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Sparse Distribution of Hepatocyte Growth Factor-producing Cells inside Hepatocellular Foci in Rats Treated with Hepatocarcinogens

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The distribution of hepatocyte growth factor (HGF)-synthesizing cells in rat liver during development of glutathione *S*-transferase P form (GST-P)-positive nodules after diethylnitrosamine initiation followed by promotion with 2-acetylaminofluorene plus partial hepatectomy (PH) was investigated using *in situ* hybridization. HGF-producing cells were non-parenchymal in nature, and were suspected to be mainly of Kupffer type. They were mostly located outside GST-P-positive lesions, in the surrounding parenchyma. In the oval cell proliferation phase 1 week after PH, they increased and they were mainly localized around the portal triads. It is concluded that HGF is directly involved in an endogenous paracrine growth pathway controlling proliferation in oval cells and in normal, but not GST-P-positive, hepatocytes.

Key words: HGF — *In situ* hybridization — Rat — Liver — Carcinogenesis

The growth of parenchymal hepatocytes during liver regeneration is strongly supported by hepatocyte growth factor (HGF) synthesized and secreted in a paracrine system.[1,2] Recently it has been reported that not only normal hepatocytes,[3,4] but also preneoplastic hepatic cells[5] can be stimulated to proliferate by administration of recombinant HGF to rats. However, it remains unclear how development of preneoplastic lesions is related to endogenous HGF production during rat hepatocarcinogenesis. Elevated expression of the growth factor in the liver might stimulate proliferation of initiated hepatocytes and predispose them to develop towards neoplastic lesions. Some authors have suggested that transforming growth factor-α (TGF-α) and its receptor epidermal growth factor receptor play an important role in an autocrine growth pathway causing growth of preneoplastic and neoplastic lesions initiated with chemical carcinogens and selected or promoted with a combination of partial hepatectomy (PH) and 2-acetylaminofluorene (2-AAF) or phenobarbital.[6,7] On the other hand, we have recently concluded that the expression of mRNAs for HGF and its receptor c-*met*, is not directly involved in growth of preneoplastic cells, based on sequential quantitation of transcriptional levels during rat hepatocarcinogenesis.[8] HGF-producing cells have been demonstrated by *in situ* hybridization histochemistry (ISH) in the livers of normal[2] and 2-AAF-treated rats,[9] but their location with respect to preneoplastic populations has not been examined in detail. In the present study, we therefore examined the distribution of HGF-producing cells in the rat liver under conditions of rapid development of glutathione *S*-transferase P form (GST-P)-positive nodules initiated with diethylnitrosamine (DEN) and promoted with 2-AAF plus PH[10] to ascertain their possible involvement in rat hepatocarcinogenesis.

Male Fischer rats, 7 weeks old at the start of the experiment, were maintained on basal diet and water *ad libitum* and housed in plastic cages in an air-conditioned room. Initially they were all treated intraperitoneally with DEN at a dose of 200 mg/kg body weight and then maintained on basal diet for the first 2 weeks. The animals were then given basal diet containing 0.015% 2-AAF for 6 weeks. Two-thirds PH was performed at the end of week 3 of the experiment. Three animals were killed under ether anesthesia at 0, 1 and 5 weeks after PH. Right lateral lobes of the livers were excised, cut into 2–3 mm thick slices and fixed in ice-cold, phosphate-buffered 4% paraformaldehyde for routine staining of sections with hematoxylin and eosin, and for ISH of HGF mRNA. The remaining portions of the right lateral lobes were immediately frozen in liquid nitrogen for immunohistochemical demonstration of HGF. The method for ISH using digoxigenin-labeled RNA probe was that described previously[11] with some modifications. Paraffin-embedded sections were deparaffinized and proteinase K treatment was carried out. The hybridization solution contained 50% formamide, 10% dextran sul-

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fate, 1×Denhardt's solution, 600 m*M* NaCl, 10 m*M* Tris-HCl pH 7.6, 0.25% SDS, 200 μg/ml of yeast tRNA and approximately 0.5 μg/ml of RNA probe. A 1.4-kb fragment of rat HGF complementary DNA (pRBC1 clone) was subcloned into the pBluescript SK(−) vector, and a digoxigenin-11-UTP-labeled antisense RNA probe was prepared using a DIG RNA Labeling Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). After hybridization at 50°C for 16 h, washing was performed in 50% formamide, 2×SSC for 30 min at 65°C followed by RNase A treatment. Hybridized digoxigenin-labeled probes were detected with a Nucleic Acid Detection Kit (Boehringer Mannheim GmbH Biochemica). Control sections, including those hybridized with the sense RNA probe or without the antisense RNA probe or the anti-digoxigenin antibody, showed no positive signals. For immunohistochemistry of HGF, frozen sections of liver tissues were cut in a cryostat and fixed in periodate-lysine paraformaldehyde solution. The peroxidase-anti-peroxidase method was performed using a monoclonal antibody against recombinant rat HGF (Toyobo, Osaka). Since expression of GST-P in focal areas of hepatocytes has been widely used as an immunohistochemical marker for the identification of preneoplastic cell populations,[12] anti GST-P antibody was purchased from Medical & Biological Laboratories Co. (Nagoya) and the avidin-biotin-peroxidase complex method was used to determine the location of GST-P-positive cells in the liver.

