Hepatocyte Growth Factor/Scatter Factor and MET Are Involved in Arterial Repair and Atherogenesis

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Several studies have shown that in the arterial wall hepatocyte growth factor/scatter factor (HGF/SF) is expressed by smooth muscle cells (SMCs) but acts on endothelial cells, not SMCs. Other studies, however, have indicated that SMCs can respond to HGF/SF. We have reinvestigated expression and activity of HGF/SF and its receptor MET in arterial SMC and endothelial cell cultures and in whole arteries after superficial or deep injury or atherogenesis. High-density cultures of SMCs produced HGF/SF but did not express MET, whereas SMCs, at the leading edge of injured cultures, expressed both ligand and receptor and showed a dramatic motility and growth response to HGF/SF. In line with these results, HGF/SF and MET expression was undetectable in the media of uninjured carotid arteries but was induced after deep arterial injury in areas of SMC migration in the neointima. Strong MET expression was also observed in the SMCs of the atherosclerotic lesions of homozygous apoE−/− mice, whereas HGF/SF was expressed by macrophage-derived foam cells. These results demonstrate that MET is induced in migrating and proliferating SMCs and that HGF/SF and MET are key mediators of the SMC response in atherogenesis. (Am J Pathol 2006, 168:340–348; DOI: 10.2353/ajpath.2006.050379)
but also leading to divergent conclusions. Early studies by Rosen and colleagues established that SMC cultures produce HGF/SF and that endothelial cells (ECs) respond to the factor. Subsequent studies revealed a major angiogenic activity for HGF/SF although failing to detect MET expression and/or a response to HGF/SF in SMCs. These studies led to the view that, in the arterial wall, SMC-derived HGF/SF acts as a paracrine effector of ECs. Other studies, however, have shown that ECs can also produce HGF/SF, that SMCs can respond to HGF/SF, and that intimal SMCs express HGF/SF and MET in rabbit carotid arteries after a balloon injury.

Why do different studies report such a variable response of SMCs to HGF/SF? We have readdressed this problem by hypothesizing that the response of SMCs to HGF/SF may depend on the phenotypic state of these cells, i.e., quiescent versus migratory. We show that, while confluent cultures of SMCs exhibit little or no MET expression, in agreement with earlier reports, sparse or injured cultures express the receptor and show a marked growth and motility response to HGF/SF. We next demonstrate that a transition from a MET- to a MET+ phenotype also occurs in SMCs in vivo during the recovery from experimental balloon injuries and during migration of SMCs from the media to the neointima. Lastly, we demonstrate that MET is highly expressed in SMCs surrounding the atherosclerotic lesions of apoE mice while the ligand is also expressed in the center of the lesion by monocyte-derived foam cells. Together the in vitro and in vivo data establish that HGF/SF and MET are involved in multiple key pathways leading to arterial remodeling and atherogenesis. They also provide a rational framework for the seemingly conflicting data previously reported.

**Materials and Methods**

**Mouse and Human HGF/SF**

A full-length cDNA encoding mouse HGF/SF was cloned from a ras-transformed 3T3 fibroblast line (D4) and subcloned into the expression vector pA71. Mouse HGF/SF was purified from culture supernatants using a combination of heparin-Sepharose affinity and Mono S chromatography. The human recombinant HGF/SF used in this study was a kind gift from R. Schwall (Genentech, San Francisco, CA).

**Antibodies**

A neutralizing polyclonal anti-mouse HGF/SF antibody (1W66) was raised in sheep using a recombinant mouse HGF/SF protein, and antibodies were purified by ion exchange chromatography. The biological activity of 1W66 was confirmed by its inhibitory effect on HGF/SF-induced dispersion of MDCK colonies. Anti-mouse-MET polyclonal antibody was purchased from Santa Cruz Biotechnology (sc-162; Santa Cruz, CA).

