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Decreased Sensitivity to Phalloidin of Normal-looking Rat Hepatocytes after Short-term 2-Acetylaminofluorene Feeding

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Male F344/DuCrj rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF) for 1 or 3 weeks, and then fed a basal diet for 2 days, 2 weeks, 8 weeks, 22 weeks or 36 weeks. Hepatocytes were isolated from the liver by collagenase perfusion, and their sensitivity to phalloidin, in terms of the formation of multiple cytoplasmic blebs, was examined. The sensitivity of gamma-glutamyltransferase (GGT)-negative hepatocytes decreased on the 22nd and 36th weeks after withdrawal of 2-AAF feeding, and that of GGT-positive cells decreased on the 36th week. Induction of a small number of foci positive for the placental form of glutathione S-transferase (GSTP) was observed in the liver of all rats on the 8th, 22nd and 36th weeks after the withdrawal of the carcinogen. However, the total area of the foci was estimated to account for less than 0.2% of liver tissues even on the 36th week. Therefore, the decrease in phalloidin sensitivity of hepatocytes, particularly of GGT-negative hepatocytes, on the 22nd and 36th weeks after 2-AAF withdrawal is suggested to be a result of a decrease in the sensitivity of otherwise normal-looking hepatocytes, which may be precursors of the cells forming the preneoplastic foci.

Key words: Phalloidin — 2-Acetylaminofluorene — Normal-looking hepatocytes — Enzyme-altered foci

It is well known that focal proliferation of "enzyme-altered" hepatocytes is induced in experimental hepatocarcinogenesis, and these cells are regarded as preneoplastic cells.[1-4] On the other hand, there is a certain latent period between carcinogen administration and the induction of the preneoplastic foci, although no information is available as to the phenotypic changes of hepatocytes during this latent period.

It was previously reported that preneoplastic hepatocytes of rats induced by carcinogens acquired a relative resistance to phalloidin,[5-7] a hepatocyte-specific mushroom toxin.[8] The resistance was suggested to be closely related with the cell membrane alteration(s) that suppressed the inward transport of the toxin into the cells.[7] On the other hand, our previous observations[9] showed a decrease in the phalloidin sensitivity of hepatocytes isolated from the livers of aged rats, in which the enzyme-altered foci were naturally induced.[10] However, the number and size of the foci were too small to account for the decrease in the sensitivity. It was suggested that the decrease in the sensitivity was accounted for by otherwise normal-looking hepatocytes, some of which were ancestors of later preneoplastic cells.

The present investigations were made in order to examine whether the phalloidin sensitivity of normal-looking hepatocytes is changed at the time when the carcinogen-induced enzyme-altered foci are induced or before the foci develop, after short-term feeding with 2-AAF.*[2]

MATERIALS AND METHODS

Male SPF F344/DuCrj rats weighing 160–180 g were purchased from Charles River Japan Inc., Atsugi. They were maintained in an SPF animal facility at 23±1° on a standard 12-hr light-dark daily cycle. They received a basal diet (M, Oriental Yeast Co., Tokyo) and drinking water *ad libitum*. They were acclimated to their environment for one week before the start of the experiments.

The experimental schedule is outlined in Fig. 1. Briefly, rats were fed a diet containing 0.02% 2-

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*[2] Abbreviations used: 2-AAF, 2-acetylaminofluorene; GGT, gamma-glutamyltransferase; GSTP, placental form of glutathione S-transferase.

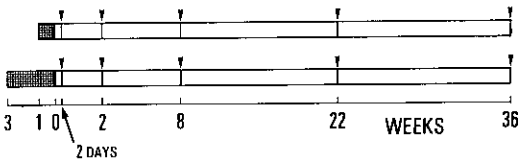

Fig. 1. Schematic representation of experiments. ▦, Period of 0.02% 2-AAF feeding; ▼, the time of sacrifice.

acetylaminofluorene (Tokyo Kasei Indust., Tokyo) for 1 week or 3 weeks. Thereafter, all rats were fed a basal diet until the time of sacrifice.

