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Three-dimensional Analysis of Glutathione S-Transferase Placental Form-positive Lesion Development in Early Stages of Rat Hepatocarcinogenesis

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The development of glutathione S-transferase placental form (GST-P)-positive lesions in the rat liver, from single cells to foci, and their locations within liver lobules were examined by a combined stereological approach for calculation of three-dimensional (3-D) data and by 3-D computer graphics for reconstruction of lesions. Two weeks after initiation with diethylnitrosamine, the rats were divided into two groups. Animals in group 1 were given 2-acetylaminofluorene, and animals in group 2 were given basal diet for 6 weeks. Partial hepatectomy (PH) was performed at week 3. The GST-P-positive single cells increased in number per liver after PH in group 1, but not in group 2, where a plateau level was maintained. The number of GST-P-positive foci per liver in group 2 also reached an almost constant low value after 4 weeks. In contrast, foci in group 1 increased greatly after PH. A 3-D reconstruction, performed with a computer graphics system using up to 180 sections at 10 μm intervals, revealed the single cells to be distributed at random. Those that grew into foci were also not preferentially localized in any particular zone of the hepatic lobule. When foci within the same lobule came into contact, they underwent fusion. The present results thus indicate that only a small proportion of GST-P-positive single cells develops into foci, and that their growth is independent of zonal factors within individual lobules in early stages of rat hepatocarcinogenesis.

Key words: Rat liver — Preneoplastic lesion — Three-dimensional analysis — Glutathione S-transferase placental form — Enzyme-altered focus

The glutathione S-transferase placental form (GST-P) is greatly increased in preneoplastic and neoplastic hepatocellular lesions in rat livers.[1–7] Since the background level is low, if not non-existent, this facilitates detection of foci and nodules by specific immunohistochemical staining using antibody to this isozyme. GST-P-positive single cells, which appear in the early stages of rat hepatocarcinogenesis, are established as the first changes detectable at the cell level, prior to the formation of focally altered populations.[8,9] However, unequivocal evidence for development of foci from all such GST-P-positive single cells is lacking.

Three-dimensional (3-D) analyses have been developed to allow better evaluation of the true numbers and volumes of foci in the liver. However, the stereological formulae applied are based on the critical assumption that all foci are independent and also spherical[10–17] and therefore care must be taken in their use. This is because foci are not always spherical and indeed develop irregular branching shapes relatively early in rat hepatocarcinogenesis.[18–20]

In the present study, development of GST-P-positive liver cells was therefore followed by a combined quantitative stereological and 3-D computer graphics reconstruction approach. The well-established medium-term assay system for hepatocarcinogenicity has clear advantages as an experimental model for this purpose.

MATERIALS AND METHODS

Chemicals Diethylnitrosamine (DEN) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo and 2-acetylaminofluorene (2-AAF) from Nacalai Tesque Co., Ltd., Kyoto.

Animals and treatments A total of 48 male F344 rats, each weighing about 160 g, were obtained from Charles River Japan Inc., Atsugi, and maintained on basal diet (Oriental MF, Oriental Yeast Co., Tokyo) *ad libitum*. They were housed, 5 to a cage, on wood-chip bedding in plastic cages in an air-conditioned room at 24±2°C and 55±5% humidity. The animals were divided into 2 groups and treated as shown in Fig. 1. Group 1 was given a single intraperitoneal injection (i.p.) of DEN at a dose of 200 mg/kg dissolved in 0.9% NaCl solution to initiate hepatocarcinogenesis. After 2 weeks on basal diet, the rats received 0.02% 2-AAF in the diet for the following 6 weeks. Group 2 was given DEN (i.p.) and then maintained on basal diet as a negative control. In both groups, all rats were subjected to two-thirds partial hepatectomy (PH) at week 3. The animals had free access to diet and

water throughout. Sub-groups of 3 rats each were killed sequentially under light ether anesthesia at hour 6, day 4, and weeks 1, 2, 3 after DEN initiation, as well as at days 1 and 3, and weeks 1, 3, and 5 after PH.