**Western Blots**

For Western blots of conditioned media, rat SMCs (rSMCs) were grown to confluence in Dulbecco’s modified Eagle’s medium (DMEM)/5% fetal calf serum (FCS), washed twice in warm phosphate-buffered saline (PBS), and incubated for 3 days in 50 ml of serum-free DMEM. The conditioned medium was collected, added to phenylmethyl sulfonl fluoride and N-methylmaleimide (1 mmol/L each), and centrifuged at 1500 x g for 10 minutes at 4°C. It was then filtered and stored at −20°C. Samples were partially purified by heparin-Sepharose chromatography before sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For arterial extracts, rats were culled by suffocation in a rising concentration of carbon dioxide and perfused via the abdominal aorta with ice-cold Krebs buffer supplemented with 1 mmol/L phenylmethyl sulfonl fluoride and 1 mmol/L N-methyl maleimide. Carotid arteries were dissected free of connective tissue, checked to be free of all blood, and snap-frozen in liquid nitrogen. Arteries were ground under liquid nitrogen using a mortar and pestle, suspended in 100 ml of gel loading buffer, and centrifuged at 13,000 rpm in a microfuge for 5 minutes to pellet and remove cell fragments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 12.5% gels at 200 V. Gels were washed in transfer buffer (20% methanol, 192 mmol/L glycine, 25 mmol/L Tris, and 0.1 g/L sodium dodecyl sulfate) for 5 minutes, and proteins were transferred onto nitrocellulose membranes at 500 mA for 1 hour in a cooled blotting tank. Membranes were washed in Tris-buffered saline for 5 minutes and incubated in 50 g/L nonfat dried milk in Tris-buffered saline overnight at 4°C. Blots were next incubated with 1W66 prepared in 25 g/L nonfat dried milk in 1 g/L Tween 20 in Tris-buffered saline for 1 hour at room temperature, washed 3 x 10 minutes, and incubated with anti-sheep IgG horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature before addition of chemiluminescent horseradish peroxidase substrate (ECL substrate, RPN-2106; Amersham, Buckinghamshire, UK) for 1 minute and exposure to film.

**Cell Assays**

Primary cultures of rSMCs were prepared by outgrowth from aortic explants, based on the method of McMurray and colleagues. Passaged cells were maintained in cultured media with 10% FCS and characterized by hills and valley morphology and α-smooth muscle actin immunofluorescence and used between passages 3 to 14. Primary cultures of human SMCs were a kind gift from D. Proudfoot (Department of Medicine, University of Cambridge, Cambridge, UK); they were prepared from medial explants of nondiseased sections of aorta using the same procedure used for rSMCs and used within passage 12. Primary cultures of rat ECs were a kind gift from H. Lauder (Department of Pharmacology, University of Cambridge); they were prepared by outgrowth from aortic...
rings and used within passage 10. All cultures were tested for and found to be free of mycoplasma.

For scrape injury of SMC or EC cultures, cells were plated at 12,000 cells per well and 8000 cells per well, respectively, onto 96-well plates in 10% FCS in DMEM and grown to confluence. Cultures were then washed in PBS and incubated with 200 ml per well of 0.05% FCS in DMEM for another 48 hours to induce quiescence. A section of cells (30 to 50% of the total cell area) was then scraped from each well using a plastic scraper adapted from the plunger of an Eppendorf AG (Hamburg, FRG) multidispenser pipette tip. The cells were washed twice in PBS at 37°C and incubated in test media diluted in 1 mg/ml bovine serum albumin in DMEM for 24 hours. Cell numbers were measured at the end of the assay using a hemocytometer.

SMC directed migration was measured in a Boyden chamber using the method of Bornfeldt and colleagues50 with PDGF as a positive control. Polyvinylpyrrolidone-free filters of 8-mm pore size (10474; Poretics, Livermore, CA) were coated with fibronectin (for rSMCs) or collagen (for human SMCs) to promote cell adhesion. Cells were harvested with trypsin and ethylenediamine tetraacetic acid, washed with 1 mg/ml bovine serum albumin in DMEM, and resuspended at a concentration of 120,000 cells/ml in 1 mg/ml bovine serum albumin in DMEM. The bottom wells of the Boyden chamber were filled with 0.5 to 500 ng/ml HGF/SF, 10 nmol/L PDGF, or DMEM/1 mg/ml bovine serum albumin as a negative control. The chamber was assembled with a filter and cells were added to the top wells. Chambers were incubated at 37°C for 6 hours, before migrated cells were visualized using a DiffQuick staining system (Gamidor Ltd., Abingdon, Oxfordshire, UK) and counted under the microscope.

