Vascular Origin of a Soluble Truncated Form of the Hepatocyte Growth Factor Receptor (c-met)

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Abstract—Hepatocyte growth factor (scatter factor) is an angiogenic growth factor that binds to its cellular transmembrane receptor, c-met. Both HGF and c-met are expressed by vascular smooth muscle and endothelial cells, where HGF may exert autocrine and paracrine effects. We have found that human aortic smooth muscle cells (HASMCs) and human umbilical vein endothelial cells (HUVECs) release a soluble, truncated form of c-met. Receptor shedding was induced by treatment of the cells with phorbol 12-myristate 13-acetate (PMA) and by the ligand, HGF. Shedding was inhibited by cycloheximide, a metalloproteinase inhibitor, and protein kinase C inhibitors. The soluble form of c-met was able to bind HGF, although with reduced affinity (Kd = 10 nmol/L) compared with the membrane bound receptor. Conditioned medium containing soluble c-met inhibited the induction of Akt phosphorylation by HGF in HUVECs. The soluble truncated form of c-met was detectable in the plasma of 5 healthy volunteers. The shedding of c-met may represent a novel mechanism for regulating the mitogenic, motogenic, and morphogenic effects of hepatocyte growth factor. (Circ Res. 2002;90:46-52.)

Key Words: angiogenesis ▪ endothelium ▪ shedding ▪ c-met ▪ hepatocyte growth factor

Hepatocyte growth factor (HGF), also known as scatter factor, is a heparin-binding growth factor with pleiotropic actions.1 HGF and its receptor (c-met) have essential bonds to a 145-kDa collateral formation in the rabbit ischemic hind limb.11 A variety of cells including hepatocytes,5,6 epithelial cells,7 and paracrine or autocrine fashion. HGF has the ability to stimulate the proliferation and migration of endothelial15 and several carcinoma cell lines.8,9

HGF is a potent angiogenic growth factor, inducing neovascularization formation in a mouse Matrigel model10 and collateral formation in the rabbit ischemic hind limb.11 Because HGF and its receptor, c-met, are both expressed in endothelial and smooth muscle cells,12–14 HGF may act in a paracrine or autocrine fashion. HGF has the ability to stimulate the proliferation and migration of endothelial15 and smooth muscle cells,16 indicating that the vascular wall is an important target for this growth factor.

The receptor for HGF, the c-met protooncogene,17 is a transmembrane protein that is derived from a 170-kDa precursor. After processing by furin,18 the mature c-met is composed of a 50-kDa α subunit that is linked by 2 disulfide bonds to a 145-kDa β subunit.19 The α subunit is extracellular and heavily glycosylated, whereas the β subunit consists of an extracellular portion to which HGF binds, a membrane spanning domain, and a cytoplasmic tyrosine kinase domain.20,21

A variety of integral membrane receptors, including c-met, can be released from the lipid bilayer by proteolysis to form soluble, truncated proteins.22–24 The proteases that generate soluble forms of membrane proteins, membrane protein secretases, are predominantly metalloproteinases or serine proteinases.23 The soluble receptors are smaller, consisting of the extracellular region of the membrane-bound receptor and, in general, are able to bind ligand, although sometimes with reduced affinity.22

In the present study, we have shown that human aortic smooth muscle cells (HASMCs) and human umbilical vein endothelial cells (HUVECs) were able to shed a soluble form of c-met after stimulation by phorbol esters and by HGF. We further demonstrate that the generation of soluble receptors by both HGF and phorbol ester is dependent on metalloproteinase and protein kinase C (PKC) activities. We also demonstrate that soluble c-met can be detected in normal plasma, possibly as a result of shedding from the vessel wall. Soluble c-met may contribute to the tight regulation of angiogenesis and other processes controlled by HGF.

