

Activation of EphB2 and Its Ligands Promotes Vascular Smooth Muscle Cell Proliferation*

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T. Cooper Woods^{‡§¶}, Chad R. Blystone[‡], Jane Yoo[‡], and Elazer R. Edelman^{‡¶}

From the [‡]Harvard University-Massachusetts Institute of Technology Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and [§]Emisphere Technologies, Inc., Tarrytown, New York 10591

EphB2 and its ligands regulate interactions between endothelial and mesenchymal cells in developing arteries. In adult arteries, the relationship between smooth muscle cells and overlying intact endothelium is responsible for maintaining the health of the vessel. Heparin inhibits vascular smooth muscle cell growth in culture and intimal hyperplasia following endothelial denudation. Using gene microarrays, we identified the tyrosine kinase receptor EphB2 as being differentially expressed in response to continuous intravenous heparin administration in the rabbit model of arterial injury. EphB2 protein levels increased in cultured bovine vascular smooth muscle cells following serum stimulation and were decreased in a dose-dependent fashion by heparin. Fc chimeras of the binding domain of the EphB2 ligands blocked the formation of the EphB2 ligand-receptor complex and reduced growth of serum-stimulated vascular smooth muscle cells in a dose-dependent fashion. Activation of the ligand by an Fc chimera to EphB2 followed a parabolic dose-response growth curve, indicating growth stimulation until the chimera begins to compete with native receptors. Co-administration of EphB2/Fc chimera with heparin shifted the dose-response curve to the right. These data indicate a possible new route of Heparin's antiproliferative effect and a role of EphB2 and its ligands in vascular smooth muscle cell proliferation.

The Eph family is the largest family of receptor tyrosine kinases. These proteins are highly conserved membrane-bound receptors that interact with cell surface ligands known as ephrins. There are two classes of Eph receptors and ephrin ligands. Type A ligands bind type A receptors, are membrane-bound, and are entirely extracellular. Type B ligands, which bind type B receptors, possess a cytoplasmic tail capable of kinase signaling activity. Thus, in the case of the type B receptor-ligand complex bidirectional signaling is possible. The extracellular portion of these receptors possesses two fibronectin domains and a cysteine-rich region both of which are implicated in homo- and heterodimerization of the receptor (1). The intracellular domain contains a sterile α motif as well as con-

sensus sequences for binding PDZ proteins (2, 3). These multiple binding capacities lead to several pathways that Eph receptors have been demonstrated to regulate, including the Src, Ras, and c-Jun N-terminal kinase families (4). The EphB2 receptor is expressed in both embryonic and adult tissues with decreased levels seen in the latter. Its functions include axonal guidance, and it may play a role in the assembly of several neural structures (5). Axonal guidance is believed to be conducted through repulsive interactions between the ligand and the receptor. In a similar manner, EphB2 has been implicated in the formation of arterial venous boundaries, vascular morphogenesis, and sprouting angiogenesis (6, 7).

Heparin, endogenous analogs, and related compounds mediate arterial injury to an extent seen with virtually no other compound, yet it is still not clear how this effect is achieved. Heparin regulates leukocyte adhesion and diapedesis (8, 9), extracellular matrix remodeling (10–13), growth factor displacement (14), cytokine production (15–17), induction of cell proliferation, protein kinase expression (18, 19), endothelium-dependent relaxation and vasodilation (20, 21), and reendothelialization (22).

Gene microarrays allowed the screening of thousands of potential genes of interest in the vascular response to injury for the potential of mediating heparin's antiproliferative effect. We observed EphB2 mRNA levels to be down-regulated by continuous intravenous heparin administration. Western blotting of vascular smooth muscle cell samples confirmed an effect of heparin on EphB2 protein levels. Fc chimeras to the binding region of the receptor and its ligand produced a growth stimulatory effect through the formation of the complex. Thus, by screening thousands of candidate genes with gene microarrays, we have discovered a new mediator of vascular smooth muscle cell growth and of heparin's antiproliferative effect and a stronger link between the biology of arterial development and vascular repair.

EXPERIMENTAL PROCEDURES

Animal Model—The iliac arteries of 18 New Zealand White rabbits were denuded with three passes of a 3F embolectomy catheter. Nine rabbits received heparin via an osmotic pump implanted subcutaneously on the animals flank with an intravenous line delivering 0.3 mg/kg/h heparin to the femoral vein. At 3, 7, and 14 days, six rabbits were sacrificed, and iliac tissue was harvested and snap-frozen in liquid nitrogen.

