Stanniocalcin 1 Is an Autocrine Modulator of Endothelial Angiogenic Responses to Hepatocyte Growth Factor*

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Stanniocalcin 1 (STC1) is a secreted glycoprotein originally described as a hormone involved in calcium and phosphate homeostasis in bony fishes. We recently identified the mammalian homolog of this molecule to be highly up-regulated in an in vitro model of angiogenesis, as well as focally and intensely expressed at sites of pathological angiogenesis (e.g. tumor vasculature). In the present study, we report that STC1 is a selective modulator of hepatocyte growth factor (HGF)-induced endothelial migration and morphogenesis, but not proliferation. STC1 did not inhibit proliferative or migratory responses to vascular endothelial growth factor or basic fibroblast growth factor. The mechanism of STC1 inhibitory effects on HGF-induced endothelial migration seem to occur secondary to receptor activation because STC1 did not inhibit HGF-induced c-met receptor phosphorylation, but did block HGF-induced focal adhesion kinase activation. In the mouse femoral artery ligation model of angiogenesis, STC1 expression closely paralleled that of the endothelial marker CD31, and the peak level of STC1 expression occurred after an increase in HGF expression. We propose that STC1 may play a selective modulatory role in angiogenesis, possibly serving as a “stop signal” or stabilizing factor contributing to the maturation of newly formed blood vessels. HGF is a mesenchyme-derived pleiotropic factor with mitogenic, motogenic, and morphogenic activities on a number of different cell types. HGF effects are mediated through a specific tyrosine kinase, c-met, and aberrant HGF and c-met expression are frequently observed in a variety of tumors. Recent studies have shown HGF to be a potent growth factor implicated in wound healing, tissue regeneration, and angiogenesis.

We recently reported that HGF,1 and more potently, HGF in combination with VEGF, synergistically induced vascular morphogenesis in vitro and angiogenesis in vivo (1). In a related study, we analyzed the gene expression profile of endothelial cells undergoing HGF- and VEGF-stimulated morphogenesis using Affymetrix oligonucleotide arrays. We identified the homodimeric secreted glycoprotein, stanniocalcin-1 (STC1), as one of the most highly up-regulated genes in this in vitro model (2). We also observed intense expression of STC1 in the vasculature of colon carcinomas, providing further evidence that this novel glycoprotein might play an important role in one or more of the processes associated with angiogenesis (2).

The objective of the present study was to determine what role STC1 played in HGF-induced responses of vascular endothelial cells. MATERIALS AND METHODS

Reagents—Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and maintained in endothelial growth medium (Clonetics) supplemented with fetal bovine serum to a final concentration of 10%. Other reagents included type I rat tail collagen (Upstate Biotechnology, Lake Placid, NY), recombinant bFGF (Collaborative Biomedical Products, Bedford, MA), and recombinant VEGF and HGF (Genentech, South San Francisco, CA). All other cell culture reagents were obtained from Invitrogen.

Isolation of STC1 and Construction of Expression Vectors—cDNA clones were isolated from a human endothelial cDNA library and sequenced in their entirety. Fc fusion proteins (immunoadhesins) were prepared by fusion of the entire open reading frame of STC1-1 in frame with the Fc region of human IgG1 using the baculovirus vector pHiF, a derivative of pVL1393 purchased from PharMingen. Fusion proteins were transiently expressed in S9 insect cells and purified over a protein A column. STC1 was also expressed as a C-terminal His tag fusion in Escherichia coli and the denatured protein was used for immunization. The identities of the purified proteins were verified by N-terminal sequence analysis.