Materials and Methods

Reagents, Antibodies, and Cell Lines

Protease inhibitor cocktail and PKC inhibitors (calphostin C and the bisindolylmaleimide I HCl) were purchased from Calbiochem. Bisindolylmaleimide I inhibits multiple PKC subtypes (α, βI, βII, γ) with an IC50 of 10 to 20 nmol/L.25 Calphostin C is a specific PKC inhibitor with an IC50 of 50 nmol/L.26 Solutions of Calphostin C were made in tissue culture grade dimethylsulfoxide and bisindolylmaleimide in sterile water. HGF, protease inhibitor cocktail, goat anti-
human HGF, cycloheximide, and the alkaline phosphatase-conjugated antibodies (anti-goat and anti-rabbit IgG) were purchased from Sigma (St Louis, Mo). Peroxidase conjugated antibodies (anti-mouse IgG and anti-rabbit IgG) were from Amersham. Rabbit anti-human HGF was from Santa Cruz Biotechnology. An antibody to phospho-Akt (Ser473) was purchased from Cell Signaling Technology. Phorbol 12-myristate 13-acetate (PMA) was from Fisher Scientific. Anti-human met, extra cellular region (mouse monoclonal IgG, Clone DL-21), was purchased from Upstate Biotechnology (Lake Placid, NY). HASMCs, HASMC medium (SmGM-2), HUVECs, and the HUVEC medium EGM-2 were purchased from Clonetics Corporation (Walkersville, Md). A431 (epidermoid carcinoma cells) and LoVo cells (human colorectal adenocarcinoma) were obtained from American Type Culture Collection (Manassas, Va). Centricron-10 concentrators were from Amicon. Alkaline phosphatase activity was detected using p-nitrophenylphosphate with diethanolamine buffer from BioRad. The hydroxamic acid–based metalloproteinase inhibitor BB-3103 was a gift from British Biotech Pharmaceuticals, LTD, Oxford, UK.

Cell Culture

HASMCs were maintained in SmGM-2 medium containing growth factors, 5% FBS, and 50 μg/mL gentamicin. HUVECs were maintained in EGM-2 medium containing growth factors, bovine brain extract (12 μg/mL), 2% FBS, 50 μg/mL gentamicin, and 50 μg/mL of amphotericin. A431 (human epidermoid carcinoma cells) were grown in modified DMEM medium containing 10% FBS. LoVo cells were grown in Ham’s F12 medium with 10% FBS.

Treatment of Cells

HASMCs were seeded in 100-mm tissue culture plates and were grown in SmGM-2 complete medium to 90% confluence, then incubated overnight in SmBM serum-free medium. The cells were then washed 3 times with PBS and once with serum-free SmBM medium, then incubated for the indicated times at 37°C in 10 mL of serum-free medium containing the defined concentrations of PMA or HGF, with or without protein kinase C inhibitors. HUVECs, A431, and LoVo cells were treated identically, except that the serum-free media were EBM-2, DMEM and Ham’s F-12, respectively. To examine the requirement for protein synthesis in shedding, cycloheximide was added at 10 μg/mL. To determine the role of metalloproteinase activity in the shedding of c-met, BB-3103 (10 to 50 μmol/L) was added. With assays examining the effect of calphostin C, DMSO at a final concentration of 0.03% was added to the control cells to exclude an effect of solvent on the generation of primary antibody (0.5 to 1 μg/mL) in 5% nonfat milk in TBST. The membranes were washed twice in TBST and incubated in secondary antibody labeled with horseradish peroxidase for 1 hour at room temperature, then washed twice in TBST for 10 minutes and developed for enhanced chemiluminescence (Super Signal, Pierce).

Reverse Transcription-Polymerase Chain Reaction

Total RNA of human aortic smooth muscle cells (passage 5) was isolated using TrizOL reagent (Gibco/BRL), following the manufacturer’s instructions. The first strand synthesis was performed with 1 μg of total RNA and cDNA specific antisense primers using cDNA cycle kit (Invitrogen). The cDNA was amplified using Taq polymerase in a Perkin Elmer DNA thermal cycler for 30 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 1 minute), and extension (72°C, 2 minutes). The sense and antisense primers used were P1 (sense): 5’-TCCATAAAACTCTGGGATTGCAT-3’ (nucleotides 1035 to 1055); P2 (antisense): 5’-ACCATTCTCTGTAGTTGGCGTT-3’ (nucleotides 3229 to 3209). Primers P1 and P2 amplify a 2194-bp product encoding amino acids 283 to 1013, which includes the transmembrane domain (residues Gly 951-Leu 973). A second primer pair was P3 (sense): 5’-ATGACCTCGTGAATCATGAGGACT-3’ (nucleotides 2935 to 2962) and P4 (antisense): 5’-AGTACTGACATGTATGTCGACAGGA-3’ (nucleotides: 4425 to 4393). Primers P3 and P4 amplify a 1490-bp fragment containing amino acid residues 917 to 1408, the stop codon, and 10 bases into the 3’ UTR. A third primer set was P5 (sense): 5’-GGAATAGGGGCAGCTCGAAT-3’ (nucleotides: 2806 to 2777). Primers P5 and P6 amplify a 806-bp product including amino acid residues 606 to 872 that does not include the transmembrane domain. The polymerase chain reaction (PCR) products were TA-cloned into the PCR 2.1 vector (Invitrogen) and sequenced.

