Hepatocyte Growth Factor Modulates Vascular Endothelial-cadherin Expression in Human Endothelial Cells

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ABSTRACT
Hepatocyte growth factor (HGF)/scatter factor (SF) is a cytokine exerting a wide range of biological functions on many cell types, including vascular endothelial cells. HGF/SF increases migration, motility, and dissociation of human umbilical vascular endothelial cells (HUVECs). This study demonstrated that such action of HGF/SF on HUVECs was achieved by regulation of the endothelial cell-specific cadherin, vascular endothelial (VE)-cadherin. HGF/SF induced a time-dependent reduction of VE-cadherin protein from HUVECs as shown by Western blotting and immunocytochemistry, accompanied by an increase in the migration of HUVECs. The change of VE-cadherin appeared at the mRNA level, as demonstrated by reverse transcription-PCR, with a decrease in the VE-cadherin signal. These results show, for the first time, that HGF/SF targets the endothelial cell-specific adhesion molecule VE-cadherin.

INTRODUCTION
HGF, otherwise known as SF, is a cytokine with numerous functions that evoke a number of diverse responses in different cells and tissues. The mature HGF/SF molecule is a heterodimer consisting of a Mr 69,000 α-chain and a Mr 34,000 β-chain (1) and becomes active after the action of serine protease. The receptor for HGF/SF is encoded by the c-met proto-oncogene. HGF/SF is secreted by fibroblasts in vivo (1) and plays a role in a number of important physiological processes including cell motility and migration, proliferation, and cell invasion. HGF/SF is able to regulate the angiogenic process and to hasten tissue repair after insult (2–4), where activation of the receptor for HGF/SF is widely published, reports on the underlying mechanisms, such as changes of endothelial-specific molecules, are limited. This study shows that HGF/SF is able to modulate the expression of the cell adhesion molecule VE-cadherin, consequently enabling stimulation of endothelial cell motility, migration, and angiogenesis.

MATERIALS AND METHODS
Reagents and Antibodies. Monoclonal mouse anti-VE-cadherin was purchased from PharMingen International. Antibody to β-catenin (rabbit) was purchased from Sigma (Poole, United Kingdom). Peroxidase-conjugated antimouse IgG for Western blotting was from Amersham International Plc (Little Chalfont, United Kingdom). Monoclonal mouse anti-HGF/SF was purchased from R&D (Abingdon, United Kingdom). HGF/SF was a generous gift from Dr. T. Nakamura (Osaka, Japan).

Cell Lines. Primary HUVECs (Clonetics, San Diego, CA) were cultured in endothelial cell basal medium (Clonetics).

Cell Motility: Colloidal Gold Assay. The colloidal gold phagokinetic assay was based on that reported previously by Albrecht-Buehler (16). BSA-coated glass coverslips were coated with colloidal gold before the addition of HUVECs. HGF/SF was then added to a final concentration of 5 ng/ml. Cells were incubated at 37°C for 24 h. The clear area resulting from phagocytosis of gold particles during migration was visualized under dark-field microscopy (Olympus) and quantified using an image analysis package (Optimas 7; Optimas UK Ltd.).

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Cell Migration Assay. HUVECs were seeded into 96-well plates and allowed to reach confluence. An area of clear cells was made using a 0.5-mm needle, and then HGF/SF was added to a final concentration of 5 ng/ml. Mineral oil was used to overlay the medium, and the cells were viewed microscopically on a heated (37°C) stage for 2 h while being recorded on videotape. Decrease in the cleared area was assessed at 5-min intervals using Optimas 6.

Endothelial Tube Formation Assay. The endothelial tube formation assay was based on that reported previously (17). Aliquots (100 μl) of 200 μg/ml Matrigel (reconstituted basement membrane; Becton Dickinson) were added to wells in a 96-well plate and gelled at 37°C. HUVECs were seeded onto the Matrigel at 10³ cell/well and allowed to attach for 2 h. The medium was aspirated, and a second layer of Matrigel was added to sandwich the cells. After gellation of the Matrigel, 50 μl of medium were added, and the cells were incubated at 37°C for 24 h. Endothelial tube formation was visualized microscopically, and the relative tube lengths were measured in millimeters.

