Basic Fibroblast Growth Factor Is an Extracellular Matrix Component Required for Supporting the Proliferation of Vascular Endothelial Cells and the Differentiation of PC12 Cells

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Abstract. Vascular endothelial cells (ECs) seeded sparsely on extracellular matrix (ECM) will proliferate in the absence of exogenous basic fibroblast growth factor (bFGF). This ECM will also stimulate neurite outgrowth in PC12 cells in the absence of exogenous growth factors. We have previously shown that bFGF is found in subendothelial ECM (Vlodavsky, I., J. Folkman, R. Sullivan, R. Fridman, R. Ishai-Michaeli, J. Sasse, and M. Klagsbrun. 1987. *Proc. Natl. Acad. Sci. USA.* 84:2292–2296) and in basement membranes (Folkman, J., M. Klagsbrun, J. Sasse, M. Wadzinski, D. Ingber, and I. Vlodavsky. 1988. *Am. J. Pathol.* 130:393–400). The actual requirement of ECM-associated bFGF for the growth of ECs and differentiation of PC12 cells was shown in two ways. First, polyclonal anti-bFGF antibodies added to subendothelial ECM inhibited both EC proliferation and PC12 neurite outgrowth. Secondly, PF-HR-9 cells, which do not synthesize bFGF and which produce an ECM not permissive for EC proliferation and PC12 neurite outgrowth, were transfected with bFGF cDNA. PF-HR-9 cells transfected with bFGF, but not with the dominant selectable marker SV2-neomycin, were found to express bFGF and to produce an ECM which did support both EC proliferation and PC12 differentiation. The ECM-mediated stimulatory effects were inhibited by anti-bFGF antibodies but not by anti-nerve growth factor antibodies or nonimmune rabbit IgG. These results indicate that bFGF associated with ECM is a required ECM component for ECM-mediated cell proliferation and differentiation.

A ROLE for cell-matrix interactions in the control of cell proliferation and differentiation has been demonstrated both in vivo and in vitro (6, 19, 21, 23, 44). The extracellular matrix (ECM)[1] deposited by cultured bovine corneal endothelial cells (ECs) stimulates proliferation of vascular ECs (17) and neurite extension in the sympathetic cell line PC12 (10, 42). Both these activities are also induced by basic fibroblast growth factor (bFGF) when added to the tissue culture medium (36, 41). Recently, we have extracted bFGF-like growth factors from the subendothelial ECM produced by cultured corneal ECs (39) and from Descemet's membranes of bovine cornea (8). We have also demonstrated that the ECM-associated bFGF is bound to heparan sulfate proteoglycans (HSPGs) (2), suggesting that HSPGs provide a natural storage depot for bFGF and possibly other heparin-binding growth factors (32). However, in view of the molecular complexity of ECM and basement membranes, we could not ascribe a specific biological activity to the ECM-bound bFGF.

The present study was undertaken to investigate the involvement of the ECM-associated bFGF in its induction of EC proliferation and PC12 differentiation. For this purpose we applied two approaches: (*a*) use of anti-bFGF antibodies which inhibit the activity of soluble bFGF (26); and (*b*) comparison of the ECM produced by PF-HR-9 mouse endodermal carcinoma cells (3, 25), which do not synthesize bFGF, and by PF-HR-9 cells transfected with the gene for bFGF. Our results indicate that ECM induction of EC proliferation and PC12 neuronal differentiation is mediated by ECM-bound bFGF.

Materials and Methods

Cells

Cultures of bovine corneal ECs were established from steer eyes as previously described (16). Stock cultures were maintained in DME (1 g glucose/liter) supplemented with 10% bovine calf serum, 5% FCS, penicillin

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; CE-ECM, corneal endothelial cell extracellular matrix; ECs, endothelial cells; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; NGF, nerve growth factor.

(50 U/ml), and streptomycin (50 μg/ml) (Gibco Laboratories, Grand Island, NY) at 37°C in 10% CO_2-humidified incubators. Partially purified, brain-derived bFGF (100 ng/ml) was added every other day during the phase of active cell growth. Cloned populations of adult bovine aortic ECs were isolated and cultured in DME containing 10% bovine calf serum, as described (41). Neomycin-resistant PC12 cells (clone PC12 NI), originally established from a transplantable rat pheochromocytoma (18), were cultured in DME (4.5 g glucose/liter) supplemented with 10% calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). PF-HR-9 cells derived from a differentiated mouse endodermal carcinoma (3) (kindly provided by Dr. R. H. Kramer, University of California, San Francisco, CA), were maintained in DME (4.5 g glucose/liter) containing 10% FCS and antibiotics, as described (25). Cells were maintained at 37°C in 10% CO_2-humidified incubators and subcultured weekly at a 1:10 split ratio after dissociation with saline containing 0.05% trypsin, 0.01 M sodium phosphate, pH 7.2, and 0.02% EDTA (Gibco Laboratories).

Preparation of Dishes Coated with ECM

Cultures of bovine corneal ECs were dissociated from stock cultures (passage 2–5) with saline containing 0.05% trypsin, 0.01 M sodium phosphate, pH 7.2, and 0.02% EDTA, and plated into 35-mm tissue culture dishes (Falcon Labware Division, Becton, Dickinson and Co., Oxnard, CA) at an initial density of 5×10^4 cells/dish. Cells were maintained as described above, except that bFGF was not added, and 5% dextran T-40 was included in the growth medium. 6–8 d after the cells reached confluency, the subendothelial ECM was exposed by dissolving (3 min at 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM NH_4OH in PBS, followed by four washes in PBS (43). The ECM remained intact and firmly attached to the entire area of the tissue culture dish. Alternatively, the ECs were removed intact by exposure (10–20 min) to 2 M urea in DME, a procedure that exposes the subendothelial ECM without lysing the cells (13). Similar results were obtained with both methods. 1 cm^2 of ECM has been shown to contain a total of ~1.8 U (~0.36 ng) of endogenous bFGF activity (39). We have previously demonstrated that the polar secretion of ECM by corneal ECs and the firm interaction of this ECM with the tissue culture plastic, provide an appropriate system to obtain a basement membrane–like substrate, free of cellular elements (17, 43). The presence of nuclei or cytoskeletal elements could not be detected on the denuded ECM when plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence, using antiactin and antivimentin antibodies, or the benzimidole derivative, Hoechst 33258, for nuclear staining (13). Moreover, ECM prepared after a 24-h exposure of subconfluent corneal EC cultures to [^{3}H]thymidine was devoid of labeled material. Likewise, no labeled cell surface components remained associated with the ECM after solubilization (Triton/NH_4OH) of lactoperoxidase-iodinated corneal ECs that were plated on ECM for 24 h. No serum proteins could be identified in the ECM (12).

