

## Isolation and characterization of a macrophage-derived heparin-binding growth factor

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Human mononuclear cells were plated in culture, and the conditioned media of these cells were analyzed by heparin-Sepharose affinity chromatography. The fractions were tested for growth factor activity as measured by the stimulation of DNA synthesis in BALB/c 3T3 cells. After 2 d in culture, two peaks of heparin-binding growth factor (HBGF) activity were detected, one eluting with 0.5 M NaCl, which could be shown to be platelet-derived growth factor (PDGF)-like, and the other eluting with 1.0 M NaCl. After 7–11 d in culture, when monocytes had clearly differentiated into macrophages, >95% of the HBGF activity in conditioned medium consisted of the 1.0 M NaCl elution peak. This activity, which was designated macrophage-derived HBGF (MD-HBGF), was found to be a cationic heat-resistant polypeptide with a molecular weight in the range of 14–25 kDa. Analysis using Western blots and specific neutralizing antisera, as well as comparative heparin affinity analysis, indicated that MD-HBGF was not identical to other heparin-binding 3T3 cell growth factors known to be produced by macrophages, such as PDGF (AB, AA, and BB forms), acidic fibroblast growth factor, and basic fibroblast growth factor. In addition to stimulating mitogenesis in 3T3 cells, MD-HBGF also stimulated the proliferation of vascular smooth muscle cells, but did not stimulate the proliferation of vascular endothelial cells.

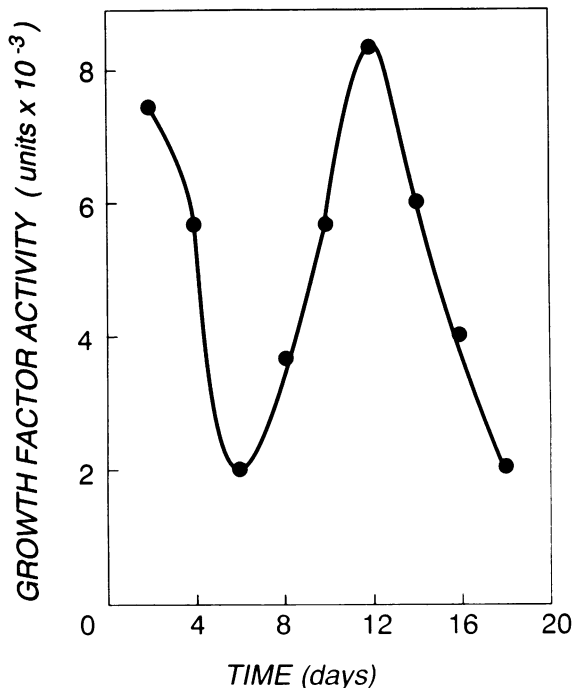
### Introduction

Macrophages are responsible for many important functions including host defense, acute and chronic inflammation, and wound healing. Some of the biological activities of macrophages, such as the stimulation of connective tissue cell pro-

liferation after injury, are thought to be mediated by macrophage-derived growth factors. Macrophages produce many growth factors, possibly more than any other cell type. Among the well-characterized macrophage-derived growth factors are platelet-derived growth factor (PDGF) (Shimokado *et al.*, 1985; Mornex *et al.*, 1986), basic fibroblast growth factor (bFGF) (Baird *et al.*, 1985), transforming growth factor-alpha (TGF- $\alpha$ ) (Madtes *et al.*, 1988), transforming growth factor-beta (TGF- $\beta$ ) (Assoian *et al.*, 1987), interleukin-1 (IL-1) (Hazuda *et al.*, 1988; Schmidt *et al.*, 1984), and tumor necrosis factor-alpha (TNF- $\alpha$ ) (Leibovich *et al.*, 1988). Additional macrophage-derived growth factors have been reported but they have yet to be fully characterized and purified (Madtes *et al.*, 1988; Singh *et al.*, 1988). With the exception of bFGF and IL-1, macrophage-derived growth factors are secreted and act ostensibly in a paracrine manner. bFGF is known to stimulate processes such as formation of granulation tissue and angiogenesis (Davidson *et al.*, 1985; Shing *et al.*, 1985; Folkman and Klagsbrun, 1987; Klagsbrun, 1989). However, as a cell-associated growth factor, the mechanism by which macrophage-derived bFGF could stimulate these processes is unclear. Alternatively, these processes may be induced by other macrophage-derived growth factors, either characterized, e.g., TGF- $\beta$ , PDGF, or as yet uncharacterized.