The livers were immediately excised and each remaining lobe after PH (right anterior, right posterior and caudal lobes) was weighed and cut with a razor blade into 2–3 mm thick slices. Tissues were fixed in ice-cold acetone for subsequent immunohistochemical staining for GST-P, and in 10% phosphate-buffered formalin solution for routine staining with hematoxylin and eosin.

Immunohistochemical staining GST-P antibody was raised and binding localized as described previously[20, 21] using the avidin-biotin-peroxidase complex (ABC) method.[22] Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin G (IgG) and ABC (Vectastain ABC kit, PK 4001) were obtained from Vector Laboratories Inc. (Burlingame, CA). Paraffin sections were routinely passed through a graded alcohol series, then treated sequentially with normal goat serum, and exposed to rabbit GST-P antibody (1:5000), biotin-labeled goat anti-rabbit IgG (1:400) and peroxidase complex. The sites of peroxidase binding were demonstrated by the 3,3′-diaminobenzidine method. Sections were then counter-stained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, pre-immune rabbit serum was used instead of antiserum.

Quantitative analysis The GST-P-positive populations were divided into single cells and foci more than 0.2 mm in diameter. For the 3-D quantitative analysis, numbers of lesions (single cells and foci) and their total volumes per liver were calculated by applying Enzmann *et al.*'s formula,[17] using a color video image processor (Spicca Computer System, Nippon Avionics Co., Ltd., Tokyo). The total numbers of GST-P-positive cells were calculated based on measured liver weights (the specific gravity of the liver was assumed to be 1).

For the 3-D reconstruction of GST-P-positive cells, paraffin-embedded livers were serially sectioned at 5 μm and immunohistochemically stained with GST-P at 10 μm steps (every 2 sections) for up to 180 sections. Reconstructions of GST-P-positive single cells and foci were performed at weeks 2, 3, 4, 6 and 8, with the assistance of a 3-D computer graphics analysis system ('TRI' Ratoc System Engineering Co., Ltd., Tokyo). Bile ducts and central veins in the liver were also reconstructed together with single cells and foci.

RESULTS

Liver weight Liver weights (total weights of right anterior, right posterior and caudal lobes remaining after PH) in group 2 were much higher than in group 1 until week 4, but after the 6 week time point the reverse was the case (Fig. 2).

Quantitative analysis of GST-P-positive cells 3-D quantitative analysis revealed that GST-P-positive single cells numbered 234×10^3 per liver at week 1 and then maintained a plateau value with elevation after PH only in group 1. GST-P-positive foci per liver in this latter case 1 showed a great increase after PH to about 65.0×10^3 at week 4. Thereafter accurate quantitative data could not be generated, because the growing foci underwent fusion. In group 2 the number of foci per liver reached

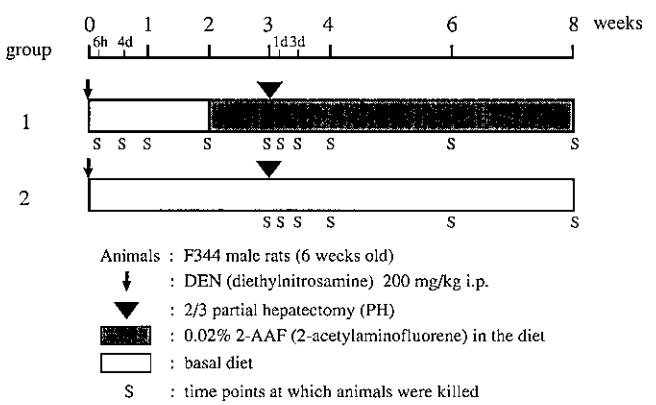

Fig. 1. Experimental design used in the medium-term bioassay for carcinogens. Group 1, DEN+2-AAF; group 2, DEN alone. All rats were subjected to PH at week 3.