For preparation of PF-HR-9 ECM, cells (10^5/35-mm dish) were seeded into tissue culture dishes precoated with fibronectin (50 μg/dish) to enforce a firm adhesion of the ECM to the plastic substratum. Ascorbic acid (50 μg/ml) was added on days 2 and 4 and the ECM denuded 6–7 d after seeding the cells, as described above for corneal ECs (25). In contrast with the ECM produced by corneal ECs (CE-ECM), some cellular debris was always left adhering to the PF-HR-9 ECM. The CE-ECM contained mainly fibronectin, laminin, collagen types I, III, and IV, elastin, and sulfated proteoglycans (i.e., HSPGs, dermatan sulfate proteoglycans, and chrondroitin sulfate proteoglycans) (15, 43). Major constituents of the HR-9-ECM were laminin, entactin, collagen type IV, and HSPGs (15, 25).

Transfection of PF-HR-9 Cells with bFGF Expression Plasmid

bFGF expression plasmid (pbFGF) was constructed by inserting the Eco RI–flanked bFGF cDNA sequence from pJJ11-1 plasmid (1) in the 5′ to 3′ orientation behind the SV-40 early promoter sequences into the Eco RI site of the pJay3 mammalian expression vector (33). The plasmid (pbFGF) was transfected into PF-HR-9 cells, as described (37). Briefly, calcium phosphate precipitate of DNA (0.1 μg pSV2-neo plus 40 μg of either NIH 3T3 DNA or pbFGF DNA) was added to 2 × 10-cm dishes of subconfluent PF-HR-9 cells in DME containing 5% calf serum. After 8 h of incubation with these DNA mixtures, the medium was aspirated and cells shocked with 15% glycerol in PBS for 3 min. Cultures were refed with DME containing 10% calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, incubated for 24 h, and subcultured (1:8) in the same medium supplemented with 0.5 mg/ml G418 (Gibco Laboratories). The selection medium was replaced every 3 d for 2 wk. About 20 colonies of PF-HR-9 cells transfected with pSV2-neo (38), with or without pbFGF, were cloned and expanded into cell lines. Lysates of these cell lines were screened for bFGF activity using (*a*) Balb/c 3T3 cell [^{3}H]thymidine incorporation assay (8, 39); and (*b*) immunoblot and immunoprecipitation analysis with anti-bFGF antibodies (7, 33, 39). PF-HR-9 clone HR9/neo transfected with pSV2-neo alone and clone HR9/bFGF transfected with pSV2-neo plus pbFGF were used in the present study.

Growth Factor Activity

Assay for DNA synthesis in 3T3 cells was performed as described (8, 39). Briefly, Balb/c 3T3 cells were plated at half confluence on 0.3-cm^2 microtiter wells in DME supplemented with 10% calf serum. After reaching confluence (2–3 d), the cells were further incubated for a minimum of 5 d. Samples and [^{3}H]thymidine (1 μCi/well) were then added to the quiescent cells and, after an incubation period of 32–40 h, DNA synthesis was assayed by measuring the radioactivity incorporated into TCA-insoluble material. A unit of 3T3 cell growth factor activity (~70,000 cpm) was defined as the amount of growth factor in 0.25 ml required to stimulate half-maximal DNA synthesis in 3T3 cells. Lysates of 10^6 bFGF-transfected HR9 cells (clone HR9/bFGF) contained 80–100 U of growth factor activity. On the basis of previous studies, it is estimated that pure bFGF has a specific activity of ~5 U/ng. Two methods were used to measure proliferation of bovine aortic ECs (9). (*a*) For measurements of DNA synthesis, ECs were seeded at a low density (1×10^3 cells per 2-cm^2 well of a 24-well plate) in 1 ml DME containing 10% calf serum. bFGF was added on days 2 and 4 and [^{3}H]thymidine (2.5 μCi/well) added on day 5 after seeding. 2 h later, the cultures were washed and assayed for [^{3}H]thymidine incorporation into DNA (9). (*b*) ECs were seeded at a clonal density (400 cells per 35-mm dish). On day 9 after seeding, the cultures were fixed with 3.7% formaldehyde and cell colonies were stained with 0.1% crystal violet (9, 39).

Column Chromatography

Heparin–Sepharose affinity chromatography was carried out as described (8, 22, 39). Cells were grown for 5–6 d until they reached confluence. The medium was removed and cells ($6–8 \times 10^7$) were resuspended at 10^7 cells/ml in 10 mM Tris-HCl, pH 7, 1 M NaCl, and lysed by sonication. Cell lysates diluted to 0.1 M NaCl were applied to heparin–Sepharose columns (3 ml) and bFGF was eluted with an 80-ml gradient of 0.2–3.0 M NaCl, at a flow rate of 30–40 ml/h at 4°C. Fractions (4 ml) were collected and tested for stimulation of DNA synthesis in 3T3 cells (22).

Neutralizing Anti-bFGF Antibodies

Anti-bFGF polyclonal antibodies that block the mitogenic effect of bFGF on ECs and the ability of bFGF to stimulate neurite outgrowth in PC12 cells were prepared as described previously (26). Briefly, New Zealand white rabbits were injected twice with human recombinant bFGF (Takeda, Chemical Industries, Osaka, Japan; 140 μg/rabbit per injection) and serum was collected from the ear 7 d after the second injection. The titer of the serum was determined using an enzyme-linked immunoabsorbent assay in which a 96-well plate was coated with 50 ng of bFGF. An anti-bFGF IgG fraction (final concentration of 4 mg/ml) was prepared by protein A–Sepharose column chromatography. The anti-bFGF IgG fraction at a concentration of 200 μg/ml completely inhibited the mitogenic activity of bFGF for 3T3 cells (26).