Heparin affinity chromatography has been used widely as a method for purifying and characterizing growth factors, in particular, members of the FGF family which have a strong affinity for heparin (Shing *et al.*, 1984; Klagsbrun and Shing, 1985; Folkman and Klagsbrun, 1987; Klagsbrun, 1989). Acidic FGF (aFGF) and bFGF bind to immobilized heparin columns and are eluted with 1–1.2 and 1.5–1.8 M NaCl, respectively. Several growth factors structurally homologous to aFGF and bFGF, including *hst/K-fgf* and KGF have an affinity for heparin as well (Delli-Bovi *et al.*, 1988; Finch *et al.*, 1989). PDGF also binds to immobilized heparin but with relatively low affinity and is eluted with 0.5 M NaCl, typical of cationic proteins in general (Shing *et al.*, 1984). Given the recent success of heparin

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**Figure 1.** Time course of growth factor secretion by human mononuclear cells. Human peripheral blood mononuclear cells were isolated and placed in cell culture. Conditioned medium (CM) was harvested every 2 d and assayed for stimulation of DNA synthesis in BALB/c 3T3 cells. Each point (in quadruplicate) represents the total number of units of growth factor activity present at that time in 500 ml of 1× conditioned medium. The same results were repeated in a second experiment.

affinity chromatography in isolating novel growth factors such as KGF (Finch *et al.*, 1989) and vascular endothelial growth factor (VEGF) (Ferrara and Henzel, 1989; Tischer *et al.*, 1989), we analyzed monocyte/macrophage-conditioned medium for the presence of secreted heparin-binding growth factors. In this report, we demonstrate that short- and long-term cultures of adherent macrophages produce a heparin-binding growth factor mitogenic for 3T3 and smooth muscle cells but not for endothelial cells. This macrophage-derived heparin-binding growth factor (MD-HBGF) is neither PDGF nor FGF and may be novel.

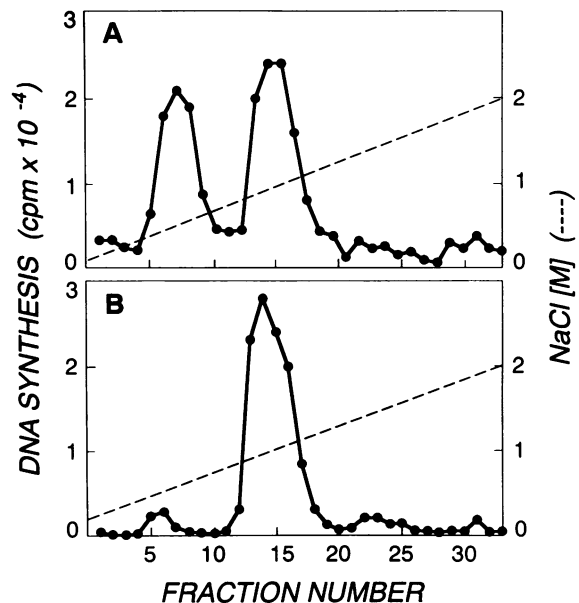
## Results

### Secretion of heparin-binding growth factors by macrophages

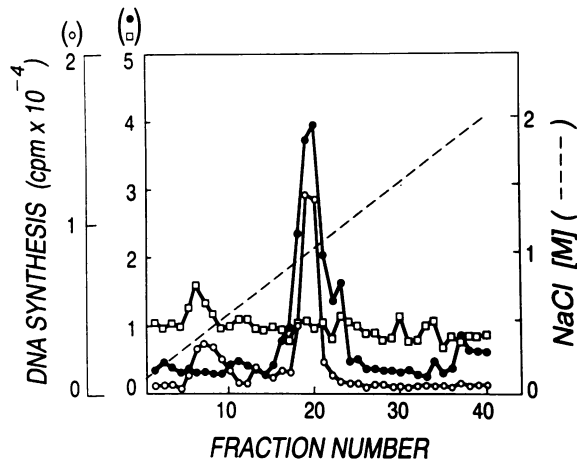
Conditioned media (CM) obtained from 1–2-d-old cultures of human mononuclear cells contained high levels of growth factor activity (~2–10 U/ml), as measured by the stimulation of

DNA synthesis in BALB/c 3T3 cells, whereas relatively little growth factor activity, ~2% as much, was found in cell lysates. When mononuclear cell CM was harvested and analyzed at 48-h intervals, growth factor activity in CM was found to fluctuate with time in culture (Figure 1). Growth factor activity was relatively high 2 d after plating, decreased at 4–6 d, increased at 8–12 d, and slowly decreased after that. The growth factor activity found a week after plating and beyond was strictly macrophage derived since the nonadherent cells had been removed, and the remaining cells were 99.8% pure macrophages as assessed by staining for  $\alpha$ -naphthyl acetate esterase (not shown).

CM collected after plating of mononuclear cells were analyzed by heparin affinity chromatography (Figure 2). At 2 d, two peaks of heparin-binding growth factor (HBGF) activity were found in CM, one eluting at 0.5 M NaCl and the other at 1 M NaCl (Figure 2A). The relative amounts of these growth factors varied in the different preparations that were obtained from the blood of various hospital volunteers. The units of mitogenic activity in the peak eluting at 0.5 M NaCl were in the range of one to five



**Figure 2.** Heparin-Sepharose affinity chromatography of mononuclear cell CM. Approximately 500 ml of crude 1× CM harvested either (A) 1–2 d or (B) 7–11 d after plating mononuclear cells in culture was applied to a column of heparin-sepharose (2 ml). Growth factor activity, as measured by the stimulation of DNA synthesis in 3T3 cells, was eluted with a gradient of 0.2–2.0 M NaCl. In each of the 2.5-ml fractions collected, 10  $\mu$ l were tested for growth factor activity. The recovery of HBGF activity from heparin-Sepharose is in the order of 20–30%.



