Comparison of Biological Properties and Transforming Potential of Human PDGF-A and PDGF-B Chains

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Human platelet-derived growth factor (PDGF) consists of two distinct but related polypeptide chains designated PDGF-A and PDGF-B. The gene encoding PDGF-B has given rise to the v-*sis* oncogene. In the present study the transforming activities of PDGF-A and PDGF-B genes are compared. The PDGF-A chain gene is markedly less efficient in inducing transformation than the PDGF-B gene under the influence of the same promoter. There are significant differences in the secretory and growth stimulating properties of the two chains. These properties appear to account for the much more potent transforming ability of the PDGF-B gene. These findings provide insights into biologic properties of a growth factor responsible for potent autocrine stimulation of abnormal cell proliferation.

HUMAN PLATELET-DERIVED GROWTH factor (PDGF), the major mitogen found in serum, is a potent growth factor for cells of mesenchymal origin (*1*). It consists of a disulfide-linked dimer of two related polypeptide chains, designated A and B, which are assembled as a heterodimer (PDGF-AB) or as homodimers (PDGF-AA and PDGF-BB) (*2*). Each chain is the product of a distinct cellular gene. The gene for PDGF-B has been identified as the human homolog of the v-*sis* oncogene (*3*). Expression of the human PDGF-B in tumor cells, which also express the cognate PDGF receptor, may play a role in the development of these malignancies (*4, 5*). The A-chain gene is frequently expressed by human tumor cells, and PDGF-AA has been identified as a product of osteosarcoma (*6*), melanoma (*7*), and glioblastoma cells (*8*). The isolation of PDGF-B and PDGF-A chain cDNAs (*9*) offered the possibility to directly compare the biologic properties of the two PDGF genes when expressed in a target cell susceptible to PDGF stimulation.

To compare their biologic activities, eukaryotic expression vectors were generated in which the cDNAs for the human PDGF-A or human PDGF-B molecules could be expressed under the transcriptional control of the same promoter. We used the mouse metallothionein (MMT) promoter, which is inducible by heavy metals including Zn^{2+} and Cd^{2+} (*10*). Eukaryotic expression vectors were constructed by inserting the cDNAs for either the human PDGF-A or PDGF-B chains downstream from the MMT promoter of p341–3 (*11*). A 0.83-kbp fragment containing the entire coding region for PDGF-B was removed from pGSS 2-7 (*9*) by digestion with Mlu I and Nco I. The complete open reading frame for the PDGF-A cDNA was excised from pUC 13 PDGF-A D1 (*9*) with Rsa I. Bam HI linkers were ligated to each DNA fragment for cloning into the Bgl II site of p341–3 to generate the 6.2-kb plasmids pMMT–PDGF-B and pMMT–PDGF-A. Thus, when PDGF-A and PDGF-B cDNAs are expressed under MMT control, the effects of different levels of expression on biological and transforming activity can be compared.

When NIH 3T3 cells were transfected with serial dilutions of either plasmid, the plasmid containing MMT–PDGF-B induced discrete foci of transformed cells that were readily seen within 7 days. In contrast, morphologically altered areas were first detectable only several days later in PDGF-A transfected cultures, and foci remained smaller and less discrete (Fig. 1). At 3 weeks, the transforming efficiency of the MMT–PDGF-A construct was at least 20-fold less than that of MMT–PDGF-B (Fig. 1). When the medium of the transfected cultures was supplemented with $ZnCl_2$, the transforming efficiency of both constructs increased, but the differences in transforming efficiency and size of individual foci persisted. Even in the absence of $ZnCl_2$, foci containing MMT–PDGF-B were larger and more aggressive than those observed with the same amount of MMT–PDGF-A DNA under induced conditions (Fig. 1).

Both MMT–PDGF-A and MMT–PDGF-B transfectants bound less PDGF-AB than untransfected controls (Table 1). In each case, there was greater receptor downregulation in the PDGF-A as compared to PDGF-B gene–transfected cultures. Thus, a lower level of PDGF-A (compared to PDGF-B expression) as measured by PDGF receptor downregulation was not likely to account for the lower transforming activity of the PDGF-A construct.