Enzyme-Linked Immunosorbent Assay

Confluent A-431 cells were incubated with or without 300 μmol/L suramin in serum-free DMEM. The medium was combined with protease inhibitor cocktail and 0.02% sodium azide and concentrated 10-fold using Centricron-10. Suramin-treated media was dialyzed in dialysis tubing (molecular weight cutoff=12 000) overnight against TBS. Concentrated medium containing soluble c-met (total protein concentration ~4 mg/mL) or Voller’s buffer (15 mmol/L Na2CO3, 35 mmol/L NaHCO3, 0.2% NaN3, pH 9.6) were added to each well of a 96-well EIA/RIA plate (100 μL) and allowed to adsorb overnight at 4°C. The plates were washed 3 times with PBS between each step. PBS containing 1% nonfat dry milk was added for 1 hour at room temperature to block the plates. For a sandwich ELISA, 96-well EIA/RIA plates were incubated with a monoclonal antibody to the extracellular β-chain of c-met (DL-21) in Voller’s buffer. The plates were blocked and concentrated medium containing soluble c-met receptor was added. Hepatocyte growth factor in PBS/1% milk was incubated on the plate for 1 hour at room temperature. The primary polyclonal antibody (anti-HGF) at 1:250 dilution was incubated for 1 hour at room temperature. Alkaline phosphatase-conjugated secondary antibody (anti-goat IgG; 1:250) was incubated for 1 hour at room temperature. Alkaline phosphatase activity was detected in 1 mol/L diethanolamine (pH 9.8), 0.5 mmol/L MgCl2, and 12 mmol/L p-nitrophenyl phosphate. Plates were read at OD405 in an ELISA plate reader. The Vmax was determined as the maximum rate of change using SOFT-MAX Pro software (version 2.1.1). Each experiment was performed at n=3.

Effect of Conditioned Medium on Akt Phosphorylation by HGF

HUVECs were cultured in serum free medium for 16 hours. The medium was harvested, centrifuged to remove cell debris, concentrated 10-fold, and immunoblotted to verify the presence of soluble c-met. Separate cultures of serum-starved HUVECs in 4 mL total volume were treated with HGF, 1 ng/mL, in the presence or absence
of varying concentrations of CM. After 5 minutes, cell lysates were prepared and immunoblotting for phospho-Akt was performed.

Results

The cell membrane form of c-met was identical in HASMCs, HUVECs, and A431 cells. LoVo cells displayed an unprocessed (190 kDa) form of membrane c-met not seen in the other cells (data not shown). In order to detect soluble c-met, immunoblotting was performed using an antibody to the extracellular domain of c-met. Figure 1A shows that concentrated medium from A431 cells, HUVECs, HASMCs (after PMA stimulation, see next paragraph) and LoVo cells all had a 135-kDa band under nonreducing conditions. This size is consistent with the disulfide-linked α chain and the truncated 85-kDa β chain of c-met. Under reducing conditions, an 85-kDa fragment was recognized for all cells (Figure 1B). LoVo cells displayed a 135-kDa band even under reducing conditions (Figure 1B, lane 1).

PMA induced the secretion of c-met from HUVECs and HASMCs in a concentration-dependent fashion, with maximum effect at 200 to 300 nmol/L (Figure 2; HUVEC data not shown). When a time course analysis was performed, the induction of soluble c-met was not visualized until the cells were exposed to PMA for 8 to 16 hours (Figure 3; HUVEC data not shown). Cycloheximide (10 µg/mL) completely inhibited PMA-induced shedding, indicating that new protein synthesis is required in this process (data not shown). PMA had no effect on the size or quantity of the membrane-bound form of c-met in whole cell lysates of HASMCs and HUVECs (data not shown). We tested the ability of the PKC inhibitor calphostin C to reverse the induction of soluble c-met by PMA. As demonstrated in Figures 4A and 4B, Calphostin C and bisindolylmaleimide I HCl were able to completely reverse the PMA effect, confirming that PKC activity is involved.