Hepatocytes were isolated by the collagenase perfusion method as previously described.[7] The hepatocytes were finally suspended in Williams' medium E (Flow Lab. Inc., Rockville, Md.) supplemented with 10% calf serum (HyClone Lab., Logan, Utah), 0.5 μg/ml insulin, 0.04 mg/ml streptomycin and 40 IU/ml penicillin-G. After the viability of the cells (85–95% in these experiments) was examined by a trypan blue exclusion test, 3×10^5 viable cells in 1.5 ml of the medium were inoculated in 35-mm plastic dishes. After a one-hour incubation in a CO_2-incubator at 37°, the cells were incubated in 1.5 ml of the medium containing phalloidin (Sigma Chemical Co., St. Louis, Mo.) for 20 min at 37°. The cells in the dishes were fixed in ice-cold ethanol for 10 min and stained for GGT-activity.[11] The sensitivity of the cells to phalloidin was assessed by counting on the same dishes the numbers of GGT-negative and GGT-positive hepatocytes, which had formed cytoplasmic blebs, as previously reported.[12]

Four to 6 rats of each group were killed after being anesthetized, and the livers were immediately removed. Slices (3 mm thick) of livers were fixed in ice-cold acetone or 10% buffered formalin. Serial sections were prepared from the acetone-fixed materials and each section was subjected to hematoxylin and eosin (HE) stainig, GGT-histochemistry, and GSTP-immunohistochemistry. The GSTP-stained preparations were photographed, and then the number of the foci which consisted of more than 5 cells was counted and the size of the foci was measured with the use of an image analyzer (Kontron Co., Munich) as previously described.[10]

Results

In rats on the 2nd day after the end of 1 week of 2-AAF feeding, the percentage of GGT-positive hepatocytes was 0.71 ± 0.21 as compared with 0.16 ± 0.05 in normal rats (control). Thereafter, the percentages on the 2nd, 8th, 22nd and 36th weeks after the withdrawal of the carcinogen returned to control values. As shown in Fig. 2, phalloidin sensitivity of both GGT-negative and GGT-positive cells on the 2nd day was decreased significantly as compared to that of the respective cells in the control. In both GGT-negative and GGT-positive hepatocytes isolated from rats on the 2nd and 8th weeks, the sensitivity was comparable to that in the control. The sensitivity of GGT-negative cells decreased significantly on the 22nd and 36th weeks, while that of GGT-positive cells decreased on the 36th week. In separate experiments to cover the age span of normal rats from 10 to 47 weeks of age, it was confirmed that there was no difference in the phalloidin sensitivity of either GGT-negative and GGT-positive hepatocytes or in the percentage of GGT-positive cells among rats of various ages.

In rats after the termination of 3 weeks of 2-AAF feeding, the increase in the percentage of GGT-positive hepatocytes on the 2nd day (2.53 ± 1.26) was much greater than that of the cells on the 2nd day after 1 week of 2-AAF feeding (0.71 ± 0.21). Furthermore, an increase was also found in rats on the 2nd weeks after the withdrawal of 2-AAF (0.48 ± 0.10). Thereafter, the percentage returned to control values. Phalloidin sensitivity of both the GGT-negative and GGT-positive cells on the 2nd day after the end of 3 weeks of 2-AAF feeding decreased more significantly than that of the respective cells on the 2nd day after the end of 1 week of 2-AAF feeding (Fig. 2). Thereafter, the sensitivity of the GGT-negative cells returned to control values until the 8th week in the cases of both 1 week and 3 weeks of 2-AAF feeding, while the sensitivity of the GGT-positive cells showed the control values until the 22nd week. The sensitivity decreased significantly on the 22nd and 36th week for the GGT-negative cells and on the 36th week for the GGT-positive cells.

Histological examinations showed that, on the 2nd day after the withdrawal of 2-AAF feeding, small hepatocytes having basophilic cytoplasm proliferated in the periportal zone (Fig. 3), especially in the liver after 3 weeks of 2-AAF feeding. These cells failed to show GSTP-positivity (Fig. 4), but many showed GGT-positivity (Fig. 5). In contrast to normal rat livers, GSTP was slightly positively