The culture medium of the MMT–PDGF-B transfectants contained little, if any, mitogenic activity, even when exposed to $ZnCl_2$ during the collection period (Fig. 2). In contrast, the MMT–PDGF-A transfectant released readily detectable mitogen, which increased three- to fourfold in concentration under conditions of $ZnCl_2$ induction (Fig. 2). The PDGF-specific nature of the secreted growth factor was shown by the fact that neutralizing antibody against PDGF inhibited its mitogenic activity by greater than 95% (Fig. 2). By this comparison, the PDGF-A chain appeared to be secreted at least 80- to 100-fold (Fig. 2) more efficiently than the PDGF-B chain.

To further investigate the compartmentalization of PDGF-A as compared to PDGF-B chains, we prepared antiserum to a COOH-terminal region of the deduced A-chain sequence (residues 185 to 199), a region analogous to that of the B chain for which antiserum was available (*12*). The transfectants were labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 hours, and total cell lysates as well as tissue culture

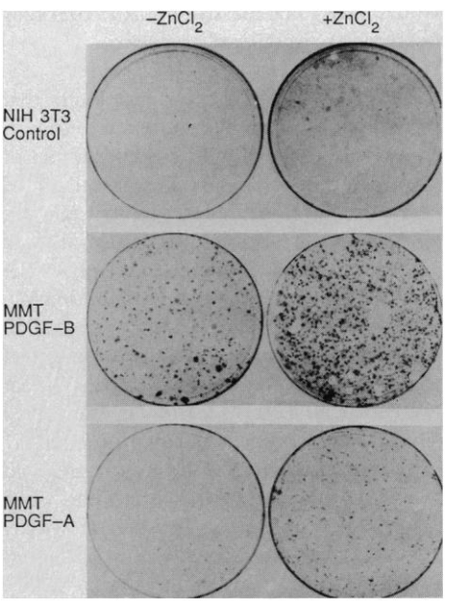

Fig. 1. Focus assay after transfection of NIH 3T3 cells with plasmid DNAs. NIH 3T3 cells were transfected, as indicated, with 0.1 μg of plasmid DNA and 40 μg of carrier calf thymus DNA by the calcium phosphate precipitation technique (*30*). Cultures were grown in the absence (−) or presence (+) of 50 μ*M* $ZnCl_2$ and stained with Giemsa at 3 weeks after transfection. Calf thymus DNA alone was used as a negative control in transfection.

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Table 1. ^{125}I-labeled PDGF binding to transfected NIH 3T3 cells. All transfectants were isolated from mass cultures that received 5 μg of the plasmid containing the construct indicated as well as 0.1 μg of pSV2neo DNA. Cultures were selected by their ability to grow in the presence of Geneticin (*28*). Target cells were grown in the absence (−) or presence (+) of 50 μ*M* $ZnCl_2$. The ^{125}I-labeled human PDGF binding assay was performed as described (*29*), except that cells were plated on human fibronectin (1 μg/cm^2). Values shown represent the average of four independent determinations. In each case, standard error was less than 5% of the mean.

Construct	PDGF binding (% of control)	
	$-ZnCl_2$	$+ZnCl_2$
MMT	100.0	100.0
MMT–PDGF-A	55.1	48.5
MMT–PDGF-B	86.2	71.3

fluids were analyzed immunologically for the presence of PDGF-A or PDGF-B chains. When immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions, neither control MMT-transfected NIH 3T3 cells nor tissue culture fluids of the same cells contained detectable levels of proteins immunologically reactive with any of the antisera (Fig. 3a).

The PDGF-B product was specifically detected in the PDGF-B transfectant cell lysates by antiserum to PDGF (anti-PDGF) and anti–PDGF-B but not by anti–PDGF-A. Thus, the primary PDGF-B product, a 26-kD protein (*5*) as well as its 20-kD processed form, was detected in MMT–PDGF-B transfectants (Fig. 3a). In contrast, PDGF-A–synthesizing NIH 3T3 cells demonstrated major products that appeared as doublets of around 22 to 23 kD and 17 to 18 kD. These were specifically recognized by anti–PDGF-A and anti-PDGF but not by anti–PDGF-B. Striking differences in compartmentalization of the two PDGF chains were observed as well (Fig. 3a). Although PDGF-B was not detected in tissue culture fluids, approximately 70 to 80% of the total PDGF-A synthesized during the 3-hour labeling period was released into the supernatant. All of these findings established that the PDGF-A gene product was much more efficiently secreted than PDGF-B under comparable conditions of expression in NIH 3T3 cells.