At the time of PH, the livers were histologically almost normal. We observed scattered polygonal HGF-producing cells in ISH sections and HGF-positive cells on immunohistochemistry, and identified them as non-parenchymal cells from their morphological characteristics. At 1 week after PH, numerous GST-P-positive hepatocellular foci had grown and proliferation of oval cells had occurred around the portal triads in the periportal zones. These immature small epithelial cells with large ovoid nuclei and scant cytoplasm appear during rat hepatocarcinogenesis in the portal spaces, undergoing morphological and functional differentiation along hepatocyte and bile ductal cell lineages.[13] The kinetics of oval cell proliferation after 2-AAF feeding and PH have been described in detail[14] and the results in the present study were in accordance. In this oval cell proliferation phase at week 1, the number of HGF-producing cells increased markedly, and these cells were mainly localized around the portal triads in the liver, rather than in or around GST-P-positive foci (Fig. 1). They could be readily distinguished from oval cells by their cytoplasmic and nuclear shape, and were suspected to be mainly Kupffer cells because of their positive ED-1 immunohistochemistry (data not shown).[15] GST-P-positive nodules had developed to occupy a large percentage of the liver at

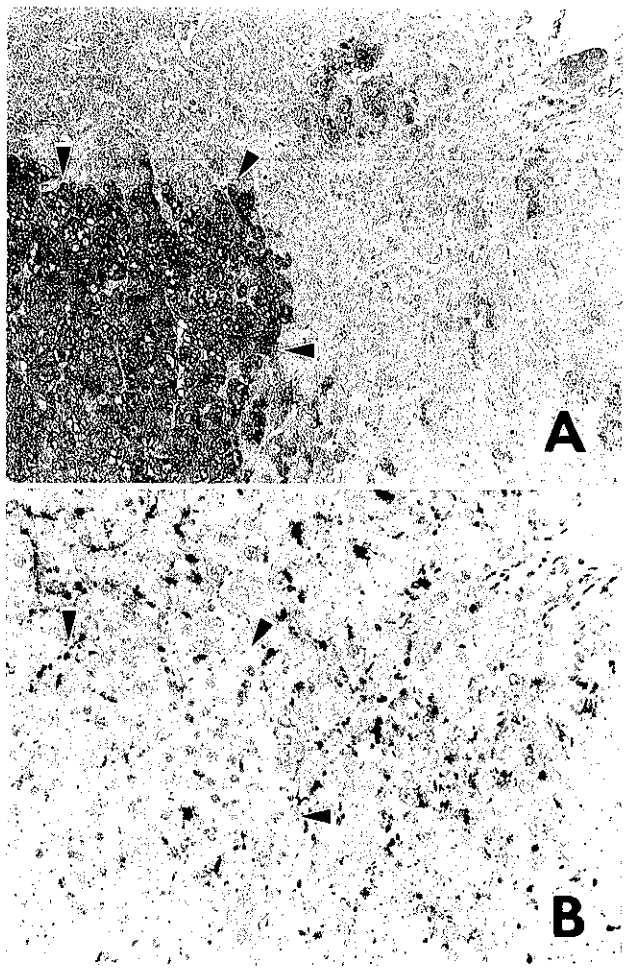

Fig. 1. A, A GST-P-positive focus (arrowheads) evident 1 week after partial hepatectomy (PH). GST-P immunohistochemistry, ×100. B, HGF-producing cells in a serial section are mainly localized in the periportal zone where oval cells are evident, rather than within the GST-P-positive focus (arrowheads). HGF mRNA *in situ* hybridization, ×100.

week 5 after PH as a result of continuous 2-AAF feeding, whereas the numbers of oval cells were reduced. In this GST-P-positive nodule-developing phase, HGF-producing cells were mainly located outside the GST-P-positive lesions (Fig. 2). No obvious differences in the distributions and numbers of HGF-producing cells evident on ISH and immunohistochemically demonstrated HGF-positive cells were observed at any time point.

