Heparin-binding EGF-like Growth Factor Stimulation of Smooth Muscle Cell Migration: Dependence on Interactions with Cell Surface Heparan Sulfate

Shigeki Higashiyama,*‡‖ Judith A. Abraham,¶ and Michael Klagsbrun‡§‖

*Department of Biochemistry, Osaka University Medical School, Osaka, 565 Japan; ‡Departments of Surgery, §Biological Chemistry and Molecular Pharmacology, ‖Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115; and ¶Scios Nova Inc., Mountain View, California 94043

Abstract. Heparin-binding EGF-like growth factor (HB-EGF), but not EGF, binds to cell surface heparan sulfate proteoglycan (HSPG). This was demonstrated in (*a*) the binding of ^{125}I-HB-EGF to mutant CHO cells deficient in HS production was diminished by 70% compared to wild-type CHO cells, (*b*) the binding of ^{125}I-HB-EGF to CHO cells and bovine aortic smooth muscle cells (BASMC) was diminished 80% by heparitinase or chlorate treatment, and (*c*) ^{125}I-EGF did not bind to CHO cells and its binding to BASMC was not diminished at all by heparitinase and only slightly by chlorate treatment. Accordingly, the role of HB-EGF interactions with HSPG in modulating bioactivity was examined. Heparitinase or chlorate treatment of BASMC diminished the ability of HB-EGF to stimulate BASMC migration by 60–80%. A similar inhibition of migration occurred when BASMC were treated with a synthetic peptide (P21) corresponding to the sequence of the putative heparin-binding domain of HB-EGF. As a control for BASMC viability, and for specificity, it was found that heparitinase and P21 did not inhibit at all and chlorate inhibited only slightly the stimulation of BASMC migration by PDGF AB. Since heparitinase, chlorate, and P21 treatment also diminished by 70–80% the cross-linking of ^{125}I-HB-EGF to the EGF receptor, it was concluded that the interaction of HB-EGF, via its heparin-binding domain, with cell surface HSPG was essential for its optimal binding to the EGF receptor on BASMC and hence for its optimal ability to stimulate migration.

HEPARIN-binding EGF-like growth factor (HB-EGF)[1] is a recently described member of the EGF family that was first identified as a secreted product of macrophages and macrophage-like cells (1, 2, 9, 10). Subsequently, HB-EGF has been found to be expressed by vascular endothelial cells (28), vascular smooth muscle cells (5, 5a), and to be a component of wound fluid (17). HB-EGF, which has been found to contain up to 86 amino acids (10) is significantly longer than EGF and the structurally homologous mitogen, TGF-α, which have sizes of 53 and 50 amino acids, respectively (4). The COOH-terminal region of HB-EGF contains six cysteine residues with the spacing characteristic of members of the EGF family (4) and shares ~40% sequence identity with EGF and TGF-α. Comparative structural examination suggests that HB-EGF is analogous to an NH_2-terminally extended form of EGF and TGF-α. Since EGF and TGF-α do not bind to heparin (2, 13) while HB-EGF binds tightly to heparin affinity columns and is eluted with 1–1.2 M NaCl (2, 9, 10), it appeared likely that the heparin-binding domain of HB-EGF would be somewhere upstream of the COOH-terminal EGF, TGF-α-like domain. Site-directed mutagenesis of HB-EGF and studies with peptide fragments have indicated that the heparin-binding sequences of HB-EGF reside primarily in a twenty one-amino acid stretch upstream of and slightly overlapping the EGF-like domain (Thompson, S., S. Higashiyama, K. Wood, M. Klagsbrun, and J. Abraham, manuscript in preparation).

HB-EGF is as active as PDGF but considerably more potent than EGF or TGF-α in stimulating bovine aortic smooth muscle cells (BASMC) proliferation (9) and migration (as shown in this report). The enhanced potency compared to EGF and TGF-α occurs despite the fact that all three EGF family members apparently bind to the same high affinity EGF receptor (4, 9). One possible explanation is differential binding to HSPG. Previous studies have shown that the interaction of two other heparin-binding growth factors, bFGF and vascular endothelial growth factor (VEGF), with cell surface heparan sulfate proteoglycan (HSPG), is an essential

Please address correspondence to Dr. M. Klagsbrun, Department of Surgery, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.

1. *Abbreviations used in this paper*: BASMC, bovine aortic smooth muscle cells; HB-EGF, heparin-binding EGF-like growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; VEGF, vascular endothelial growth factor.

requirement for binding to high affinity receptors and for mitogenic activity (7, 15, 24, 27). These conclusions were based on experiments that used (*a*) cells mutant in heparan sulfate biosynthesis, (*b*) cells whose cell surface HSPG was depleted by heparitinase digestion of heparan sulfate, and (*c*) cells incubated with chlorate to block the sulfation of heparan sulfate. These results suggested that heparin-binding growth factors in general might be dependent on a dual receptor system consisting of HSPG and a high affinity tyrosine kinase receptor that mediates cell signaling. Given that HB-EGF has a strong affinity for immobilized heparin (9), we wanted to determine whether it could bind to cell surface HSPG and whether its bioactivity, in this case the ability to stimulate cell migration, was dependent on interaction with cell surface HSPG. Accordingly, BASMC surface HSPG was altered by heparitinase or chlorate treatment, and a synthetic peptide corresponding to the putative heparin-binding domain of HB-EGF was used to compete for HB-EGF binding to cell surface HSPG. We report here that HB-EGF binds to cell surface HSPG and that the binding of HB-EGF to the EGF receptor on BASMC and its ability to stimulate BASMC migration are considerably diminished when HB-EGF–HSPG interactions are abrogated.

Materials and Methods

Growth Factors

HB-EGF was prepared from U-937 cell conditioned medium as previously described (9, 10). PDGF AB (23) was obtained from Creative Biomolecules (Hopkinton, MA). EGF (4) was obtained from Collaborative Research (Bedford, MA). HB-EGF (2 μg) was radiolabeled with $Na^{125}I$ (200 μCi/2 μl; ICN Biomedicals, Costa Mesa, CA) using IODO-BEADS (Pierce, Rockford, IL) as previously reported (10). The specific activity of ^{125}I-HB-EGF was 55,500 cpm/ng, as calculated based on the mitogenic activity of radiolabeled HB-EGF for 3T3 cells compared to HB-EGF standards. ^{125}I-EGF (102,800 cpm/ng) was purchased from Collaborative Research. ^{125}I-bFGF (77,000 cpm/ng) was a kind gift of Dr. Brad Olwin (University of Wisconsin).

