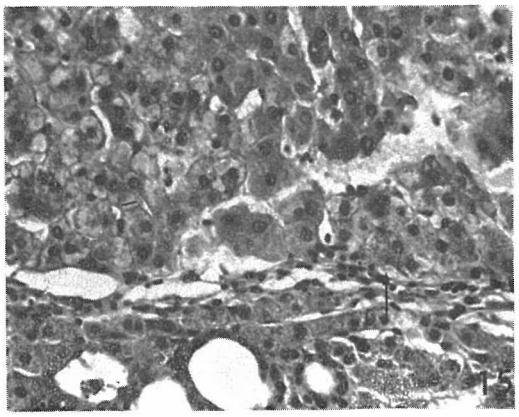
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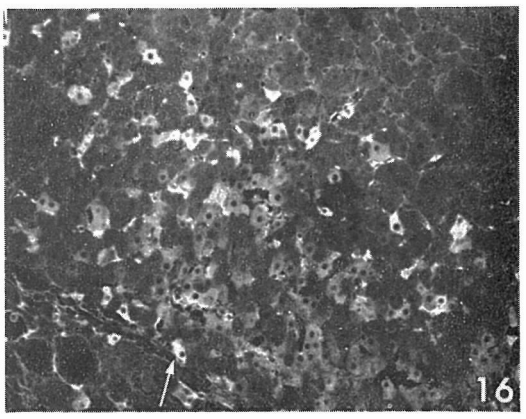
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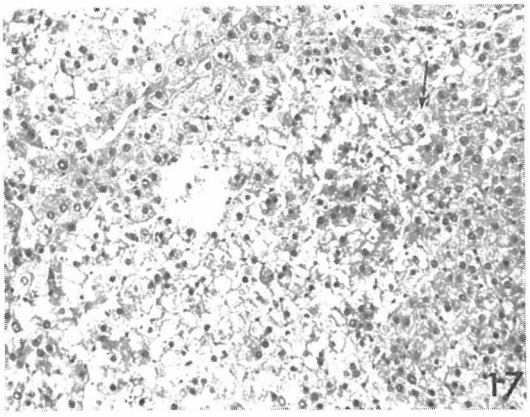
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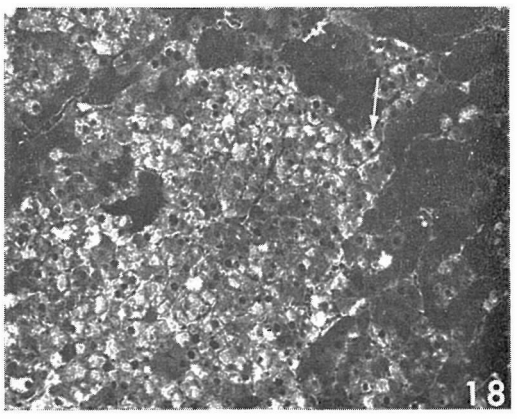
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Figs. 1 to 6

Photomicrographs of the liver sections from rats sacrificed in the 5th week of the 2-AAF diet.

Fig. 1 Note the disorganization of the hepatocytes around the portal triad (▲). (H & E,×400)

Fig. 2 Distribution of AFP containing cells around the portal triad (△) in the same sample shown in Fig. 1. (AFP by immunofluorescence,×400)

Figs. 3 and 4 More highly magnified views of Figs. 1 and 2. The arrow in Fig. 3 (H & E,×800) corresponds to the arrow in Fig. 4 (AFP by immunofluoreescnce,×800). AFP containing cells are 2 cells thick and similar to the hepatoytes, as shown by the H & E stain.

Figs. 5 (H & E) and 6 (AFP by immunofluorescence)
The localization of AFP in the liver from another rat. Note AFP containing cells around the portal triads. The symbols (▲, △) indicate the central vein. (×400)

Figs. 7 to 14

Photomicrographs of the liver sections from the rats sacrificed in the 8th week of the 2-AAF diet.

Figs. 7 (H & E) and 8 (AFP by immunofluorescence)
Note AFP containing cells forming a nodule (white arrow) in Fig. 8, which is identified with black arrow in Fig. 7. (×400)

Figs. 9 to 12

Figs. 9(H & E) and 10 (AFP by immunofluorescence). (×400)

Figs. 11(H & E) and 12 (AFP by immunofluorescence). (×800)
The hyperplastic nodule, which occupies over half of Fig. 9, is evenly stained for AFP by immunofluorescence (Fig. 10). The symbols (▲, △) indicate the identical portion. A more highly magnified view shows that AFP containing cells are hyperbasophilic when compared to hepatocytes adjacent to the nodules. The arrows in Figs. 11 and 12 show theidentical portion.

Figs. 13 (H & E) and 14 (AFP by immunofluorescence)
The ocalization of AFP is seen in the wol forming hyperplastic nodules. The arrow indicates a hyperplastic liver nodlue. (×400)

Figs. 15 to 18

Photomicrographs of the liver sections from the rats sacrificed in the 13th week of the 2-AAF diet.

Figs. 15 (H & E,×800) and 16 (AFP by immunofluorescence,×400)
AFP containing cells are distributed un-uniformly in the hyperplastic nodule.

Figs. 17 (H & E) and 18 (AFP by immunofluorescence)
Fig. 17 shows the inside of the hyperplastic nodule. The hepatocytes with ground glass like cytoplasms stain for AFP through immunofluorescence, even in the nodule. (×400)