**Immunofluorescence and Immunohistochemistry**

Subconfluent cells plated on fibronectin-coated glass slides were fixed in ice-cold acetone for 5 minutes, air-dried, and washed in PBS before blocking nonspecific binding for 15 minutes in Protein Block (X0909; DAKO, Carpinteria, CA). Cells were incubated in 50 µg/ml 1W66 or X µg/ml anti-MET antibody for 1 hour at 37°C, washed in PBS, and incubated for another hour at 37°C in antishift or anti-rabbit fluorescein isothiocyanate isothiocyanate-conjugated secondary antibody, respectively. Cells were counterstained with propidium iodide and aqueously-mounted before examination with appropriate filters using a fluorescence microscope. Specificity of staining with the 1W66 antibody was confirmed by assessing its reactivity with cell lines known to express (or fail to express). For immunofluorescence of tissue sections, arteries were snap-frozen, mounted in OCT, and 10-mm sections were cut using a cryostat. Sections were fixed in acetone at −20°C for 10 minutes, air-dried, and processed as above.

**In Vivo Injury Models**

For balloon injury, 300-g rats were anesthetized and a 2-cm longitudinal midline incision was made through the skin. The left external carotid artery was located using blunt dissection and ligated distally, while proximally the artery was held with an untied ligature. A small hole was made in the vessel and a 2-French Fogarty balloon catheter inserted into the vessel and down to the aortic arch. Balloon injury of the common carotid artery was performed by three passes of the partially inflated balloon before the proximal ligature on the external carotid artery was ligated and the skin wound sutured.

For defined filament injury, rats were anesthetized and the external carotid artery located as described for balloon injury; however, in this case a 0.25-mm filament with a curved end, and housed retracted in a section of PE10 tubing, was inserted into the vessel. Once the end of the injury device had reached the aortic arch, the curved end of the filament was pushed out and the device pulled out of the vessel, with the aim of causing a single track of endothelial denudation approximately one third of the width of the opened out vessel, along the length of the common carotid artery. The external carotid artery was then tied off. The rat was then placed on its stomach and an area of the shoulder blades was shaved and swabbed before a 2-cm skin incision was made. Blunt dissection was used to create a skin pocket large enough to insert a 2-ml osmotic minipump filled with 3 mg/ml of human HGF/SF (hHGF/SF) in 20 mmol/L Tris-HCl and 0.5 mol/L NaCl, pH 7.6, buffer or buffer alone. The dosing schedule for hHGF/SF was determined in pilot studies. The tubing from the minipump was passed under the skin and cannulated into the right jugular vein and all skin wounds were sutured. Seventeen hours before termination, rats were dosed with a bromodeoxyuridine (BrdU) tablet. Rats were killed by suffocation in a rising concentration of carbon dioxide, perfused with Krebs buffer via the abdominal aorta, and the carotid arteries gently removed by dissection. Arteries were opened longitudinally, and pinned luminal side up for end face immunohistochemical staining for BrdU expression with diaminobenzidine substrate.

**ApoE−/− Mice**

The ApoE−/− mice were a kind gift of C. Long (Pfizer Central Research, Sandwich, UK) via Charles River Laboratories (Wilmington, MA). The mice were rederived onto a 129ola x C57BL/6 x BALB/c background and genotyped by Charles River. Mice were weaned at the age of 4 weeks, fed a normal chow diet for 1 week before being given a high-fat diet as described by Nakashima and colleagues.51 For histology and histochemistry, arteries that had been perfusion-fixed (40 g/L phosphate-buffered paraformaldehyde) were cut transversely into three to four pieces, fixed overnight, washed in saline, and paraffin-embedded. Sections (5 µm) were cut and mounted onto Vectabond (SP1800; Vector, Burlingame, CA). For HGF/SF and MET immunohistochemistry, paraf-
fin-embedded sections were stained with primary antibodies as described above followed by biotinylated secondary antibodies and avidin-biotin reagent (SC-2018; Santa Cruz Biotechnology) before diaminobenzidine substrate. In addition to staining for HGF/SF and MET, sections were also stained for \( \alpha \)-H9251-smooth muscle actin and for the macrophage marker CD68.

