# Basic fibroblast growth factor in human rhabdomyosarcoma cells: Implications for the proliferation and neovascularization of myoblast-derived tumors

(fibroblast growth factor gene expression/fibroblast growth factor receptor/heparin binding/angiogenesis)

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**ABSTRACT** Cultured human embryonal rhabdomyosarcoma cells express the basic fibroblast growth factor (bFGF) gene and they produce bFGF, which is apparently composed of two microheterogenous forms with $M_r$s of 16,500 and 17,200, respectively. bFGF derived from the rhabdomyosarcoma cells stimulates their own proliferation and that of human or bovine vascular endothelial cells. It is conceivable that the rhabdomyosarcoma-derived bFGF stimulates the growth and neovascularization of human rhabdomyosarcomas and that it may thereby contribute to the development of these tumors.

Basic and acidic fibroblast growth factors (bFGF and aFGF, respectively) are closely related peptide mitogens of $M_r$ 15,000–16,000 (1–6). Both factors are present in neural tissues, and bFGF is also found in a wide variety of other tissues (1–6). bFGF and aFGF have a 55% amino acid sequence homology, they can be purified by heparin-Sepharose (HS) affinity chromatography (1–6), and they bind to the same cell-surface receptor (7). *In vitro*, both can stimulate the proliferation of the same variety of mesoderm- or neuroectoderm-derived cells, including myoblasts and capillary endothelial cells (1), and *in vivo*, both can stimulate the formation of new blood vessels (1, 4, 5), a process termed angiogenesis (8, 9).

When cultured normal cells are transformed into malignant cells, they acquire the ability to proliferate with little or no exogenous growth factors. This is thought to be due to the ability of malignant cells to produce, and to respond to, their own growth factors (10, 11). We have demonstrated that cultured normal myoblasts (12), but not their malignant counterparts, embryonal rhabdomyosarcoma cells (13), require exogenous bFGF to proliferate. This suggested that the embryonal rhabdomyosarcoma cells might produce bFGF and that they might use this mitogen to stimulate their own proliferation. If these cells indeed would synthesize bFGF, they might further be able to induce angiogenesis. This might explain the rich vascularization observed in human embryonal rhabdomyosarcomas (14).

In the present study, we have examined whether cultured human embryonal rhabdomyosarcoma cells might produce bFGF and, if so, whether this mitogen could stimulate their own proliferation and that of vascular endothelial cells.

## MATERIALS AND METHODS

**Materials.** The human embryonal rhabdomyosarcoma cell lines A-204, RD, and A-673 were obtained, respectively, from Naval Biosciences Laboratories (Oakland, CA), American Type Culture Collection, and M. Schwab (University of California, San Francisco). bFGF and aFGF were prepared from bovine pituitaries and brains, respectively, as described (3, 5, 15). Seven free fatty acids/bovine serum albumin complexes (7FFA/BSA) were prepared as follows. The free fatty acids (palmitic, palmitoleic, linoleic, linolenic, oleic, arachidonic, and stearic) were combined in ethanol and the solution was dropped into a swirling solution of phosphate-buffered saline (PBS) containing BSA, to give a final ratio of 0.9 mol of FFA bound to 1 mol of BSA (= 15 mol of FFA per mg of BSA). Tissue culture dishes (530 cm²) were from Nunc. All other materials were from sources given previously (3, 5, 7, 15, 16).

**Cell Culture.** Rhabdomyosarcoma or bovine vascular endothelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and antibiotics (13, 17) (growth medium). Human umbilical vein endothelial cells were grown in medium 199 supplemented with 20% fetal calf serum, Hepes (25 mM), and antibiotics (3) (growth medium). Vascular endothelial cells received bFGF (1 ng/ml) every other day until cultures had become confluent.