Gene Microarray Experiments—Total RNA was prepared from each frozen tissue sample, and 1 μ g of sample was used to prepare ³³P-labeled cDNA that was hybridized to the Research Genetics Human "Named Genes" GeneFilters® Microarrays Release 1. Paired experiments were prepared sequentially on the same membrane. The spot intensities and identities were determined by Research Genetics Pathways™ software. Fold changes greater than 3 were considered significant, and intensity values within 2-fold of background were considered insignificant. The ratios of spot intensities between samples hybridized to the same array were calculated and averaged across each time point.

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¶ To whom correspondence should be addressed: Harvard University-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 16-343, 400 Main St., Cambridge, MA 02139. Tel.: 617-253-2569; Fax: 617-258-2514; E-mail: cwoods@mit.edu.

¶ An Established Investigator of the American Heart Association.

Cell Culture—Smooth muscle cells were isolated from bovine aortas obtained through Research 87 (Cambridge, MA). Small explants of tissue with removed endothelium were placed in 15 × 30 tissue culture dishes. Smooth muscle cells were grown and expanded from these explants to create cell stocks for future experiments. Dulbecco's modified Eagle's medium supplemented with 1% Pen-Strep and L-glutamine and calf serum (Life Technologies, Inc.) was used for normal cell growth and experiment. Cells between passages 3 and 6 were seeded at 1,000 cells/well of a 48-well plate in media supplemented with 10% calf serum for cell growth assays. The next day, these cells were starved at 0.1% calf serum-supplemented media for 24 h. Then ephrin B1/Fc, ephrin B2/Fc, and EphB2/Fc chimeras (R & D Systems) and heparin were added at the indicated concentrations to the cells in 1–2.5% calf serum-supplemented Dulbecco's modified Eagle's medium. Each condition was performed in triplicate. After a 3-day incubation period, the smooth muscle cell population for each condition was counted using a Z1 Coulter Counter.

Heparin's Effect on EphB2—To study heparin's effect on EphB2 protein levels, vascular smooth muscle cells were plated in 100-mm tissue culture (Costar) dishes at subconfluent levels (1–1.25 million cells) and then serum-starved the following day in 0.1% calf serum-supplemented medium. After 24 h, the cells were given a dose of 100 µg/ml heparin in 5% calf serum-supplemented medium after the starvation period. Cells were harvested at 3, 6, 9, 12, and 24 h. The cells were harvested using radio-immune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl) supplemented with Complete MiniTablet protease inhibitors (Roche Molecular Biochemicals). Relative EphB2 protein levels were determined using Western blotting and antibodies to EphB2 (R & D Systems). A separate set of cultures prepared in the same manner were grown in 5% calf serum containing 1, 10, or 100 µg/ml heparin and EphB2 levels were measured as above.

RESULTS

Gene Microarray Screening—Using gene microarrays to perform differential display, *EphB2* and other members of the *Eph* receptor family were identified as novel candidate mediators of the arterial repair. In the rabbit iliac artery model of arterial injury, *EphB2* levels were seen to increase from insignificant levels at 3 days to 3.6-fold over uninjured arteries at 7 days. At 14 days, the mRNA for *EphB2* returned to insignificant levels. Continuous intravenous heparin administration blocked the increase in *EphB2* mRNA level seen in untreated tissues. Analysis of the levels of mRNA versus control of the array showed a similar profile for *EphB2* and vascular endothelial growth factor (VEGF),¹ which showed insignificant levels at 3 and 14 days and a 4.2-fold increase over the uninjured arteries at 7 days. Again heparin administration attenuated the increase, limiting VEGF mRNA to 3-fold over control at 7 days.

EphB2's Response to Heparin—To confirm heparin's effect on EphB2 expression in smooth muscle cells, bovine aortic smooth muscle cells were exposed to heparin and harvested at various time points (Fig. 1). A dose-dependent response to heparin was observed with 100 µg/ml heparin causing a nearly 6-fold decrease in protein levels. Peak down-regulation was observed at 6 h with a return to near normal expression levels at 12 h.

