Expression of hepatocyte growth factor and c-met mRNAs during rat chemically induced hepatocarcinogenesis

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The receptor for hepatocyte growth factor (HGF), a potent mitogen for hepatocytes, is the product of the proto-oncogene c-met. In order to cast light on their significance for hepatocarcinogenesis, levels of both HGF and c-met mRNA were evaluated in rat livers during development of 2-acetylaminofluorene (2-AAF)-selected preneoplastic nodules and carcinomas following diethylnitrosamine (DEN) injection. Rats were given a single i.p. injection of 200 mg/kg body wt DEN and, starting 2 weeks later, were administered 0.015% 2-AAF in the diet for up to 6 weeks. All rats were subjected to partial hepatectomy (PH) at week 3. Additional animals undergoing the DEN, 2-AAF and PH regimen were sacrificed at week 40 to allow evaluation of carcinomas. Oval cell proliferation, glutathione S-transferase placental form (GST-P)-positive preneoplastic lesion development and HGF and c-met mRNA levels were sequentially analyzed after PH. Numerous oval cells were observed 1 week after PH, but were remarkably reduced 2 weeks thereafter. The areas of GST-P-positive foci and nodules rapidly increased with time not only during 2-AAF feeding, but also to the same degree for at least 2 weeks after cessation of carcinogenic insult. Dot blot analysis showed HGF transcripts to be elevated after PH and during the selective growth conditions of 2-AAF feeding, dropping after cessation of carcinogenic insult. In the c-met transcript case transient increases were observed after PH, followed by a decrease. c-met over-expression in nodular livers did not correlate with the presence of 2-AAF or lesion development. In most hepatocellular carcinoma samples expression of both HGF and c-met mRNAs was below levels in non-neoplastic regions. These data suggest that HGF and c-met are directly involved in a paracrine growth pathway controlling proliferation in normal hepatocytes and oval cells, but not in preneoplastic and neoplastic cells.

Introduction

In the process of chemically induced hepatocarcinogenesis an established mechanism of promotion is 'differential inhibition' of cell proliferation (1). By providing a growth stimulus and at the same time inhibiting cell division in the vast majority of uninhibited hepatocytes by exposure to the growth-inhibitory action of hepatotoxins the few rare resistant initiated hepatocytes are able to respond to the mitogenic stimulus and rapidly generate preneoplastic foci and nodules. Thus in the resistant hepatocyte model promotion is essentially selective inhibition, not selective stimulation (1).

It is not clear how development of preneoplastic lesions is related to endogenous growth factors during rat hepatocarcinogenesis. Elevated expression of growth factor production in the liver might stimulate the proliferation of initiated hepatocytes and predispose these cells to develop towards neoplastic lesions by selective stimulation. Some authors have suggested that transforming growth factor-α (TGF-α) and its receptor, epidermal growth factor receptor, play an important role in the growth of preneoplastic and neoplastic lesions initiated with chemical carcinogens and selected or promoted with a combination of two-thirds partial hepatectomy (PH) and 2-acetylaminofluorene (2-AAF) or phenobarbital (2,3). On the other hand, it was recently reported that TGF-α expression might not necessarily confer a growth advantage on hepatocellular preneoplastic and neoplastic lesions in a peroxisome proliferator-treated model (4).

Hepatocyte growth factor (HGF), first demonstrated in the serum of partially hepatectomized rats (5,6) and in rat platelets (7), is a potent mitogenic growth factor for mature hepatocytes in culture. Liver is one of the major organs producing HGF in vivo and the growth of parenchymal hepatocytes during liver regeneration is supported by HGF synthesized and secreted in a paracrine system (8,9). The receptor for HGF has been characterized as the product of the proto-oncogene c-met, with tyrosine kinase activity (10,11). Primary structures have been deduced from cDNA clones of both rat HGF and mouse c-met (12,13). On the basis of expression of HGF and c-met mRNA in rat liver after injury by hepatotoxins or PH (8,14-17) it has been concluded that HGF might be a major early signal that triggers hepatocyte proliferation during liver regeneration. However, levels of HGF mRNA transcripts during hepatocarcinogenesis and the role of this factor during liver tumor development remain unclear.

The resistant hepatocyte model is particularly suitable for sequential analysis of the relations between growth factors and rat hepatocarcinogenesis. The expression of glutathione S-transferase placental form (GST-P) in focal areas of hepatocytes has been widely used as an immunohistochemically demonstrable marker for the identification of preneoplastic cell populations (18). In the present study we examined the relation between levels of HGF and c-met mRNAs in the rat liver under conditions of rapid development of GST-P-positive
preneoplastic nodules after PH in the Solt–Farber model to ascertain possible involvement in rat hepatocarcinogenesis.

