Serum, Phorbol Ester, and Polypeptide Mitogens Increase Class 1 and 2 Heparin-binding (Acidic and Basic Fibroblast) Growth Factor Gene Expression in Human Vascular Smooth Muscle Cells

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Abstract

Vascular smooth muscle cell proliferation is regarded as a key early event in the pathogenesis of atherosclerosis. Heparin-binding growth factor (HBGF)-1 and HBGF-2, also referred to as acidic and basic fibroblast growth factor, are potential mitogens for human vascular smooth muscle cells. These cells coexpress HBGF-1 and HBGF-2 and thus represent a vessel wall source for both polypeptides. In this report, we demonstrate that HBGF-1 and HBGF-2 expression is increased when quiescent human smooth muscle cells are treated with fetal bovine serum. The kinetics of HBGF-1 and HBGF-2 mRNA accumulation following serum treatment are distinct. In addition, HBGF-1 transcripts remain elevated for a longer time period; this may reflect the different decay rates of the HBGF-1 and HBGF-2 mRNAs. Serum-inducible HBGF-1 and HBGF-2 mRNA expression does not occur when RNA synthesis is repressed by actinomycin D but can occur in the presence of cycloheximide, an inhibitor of protein synthesis. Immunoprecipitation experiments indicate that serum treatment also increases HBGF-1 and HBGF-2 production. Smooth muscle cells treated with phorbol 12-myristate 13-acetate or certain combinations of polypeptide growth factors also express increased levels of HBGF-1 and HBGF-2 transcripts. Potential sources for these growth factors in vivo include platelets, macrophages, and T lymphocytes; thus, smooth muscle cells located at sites of vascular injury or inflammation may express elevated levels of HBGF-1 and HBGF-2.

Introduction

The major cell types present in the normal arterial wall are endothelial cells, which form a nonthrombogenic monolayer lining the lumen, and underlying SMC, which are responsible for contraction and maintenance of vascular tone. SMC proliferation is associated with a variety of vascular pathologies; for example, growth (hyperplasia and hypertrophy) of medial SMC is an important feature of hypertension (reviewed in Ref. 1). In addition, SMC migration and intimal proliferation occur during the pathogenesis of both transplant-associated arteriosclerosis (2) and restenosis (reviewed in Ref. 3). SMC proliferation also contributes to the development of restenosis after coronary angioplasty (4) and to the occlusion of synthetic bypass grafts (5).

Vascular SMC in culture will proliferate in response to numerous polypeptides (reviewed in Ref. 6). The relative importance of these various mitogens in SMC proliferative disease is unknown; however, the biological properties of HBGF-1 and HBGF-2 indicate that they may play an important role (reviewed in Ref. 7). HBGF-1 and HBGF-2, also commonly known as acidic and basic fibroblast growth factor, respectively, are structurally related members of the HBGF (fibroblast growth factor) family of proteins (reviewed in Ref. 8). HBGF-1 is a chemotactic factor (9), and both polypeptides are mitogenic (10) for SMC cultured in vitro. Also, the administration of HBGF-2 after balloon catheter injury of the rat carotid artery induces SMC proliferation and intimal thickening (11). SMC are not only respond to HBGF-1 and HBGF-2 but are a vessel wall source for these mitogens. Bovine aortic SMC express predominantly HBGF-2 (12, 13), whereas SMC derived from human vessels coexpress both HBGF-1 and HBGF-2 (13–15). The expression of HBGF-1 and HBGF-2 by SMC is not restricted to cells grown in tissue culture. The mRNAs encoding both HBGFs are present in rat aorta (16), and HBGF-2 protein has been detected in this same tissue by Western blot analysis (11). In addition, HBGF-2 immunostaining has been noted in SMC of human (17) and rat (18) vessels.

Taken together, these results indicate that HBGF-1 and HBGF-2 may regulate SMC growth in vivo by both autocrine and paracrine mechanisms. For this reason, we are investigating the regulation of HBGF-1 and HBGF-2 gene expression in SMC. Our previous study using human saphenous vein SMC indicated that the inflammatory cytokines interleukin 1 and tumor necrosis factor α induced HBGF-2 expression but had no detectable effect.