The molecular mechanisms underlying preneoplastic foci development during the promotion stage, including the roles played by growth factors, still remain a major research topic in the field of rat hepatocarcinogenesis. As well as HGF, the hepatocyte mitogenic growth factor

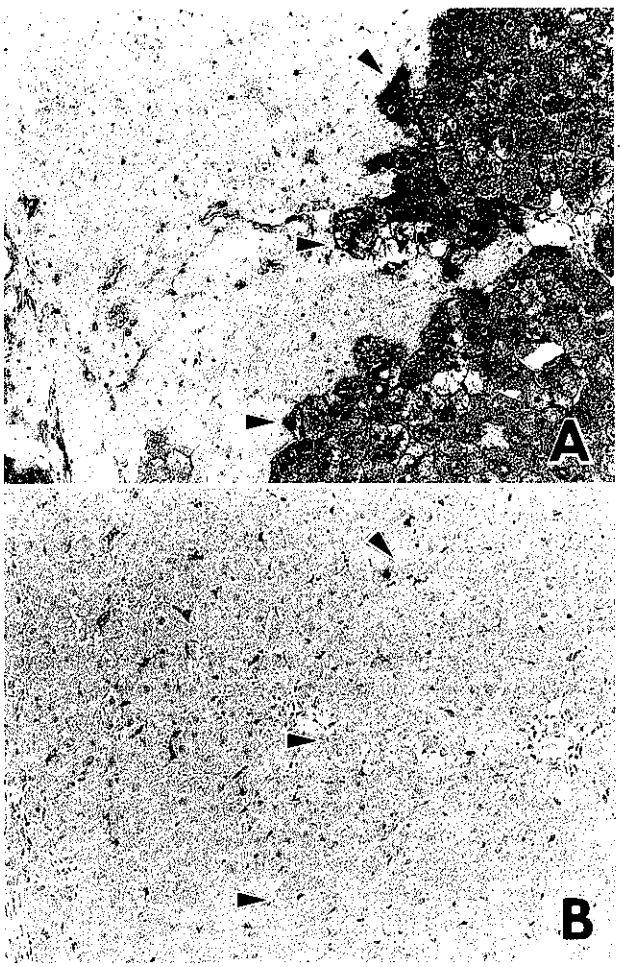

Fig. 2. A, GST-P-positive lesions (arrowheads) developing after continuous 2-AAF feeding. GST-P immunohistochemistry, ×50. B, In a serial section, HGF-positive polygonal cells are only evident outside the GST-P-positive lesions (arrowheads). HGF immunohistochemistry, ×50.

TGF-α has a possible role in the early stages of rat hepatocarcinogenesis.[7] To our knowledge, the present paper is the first to focus on the relevance of HGF to GST-P-positive hepatocellular lesion development using ISH to demonstrate HGF-producing cells in the resistant hepatocyte model. Previous reports described HGF to be synthesized and secreted by Kupffer and endothelial cells in normal and damaged rat liver,[2] or Ito cells in 2-AAF-treated rat liver,[9] acting to repair the liver tissue or to induce proliferation of oval cells in paracrine fashion. The present experiment also demonstrated HGF to be produced by non-parenchymal cells, suspected to be mainly Kupffer cells, in a paracrine growth pathway during GST-P-positive lesion development in rat liver. However, a marked spatial dissociation between the HGF-producing and GST-P-positive hepatocellular populations was found. In the literature, it has been documented that HGF-positive cells are generally localized in areas where regeneration of hepatocytes occurs, around the portal triads in rat liver bearing spontaneous fulminant hepatitis, and diffusely in the tissue of the rat liver after PH.[16] In the present model, HGF-producing cells were mainly located outside GST-P-positive hepatocellular lesions, suggesting that the paracrine influence exerted on normal hepatocytes and oval cells[8,9] must be much weaker in the case of GST-P-positive foci and nodules. This implies a certain degree of independence of the latter from HGF for growth. Moreover, it was recently reported that the HGF receptor Met is expressed in oval cells, but not in GST-P-positive foci during chemically induced rat hepatocarcinogenesis.[17] While further study, using *in situ* hybridization, is needed to identify any cells synthesizing HGF within hepatic neoplasms, our previous data at least suggest that this growth factor does not play an important role, since in most hepatocellular carcinomas examined, HGF and its receptor c-*met* transcripts have been found to be present at levels below those in the surrounding non-neoplastic parenchyma.[8] In conclusion, the present results imply that HGF is directly involved in an endogenous paracrine growth pathway controlling proliferation in oval cells and normal, but not GST-P-positive, hepatocytes.

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