**Cell Proliferation Assays.** Vascular endothelial or A-204 rhabdomyosarcoma cells were seeded, at the densities indicated, into 35-mm plastic tissue culture dishes containing growth medium or into extracellular matrix-coated dishes (18) that contained DMEM supplemented with 7FFA/BSA complexes (1 mg/ml), transferrin (25 μg/ml), insulin (15 μg/ml), and antibiotics. Dishes received bFGF, samples to be tested, or no additions. After various time intervals, cultures were trypsinized and cell densities were determined with a Coulter particle counter as described (17). Values of cell densities represent the means of duplicate determinations, which varied by <10%.

**Transfer Blot Analysis of poly(A)⁺ mRNA.** A-204 cells grown to confluency were trypsinized and then lysed in 5 M guanidinium thiocyanate; poly(A)⁺ mRNA was isolated by oligo(dT)-cellulose affinity chromatography (19). Aliquots of poly(A)⁺ mRNA were separated on 1.2% agarose/formaldehyde gels (20) and transferred to nitrocellulose filters (21). The filters were probed either with a $^{32}$P-labeled 1.4-kilobase (kb) *Eco*RI fragment isolated from a bovine bFGF cDNA clone (22) or with a $^{32}$P-labeled 2.3-kb *Hin*dIII fragment isolated from a human aFGF genomic clone (A. Mergia, A. Tumolo, J.A.A., J. L. Whang, D.G., and J.C.F., unpublished data).

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Abbreviations: bFGF and aFGF, basic and acidic fibroblast growth factor(s); HS, heparin-Sepharose; kb, kilobase(s); 7FFA/BSA, seven free fatty acids/bovine serum albumin complexes; BCE cells, bovine brain-derived capillary endothelial cells.
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**Preparation and Purification of Cell Extracts.** A-204 cells grown in 530-cm² dishes were trypsinized, washed, and resuspended in 10 mM Tris·HCl buffer (pH 4.5) containing 250 mM NaCl, and the suspension was sonicated (2 min, 4°C). The pH was readjusted to 4.5 with HCl (1 M), and the crude lysate was stirred (30 min, 4°C) and centrifuged (5 min, 300 × g, 4°C).

The resulting supernatant (S1) was set aside, while the pellet (P1) was resuspended in 10 mM Tris·HCl (pH 7.0) containing 2 M NaCl, stirred (30 min, 4°C), and diluted 1:7 with distilled water, and its pH was readjusted to 7.0; the supernatant obtained after another centrifugation step (15 min, 50,000 × g, 4°C), which is referred to as "nuclear" extract, was filtered and stored at −70°C.

The S1 supernatant received solid NaCl (final concentration, 2 M), and it was stirred (30 min) and centrifuged (15 min, 50,000 × g) at 4°C. The resulting supernatant (S2) was set aside, while the pellet (P2) was resuspended in PBS. The S2 and P2 preparations (referred to as "cytosol" or "membrane" extracts, respectively) were adjusted to pH 7.0; both extracts were dialyzed against PBS, filtered, and stored at −70°C.

To purify A-204 cell extracts, nuclear and cytosol extracts were pooled and applied to a HS affinity column (1.2 × 1.3 cm) that had been preequilibrated with 10 mM Tris·HCl buffer (pH 7.0) containing 0.6 M NaCl. The column was washed with the same buffer and eluted with a gradient of 0.6–3.0 M NaCl in the same buffer (15). The flow rate was 22.5 ml/hr. Fractions of 0.75 ml were collected and aliquots thereof were assayed for their abilities to stimulate cell proliferation. Protein was determined as described (15).