Immunoblotting

Electrophoretic transfer blots were carried out as previously described (8, 22, 39). Proteins within active fractions isolated by heparin-affinity chromatography were dialyzed, lyophilized, and electrophoresed on 15% SDS-polyacrylamide gels. The gel was equilibrated for 30 min in transfer buffer containing 1% SDS, rinsed briefly in transfer buffer without SDS, and overlaid with 0.1-μm-pore nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH). The electrophoretic transfer was performed at 40 V (Transblot cell; Bio-Rad Laboratories, Richmond, CA) overnight at 4°C. The nitrocellulose paper was either stained for protein with Aurodye–colloidal gold reagent (Janssen Life Sciences Products, Beerse, Belgium) or incubated with anti-bFGF antisera raised against synthetic peptide representing an internal region (positions 33–43) of bFGF, as previously described (8, 22). Immunoreactivity was visualized by successive incubations with biotinylated goat anti-rabbit antibodies, peroxidase-conjugated strepavidin, and 4-chloronaphthol substrate (22, 39).

Table I. Effect of Anti-bFGF Antibodies on bFGF- and ECM-induced Stimulation of EC Proliferation

	EC [^{3}H]thymidine incorporation		
	Plastic	Plastic + FGF	ECM
	cpm × 10^{-3}	*cpm* × 10^{-3}	*cpm* × 10^{-3}
Control	8.3	56.5	84.0
+ Anti-bFGF	6.8	9.7	20.5
+ Nonimmune IgG	8.1	55.3	82.5

Bovine aortic ECs were seeded (5×10^2 cells/cm^2) in DME plus 10% calf serum into regular and CE-ECM-coated wells of 24-well tissue culture plates. Recombinant bFGF (2 ng/ml), rabbit anti-bFGF IgG (40 μg/ml), or nonimmune rabbit IgG were added to some of the ECM-coated wells on days 1 and 3 after seeding. [^{3}H]Thymidine (2.5 μCi/well) was added on day 5 for 2 h and the amount of thymidine incorporation into TCA-insoluble material was determined. Each data point represents the mean of six determinations and the variation between different determinations did not exceed 15% of the mean.

Immunoprecipitation

Cells cultured in 10-cm dishes were radiolabeled for 16 h using 5 ml of cysteine-free DME supplemented with 5% calf serum and 50 μCi of [^{35}S]cysteine (sp act >1,000 Ci/mmol; New England Nuclear, Boston, MA). The cells were washed twice with PBS and scraped into 1 ml cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% NP-40 detergent). After a 5-min incubation on ice, the nuclei were pelleted by a 5-min centrifugation in an Eppendorf centrifuge (Brinkman Instruments Co., Westbury, NY). The supernatant was preadsorbed with 5 μl normal rabbit serum and 40 μl protein A-Sepharose for 4 h at 4°C. The protein A-Sepharose was centrifuged and the supernatant incubated for 16 h at 4°C with 5 μl of anti-amino terminus peptide rabbit antibody (22). 20 μl protein A-Sepharose was added for 1 h of further incubation. The pellet was washed four times with cell lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 1% aprotinin solution. 20 μl SDS-PAGE sample buffer was added to the washed pellet and the sample electrophoresed on SDS-PAGE.

Results

Effect of Anti-bFGF Antibodies on ECM Stimulation of EC Proliferation and PC12 Differentiation

We have previously demonstrated that the ECM produced by cultured corneal ECs replaces the requirement that vascular ECs have for bFGF in order to proliferate and to express their normal phenotype (9, 17). Incubation of this ECM with polyclonal anti-bFGF antibodies inhibited by 70–80% the ECM-stimulated incorporation of [^{3}H]thymidine into sparsely seeded vascular ECs (Table I). These antibodies also inhibited the mitogenic activity of soluble bFGF (Table I) (26). Nonimmune rabbit IgG had no inhibitory effect. The basal incorporation of [^{3}H]thymidine into ECs maintained on regular tissue culture plastic in the absence of bFGF did not exceed 10% of that obtained on ECM and was either little or not affected by the anti-bFGF antibodies (Table I). Inhibition (65–80% in different experiments) of EC proliferation was also obtained when the cells were seeded at a clonal cell density on ECM that was pretreated (30 min) with the anti-bFGF antiserum. Antibodies (40 μg/ml) were added on days 2 and 4 and formation of cell colonies was evaluated 8 d after seeding.

Soluble bFGF induces neurite extension in PC12 cells (36). This neuronal differentiation was inhibited by the polyclonal anti-bFGF antibodies (Fig. 1). PC12 cells plated on ECM in the absence of added bFGF or nerve growth factor (NGF) responded by extension of neuronal processes 24–48 h after seeding. At 3–4 d after seeding, these neurites were at least tenfold longer than the cell diameter (Fig. 2 *A*). Neurite outgrowth ceased after 5–7 d in culture when some cell rounding and detachment was observed. Addition of anti-bFGF antibodies, 12 and 36 h after seeding, resulted in a complete inhibition of neurite outgrowth (Fig. 2 *B*). The cells remained flattened and firmly bound to the ECM, followed by 20–30% cell detachment 6–8 days after seeding. There was no effect to anti-NGF antibodies (Fig. 2 *D*) and to nonimmune rabbit IgG (Fig. 2 *C*). Addition of anti-bFGF antibodies 48–72 h after seeding the PC12 cells on ECM, resulted in retraction of preextended neurites. These results suggest that stimulation of both EC proliferation and PC12 morphological differentiation by ECM are mediated by the ECM-associated bFGF-like molecules.