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* The abbreviations used are: SMC, smooth muscle cell(s); cDNA, complementary DNA; EGF, epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBGF, heparin-binding growth factor; IGF, insulin-like growth factor; kb, kilobase(s); kDa, kilodalton(s); PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; TGF, transforming growth factor.
Results

Coexpression of HBGF-1 and HBGF-2 mRNA by Human Vascular Cells. The human saphenous vein SMC used in the present study express both HBGF-1 and HBGF-2 transcripts (Fig. 1, A and B). Our size estimates for the major HBGF transcripts in SMC (HBGF-1 mRNAs of 4.3, 3.2, and 2.7 kb; HBGF-2 mRNAs of 7.0 and 3.7 kb) agree with those reported previously for human fibroblasts (15, 20, 21). Additional HBGF-1 and HBGF-2 transcripts of a smaller size can be detected using longer film exposures and have been noted by others (20, 21). Although we have noticed some experimental variation in the relative signal intensities of the 7.0- and 3.7-kb HBGF-2 transcripts, this is likely to reflect different transfer efficiencies of the larger mRNA species. In this and all other RNA gel blot analyses, we verified that similar amounts of RNA were applied to each lane by rehybridizing the HBGF blots with a GAPDH cDNA probe.

When assayed by RNA gel blot hybridization, we could not detect HBGF-1 or HBGF-2 transcripts in human umbilical vein endothelial cells using film exposure times sufficient for visualizing these mRNAs in SMC (Fig. 1, A and B). However, evidence that both HBGF-1 and HBGF-2 transcripts are expressed at a low level in endothelial cells was obtained using the reverse transcription-polymerase chain reaction technique. Endothelial cell RNA was incubated with reverse transcriptase, and the resultant cDNA was amplified using HBGF-1- or HBGF-2-specific oligonucleotide primers. The primers were chosen so that different sized amplification products would be synthesized; in addition, since each primer pair spanned at least one intron within the respective gene, amplification from any contaminating genomic DNA would result in significantly larger products. The amplification products generated were of the predicted size (489 and 243 base pairs, respectively; Fig. 1C) and hybridized with the appropriate radiolabeled DNA probe in a Southern blot assay (data not shown). These products were not detectable when cDNA template was omitted from the reaction. Together, these results indicate that HBGF-1 and HBGF-2 transcripts are present in both of the major vascular cell types but are more abundant in SMC.

Serum Increases HBGF-1 and HBGF-2 mRNA Levels in SMC. To determine whether serum could induce HBGF-1 and/or HBGF-2 mRNA expression in SMC, cells were serum-starved for 72 h and then treated with 10% FBS for different periods of time. Using cellular autoradiography, we determined that the cells become growth arrested in response to serum starvation (<5% of the cells will replicate during the subsequent 48-h period; data not shown). RNA was prepared, and HBGF-1 and HBGF-2 mRNA levels were assayed by RNA gel blot analysis. HBGF-1 transcripts were undetectable in quiescent cells; in contrast, HBGF-2 transcripts were expressed at a detectable level (Fig. 2). Serum treatment of quiescent cells increased both HBGF-1 and HBGF-2 mRNA expression. Increased HBGF-1 mRNA levels were evident 2 h after serum addition, and maximal expression occurred at 4–12 h. Increased HBGF-2 mRNA levels were evident earlier, at 1 h after serum addition, and expression was more transient, with maximal levels observed at 4–6 h. Thus, the HBGF-1 and HBGF-2 mRNAs accumulated to their maximal levels with similar kinetics but returned to basal levels at significantly different rates.

Serum Induction of HBGF-1 and HBGF-2 mRNA Expression Occurs in the Presence of Cycloheximide but not Actinomycin D. We investigated whether de novo protein synthesis was required for serum-induced HBGF-1 or HBGF-2 mRNA accumulation by treating quiescent SMC with serum for 6 h in the absence or presence of cycloheximide, an inhibitor of protein synthesis (22). RNA was prepared, and HBGF mRNA levels were analyzed by RNA gel blotting. HBGF-1 and HBGF-2 mRNA induction still occurred in the absence of protein synthesis (Fig. 3). The HBGF-1 mRNA induction level was equivalent in the absence or presence of cycloheximide; in contrast, HBGF-2 mRNA expression was 2-fold higher in the presence of cycloheximide. This observation is consistent with the differential effect of cycloheximide on HBGF-1 and HBGF-2 gene expression; cycloheximide treatment alone increased HBGF-2 mRNA levels, but not HBGF-1 mRNA levels (Fig. 3).

To determine whether cycloheximide induced HBGF-2 mRNA levels by increasing the half-life of HBGF-2 transcripts, quiescent SMC were first treated with serum and cycloheximide for 6 h. They were then incubated in medium containing serum, the RNA synthesis inhibitor actinomycin D (23), and, in some cases, cycloheximide. Cells were collected 4, 8, or 16 h later, and RNA was prepared. HBGF-1 and HBGF-2 mRNA levels were ana-
lyzed by RNA gel blot hybridization. No significant decrease in HBGF-1 or GAPDH mRNA levels was observed during the actinomycin D treatment (Fig. 4). In contrast, HBGF-2 transcripts decayed with an estimated half-life of 5.2 h. When HBGF-2 mRNA levels were elevated by serum addition alone and shorter periods of actinomycin D treatment were used, a half-life of 4.9 h was calculated (data not shown). In the absence of protein synthesis, HBGF-2 mRNA was stabilized, decaying with a half-life of &gt;16 h (Fig. 4).