Results

Expression of HGF/SF and MET in SMCs and ECs

Early passage cultures of rat aortic ECs, SMCs, and adventitial fibroblasts were studied initially for expression of HGF/SF by immunofluorescence, Western blot, and biological activity on MDCK colonies, and for MET by immunofluorescence. Adventitial fibroblasts expressed HGF/SF and did not respond to the factor. The behavior of these cells, therefore, appeared to be typical of fibroblast isolates from other tissues\(^{13}\) and was not investigated further.

Figure 1, a and d, shows the appearance of typical EC and SMC cultures from rat aorta. A number of ECs expressed HGF/SF, as shown by green fluorescence with the 1W66 anti-mouse HGF/SF polyclonal antibody (Figure 1b) and secretion of MDCK colony-dispersing activity (data not shown). Expression was not homogenous throughout the cell population, with some cells showing more intense fluorescence than others (Figure 1b). As expected, ECs expressed the MET receptor (Figure 1c). SMCs showed strong expression of HGF/SF (Figure 1e), and the SMC-derived factor was indistinguishable from the factor originally isolated from a 3T3 line.\(^{14}\) g and h: Expression of MET in SMCs. Confluent cultures of SMCs do not express MET (g) but SMCs migrating at the edge of a scrape injury (as shown in Figure 2) show clear, positive staining for MET (h). i: MET-expressing cells from SMC cultures are positive for \( \alpha \)-smooth muscle actin. Immunofluorescence of HGF/SF or MET is demonstrated by green fluorescence with nuclear counterstain in red.

HGF/SF Causes Migration and Growth of SMCs

To clarify the functional role, if any, of MET expression in SMCs, a series of experiments was performed in which the response of these cells to HGF/SF was assessed in terms of wound recovery (Figure 2, a–d), growth (Figure 2e), and motility stimulation on individual cells (Figure 2, f–h). Figure 2a shows a control culture in basal medium 24 hours after wounding. The culture shows little regrowth in the free area compared to parallel cultures in 5% FCS (Figure 2b). HGF/SF alone induced regrowth at a level comparable with that of 5% FCS (Figure 2c) and considerably enhanced the regrowth caused by 5% FCS (Figure 2d).
2d). HGF/SF also increased SMC growth (cell number) in scrape-injured cultures to the same level achieved by 10% FCS (Figure 2e). This effect on SMC proliferation after injury was more potent than the one induced on scrape-injured EC cultures (Figure 2i). A direct effect of HGF/SF on SMC motility was next assessed in Boyden chamber assays. HGF/SF induced a dramatic motility response (Figure 2f) that resulted primarily from stimulation of directional (chemotactic) rather than random (chemokinetic) cell movement (Figure 2g). Human SMCs also exhibited a strong motility response to HGF/SF demonstrating that the effect of HGF/SF on SMCs translates across species (Figure 2h). Thus, the MET expression observed in scrape-injured cultures (Figure 1h) induces a strong migratory (Figure 2c, d, and f–h) and growth (Figure 2d) response to HGF/SF in SMCs, raising the possibility of a much broader role of HGF/SF in arterial repair than previously postulated. This hypothesis was tested next through a series of expression and functional studies in vivo.