Materials and methods

Animals and treatments

A total of 103 male Fischer rats (Charles River Japan Inc., Kanagawa, Japan) weighing 110–130 g were maintained on basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water ad libitum and housed in plastic cages in an air-conditioned room at 24°C. They were divided into five groups. Initially all rats were treated intraperitoneally with diethylnitrosamine (DEN; Tokyo Chemical Industry Co., Tokyo, Japan) at a dose of 200 mg/kg body wt (groups 3–5) or its saline solvent (groups 1 and 2) and given basal diet for the first 2 weeks. The animals in groups 2, 4 and 5 were then given basal diet containing 0.015% 2-AAF (Tokyo Chemical Industry Co., Tokyo, Japan) for 2 (groups 2 and 4) or 6 weeks (group 5). Animals in groups 1 and 3 received unsupplemented basal diet. All groups other than group 5 were maintained on basal diet during the period from week 4 until 8. PH was performed on all rats at week 3 of the experiment. Group 1 underwent PH alone as a control. Three animals from each group were killed under ether anesthesia at 0, 1, 8, 18, 48 h and 1, 3 (only groups 4 and 5) and 5 weeks after PH (Figure 1). Ten additional animals in group 5 were maintained on basal diet until week 40 for collection of carcinoma samples.

Histopathological observations

Right lateral lobes of the livers were excised, cut into 2–3 mm thick sections with a razor blade and fixed in ice-cold acetone for routine staining with hematoxylin and eosin and immunohistochemical demonstration of GST-P. Remaining liver of the right lateral lobes was immediately frozen in liquid nitrogen for RNA isolation. Areas of oval cell proliferating zones, defined as areas mainly consisting of oval cells clearly distinguishable from the surrounding preneoplastic lesions and normal parenchyma, and numbers and areas of GST-P-positive preneoplastic foci and nodules >0.2 mm in diameter, both demonstrated in GST-P sections (18,20), were measured using a color image processor (SPICCA, Nippon Avionics Co., Tokyo, Japan). Oval cells are immature small epithelial cells with large ovoid nuclei and scant cytoplasm which appear during rat hepatocarcinogenesis in the portal spaces, undergoing morphological and functional differentiation along the hepatocyte and bile ductal cell lineages (19). The oval cell proliferating zones were analyzed by assessment of 20 portal areas per animal; tissue comprising >80% oval cells being measured. The number and volume of GST-P-positive lesions per mm² were assayed from stereological analysis as described previously (21).

Sequential quantitation of oval cell proliferating zones

Histological findings after 2-AAF feeding and PH following DEN administration resembled those previously described (24). At the time of PH the livers of groups 2, 4 and 5 were histologically almost normal. At 1 week after PH numerous altered hepatocellular foci had grown and proliferation of numerous oval cells induced in the portal triads and perportal zones occurred in group 5 (in common with group 4). A milder degree of proliferation of oval cells and slight basophilia of hepatocytes were also evident in group 2, without altered hepatocellular foci development. Three weeks after PH numbers of oval cells were very much reduced not only in group 2, but also in groups 4 and 5. All the livers of groups 1 and 3 were histologically normal during the experiment. Seven of 10 group 5 animals had hepatocellular carcinomas at week 40. The latter showed a mainly trabecular growth pattern with some pseudoglandular elements and contained various degrees of bile duct proliferation and/or carcinoma cell degeneration.

Sequential quantitation of oval cell proliferating zones

Sequential changes in areas of oval cell proliferating zones in groups 2, 4 and 5 (Figure 2b and c) are summarized in Figure 3. The kinetics of oval cell proliferation after 2-AAF feeding and PH have been previously described in detail and the results in the present study were in accordance (25). At 1 week after PH the areas of the oval cell proliferating zones reached a peak in groups 2 and 5 (in common with group 4), with values in groups 4 and 5 being significantly larger (P < 0.01) than
HGF in rat hepatocarcinogenesis

0 1 3 5 week after partial hepatectomy

Fig. 3. Sequential changes in the areas of oval cell proliferating zones in groups 2, 4 and 5. Results are expressed as means ± SD and represent: □, group 2; ■, group 4; ●, group 5. *P < 0.01 versus group 5 (in common with group 4); †P < 0.01 versus groups 4 and 5.

in group 2. At 3 weeks after PH the areas of the oval cell proliferating zones were remarkably reduced. Almost no oval cell proliferation was observed at any time point in groups 1 and 3 (Figure 2a).

