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Expression of hepatocyte growth factor and its receptor c-met, correlates with severity of pathological injury in experimental alcoholic liver disease

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Abstract. Expression of hepatocyte growth factor (HGF) and its receptor, c-met is up-regulated in various forms of liver injury. This study evaluated the relationship between HGF and c-met expression and pathological changes in experimental alcoholic liver disease. Rats (5 per group) were fed ethanol and a diet containing saturated fat corn oil or fish oil by intragastric infusion. Dextrose isocalorically replaced ethanol in controls. In a second set of experiments, Kupffer cells, endothelial cells and hepatocytes were isolated from rats in each group. Pathological evaluation and analysis of HGF and c-met expression were performed in liver and the different cell types. Increased expression of HGF and c-met expression was detected in the liver of rats showing necroinflammatory changes. The Kupffer and endothelial cells were primarily responsible for the increase in HGF, c-met expression was seen only in hepatocytes. Thus, up-regulation of HGF and c-met occurred in the presence of the necrosis and inflammation suggesting that HGF may be acting to protect against liver injury or accelerate the regenerative process.

Introduction

Hepatocyte growth factor (HGF) is the most powerful hepatocyte mitogen in vitro (1) and it is also an extremely potent mitogen, motogen, and morphogen in vivo (2-4). HGF was first detected in the serum of both normal and partially hepatectomised rats and was subsequently cloned and sequenced by Nakamura et al (5). It has since been established that HGF is expressed by various mesenchymal-derived cells throughout the body, but is produced in the liver by Kupffer, endothelial and hepatic stellate cells (6). HGF produces its biological effects by activating the transmembrane c-met receptor (7-9), which on binding HGF, dimerises and cross phosphorylates its intracellular tyrosine kinase sites, initiating the signal transduction cascade which culminates in effecting the mitogenic, motogenic and morphogenic properties of its ligand (10). The exact mechanisms of these downstream effects are not as yet fully elucidated.

The presence of necrosis and an inflammatory response characterizes alcoholic liver disease (ALD). Since several lines of investigation indicate that HGF is up-regulated in response to hepatic injury (3), the aim of this study was to identify the pattern of HGF and c-met expression in liver tissue and the different cell types in alcohol-treated rats. We utilized the rat model of intragastric feeding to induce alcoholic liver injury (11,12) and to determine whether any such relationship might exist between ALD and expression of HGF and c-met. This strategy was employed primarily because the severity of injury induced in the intragastric feeding model can be varied simply by changing the type of dietary fat. For instance, based on epidemiological studies relating to the nature of the dietary fat and incidence of ALD (13) experimental studies have demonstrated that rats fed saturated fatty acids and ethanol develop none of the histological features of ALD (14) whereas rats fed polysaturated fatty acids and ethanol show fatty liver, necrosis, inflammation and fibrosis (15). Moreover, if fish oil is fed with ethanol, the necrosis, inflammation and fibrosis is much more severe than is seen in rats fed corn oil and ethanol (16-18).

Thus, the ability to induce various grades of alcoholic liver injury enabled us to relate the severity of liver damage to any concomitant fluctuation in HGF or c-met expression between the different dietary groups. Analysis of transcription of both HGF and c-met genes was performed by in situ hybridization (ISH) on formalin-fixed paraffin-embedded liver, in addition to mRNA analysis by reverse transcription polymerase chain reaction (RT-PCR) of liver tissue and purified parenchymal
Analysis of HGF, c-met and β-actin expression in whole tissue and cells by RT-PCR. Total RNA was isolated from both animals. The purified cell populations were identities confirmed using morphological criteria and immunohistochemical analyses (19). The sequences of the primers used in this study have been previously published (20, 21). Negative controls for each experiment consisted of analyzing a sample of total RNA which had not undergone reverse transcription.

PCR products and appropriate molecular weight markers were electrophoresed on a 1% agarose gel and after staining with ethidium bromide were analysed by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). None of the negative control samples yielded any product, confirming the absence of contaminating DNA species.

Histopathological analysis. Small samples of exsanguinated livers were fixed overnight in formalin, routinely processed and embedded in paraffin wax. Five micron sections were cut from these blocks, processed for routine haematoxylin and eosin (H&E) staining and the severity of pathological changes (i.e. extent of steatosis, necrosis and inflammation) were ascertained as previously described (14, 18). Briefly the degree of steatosis was graded according to the percentage of liver cells containing fat and necrosis and inflammation according the number of foci per power field.

