Expression of hepatocyte growth factor receptor (c-met) mRNA in primary cultures of human hepatocytes

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Abstract

Aim—To investigate the regulation of hepatocyte growth factor (HGF) receptor (c-met) gene expression in isolated primary human hepatocytes.

Methods—Primary hepatocytes were maintained in monolayer culture for up to 72 hours in serum-free medium. They were treated with growth factors and the level of HGF, c-met and reduced glyceraldehyde-phosphate dehydrogenase mRNA expression determined by northern blot analysis.

Results—Hepatocytes expressed a single 9 kilobase c-met gene transcript whilst HGF mRNA analysis was negative. Addition of HGF and epidermal growth factor, both potent mitogens for human hepatocytes, enhanced c-met mRNA expression approximately twofold within 24 hours, after which levels returned to normal. In non-growth factor treated cells, transforming growth factor- β (TGF β) had little effect upon c-met mRNA levels. However, TGF β inhibited the HGF induced increase in c-met mRNA levels.

Conclusions—These results indicate that hepatocytes which proliferate in response to HGF demonstrate levels of c-met mRNA which are subject to growth factor modulation and suggest an important means of growth regulatory control.

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Keywords: Hepatocyte growth factor, c-met receptor, human hepatocytes, growth control.

Hepatocyte growth factor (HGF) is a disulfide linked heterodimeric polypeptide consisting of a heavy α subunit of 60 kDa and a light β subunit of 30 kDa.^{1,2} Molecular cloning of human and rat HGF cDNAs have been reported.¹⁻³ HGF is a potent mitogen not only for isolated rat and human hepatocytes,⁴⁻⁶ but also for a variety of other epithelial cells including human biliary epithelial cells.^{7,8} In addition to its mitogenicity, HGF is also known to induce motility in certain cell types⁹ and to act as a tissue morphogen.¹⁰

Circulating concentrations of HGF rise following partial hepatectomy^{11,12} or administration of carbon tetrachloride in rats,¹³ and a direct correlation between circulating HGF concentrations and severity of human liver diseases has also been shown.¹⁴ Furthermore, several groups have reported some biological growth stimulatory activity in the

liver following in vivo infusion.^{15,16} HGF messenger RNA (mRNA) has been localised in the liver by in situ hybridisation predominantly in lipocytes^{17,18} but is also present in Kupffer cells¹⁹ and sinusoidal endothelial cells.²⁰ Taken together, this evidence indicates that HGF plays a fundamental role in the control of liver growth.

The HGF receptor has been identified as the c-met proto-oncogene^{21,22} encoding a 50 kDa extracellular α subunit and a transmembrane 145 kDa β subunit with an intra-cellular tyrosine kinase domain. Following partial hepatectomy, c-met receptors are rapidly downregulated (within six hours) as a result of raised circulating HGF concentrations and receptor/ligand internalisation.²³ However, during liver regeneration induced by carbon tetrachloride, c-met mRNA transcripts are elevated at six hours.²⁴ Although the c-met post-receptor signalling events have been extensively studied in many cell types,²⁵ less is known about the factors which control its expression in cells. Here, using a hepatocyte primary culture model, we have investigated the expression of c-met mRNA and its possible regulation by exogenous growth factors.

Methods

Materials used included: Collagenase A, dispase, hyaluronidase and DNAase supplied by Boehringer Mannheim. Epidermal growth factor (EGF) was purchased from Sigma, Poole, Dorset, and transforming growth factor- β (TGF β) from R & D Systems, Abingdon, Oxfordshire. Recombinant human HGF was from Dr T Ishii, Mitsubishi Kasei Corporation, Yokohama, Japan. Oligo(dT)-latex (Oligotex-dT30 super) was from Roche Pharmaceuticals, Welwyn Garden City, Herts. Rapid-Hyb buffer and Hybond-N membranes were from Amersham Life Science, Little Chalfont, Bucks. Human c-met cDNA (clone Pmet5) was obtained from the American type tissue collection and the human reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA was kindly provided by Dr S Sakiyama (NIC, Tokyo, Japan).