EphB2's Effect on Proliferation—Based on previously published data, an Fc chimera of ephrin B1 served as a competitive antagonist to the activation of the receptor (Fig. 2) (23). Administration of ephrin B2/Fc chimera yielded a similar growth inhibition about 70% of that seen with the ephrin B1/Fc chimera. The concentrations required to achieve this growth inhibition were comparable with previous studies (24–26). The addition of control Fc fragments had no significant effect on growth. Bovine aortic smooth muscle cells grown in the presence of these chimeras demonstrated dose-dependent growth inhibition, the maximum growth inhibition being comparable with that seen in heparin-treated cells, at a molar ratio of heparin to chimera of ~2.5.

Similarly, an Fc chimera of the ligand-binding portion of the

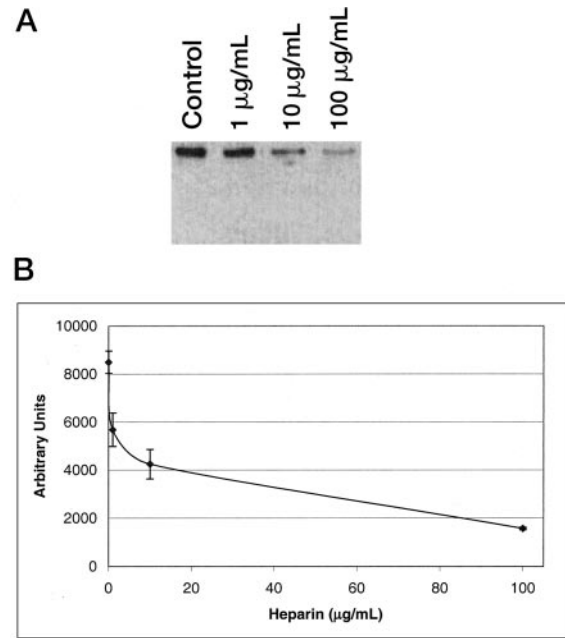


FIG. 1. Dose-dependent response of EphB2 levels to heparin. Vascular smooth muscle cells were grown following starvation in 5% calf serum supplemented with 1, 10, and 100 µg/ml heparin. A, Western blotting was used to measure EphB2 protein levels. The average densitometric measurements of three separate experiments are shown (B).

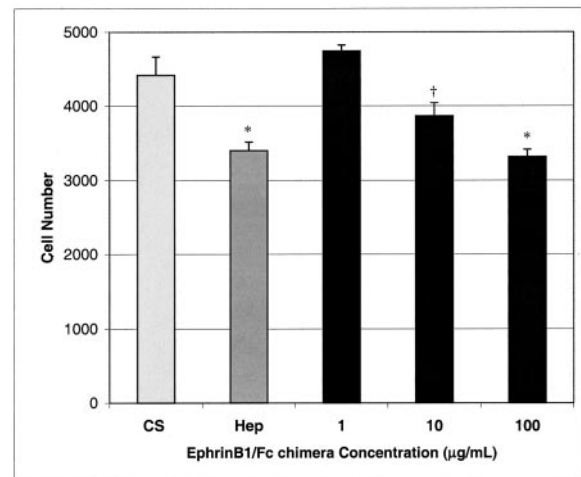


FIG. 2. Dose-dependent response of cell growth to ephrin B1 Fc chimera. Vascular smooth muscle cells were grown for 3 days following starvation in 2.5% calf serum alone (CS) or supplemented with 1, 10, and 100 µg/ml of ephrin B1/Fc chimera. Cells were also grown in 2.5% calf serum supplemented with 100 µg/ml heparin as a standard for growth inhibition. *, $p < 0.001$; †, $p < 0.05$ when compared with serum alone.

EphB2 receptor has been shown to stimulate ligand activity (27–29). When aortic smooth muscle cells are grown in the presence of this chimera, a parabolic dose-dependent cell growth curve was observed (Fig. 3). The cell number increased to a maximum, above which less growth stimulation was seen. This maximum probably represents the point at which all free ligand has been bound either by either native receptor or by the exogenous chimera. At concentrations above this level, the native EphB2 receptor is probably competing with the chimera for free ligand, causing a decrease in the growth stimulation. When heparin was added to this medium, reducing the number of native receptors and thereby freeing more ligand for binding to the chimera, the percentage of stimulation was similar, but the amount of chimera that was added before a maximum was

¹ The abbreviation used is: VEGF, vascular endothelial growth factor.