Table I. Blood alcohol levels (mean ± SE) and pathological changes in the different experimental groups.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Blood alcohol (mg/dl)</th>
<th>Fatty liver (0-4)</th>
<th>Necrosis (foci/LPF)</th>
<th>Inflammation foci/LPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCTD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCTE</td>
<td>242±47</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CE</td>
<td>249±37</td>
<td>3.4±0.5</td>
<td>1.2±0.4b</td>
<td>1.2±0.4b</td>
</tr>
<tr>
<td>FD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FE</td>
<td>244±45</td>
<td>4.0±0.0</td>
<td>2.0±0.0b</td>
<td>2.0±0.0b</td>
</tr>
</tbody>
</table>

*p<0.05 vs. CE; p<0.01 vs MCTE; b p<0.01 vs MCTE. MCT, medium chain triglycerides; C, corn oil; F, fish oil; D, dextrose; E, ethanol.

and non-parenchymal liver cell populations obtained from the rats in the different dietary groups.

Materials and methods

Animals. All animals used for this study were cared for in accordance with the National Institutes of Health criteria for care of laboratory animals. Six groups of male Wistar rats (five rats per group) weighing 275-300 g were fed by continuous infusion of a liquid diet via permanently implanted gastric tubes, as previously described (11, 12). The six diets were constituted as follows: medium chain triglycerides, corn oil or fish oil, each being supplemented with either dextrose (MCTD, CD and FD) or ethanol (MCTE, CE and FE) respectively. Dietary fatty acids contributed 35% of total caloric intake and the fatty acid composition of these diets has been described previously (17). Ethanol was initially administered at a dose of 10 g/kg/day and was gradually increased to 16 g/kg/day as tolerance developed. Blood alcohol levels were maintained at between 150 and 300 mg/dl; blood was collected from the tail vein and ethanol concentration measured using an alcohol dehydrogenase assay (Sigma). All rats were sacrificed four weeks after initiating each feeding regime and the liver was processed as described below.

Isolation of hepatocytes, Kupffer cells and endothelial cells. Detailed descriptions of the cell isolation and purification procedures have already been published (19, 30). Briefly, livers were exsanguinated in situ, excised and finely minced and incubated in 0.05% collagenase at 37°C for 45 min. Each of the three cell populations were thereafter isolated and their identities confirmed using, morphological criteria and immunohistochemical analyses (19). The purified cell populations were stored at -70°C until required for RNA isolation and RT-PCR.

Analysis of HGF, c-met and β-actin expression in whole tissue and cells by RT-PCR. Total RNA was isolated from both whole liver tissue and the purified cell populations according to the guanidium isothiocyanate method (8). The integrity of isolated RNA was determined by agarose gel electrophoresis and ethidium bromide staining. Total RNA (0.5-1.0 μg) was reverse transcribed and the resultant HGF, c-met and β-actin cDNAs were amplified using the conditions described previously (19). The sequences of the primers used in this procedure have already been published (19). The sequences of the primers used in this study have been previously published (20, 21). Negative controls for each experiment consisted of analyzing a sample of total RNA which had not undergone reverse transcription.

PCR products and appropriate molecular weight markers were electrophoresed on a 1% agarose gel and after staining with ethidium bromide were analysed by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). None of the negative control samples yielded any product, confirming the absence of contaminating DNA species.

Histopathological analysis. Small samples of exsanguinated livers were fixed overnight in formalin, routinely processed and embedded in paraffin wax. Five micron sections were cut from these blocks, processed for routine haematoxylin and eosin (H&E) staining and the severity of pathological changes (i.e. extent of steatosis, necrosis and inflammation) were ascertained as previously described (14, 18). Briefly the degree of steatosis was graded according to the percentage of liver cells containing fat and necrosis and inflammation according the number of foci per power field.

In situ hybridization for HGF and c-met mRNAs. The method described is based on that by Senior et al (22) with slight modifications (23). Each sample was prepared in duplicate so that two exposure times to the emulsion (14 and 28 days) could be performed. Plasmids containing cDNA for regions of rat HGF (rHGF) (Eco RI subclone from RBC1 in pBluescript SK vector) were kindly provided by Dr T. Nakamura, Kyushu University, Fukuoka, Japan; the mouse c-met (Sphl - SacI subclone into Bluescribe), was kindly provided by Dr Laki Buluwela, Charing Cross & Westminster Medical School, London, UK; and the rat β-actin control was produced and provided by Dr Rebecca Chinery, ICRF Histopathology Unit, London, UK. These plasmids were used to produce antisense riboprobes internally labeled with approximately 800 Ci/mmol of (24) serum.