ISOLATION OF HUMAN HEPATOCYTES

Normal liver tissue was obtained from the paediatric liver transplantation programme. Tissue had been preserved with University of Wisconsin fluid and maintained aseptically at 4°C for 12 to 24 hours before cell isolation. Hepatocytes were isolated by enzyme perfusion as

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previously described.^{6,26} Briefly, about 200 g of tissue was perfused at 50 ml/minute, sequentially with 1000 ml $\text{Ca}^{2+}/\text{Mg}^{2+}$ free phosphate buffered saline (PBS) containing 10 mM HEPES (pH 7.4), 500 ml 0.5 mM EGTA (ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid) in PBS and another 500 ml of PBS. This was followed by 300 ml of enzyme solution (0.05% collagenase A, 0.038% hyaluronidase, 0.075% dispase, 0.005% DNAase in Hank's balanced salt solution with 5 mM CaCl_2), which was recirculated for seven to eight minutes. The tissue was then minced with scissors in 200 ml DMEM (Dulbecco's modified Eagle's medium) with 10% fetal calf serum and hand-stirred for a further 10 minutes. The cell suspension was filtered through 250 μm and 60 μm nylon meshes, washed three times in DMEM/ CaCl_2 /10% FCS by centrifugation at $50 \times g$ for five minutes, and viability and yield assessed by trypan blue exclusion and hemocytometer counting. All

solutions were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin or 50 mg/ml gentamycin.

CULTURE OF HEPATOCYTES

Hepatocytes were cultured on rat-tail collagen coated 35 mm or 100 mm tissue culture dishes ($\times 10^5$ cells/ml) in 2 ml and 15 ml DMEM with 10% FCS, respectively.^{6,26-28} After two hours, attached cells were washed twice with PBS, and were refed serum- and arginine-free Williams medium E supplemented with ornithine (0.4 mM), insulin (1 mM), hydrocortisone (5.5 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). Medium was renewed every 24 hours and growth factors added at the times indicated.

EXTRACTION OF POLY(A)⁺ mRNA AND NORTHERN BLOTTING

Cultures were washed twice with PBS at 4°C and then harvested and stored at -70°C. Total RNA was extracted by the acid-guanidinium thiocyanate-phenol-chloroform method.²⁹ Poly(A)⁺ mRNA was prepared using oligo dt-latex (Oligotex dt30 Super), samples were electrophoresed through formaldehyde-1% agarose gels and blotted onto nylon membranes by the downward alkaline transfer method.³⁰ Hybridisation was performed using a nick translated (³²P)-labelled 500 base pair *EcoRI*/*HindIII* fragment of human c-met cDNA, a 2.2 kilobase *BamHI*/*KpnI* HGF cDNA or 1.5 kilobase pair *EcoRI* fragment of human GAPDH cDNA. Hybond-N membranes were pre-hybridised in Rapid-Hyb buffer at 65°C for 30 minutes. Denatured probes were added to the pre-hybridisation buffer, mixed well and incubated for two hours at 65°C. Membranes were then washed in $2 \times \text{SSC}$, 0.1% SDS for 15 minutes at room temperature, then twice in $0.1 \times \text{SSC}$ for 15 minutes at 65°C. Hybridised membranes were autoradiographed for one to 14 days with intensifying screens at -70°C. Scanning laser densitometry was performed within the linear range of intensity of the film to quantify differences in mRNA expression. Membranes were stripped by washing in 0.1% SDS at 95°C for 30 minutes and then re-hybridised with additional probes. Each experiment was repeated with cells isolated from three separate donors. Data presented are from one representative experiment.

Results

Human hepatocytes formed sub-confluent monolayers and morphologically were indistinguishable from rat hepatocytes as previously characterised.^{6,26,27} The preparations were highly hepatocyte enriched, with >95% of cells immunostaining positively with an antibody directed against the asialoglycoprotein receptor (data not shown). Although other cell types were present, they represented no more than 2% of the population.

In previous experiments with both rat and human hepatocytes exposure of cells to growth

Figure 1 Northern blot analysis of *c-met* and *GAPDH* mRNA prepared from human hepatocytes cultured with basal medium. Poly(A)⁺ RNA (approximately 10 μg) was prepared from cells at the times indicated and membranes hybridised with *c-met* and *GAPDH* mRNA probes (A). Autoradiograms were scanned by laser densitometry and levels of *c-met* mRNA expressed as the ratio of *c-met*/*GAPDH* (B).

Figure 2 Northern blot analysis of *c-met* and GAPDH mRNA (A) and laser scanning densitometry analysis (B) in cultured human hepatocytes treated with growth factors for the times indicated. C = control (no addition); H = HGF (10 ng/ml); E = EGF (10 ng/ml); Tb = TGFβ (0.25 ng/ml).

factors during the first 24 hours failed to elicit a growth stimulatory response.^{6,31} Therefore, we cultured the cells with basal medium for 24 hours before the addition of growth factors and monitoring mRNA expression. In control cells a major 9 kilobase *c-met* transcript was detected (fig 1A), the expression of which increased slightly 72 hours after plating compared with the level of GAPDH mRNA expression (fig 1B). Using the same RNA preparations, we have been unable to detect specific HGF mRNA transcripts (data not shown).