The PDGF-A coding sequence was isolated from a human glioma cell line, U-343 MGa C12:6, which also expresses the transcript of an alternative form (*9*). The two transcripts arise from the alternative usage of a 69-bp exon, the presence of which predicts a 15–amino acid COOH-terminal extension of the A-chain precursor with an extremely high proportion of basic residues (*9, 13, 14*). The short PDGF-A transcript appears to predominate in endothelial cells and in certain tumor cells (*13, 15, 16*). To compare the biological properties of the two PDGF-A variants, we attempted to express the short PDGF-A cDNA by using a number of different expression vectors, including the vector containing the MMT promoter. None gave sufficiently high levels of expression to allow measurement of transforming activity. However, use of the pLJ vector (*17*) provided sufficient expression for detection of the short PDGF-A product.

Expression of the short PDGF-A variant led to the detection by anti-PDGF of two major products of approximately 20 and 16 kD (Fig. 3b). Although the antiserum directed against a COOH-terminal region specific to the longer variant efficiently detected both its 23 and 18 kD products, the same antiserum failed to recognize either of the short PDGF-A products (Fig. 3b). These findings identified the product of the short PDGF-A transcript. We observed no differences in compartmentalization of the longer and shorter PDGF-A products. As was the case with the longer form, approximately 70 to 80% of the short PDGF-A product was detected in tissue culture fluids after a 3-hour labeling period (Fig. 3b).

To compare the mitogenic activities of the PDGF-A with PDGF-B or native PDGF, we used two different approaches. There were sufficient amounts of the secreted long PDGF-A product to standardize its concentration on the basis of its ability to compete for binding of ^{125}I-labeled PDGF to control NIH 3T3 cells. The long PDGF-A product, PDGF, and PDGF-B each competed with similar slopes, although PDGF and PDGF-B were more efficient competitors at saturating concentrations (Fig. 4a). When compared on this basis, the long PDGF-A product was significantly less efficient than either PDGF or PDGF-B at inducing DNA synthesis. At saturating concentrations, the longer PDGF-A variant induced approximately one-third as much synthesis as PDGF or PDGF-B. That this was not due to toxicity in the PDGF-A preparation was established by mixing experiments in which saturating levels of PDGF-A and either PDGF-B or platelet PDGF increased DNA synthesis to the maximum level obtained with either of the more potent growth factors.

There were sufficient amounts of the short PDGF-A product to compare its mitogenic activity with that of the longer variant standardized on the basis of the immunoreactivity of each preparation as determined by protein immunoblot analysis with the use of anti-PDGF. We observed no significant differences in the specific mitogenic activities of two PDGF-A variants. Thus, although low levels of expression of the short PDGF-A product relative to the longer variant precluded a direct comparison of their transforming activities, the indistinguishable mitogenic and secretory properties of these PDGF-A gene products suggest that their transforming activities would be similar if expressed at equivalent levels.

Collins *et al.* (*15*) reported expression of the short and long PDGF-A cDNAs in COS-1 cells. However, they did not detect secretion of the short PDGF-A product, despite ample expression of its transcript. Therefore, they concluded that alternative splicing played an important role in determining the functional properties of the two PDGF-A variants. The lack of short PDGF-A secretion from COS-1 cells is consistent with our findings that its expression was low as compared to the long PDGF-A gene product in NIH 3T3 cells. However, at levels of expression where the short PDGF-A product was detectable in cell lysates, we demonstrated that its secretory and mitogenic properties were indistinguishable from those of the longer PDGF-A protein. Thus, our findings are consistent with the well-documented detection of PDGF-like activities secreted from normal cells in which