Cells

BASMC were routinely grown in DMEM supplemented with 10% BCS, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (DMEM/10% BCS/PS) as previously described (9, 29). Wild-type (Kl) CHO cells and mutant CHO 803 cells, which synthesize ~5–10% of the normal amount of HSPG, were routinely grown in Ham's F12 medium supplemented with 10% BCS as previously described (27).

BASMC Migration

The migration of BASMC was assayed in a Boyden chamber (3, 8). Subconfluent BASMC were detached from 10-cm culture dishes by incubation with trypsin-EDTA (0.05% trypsin, 0.5 mM EDTA, GIBCO BRL, Gaithersburg, MD) at 37°C. To avoid excessive exposure to trypsin, the incubations were carried out for as short a time as possible, usually 1 min or less. The cells were washed first in DMEM, 10% BCS, and then in binding buffer A (DMEM, 20 mM Hepes, pH 7.4, 0.5% BSA) twice, resuspended in the same medium, and the cell number was then adjusted to 10^5 cells/ml. To set up the migration assay, samples to be tested for stimulation of BASMC migration were first added to the bottom wells of a 48-well Boyden chamber (Neuro Probe Inc., Cabin John, MD) and an 8-μm pore size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe Inc.) precoated with fibronectin (100 μg/ml in PBS, Cappel Organon Teknika Corp., West Chester, PA) was placed above these wells. 50 μl of the BASMC suspension were then added to the upper wells of the 48-well chamber and the chamber was assembled and incubated for 4 h at 37°C in a humidified atmosphere of air/10% CO_2. Cells adhering to the upper surface of the filter membrane were removed by scraping with a rubber blade. Cells that had migrated through the filter were fixed with buffered formalin overnight, and then stained with Gill's hematoxylin overnight. The filter was then mounted between two glass slides with 90% glycerol, and the number of BASMC per well that had migrated across the filter was determined by counting the stained BASMC under a microscope.

Binding Assays

The ability of ^{125}I-HB-EGF to bind to BASMC and CHO cells was measured in binding assays carried out in 6-well plates. Briefly, confluent cells (3×10^5 cells/well) in each well were washed with ice-cold PBS/0.5% BSA, and incubated for 10 min at 4°C with 750-μl of ice-cold binding buffer A. ^{125}I-EGF (0.125 ng/750 μl/well, 12,800 cpm) or ^{125}I-HB-EGF (0.25 ng/750 μl/well, 13,900 cpm) were added to wells of the plates and the plates were incubated on a shaker at 4°C for 2 h. The wells were then washed three times with ice-cold binding buffer A, and twice with ice-cold PBS/0.5% BSA. Total binding was measured by determining the amount of radioactivity eluted with a wash of 2 M NaCl, 20 mM Na-acetate, pH 4.0 (18, 19, 27).

Heparitinase and Chlorate Treatment of Cells

BASMC and CHO cells were treated as previously described with heparitinase (24), in order to digest cell surface heparan sulfate (HS), or with chlorate, an inhibitor of HS sulfation (12, 24). For heparitinase treatment, BASMC and CHO cells were washed three times with binding buffer A and incubated for 1 h at 37°C with the binding buffer (750 μl/6-well plate well, 5 ml/10-cm dish or 10 ml/15-cm dish) containing 0.01 U/ml of heparitinase (Seikagaku America, Inc., Rockville, MD). A second aliquot of heparitinase was then added to each well or dish to bring the concentration to 0.02 U/ml and the incubation was allowed to proceed for another 2 h. For chlorate treatment, BASMC and CHO cells were incubated with DMEM/10% dialyzed BCS/PS supplemented with 30 mM Na-chlorate for 48 h. Dialyzed BCS was used to diminish the concentration of cysteine (a source of sulfate) to 50 μM (11, 12). Heparitinase and chlorate-treated cells were used directly for binding and cross-linking studies, or were trypsinized and analyzed for response to migration factors in a Boyden chamber. It should be noted that chlorate appears to exert a mild toxic effect on cells, possibly due to the long exposure, 48 h, of cells to low cysteine media (12).

Receptor Cross-linking

Cross-linking of HB-EGF and EGF to the EGF receptor was carried out using methods previously described (26, 27). Briefly, confluent monolayers of BASMC (10^7 cells) in 15-cm dishes were washed twice with ice-cold 2 M NaCl, 20 mM Na-acetate, pH 4.0, in order to remove any bound endogenous BASMC-derived HB-EGF or EGF. The cells were then washed three times with ice-cold binding buffer B (20 mM Hepes, pH 7.4, 0.5% BSA, 1 mM $MgCl_2$, 1 μM KI in DMEM) and the monolayers were incubated at 4°C for 2 h in binding buffer B containing ^{125}I-HB-EGF (13.3 ng/7.5 ml/15-cm dish, 738,000 cpm) or ^{125}I-EGF (9.4 ng/7.5 ml/15-cm dish, 966,000 cpm). After the incubation, the cells were washed twice with ice-cold PBS, pH 7.4, containing 0.5% BSA, 1 mM $MgCl_2$, and 1 μM KI, and then twice with ice-cold PBS. Bound ^{125}I-growth factor was cross-linked to cells by the addition of disuccinimidyl suberate (DSS; Pierce, Rockford, IL) at a final concentration of 30 μM in PBS for 20 min at room temperature. After free-reactive DSS was quenched with a large excess of 10 mM Tris-HCl, pH 7.5, 150 mM glycine, the cells were washed with ice-cold PBS, and then scraped and lysed in 400 μl of lysis buffer (1% Nonidet P-40 [Pierce], 1% deoxycholate [Aldrich Chem. Co., Milwaukee, WI], 0.1% SDS, 1% aprotinin [Boehringer Mannheim Corp., Indianapolis, IN], 1 mM PMSF [Pierce], 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.4 mM sodium orthovanadate, 10 mM iodoacetoamide [Aldrich] in PBS). Samples (30 μl) were subjected to SDS-PAGE and ^{125}I-labeled bands were visualized and quantitated by analysis using a PhosphorImager device (Molecular Dynamics, Sunnyvale, CA).