Within 24 hours of treatment with either HGF or EGF, *c-met* mRNA expression was elevated 2 to 2.4-fold in hepatocytes (figs 2A and 2B). HGF and EGF added together had no further effect. On the other hand, TGFβ treated cells demonstrated similar or slightly decreased *c-met* mRNA levels compared with controls. The growth factor induced elevation of *c-met* mRNA expression had returned to control levels 48 hours after addition (figs 2A and 2B).

Finally, we investigated the effect of TGFβ on HGF induced *c-met* mRNA expression. In this series of experiments HGF was added to

Figure 3 Effect of TGFβ on HGF enhanced *c-met* mRNA expression in hepatocytes. HGF was added to medium 24 hours after plating and RNA prepared at the times indicated. *TGFβ was added after a further delay of 18 hours (12 and 24 hours exposure to TGFβ, respectively). Northern blot hybridisation was performed as before (A). Levels of *c-met* mRNA are expressed as the ratio of *c-met*/GAPDH and compared with controls (expressed as 100%) at each indicated time (B).

the medium at 24 hours after plating and TGFβ added after a further 18 hours. As shown in figs 3A and 3B, HGF increased *c-met* mRNA expression by 2.8, 1.6 and 1.5-fold at 18, 30 and 42 hours, respectively. Addition of TGFβ resulted in a decrease in *c-met* mRNA levels to below that of cells at 30 and 42 hours after addition of HGF (12 and 24 hours after addition of TGFβ, respectively).

Discussion

In the present study, we have sought to investigate the regulation of HGF receptor (*c-met*) expression in cultured human hepatocytes. We have successfully demonstrated a single specific *c-met* mRNA transcript (9 kilobases) in cells and that the mRNA levels appear to be upregulated by both EGF and HGF and downregulated by TGFβ. The failure to detect HGF transcripts indicates a low level of non-parenchymal cell contamination of these cultures, as the latter are responsible for HGF expression in the liver.¹⁷⁻²⁰ Thus, it seems likely that transcriptional control of *c-met* is one

means whereby hepatocytes regulate their response to HGF.

Our findings are consistent with recent observations made by Boccacchio *et al.*³² who reported a similar upregulation of c-met mRNA within four hours of HGF addition to A549 human lung adenocarcinoma cells. Interestingly, it has also been found that the rise in c-met expression is transient, with levels falling to basal after a further 16 hours³³ and that c-met was also induced by EGF (albeit rather more modestly). Although with a more rapid time course, these changes may reflect a common mechanism of regulation in the two cell types. This mechanism may not be universal, however, because c-met mRNA expression was downregulated up to eight hours after HGF addition to MDCK cells and returned to the control level at 27 hours.³⁴ This observation is perhaps not surprising as the biological responses of MDCK cells and hepatocytes to HGF are quite different. MDCK cells become more motile and disperse in culture in response to HGF⁹ in contrast to hepatocytes which proliferate.⁴⁻⁶ Thus, it is possible this reflects a different mechanism of receptor regulation.

TGF β is well recognised as a potent growth inhibitor for hepatocytes both *in vitro*^{4,30,35} and *in vivo*.³⁶ The mechanism of action, however, is not understood. The ability of TGF β to abrogate the growth factor induction of c-met in human hepatocytes suggests an interesting regulatory mechanism of growth inhibition *in vitro* which has previously not been reported in primary cultured hepatocytes. This mechanism is currently under investigation.

Clearly, it is also important to determine receptor expression at the translational level by ligand binding studies before identifying the major control step. In rat hepatocyte cultures HGF ligand binding is rapidly downregulated within two hours of HGF treatment reflecting receptor internalisation followed by recovery within a further 20 hours,³⁷ a pattern which is also seen in the regenerating liver following partial hepatectomy.²³ The recovery *in vitro* was reduced by cycloheximide suggesting that *de novo* protein synthesis was necessary.²³ The time scale of this response in rat hepatocytes is consistent with the increase in human hepatocyte c-met mRNA expression observed in the present study and therefore with the possibility that this increase may also result in enhanced synthesis of receptor protein. Using the human hepatocyte culture system, determination of c-met protein expression is currently underway. These studies offer an insight into the means whereby cells may regulate their responsiveness to growth factors.

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