Preparation of Monoclonal Antibodies to STC1—Ten Balb/c mice (Charles River Laboratories, Wilmington, DE) were hyperimmunized with recombinant polyhistidine-tagged (HIS8) human STC1 expressed in E. coli (Genentech) in Ribi adjuvant (Ribi Immunocen Research, Inc., Hamilton, MO). B-cells from five mice demonstrating high anti-STC1 antibody titers were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection, Rockville, MD) using a modified protocol analogous to one described previously (3, 4). After 10–14 days, the supernatants were harvested and screened for antibody production by direct enzyme-linked immunosorbent assay (ELISA). Five positive clones, showing the highest immunobinding after the second round of subcloning by limiting dilution, were injected into Pristane primed mice (5) for in vivo production of the monoclonal antibody. The ascites fluids were pooled and purified by protein A affinity chromatography (fast protein liquid chromatography, Pharmacia, Uppsala, Sweden) as described previously (3). The purified antibody preparations were sterilized (0.2-μm pore size; Nalgene, Rochester, NY) and stored at 4 °C in phosphate-buffered saline. For ELISA for STC1, high binding, flat-bottom polypolyethylene 96-well plates (NUNC, Naperville, IL) were coated overnight at 4 °C with 100 μl monoclonal STC1 antibody 2734 (250 ng/ml). The plates were washed (phosphate-buffered saline containing 0.05% Tween), blocked (phosphate-buffered saline containing 0.5% BSA), and washed again before adding 100 μl of supernatant or STC1 onto duplicate wells. After subsequent washing steps, a second biotinylated STC1 monoclonal antibody (2733; 250 ng/ml) was added to the wells. After a 2-h incubation and a wash step, a 1:10,000 dilution of streptavidin-horseradish peroxidase (Amersham Biosciences) was added to the plates. Tetramethyl benzidine (Kirke-
were pretreated with native or boiled STC1 (50 ng/ml). Confluent HUVEC were incubated overnight in basal medium. Cells from both the ligated and sham animals. Six animals were used for the experiments. Animals were allowed to recover on a warm water heating pad until the end of the experiment.

Spectra Max Gemini (Molecular Devices), with the OD excitation at 535 nm and the absorbance at 450 nm was determined (Spectra Max 250, Molecular Devices, Sunnyvale, CA). The minimum level of STC1 that could be reliably detected by the ELISA was 20 pg/ml. For RNA isolation and quantitative reverse transcriptase-PCR (ABI PRISM TaqMan), Tri-reagent-LS (Molecular Research Center, Cincinnati, OH) was added to the cells, and total RNA was extracted according to manufacturer’s protocol. Gene-specific oligonucleotide primer pairs and a specific probe (labeled with a fluorescent dye at the 5’ end and a quencher fluorescent dye at the 3’ end) were designed using Oligo version 4.0 software (National Biosciences, Plymouth, MN) and levels of STC1 mRNA were determined by real-time quantitative PCR (ABI PRISM TaqMan), as described previously (6).

Collagen gels containing HUVECs were prepared as described previously (1). The gels were overlaid with 1x basal media (Medium 199 supplemented with 1% fetal bovine serum, 1% ITS (insulin, selenium and transferrin, Invitrogen), and 2 mmol/liter L-glutamine), 100 units/ml penicillin, and 100 units/ml streptomycin containing 200 ng/ml HGF and 200 ng/ml VEGF to elicit tube formation, as described previously (1). For “film” experiments, endothelial cells were seeded onto the surface of a collagen gel and incubated in the identical media as that described for the gel experiments. To evaluate endothelial morphogenesis, HUVECs were cultured in Matrigel on glass coverslips and were incubated in 1x basal media in the presence of various factors, as described. Network formation was quantitated at 8 h by photographing three random fields of each well and then determining the total network area/field using OpenLab software (Improvement).

Cell Migration Assay—HTS multi-well insert 24-well plates (BD Biosciences) were coated with cell attachment factor (BD Biosciences) on the bottom layer and type 1 collagen on the membrane surface. 25,000 cells were seeded into each chamber and incubated for 18 h at 37 °C in 5% CO₂. The collagen and unimmigrated cells were scraped off the membrane surface with a plastic Pasteur pipette, and then all the media were aspirated. Absolute methanol was added to the membranes, and the membrane chamber was fixed at room temperature for 30 min. The methanol was aspirated off, and a 10 µl solution of YO-PRO-1 (Molecular Probes, Eugene, OR) was added. Cells were counted under fluorescence isocyanate optics using OpenLab version 2.5 (Improvement).