**Figure 3. Mitogenic activity of heparin affinity purified fractions for 3T3, smooth muscle, and endothelial cells.** Fractions (0.5 ml) were obtained after TSK-heparin FPLC of macrophage-conditioned medium (7–11 d in culture) and 5- $\mu$ l aliquots were analyzed for the ability to stimulate DNA synthesis in BALB/c 3T3 cells ( $\bullet$ ), bovine aortic smooth muscle cells ( $\circ$ ), and bovine capillary endothelial cells ( $\square$ ). The positive control for BALB/c 3T3 is 20% serum (135 000 cpm), for smooth muscle cells it is 20% bovine serum (51 000 cpm), and for endothelial cells it is 2 ng/ml basic FGF (42 000 cpm).

times that of the peak eluting at 1 M NaCl. By 7–11 d after plating, the macrophages secreted the HBGF eluting at 1 M NaCl almost exclusively (Figure 2B), and this pattern persisted thereafter. Cells grown in serum-free medium for 72 h also contained two HBGFs eluting from a heparin affinity column at 0.5 and 1 M NaCl showing that these HBGFs were macrophage-derived and not serum-derived. Growth factor activity that did not bind to heparin was also measured at intervals following plating. At 2 d, non-HBGF activity constituted  $\sim$ 65% of the total activity, but by day 12, non-HBGF was only  $\sim$ 5% of the total (data not shown). Taken together, these results suggested that as the cultured monocytes differentiated into macrophages, most of their secreted growth factor activity, as measured by the stimulation of 3T3 DNA synthesis, was comprised of the HBGF eluting at 1 M NaCl.

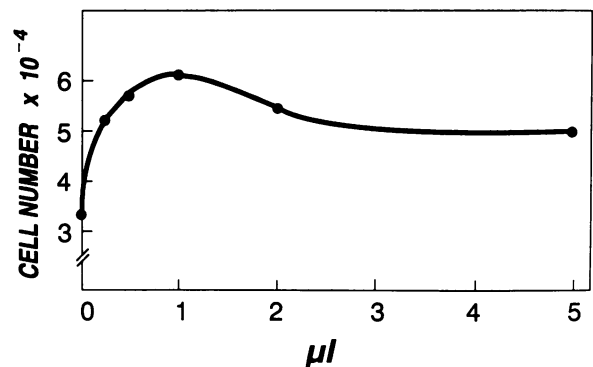
The fractions collected after heparin affinity chromatography of macrophage CM (7–11 d in culture) were also analyzed for their ability to stimulate smooth muscle cell and endothelial cell proliferation (Figure 3). The HBGF eluting at 1 M NaCl, previously shown to be mitogenic for 3T3 cells, was also mitogenic for smooth muscle cells but was not mitogenic for endothelial cells that were responsive to basic FGF. A dose-dependent stimulation of smooth mus-

cle cell proliferation by the HBGF eluting at 1 M NaCl is shown in Figure 4.

#### **Analysis of macrophage CM for PDGF and FGF**

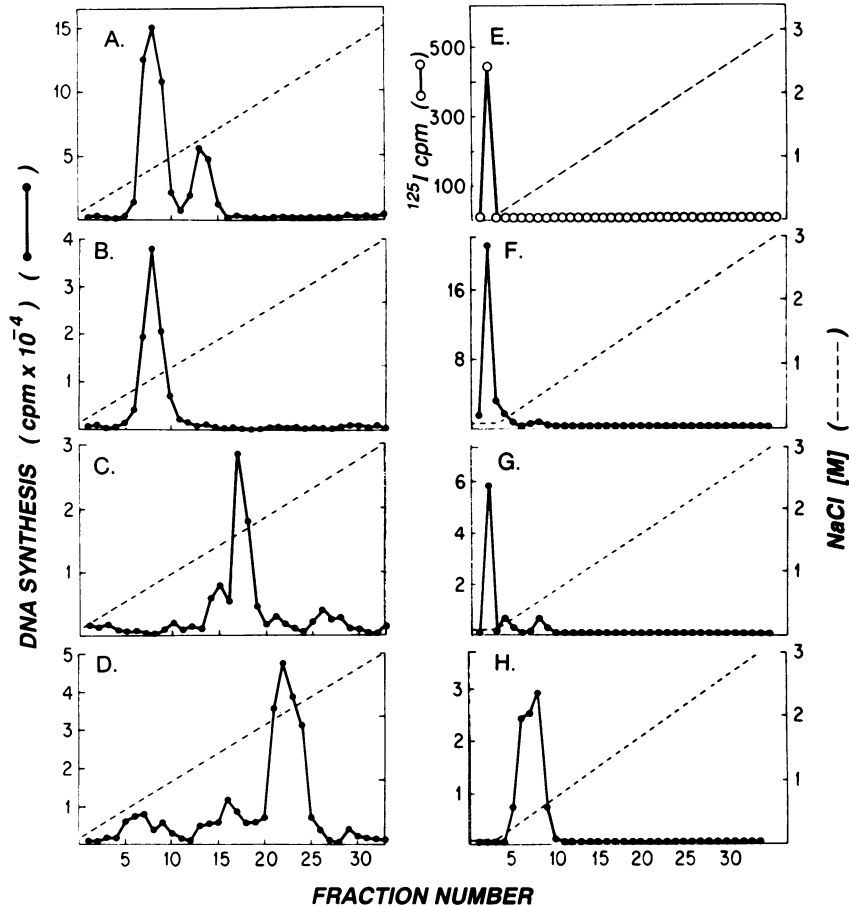
PDGF, aFGF, and bFGF have previously been shown to be HBGFs (Klagsbrun, 1989; Shing *et al.*, 1984; Klagsbrun and Shing, 1985). In addition, macrophages synthesize PDGF (Shimokado *et al.*, 1985; Mornex *et al.*, 1986) and bFGF (Baird *et al.*, 1985). To characterize the macrophage CM-derived HBGFs in a preliminary way, their heparin column elution profiles were compared with those of PDGF, aFGF, bFGF, and other growth factors previously shown to be produced by macrophages (Figure 5). TGF- $\alpha$  (5F), TGF- $\beta$  (5E), and IL-1 (5G) did not bind to heparin, TNF- $\alpha$  (5H) was eluted with 0.1–0.2 M NaCl, PDGF (5B) (AB, AA, or BB forms) was eluted with 0.5 M NaCl, aFGF (5C) with 1.5 M NaCl, and bFGF (5D) with 2 M NaCl. Of the two HBGFs secreted by macrophages (5A), the one eluting at 0.5 M NaCl coincided with PDGF (5B), whereas the HBGF eluting at 1 M NaCl did not coincide with any other of the growth factors. Antisera that inhibited the mitogenic activity of human PDGF (whether AB, AA, or BB dimeric forms) for BALB/c 3T3 cells inhibited the mitogenic activity of the HBGF eluting with 0.5 M NaCl but not of the HBGF eluting at 1 M NaCl (Table 1). These results suggested that the macrophage-derived HBGF eluting at 0.5 M NaCl was PDGF-like, whereas the HBGF eluting at 1 M NaCl was not.