Characterization of a Synthetic Peptide Corresponding to the Heparin-binding Domain of HB-EGF

A synthetic peptide corresponding to the putative 21-amino acid heparin-binding sequence KRKKKGKGLGKKRDPCLRKYK of human HB-EGF (amino acids 93-113 of the 208 residue precursor [9]) was synthesized using an Applied Biosystems Peptide Synthesizer (courtesy of Margaret Ehrhardt [Brigham and Women's Hospital, Boston, MA]). To determine the effect of P21 on the binding of HB-EGF to heparin-Sepharose, 40 μl of 50% heparin-Sepharose (Pharmacia) in 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, were

mixed with 40 μl of the same buffer containing increasing amounts of P21 and constant amounts of ^{125}I-HB-EGF (0.25 ng, 13,900 cpm), ^{125}I-EGF (0.125 ng, 12,800 cpm), and ^{125}I-bFGF (0.27 ng, 20,800 cpm). The mixtures were incubated for 5 min at room temperature, after which the heparin-Sepharose was washed four times with 400 μl of 10 mM Tris-HCl, pH 7.4 and counted in a gamma counter (Beckman Gamma 5500). To monitor the effects of P21 on the stimulation of migration by HB-EGF, BASMC (60–70% confluent) were trypsinized, washed with DMEM, 10% BCS, incubated with binding buffer A, incubated with 25 μg/ml P21 in binding buffer A for 10 min at 37°C, and washed three times with the binding buffer to remove nonbound P21. The BASMC pretreated with P21 were analyzed for the response to migration factors in a Boyden chamber. To monitor the effects of P21 on cross-linking, BASMC were incubated with 25 μg/ml P21 and either ^{125}I-HB-EGF or ^{125}I-EGF on ice, and cross-linking was measured as described above.

Results

HB-EGF Binds to Cell Surface HSPG

HB-EGF binds to immobilized heparin (2, 9, 10). To demonstrate that HB-EGF can also bind to cell surface HSPG, the ability of ^{125}I-HB-EGF to bind to CHO cells was measured in several ways (Fig. 1). CHO cells possess cell surface HSPG but little if any EGF receptor. The binding of ^{125}I-HB-EGF to mutant CHO cells which are deficient in HSPG biosynthesis was diminished by ~70% in comparison to the binding of ^{125}I-HB-EGF to wild-type CHO cells (Fig. 1 *A*). In addition, treatment of CHO cells with heparitinase which degrades cell surface HSPG, or chlorate which inhibits HSPG sulfation, diminished ^{125}I-HB-EGF binding by ~80% (Fig. 1 *B*). Taken together, it appears that the binding of HB-EGF to CHO cells is markedly reduced in the absence of HSPG and that therefore HB-EGF binds to CHO cell surface HSPG. On the other hand, ^{125}I-EGF did not bind at all to CHO cells consistent with its inability to bind to immobilized heparin (2) (Fig. 1 *C*).

Since we were interested in analyzing the role of HSPG in modulating HB-EGF biological activity towards SMC,

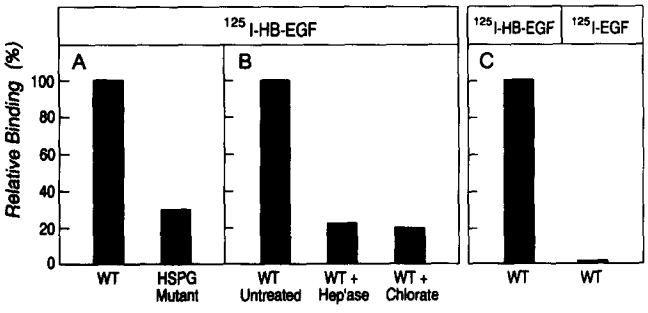

Figure 1. Binding of HB-EGF to CHO cells. The relative binding of total ^{125}I-HB-EGF and ^{125}I-EGF to CHO cells was measured under different conditions. (*A*) Binding of ^{125}I-HB-EGF (0.25 ng, 13,900 cpm) to 3 × 10^5 confluent wild-type (*WT*) CHO cells and mutant CHO 803 cells deficient in HSPG synthesis (*HSPG Mutant*). (*B*) Binding of ^{125}HB-EGF (0.25 ng, 13,900 cpm) to wild-type CHO cells (*Untreated*) and CHO cells incubated with heparitinase (*Hep'ase*) and chlorate as described in Materials and Methods. (*C*) Binding of ^{125}I-HB-EGF (0.25 ng, 13,900 cpm) and ^{125}I-EGF (0.125 ng, 12,800 cpm) to wild-type CHO cells. Under these conditions, wild-type CHO cells bind ~5,000 cpm of ^{125}I-HB-EGF and 50 cpm of ^{125}I-EGF.

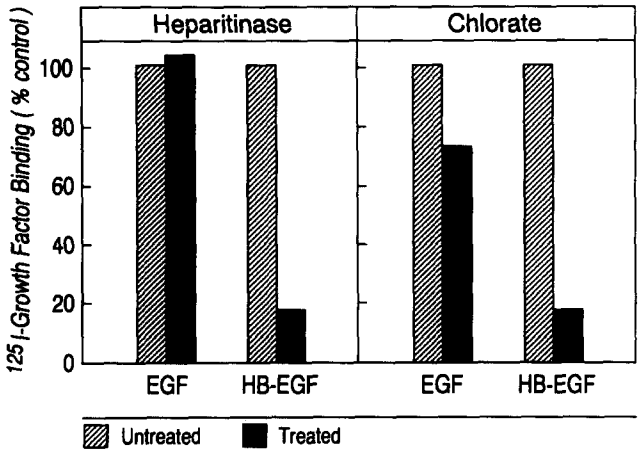

Figure 2. Effects of heparitinase and chlorate on binding of ^{125}I-HB-EGF to BASMC. BASMC treated (*black bars*) with heparitinase or chlorate were incubated with ^{125}I-HB-EGF or ^{125}I-EGF as in Fig. 1 and total binding relative to untreated cells (*gray-hatched bars*) was measured. (*LEFT*) Binding of ^{125}I-HB-EGF or ^{125}I-EGF to heparitinase-treated or untreated cells. (*RIGHT*) Binding of ^{125}I-HB-EGF or ^{125}I-EGF to chlorate-treated or untreated cells. Total binding of ^{125}I-HB-EGF and ^{125}I-EGF to untreated cells was ~3,600 cpm and 1,500 cpm, respectively. The background count in the absence of ^{125}I-growth factor was ~40 cpm.