The increase in HBGF-1 and HBGF-2 mRNA levels after serum addition could be due to enhanced transcription and/or increased transcript stability. It is probable that at least part of the response may reflect transcriptional activation, since HBGF-1 and HBGF-2 mRNA levels do not increase if actinomycin D is included during serum treatment (Fig. 3). Actinomycin D treatment did not reduce the basal level of HBGF-2 mRNA or the constitutive level of GAPDH mRNA, consistent with the relatively long half-lives of these transcripts. The above results indicate that (a) serum-induced HBGF-1 and HBGF-2 gene expression requires de novo RNA synthesis but not protein synthesis; (b) the HBGF-1 and HBGF-2 mRNAs have different half-lives, with HBGF-1 transcripts being relatively long lived; (c) cycloheximide increases HBGF-2, but not HBGF-1, mRNA levels; and (d) the cycloheximide effect is due, at least in part, to HBGF-2 mRNA stabilization.

Serum Increases HBGF-1 and HBGF-2 Synthesis in SMC. We determined whether the increased HBGF-1 and HBGF-2 mRNA levels observed after serum treatment of SMC resulted in elevated HBGF-1 and HBGF-2 synthesis by comparing the levels of immunoprecipitable protein in quiescent and serum-treated cells. The antibody reagents were either prepared (anti-HBGF-1) or purchased from a commercial source (anti-HBGF-2). Although it has been demonstrated previously that the HBGF-2 monoclonal antibody used in this study does
not cross-react with bovine HBGF-1 in radioimmunoassays (24), properties of the HBGF-1 antiserum have not yet been reported. The reactivity and specificity of both antibody reagents was investigated by incubating equivalent amounts of antibody or nonimmune IgG with 

\[ ^{125}I \text{tagged HBGF-1 or } ^{125}I \text{tagged HBGF-2. Immunoprecipitable protein was recovered with protein A- or protein G-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. This immunoprecipitation assay was performed under the same conditions as those used for analyzing extracts prepared from metabolically labeled SMC (see below). Each antibody reacted specifically with the appropriate growth factor; however, the reactivity of the HBGF-1-specific polyclonal antiserum was relatively weak when compared to the HBGF-2-specific monoclonal antibody (Fig. 5).

To assay HBGF-1 and HBGF-2 synthesis in SMC, an equivalent number of quiescent cells were either left untreated or treated for 8.5 h; \(^{15}S\) cysteine was included in the tissue culture medium during the last 6 h of this incubation. Cell extracts were preclarified and then incubated with nonimmune IgG, affinity-purified HBGF-1 antiserum, or the HBGF-2 monoclonal antibody. Immunoprecipitable protein was recovered with protein

![Fig. 4. Effect of cycloheximide (CHX) on HBGF-1 and HBGF-2 mRNA stability in SMC. Quiescent SMC were either left untreated (No Addition) or treated for 6 h with FBS and cycloheximide. They were then either collected immediately or treated for the indicated time periods with actinomycin D (ACT.D) alone or actinomycin D and cycloheximide. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis.](image-url)

![Fig. 5. Specificity of the HBGF-1 and HBGF-2 antibodies in an immunoprecipitation assay. Labeled HBGF-1 or HBGF-2 was incubated with equivalent amounts of nonimmune (NI) IgG [rabbit (r) or mouse (m)], rabbit anti-human HBGF-1 antiserum, or mouse anti-bovine HBGF-2 antibody. Immunoreactive protein was adsorbed to protein A- or protein G-Sepharose and analyzed on a 15% polyacrylamide- SDS gel. Immunoprecipitates were visualized by autoradiography: only the region of the autoradiogram that contained \(^{125}I\)-labeled HBGF is shown.](image-url)

A- or protein G-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Synthesis of immunoreactive HBGF-1 could not be detected in quiescent cells (data not shown). However, serum-treated cells synthesized a polypeptide of \(~17\) kDa that was immunoprecipitated specifically by the HBGF-1 antiserum (Fig. 6). In contrast, both quiescent and serum-treated cells synthesized a polypeptide of \(~18\) kDa that was immunoprecipitated specifically by the HBGF-2 monoclonal antibody. The amount of immunoprecipitable HBGF-2 was 8-fold higher after serum addition, as estimated by densitometry. These results indicate that the increased expression of HBGF-1 and HBGF-2 mRNA in serum-treated SMC is accompanied by increased synthesis of the two polypeptides.