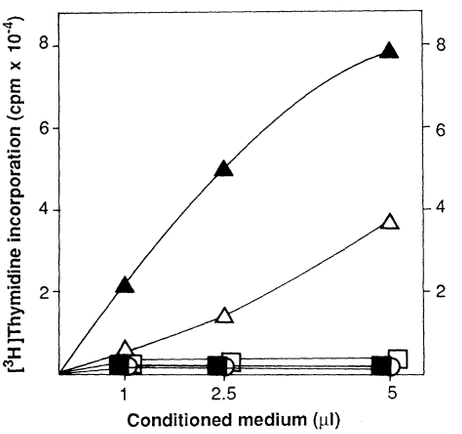


Fig. 2. Mitogenic activity secreted by PDGF-B or PDGF-A transfectants. NIH 3T3 cells were transfected and selected as indicated in the Table 1 legend. Equal numbers (2×10^7) of cells transfected with pMMT–PDGF-A (▲), pMMT–PDGF-B (□), or pMMT vector alone (○) were incubated in 5 ml of serum-free medium containing 25 μ*M* $ZnCl_2$. The pMMT–PDGF-A transfectants were incubated in the same medium but without $ZnCl_2$ (△). Media were tested for mitogenic activity on quiescent NIH 3T3 cells, as described (*24*). In one case, medium was supplemented with human PDGF antibody before addition to indicator cells (■). Each point represents the mean value of three separate determinations. Standard error was less than 6% of the mean, in the case of the two upper curves, and less than 20% of the mean for the three lower curves.

the short PDGF-A variant represents the only detectable transcript or is in vast excess to the longer variant (*13–16*).

Little is known concerning the mechanisms by which a ligand transmits a mitogenic signal through its receptor. Some rapid PDGF-induced effects include changes in ion movements (*18*), enhanced phosphorylation of membrane proteins including autophosphorylation of the PDGF receptor (*19*), and changes in cell morphology and cytoskeletal reorganization (*20*). Another growth factor family includes insulin and the insulin-like growth factors (IGFs). Insulin-like growth factor I (IGF-I) shows structural and functional similarity to insulin (*21*). While distinct high-affinity receptors for IGF-I and insulin have been identified (*22*), insulin can act as a partial agonist for the IGF-I receptor (*22*). Our present findings are compatible with the concept that PDGF-A may be a weaker agonist than either PDGF or PDGF-B for interaction with a common receptor or that different classes of receptors normally mediate the actions of these related growth factors. Evidence for two distinct receptor types in human fibroblasts has recently been obtained. One receptor (type A) appears to bind all three PDGF dimers, whereas the other (type B) only binds B-chain–containing dimers (*23*).

The differences between the PDGF-A and PDGF-B chain gene constructs in inducing the transformed phenotype is likely to relate to the different biological properties of the two homodimeric forms of PDGF, which in turn may reflect their different binding properties to two PDGF receptor types. In addition, it has been established that the v-*sis*/PDGF-B product, which induces clonal proliferation and confers the malignant phenotype to NIH 3T3 cells (*24*), is tightly cell associated (*25*). Whereas the secreted PDGF-A molecule may have a primary role in stimulating cell proliferation at a distance, the v-*sis*/PDGF-B product could have a role in stimulating adjacent cells or in autocrine growth stimulation. Transforming growth factor α (TGF-α) is an example of an efficiently secreted growth factor (*26*) that lacks the ability to induce clonal proliferation or the neoplastic phenotype in NIH 3T3 cells, even when expressed at high levels (*27*). As PDGF-A expression leads to a phenotype intermediate between those induced by TGF-α and PDGF-B, sufficient PDGF-A may remain cell-associated to provide a selective growth advantage to the producing