Reverse transcription (RT)-PCR was performed using primer sets P1-P2, P3-P4, and P5-P6. Only a single band of the expected size was seen by RT-PCR using total RNA from HASMCs, and each PCR product was a perfect match with the published sequence (data not shown). After treatment of HASMCs with PMA (150 nmol/L for 16 hours), RT-PCR

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)

![Figure 4](image4.png)
was repeated demonstrating no difference from the untreated cells. The metalloproteinase inhibitor BB-3103 (at 10 μmol/L concentration) was able to completely inhibit the shedding of c-met induced by PMA in both HASMCs and HUVECs (Figures 5A and 5B). Therefore, the soluble form of c-met occurs through proteolytic processing (rather than alternative splicing).

We found that HGF stimulated the shedding of c-met in a dose-dependent manner (Figure 6A). To determine whether HGF induction is dependent on PKC activity, calphostin C was added to HGF in some experiments. As shown in Figure 6B, calphostin C at 125 nmol/L was able to completely inhibit the induction of soluble c-met shedding by HGF. HGF-induced shedding was also inhibited by 10 μmol/L BB-3103 (data not shown).

To determine whether HGF binds to the soluble, truncated receptor, concentrated, serum-free medium was treated with suramin, an inhibitor of the HGF/c-met interaction.28 Suramin-treated medium or Voller’s buffer were adsorbed to an EIA/RIA plate (Figure 7A). Increasing concentrations of HGF (0 to 40 nmol/L) were then added, and HGF was detected with a polyclonal antibody, followed by an alkaline phosphatase-conjugated anti-goat IgG. The OD405 was monitored using an ELISA plate reader, and the V_max was used to...
quantify the amount of HGF bound to soluble c-met. HGF binding was detected with a \( K_d \) of approximately 10 nmol/L with saturation achieved at around 20 nmol/L.

The specificity of the binding of HGF to c-met was verified with the sandwich ELISA (Figure 7A, dashed line), using a monoclonal antibody to c-met attached to the plate, followed by the addition of concentrated soluble c-met receptor. The specificity of the interaction was further verified by demonstrating that suramin blocks HGF binding to c-met with an \( IC_{50} \) of \( \approx 50 \mu \text{M} \) (not shown).

HGF induced the phosphorylation of Akt (Figure 7B), as has been previously shown.\(^{29}\) Conditioned medium containing soluble c-met inhibited HGF-induced Akt phosphorylation (Figure 7B).

Plasma from 5 normal donors was separated by SDSPAGE and immunoblotted using the antibody to the c-met \( \beta \)-chain. As shown in Figure 8, we found that all 5 donors had an 85-kDa band of identical size to that from the A431 medium.

**Discussion**

In this work, we have shown that endothelial cells and human aortic smooth muscle cells in culture produce a soluble form of the hepatocyte growth factor receptor. To our knowledge, this is the first demonstration of the shedding of c-met by these vascular cells. Prat et al.\(^{27}\) showed that the human gastric carcinoma cell line GTL-16, which over-expresses c-met, secretes a soluble form of the receptor (75-kDa \( \beta \) chain and 50-kDa \( \alpha \) chain). The production of the soluble form of the receptor was increased by treatment with tetracosanoyl phorbol acetate (TPA). Galvani et al.\(^{27}\) confirmed that GTL-16 cells shed c-met, with the estimated molecular weights of 83 kDa (\( \beta \) chain, reducing conditions) and approximately 133 kDa (nonreducing conditions), which are similar to our estimates. Furthermore, this group demonstrated that 2 other carcinoma cells lines, that do not overexpress c-met (A549 and A431), also shed c-met with identical size. However, neither group looked at the shedding of c-met by vascular cells or the effect of HGF ligand on shedding.

We found that PMA stimulated the production of soluble c-met in HASMCs and HUVECs culture. The activity of nearly all known secretases can be enhanced by treatment of the cells with phorbol esters.\(^{23}\) The mechanism for this augmentation is unknown, however, and could possibly be due to an increase in production or activity of the secretase or enhanced colocalization of the substrate with the secretase. Alternatively, PMA could result in induction of the membrane bound receptor, the substrate for the secretase, but we found no evidence for this mechanism.