**Polypeptide Growth Factors or Phorbol Ester Can Also Increase HBGF-1 and HBGF-2 mRNA Levels in SMC**

Whole blood serum is a complex mixture containing plasma constituents and factors released from platelets during the process of coagulation. Four of the polypeptide growth factors in serum are PDGF-BB, TGF-β, EGF, and IGF-1; the first three factors are platelet derived (25-27), whereas IGF-1 is a plasma protein (28). All of these polypeptide growth factors can stimulate human SMC proliferation in vitro (10, 29-31). We determined whether one or more of these mitogens could induce HBGF-1 or HBGF-2 expression in a manner similar to whole serum. Since many serum mitogens may function at least in part through stimulation of protein kinase C, we also treated cells with the tumor-promoting phorbol ester PMA, an activator of this enzyme (reviewed in Ref. 32). Quiescent SMC were either left untreated or treated for 6 h with serum, each purified growth factor, or PMA. RNA was prepared, and HBGF mRNA levels were analyzed by RNA gel blot hybridization. At the time interval tested and at the doses used, only serum or PMA increased both HBGF-1 and HBGF-2 mRNA levels (Fig. 7). TGF-β, treatment increased HBGF-2 mRNA levels 3-fold but did not induce HBGF-1 mRNA expression.
We next determined whether various combinations of the four polypeptide growth factors could induce HBGF-1 or HBGF-2 mRNA expression. Quiescent SMC were either left untreated or treated for 6 h with serum or combinations of growth factors. RNA was prepared, and HBGF mRNA levels were analyzed by RNA gel blot hybridization. In this experiment, serum treatment increased HBGF-1 and HBGF-2 mRNA expression 14-fold and 11-fold, respectively (Fig. 8). TGF-β, EGF, and IGF-1 in combination induced HBGF-1 mRNA levels 12-fold and HBGF-2 mRNA levels 9-fold. PDGF, EGF, and IGF-1 or EGF and IGF-1 in combination increased both HBGF-1 and HBGF-2 mRNA levels by 6-fold and 5-fold, respectively. These results indicate that serum polypeptide growth factors can act cooperatively to induce HBGF-1 and HBGF-2 gene expression.

Discussion

The studies reported here demonstrate that (a) although both human vascular SMC and endothelial cells coexpress HBGF-1 and HBGF-2 transcripts, they are present at higher levels in SMC; (b) serum treatment of quiescent SMC increases both HBGF-1 and HBGF-2 gene expression; and (c) combinations of purified polypeptide growth factors or phorbol ester can also increase HBGF-1 and HBGF-2 mRNA levels.

Human SMC derived from splenic artery (13), abdominal aorta (14), and, as shown in this report, saphenous vein coexpress HBGF-1 and HBGF-2 transcripts. Multiple mRNA species are transcribed from both of the HBGF genes; the various HBGF mRNAs result from transcriptional termination at different polyadenylation sites (33-35) and, at least for HBGF-1 mRNA, alternative splicing of 5'-noncoding exons (33, 36). We were unable to detect significant levels of HBGF-1 or HBGF-2 mRNA in human umbilical vein endothelial cells by RNA gel blotting. Previous studies using this technique have indicated that these cells do not express HBGF-1 mRNA (13-15, 37, 38) but may (13, 14, 19, 39) or may not (37, 38) express HBGF-2 mRNA. The inconsistent results obtained in studies analyzing HBGF-2 mRNA expression in this cell type may indicate that the expression level is
mRNA expression was evident at 2 h, and maximal levels were present at 4–12 h; in contrast, HBGF-2 mRNA induction was evident 1 h earlier, and expression was maximal at 4–6 h. Two previous studies noted that HBGF-2 mRNA expression was increased after serum treatment of quiescent cells. Sternfeld et al. (21) reported that HBGF-2 mRNA levels were elevated 4 h after serum treatment of human fibroblasts; however, a time course was not presented. In human astrocytoma cells, HBGF-2 mRNA induction was apparent at 3 h after serum addition, and the expression level peaked at 6–12 h (41). The above studies did not examine HBGF-1 gene expression; however, Cook et al. (42) reported a modest increase in HBGF-1 mRNA levels following serum addition to rapidly growing human fibroblasts.