**Preparation and Purification of Conditioned Medium.** A-204 cells grown to confluence in growth medium were incubated in DMEM containing 0.01% BSA and antibiotics. After 2 days, the medium was discarded and replaced. Thereafter, the conditioned medium was collected every 3–4 days as long as the cells appeared viable. The collected medium was centrifuged (20,000 × g) and the supernatants were stored at −20°C. For purification, the conditioned medium (4.5 liters) was thawed, pooled, adjusted to pH 4.5, and stirred (1 hr, 4°C). The pH was adjusted to 6.0, and the conditioned medium was mixed (24 hr, 4°C) with 100 ml of a packed suspension of O-carboxymethyl-Sephadex C-50 (CMS) in 0.1 M sodium phosphate buffer (pH 6.0). The CMS was allowed to settle down and was poured into a column; the adsorbed material was eluted stepwise with 0.1 M sodium phosphate buffer (pH 6.0) containing 0.1 M, 0.15 M, or 0.6 M NaCl. Material eluted with 0.6 M NaCl was further purified by HS affinity chromatography as outlined for the cell extract.

**Radioiodination of Purified Cell Extract.** One hundred microliters of purified A-204 extract (50 ng of protein) and Na$^{125}$I (50 μCi; 1 Ci = 37 GBq) were incubated for 15 min at 21°C in a tube coated with Iodo-Gen (16 μg). The tube then received 100 μl of a buffer containing 30% glycerol (wt/vol), 4% NaDodSO₄ (wt/vol), 150 mM Tris·HCl (pH 6.8), 4 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, 2 mM N-ethylmaleimide, 2 mM iodoacetic acid, 0.1% bromphenol blue, and 0.3 M dithiothreitol. The solution was boiled for 5 min, 10-μl aliquots were subjected to NaDodSO₄ electrophoresis on 10–18% polyacrylamide gels (15), and the gels were developed by autoradiography (7, 16).

**Radioreceptor-Binding Assay.** $^{125}$I-labeled bFGF ($^{125}$I-bFGF) and BHK-21 cell membranes were incubated for 30 min at 21°C (16). Specific $^{125}$I-bFGF binding, which represents bound $^{125}$I-bFGF that was displaced by 200 ng of unlabeled bFGF per ml, was then determined as described (16). Values are the means of duplicate determinations, which varied by <10%.

**Crosslinking Experiments.** Experiments were performed essentially as described (7). In brief, cultures of BHK-21 or

A-204 cells were grown to confluence in 5-cm dishes. The cultures were incubated for 3 hr at 4°C with $^{125}$I-bFGF or $^{125}$I-labeled aFGF ($^{125}$I-aFGF) in the absence or presence of various unlabeled peptides. After the cultures were washed, crosslinking of cell-bound radioactivity was started by the addition of disuccinimidyl suberate (final concentration, 0.15 mM) and the reaction was terminated after 15 min as described (7). The cells were scraped off and lysed, the lysate was boiled, and aliquots of the lysate were analyzed by NaDodSO₄ electrophoresis on 6% polyacrylamide gels and autoradiography (7).

## RESULTS

The rich vascularization observed in human embryonal rhabdomyosarcomas (14) suggested that cultured cells derived from these tumors might contain material able to support the proliferation of capillary endothelial cells. Indeed, as shown in Fig. 1A, extracts from all human embryonal rhabdomyosarcoma cell lines tested (A-204, RD, and A-673) were able to stimulate capillary endothelial cell proliferation. Extracts from the A-204 cell line appeared to be most potent and this cell line was further examined.

All subcellular A-204 extracts were able to stimulate capillary endothelial cell proliferation (Fig. 1B). The cytosol and nuclear extracts seemed to contain most of the bioactivity and their potencies were comparable to that of bFGF.