bFGF and aFGF are cell-associated growth factors and are not secreted (Folkman and



**Figure 4. Dose-dependent stimulation of smooth muscle cells.** The peak fractions of HBGF activity eluting with 1 M NaCl shown in Figure 2B were pooled and analyzed for the ability to stimulate the proliferation of smooth muscle cells in a dose-dependent manner. Points represent wells in triplicate. The experiment was repeated four times.

**Figure 5. Heparin-Sepharose elution profiles of known macrophage-secreted factors.** Growth factor samples were applied to columns of heparin-Sepharose. Columns were washed with equilibration buffer and eluted with a linear gradient of 0.1 to 3.0 M NaCl. Fractions were collected and assayed for stimulation of [<sup>3</sup>H]-thymidine incorporation in BALB/c 3T3 cells, with the exception of <sup>125</sup>I-TGF- $\beta$ , where radioactivity was measured. (A) Conditioned medium harvested on days 1–2 of mononuclear cell culture, (B) PDGF, (100 ng human PDGF AB is shown, but similar elution profiles were found for PDGF AA and PDGF BB), (C) aFGF (1000 U, 2–5 U/ng), (D) bFGF (1000 U, 2–5 U/ng), (E) <sup>125</sup>I-TGF- $\beta$  (209  $\mu$ Ci/ $\mu$ g, 10 000 cpm), (F) TGF- $\alpha$  (10  $\mu$ g) (G) IL-1 (200 U), and (H) TNF- $\alpha$  (10  $\mu$ g). Although not shown, <sup>125</sup>I-GM-CSF (70 ng) also elutes in the void volume.



Klagsbrun, 1987). Nevertheless, the fractions collected after heparin affinity chromatography of macrophage CM were tested for the presence of aFGF and bFGF. The fractions were not mitogenic for endothelial cells (Figure 3), suggesting the absence of bFGF or aFGF, both potent endothelial cell mitogens. In a more direct analysis, it was found that neither the HBGF eluting at 0.5 nor 1 M NaCl was inhibited by anti-bFGF neutralizing antibodies (Table 1). aFGF neutralizing antibodies were not available for such analysis. However, Western blot analysis of 100 U of HBGF eluting at 1 M NaCl using antisera that can easily detect 10 ng (20–50 U) amounts of acidic FGF and basic FGF, clearly indicated that it was neither aFGF nor bFGF (Figure 6). Taken together, the lack of endothelial cell mitogenic activity and of interaction with anti-aFGF or anti-bFGF antibodies strongly suggested that macrophages did not secrete biologically active or immunoreactive FGF-like growth factors.

#### Biochemical characterization

The macrophage-derived HBGF eluting with 1.0 M NaCl was partially characterized. Incubation

with 50  $\mu$ g/ml trypsin for 30 min destroyed >96% of the mitogenic activity of this growth factor. Both HBGF eluting at 0.5 M NaCl (PDGF) and HBGF eluting at 1 M NaCl were exposed to 100°C for 10 min and to 5 mM dithiothreitol (DTT). Both HBGF were resistant to the heat treatment. On the other hand, the HBGF eluting with 1.0 M NaCl was resistant to inactivation by DTT under conditions that inactivated PDGF (not shown). The macrophage-derived HBGF eluting with 1.0 M NaCl adhered to the cation exchange column Mono-S and was eluted with 0.7 M NaCl, suggesting it to be a highly cationic protein (Figure 7A). This HBGF was shown to have a molecular mass of 14–25 kDa by gel filtration chromatography (Figure 7B). A combination of heparin-Sepharose, Mono-S, and gel filtration chromatography (each of which has about a 20–30% recovery) was insufficient to purify the higher affinity HBGF.