Expression of bFGF by PF-HR-9 Cells Transfected with bFGF cDNA

PF-HR-9 cells secrete an underlying basement membrane-like ECM (15, 25) which is not mitogenic for vascular ECs. This ECM also failed to induce extension of neuronal processes in PC12 cells. Similar results were obtained with the ECM produced by a neomycin-resistant clone (HR9/neo) of PF-HR-9 cells transfected with the dominant selectable marker pSV2-neo (38) (Figs. 4 *B* and 5 *D*; see next section). When lysates of HR9/neo cells were subjected to heparin-Sepharose chromatography, no growth factor activity for 3T3 fibroblasts and vascular ECs was eluted with a salt gradient ranging from 0.2 to 3.0 M NaCl (Fig. 3 *A*). Metabolic labeling of HR9/neo cells with [^{35}S]cysteine followed by immunoprecipitation of cell lysates with anti-bFGF antibodies failed to reveal any bFGF synthesized by these cells (Fig. 3 *C*). In contrast, a clonal cell population (HR9/bFGF) derived by cotransfecting the expression vector pbFGF with pSV2-neo (33) expressed bFGF, as determined by (*a*) elution of growth-promoting activity from heparin-Sepharose at 1.6 M NaCl (Fig. 3 *A*); (*b*) Western blot analysis of the peak fractions with anti-bFGF antibodies directed against an internal region of bFGF (Fig. 3 *B*); and (*c*) immunoprecipitation of [^{35}S]cysteine-labeled cells (Fig. 3 *C*). The immuno-detected bFGF could be displaced by an excess of the immunizing peptide (corresponding to positions 1–12 and 33–43 of bFGF) (Fig. 3, *B* and *C*).

Growth- and Differentiation-promoting Activities of ECM Produced by bFGF-expressing PF-HR-9 Cells

ECM produced by control PF-HR-9 cells (clone HR9/neo) transfected with pSV2-neo alone and by PF-HR-9 cells (clone HR9/bFGF) transfected with both pSV2-neo and pbFGF were compared for their ability to support proliferation of vascular ECs and neurite extension in PC12 cells. As demonstrated in Fig. 4 *B*, the ECM produced by HR9/neo cells had little or no effect on clonal growth of vascular ECs. Both the number and size of EC colonies were similar or at most slightly higher than those obtained on regular plastic tissue culture dishes (Fig. 4 *A*). In contrast, the ECM produced by PF-HR-9 cells which expressed bFGF induced an extensive clonal growth of vascular ECs (Fig. 4 *C*), similar to that observed with the ECM produced by corneal ECs (Fig. 4 *D*). Stimulation of both clonal growth and DNA synthesis in vas-

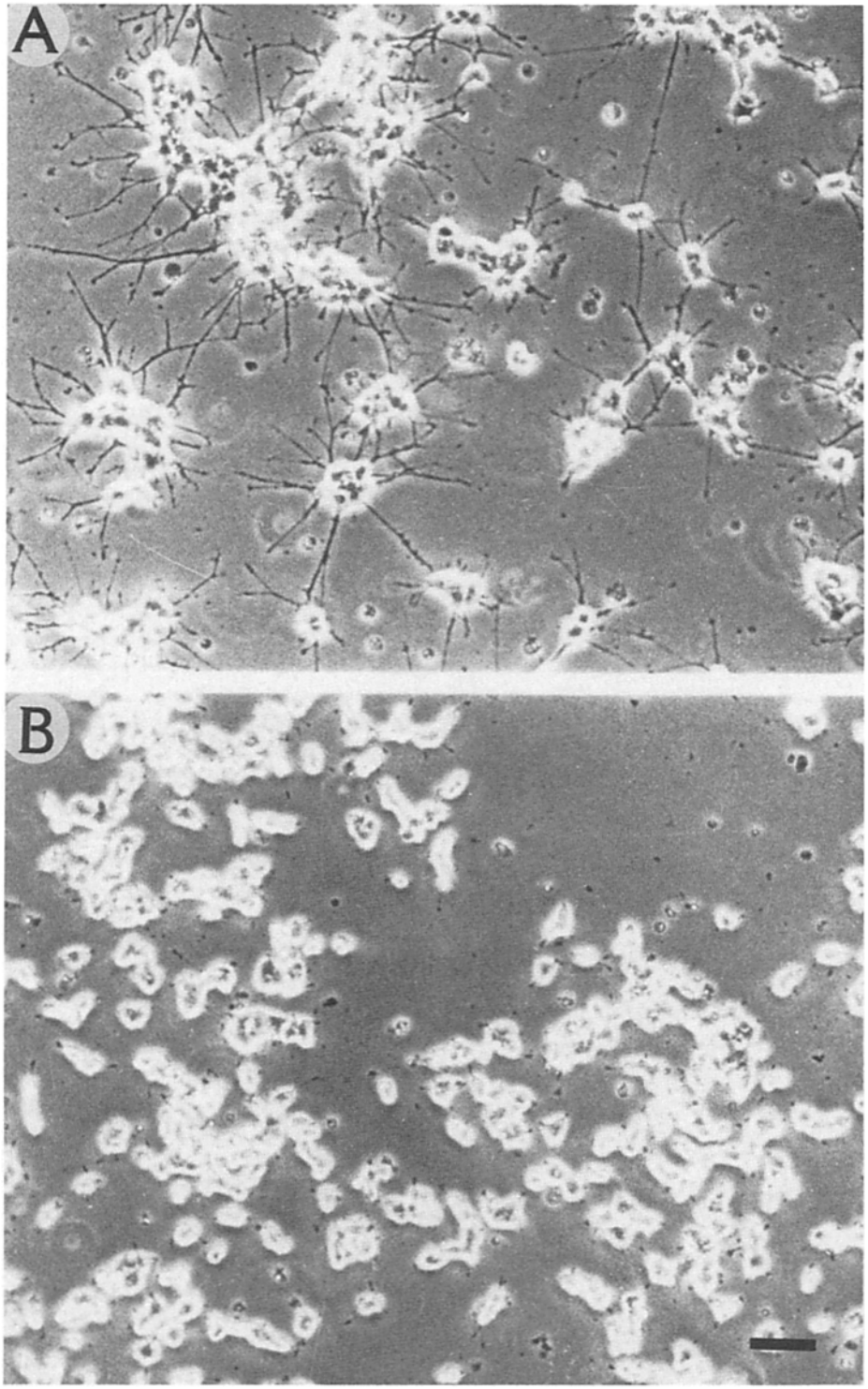

Figure 1. Effect of anti-bFGF antibodies on bFGF-induced neurite outgrowth in PC12 cells. PC12 cells were seeded (10^5 cells/dish) in DME plus 10% calf serum into 35-mm culture dishes. Cultures were incubated with recombinant bFGF (15 ng/ml) in the absence (*A*) or presence (*B*) of polyclonal anti-bFGF antibodies (40 μg IgG/ml). Phase-contrast micrographs were taken 5 d after seeding. Bar, 50 μm.

cular ECs plated on the ECM produced by HR9/bFGF cells was inhibited by anti-bFGF antibodies to an extent which was similar to that observed with cells seeded on the ECM produced by corneal ECs.