The possibility that the growth-stimulatory activity present in A-204 cells was due to bFGF was further examined. The cytosol and nuclear extracts (see Fig. 1B) were pooled and subjected to HS affinity chromatography, a method suitable for the selective and rapid purification of FGF (1–6, 8, 9). Most of the protein (>99%) either was not retained by the HS column (not shown) or was eluted in the 0.6 M NaCl wash (Fig. 2). The unadsorbed material had little biological activity (Fig. 3A). When the column was subjected to gradient elution, most (>75%) of the bioactivity eluted with 1.4–1.7 M NaCl (Fig. 2) and the eluted material (fractions 24–28) was able to elicit maximal stimulation of capillary endothelial cell proliferation at a concentration of about 1 ng/ml (Fig. 3A). Gel electrophoretic analysis of the bioactive, radioiodinated material revealed that it was apparently composed of two
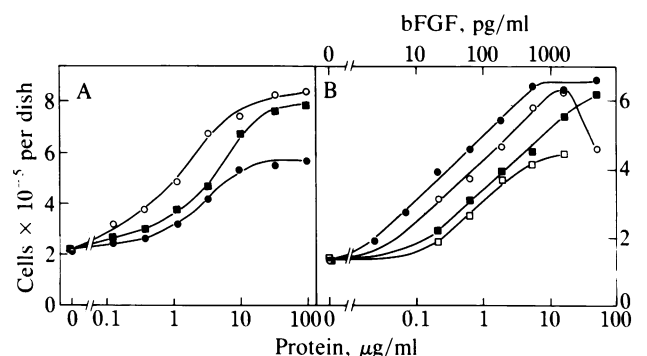


FIG. 1. Effect of crude extracts from various rhabdomyosarcoma cells (A) and of subcellular extracts from A-204 rhabdomyosarcoma cells (B) on capillary endothelial cell proliferation. (A) A-204 (○), RD (●), or A-673 (■) human embryonal rhabdomyosarcoma cells were trypsinized and centrifuged (300 × g, 4°C). The cell pellets were resuspended in PBS and sonicated (2 min, 4°C). The resulting lysates were centrifuged (15 min, 50,000 × g, 4°C) and the indicated amounts of the filtered supernatants were added every other day to bovine brain-derived capillary endothelial cells (BCE cells) that had been seeded at a density of 20,000 per dish. Cells were counted after 5 days. (B) Membrane (□), cytosol (■), or nuclear (○) extracts were prepared from A-204 cells, and aliquots thereof or of bFGF (●) were examined for their abilities to stimulate the growth of BCE cells as outlined in A.
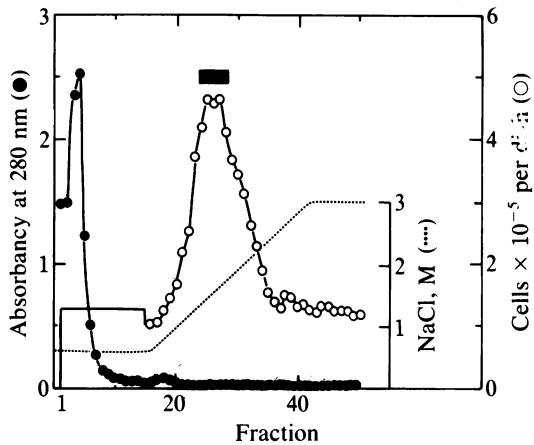
FIG. 2. Purification of rhabdomyosarcoma cell extract by HS affinity chromatography. Cytosol and nuclear extracts prepared from A-204 cells (see Fig. 1B) were pooled and applied to a HS column, and material bound to the column was eluted with a 0.6–3.0 M NaCl gradient, as indicated. The fractions eluted were diluted 1:5 with DMEM containing 0.5% BSA and 10-$\mu$l aliquots thereof were added every other day to BCE cells that had been seeded at a density of 10,000 per dish. Cells were counted after 5 days. Fractions 24–28 (indicated by the solid bar) were pooled for further examination.

peptides with $M_r$s of 16,500 and 17,200, respectively (Fig. 3B).