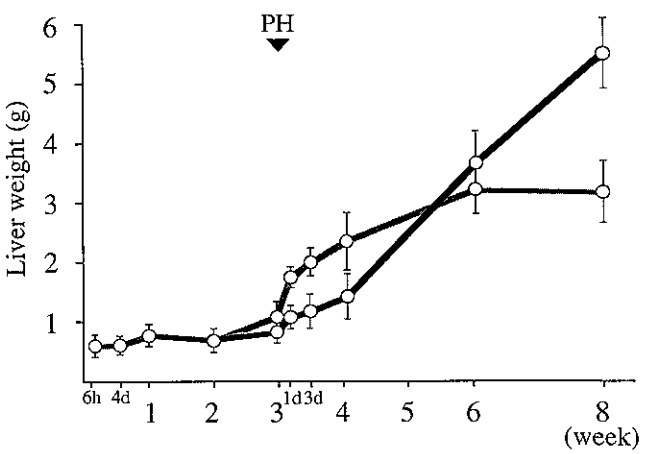

Fig. 2. Total liver weights of the remaining 3 lobes after PH (right anterior, right posterior and caudal lobes). ○—○, DEN→2-AAF+PH (group 1); ○═○, DEN+PH (group 2). Note group 2 (○═○) values are much higher until week 4 than group 1 values (○—○), while the opposite is the case after 6 weeks.

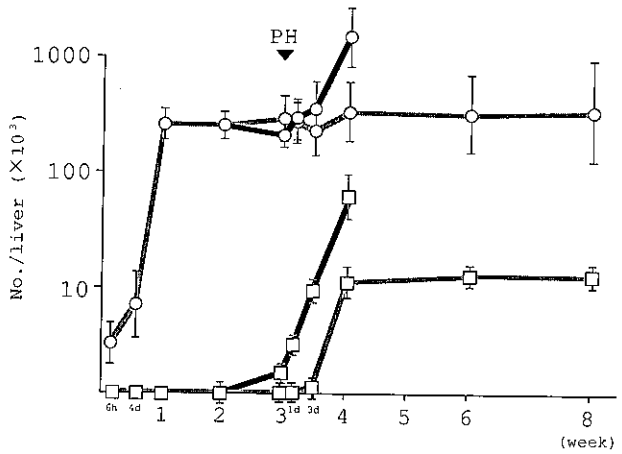

Fig. 3. Numbers of GST-P-positive single cells and foci per liver calculated by 3-D quantitative analysis. ○—○, DEN → 2-AAF+PH (single cells in group 1); ○═○, DEN+PH (single cells in group 2); □—□, DEN → 2-AAF+PH (foci in group 1); □═□, DEN+PH (foci in group 2). The single cells in group 2 (○═○) persist at a plateau value after 1 week.

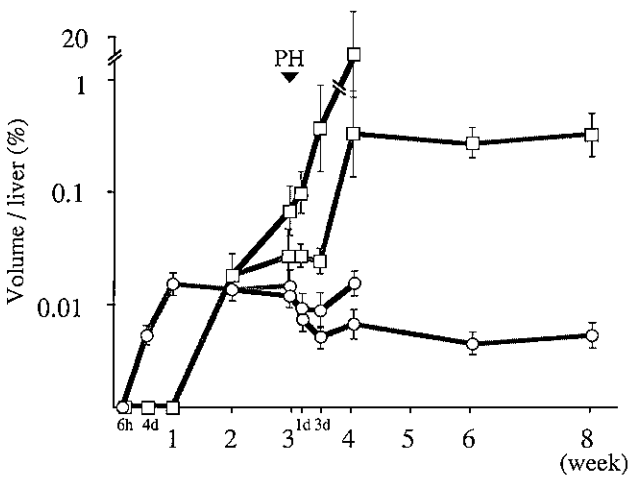

Fig. 4. Calculated volumes of GST-P-positive single cells and foci per total remaining liver (%). ○—○, DEN → 2-AAF+PH (single cells in group 1); ○═○, DEN+PH (single cells in group 2); □—□, DEN → 2-AAF+PH (foci in group 1); □═□, DEN+PH (foci in group 2). The single cells and foci in group 2 (○═○, □═□) demonstrate plateau values after week 4.

about 12.1×10^3 at week 4, and then remained constant (Fig. 3).

Similar findings were gained for quantitative calculated volumes of GST-P-positive cells per total remaining liver (%) (Fig. 4).