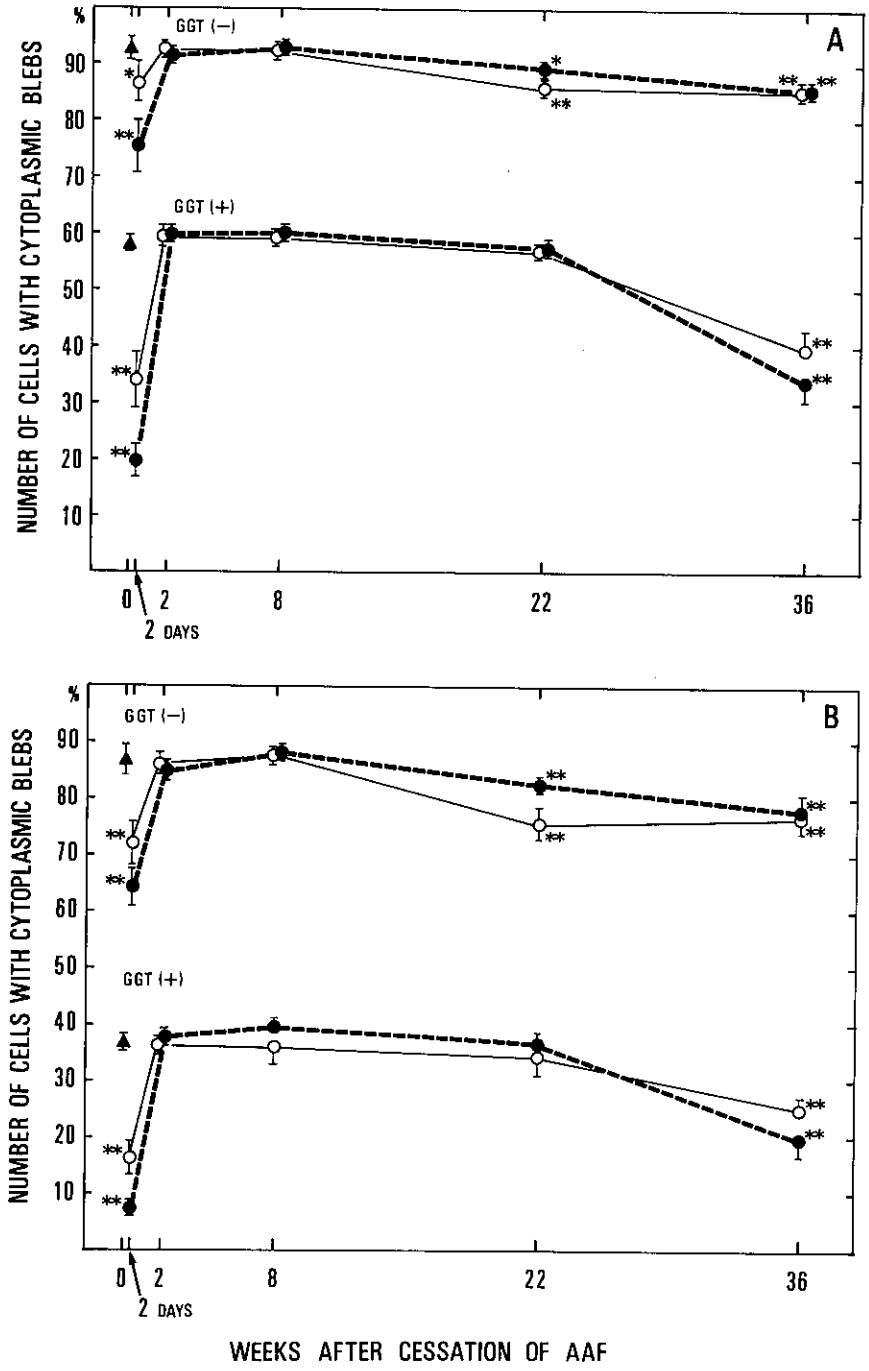

Fig. 2. Phalloidin sensitivity of rat hepatocytes after 2-AAF feeding. The concentration of phalloidin was 10 μg/ml (A) or 5 μg/ml (B). Open circles (○) show the values after 1 week of 2-AAF feeding, and closed circles (●) show those after 3 weeks of 2-AAF feeding. Closed triangles (▲) show the control values. Bars represent ±SD. Significantly different from control: $^{*}P<0.05$; $^{**}P<0.01$.