HGF/SF and MET in Arterial Repair in Vivo

Figure 3 shows that HGF/SF promotes re-endothelialization in the rat carotid artery after superficial, scrape injury in vivo. Electron micrographs of the en face surface of uninjured (a) and injured (b) rat carotid arteries. The scrape injury causes denudation (D) and platelet adhesion and aggregation but no damage to the underlying internal elastic lamina and media layers. c: Replicating cells (RCs) in the endothelium adjacent to the scrape injury are visualized by BrdU incorporation into DNA and shown as brown coloration under the light microscope. Nonreplicating (NR) ECs further away from the injury only show background staining. d: The en face area of the artery without ECs (labeled with NC), is compared in control and HGF/SF-treated rats 48 hours after injury and expressed as a percentage of the total en face area of each artery. Data are mean ± SEM from seven rats per group. Statistical significance is represented at \( P < 0.05 \) (*).
Injury Induces MET Expression in SMCs in Vitro and in Vivo

Although SMCs in culture express HGF/SF (Figure 1, e and f), expression by SMCs in vivo in uninjured arteries is barely detectable (Figure 3a). Thus the HGF/SF gene is one of several genes that become transcriptionally activated in SMCs during the phenotypic transition that occurs in culture. In vitro expression of HGF/SF in SMCs is not influenced by cell density or injury and appears constitutive. Expression of MET in SMCs, like HGF/SF, is undetectable in vivo in uninjured arteries (Figure 3b). However, in vitro, MET expression is remarkably regulated, being negligible in confluent cultures (Figure 1g), low in sparse cultures (data not shown), and high after scrape injury in cells at the leading edge of the wound.

Discussion

This study presents data that define new, major roles for HGF/SF and MET in vascular physiology and pathology and explain previous inconsistencies in the literature on the SMC response to HGF/SF. The experiments confirm that SMCs in culture produce HGF/SF (Figure 1, e and f), and that ECs express the MET receptor (Figure 1c) and respond to HGF/SF (Figure 2), in line with earlier reports. The study thus supports a paracrine role for SMC-derived HGF/SF on vascular ECs in normal, adult arteries but also suggests novel roles for HGF/SF and MET in SMC-dependent arterial remodeling and atherogenesis after injury.

HGF/SF and MET in Atherogenesis

Endothelial damage and migration of SMCs from the media to the neointima are features of both arterial remodeling after mechanical injury and atherogenesis. Atherosclerotic lesions, however, are characterized by additional processes, such as lipid deposition, and the presence of a third and critical cell type, namely the monocyte-derived macrophage. We analyzed the expression of HGF/SF and MET during atherogenesis in the apoE<sup>−/−</sup> mouse, a model of atherogenesis that closely resembles the development and progression of human lesions. Figure 5, a, b, and c, show typical early, intermediate, and advanced lesions in such mice and, noticeably, the presence of a typical fibrous cap and lipid core in the advanced lesion similar to those of human lesions (Figure 5c). Expression of HGF/SF in the arterial lesions of apoE<sup>−/−</sup> mice occurred in lumen-lining cells and in foam cells within the lesions (Figure 5, d and e). These foam cells expressed CD68 and lacked expression of α-smooth muscle actin (data not shown); they were therefore HGF/SF-expressing macrophage-derived foam cells. In contrast, MET expression was strong in SMCs surrounding and entering the lesions, namely in the underlying media (Figure 5f), the shoulder region and the fibrous cap (Figure 5g). In summary, in the arterial lesions of apoE<sup>−/−</sup> mice, HGF/SF is expressed predominantly in macrophage-derived foam cells in the central area of the lesions whereas MET is expressed in SMCs at the periphery of and underneath the lesion.

Migration and proliferation of SMCs was investigated next. For this, balloon injuries were produced and the expression of HGF/SF and MET in the artery was studied throughout the arterial repair reaction. Expression of HGF/SF in vivo in uninjured carotid arteries was confined to lumen-lining cells (ECs) and to certain areas of the adventitia (A) but was barely detectable in the media of uninjured arteries (Figure 4a). Fourteen days after a deep arterial injury, a thick neointima develops containing a large number of SMCs migrated from the media and undergoing mitosis and extensive synthesis of the extracellular matrix. The SMCs of the neointima showed dramatic expression of both HGF/SF (Figure 4c) and MET (Figure 4d), and a time course of HGF/SF protein indicated striking changes, namely initial conversion of pro-HGF/SF (αβ) to active, two-chain HGF/SF (α chain is shown) followed by new synthesis of pro-HGF/SF and the subsequent activation. The Western blots are representative of three separate balloon injury experiments.
Further, these MET-positive SMCs show a potent response to HGF/SF (Figure 2, c–h).