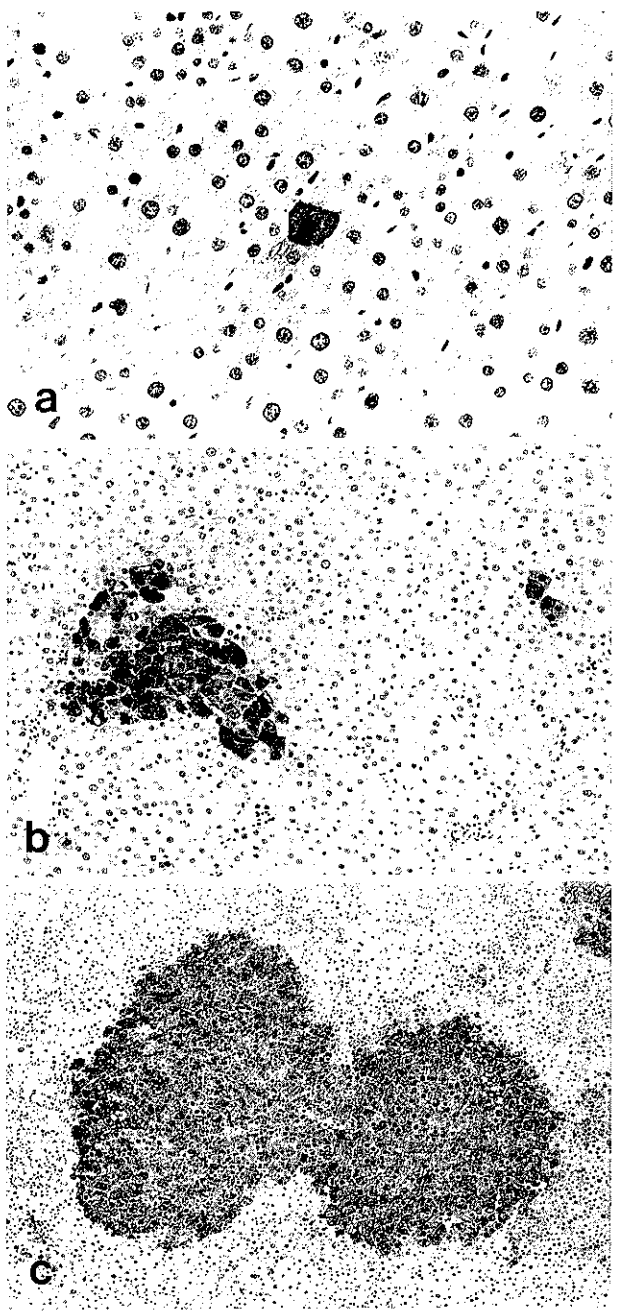

Fig. 5. 2-D sections of a GST-P-positive single cell and foci. (a) A GST-P-positive cell at 6 h after DEN initiation. (b) Small GST-P-positive foci at week 3 in group 1. (c) GST-P-positive foci at week 4 in group 1. They are fused with each other.

Three-dimensional reconstruction of GST-P-positive cells and hepatic lobules Six hours after DEN initiation, only a few GST-P-positive single cells (Fig. 5a) had