The rHGF probe of approximately 1.4 kb was generated from Eco RV restriction enzyme linearised plasmid; mouse c-met probe of 0.79 kb was generated from HindIII linearised plasmid, and rat β-actin probe of 0.24 kb was generated from Eco RI restriction enzyme linearised plasmid. The probes were transcribed using T3 (rHGF and β-actin) or T7 (c-met) RNA polymerases (Promega). In vitro transcription using 0.5 mg
Figure 1. Densitometric analysis for HGF mRNA normalized using β-actin in (A) livers, (B) endothelial cells and (C) Kupffer cells in the different treatment groups. Ethanol administration led to significant increases (p<0.01) in HGF mRNA in the liver, endothelial cells and Kupffer cells in the corn oil and fish oil groups but not in the MCT group. HGF mRNA was not detected in hepatocytes. *p<0.05 vs. MCTD, MCTE, CD; *p<0.05 vs. all other groups.

Figure 2. Densitometric analysis for c-met mRNA normalized using β-actin in (A) liver and (B) hepatocytes in the different treatment groups. Ethanol administration led to significant increases (p<0.01) in c-met mRNA in the liver and hepatocytes in the corn oil-ethanol and fish oil-ethanol group. c-met mRNA was not detected in Kupffer and endothelial cells. *p<0.01 vs. dextrose fed groups and MCTE.

Statistical analysis. All data are expressed as means ± SD unless stated otherwise. Differences between the groups were analyzed using analysis of variance with post hoc analysis using the Bonferroni test.

Results

Weight gain, blood alcohol levels and histopathology. All of the rats gained weight over the experimental period and no significant difference in weight gain was detected between the ethanol-treated and control groups, nor was there any significant difference in the mean blood alcohol levels in the ethanol treated rats (Table I).

As previously observed (19), no pathological changes were seen in either the dextrose-fed control groups (MCTD, FD and CD) or in those animals receiving medium chain triglycerides and ethanol (MCTE). Injury was detected in corn oil and fish oil, ethanol-fed animals with the pathological profile being more severe in those rats which had received fish oil and ethanol (FE) (Table I).

Expression of HGF and c-met mRNAs. The relative abundance and cellular origin of HGF and c-met transcripts were examined by RT-PCR analysis of whole liver homogenates...
and purified populations of hepatocytes, endothelial cells and Kupffer cells. Fig. 1 displays the relative abundance of HGF mRNA in whole liver and demonstrates that HGF expression increased significantly only in rats fed ethanol in combination with either fish oil or corn oil. The FE diet was clearly the most significant inducer of HGF expression. Moreover, similar analyses of c-met expression showed an identical pattern with significant increases in the CE and FE groups and the largest increase was in the FE group (Fig. 2).

These observations in whole liver are further supported by the RT-PCR results pertaining to the purified cell populations (Figs. 1 and 2), which clearly demonstrate that HGF is expressed by the endothelial and Kupffer cells. As was seen with whole liver, the level of expression in both cell types was highest in the FE treated livers. Expression of c-met on the other hand was confined only to hepatocytes which had been isolated from ethanol fed rats (Fig. 2). Of note is that both HGF and c-met mRNAs were detected in all of the whole liver homogenates, yet neither mRNA species was detectable in any of the purified cell populations isolated from those rats receiving the dextrose containing diets. This apparent anomaly can, however, be explained by the fact that HGF and c-met transcripts are expressed in normal liver principally by the stellate (Ito) cells (14) and cholangiocytes (15) respectively, cell types which had not been purified in this study.

In situ hybridization for HGF confirmed the results obtained by RT-PCR analysis and revealed that in animals fed ethanol in combination with either fish oil or corn oil, there was strong labeling for HGF of non-hepatocyte cells scattered in the parenchyma (Fig. 3). Higher power fields revealed that these cells were adjacent to the sinusoids, in a distribution consistent with Kupffer and sinusoidal endothelial cells, although there were identifiable unlabelled sinusoidal endothelial cells (Fig. 4).

c-met expression was only consistently detected in the same groups of animals in which HGF was demonstrable by ISH (FE and CE) (Fig. 5). Livers from animals in both these groups showed c-met labeling predominantly over hepatocytes with no enhancement of labeling of sinusoidal lining cells. There was very little background labeling. There was no labeling for c-met in the dextrose-fed groups or rats fed ethanol with medium chain triglycerides (data not shown).

Discussion

HGF was originally discovered to be a potent mitogenic factor for hepatocytes, and it is known to additionally possess a multitude of functions, inducing mitogenic, motogenic, morphogenic and tumoricidal effects upon a variety of cell types. HGF transduces these multiple biologic effects via activation of the transmembrane tyrosine kinase c-met receptor (7-9). In the liver, c-met is only expressed in hepatocytes and cholangiocytes, while HGF expression is restricted to the mesenchymal cells, primarily the stellate, Kupffer and endothelial cell populations (25,26).