Proliferation Assay—5,000 cells were seeded on gelatin-coated 96-well plates and incubated overnight with endothelial growth medium. The cells were then starved for 3 days with M199 containing 1% fetal bovine serum, 2 mmol/liter L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. 20 ng/ml of various growth factors were added to the starvation media, and the cells were incubated for 4 days. Alamar blue solution (BIOSOURCE International, Camarillo, CA) was added to the wells, and an amount equal to 10% of the culture volume and incubated for 4–6 h at 37 °C in 5% CO₂. The plates were read on a Spectra Max Gemini Molecular Devices, with the OD excitation at 535 nm and emission at 590 nm.

Femoral Ligation Surgery—Femoral artery ligation was performed under isoflurane (Aerrane, Fort Dodge, CO) inhalation anesthesia on 8-week-old male C57/B6J mice (Charles River Laboratories). Briefly, the femoral artery was isolated at the level of the inguinal ligament and ligated with 7–0 silk suture (Ethicon, Somerville, NJ). Animals were allowed to recover on a warm water heating pad until ambulatory. Total RNA was isolated from the gastrocnemius muscle of both the ligated and sham animals. Six animals were used for the control (sham) and six animals for the ligated group for each time point. The RNA samples were reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA samples were then used in real-time PCR experiments.

RESULTS

Regulation of STC1 Production in Endothelial Cells in Monolayer Culture—To evaluate the effects of various cytokines and growth factors on the release of STC1 from HUVEC, we developed an ELISA-based assay (see “Materials and Methods” for details) which was capable of measuring STC1 levels as low as 20 pg/ml. There was no detectable STC1 release from unstimulated HUVEC (not shown). To survey for the possible effects of various cytokines and growth factors on STC1 release, confluent endothelial cells cultured in 96-well tissue culture plates were incubated with these factors for 24, 48, and 72 h, and STC1 levels were determined in the cell supernatants. The majority of factors evaluated had either no effect (e.g., VEGF, TGF, bradykinin, histamine, and TNF) or very modest effects (IL-1) on STC1 release at these time points. The concentrations shown in Table I are the highest concentration tested (for each drug we tested, the indicated dose and at least two lower doses (e.g. 1:10 and 1:100) of that shown). Of the growth factors examined, only bFGF and HGF stimulated significant STC1 release. The cytokines IL-6 and IL-4 also stimulated STC1 release.

Levels of STC1 mRNA and Protein Secretion Are Much Greater in Three-dimensional Cultures Compared with Two-dimensional Cultures—Equal numbers (1.5 x 10⁵) of HUVEC were plated either onto type I collagen gels (film experiments) or suspended in type I collagen gels (gel experiments) and incubated with HGF and VEGF (200 ng/ml) for various times as indicated in Fig. 1. It should be noted that the combination of HGF and VEGF was required in three-dimensional gels for survival; as described previously, neither growth factor alone was capable of supporting survival and tubulogenesis in three-dimensional collagen gels (1). The mRNA levels (Fig. 1A) for STC1 were dramatically in the gel versus film environment, such that at 24 h, the mRNA levels for STC1 were 10- to 20-fold higher in the three-dimensional cultures. Levels of STC1 protein (Fig. 1B) in the supernatants were 2- to 6-fold higher in the three-dimensional cultures. Because the cells are embedded in three-dimensional collagen gels, any protein in the supernatants must be "released" from the gel to be detected, and this may account for the discrepancy in relative ratios of mRNA versus protein in the two environments.
Effects of STC1 on Endothelial Proliferation—STC1 (0.001–5 μg/ml) had no effect on bFGF-stimulated (10 ng/ml), VEGF-stimulated (10 ng/ml), or HGF-stimulated (10 ng/ml) endothelial proliferation (data not shown). Additionally, STC1 did not stimulate endothelial proliferation when tested in the absence of growth factors (not shown).