^{125}I-HB-EGF binding to BASMC was measured. Unlike CHO cells, SMC mutant in HSPG biosynthesis are not available and these cells possess EGF receptor (9). Nevertheless, both heparitinase and chlorate diminished total binding of ^{125}I-HB-EGF to BASMC by 80% (Fig. 2), an amount similar to that produced by these treatments of CHO cells. Thus, it is apparent that HB-EGF binds to BASMC HSPG in addition to the EGF receptor. On the other hand, heparitinase did not diminish at all ^{125}I-EGF binding to BASMC while chlorate reduced ^{125}I-EGF binding by 25%. Taken together, these results suggest that EGF, unlike HB-EGF, binds very poorly, if at all, to BASMC HSPG.

The Ability of HB-EGF to Stimulate BASMC Migration Is Dependent on Interactions with Cell Surface HSPG

It had been previously shown that HB-EGF is a potent mitogenic factor for BASMC, with half-maximal activity at 100 pg/ml (9). The mitogenic activity of HB-EGF was comparable to PDGF but its specific activity was 30–40 times greater than EGF. Using a Boyden chamber migration assay, it is apparent that this is true for migration as well. HB-EGF is a potent stimulator of BASMC migration, with a half-maximal stimulation at 150 pg/ml (Fig. 3), and 100 pg/ml HB-EGF is as active as 330 pg/ml PDGF AB and 3.3 ng/ml EGF.

Since HB-EGF, but not EGF, binds to HSPG, the relationship between growth factor-mediated biological activities and interactions with cell surface HSPG was subsequently analyzed. Migration rather than mitogenic activity was measured in this analysis because the shorter duration of migration assays (4 h) compared to mitogenic assays (72 h) minimized the amount of time needed to measure the effects of growth factors on BASMC treated in various ways. Ac-

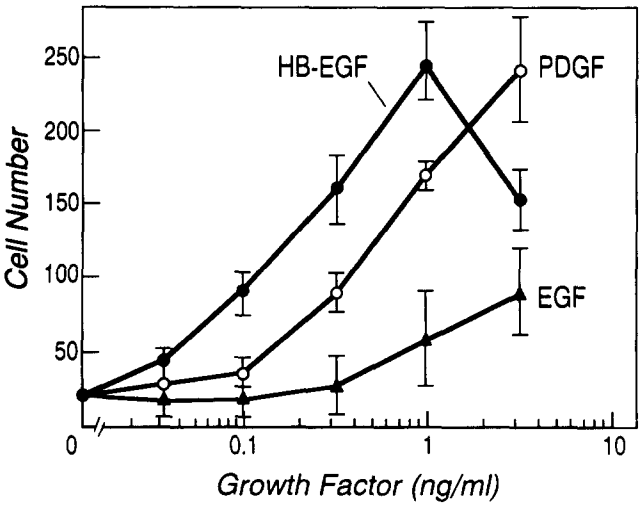

Figure 3. Stimulation of BASMC migration. Migration was measured in a Boyden chamber assay. BASMC were exposed to increasing concentrations of HB-EGF (*closed circles*), PDGF AB (*open circles*), and EGF (*triangles*) in the lower wells of a Boyden chamber and the number of cells per well migrating across the polycarbonate membrane in the chamber was measured. Each point is the average of quadruplicate measurements. The background migration in the absence of growth factor was 20 ± 5 cells/well.

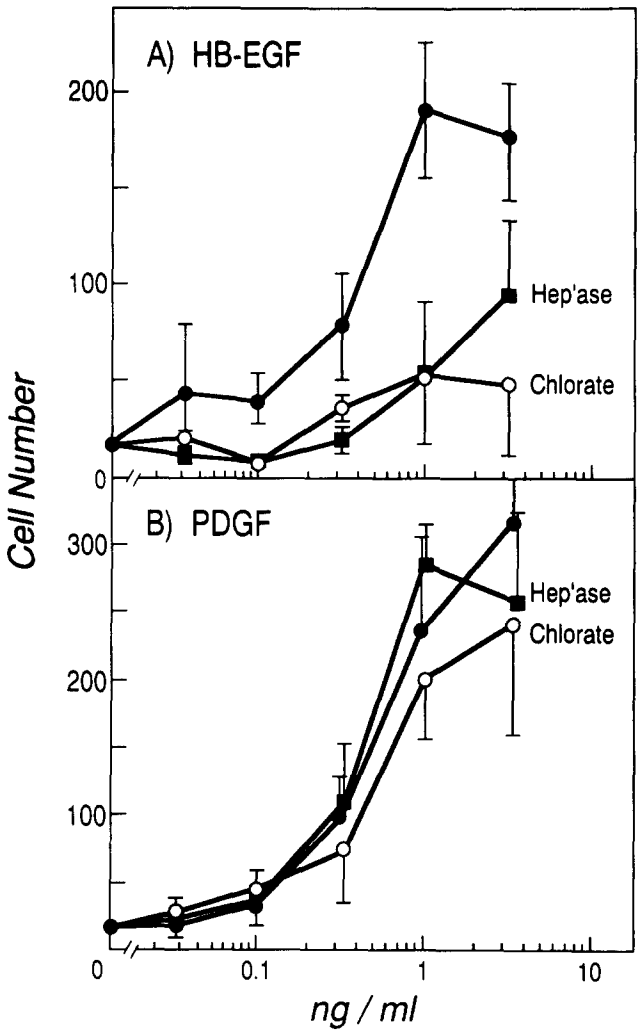

Figure 4. Migration of BASMC preincubated with heparitinase or chlorate. BASMC were either left untreated (*closed circles*), pretreated with heparitinase (*Hep'ase*) for 3 h (*squares*), or pretreated with chlorate for 48 h (*open circles*). Migration of these cells in response to increasing concentrations of (*A*) HB-EGF and (*B*) PDGF AB was measured as in Fig. 3. Each point is the average of quadruplicate values. The background migration in the absence of HB-EGF or PDGF was 17 ± 5 and 15 ± 4 cells/well, respectively.

cordingly, stimulation of migration by HB-EGF was measured on BASMC that had been incubated for either 3 h with heparitinase or for 48 h with chlorate (Fig. 4 *A*). Both treatments markedly inhibited the ability of HB-EGF to stimulate migration at all concentrations tested. At the concentration of maximal HB-EGF stimulation, 1 ng/ml, heparitinase or chlorate-treated BASMC migration was diminished by 80%. 1 ng/ml HB-EGF was needed to stimulate the migration of treated BASMC to the same extent as did 150 pg/ml HB-EGF for untreated BASMC. Indeed, the ability of HB-EGF to stimulate BASMC depleted of HSPG was more comparable to that typically seen for EGF on BASMC with a normal complement of cell surface HSPG. Taken together, these results suggested that the ability of HB-EGF to induce migration of BASMC is reduced markedly if the cells are diminished in available or intact cell surface HSPG. For comparison and as a control for SMC viability, the activity of PDGF AB, a potent stimulator of SMC migration (23), was measured on BASMC treated with heparitinase or chlorate (Fig. 4 *B*). The ability of PDGF AB to stimulate BASMC migration was not diminished at all by heparitinase suggesting that this treatment was not toxic for SMC nor was the availability of cell surface HSPG an absolute requirement for migration. Chlorate, on the other hand, did diminish PDGF AB-stimulated migration but by only ~20%.