Seeding of PC12 cells on ECM produced by transfected clones of PF-HR-9 cells resulted in neurite outgrowth on the ECM produced by cells (clone HR9/bFGF) which expressed bFGF (Fig. 5 *A*), but not by control cells (clone HR9/neo) which showed no detectable synthesis of bFGF (Fig. 5 *D*). Induction of neuronal differentiation was inhibited by anti-bFGF antibodies (Fig. 5 *B*), but not by anti-NGF antibodies (Fig. 5 *C*).

Sequestration of bFGF by heparan sulfate in the ECM may take place when the cell layer is solubilized and the ECM exposed. The following results suggest that this is not the case and that bFGF is deposited into the ECM by intact cells before the actual removal of the cell layer. First, clonal growth of vascular ECs was stimulated to a similar extent regardless

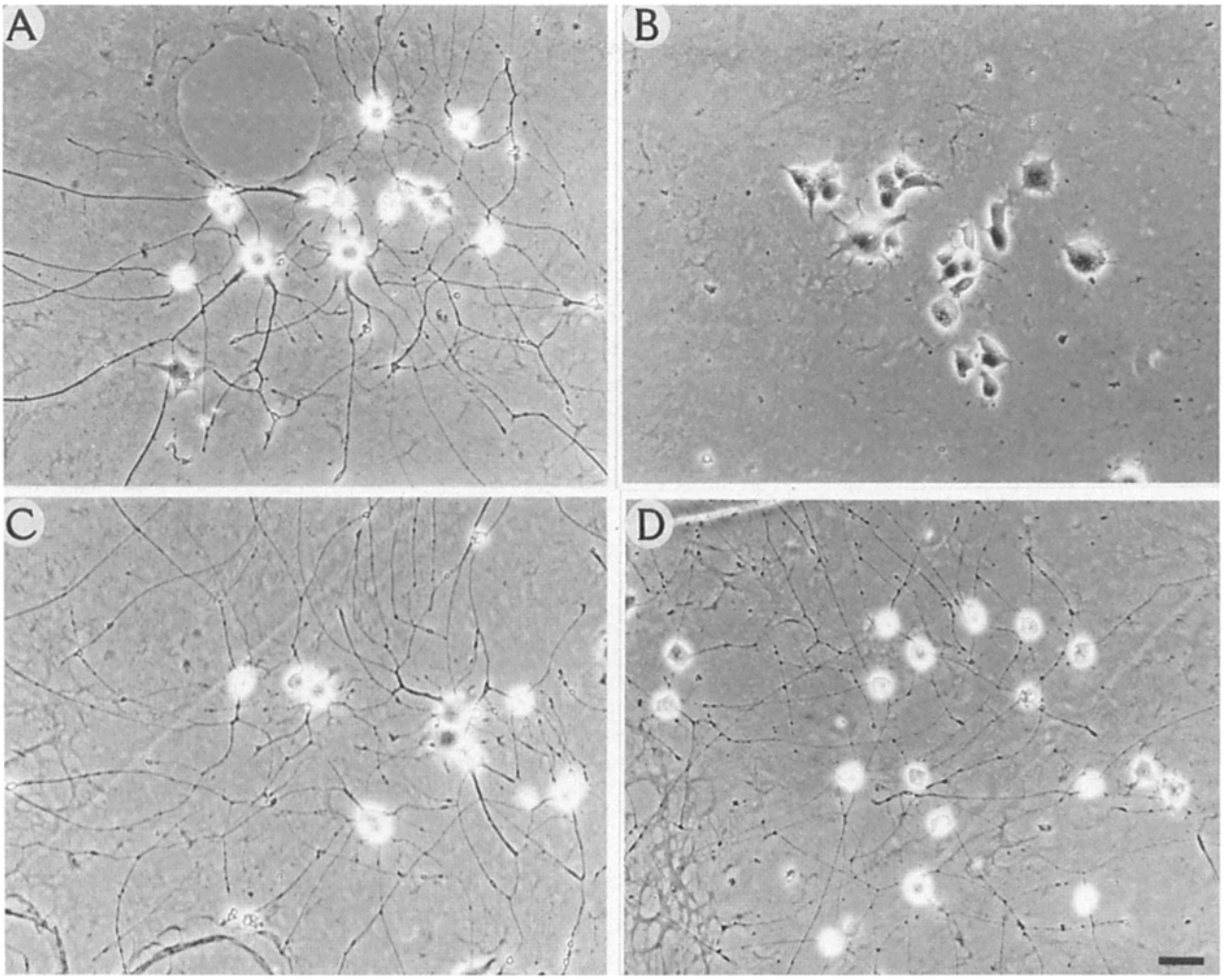

Figure 2. Effect of anti-bFGF antibodies on CE-ECM-induced extension of neuronal processes in PC12 cells. PC12 cells were seeded (10^5 cells per dish) in DME plus 10% calf serum into 35-mm culture dishes coated with CE-ECM. Rabbit anti-bFGF antibodies (*B*), nonimmune rabbit IgG (*C*), or anti-NGF antibodies (*D*) were added (40 μg/ml) to some of the dishes 12 and 36 h after seeding. Control PC12 cells were maintained on ECM alone (*A*). Phase-contrast micrographs were taken on day 4. Bar, 20 μm.

of whether the ECM was exposed by lysing the cells in the absence or presence of heparin. Heparin (50 μg/ml) was included in the lysis solution to inhibit binding of bFGF to the ECM's heparan sulfate when the corneal ECs are lysed (2). In a second experiment corneal ECs were seeded at a confluent density (10^6 cells per 35-mm dish) on top of PF-HR-9 ECM. A short time (10–20 min) after cell attachment and flattening, the cells were lysed with Triton and NH_4OH and the underlying ECM tested for mitogenic activity. The exposed ECM failed to promote clonal growth of vascular ECs. In contrast, ECM exposed 7 d after seeding the same corneal ECs on top of the nonpermissive HR-9 ECM, was highly mitogenic for vascular ECs. Similar amounts of bFGF were determined in lysates of confluent corneal ECs prepared a short time and 7 d after seeding the cells (data not shown).