The different HBGF-1 and HBGF-2 mRNA expression patterns observed in serum-treated SMC may reflect, at least in part, the different half-lives of these transcripts. HBGF-1 mRNA decay did not occur in cells treated with actinomycin D for periods of up to 16 h; in contrast, HBGF-2 transcripts decayed with a half-life of ~5 h. In two human tumor cell lines, designated T98-G and U87-MG, HBGF-2 mRNA decays with a half-life of 10 min or >5 h, respectively (43). Thus, HBGF-2 gene expression can be regulated at the posttranscriptional level in a cell type-specific manner. It is of interest to note that the 3′-untranslated region of the HBGF-1 and HBGF-2 transcripts contains one or more copies of the pentanucleotide AUUUA (34, 35). These motifs, frequently positioned within a larger AU-rich domain, confer instability to various growth-regulated mRNAs (reviewed in Ref. 44). The relatively long estimated half-lives of HBGF-1 transcripts in SMC and HBGF-2 transcripts in both SMC and U87-MG cells demonstrate that these AU-rich regions do not by themselves result in rapid mRNA turnover.

The elevation in HBGF-1 and HBGF-2 mRNA levels following the addition of serum to quiescent SMC can occur in the presence of cycloheximide and thus does not require active protein synthesis. Therefore, the genes encoding HBGF-1 and HBGF-2 can be included in the family of serum-inducible, immediate-early genes (reviewed in Ref. 45). Cycloheximide treatment alone increased HBGF-2 mRNA levels, and the simultaneous addition of both serum and cycloheximide elevated HBGF-2 mRNA levels to a greater degree than serum treatment alone. These effects may be due, at least in part, to HBGF-2 mRNA stabilization. Cycloheximide increased HBGF-2 mRNA stability at least 3-fold, indicating that HBGF-2 mRNA is normally degraded by a labile ribonuclease and/or its degradation is coupled to its translation.

Immunoprecipitation experiments indicate that serum treatment of quiescent SMC increases HBGF-1 and HBGF-2 synthesis. It was relatively difficult to detect HBGF-1 synthesis in SMC by immunoprecipitation. Newly synthesized, radiolabeled HBGF-2 could be detected in both quiescent and serum-treated cells after a 2-day autoradiogram film exposure, but a 7-fold longer exposure time was necessary for detecting the HBGF-1 synthesized by serum-treated cells. This difference could be due to a combination of the following: (a) the reactivity of the HBGF-1 polyclonal antiserum is relatively weak in comparison to the HBGF-2 monoclonal antibody; (b) HBGF-1 transcripts may be less abundant than HBGF-2 transcripts; (c) HBGF-1 may be relatively unstable; or (d)

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**Fig. 8.** Expression of HBGF-1 and HBGF-2 mRNA in SMC treated with serum or combinations of polypeptide growth factors. Quiescent SMC were either left untreated (No Addition) or treated for 6 h with FBS or the growth factor combinations indicated. RNA was prepared, and equivalent amounts of RNA were used for Northern analysis.
HBGF-1 expression may be subject to translational control. In regard to the latter possibility, Crumley et al. (36) have noted that there are four translation initiation codons in one of the alternatively spliced 5'-untranslated exons of the HBGF-1 gene. Most eukaryotic mRNAs do not contain upstream AUG codons, and studies indicate that they can repress translation initiation at 3'-proximal AUGs (reviewed in Ref. 46). Transcripts encoding two other members of the HBGF family of proteins, int-2 and fibroblast growth factor 5, also contain upstream AUG codons, and these have been shown to suppress translation of the major open reading frame (47, 48).

In addition to whole blood serum, the phorbol ester PMA can induce HBGF-1 and HBGF-2 mRNA expression. PMA has a structure similar to diacylglycerol and activates protein kinase C both in vitro and in vivo (reviewed in Ref. 32). Castellot et al. (10) reported that various concentrations of PMA were unable to stimulate the proliferation of human saphenous vein SMC, the same cell type used in our experiments. Thus, elevated expression of HBGF-1 and HBGF-2 can occur in response to either mitogenic (serum) or nonmitogenic (PMA) stimulation. Murphy et al. (49) reported that phorbol 12,13-dibutyrate could induce HBGF-2 mRNA expression in human astrocytoma cells, but they did not analyze HBGF-1 mRNA levels. In human umbilical vein endothelial cells, the HBGF-1 and HBGF-2 genes are differentially regulated by PMA; Bikalvi et al. (37) reported that PMA increases only HBGF-2 mRNA levels.