Inhibition of HB-EGF-stimulated BASMC Migration by a Heparin-binding Domain Synthetic Peptide Analogue

Another approach to determining the importance of HB-EGF interactions with SMC HSPG would be to interfere with the ability of HB-EGF to bind to cells via its heparin-binding domain. A putative heparin-binding domain in human HB-EGF has been identified by site-directed mutagenesis and peptide fragment studies (Thompson, S., S. Higashiyama, K. Wood, M. Klagsbrun, and J. Abraham, manuscript in preparation). The results of this study are summarized in Fig. 5 *A* which shows a putative heparin-binding domain of 21 amino acids (amino acids 31-51 of the mature 86-amino acid form of HB-EGF [10]). A 21-amino acid synthetic peptide corresponding to this domain, designated as P21, was prepared and tested for the ability to interfere with the binding of HB-EGF to heparin-Sepharose (Fig. 5 *B*). The peptide inhibited half-maximally binding of ^{125}I-HB-EGF to heparin-Sepharose at a concentration of ~2 mg/ml. On the other hand, ^{125}I-EGF did not bind to heparin-Sepharose at all consistent with previous observations (13) and therefore was unaffected by P21. Interestingly, the binding of ^{125}I-bFGF, a growth factor with a very strong affinity for heparin and HSPG (13), to heparin-Sepharose

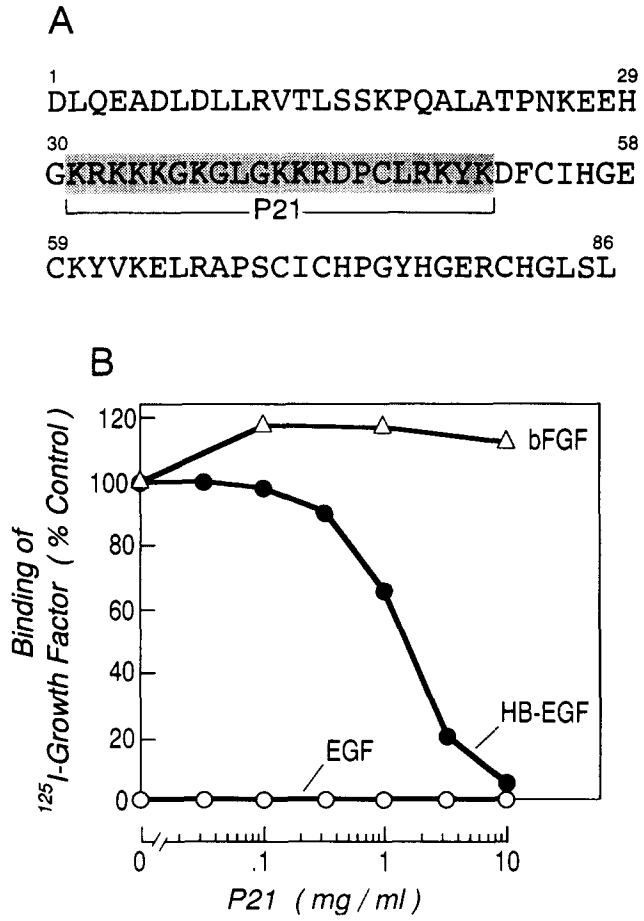

Figure 5. Inhibition of HB-EGF binding to heparin-Sepharose by a synthetic peptide corresponding to a putative heparin-binding domain of HB-EGF. (*A*) The sequence of mature HB-EGF (9, 10) with the location of its 21-amino acid putative heparin-binding domain (residues 31–51 of HB-EGF) outlined in gray. (*B*) The synthetic peptide (P21) corresponding to residues 31–51 was tested in a dose-dependent manner for its effect on the binding of ^{125}I-HB-EGF (*closed circles*), ^{125}I-EGF (*open circles*) or ^{125}I-bFGF (*triangles*) to heparin-Sepharose as described in Materials and Methods. Binding of ^{125}I- growth factor in the presence of P21 is plotted relative to control values in the absence of P21 except ^{125}I-EGF which is plotted relative to ^{125}I-HB-EGF. Under the conditions of these experiments, the control binding of ^{125}I-bFGF (0.27 ng, 20,800 cpm), ^{125}I-HB-EGF (0.25 ng, 13,900 cpm), ^{125}I-EGF (0.125 ng, 12,800 cpm) to heparin-Sepharose in the absence of P21 was 15,670, 5,600, and 50 cpm, respectively.