Effects of STC1 on HGF-induced Endothelial Migration—HGF is a known potent stimulus for endothelial migration. Therefore, we determined the role of STC1 in HGF-induced endothelial cell migration. As shown in Fig. 2A, when rSTC1 (Ig fusion protein) was added to the Boyden chambers, it markedly inhibited the migratory response of the endothelial cells to HGF. Denaturation of the recombinant protein by boiling completely eliminated this inhibitory activity. Additionally, the inhibitory effects of STC1 were not observed at lower (2.5 ng/ml) concentrations of the protein. The inhibitory effects of STC1 on HGF cell migration were also blocked by the inclusion of 25 μg/ml of the anti-STC1 monoclonal, 2734 (Fig. 2B), in contrast to the lack of effect of an isotype-matched non-immune IgG. To further evaluate the selective effects of STC1 on endothelial cell migration, we evaluated the effects of rSTC1 on bFGF-induced (10 ng/ml) and VEGF-induced (10 ng/ml) endothelial cell migration. These doses of bFGF and VEGF elicited a similar magnitude of cell migration as 20 ng/ml HGF, yet none of the STC1 reagents tested (native STC1, boiled STC1, STC1 monoclonal antibody 2734 (25 μg/ml)) had a significant
Effects of STC1 on HGF-induced Endothelial Morphogenesis—To evaluate the possible effects of STC1 on endothelial morphogenesis, we tested the effects of native rSTC1 and boiled STC1 on HGF-induced endothelial branching network formation on growth factor-depleted Matrigel™. In this model, incubation of endothelial cells without a growth factor such as HGF results in little or no cord formation (not shown). Addition of HGF (20 ng/ml) results in an elaborate network of endothelial branching structures (Fig. 3A). Addition of rSTC1 (1 μg/ml) markedly reduced the overall area of endothelial networks (Fig. 3B), and the structures that did form were discontinuous and poorly formed. Heat denaturation abrogated the effects of rSTC1 (Fig. 3B).

Effects of STC1 on HGF-induced c-met and FAK Phosphorylation—HGF induced the phosphorylation of c-met (Fig. 4). Pretreatment or cotreatment of HUVEC with 5 μg/ml STC1 did not reduce the phosphorylation response of c-met to added HGF (Fig. 4), suggesting that STC1 did not inhibit HGF binding to its receptor. To determine possible effects downstream of c-met phosphorylation, we examined the effects of STC1 HGF-induced FAK activation. STC1 abrogated HGF-induced phosphorylation of FAK (Fig. 5). Preliminary experiments indicated that maximal FAK phosphorylation after HGF treatment occurred at 60 min. Pretreatment of the endothelial cells with 5 μg/ml STC1 completely blocked HGF-induced FAK phosphorylation. Although difficult to detect, the modest FAK phosphorylation induced by bFGF or VEGF (Fig. 5) was not inhibited by pretreatment with STC1.
Expression of STC1 in the Hind-limb Ischemia Model of Angiogenesis—We also evaluated the expression of STC1 in the mouse femoral ligation assay. In this model, the femoral artery is ligated, dramatically reducing blood flow to the lower limb including the gastrocnemius muscle (7). This hypoxic insult results in the rapid development of new vessels in the hypoxic muscle as a component of the spontaneous recovery of perfusion, which is also associated with proximal arteriogenesis (8, 9). Because the newly developing vessels are not readily visualized in this assay, we also measured the mRNA levels of the endothelial marker, CD31, as an index of new vessel formation. As shown in Fig. 6, A and B, both CD31 mRNA and STC1 mRNA expression peaked at day 3 and then returned to lower levels at later time points. We also measured the mRNA levels of HGF. Interestingly, the expression of HGF also increased markedly in this in vivo model, peaking at day 3 (Fig. 6B).

FIG. 5. STC1 inhibits FAK phosphorylation induced by HGF. HUVECs were pretreated for 30 min with 5 μg/ml STC1 and then challenged with HGF (10 ng/ml), VEGF (10 ng/ml), or bFGF (10 ng/ml) for 60 min. Lysates were subjected to immunoprecipitation for FAK, separated by gel electrophoresis, and transferred to nitrocellulose. The resulting blots were immunoblotted with antibodies to phosphotyrosine.

FIG. 6. STC1 is up-regulated in the hind limb ischemia model of angiogenesis. A, STC1 and CD31 mRNA in gastrocnemius muscles removed from mice after sham surgery or femoral ligation. Values are expressed as the mean ± S.E.; n = 6 mice/time point and treatment. *, significantly different from sham at the same time point; p < 0.05.
role in the regulation of cellular metabolism (14, 31). Thus the elevated expression of STC1 during angiogenesis may play an additional role in the metabolic requirements of endothelial cells and other cells involved in the formation of new blood vessels.

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