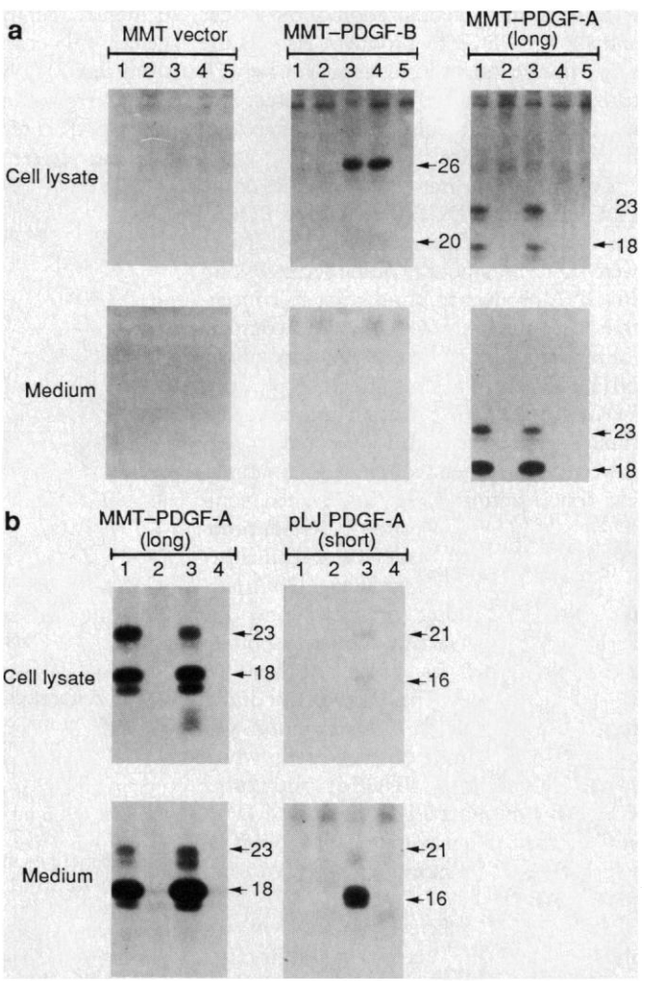
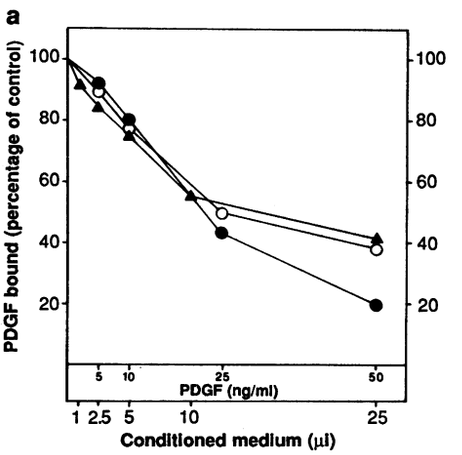


Fig. 3. (**a**) Detection of PDGF-B and PDGF-A translational products in transfected NIH 3T3 cells. Mass populations of 10^7 cells transfected with the vector containing MMT, MMT–PDGF-B, or MMT–PDGF-A (long) were preincubated overnight in Dulbecco's minimum essential medium (DMEM) containing 10% calf serum and 50 μ*M* $ZnCl_2$. Medium was replaced with methionine- and cysteine-free DMEM containing [^{35}S]methionine and [^{35}S]cysteine (each at 125 μCi/ml), as well as 25 μ*M* $ZnCl_2$. Conditioned media or cell lysates were immunoprecipitated with anti–PDGF-A (lanes 1 and 2), anti-PDGF (lanes 3), or anti–PDGF-B (lanes 4 and 5). In some cases (lanes 2 and 5), antibodies were preincubated with excess homologous peptide. Immunoprecipitates were collected with the aid of protein A–Sepharose and analyzed by SDS-PAGE on 14% gels under reducing conditions. (**b**) Expression of PDGF-A (short) and PDGF-A (long) translational products in transfected NIH 3T3 cells. NIH 3T3 cells transfected with vectors containing the variant indicated were metabolically labeled as detailed in (a). Cell lysates or conditioned media were immunoprecipitated with anti–PDGF-A (lanes 1 and 2), anti-PDGF (lanes 3), or preimmune goat serum (lanes 4). In some cases, anti–PDGF-A was preincubated with excess PDGF-A peptide (lanes 2). Immunoprecipitates were analyzed as described in (a).



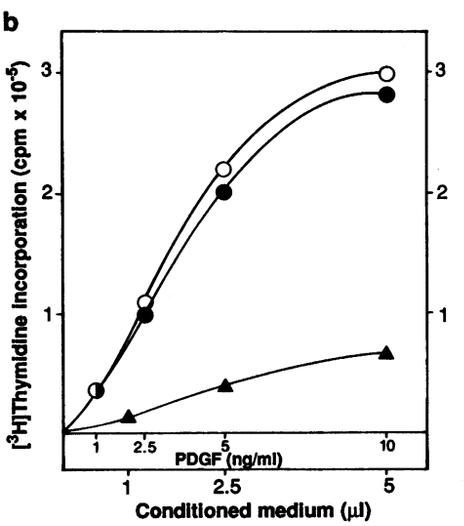


Fig. 4. (**a**) PDGF receptor-competing activity of medium conditioned by MMT–PDGF-A transfectants. Medium conditioned by MMT–PDGF-A transfectants grown in the presence of 25 μ*M* $ZnCl_2$ (▲) was compared with purified human PDGF (●) or PDGF-B (Amgen) (○) for its ability to compete with ^{125}I-labeled human PDGF for NIH 3T3 cell receptor binding as described (*29*). Values, determined in triplicate for each concentration tested, varied by no more than 5%. Results shown represent the average values from four independent experiments. Variation among experiments was less than 5% at each concentration tested. (**b**) Mitogenic potency of medium conditioned by MMT–PDGF-A relative to that of PDGF or PDGF-B. Samples standardized on the basis of receptor competition were assayed for mitogenic activity on quiescent NIH 3T3 cells. Values represent averages of four independent experiments. Designation of symbols is the same as in (a). Variation among experiments was less than 5% at each concentration tested.

cell under conditions of high-level expression.