SDS-PAGE and Western Blotting. For preparation of total cells lysate, cells were pelleted and lysed in HCMF buffer containing 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl₂, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml apro- tinin, and 10 mM sodium orthovanadate for 40 min; sample buffer was added; and the protein was boiled at 100°C for 5 min before clarification at 13,000 × g for 10 min. Equal amounts of protein from each cell sample (controls and those treated with matrix-bound fibroblasts) were added onto an 8% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min before probing with peroxidase-conjugated secondary antibody (1:2000). Protein bands were visualized with an enhanced chemiluminescence system (Amersham International Plc). The band intensities on photographic film were analyzed with densitometry software (Optimas 6.0; Optimas UK Ltd.) and shown as relative values.

For a comparison of protein levels in the Triton-soluble and -insoluble fractions of the cell lysate, protein was prepared as described above but without SDS and with an increased Triton X-100 concentration of 20%. After cell lysis for 40 min, the lysate was centrifuged at 13,000 × g for 25 min to remove the Triton-insoluble fraction. The supernatant containing the Triton-soluble fraction was then prepared as usual. The Triton-insoluble fraction was extracted with SDS lysis buffer (as described for total cell lysate) and prepared as usual. In addition, the conditioned media surrounding the treated cells were collected and prepared for protein analysis (as described for total cell lysate) to observe any VE-cadherin shed from the cell surface. PAGE and Western blotting were then carried out as described above.

RNA Extraction and RT-PCR. RNA was isolated from HUVECs using standard RNAzol procedure. For RT-PCR, cDNA was synthesized in a 25-μl reaction mixture as described in the protocol (Promega Reverse Transcription System). The cDNA obtained was amplified by a standard PCR mixture (as supplied in Pre- aliquoted Ready-Load Mix; Advanced Biotechnologies) at 1.5 mM MgCl₂ using PCR primers specific for VE-cadherin. Cycling conditions for the 25-μl reaction mixture were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This was followed by a final extension of 5 min at 72°C. The products were visualized on a 0.8% agarose gel after staining with ethidium bromide. The PCR primers used were as follows: (a) VE-cadherin (891-bp fragment), VECADF1 (ATGCAGAGGCTCATGATG) and VECADR1 (CTACGACATGAACCAGTA); and (b) β-catenin, (227-bp fragment), BCA TF1 (CGT GATTTGATGGAGTTG) and BCA TR1 (CGATGAAACAAGACTC A CT).

Immunohistochemical Microscopy. HUVECs were cultured in glass 16-well chamber slides until confluence and treated accordingly. Media were removed, and the cells were fixed with 4% formaldehyde for 5 min, followed by permeabilization with 0.05% Triton X-100 for 3 min. After washing with buffered saline solution, the cells were blocked with 10% FCS, 10% HS, and 10% skimmed milk in Tris-buffered saline for 60 min. Proteins were probed with VE-cadherin antibody (1:250) followed by peroxidase-conjugated secondary antibody (1:1000). Protein distribution was visualized with the addition of DAB, analyzed using densitometry software to evaluate the intensity of the stain, and shown as relative values (Optimas).

Statistical Analysis. Statistical analysis was performed by MINITAB version 9.2 (Minitab Inc., State College, PA) using a two-sample Student’s t test.

RESULTS

HGF/SF Modulation of VE-cadherin Protein Expression. HUVECs cultured with 5 ng/ml HGF/SF showed a decrease in VE-cadherin protein expression from total cell lysate from 1 h onward (from a relative level of 237 at 0 h to 194 at 4 h and 152 at 24 h), as shown in Fig. 1. There was no corresponding change in the level of β-catenin over the 24-h period (Fig. 2). The level of VE-cadherin shown in the Triton-insoluble (SDS-soluble) fraction of the HUVEC cell lysate showed a steady decrease after the addition of 5 ng/ml HGF/SF (from a relative level of 87 at 0 h to 30 at 24 h), as shown in Fig. 3. The VE-cadherin protein level in the Triton-soluble fraction of the cell lysate was also probed with VE-cadherin antibody, as shown in Fig. 1. HGF/SF regulates total protein expression of VE-cadherin as assessed using Western blotting. Human vascular endothelial cells were cultured with HGF/SF at 5 ng/ml for varying time points up to 24 h. A shows the blot after immunoprobing with a monoclonal VE-cadherin antibody. This is summarized graphically in B, which shows the decrease of VE-cadherin expression in total cell lysate.