Sequential quantitation of GST-P-positive hepatocellular lesions

Sequential changes in numbers, areas and volumes of GST-P-positive lesions, including foci and nodules in groups 4 and 5 (Figure 4a and b), are summarized in Table I and Figure 5. The number of GST-P-positive lesions had rapidly increased by 1 week after PH and then slightly decreased, due to their fusion by rapid growth. The percentage of GST-P-positive lesions rapidly increased with time in group 5 from 1 to 5 weeks after PH as a result of continuous 2-AAF feeding. Finally, the livers in group 5 consisted almost entirely of GST-P-positive lesions (89.60 ± 13.97 vol. %) at week 5 after PH.
Table I. Numerical density and volume percentage of GST-P-positive lesions selected by 2-AAF following DEN initiation

<table>
<thead>
<tr>
<th>Week after</th>
<th>PH group</th>
<th>N</th>
<th>No./cm²</th>
<th>Volume (%)</th>
<th>N</th>
<th>No./cm²</th>
<th>Volume (%)</th>
<th>N</th>
<th>No./cm²</th>
<th>Volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td></td>
<td>(In common with group 5)</td>
<td></td>
<td>3</td>
<td>31.72±54.93</td>
<td>0.03±0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>3</td>
<td>3735.80±1224.17</td>
<td>26.30±11.59</td>
<td>3</td>
<td>2457.29±347.11</td>
<td>52.27±6.31</td>
<td>3</td>
<td>1608.63±362.55</td>
<td>89.60±13.97</td>
</tr>
</tbody>
</table>

Values presented are Means ± SD. N, effective no. of animals.

Fig. 5. Sequential changes in the numbers and areas of GST-P-positive lesions (foci and nodules) in groups 4 and 5. Results are expressed as means ± SD and represent: O, numbers of GST-P-positive lesions in group 4; •, numbers of GST-P-positive lesions in group 5; ◼, areas of GST-P-positive lesions in group 4; ■, areas of GST-P-positive lesions in group 5. *P < 0.01 versus group 5.

After release from selective growth stimulation GST-P-positive lesions in group 4 developed almost as in group 5, at least until week 3 after PH, then declining, so that differences between groups 4 and 5 were statistically significant (P < 0.01) at week 5. The numbers (no./cm²) and areas (mm²/cm²) of GST-P-positive lesions 1 week after PH were 4.5 ± 2.3 and 0.3 ± 0.1 in group 3 and at 5 weeks after PH were 6.7 ± 1.9 and 0.5 ± 0.3 in group 3 and 1.7 ± 1.4 and 0.2 ± 0.2 in group 2. No GST-P-positive lesions were detected in group 1.

Sequential quantitation of HGF and c-met mRNA

Data for relative HGF and c-met mRNA amounts are summarized as fold increase as compared with that of normal rat livers (group 1, at the time of PH) in Figure 6.

HGF mRNA ~6.0 kb in size was detected by Northern blot analysis (Figure 7a; 12). Dot blot analysis showed that sequential changes in HGF mRNA after PH in group 1 resembled those previously described (14,15), with an increase to a peak at 8 h after PH. In group 2 the levels of HGF mRNA rapidly increased within 1 h after PH, remained high until 18 h, declined and then rose to a maximum level at 1 week after PH. In group 3 HGF mRNA rapidly increased and reached the maximum level at 1 h after PH, then declining to normal by 48 h. In group 5, the expression of HGF transcripts had already increased more than 5-fold at the time of PH and then reached a maximum level 1 h after PH (>10-fold), which was essentially maintained until 5 weeks after PH, whereas in group 4 it declined after cessation of 2-AAF feeding, reaching a normal level by 3 weeks.

The c-met mRNA signal was detected at ~8.5 kb in size by Northern blot analysis (Figure 7b; 13). Dot blot analysis showed that expression of c-met transcripts remarkably decreased around 18 h after PH in groups 2 and 5 (in common with group 4). The level of c-met mRNA increased and reached a peak at 1 week after PH, gradually returning to normal by 5 weeks after PH in groups 2 and 5, whereas it maintained a high level after cessation of 2-AAF feeding in group 4.