The pattern of expression of HGF/SF and MET during arterial remodeling after deep balloon injury confirmed that injury causes a dramatic increase in HGF/SF in vivo (Figure 4, c and e) and that the SMCs that migrated from the media to the neointima express MET (Figure 4d). Thus, HGF/SF appears to be critically involved in the SMC’s response to injury. The signals responsible for activation of the MET gene in SMCs after injury remain to be established, but the fact that the transition from a MET+/H11002 phenotype can be achieved in vitro in scrape-injured cultures suggests ways in which such signal(s) may be examined, for example by microarray experiments.

HGF/SF and MET in Atherogenesis

There is now broad agreement that atherosclerosis constitutes an inflammatory response of the arterial wall to chronic injury. This “response to injury” hypothesis, first proposed in the 19th century by Rudolf Virchow,52 has been strongly advocated and extended by Russell Ross1–4 throughout the last 3 decades and is now widely accepted.5 Endothelial injury is thought to be a key, and possibly initiating feature, of early lesion development.53 Whereas small areas of an endothelial injury may be repaired without disease progression,54 chronic damage to the endothelium leads to platelet adhesion and aggregation and to macrophage recruitment. This, in turn, elicits SMC migration to the intima and the development of advanced lesions.

Platelets were one of several original sources of HGF/SF,55 along with macrophages in several tissues.56,57 Interestingly, HGF/SF expression by liver macrophages is essential for a full regenerative response of hepatocytes after injury.58 We propose that a similar course of events occurs in the arterial wall where HGF/SF is expressed in macrophage-derived foam cells in atherosclerotic lesions of apoE−/− mice (Figure 5, d and e) and MET is expressed in neighboring SMCs (Figure 5, f and g). Expression of HGF/SF has also been reported in macrophages in human arterial lesions.59,60 Thus, macrophage-derived HGF/SF may act as a key chemoattractant and mitogen for SMCs in atherogenesis and platelets (Figure 3b) and/or ECs (Figure 1b) may constitute additional sources of the factor. In contrast, in the macrophage- and EC-free lesions caused by deep mechanical injury (Figure 4), platelets may constitute the primary local source of HGF/SF in the subendothelium.

The finding that SMCs are a key target of HGF/SF in arterial injury and atherogenesis has implications for the clinical use of HGF/SF in vascular disease. It is widely accepted that endothelial integrity and repair prevents lesion development while SMC migration and proliferation in the intima are typical features of advanced lesions.2 HGF/SF promotes endothelial repair (Figure 3) but appears to be equally and critically involved in the SMC response (Figures 4 and 5). We argue that endothelial repair, on the one hand, and SMC migration and proliferation, on the other, are complementary aspects and stages of the arterial response to injury. From an evolutionary view point we believe it should be no surprise that the same signaling system may be involved in both pro-
cesses. This, however, has interesting implications for both diagnosis and therapy of arterial disease.

There is probably no clinical counterpart to the localized, superficial endothelial injury illustrated in Figure 3b, whose repair is greatly enhanced by HGF/SF (Figure 3d). Chronic endothelial damage however, with or without cell loss, is probably critical for lesion formation and for SMC activation. Our data suggest that activated SMCs express MET and may migrate toward a source of HGF/SF in the endothelial or subendothelial area. We are aware of data indicating that HGF/SF reduces intimal thickening in balloon-injured rabbit arteries.61 Further studies will be required, however, to establish complete role of HGF/SF and MET in atherogenesis, and we suggest that mouse models in which the HGF/SF or MET genes are overexpressed or ablated in either the SMC or EC lineages may contribute considerably.

References

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