Fig. 1  HGF/SF regulates total protein expression of VE-cadherin as assessed using Western blotting. Human vascular endothelial cells were cultured with HGF/SF at 5 ng/ml for varying time points up to 24 h. A shows the blot after immunoprobing with a monoclonal VE-cadherin antibody. This is summarized graphically in B, which shows the decrease of VE-cadherin expression in total cell lysate.
HGF Modulates VE-cadherin Expression

Fig. 2  HGF/SF regulates total protein expression of β-catenin as assessed using Western blotting. Human vascular endothelial cells were cultured with HGF/SF at 5 ng/ml for varying time points up to 24 h.

Fig. 3  HGF/SF regulates Triton-insoluble protein expression of VE-cadherin as assessed using Western blotting. Human vascular endothelial cells were cultured with HGF/SF at 5 ng/ml for varying time points up to 24 h. A shows the blot after immunoprobing with a monoclonal VE-cadherin antibody, which is summarized in the graph (B). VE-cadherin expression in the Triton-insoluble fraction of the cell lysate decreased over 24 h of coculture.

Fig. 4  HGF/SF regulates Triton-soluble protein expression of VE-cadherin as assessed using Western blotting. Human vascular endothelial cells were cultured with HGF/SF at 5 ng/ml for varying time points up to 24 h. A shows the blot after immunoprobing with a monoclonal VE-cadherin antibody, which is summarized in the graph (B). VE-cadherin expression in the Triton-soluble fraction showed an increase (from levels of 30 at 0 h to levels of 72 at 24 h). The trends in this data were consistent after repeated blotting. Western blotting was also carried out on supernatant from HUVECs cultured with HGF/SF at 5 ng/ml. VE-cadherin was not observed, suggesting that the molecule was not shed from the cells.

Changes in Gene Expression of VE-cadherin by HGF/SF. Levels of VE-cadherin after RT-PCR over 24 h are shown in Fig. 5. The signal for VE-cadherin decreased gradually after culture with HGF/SF at 5 ng/ml. There was little change in the signal for β-catenin over the 24-h period (data not shown).

Effect of HGF/SF on Intensity of Immunocytochemical Staining of VE-cadherin and β-Catenin. Over a 24-h culture with HGF/SF, the general level of staining of VE-cadherin increased from 0.28 ± 0.03 to 0.35 ± 0.08 (95% CI, −0.36 to −0.085; \( P = 0.0045; n = 4 \)). However, when individual time points were considered, this increased staining followed a gradual decrease in staining over a 4-h period, from 0.2 ± 0.05 at 2 h to 0.12 ± 0.06 at 4 h (data not shown). This appears to indicate a change in the distribution of VE-cadherin. Again, as with Western blotting, there was no change in the intensity of β-catenin in HUVECs cultured with 5 ng/ml HGF/SF.

Effect of HGF/SF on Capillary Tubule Formation by HUVECs. The addition of HGF/SF to the endothelial tube formation assay caused an increase in tubule formation from 0.236 ± 0.05 in the control to 0.416 ± 0.09 (95% CI, −0.37 to −0.121; \( P = 0.0017; n = 5 \)) with HGF/SF (data not shown).

HGF/SF Increased HUVEC Motility. The results for the colloidal gold motility assay were calculated as the area (in \( \text{mm}^2 \)) cleared by the endothelial cell and were 4.49 ± 1.01 \( \text{mm}^2 \) for the control and 95.58 ± 2.68 \( \text{mm}^2 \) for HGF/SF (95% CI, −101.990 to −88.70; \( P = 0.0001; n = 3 \); data not shown).