#### Discussion

Conditioned media of human peripheral blood monocytes cultured for 1–2 d contain two dis-

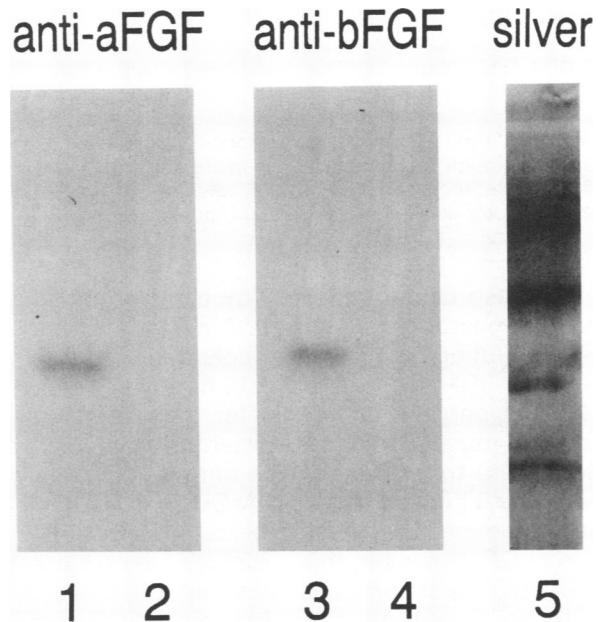
**Table 1.** Effects of anti-PDGF and anti-bFGF antisera on macrophage-derived heparin-binding growth factors

	Antisera to human PDGF		% Inhibition
	- Anti-PDGF	+ Anti-PDGF	
PDGF	49 769 ± 5 499	10 907 ± 1 680	78
HBGF-0.5	111 608 ± 6 585	23 047 ± 24	79
HBGF-1.0	99 968 ± 9 768	90 828 ± 3 750	9
	Antisera to human bFGF		% Inhibition
	- Anti-bFGF	+ Anti-bFGF	
bFGF	80 384 ± 10 456	18 032 ± 3 748	78
HBGF-0.5	29 450 ± 495	25 850 ± 71	12
HBGF-1.0	42 550 ± 4 718	37 750 ± 1 485	11

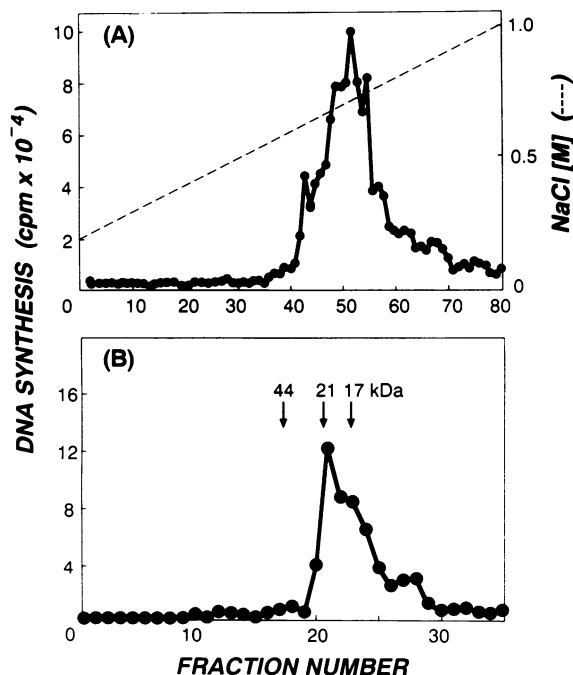
Heparin-binding growth factors eluting at 0.5 and 1.0 M NaCl (HBGF-0.5 and HBGF-1.0, respectively) from a heparin affinity column (Figure 2A) were tested for their ability to stimulate DNA synthesis in BALB/c 3T3 cells in the presence (+) or absence (-) of blocking antisera to human PDGF (1:1000 dilution) or human bFGF (1:10 dilution). Control wells received the same concentration of antisera plus either 0.5 ng/ml human PDGF or 0.5 ng/ml bFGF. The data shows the mean ± SEM of triplicate determination of [<sup>3</sup>H]thymidine incorporation (in cpm), which in the absence of any addition of growth factor was 1000 ± 100 cpm. Nonimmune serum gave the same results as absence of neutralizing antisera.

tinct growth factors that bind to heparin. One of these HBGFs elutes from immobilized heparin with 0.5 M NaCl and can be shown to be PDGF-like, confirming earlier reports of macrophage-derived PDGF (Shimokado *et al.*, 1985; Mornex *et al.*, 1986). The evidence that the HBGF eluting at 0.5 M NaCl is PDGF-like includes coelution with human PDGF on immobilized heparin columns, neutralization of growth activity by antisera that neutralize PDGF, inactivation by disulfide reducing agents, and resistance to heating at 100°C. It is not known at present whether the macrophage-derived PDGF is the AB, AA, or BB heterodimeric form. The second HBGF has a stronger affinity for heparin and is eluted from immobilized heparin with 1 M NaCl. It is not neutralized by anti-PDGF antisera, suggesting that it is not a PDGF-like growth factor. At 1–2 d in culture, it is difficult to ascertain the cellular origin of these two HBGFs because at this point mononuclear cell preparations are contaminated by other cells such as platelets, which are known producers of growth factors such as PDGF (Shimokado *et al.*, 1985; Mornex *et al.*, 1986) and platelet-derived endothelial cell growth factor (PD-ECGF) (Ishikawa *et al.*, 1989). In general, there is about one to five times more PDGF compared with the higher affinity-binding