The fact that the bioactive material could (*i*) bind to HS and elute at 1.6 M NaCl, (*ii*) stimulate capillary endothelial cell proliferation, and that (*iii*) its molecular weight was similar to that of bFGF ($M_r$ = 16,500) (3, 15) suggested that it might be related to bFGF. To assess this possibility, we further examined the material using a specific FGF radioreceptor-binding assay (16). Indeed, as seen in Fig. 4A, the material was able to inhibit specific ^{125}I-bFGF binding to the FGF receptor and its potency was very similar to that of bFGF. Like bFGF, and unlike aFGF (Fig. 4B, left; ref. 7), increasing amounts of the A-204-derived material displaced receptor-bound ^{125}I-bFGF equally well from the $M_r$ 145,000 and $M_r$ 125,000 receptor species. Furthermore, like native bFGF, and unlike aFGF (Fig. 4B, right; ref. 7), increasing amounts

of the material had only little effect on ^{125}I-aFGF binding to the $M_r$ 125,000 receptor species. Thus, the A-204-derived material is indistinguishable from bFGF.

To confirm the synthesis of bFGF in A-204 cells, poly(A)$^+$ mRNA obtained from the cells was subjected to RNA transfer blot analysis and hybridization with a bFGF-specific cDNA probe (22). As seen in Fig. 4C, A-204 cells contain 7.0- and 3.7-kb transcripts of the bFGF gene, which are identical in size to those identified in bovine hypothalamus and in the human hepatoma cell line SK-Hep1 (22). [In a previous paper (22), the sizes of the bFGF gene transcripts were incorrectly reported as being 4.6 and 2.2 kb, respectively. The correct sizes are 7.0 and 3.7 kb, respectively.] Transcripts of the aFGF gene could not be detected (results not shown).

The A-204 cell-derived bFGF was able to stimulate the proliferation of various vascular endothelial cell species, including vascular endothelial cells derived from bovine brain (see Fig. 3A) or adrenal cortex (Fig. 5A) capillaries or from human umbilical vein (Fig. 5A).

To determine whether medium conditioned by A-204 cells might contain bFGF, serum-free conditioned medium was subjected to HS chromatography. In fact, the conditioned medium did contain bFGF, which, like the bFGF from A-204 cell extract, eluted from HS columns with 1.4–1.7 M NaCl (data not shown) and could stimulate the proliferation of BCE cells (Fig. 5B).

HS-purified bFGF derived from A-204 extract or conditioned medium could also stimulate the proliferation of A-204 cells (Fig. 6A), and the extract-derived bFGF exerted its maximal effect at a concentration of about 1 ng/ml. To determine whether the A-204-derived bFGF might stimulate A-204 cell proliferation by its interaction with specific FGF cell-surface receptors, we performed crosslinking experiments using pituitary-derived ^{125}I-bFGF as a ligand. Indeed, ^{125}I-bFGF labeled two $M_r$ 145,000 and $M_r$ 125,000 macromolecular species on the A-204 cell surface and the labeling intensity of these species decreased when incubations were done in the presence of unlabeled bFGF (Fig. 6B, lanes 1 and 2, respectively). The labeled macromolecular species are identical in size to those identified on BHK-21 cells (see Fig. 4B and refs. 7 and 16). These results indicate the presence of
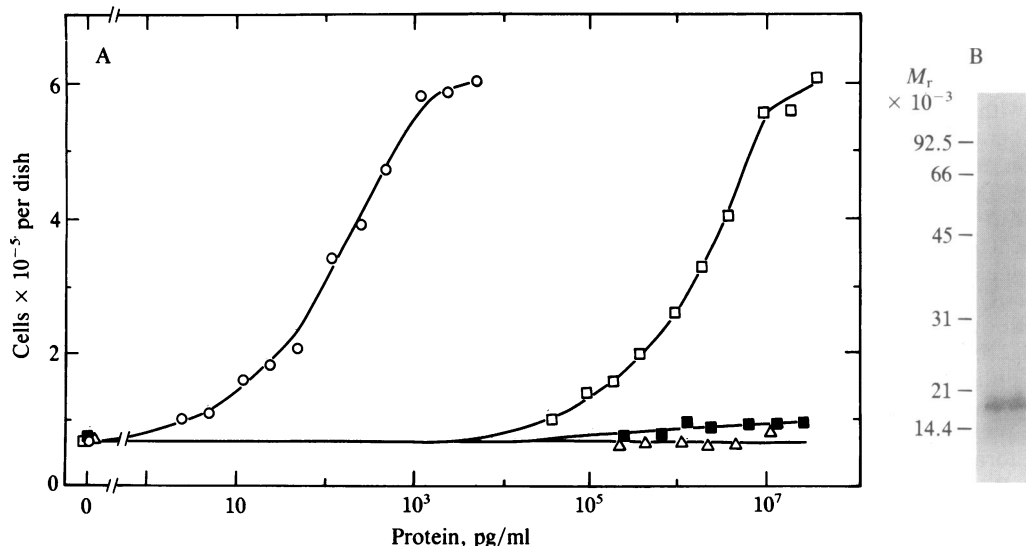