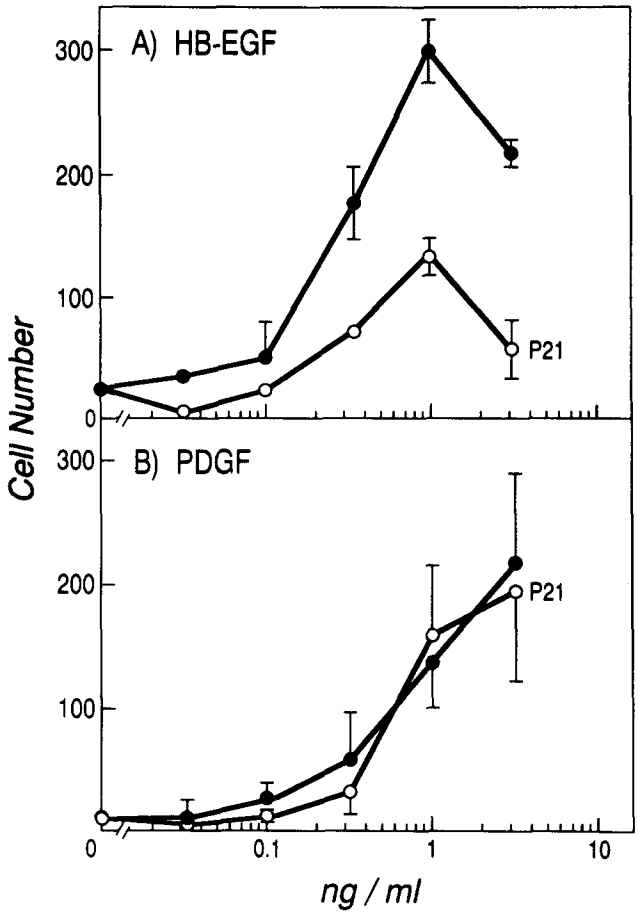

Figure 6. Effect of the synthetic peptide, P21, on BASMC migration. Stimulation of BASMC migration in response to increasing concentrations of (*A*) HB-EGF or (*B*) PDGF AB, was measured in the absence of (*closed circles*) and in the presence of 25 μg/ml P21 (*open circles*). Each point is the average of quadruplicate values. The background migration in the absence of HB-EGF and PDGF AB was 23 ± 6 and 12 ± 4 cells/well, respectively.

was not inhibited by P21 suggesting that bFGF has a different heparin-binding domain than HB-EGF.

At the concentration of maximal HB-EGF stimulation, 1 ng/ml, the P21 peptide at 25 μg/ml inhibited the ability of HB-EGF to stimulate BASMC migration by ~60% (Fig. 6 *A*). One ng/ml HB-EGF was needed to stimulate the migration of P21-treated BASMC to the same extent as did 200 pg/ml HB-EGF for untreated BASMC. On the other hand, P21 did not inhibit the ability of PDGF to stimulate BASMC migration demonstrating that the synthetic peptide was not toxic and did not interfere with BASMC migration in a non-specific manner (Fig. 6 *B*). Nor did P21 inhibit any further the relatively poor ability of EGF to stimulate BASMC migration (not shown). Taken together, these results demonstrated further that the ability of HB-EGF to stimulate BASMC migration is modulated by its heparin-binding properties.

Effects of Heparitinase, Chlorate, and P21 on Receptor Cross-linking

^{125}I-EGF and ^{125}I-HB-EGF could both be cross-linked to BASMC to form labeled complexes of ~170 kD (Fig. 7). These complexes comigrated with ligand-EGF receptor complex standards prepared by cross-linking ^{125}I-EGF or ^{125}I-HB-EGF to A431 cells (not shown). ^{125}I-HB-EGF cross-linking was inhibited by excess cold EGF and ^{125}I-EGF cross-linking was inhibited by excess cold HB-EGF supporting the previous conclusion that EGF and HB-EGF bind to the same EGF receptor (9) (not shown). The synthetic peptide, P21, inhibited the cross-linking of ^{125}I-HB-EGF (lane *2*) to BASMC by ~75%, comparable in degree to the inhibition of cross-linking caused by chlorate (lane *3*) and heparitinase (lane *4*) treatment of BASMC. On the other hand, P21 did not inhibit ^{125}I-EGF cross-linking to BASMC (lane *6*) nor did heparitinase (lane *8*). Chlorate inhibited

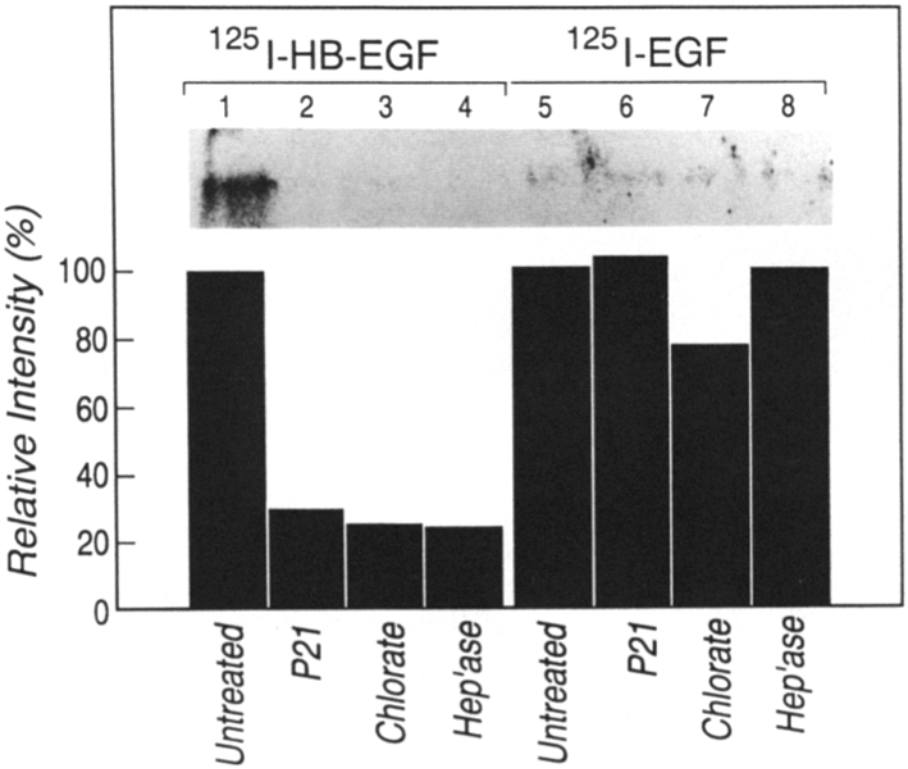

Figure 7. Cross-linking of HB-EGF and EGF to the EGF receptor on treated and untreated BASMC. BASMC were either untreated or exposed to heparitinase, chlorate, or 25 μg/ml of the synthetic peptide, P21. The cells were incubated with either ^{125}I-HB-EGF (lanes *1–4*) or ^{125}I-EGF (lanes *5–8*). After cross-linking, samples were subjected to SDS-PAGE, and bands were visualized (*Top*) and quantitated by phosphor-Imaging (*Bottom*) as described in Materials and Methods. The growth factor complexes formed comigrated with an EGF–EGF receptor complex standard prepared by cross-linking ^{125}I-EGF to A431 cells (data not shown).

^{125}I-EGF cross-linking slightly, by ~20% (lane 7), consistent with the EGF-BASMC binding studies shown in Fig. 2.

In conclusion, using several different approaches, it is apparent that the binding of HB-EGF to BASMC surface HSPG is required for its optimal binding to the EGF receptor and that these HB-EGF–HSPG interactions facilitate the ability of HB-EGF to stimulate migration of BASMC.