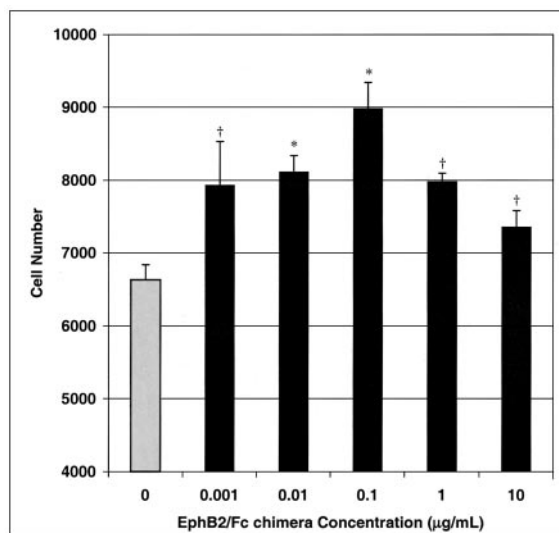


FIG. 3. **Dose-dependent response of smooth muscle cell growth to EphB2 Fc chimera.** Vascular smooth muscle cells were grown for 3 days following starvation in 1% calf serum alone (CS) supplemented with 0.001, 0.01, 0.1, 1, and 10 µg/ml EphB2/Fc chimera. *, $p < 0.001$; †, $p < 0.05$ when compared with serum alone.

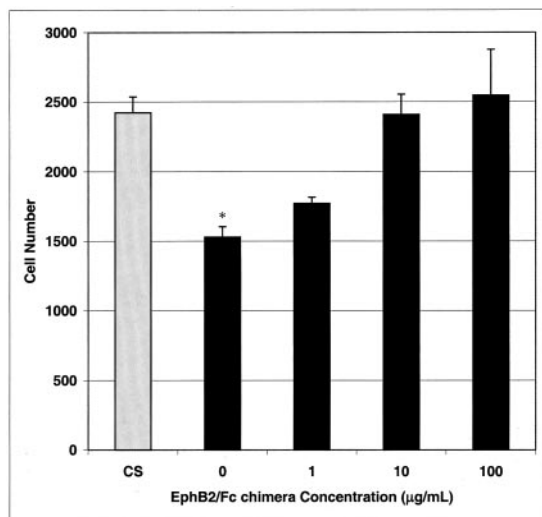


FIG. 4. **EphB2-Fc chimera effect in the presence of heparin.** Vascular smooth muscle cells were grown for 3 days following starvation in 1% calf serum alone (CS) and with 100 µg/ml heparin supplemented with 1, 10, and 100 µg/ml EphB2/Fc chimera. *, $p < 0.01$ when compared with serum alone.

reached increased (Fig. 4). This manifests itself as a shift in the dose response to the right.

DISCUSSION

In an adult artery, the interactions between the endothelium, circulating platelets, and inflammatory cells and the underlying layer of smooth muscle cells help maintain vascular homeostasis. The removal of or disease in the endothelial monolayer leads to a cascade of surface and intramural events that includes cell adhesion and activation, inflammation, thrombosis, proliferation, cell migration, and matrix remodeling that can create a neointima that limits luminal integrity. Continuous intravenous heparin administration inhibits virtually all of these processes in animal models (30). In examining the potential molecular modification of vascular repair, we found increased mRNA expression of the Eph family of ligands and receptors after arterial denudation and attenuation of this family with heparin administration. Similar results were ob-

tained in cultured vascular smooth muscle cells. EphB2 was not detected in growth-arrested smooth muscle cells, but Western blotting indicated an increase in EphB2 protein levels within 3 h of the addition of serum, which was reduced by heparin in a dose-dependent fashion.

EphB2 and its ligands have been linked with vascular development (4, 6, 7). Expression of ephrin B2 and EphB2 at the interface between the mesenchyme and endothelium in developing embryos suggests that they play a role in the regulation of the interactions between these two arterial layers and, as a result, may well help control the vascular response to injury and vascular repair. Signaling of the class B Eph ligand receptor complex is bidirectional, allowing for a phosphorylation of both the ephrin and the Eph receptor. Activation of the ligand is achieved by the binding to a single receptor, while the receptor requires a clustering of multiple ligands to produce an effect (31). Fc chimeras of the ephrin binding domain blocked the binding of native ligand to its receptor and therefore prevented the activation of the receptor. When added to serum, these chimeras inhibited vascular smooth muscle cell growth in a dose-dependent manner. At high concentrations, this inhibition equaled that of the heparin. Increases in the concentration of serum lead to a shift in the dose-response curve to the right. Since the increases in cell number will be accompanied by increases in the number of receptors present, it follows that higher dosing levels would be required at higher serum concentrations. It is also interesting to note that free ephrins have been detected in the conditioned media of some epithelial cell lines (25). It is possible that the release of free ligand from an overlying endothelial layer could prevent the formation of the receptor-ligand complex between adjacent smooth muscle cells in a normal artery.