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Site-Directed Neovessel Formation in Vivo

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Angiogenesis is an important component of organogenesis and wound repair and occurs during the pathology of oncogenesis, atherogenesis, and other disease processes. Thus, it is important to understand the physiological mechanisms that control neovascularization, especially with methods that permit the molecular dissection of the phenomenon in vivo. Heparin-binding growth factor–1 was shown to bind to collagen type I and type IV. When complexed with gelatin, heparin-binding growth factor–1 can induce neovascularization at polypeptide concentrations that are consistent with the biological activity of the mitogen in vitro. The adsorption strategy induces rapid blood vessel formation at and between organ- and tissue-specific sites and permits recovery of the site-specific implant for examination and manipulation by molecular methods.

ANGIOGENESIS IS THE FORMATION of blood vessels in situ and involves the orderly migration, proliferation, and differentiation of vascular cells (*1, 2*). The initiation of angiogenesis by the direct stimulation of endothelial cell proliferation is the presumed responsibility of two polypeptide mitogens (*1, 2*): the class I heparin-binding growth factor (HBGF-I), also known as acidic fibroblast growth factor, and class II heparin-binding growth factor (HBGF-II) or basic fibroblast growth factor. These polypeptides are mitogens for endothelial cells in vitro (*1, 3, 4*) and angiogenesis signals in vivo (*4, 5*); they exert their biological response in vitro through high-affinity cell surface receptors (*6–8*). HBGF-I and HBGF-II are similar in structure (*9, 10*), and both are synthesized as polypeptides lacking a consensus signal peptide sequence (*11, 12*). Cells that express HBGF-I do not secrete the polypeptide in vitro (*13*). Furthermore, HBGF-II is associated with the extracellular matrix (*14*), and heparin protects HBGF-I from proteolytic inactivation (*15*). To define further the angiogenic action of HBGF-I in vivo, we used the affinity of HBGF-I for polypeptide components of the extracellular matrix in a manner that permits the construction of site-specific neovessels in vivo.

Gelatin-Sepharose and collagen type IV–Sepharose adsorb radiolabeled HBGF-I (Fig. 1). Most (approximately 80%) of the growth factor binds to immobilized gelatin and to collagen type IV and can be eluted with 1.5*M* NaCl (Fig. 1, C and G). HBGF-I labeled with ^{125}I can also be eluted with 0.5*M* NaCl (*16*) and with heparin (Fig. 1, A and E). Approximately 20% of the growth factor, which remains bound after heparin elution, can be eluted with 1.5*M* NaCl (Fig. 1, A and E). Pretreatment of the gelatin and collagen type IV matrix with heparin (50 U) significantly reduces the ability of either matrix to adsorb ^{125}I–HBGF-I (Fig. 1, B and F). Regeneration of either matrix by washing with 1.5*M* NaCl permits ^{125}I–HBGF-I adsorption (Fig. 1, B and F). Denaturation of ^{125}I–HBGF-I by heat (90°C for 1 min) significantly reduces the ability of the polypeptide to bind to immobilized gelatin and collagen type IV (Fig. 1, C and G). Furthermore, bovine serum albumin (1 mg/ml) and human fibronectin (1 mg/ml) do not elute significant quantities of ^{125}I–HBGF-I adsorbed to either matrix (Fig. 1, D and H).

Since HBGF-I binds to immobilized gelatin and collagen type IV, we evaluated the possibility that direct implantation of commercial gelatin sponges [Gelfoam (Upjohn)] treated with HBGF-I could be used to induce angiogenesis in situ. HBGF-I–treated Gelfoam was independently placed in the neck (Fig. 2, A and B) and peritoneal cavities (Fig. 2, C to F) of the rat. We observed a significant angiogenic response in situ 1 week after surgery with HBGF-I (1

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