HBGF-BB, TGF-β, EGF, and IGF-1 are polypeptide growth factors present in serum which have been shown previously to regulate human SMC growth. When quiescent SMC were treated with each growth factor alone, TGF-β induced HBGF-2 mRNA levels but had no detectable effect on HBGF-1 mRNA expression. This induction was weak relative to serum or PMA treatment. TGF-β has been shown previously to induce HBGF-2 mRNA expression in human fibroblasts (21) and keratinocytes (42). Although the other three polypeptides tested did not significantly increase HBGF-1 or HBGF-2 mRNA levels, we should emphasize that our assay tested one concentration at a single time point. It is possible that dose-response and time-course experiments using each of the mitogens would reveal that these growth factors can also regulate HBGF-1 or HBGF-2 gene expression. Interestingly, serum growth factors in combination, particularly TGF-β, EGF, and IGF-1, could induce both HBGF-1 and HBGF-2 mRNA expression to levels approaching those detected with whole serum. It is possible that other combinations that we have not tested could give a similar response. Thus, it appears that cooperative interactions between polypeptide growth factors may be responsible for serum-induced HBGF-1 and HBGF-2 gene expression.

If the level of HBGF-1 and HBGF-2 expression by SMC is regulated in vivo by these same polypeptide growth factors, then it may be elevated in situations where these factors are present within the vessel wall. Vascular cells themselves are potential sources for two of these mitogens; PDGF and TGF-β are secreted by endothelial cells (39, 50, 51) and SMC (52, 53) cultured in vitro. Following vascular injury, SMC could interact with plasma constituents (e.g., IGF-1) as well as factors released from adherent platelets (e.g., PDGF, TGF-β, EGF) in areas of exposed subendothelium. In regard to atherogenesis, two of the cell types commonly associated with atherosclerotic plaques are activated macrophages and T lymphocytes (54). Macrophages express PDGF (55, 56), and both macrophages (57) and T lymphocytes (58) produce TGF-β.

Increased HBGF-1 and/or HBGF-2 production in response to inflammatory cytokines (19) or polypeptide growth factors may promote SMC proliferation by an autocrine mechanism. Although neither protein contains a hydrophobic signal peptide required for secretion via the classical pathway, there is evidence that they can act in an autocrine manner to stimulate cell proliferation (59–63). The autocrine effects may reflect an intracellular mode of action, active release via a novel secretory mechanism, or passive release as a consequence of cell injury. Studies are currently in progress to assay levels of HBGF-1 and HBGF-2 expression by SMC in vivo and to determine whether high levels of HBGF synthesis promote SMC growth.

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells were kindly provided by Dr. M. Gimbrone, Harvard Medical School, Boston, MA. They were grown at 37°C on fibronectin-coated 150-mm plates (2 μg/cm²) in medium 199 (Meditech) supplemented with 10% (v/v) heat-inactivated FBS (HyClone Laboratories), 1X antibiotic/antimycotic (GIBCO Laboratories), 10 units/ml heparin (Upjohn Co.), and 10 ng/ml recombinant human HBGF-1 (a generous gift of Dr. W. Burgess, American Red Cross). Cultures were routinely expanded by trypsin treatment and subculturing at a 1:5 split ratio. Human saphenous vein SMC were generously supplied by Dr. P. Libby, Brigham and Women’s Hospital, Boston, MA. They were grown at 37°C on fibronectin-coated T175 flasks in Dulbecco’s modified Eagle’s medium (low glucose, buffered with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Whittaker Bioproducts, Inc.), supplemented with 10% (v/v) heat-inactivated FBS, 2 mm L-glutamine (GIBCO Laboratories), and 1X antibiotic/antimycotic. Cultures were routinely expanded by trypsin treatment and subculturing at a 1:3 split ratio. To induce a relatively quiescent SMC population, cells were incubated for 72 h in normal growth medium containing a reduced serum concentration (0.5%). All experiments used cells between passage levels 5 and 8.