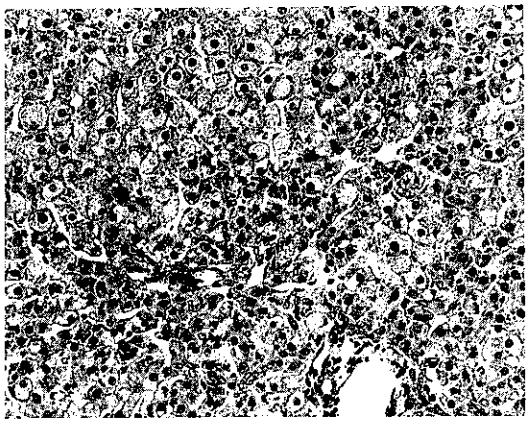

Fig. 3. Periportal zone of liver lobules in a rat 2 days after the end of 3 weeks of 2-AAF feeding. Small hepatocytes are shown. HE-staining (formalin-fixed preparation). ×170.

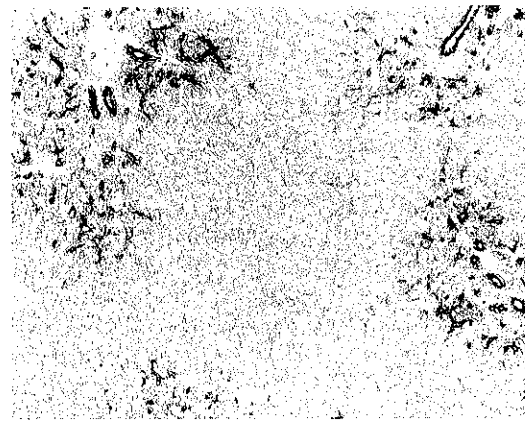

Fig. 5. GGT-staining of a liver section. Many hepatocytes and epithelial cells of bile ducts stained strongly for GGT in the periportal zone of liver lobules. The section was adjacent to that shown in Fig. 4. ×85.

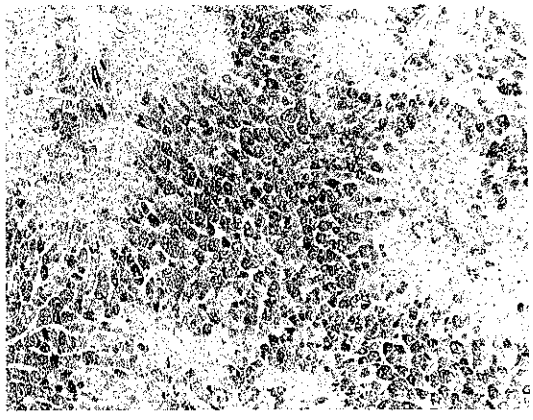

Fig. 4. GSTP-staining of a liver section of a rat 2 days after the end of 3 weeks of 2-AAF feeding. In the periportal zone of liver lobules, small hepatocytes were GSTP-negative, while epithelial cells of bile ducts were GSTP-positive. Hepatocytes in the mid and central zones of liver lobules were slightly GSTP-positive (acetone-fixed preparation). ×85.

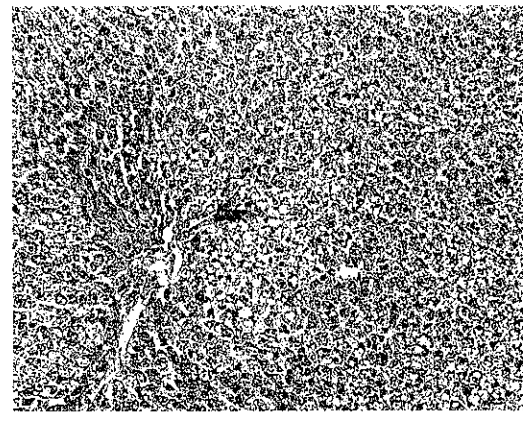

Fig. 6. A focus of altered hepatocytes with clear cytoplasms in a rat 36 weeks after the end of 3 weeks of 2-AAF feeding. HE-staining (acetone-fixed preparation). ×85.