FIG. 3. Analysis by cell proliferation assay (A) or gel electrophoresis (B) of different fractions obtained by HS chromatography of A-204 rhabdomyosarcoma cell extract. (A) Input (i.e., the combined nuclear and cytosol extracts) (□), breakthrough (△), or the combined fractions 1–15 (■) or 24–28 (○) from the experiment depicted in Fig. 2 were diluted in DMEM/0.5% BSA, and aliquots (10 $\mu$l) of the dilutions that contained the indicated amounts of protein were added every other day to BCE cells. Seeding and counting of BCE cells were as described in the legend to Fig. 2. (B) An aliquot of the combined fractions 24–28 from the experiment shown in Fig. 2 was radioiodinated and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. An autoradiogram of the gel with the corresponding molecular size markers is shown.
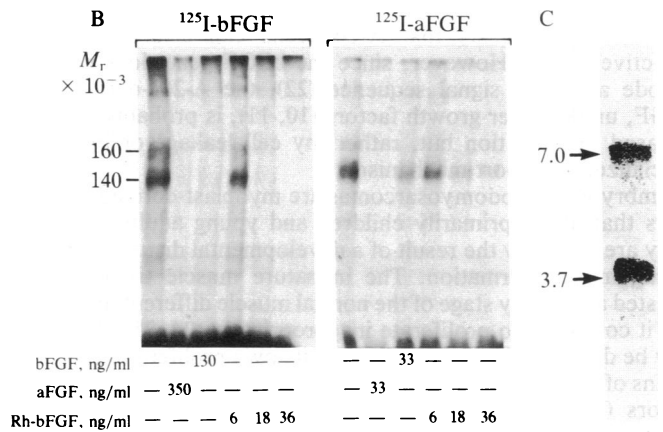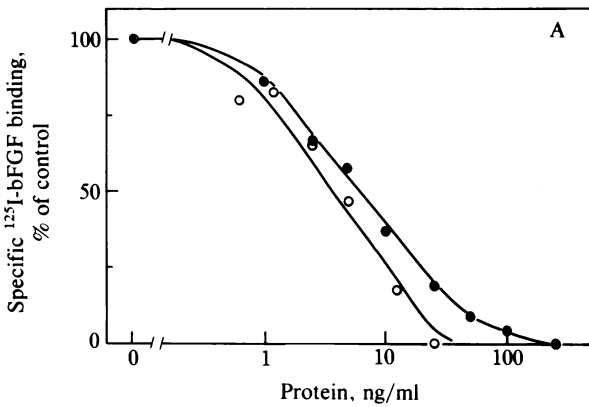
FIG. 4. Analysis of bFGF purified from A-204 extract by radioreceptor-binding assay (*A*) or crosslinking experiments (*B*) and analysis of A-204-derived poly(A)$^+$ mRNA by RNA transfer blot and hybridization with a bFGF-specific cDNA probe (*C*). (*A*) $^{125}$I-bFGF (250 pM) and BHK-21 cell membranes (16 $\mu$g of protein) were incubated with the indicated amounts of dialyzed A-204-derived bFGF (o) or native bFGF (●), respectively, and specific $^{125}$I-bFGF binding was determined. (*B*) $^{125}$I-bFGF (50 ng/ml) (left) or $^{125}$I-aFGF (20 ng/ml) (right) were incubated with BHK-21 cells (5 × 10$^6$ per dish) for 3 hr at 4°C in the absence or presence of the indicated amounts of unlabeled bFGF or aFGF or dialyzed A-204-derived bFGF (Rh-bFGF), respectively. See text for discussion of crosslinking of bound radioactivity and analysis of the crosslinked species. Shown are autoradiograms of gels with the molecular sizes of the binding site species ($M_r$ = 160,000 or 140,000, respectively) prior to subtraction of the $M_r \approx 16,000$ of the $^{125}$I-labeled ligands. (*C*) Aliquots of poly(A)$^+$ mRNA prepared from A-204 cells (5 $\mu$g per lane) were analyzed for the presence of bFGF gene transcripts by RNA transfer blot. An autoradiogram of a gel with the sizes of the hybridizing mRNAs (in kb) is shown. [In a previous paper (22), the sizes of the bFGF gene transcripts were incorrectly reported as being 4.6 and 2.2 kb, respectively. The correct sizes are 7.0 and 3.7 kb, respectively.]