Discussion

It has now been widely accepted that ECMs play a critical role in the control of cell growth, migration, and differentiation (6, 19, 21, 33, 44). Apparently, the substratum to which a cell is attached can determine the final shape that a cell will adopt and this shape can play a role in the response of cells to serum and plasma factors (7, 14). Whereas some of these responses are mediated by various macromolecular constituents of the ECM (i.e., collagen, laminin, fibronectin, HSPGs) (4, 23), active molecules such as growth factors and enzymes that are firmly associated with the ECM may be involved as well. In previous studies we have reported on the identification and localization of bFGF in basement membranes of the cornea in vivo (8) and in the subendothelial ECM produced by cultured corneal ECs (39). bFGF binds to heparan sulfate in the ECM (2), as was also reported for both granulocyte/macrophage colony-stimulating factor and Interleukin-3 (32). Heparin-binding growth factors have also been identified in bone particles (20, 35).

In the present study we have applied two different approaches to investigate the involvement of the ECM-associated bFGF in its induction of EC proliferation and PC12 neuronal differentiation. Using the ECM produced by cultured

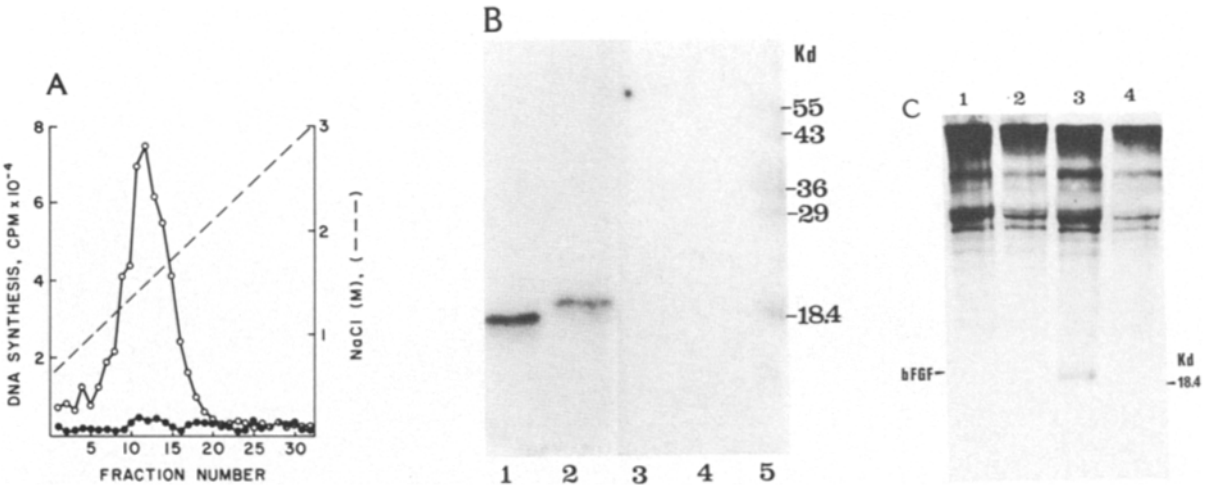

Figure 3. Analysis of bFGF expression in PF-HR-9 cells transfected with bFGF expression plasmid. Clonal cell lines of PF-HR-9 cells transfected with pSV2-neo alone (clone HR9/neo) or pSV2-neo plus pbFGF (clone HR9/bFGF) were obtained as described in Materials and Methods. (*A*) Heparin–Sepharose chromatography. Lysates of control PF-HR-9 cells (clone HR9/neo) transfected with pSV2-neo alone (●) and cells transfected with pSV2-neo plus pbFGF (clone HR9/bFGF) (○) were subjected to heparin–Sepharose affinity chromatography, as described in Materials and Methods. Fractions (4 ml) eluted with a salt gradient (0.2–3.0 M NaCl) were collected and aliquots (10–40 μl) tested for ability to stimulate DNA synthesis ([^{3}H]thymidine incorporation) in 3T3 cells. (*B*) Western immunoblot analysis. Lanes *1* and *3*, recombinant bFGF; lanes *2* and *4*, active fractions (pooled, dialyzed, and lyophilized) eluted from heparin–Sepharose and derived from lysates of clone HR9/bFGF; lane *5*, molecular weight markers. Recombinant bFGF is the truncated 146 amino acid form of bFGF and therefore has a lower molecular weight than native bFGF (154 amino acids). Incubation of the nitrocellulose paper with anti-bFGF antibodies directed against a synthetic peptide representing an internal region (position 33–43) of bFGF (22), was performed in the absence (lanes *1* and *2*) and presence (lanes *3* and *4*) of excess peptide representing amino acids 33–43 of bFGF. (*C*) Immunoprecipitation. Cells were subjected to metabolic labeling with [^{35}S]cysteine and immunoprecipitation with anti-bFGF amino-terminal peptide rabbit antibody (22), as described in Materials and Methods. As a control, 5 μg of the immunizing peptide (representing amino acids 1–12 of bFGF) were added to an identical sample. Lane *1*, parental PF-HR-9 cells; lane *2*, peptide control (PF-HR-9 cells immunoprecipitated in presence of 5 μg peptide *1–12*); lane *3*, HR9/bFGF cells (pSV2-neo plus pbFGF); lane *4*, peptide control of lane *3*.

ECs we have demonstrated the ability of polyclonal anti-bFGF antibodies to inhibit the ECM-mediated stimulation of both EC proliferation and neurite outgrowth in PC12 cells. Experiments performed with the PF-HR-9 mouse endodermal carcinoma cell line demonstrated that PF-HR-9 cells that do not synthesize bFGF deposit ECM which do not promote EC proliferation and PC12 differentiation. In contrast, PF-HR-9 cells that synthesize bFGF after transfection with the bFGF cDNA deposit ECM which induces both clonal growth of vascular ECs and extension of neuronal processes in PC12 cells. These results clearly indicate that ECM-bound bFGF is playing a major role in stimulation of EC proliferation and PC12 differentiation by ECM.

Macromolecular constituents of the ECM and in particular laminin and HSPGs have been reported to promote neurite outgrowth and to have profound effects within the developing nervous system (27). The heparin-binding fragment of laminin promotes nerve cell survival and neurite outgrowth (5). It may, therefore, well be that the interaction of macromolecules (i.e., laminin) and lower molecular weight constituents (i.e., bFGF) of the ECM with cell surface HSPG is playing a role in the initiation and maintenance of morphological differentiation in nerve cells. Since the polyclonal anti-bFGF antibodies inhibit binding of bFGF to high affinity cell surface receptors (30) but not to heparin (26), interaction with specific cell surface sites is likely to be involved in the observed effects of ECM-bound bFGF.