HBGF in the conditioned medium of 1–2-d-old mononuclear cells. The variation might be due to nonmononuclear cell contamination. On the other hand, after longer term culture of 1 wk and beyond, when the cell population is virtually all adherent macrophages and potential contaminating cells are absent, almost of all the growth factor activity secreted into conditioned medium is due to the HBGF that has a higher affinity for heparin than PDGF. The decrease in PDGF activity with time in culture is of interest. PDGF found in early cultures may be produced by contaminating cells and removal of these cells would diminish PDGF levels. Alternatively, PDGF secretion may decrease as monocytes differentiate into macrophages in the absence of any inducer of PDGF synthesis. Although, it can not be definitively demonstrated that the HBGF eluting at 1 M NaCl is synthesized by monocytes, it certainly appears to be synthesized by adherent macrophages. The mechanism of activation of this HBGF is unclear at this point. Its synthesis is not stimulated any further by treatment of macrophages with PMA but other activating factors can not be ruled out.



**Figure 6.** Western blot analysis with anti-FGF antibodies. Macrophage-derived HBGF eluting from heparin-Sepharose at 1 M NaCl as shown in Figure 2B was analyzed by SDS-PAGE. Western blot analysis was carried out using anti-acidic FGF antisera (lanes 1 and 2) and anti-basic FGF antisera (lanes 3 and 4). Silver stain was used to confirm that the sample of macrophage-derived HBGF contained significant amounts of protein (lane 5). The following samples were applied: lane 1, 12 ng bovine brain acidic FGF; lanes 2, 4 and 5, 100 U of macrophage-derived HBGF eluting at 1 M NaCl; lane 3, 12 ng human recombinant basic FGF.



**Figure 7. Biochemical characterization of macrophage-derived heparin-binding growth factor.** Macrophage-derived HBGF eluting from heparin affinity columns with 1 M NaCl (Figure 2B) was (A) applied to an FPLC Mono-S column and eluted with a gradient of 0.02–1 M NaCl, or (B) applied to a Sephadex G75 column equilibrated in 0.6 M NaCl and 0.02 M Tris-HCl, pH 7.5. Fractions were collected (0.5 ml for Mono S and 1 ml for G-75) and tested (5 and 10  $\mu$ l aliquots, respectively) for the ability to stimulate 3T3 cell DNA synthesis. The recovery from the Mono-S column was 506 units of growth factor activity out of the 2400 U applied (21%). The recovery from the G-75 column was 146 U of growth factor activity out of the 500 U applied (29%).

Because of its secretion by cultures of adherent macrophages, we have designated the HBGF eluting from heparin columns with 1 M NaCl as MD-HBGF, which is a cationic, 14–25 kDa polypeptide mitogenic for 3T3 cells and smooth muscle cells but not endothelial cells. It appears to be heat stable and is not inactivated by DTT. The affinity of MD-HBGF for heparin suggests that it may be related to aFGF and bFGF. However, this is highly unlikely since MD-HBGF is secreted, is not an endothelial cell mitogen, is not neutralized by anti-bFGF antisera, and is not immunoreactive with anti-aFGF or anti-bFGF antisera. It is also unlikely that MD-HBGF is another member of the FGF family, e.g., *int-2*, *hst/Kgf*, FGF 5, and KGF. *int-2* has not been shown to be mitogenic for any cell type, *hst/Kgf* and FGF 5 are mitogenic for endothelial cells, and KGF is specific for epithelial cells (Delli-Bovi *et al.*, 1988; Finch *et al.*, 1989; Zhan *et al.*, 1988). Other known macrophage-derived

growth factors differ from MD-HBGF in that either they do not bind to heparin, e.g., TGF- $\beta$ , TGF- $\alpha$ , TNF- $\alpha$  (Figure 5); they are endothelial cell mitogens, e.g., TGF- $\alpha$  (Schreiber *et al.*, 1986); or they are endothelial cell inhibitors (TGF- $\beta$ , TNF- $\alpha$ ) (Baird and Durkin, 1986; Heimark *et al.*, 1986; Frater-Schroder *et al.*, 1987; Muller *et al.*, 1987; Schweigerer *et al.*, 1987). The macrophage inflammatory proteins (MIP), although heparin-binding, are low molecular weight (8 kDa) anionic polypeptides that are pyrogenic and chemotactic for leukocytes but do not appear to be mitogenic (Wolpe *et al.*, 1988; Wolpe *et al.*, 1989). The monocyte-derived growth factor described by Singh and Bonin (1988) is an anionic 40-kDa polypeptide. Given all the evidence so far, direct and circumstantial, MD-HBGF does not appear to be one of the well-characterized macrophage-derived growth factors and may indeed be novel. However, in the absence of sequence data, we can not yet rule out that MD-HBGF may be a precursor or an altered form of one of the well-characterized macrophage-derived growth factors.