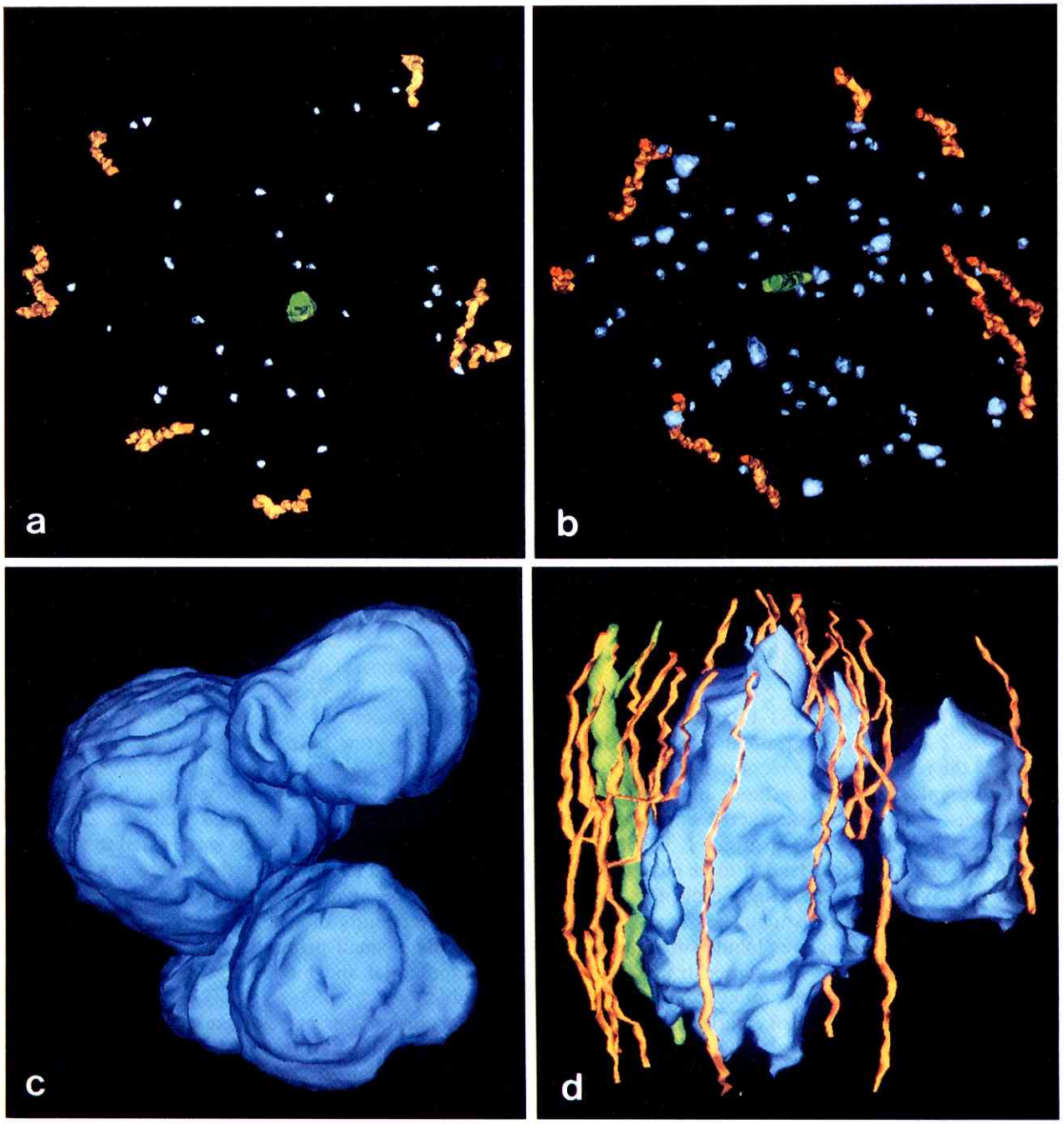

Fig. 6. 3-D reconstruction of GST-P-positive cells and liver structures. The central vein is indicated in green, the bile ducts in brown and the single cells and foci in blue. (a) 3-D reconstruction at week 2. Single cells appear at random in the liver lobule without zonal bias. (b) 3-D reconstruction at week 3 in group 1. Both single cells and foci appear at random within the liver lobe without any reduction in the single cells. (c) 3-D reconstruction at week 4 in group 1. The foci are inclined to be enlarged and fused with each other. (d) 3-D reconstruction of foci and bile ducts at week 6 in group 1. The foci within the same liver lobule are fused, exhibiting a complicated shape and are surrounded by bile ducts.

appeared. A 3-D reconstruction at week 2 of GST-P-positive single cells and rat liver structures in group 2 is shown in Fig. 6a. In this figure, the central vein is indicated in green, the bile ducts in brown and the single cells in blue. The GST-P-positive single cells were found to be distributed at random in the liver lobule without