ECM-associated bFGF supports growth and differentiation, but it is not known whether ECM-bFGF must be bound or released to be biologically active. bFGF in ECM is bound to HSPGs with a low affinity (K_d = 6.1 × 10^{-7} M) (2). The heparan sulfate might act to stabilize bFGF (11, 34). Release of bFGF from ECM can be brought about by heparan sulfate-degrading enzymes, by heparin and heparan sulfate, and by proteases that degrade ECM such as collagenase and tryspin (2). ECs synthesize HSPGs and a small proportion of these molecules have the disaccharide structure of heparin (29). These cells also produce plasminogen activator and collagenase whose synthesis is stimulated by bFGF (28). Thus, ECs are capable of solubilizing bFGF from ECM. Alternatively, bFGF might act while still bound to the ECM. Of interest is our finding that PC12 neurites retract when anti-bFGF antibodies are added to ECM. The continuous availability of ECM-bound bFGF might be important in maintaining differentiation of PC12 cells and maybe ECs as well.

We propose that storage of bFGF in ECM provides a novel mechanism for regulation of capillary and connective tissue growth. For example, under normal conditions, ECs form a confluent, contact-inhibited monolayer in blood vessels and may not be responsive to ECM-bFGF. bFGF might also be mostly sequestered so as not to have access to ECs. In response to alterations in basement membrane structure and turnover associated with normal (morphogenesis) and pathological (wounding, tumor progression) processes, bFGF might be released from ECM, possibly by proteases and

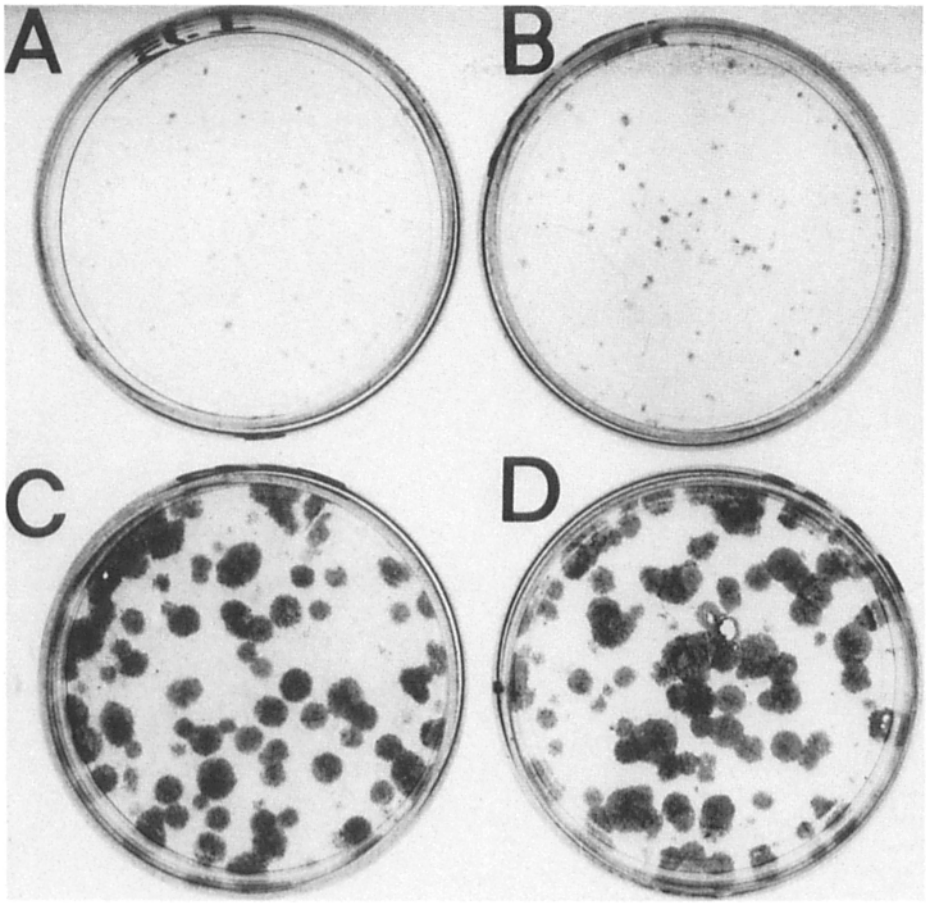

Figure 4. Effect of CE-ECM and HR9-ECM on clonal growth of vascular ECs. Bovine aortic ECs were seeded (300 cells/35-mm dish) on (*A*) regular tissue culture plastic; (*B*) ECM produced by HR9/neo cells (PF-HR-9 cells transfected with pSV2-neo alone); (*C*) ECM produced by HR9/bFGF cells (transfection with pSV2-neo plus pbFGF); and (*D*) ECM produced by corneal ECs. Cell colonies were stained with 0.1% crystal violet 9 d after seeding.

heparan sulfate–degrading enzymes. Thus, it is not necessary to argue that exogenous growth factors acting in a hormonal manner are necessary for proliferative and differentiating events. On the contrary, tissues such as the cornea (8) or blood vessels might be capable of mobilizing their own bound growth factors for repairing damage.

A striking feature of bFGF is that it is mostly an intracellular protein (39, 40) consistent with the lack of a consensus signal peptide in its gene (1). The identification of bFGF in basement membranes of the cornea (8) and of blood vessels (our unpublished observations) in vivo, and in the ECM produced by cultured corneal ECs (39), suggest that bFGF is deposited into ECM in a polar fashion, perhaps by forming an intracellular complex with HSPG. Although no demonstration of such complex formation and bFGF deposition into ECM is presented, our preliminary results on the growth-promoting activity of ECM denuded in the absence and presence of heparin, and as a function of time in culture, strongly suggest that bFGF is in fact being secreted by intact ECs rather than sequestered by the ECM's heparan sulfate when the cell layer is removed and the ECM exposed. Such deposition of bFGF during cell maintenance in culture may occur by means of a nontraditional secretory pathway that does not involve the consensus signal peptide. It is hoped that studies with PF-HR-9 cells transfected with the bFGF cDNA, with or without fusion with a signal sequence (33), will further clarify the mode of bFGF deposition.