The physiological role of MD-HBGF remains to be determined but two possibilities are wound healing and smooth muscle cell proliferation *in vivo*. The ability of MD-HBGF to stimulate fibroblast growth suggests a possible role in macrophage-mediated formation of granulation tissue, whereas the ability to stimulate aortic smooth muscle cell growth suggests a possible role in blood vessel development. It has been suggested that the smooth muscle cell hyperplasia associated with atherosclerosis is induced by a smooth muscle mitogen, possibly of monocyte/macrophage origin (Ross, 1986; Schwartz and Reidy, 1987). Furthermore, it has been suggested that this mitogen is PDGF, which is produced by macrophages (Shimokado *et al.*, 1985). However, we find that PDGF production diminishes relative to MD-HBGF as monocytes differentiate into macrophages. Thus, MD-HBGF might be an attractive candidate as a macrophage-derived stimulator of smooth muscle cell proliferation, both in normal physiology and in pathology. The affinity of MD-HBGF for heparin might be significant. Heparan sulfate proteoglycans (HSPG) are found in blood vessels and have been shown to be low affinity binding sites for bFGF (Vlodavsky *et al.*, 1987; Moscatelli, 1988; Bashkin *et al.*, 1989). We speculate that MD-HBGF could also bind to HSPG in blood vessels. Perhaps, the combination of heparin-binding growth factors such as bFGF or aFGF, which are endothelial cell mitogens, and MD-HBGF which is a smooth mus-

cle cell mitogen, could act as stimuli for blood vessel development.

## Materials and methods

### Materials

Bovine aFGF was a gift from Dr. Pat D'Amore, The Children's Hospital, Boston, MA. Recombinant human bFGF was supplied by the Takeda Chemical Company, Osaka, Japan. Human PDGF was obtained from PDGF Inc., Boston, MA. Recombinant homodimer and heterodimer forms of human PDGF (AA, AB, and BB), TGF- $\alpha$  and EGF were kindly supplied by Dr. Mark Charette, Creative Biomolecules, Hopkinton, MA. Iodinated human granulocyte macrophage-colony stimulating factor (GM-CSF) was supplied by Dr. Colin Seiff, Dana-Farber Cancer Institute, Boston, MA. Iodinated porcine TGF- $\beta$  was obtained from R & D Systems, Minneapolis, MN. Human cell line-derived IL-1 and recombinant human TNF- $\alpha$  were obtained from Genzyme, Boston, MA. Neutralizing antibodies to bFGF (Kurokawa *et al.*, 1989) were prepared in rabbits immunized with human recombinant bFGF. Neutralizing antibody to PDGF was obtained from Collaborative Research (Bedford, MA).

### Preparation of macrophage-conditioned medium

Buffy coats prepared from whole blood donated by healthy human volunteers were obtained from The Children's Hospital, Boston, MA, layered onto Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and centrifuged at  $4 \times g$  for 20 min at room temperature, using a slight modification of the technique of Boyüm (1968). Mononuclear cells located at the Ficoll-Hypaque/PBS interface were harvested, washed twice in phosphate-buffered saline (PBS), and resuspended in RPMI 1640 medium (Gibco Inc., Grand Island, NY), containing 1% GPS (glutamine, 29.2 mg/ml; penicillin-G, 10 000 U/ml; streptomycin sulfate, 10 000  $\mu$ g/ml) (Irvine Scientific, Santa Anna, CA), to a final concentration of  $1 \times 10^7$  cells/ml. Cells ( $50 \text{ ml}$ ,  $5 \times 10^8$  cells/flask) were plated in T 150 culture flasks in RPMI 1640 containing 10% fetal calf serum (FCS) and 1% GPS and incubated at 37°C. Conditioned medium was harvested every 2 d and replaced with fresh medium. Once macrophages were adherent to the tissue culture flasks (typically 5–7 d), nonadherent cells were washed away, yielding purified macrophage cultures. Cells were maintained in culture in this way for up to 4 wk, with continuous secretion of growth factors.

### Cell proliferation

The proliferation of BALB/c 3T3 cells, bovine capillary endothelial cells, and bovine aortic smooth muscle cells was assayed by measuring the incorporation of [ $^3\text{H}$ ]-thymidine into DNA.

**BALB/c 3T3 cells.** Measurement of DNA synthesis in 3T3 cells was performed as previously described (Shing *et al.*, 1984; Klagsbrun and Shing, 1985). Briefly, BALB/c 3T3 cells were plated in 96 well plates (20 000 cells/well, 6.4 mm in diameter per well, 200  $\mu$ l of medium). After 7 days, the cells formed a confluent quiescent monolayer. Samples (from 1–50  $\mu$ l) were added along with [ $^3\text{H}$ ]-thymidine (1  $\mu$ Ci/well, 4  $\mu$ Ci/ml final concentration, 6.7 Ci/mmol, ICN). After 36–48 h with no change of media, cells were fixed with methanol, DNA was precipitated with 5% trichloroacetic acid, and 0.3 M NaOH was added to solubilize the cells, which were then transferred to scintillation vials. One unit of 3T3 stimulation activity is defined as the amount of growth factor required

to stimulate half-maximal DNA synthesis in BALB/c 3T3 cells.