The shedding of soluble c-met was induced by the ligand, hepatocyte growth factor. There are several examples of ligands inducing the shedding of receptors, such as the Fc receptor for IgA\(^{31}\) and the \( \phi 80 \) receptor for TNF\( \alpha \).\(^{32}\) The induction of shedding by HGF, like that of phorbol esters, was inhibited by protein kinase C inhibitors. This result suggests that the binding of HGF to c-met does not simply induce a conformational change in the receptor that makes it more accessible to the secretase; rather, intracellular PKC-dependent signaling pathways are involved.

The shedding of c-met induced by either PMA or HGF could be inhibited by the zinc-dependent metalloproteinase inhibitor BB-3103. BB-3103 has previously been used to block receptor shedding.\(^{33}\) This finding is consistent with previous studies demonstrating that the sheddases or secretases that cleave membrane proteins are often metalloproteinases.\(^{33}\) Currently, it is unknown whether cycloheximide blocks the synthesis of the c-met secretase or some other protein that is essential for the shedding process.

We also found that the shedding of c-met was not dependent on the complete processing of the receptor by furin. c-met consists of \( \alpha \) and \( \beta \) chain that are derived from a single chain precursor by proteolytic processing by furin.\(^{18}\) LoVo cells, derived from human colon carcinoma, have 2 distinct mutant furin alleles\(^{34,35}\) and lack furin-catalyzed processing activity for a variety of substrates.\(^{35}\) Despite the lack of furin activity, LoVo cells demonstrate partial processing of c-met with conversion of the uncleaved 190-kDa receptor to the 145-kDa \( \beta \) chain and the 50-kDa \( \alpha \) chain (data not shown).\(^{36}\) This incomplete processing may be due to partial residual furin activity or to other processing enzymes such as PACE 4 and PC6 that are expressed in many tissues.\(^{37}\) We found that LoVo cells shed both the processed and the unprocessed forms of the receptor, producing products of 135 kDa (50-kDa \( \alpha \) chain plus truncated 85-kDa \( \beta \) chain) and 85 kDa (truncated \( \beta \) under reducing conditions. This finding indicates that processing by furin is not required for proteolysis by the membrane secretase. This finding does not eliminate the possibility that HGF is the agonist for inducing soluble c-met in LoVo cells because the unprocessed receptor in LoVo cells still binds HGF and undergoes tyrosine phosphorylation in response to the ligand.\(^{36}\)

We demonstrated that conditioned medium from HUVECs could inhibit the induction of the phosphorylation of Akt, an anti-apoptotic signal.\(^{29}\) This finding suggests that the shedding of soluble c-met could produce an antiangiogenic effect locally or at remote sites. Previous studies have demonstrated that soluble receptors can either inhibit or enhance the ligand signaling pathway.\(^{24}\) In some cases, soluble receptors can compete with membrane-bound receptors for ligand.\(^{24}\) We found that the soluble receptor has a lower affinity (\( K_d \approx 10 \) nmol/L) for HGF than the membrane bound receptor (\( K_d \approx 24 \) to 32 pmol/L; see Higushi and Nakamura\(^{38}\)), although our studies did not account for the ability of the receptor to dimerize. It is therefore possible that a significant molar excess of soluble receptor would be required to compete for HGF ligand. Soluble receptors can also disrupt signaling by...
forming inactive heterodimers with membrane receptors. Therefore, the inducible shedding of c-met also supports an important influence of SMCs on angiogenesis. The role of shedding of c-met by VSMCs represents an important mechanism for regulating SMC migration and proliferation. Increasing evidence also supports an important influence of SMCs on angiogenesis. Therefore, the inducible shedding of c-met by SMCs may not only regulate SMC responses to HGF but also influence angiogenesis by a paracrine effect on endothelial cells.

HGF levels are elevated in a variety of disease states, such as proliferative vitreoretinal disease, solid tumors, and hypertension. Our demonstration that HGF can induce the shedding of c-met and that the soluble c-met receptor can bind to and inhibit HGF suggests that HGF may also circulate in a complex with c-met in human plasma. Assays that distinguish between free and soluble receptor-bound HGF may provide improved insight into the role of HGF in various disease states.

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References


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