The important finding in this study was that HGF gene expression was up-regulated in ethanol-fed rats, which develop liver injury. Furthermore, RT-PCR analysis of purified cell populations confirmed that the Kupffer and endothelial cells were responsible for this increased HGF expression. Although there are criticisms of PCR-based approaches to the quantitation of specific mRNAs, our results by RT-PCR are supported by in situ hybridization studies on liver tissue. The hepatocytes concomitantly up-regulated c-met expression,
suggesting the possibility that increased c-met was responsive to the increased levels of HGF.

Studies using other models of liver injury have also shown HGF expression to be up-regulated in both Kupffer and endothelial cells (27,28). For example, carbon tetrachloride administration stimulates a dramatic increase in HGF expression by endothelial cells but not in Kupffer or stellate cells (29). However, the present study showed that both endothelial and Kupffer cells (stellate cells were not isolated) up-regulated HGF expression in this model, an inconsistency
possibly related to the different time scales of the two studies. For instance, Maher sampled livers between 2 and 48 h after administration of the hepatotoxin (29), whereas our study analysed cells one month after ethanol administration. It is therefore possible that HGF expression by specific cell types after injury is sequential, and thus time-dependent, and/or is governed by the experimental model employed.

The exact mechanism(s) leading to hepatic HGF up-regulation in ethanol-fed rats is unknown, although increased expression of this growth factor is a phenomenon commonly associated with liver damage. For example, HGF expression increases dramatically in Long Evans Cinnamon rats which develop fulminant hepatitis (30), and similarly in rats after galactosamine or carbon tetrachloride poisoning where the extent of liver damage has been shown to correlate directly with the level of HGF expression (29-31). It has also been demonstrated that the production of HGF activator, a serine protease, is increased in response to tissue injury as a result of an inflammatory response. HGF activator is responsible for localized activation of HGF in injured tissue (32). The discovery that transcription of the HGF and c-met genes is promoted in stromal and epithelial cells respectively, by inflammatory cytokines such as TNF-α and IL-1, supports the notion that both HGF and its receptor are involved, at least in part, in the mediation of inflammatory responses associated with liver injury (33). TNF-α and IL-1 levels are increased during ALD in man (34,35) and TNF-α mRNA expression also is increased in the livers of rats receiving unsaturated fatty acids and ethanol (36).

In addition to its described hepatotrophic and biological activities, HGF may also have a cytoprotective role to play during a hepatotoxic insult. For example, HGF antagonizes interferon-γ-induced hepatotoxicity in cultured mouse hepatocytes (37), and an in vitro study has shown that HGF antagonizes the effects of IL-6 in inducing acute phase proteins in the liver (38). Similar effects have also been noted in vivo; a continuous supply of circulatory HGF produced by intrasplenically transplanted fibroblasts prevents carbon tetrachloride-induced liver damage in rats (39) and intravenous injections of recombinant human HGF suppress the progression of dimethylnitrosamine-induced hepatic injury and fibrosis (27). HGF also protects against liver injury caused by galactosamine and lipopolysaccharide (24,40). Although the exact mechanism for the protective effect of HGF is unknown, HGF increases glutathione in the liver (41) and also inhibits hepatocyte apoptosis (42).

The increased expression of c-met in hepatocytes of ethanol-fed rats is likely to be secondary to increased HGF production (43). HGF increases the expression of c-met which consequently amplifies the stimulus and, therefore, response of its target cells (43). Indeed, such an augmented response has also been observed in the regenerating livers of rats treated with carbon tetrachloride (44).

Overall, our results show that up-regulation of HGF and its receptor c-met, is most prominent in rats developing the most severe injury. Although the casual relationships between HGF and c-met expression and liver injury are uncertain, we can speculate however, that HGF may be acting to protect the liver from further damage (45). Additionally, HGF may accelerate the regenerative process, a view supported by others (12,46). We should, however, point out that the degree of cell regeneration was not evaluated in the present study but other investigators have shown that hepatic regeneration occurs in rats fed unsaturated fatty acids and ethanol (47). Clearly, HGF is not the only mediator of such responses, as several factors have been implicated to be of importance in alcoholic liver injury. Thus, the complexity of the multitude of interactions taking place between these molecules must be taken into account. Hepatocyte growth factor is only one of the many mediators which regulate the liver's response to alcoholic insult, and thus its interplay with the other regenerative factors is a particularly important area for continued investigation.

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