**Capillary endothelial cells (CEC).** CEC (Shing *et al.*, 1984; Klagsbrun and Shing, 1985) were grown in DMEM, 10% bovine calf serum (BCS), 1% GPS, and plated into gelatinized plates (48 well, 11.3 mm diameter,  $1 \times 10^4$  cells/well). After 24 h, the CEC were refed with DMEM, 2% BCS, 0.5% bovine serum albumin (BSA), and 1  $\mu$ M cold thymidine and incubated for another 24 h. Samples containing growth factor diluted in 0.2% BSA/PBS were added, and 18 h later the cells were pulsed with [ $^3\text{H}$ ]-thymidine (0.6  $\mu$ Ci/well) for 4–6 h. Tritiated thymidine incorporation into CEC DNA was measured in the same manner as 3T3 cells (Shing *et al.*, 1984; Klagsbrun and Shing, 1985).

**Bovine aortic smooth muscle cells (ASMC).** ASMC were obtained from Dr. H. Weich (Children's Hospital, Boston, MA). The ASMC were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% BCS and 1% GPS and plated in 48-well plates at  $1 \times 10^4$  cells/well. The cells were grown for 5 d (with refeeding on day 2), at which point they were confluent. On day 5 the medium was changed to DMEM containing 2% BCS and 1% GPS and cells were incubated for another 24 h. Growth factor samples were added and after 18 h of incubation, the ASMC were pulsed for 6 h with [ $^3\text{H}$ ]-thymidine (1  $\mu$ Ci/well) and incorporation of labeled thymidine was measured as for 3T3 cells and CEC. ASMC proliferation was also determined by counting cell number. In these experiments, ASMC were plated sparsely in 24-well plates in DMEM containing 10% BCS and 1% GPS at  $10^4$  cells/well. Growth factor samples were added and after 3 d cells were removed by trypsinization and counted in a Coulter counter (Coulter, Hialeah, FL).

### Chromatography

**Heparin-Sepharose chromatography.** CM was collected and centrifuged at  $15\,000 \times g$  for 30 min at 4°C. 500–1000 ml of  $1 \times$  CM was applied to a 2-ml column of heparin-Sepharose (H-S) (Pharmacia, Uppsala, Sweden), pre-equilibrated with 0.2 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5. The column was washed with 10 column volumes of equilibration buffer, and then eluted with an 80 ml gradient of 0.2–2.0 M NaCl, 0.01 M Tris-HCl, pH 7.5 at 4°C. (When indicated, gradients of 0.1–3.0 M NaCl were used.) Fractions (2.5 ml) were collected and assayed for stimulation of [ $^3\text{H}$ ]-thymidine incorporation in BALB/c 3T3 cells.

**TSK-heparin FPLC.** Samples were applied to a TSK gel heparin-5PW column (7.5 cm  $\times$  8 mm) (TosoHaas Corp., Woburn, MA), using a Pharmacia FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden). Elution was performed with a linear gradient of 0.2–2.0 M NaCl, and fractions (0.5 ml) were collected and assayed for biological activity.

**Gel filtration chromatography.** Active fractions from the 1.0 M NaCl elution peak of H-S were pooled and concentrated using Centri 10 concentrators followed by Centricon 10 microconcentrators (Amicon Co., Danvers, MA) to a final volume of 200  $\mu$ l. The retentate was then applied to a Sephadex G-75 size exclusion column (Pharmacia), pre-equilibrated with 0.6 M NaCl, 0.02 M Tris-HCl, pH 7.5. Fractions (1 ml) were collected and assayed for 3T3 cell mitogenic activity.

**Cation exchange chromatography.** Active fractions from the 1.0 M NaCl elution peak of H-S were pooled, diluted with 0.01 M Tris-HCl, pH 7.5 to reduce the NaCl concentration to 0.2 M NaCl, and applied to a Mono S cation exchange FPLC column (Pharmacia), previously equilibrated with 0.02 M 2(*n*-morpholino)ethanesulfonic acid (MES), pH

6. The column was washed with equilibration buffer and eluted with a linear gradient of 0.2–1.0 M NaCl in 0.02 M MES, pH 6. Fractions (0.5 ml) were collected and assayed for 3T3 cell mitogenic activity.

### Biochemical characterization

Macrophage-derived HBGF partially purified by heparin-Sepharose chromatography was treated as follows: 1) incubation with 50  $\mu$ g/ml pancreatic trypsin (Sigma) in PBS for 30 min at 37°C; before bioassay on BALB/c 3T3 cells, trypsin was inactivated by addition of 140  $\mu$ g/ml soybean trypsin inhibitor; macrophage-derived HBGF in the absence of trypsin was not inactivated under these conditions; 2) heating to 100°C for 10 min; and 3) incubation with 5 mM DTT (Sigma).

### Polyacrylamide gel electrophoresis

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot and silver stain as previously described (Wadzinski *et al.*, 1987; Vlodayvsky *et al.*, 1987; Iberg *et al.*, 1989). For Western blot, antisera directed against residues 33–43 of bFGF and against residues 59–90 of aFGF were used (Wadzinski *et al.*, 1987).

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