stained on hepatocytes in the mid and central zones of liver lobules (Fig. 4). Mitotic figures were encountered in the periportal zone. These changes disappeared in the livers at the 2nd week after the cessation of the carcinogen. However, foci of altered hepatocytes were observed in the livers of all rats on the 8th, 22nd and 36th weeks after the 2-AAF feeding. The altered cell foci were observed to consist of clear or eosinophilic hepatocytes (Fig. 6). All of the foci observed showed GSTP-positivity (Fig. 7), and many of the foci were also positive for GGT (Fig. 8). The number per cm^2 and the size of GSTP-positive foci on the 36th week after 1-week of 2-AAF feeding were 2.5 ± 1.8 and 0.016 ± 0.014 mm^2,

Fig. 7. A focus of GSTP-positive hepatocytes. The section was adjacent to that shown in Fig. 6. ×85.

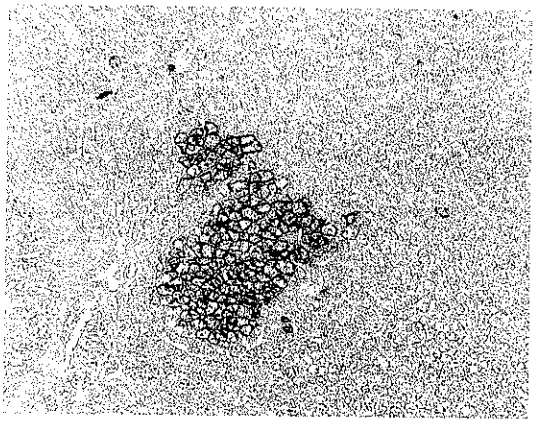

Fig. 8. A focus of GGT-positive hepatocytes. The section was adjacent to that shown in Fig. 7. ×85.

respectively, while the number per cm^2 and the size of those on the 36th week after 3 weeks of 2-AAF feeding were 4.9 ± 1.4 and 0.024 ± 0.011 mm^2, respectively. Therefore, the total area occupied by the foci was calculated as less than 0.2% of the total liver area even on the 36th week after the end of 3 weeks of 2-AAF feeding.

Discussion

Several phenotypic markers, including phalloidin sensitivity, have been reported for focally proliferating preneoplastic hepatocytes of rodents.[1-4, 6, 7] However, little information is available at present about phenotypic changes in hepatocytes during carcinogenesis before the focal proliferation of the preneoplastic cells appears, or those in the early phase of preneoplastic transformation. It was reported that a single hepatocyte or a small cluster of the cells was positively stained for GSTP in the very early stages of the carcinogenesis,[13] resulting in the assumption that these cells are "initiated" cells or precursor cells of the later preneoplastic cells. On the other hand, in aged rats, the phalloidin sensitivity of normal-looking hepatocytes was observed to decrease, and it was proposed that these cells, relatively resistant to the toxin, are precursors of the naturally occurring preneoplastic cells.[9]

Although the period of 2-AAF feeding was different, the phalloidin sensitivity of GGT-negative hepatocytes decreased apparently on the 22nd and 36th weeks after the cessation of the carcinogen. It was previously reported that GGT-negative cells in the putative preneoplastic lesions were relatively resistant to phalloidin.[7] However, the decrease in the sensitivity in the present experiments is considered to be due to a decrease in otherwise normal-looking hepatocytes, because the total area of the foci was too small to account for the changes in the sensitivity of the cells isolated from the whole liver. This finding is closely similar to that in hepatocytes of aged rats.[9] The decrease in the sensitivity of the GGT-positive cells on the 36th week after the withdrawal of 2-AAF feeding might be attributable to the contribution of the cells derived from GGT-positive foci, since the sensitivity of the GGT-positive hepatocytes isolated from preneoplastic nodules was reported to be significantly low.[7]

The decreased sensitivity to phalloidin of hepatocytes was also observed on the 2nd day after the cessation of 2-AAF feeding. This might be ascribed to immaturity of hepatocytes after an enhanced renewal of the cells in the periportal zone of the liver lobules. In fact, decreases in the sensitivity were reported in hepatocytes of baby rats or regenerating rat livers.[14]

The population of hepatocytes relatively resistant to phalloidin may be too small to be detected at the time when or before the

altered foci appear. However, the precursors of the cells forming the foci are assumed to be hepatocytes, the cell membranes of which have been altered as in preneoplastic hepatocytes.

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