Discussion

HB-EGF, but not the structurally homologous protein EGF, is a heparin-binding growth factor (2, 9). The fact that both HB-EGF and EGF bind to the EGF receptor (9) combined with the possibility that HB-EGF, but not EGF, binds to cell surface HSPG, affords an excellent opportunity to ask the question of whether HB-EGF binding to HSPG modulates its bioactivity. The ability of HB-EGF to bind to cell surface HSPG can be demonstrated by using mutant CHO cells that synthesize only 5–10% of their normal cell surface HSPG. These HSPG deficient cells bind ^{125}I-HB-EGF only 30% as well as do the wild-type CHO cells. In addition, binding to wild-type CHO cells is diminished by 80% after treatment of cells with heparitinase which destroys cell surface HSPG and with chlorate which blocks sulfation of HSPG. Since we have found that the CHO cells express little if any EGF receptor, these experiments suggest strongly that HB-EGF is capable of binding to cell surface HSPG. On the other hand, ^{125}I-EGF does not bind to CHO cells suggesting a lack of interaction with cell surface HSPG.

To analyze the effects of HSPG on HB-EGF bioactivity, we chose to study BASMC because of the previous demonstration that HB-EGF is a far more potent mitogenic factor for these cells than EGF (9) and the subsequent demonstration (Fig. 3) that this enhanced potency is true for HB-EGF stimulation of BASMC migration as well. It is apparent that HB-EGF binds to BASMC HSPG since treatment of these cells with heparitinase or chlorate diminishes ^{125}I-HB-EGF binding by ~80%. Using the migration assay, it appears that methods that interfere with HB-EGF–SMC HSPG interactions also inhibit HB-EGF activity. For example, the ability of HB-EGF to stimulate BASMC migration is considerably diminished, by ~70–80%, when these cells are treated with heparitinase or with chlorate. Furthermore, a synthetic peptide, P21, corresponding to the putative heparin-binding domain of HB-EGF (a 21-amino acid stretch just upstream and slightly overlapping the EGF-like domain [Thompson, S., S. Higashiyama, K. Wood, M. Klagsbrun, and J. Abraham, manuscript in preparation] that blocked HB-EGF, but not bFGF, binding to heparin-Sepharose), inhibited HB-EGF stimulation of BASMC migration by ~60%. As a control for the possibility that heparitinase, chlorate, or P21 treatment might be toxic to BASMC and inhibit migration directly, it was demonstrated that heparitinase and P21 did not diminish at all, while chlorate diminished slightly, the potent ability of PDGF AB to stimulate BASMC migration. It should be pointed out, however, that chlorate treatment resulted consistently in a 20–25% inhibition of binding and migration, possibly due to cell toxicity caused by the long exposure, 48 h, of BASMC to low cysteine media. The lack of PDGF dependence on HSPG-binding is consistent with the relatively low affinity of PDGF for heparin-Sepharose. PDGF which has a pI of 9.8, is eluted from heparin-Sepharose with 0.5 M NaCl, typical of basic proteins that bind to heparin due to cation-anion charge interactions rather than to any intrinsic heparin affinity (13). Treatments aimed at abrogating growth factor–HSPG interactions also had no effect on the relatively poor ability of EGF to stimulate BASMC migration consistent with the inability of EGF to bind to immobi-

lized heparin or cell surface HSPG. Taken together, it appears that if HB-EGF is prevented access to intact BASMC HSPG by either HSPG degradation, HSPG desulfation, or by a peptide that competes for HB-EGF binding to HSPG, it is no longer optimally bioactive and acts more like EGF, a relatively poor stimulator of BASMC migration.

How do HB-EGF–HSPG interactions modulate bioactivity? In cross-linking experiments in which formation of growth factor-EGF receptor complexes can be visualized and quantitated, it was found that heparitinase-, chlorate-, or P21-treated BASMC display considerably diminished, by ~70–80%, HB-EGF covalent cross-linking to the tyrosine kinase EGF receptor. On the other hand, heparitinase and P21 had no inhibitory effect and chlorate a slight inhibitory effect on EGF cross-linking to the BASMC EGF receptor consistent with the relative inability of EGF to bind to immobilized heparin or to cell surface HSPG and consistent with the lack in EGF of the heparin-domain present in HB-EGF. These results suggest that the ability of HB-EGF, but not EGF, to bind to the EGF receptor is dependent on its ability to bind to cell surface HSPG. Dependence of HB-EGF binding and bioactivity on a dual receptor system consisting of cell surface HSPG, perhaps acting as low affinity receptors, and the EGF tyrosine kinase receptor, is consistent with results found previously for two other heparin-binding growth factors, bFGF and VEGF (7, 15, 24, 27).

The actual mechanisms by which binding to cell surface HSPG modulates the bioactivity of heparin-binding growth factors such as HB-EGF, bFGF, or VEGF are not well understood. Possible mechanisms are that: (*a*) HSPG concentrates heparin-binding growth factors on the cell surface making them more available to the cell (13), (*b*) HSPG stabilizes or alters the conformation of heparin-binding growth factors and/or their receptors, thereby increasing ligand-receptor affinity (27), (*c*) HSPG stabilizes heparin-binding growth factor dimerization in turn facilitating receptor dimerization as suggested for bFGF (22), and (*d*) HSPG lowers the off-rate component of binding to high affinity receptor by heparin-binding growth factors. For example, it has been demonstrated that the loss of HSPG increases the off-rate of binding of bFGF to its high affinity tyrosine kinase receptor (20).

The identification of growth factors that are potent stimulators of SMC migration and proliferation could be significant in delineating the causes of pathological SMC hyperplasia that occurs in restenosis after angioplasty, in atherosclerosis, and in hypertension (14, 25). It has been proposed that growth factors such as PDGF and bFGF may be involved in the SMC migration and proliferation that occurs, for example, after intra-arterial injury induced by a balloon catheter (6, 16, 21). HB-EGF might play a role in these migration and proliferative processes as well since preliminary analysis indicates that the levels of HB-EGF mRNA rise markedly as early as 2 h postballoon injury in the rat carotid artery (Chao, S., M. Reidy, S. Dluz, D. Damm, J. Abraham, and M. Klagsbrun, manuscript in preparation). Analysis of how HB-EGF structure affects its biological activity for SMC, for example via heparin-binding, might be useful in learning how SMC growth is regulated and in developing strategies for antagonizing SMC migration and proliferation under pathological conditions.