HGF/SF Increased Migration of HUVECs. The migration assay was measured as speed of closure (in \( \mu \text{m/h} \)). HUVECs and HGF/SF exhibited a speed of 20.09 ± 1.24 \( \mu \text{m/h} \) (data not shown), and the control exhibited a speed of 10.97 ± 1.03 \( \mu \text{m/h} \) (95% CI, −14.11 to −8.5; \( P = 0.0001; n = 4 \)).

DISCUSSION

This study has shown that HGF/SF modulates expression of the endothelial cell-cell adhesion molecule VE-cadherin. Human endothelial cells treated with HGF/SF showed a decrease in total protein expression of VE-cadherin. There was a change from insoluble membrane-bound VE-cadherin to soluble VE-cadherin. This was also illustrated by a change in distribution of VE-cadherin in the cell, as observed by immunocytochemistry, indicating that the protein was also functionally affected. In contrast, HGF/SF did not modulate the protein expression of the cell adhesion and signaling molecule β-catenin.

HGF/SF has been shown to regulate motility, proliferation, morphogenesis, and cell-cell adhesion in tumor cells. It has been shown to be a powerful inducer of angiogenesis in cancer cells (6) and a stimulator of endothelial cell motility and migration in vivo. HGF/SF has also been shown to disrupt the cell-cell adhesion junctions of epithelial cells by modulating catenin phosphorylation and modulating E-cadherin function (18).

VE-cadherin and cadherins in general are located predominantly in the cell-cell adherens junctions, where they mediate the physical attachment between cell membranes and the intercellular network of cytoplasmic proteins and actin microfilaments (19). In addition to their function in cell-cell adhesion, such interactions...
between endothelial cells are known to initiate cell type-specific intracellular signal cascades, resulting in diverse endothelial effects (20), such as changes in migration and morphology.

Cadherin molecules have already been shown to be of importance in both developmental and disease processes, where suspension of cadherin expression correlates with migration, and regulated cadherin expression facilitates numerous morphological events such as separation and formation of cell layers and maintenance of cell proliferation (14). Dejana et al. (21) have shown that endothelial cells migrating into a wounded area undergo a loss of catenins and VE-cadherin as the cell junctions become disorganized, enabling cell motility to be achieved. Cadherins also play a role in proliferation of cells because the formation of cell-cell contacts inhibits proliferation after the interaction of VE-cadherin with catenins that transmit adhesion signals to the cell, and cadherins contribute to the anchorage of cadherin to the actin cytoskeleton (22). This cadherin-catenin complex behaves as a switch enabling regulation of density-dependent inhibition of cell growth.

It has been suggested that VE-cadherin, when recruited to form cell-cell contacts, actively sequesters catenins from the intracellular free pool. This would then reduce the catenins available for linking signal transduction pathways (21) and thus contribute to control of cell growth and differentiation. A decrease in cadherin expression suggests a decrease in sequestration of catenins.

Endothelial cells are able to use changes in the cadherin-catenin complex to modify the architecture of their intracellular junctions, enabling the passage through the endothelium of plasma constituents and blood cells; moreover, this effect is quickly reversible, and the endothelium is able to disorganize and reorganize the junctions within minutes (19).

This study shows that HGF/SF: (a) causes a decrease in total protein expression of endothelial cell-specific VE-cadherin, as investigated using Western blotting; (b) produced a decrease in levels of the mRNA signal of VE-cadherin, as observed using RT-PCR; (c) caused a change in distribution of Triton-insoluble and -soluble fractions of VE-cadherin as shown using Western blotting, suggesting a change in the location of VE-cadherin; and (d) that the change in location of VE-cadherin was visible by a change in intensity of VE-cadherin staining after immunocytochemistry. In addition, we have demonstrated that HGF/SF increased both motility and migration of these endothelial cells. From the data presented, we conclude that HGF/SF effects the changes in endothelial cell motility and migration by decreasing the expression of VE-cadherin in these cells.

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