Blocking the formation of the receptor-ligand complex inhibits smooth muscle cell growth, but since signaling is complex and might follow from activation of the ligand as well as the receptor complex, it is not clear exactly whether one or both signaling pathways are critical for growth control. Our data support the importance of both the ligand and receptor in smooth muscle cell proliferation and suggest that the specific effect of heparin is probably limited to down-regulation of the receptor alone. To determine the effect of activation of the ligand, Fc chimeras of the binding domain of the receptor were used to activate ephrin B1 and ephrin B2 without receptor complex formation (32, 33). The addition of this chimera to the serum of cultured smooth muscle cells increased cell growth in a dose-dependent manner, suggesting that the activation of the ligand does indeed play a role in the proliferative response. However, as the dose was increased, a dose-dependent decrease in growth was seen. If only the ligand's activation affected growth, a plateau in growth would be seen as we saturated the available ligands with chimera. Instead, a decrease in growth stimulation was seen, probably due the chimera sequestering ligand that would have otherwise been bound by native receptor. This reduces the number of native receptors activated and leads to the decrease in growth stimulation. Furthermore, when heparin is included in the growth medium, a shift in the dose response to the right is seen. Heparin's down-regulation of the native receptors creates more free ligand for the chimera to bind. The growth stimulation continues to increase to doses well above the previous initial dose, demonstrating that the previous loss of stimulation was due an antagonistic effect of the chimera on the ligand binding native receptor. Therefore, we conclude that activation of both the ephrin ligand and Eph receptor are capable of stimulating smooth muscle cell proliferation.

The Eph receptors have been linked to a large number of

pathways, and there is significant overlap with those pathways identified as sensitive to heparin. Heparin inhibits activation of mitogen-activated protein kinase; however, the effect appears to be proximal to this kinase and perhaps through negative feedback (19, 34). In a similar fashion, Eph receptors have been linked with numerous signaling proteins that function proximal to mitogen-activated protein kinase. Activation of Eph receptors is known to lead to the docking of adapter proteins, including members of the Grb and Src family members (23, 26). Binding of these adapter proteins leads to a kinase cascade that can ultimately effect the activation of mitogen-activated protein kinase and the proto-oncogenes associated with its activation. Heparin is also known to increase α smooth muscle actin in proliferating smooth muscle cells (35). Likewise, EphB2 binds RasGAP, an inhibitor of the Ras cascade that can also drive a reduction in α smooth muscle actin expression. These mechanisms serve as examples of how Eph signaling and heparin function may be related.

The gene array data suggest a possible functional link between heparin and EphB2. Vascular endothelial growth factor was seen to follow the expression pattern of EphB2. Numerous studies have implicated VEGF as a key growth involved in angiogenesis (36, 37). Recently, Helbling *et al.* (38) suggested that angiogenesis proceeds in three phases with the initial step, sprouting, being under control of VEGF, and the second phase, invasion, being regulated by EphB receptors and their ligands. Heparin has been utilized as an antiangiogenic factor. When co-administered with steroids, heparin functions synergistically to inhibit angiogenesis in tumors (39, 40). Heparin formulations enriched in 2.5- and 5-kDa fragments was found to block angiogenesis in a tumor model without the additional steroid (41). Since the expression profile of both EphB2 and VEGF both show a similar response both to injury and to heparin treatment, it is possible that heparin's effect seen here is largely that of an antiangiogenic factor. Further studies into VEGF's relationship to heparin and EphB2 will elucidate whether these factors are functioning together in vasculoproliferative disease.

The mechanism by which heparin down-regulates EphB2 levels is still unclear, but we have shown that EphB2 is capable of modulating the growth of vascular smooth muscle cells and that one effect of heparin on these cells is a decrease in this receptor. Determination of whether other members of the Eph family of tyrosine kinases possess similar activity requires further investigation, but it is a new area in which heparin may exert its antiproliferative activity and a further link between the biology or vascular development and arterial repair.

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