Samples were selected from 14 hepatocellular carcinomas collected at week 40, so as to minimize areas of degeneration and bile duct proliferation. The mRNA expression of HGF and c-met are shown as fold increase as compared with that of a non-neoplastic region for each animal in Figure 8. Carcinoma HGF mRNA levels were 0.66 ± 0.54-fold (mean ± SD) and carcinoma c-met mRNA levels were 0.78 ± 0.49-fold that in non-neoplastic regions of rat liver. For both
between HGF and its receptor c-met during preneoplastic and neoplastic growth. The present paper is the first to describe an association in terms of early stages of rat hepatocarcinogenesis. Oncogene products, for instance c-myc, are highly expressed in hepatocellular carcinomas and foci, but no specific pattern has yet been established for preneoplastic lesions (26–28). Recently a mitogenic growth factor for hepatocytes, TGF-α, has attracted attention in terms of early stages of rat hepatocarcinogenesis (2–4). The present paper is the first to describe an association between HGF and its receptor c-met during preneoplastic and neoplastic lesion development in the resistant hepatocyte model, a paracrine growth pathway apparently involved in control of normal hepatocyte proliferation.

At the time of PH the livers were histologically almost normal in all groups, while HGF mRNA had remarkably increased in the DEN + 2-AAF and 2-AAF alone groups and tended to increase in the DEN alone group. Several authors have recently emphasized that HGF mRNA transcripts gradually increase with oval cell proliferation in 2-AAF models (29,30), although the present experiment demonstrates that histological changes are not necessary for HGF elevation during 2-AAF treatment. Continuous 2-AAF feeding is suggested as stimulating HGF mRNA transcription and persistent cytotoxicity of DEN might provide an additional influence. Previously it was shown that the liver HGF mRNA level increases within 12 h after PH and reaches a maximum at 12–24 h (14,15). Sequential changes in HGF mRNA after PH in the control (PH alone) group of the present experiment resembled those previously described, whereas in the DEN + 2-AAF and DEN alone groups a remarkable increase was observed, reaching a peak within 1 h after PH. Such a rapid change in HGF mRNA has not been previously reported and the mechanism remains to be elucidated. The fact that expression of c-met transcripts was remarkably decreased around 18 h after PH in the DEN + 2-AAF and 2-AAF alone groups suggests some role in the mito-inhibitory effects on non-initiated hepatocytes.

One week after PH numerous GST-P-positive lesions had developed in the DEN + 2-AAF group and proliferation of oval cells was pronounced in the DEN + 2-AAF group and less evident in the 2-AAF alone group. In both cases this was associated with high levels of HGF mRNA and peaks for c-met, albeit lower in the 2-AAF alone case. In the literature it has been documented that after in situ hybridization c-met mRNA is strongly expressed in oval cells, suggesting that they are sensitive to HGF (30). Virtually all the dynamic changes in HGF and c-met mRNA at this stage of the experiment might have been related to oval cell proliferation.

In normal mature hepatocytes DNA synthesis peaks 24 h after PH, 12 h after the peak of HGF mRNA expression (15). If HGF stimulates the initial step in hepatocyte proliferation, GST-P-positive lesion development should be almost coincidental with the increase in HGF. In this experiment the GST-P-positive lesions were almost as large at least 2 weeks after release from 2-AAF feeding as in the 2-AAF continuous feeding group, despite a marked drop in the levels of HGF mRNA. The apparent lack of correlation between GST-P-positive lesion development and elevation of HGF suggests that this factor may not be necessary for the growth of preneoplastic lesions under 2-AAF selection conditions. GST-P-positive lesions made up almost the entire livers 5 weeks after PH in the DEN + 2-AAF (6 weeks) group, while transcription of c-met mRNA was at the same level as in control livers. These data indicate that c-met is not over-expressed in GST-P-positive lesions. On the other hand, interestingly, c-met mRNA was maintained at high levels after cessation of 2-AAF feeding. With this model phenotypic maturation of remodeling nodules is observed with γ-glutamyltransferase histochemistry during this phase (31,32). While this is not due to replacement by proliferating normal cells (32), the c-met transcription data described above together might indicate that relaxation of 2-AAF mito-inhibition results in some c-met expression and cell division of non-focal hepatocytes. Further study using in situ hybridization is needed.
to identify what cell type synthesizes c-met after cessation of 2-AAF feeding in the DEN + 2-AAF (2 weeks) group.

In humans HGF increase has been shown in the serum of patients after hepatic resection for tumors (33) and with fulminant hepatitis (34), though HGF expression itself is less in the tumors than in the adjacent normal liver of patients bearing hepatocellular cancers (35). It has also been reported that HGF inhibits growth of hepatocellular carcinoma cells in vitro (36) and cell proliferation of hepatocellular carcinoma in vivo (37). This is in line with our finding of decreased levels of both HGF mRNA and c-met in our Solt–Farber model hepatocellular carcinomas. In conclusion, the present data suggest that HGF and c-met are not directly related to the proliferative activity of preneoplastic and neoplastic hepatocytes.

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References