RNA Isolation. For the experiment reported in Fig. 1, endothelial cells and SMC were cultured in their respective growth media and collected. For all additional experiments, quiescent SMC were either left untreated or treated with FBS (to 10% final concentration), PMA (100 ng/ml; Sigma Chemical Co.), or purified polypeptide growth factors. The growth factors used were recombinant human PDGF-BB (20 ng/ml; Bachem, Inc.), natural human TGF-β1 (10 ng/ml; Genzyme Corp.), recombinant human EGF (20 ng/ml; Genzyme Corp.), and recombinant human IGF-1 (20 ng/ml; Genzyme Corp.). In some cases, cells were also treated with actinomycin D (2 μg/ml; CalBiochem Corp.) and/or cycloheximide (10 μg/ml; Sigma Chemical Co.). Cells were harvested, and RNA was prepared as follows. Cells were lysed in 4.2 mm guanidine isothiocyanate-0.5% sarkosyl-0.1% 2-mercaptoethanol-25 mm Tris-HCl (pH 8.0). The homogenate was then added to an equal volume of 100 mm Tris-HCl (pH 7.5) and treated with 0.5% sodium dodecyl sulfate at 65°C for 5 min. The homogenate was then centrifuged at 14,000 x g for 10 min to sediment nuclei, and the RNA pellet was washed with 75% ethanol and dried. Total RNA was dissolved in water and quantified by ultraviolet absorbance at 260 nm.
8.0)-10 mM EDTA-1% SDS layered over 2 volumes of phenol:chloroform (1:1). After addition of phenol:
chloroform and chloroform extractions, nucleic acid was precipitated with an equal volume of isopropanol,
washed with 70% ethanol, dried, and resuspended in 10 mM Tris-HCl (pH 7.6)-1 mM EDTA-5 mM MgCl2. Nucleic acid was then incubated for 15 min at room temperature with 10 μg/ml RNase-free DNase (Worthington Biochemical Corp.). One-ninth volume of 1 mM Tris-HCl (pH 8.0)-0.1 mM EDTA-10% SDS was added, and a phenol:chloroform extraction was performed. RNA was then isopropanol precipitated, washed once with 70% ethanol, dried, and resuspended in 10 mM Tris-HCl (pH 7.6)-1 mM EDTA. The amount of total RNA was quantitated by UV absorbance at 260 nm.

RNA Gel Blot Hybridization. Ten μg of each RNA sample were denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 μM formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted onto Zetabind nylon membranes (Cuno, Inc.) and cross-linked by UV irradiation (120 mJ) using a Stratallinker (Stratagene). The restriction fragments used and the source of DNA probes were as follows: (a) human HBGF-1, 2.2-kb EcoRI fragment of pDH15; American Type Culture Collection, Rockville, MD; (b) bovine HBGF-2, 1.4-kb EcoRI fragment of pl11-1; gift of Dr. J. Abraham, California Biotechnology, Inc., Mountain View, CA; (c) human GAPDH, 0.8-kb PstI/XbaI fragment of pHcGAP; American Type Culture Collection. The probes were labeled to high specific activity with [32P]dCTP (3000 Ci/mmol; Amersham Corp.), using a random primer labeling kit (Boehringer Mannheim). Unincorporated radiolabel was removed using Sephadex G-50 spin columns (Boehringer Mannheim). Blots were prehybridized (2 h) and hybridized (18 h) at 65°C in 1% bovine serum albumin-1 mM EDTA-0.5 mM NaHPO4 (pH 7.2)-7% SDS-100 μg/ml denatured salmon sperm DNA-20% formamide. They were then washed at the same temperature in 0.5% bovine serum albumin-1 mM EDTA-40 mM NaHPO4 (pH 7.2)-5% SDS (2x, 15 min each)-1 mM EDTA-40 mM NaHPO4 (pH 7.2)-1% SDS (2x, 15 min each), and finally in 0.2 mM EDTA-36 mM NaCl-2 mM NaHPO4 (pH 7.2) (2x, 15 min each). Blots were then air dried and exposed to film (XAR-5; Eastman Kodak Co.) with an intensifying screen at ~80°C. To calculate HBGF-1 and HBGF-2 mRNA levels and HBGF-2 mRNA decay rates, autoradiographic signals were quantitated using a laser densitometer (Pharmacia LKB). HBGF mRNA signals were normalized to the signal intensity obtained for GAPDH mRNA to correct for slight differences in the amount of total RNA loaded per lane. To estimate the half-life of HBGF-2 mRNA, normalized densitometry values were used to determine the percentage of HBGF-2 mRNA remaining at various times following actinomycin D addition. Best-fit lines were generated by linear regression using the time values and the natural logarithm of the percentages; the slope and y-intercept were used to estimate half-lives.

cDNA Synthesis-Polymerase Chain Reaction. Total RNA was prepared from human umbilical vein endothelial cells, and 5 μg were incubated at 37°C for 1 h in reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3)-10 mM dithiothreitol-75 mM KCl-3 mM MgCl2-0.5 mM deoxyribonucleotide triphosphates] containing 80 ng of random hexamer primers (Boehringer Mannheim) and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Reverse transcription was terminated by heating at 95°C for 10 min, and 10% of the resulting cDNA was removed for the polymerase chain reaction. These reactions were performed in 50 mM Tris-HCl (pH 8.0)-1.5 mM MgCl2-10 mM KCl-0.2 mM each deoxyribonucleotide triphosphate and included 0.5 μg of each primer and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus). Samples were subjected to 40 cycles of amplification using a thermocycler from Perkin-Elmer/Cetus. Each cycle included denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and primer extension at 72°C for 3 min. The human HBGF-1 oligonucleotide primers were 5′-CCTGCTGAGCCATGGCTGAAA ’-3′ (sense) and 5′-ACAGATCTCTTATGTTGAGAGACTG-3′ (antisense). They are predicted to amplify a 489-base pair fragment [nucleotides -11 to 478 (64)]. The human HBGF-2 oligonucleotide primers were 5′-GGAGATTGTATCCCGCTCTGGCTCATG-3′ (sense) and 5′-TCACGCTCTAGCAGACATTGGAAGA-3′ (antisense). They are predicted to amplify a 243-base pair fragment [nucleotides 565 to 807 (35)]. An aliquot of each amplification mixture was subjected to electrophoresis on a 1.2% agarose gel, and DNA was visualized by ethidium bromide staining. ØX174 DNA Haelll restriction fragments (Clontech Laboratories, Inc.) were used for size standards.