This study was supported by grants GM 47397 and CA37392 from the National Institutes of Health to M. Klagsbrun.

Received for publication 5 January 1993 and in revised form 10 June 1993.

References

1. Abraham, J. A., D. Damm, A. Bajardi, J. Miller, M. Klagsbrun, and R. A. B. Ezekowitz. 1993. Heparin-binding EGF-like growth factor: characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. *Biochem. Biophys. Res. Comm.* 190:125–133.
2. Besner, G., S. Higashiyama, and M. Klagsbrun. 1990. Isolation and characterization of a macrophage-derived heparin-binding growth factor. *Cell Regul.* 1:811–819.
3. Boyden, S. 1962. The chemotactic effect of mixtures of antibodies and antigens on polymorphonuclear leukocytes. *J. Exp. Med.* 115:435–466.
4. Carpenter, G., and M. I. Wahl. 1990. *In* Peptide Growth Factors and Their Receptors. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, Berlin. Vol. 95. 1. 69–171.
5. Dluz, S. M., S. Higashiyama, D. Damm, J. A. Abraham, and M. Klagsbrun. 1993. Heparin-binding EGF-like growth factor expression in cultured fetal human vascular smooth muscle cells: induction of mRNA levels and secretion of active mitogen. *J. Biol. Chem.* In press.

5a. Temizer, D. H., M. Yoshizumi, M.-A. Parella, E. S. Svzami, T. Quertermous, and M. E. Lee. 1992. Induction of heparin-binding epidermal growth factor-like growth factor by phorbol ester and angiotensin II in rat aortic smooth muscle cells. *J. Biol. Chem.* 267:24892–24896.

6. Ferns, G. A. A., E. W. Raines, K. H. Sprugel, A. Y. Motani, M. A. Reidy, and R. Ross. 1991. Inhibition of neointimal smooth muscle cell accumulation after angioplasty by an antibody to PDGF. *Science (Wash. DC).* 253:1129–1132.
7. Gitay-Goren, H., S. Soker, I. Vlodavsky, and G. Neufeld. 1992. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J. Biol. Chem.* 267: 6093–6098.
8. Grotendorst, G. R. 1987. Spectrophotometric assay for the quantitation of cell migration in the Boyden chamber chemotaxis assay. *Methods Enzymol.* 147:144–152.
9. Higashiyama, S., J. A. Abraham, J. Miller, J. C. Fiddes, and M. Klagsbrun. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science (Wash. DC).* 51:936–939.
10. Higashiyama, S., K. Lau, G. E. Besner, J. A. Abraham, and M. Klagsbrun. 1992. Structure of heparin-binding EGF-like growth factor. Multiple forms, primary structure, and glycosylation of the mature protein. *J. Biol. Chem.* 267:6205–6212.
11. Keller, J. M., and K. M. Keller. 1987. Amino acid sulfur as a source of sulfate for sulfated proteoglycans produced by Swiss mouse 3T3 cells. *Biochem. Biophys. Acta.* 926:139–144.
12. Keller, K. M., P. R. Brauer, and J. M. Keller. 1989. Modulation of cell surface heparan sulfate structure by growth of cells in the presence of chlorate. *Biochemistry.* 28:8100–8107.
13. Klagsbrun, M. 1990. The affinity of fibroblast growth factors (FGF) for heparin. FGF-heparan sulfate interaction in cells and extracellular matrix. *Curr. Opin. Cell Biol.* 2:857–863.
14. Klagsbrun, M., and E. Edelman. 1989. The biological and biochemical properties of basic fibroblast growth factor: implications for the pathogenesis of atherosclerosis. *Arteriosclerosis.* 9:269–278.
15. Klagsbrun, M., and A. Baird. 1991. A dual receptor system is required for basic fibroblast growth factor activity. *Cell.* 67:229–231.
16. Lindner, V., and M. A. Reidy. 1991. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 88:3739–3743.
17. Marikovsky, M., K. Breuing, P.-Y. Liu, E. Eriksson, S. Higashiyama, P. Farber, J. A. Abraham, and M. Klagsbrun. 1993. Appearance of heparin-binding-EGF (HB-EGF) mRNA in wound fluid as a response to injury. *Proc. Natl. Acad. Sci. USA.* 90:3889–3893.
18. Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* 131:123–130.
19. Moscatelli, D. 1988. Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell Biol.* 107:753–758.
20. Nugent, M. A., and E. R. Edelman. 1992. Kinetics of basic fibroblast growth factor binding to its receptor and heparan sulfate proteoglycan: a mechanism for cooperativity. *Biochemistry.* 31:8876–8883.
21. Olson, N. E., S. Chao, V. Lindner, and M. Reidy. 1992. Intimal smooth muscle cell proliferation after balloon catheter injury. The role of basic fibroblast growth factor. *Am. J. Path.* 140:1017–1023.
22. Ornitz, D. M., A. Yayon, J. G. Flanagan, C. M. Svahn, E. Levi, and P. Leder. 1992. Heparin is required for cell-free binding of basic fibroblast

growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* 12:240–247.
23. Raines, E. W., D. F. Bowen-Pope, and R. Ross. 1990. Platelet-derived growth factor. *In* Peptide Growth Factors and Their Receptors. M. B. Sporn and A. B. Roberts, editors. Vol. 95. I. Springer-Verlag, Berlin. 173–262.
24. Rapraeger, A. C., A. Krufka, and B. B. Olwin. 1991. Requirement of heparin sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science (Wash. DC).* 252:1705–1708.
25. Ross, R. 1986. The pathogenesis of atherosclerosis: an update.*N. Engl. J. Med.* 314:488–500.
26. Yayon, A., and M. Klagsbrun. 1990. Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: reversal by suramin. *Proc. Natl. Acad. Sci. USA.* 87:5346–5350.
27. Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841–848.
28. Yoshizumi, M., S. Kourembanas, D. H. Temizer, R. P. Cambria, T. Quertermous, and M.-E. Lee. 1992. Tumor necrosis factor increases transcription of the heparin-binding epidermal growth factor-like growth factor gene in vascular endothelial cells. *J. Biol. Chem.* 267:9467–9469.
29. Weich, H. A., N. Iberg, M. Klagsbrun, and J. Folkman. 1990. Expression of acidic and basic fibroblast growth factor in human and bovine vascular smooth muscle cells. *Growth Factors.* 2:313–320.