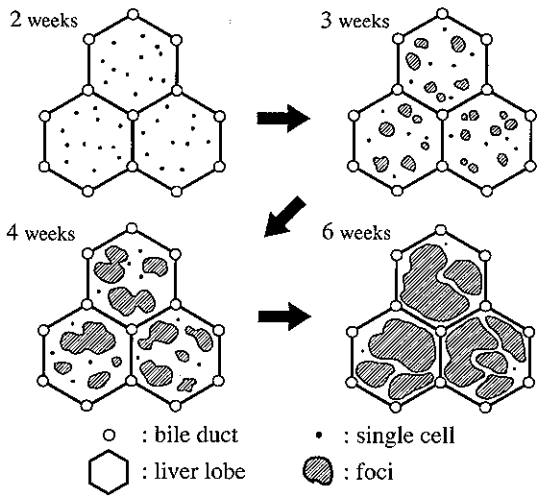

Fig. 7. The schematic development of GST-P-positive cells from single cells to foci. The single cells appear at random in the hepatic lobule until week 2, a small proportion of them grow to foci at week 3, and at 4 weeks foci are enlarged and fused in the same liver lobule. After 6 weeks, the foci in different lobules make contact with each other, but without any obvious fusion.

any zonal bias. Fig. 5b shows GST-P-positive single cells and foci in the 2-AAF treated group at week 3, and Fig. 6b illustrates the result of a 3-D reconstruction. The foci were also randomly distributed without any reduction in the single cells. The 3-D reconstructed appearance of GST-P-positive foci at week 4, in group 1, is shown in Fig. 6c. At this stage, the foci tended to be enlarged and fused with each other (Fig. 5c). Fig. 6d displays foci at week 6 which could not be distinguished from one another due to fusion within a liver lobule surrounded by bile ducts indicated in brown. The demonstrated shape is complicated. Fusion of foci was found to be generally intralobular with little evidence of interlobular mixing.

DISCUSSION

It is now generally accepted that GST-P-positive single cells, which appear from the very early stages of rat liver carcinogenesis, are of relevance to initiation and can develop into GST-P-positive foci and nodules.[8, 9, 23]

The present experiments revealed that, within the first week after an administration of DEN, single GST-P-positive cells markedly increased, in agreement with previous findings.[23] After PH alone they were not significantly altered in terms of 3-D quantitative values.

The numbers of single cells per liver were at all time points at least twenty times the values for foci. If all GST-P-positive single cells were transformed into foci, as the number of foci increased, the number of single cells should have been reduced correspondingly without showing a plateau value in this experiment. Thus, from the present experiment, it can be concluded that only a small proportion of GST-P-positive single cells appearing in the early stages of rat hepatocarcinogenesis actual develop into foci. In the 2-AAF case the situation is complicated by the fact that this carcinogen is also able to initiate carcinogenesis and cause the development of GST-P-positive focal populations. Also, a promotion effect on foci induction was already apparent at week 3, before performance of PH.

An important finding in this study was that 3-D reconstruction revealed GST-P-positive single cells to appear at random within the hepatic lobule. Fig. 7 is a schematic illustration of development of GST-P-positive cells from single cells to foci. Thus, single cells appeared at random in the 3 zones of the hepatic lobule until week 2, then some of the single cells grew to form foci by week 3, and by week 4 foci had become enlarged and fused in the same liver lobule. After 6 weeks, the foci became as big as liver lobules and the foci in different lobules come in contact with each other, but with little fusion.

The development of GST-P-positive lesions, in general, only occurred within hepatic lobules, and there was little evidence of fusion of foci from neighboring lobular structures. If foci grew beyond the confines of lobules, this phenomenon would result in a kind of invasive appearance, and would imply true neoplastic character. However, since our results indicated that the autonomous growth of GST-P-positive cells was limited to within liver lobules, it is indicative that they were not sufficiently changed to be neoplastic. This is also in line with the known reversibility of even GST-P-positive nodules.[24, 25] Thus, only a small proportion of foci might have neoplastic potential and indeed almost all of them may be reversible.

In conclusion, our present results suggest that GST-P-positive foci developed from only a limited number of the single cells which arise in early stages of rat hepatocarcinogenesis, and their growth tends to be confined within individual hepatic lobules.

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