Immunoprecipitation. Immunoprecipitations were performed using affinity-purified anti-human HBGF-1 polyclonal antiserum (kindly provided by K. Engleka, American Red Cross) and an anti-bovine HBGF-2 monoclonal IgG antibody (Upstate Biotechnology, Inc.). The specificity of each reagent in an immunoprecipitation assay was determined as follows. Bovine brain-derived HBGF-1 (a gift of Dr. W. Burgess, American Red Cross) and recombinant human HBGF-2 (Amgen Biologicals) were iodinated by the chloramine-T method as described (65). Specific activities obtained were 1.5 x 106 cpm/ng (HBGF-1) and 9.0 x 106 cpm/ng (HBGF-2). Each growth factor (106 cpm) was then incubated with an equivalent amount of each antibody or the appropriate control IgG (Sigma Chemical Co.) for 2 h at 4°C in modified RIPA buffer (20 mM 3-(N-morpholino)propanesulfonic acid-150 mM NaCl-1 mM EDTA-1% Nonidet P-40-1% deoxycholate-0.1% SDS) containing phenylmethylsulfonyl fluoride (174 μg/ml; Sigma Chemical Co.), leupeptin (0.5 μg/ml; Sigma Chemical Co.), and aprotinin (2 μg/ml; Boehringer Mannheim). The control rabbit IgG and HBGF-1 antisera samples were then incubated for 1 h at 4°C with protein A-Sepharose beads (Sigma Chemical Co.). The control mouse IgG and HBGF-2 antibody samples were incubated similarly with protein G-Sepharose beads (Pharmacia LKB). The beads were washed five times with modified RIPA buffer and once with modified RIPA buffer containing 2.5 mM KCl. Immunocomplexes were suspended in 0.125 mM Tris-HCl (pH 6.8)-4% SDS-20% glycerol-10% 2-mercaptoethanol, heated at 95°C for 5 min, and analyzed on a 15% polyacrylamide-SDS gel. The gel was fixed in water:methanol:acetic acid (5:4:1), dried, and subjected to autoradiography.

Immunoprecipitation analysis of HBGF-1 synthesis in SMC was performed as follows. SMC were incubated for 72 h in medium containing 0.5% FBS, and an equivalent number of cells were either left untreated or treated with
10% FBS for 2 h. The cells were rinsed twice with serum-free/cytosine-free medium and then incubated for 6.5 h in this medium with or without 10% FBS. The medium contained 166 μCi/ml [35S]cysteine (1200 Ci/mmol; Amersham Corp.) during the last 6 h of this incubation. The cells were washed twice with cold Hanks’ balanced salt solution (GIBCO Laboratories), harvested by gently scraping the flasks with a rubber policeman, and solubilized in a modified RIPA buffer containing protease inhibitors (see above). Following one freeze-thaw step, lysates from untreated and 10% FBS-treated SMCs were precleared with nonimmune rabbit IgG and protein A-Sepharose beads for 1 h at 4°C. Centrifugation supernatants were then incubated at 4°C for 6 h with either control rabbit IgG or affinity-purified HBGF-1 antisera and subsequently incubated with protein A-Sepharose beads for 1 h at 4°C. The beads were washed as described above, and immune complexes were analyzed on a 15% polyacrylamide-SDS gel. The gel was fixed and treated with Enlightening (New England Nuclear) prior to drying and autoradiography. Immunoprecipitation analysis of HBGF-2 synthesis was performed similarly, except that [15S]cysteine was added to a final concentration of 250 μCi/ml, mouse IgG was used for preclearing and the control incubation, the HBGF-2 monoclonal antibody was used, and protein G-Sepharose was used for collection of immune complexes. Molecular weight estimates were based on the migration of 14C-labeled protein molecular weight markers (Bethesda Research Laboratories).

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References