Our studies with the ECM produced by corneal ECs and by HR9/bFGF cells demonstrated that the ECM was more effective than soluble bFGF in stimulating the proliferation of vascular ECs. It was also noted that the mitogenic effect of the ECM was not reduced to control levels by antibodies against bFGF. In previous studies, we were unable to detect in ECM extracts any growth-promoting activity which does not bind to heparin–Sepharose or which elutes at a salt concentration <1.5 M NaCl (8, 39). Although this result does not exclude the existence in ECM of growth factors and mitogenic matrix constitutents other than bFGF, it may well be that the matrix is more effective than soluble bFGF due to stabilization and protection of the ECM-bound bFGF (11). It is also conceivable that the anti–bFGF antibodies did not reduce the mitogenic effect of the ECM to control levels because they failed to interact (steric hindrance) with all the ECM-associated bFGF molecules.

Apart from bFGF, the ECM has been shown to contain plasminogen activator (both tissue type and urokinase type) and plasminogen activator inhibitor (reference 31 and our unpublished results). It has also been reported that both plasminogen (24) and thrombin (1a) bind firmly to ECM and are protected from interaction with their natural inhibitors, α_2-plasmin inhibitor and antithrombin III, respectively. These results further emphasize the significance of the ECM as a storage depot for biologically active molecules which bind to ECM and express functional properties that differ from those expressed by the same molecules in a fluid phase. The ECM may thus function as a solid-phase domain for anchor-

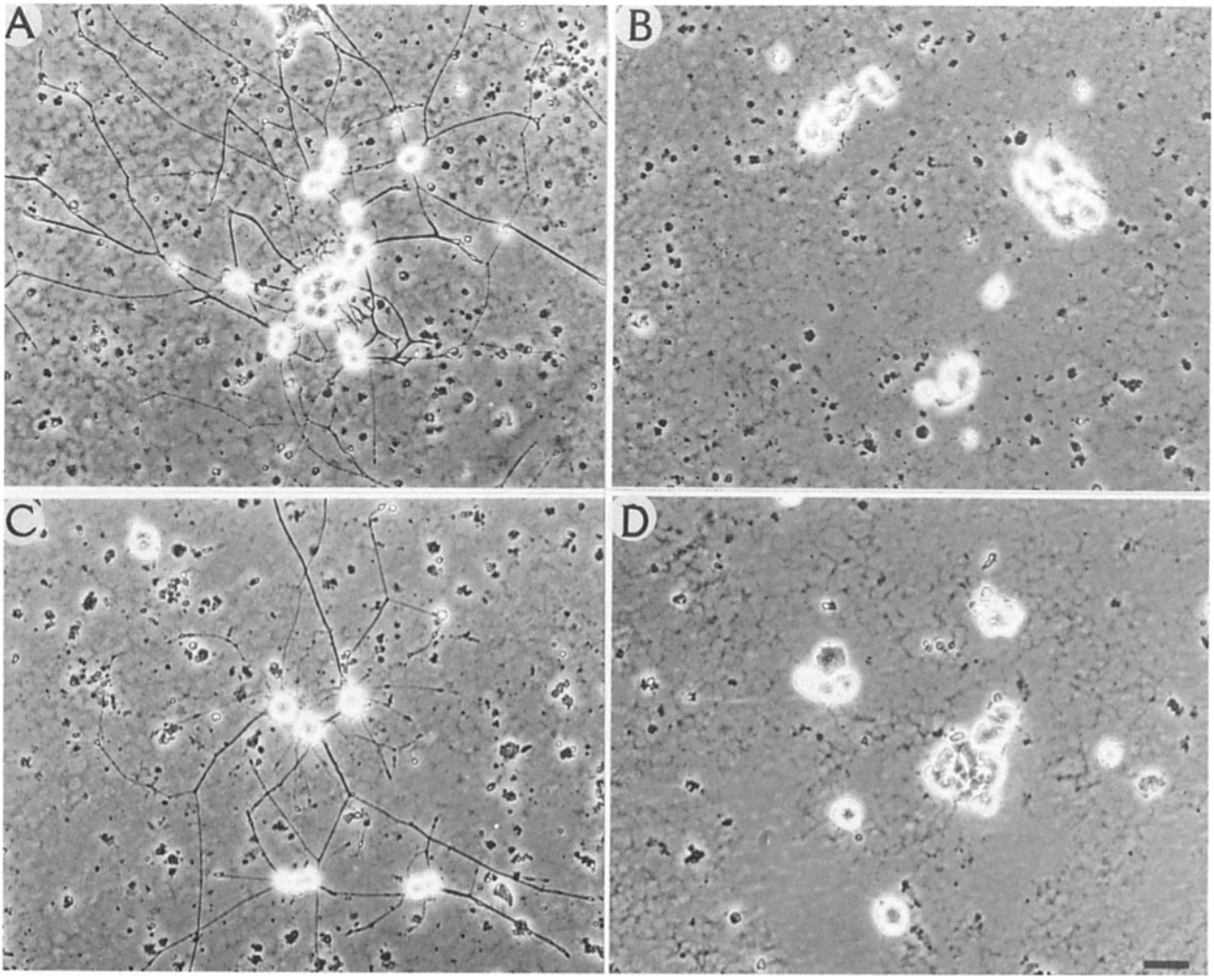

Figure 5. Induction of neurite outgrowth in PC12 cells seeded on ECM produced by PF-HR-9 cells. PC12 cells were seeded on ECM deposited by HR9/bFGF cells (PF-HR-9 cells transfected with pSV2-neo plus pbFGF). Cultures were either untreated (*A*), or incubated with polyclonal anti-bFGF antibodies (*B*), or anti-NGF antibodies (*C*). Antibodies were added (40 μg/ml) 12 and 36 h after seeding and phase-contrast micrographs taken 3 d afterwards. PC12 cells were also seeded on the ECM produced by control PF-HR-9 cells transfected with pSV2-neo alone (clone HR9/neo) (*D*). Bar, 20 μm.

age of active molecules and cellular modulators, resulting in their localization, stabilization, and protection.

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