specific FGF-binding sites (receptors) on the A-204 cell surface.

In conclusion, A-204 rhabdomyosarcoma cells produce and release material indistinguishable from bFGF that can stimulate the proliferation of vascular endothelial cells; interestingly, it can also stimulate the proliferation of A-204 cells, presumably by means of binding to specific FGF receptors. Thus, the A-204-derived bFGF can act as a self-stimulating growth factor for cultured rhabdomyosarcoma cells.

## DISCUSSION

We have demonstrated here the presence in the human embryonal rhabdomyosarcoma-derived A-204 cell line of a mitogen indistinguishable from bFGF, as judged by a variety of criteria. These include its chromatographic behavior on HS, its biological activity and potency, and its ability to crossreact with bFGF in a specific FGF radioreceptor-

binding assay. Furthermore, its interaction with the FGF receptor species is indistinguishable from that of bFGF. That this mitogen is synthesized in A-204 cells and does not represent accumulated exogenous bFGF is demonstrated by the presence of bFGF gene transcripts in A-204 cells.

Analysis of the A-204-derived bFGF by NaDodSO$_4$ gel electrophoresis revealed that it is apparently composed of two microheterogenous forms with $M_r$s of 16,500 and 17,200. The latter form differs from that of bFGF ($M_r$ = 16,500) (1, 3) and might represent an extended form of bFGF. This possibility would be consistent with the demonstration that cells can synthesize *N*-terminally extended bFGF versions (23).
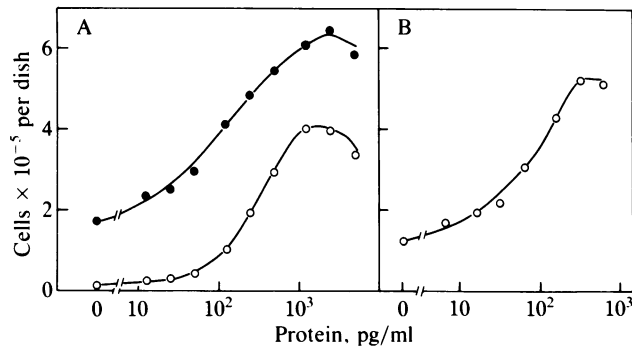


FIG. 5. Effect of bFGF purified from A-204 cell extract on the proliferation of bovine adrenal cortex-derived capillary endothelial (ACE) or human umbilical vein endothelial (HUVE) cells (*A*) and effect of bFGF from A-204-conditioned medium on the proliferation of BCE cells (*B*). ACE (●), HUVE (o), or BCE cells were seeded at densities of 20,000, 40,000, or 10,000, respectively, per dish. Every other day, cells received the indicated amounts of A-204-derived bFGF. ACE, HUVE, or BCE cells were counted after 4, 7, or 5 days, respectively.
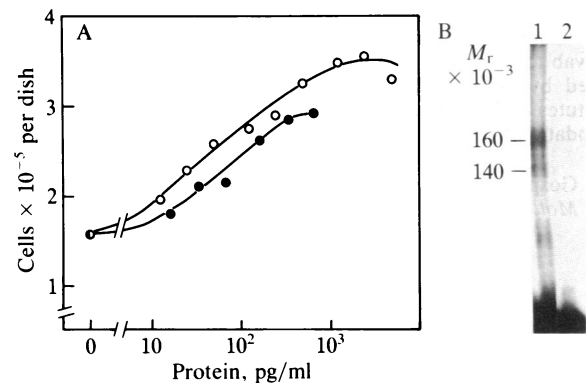


FIG. 6. Effect of bFGF isolated from A-204 cell extract or conditioned medium on the proliferation of A-204 cells (*A*) and crosslinking of $^{125}$I-bFGF to A-204 cells (*B*). (*A*) A-204 cells were seeded at a density of 40,000 per extracellular matrix-coated dish containing serum-free medium. Every other day, they received the indicated amounts of bFGF derived from A-204 cell extract (o) or conditioned medium (●). Cells were counted after 7 days. (*B*) A-204 cells grown in growth medium were incubated with $^{125}$I-labeled pituitary bFGF (10 ng/ml) in the absence (lane 1) or presence (lane 2) of unlabeled pituitary bFGF (1 $\mu$g/ml). See text for discussion of crosslinking and analysis of the crosslinked material. Shown is an autoradiogram of a gel with the molecular sizes of the binding-site species ($M_r$ = 160,000 or 140,000, respectively) prior to subtraction of the $M_r$ = 16,500 of bFGF.

The presence in A-204 cell-conditioned medium of bFGF-like activity suggests that A-204 cells can also release bioactive bFGF. However, since the bFGF gene does not encode a typical signal sequence (22), the A-204-derived bFGF, unlike other growth factors (10, 11), is probably not released by secretion but, rather, by cell leakage or by a specialized transport mechanism.

Embryonal rhabdomyosarcomas are myoblast-derived tumors that affect primarily children and young adults (14). They are probably the result of a developmental disturbance during muscle formation. The immature muscle tissue is arrested at an early stage of the normal muscle differentiation and it continues to proliferate inappropriately (24, 25). This may be due to its ability to stimulate its own proliferation by means of inappropriate production of self-stimulating growth factors (10, 11). In fact, as demonstrated here, cultured embryonal rhabdomyosarcoma cells can produce and release bFGF and the released bFGF can stimulate their own proliferation, presumably by means of binding to specific FGF cell-surface receptors. Our results indicate that bFGF might act as a self-stimulating growth factor for embryonal rhabdomyosarcomas and suggest that bFGF may play a key role in the development of human embryonal rhabdomyosarcomas.

bFGF may also be involved in the progression of these tumors. Our results show that the rhabdomyosarcoma-derived bFGF can stimulate the proliferation of various vascular endothelial cell species, indicating that it might be able to induce the vascularization of these tumors *in vivo*. This would not only allow their increased supply with nutrients required for further tumor growth but also their metastasis. That bFGF-induced angiogenesis could occur *in vivo* is supported by the rich vascularization of human embryonal rhabdomyosarcomas observed *in vivo* (14).

In conclusion, our results suggest that human embryonal rhabdomyosarcomas can support their own growth as well as their vascularization by using bFGF as a mediator. This 2-fold growth advantage might endow the tumors with an excessive proliferative potential as well as with the ability to get access to the vascular system and to metastasize. Thus, these mechanisms might contribute to the poor prognosis of embryonal rhabdomyosarcomas (14, 26).

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