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Cell Biology

Transforming and nontransforming growth factors are present in medium conditioned by fetal rat calvariae

(fibroblast growth/bone growth factors/transforming growth factors)

MICHAEL CENTRELLA* AND ERNESTO CANALIS

Department of Medicine (Endocrine Section) and Research Laboratory, Saint Francis Hospital and Medical Center, Hartford, CT 06105; and The University of Connecticut Health Center, Farmington, CT 06032

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ABSTRACT **Conditioned medium recovered from fetal rat calvarial cultures contains an autocrine factor termed bone-derived growth factor (BDGF); this factor has been purified by acid extraction, gel-permeation chromatography, and two reversed-phase HPLC steps and examined for mitogenicity on normal rat kidney fibroblasts (NRK, clone 49F). HPLC-purified BDGF caused a dose-related increase in cell number, DNA content, and [^{3}H]thymidine incorporation into acid-insoluble material. Since highly purified BDGF appeared less mitogenic than cruder preparations, the latter were tested for additional growth factors, with particular attention to those required for anchorage-independent colony formation in soft agar. BDGF did not displace ^{125}I-labeled epidermal growth factor (EGF) in a radioligand–receptor assay, indicating the absence of EGF and transforming growth factor α (TGF-α). Without EGF, no BDGF preparation induced NRK cells to form soft agar colonies. However, calvarial conditioned medium contained a factor which, like TGF-β, induced large soft-agar colonies in the presence of EGF; this TGF-β-like factor did not copurify with BDGF. Polyclonal antibodies against platelet-derived growth factor did not neutralize the effects of BDGF on NRK cells. BDGF is a potent mitogen for nonskeletal-tissue-derived fibroblasts. Although crude BDGF preparations do contain TGF-β, BDGF is distinct from this factor and others necessary for NRK cell transformation to anchorage-independent growth.**

Studies with conditioned medium (CM) obtained from fetal rat calvarial cultures suggested the presence of biologically active molecules that stimulated DNA and protein synthesis in subsequently cultured calvariae (1). The major bone growth promoter in these preparations, bone-derived growth factor (BDGF), also stimulates cartilage growth in rabbit costal chondrocyte cultures (2), but the effect of BDGF on nonskeletal tissue has not been reported.

NRK-49F are continuously cultured cells cloned from a normal rat kidney fibroblast (3); they exhibit density-dependent growth arrest after reaching confluence in culture but can be stimulated to divide further by fresh serum or mitogenic factors. Some of these factors have been termed transforming growth factors (TGFs; refs. 4 and 5) because they induce alteration from normal cell growth on a culture surface to anchorage-independent colony formation in soft-agar suspensions (6). This characteristic has been consistently correlated with tumorigenicity (7). TGFs have been isolated directly from normal and neoplastic tissues or from their culture medium (6, 8–12) and have been classified with relation to mouse epidermal growth factor (EGF). Rat TGF-α, a polypeptide of M_r 5600, binds to the EGF receptor and shares extensive sequence homology and biological properties with EGF (5); both factors can induce small NRK cell colony formation in soft agar (11). TGF-β, a polypeptide dimer of M_r 24,000, alone is inactive but in the presence of EGF or TGF-α produces larger soft-agar colonies when assayed on NRK-49F cells (6). Both TGF-α and TGF-β have at times been isolated from the same tissue sources (11, 13); the mechanism of TGF-β action appears related to the enhanced expression of EGF/TGF-α receptors (14). In addition, EGF-dependent cell transformation requires serum or low levels of some serum factors, including platelet-derived growth factor (PDGF; ref. 15) and somatomedin (16). Other TGFs (termed TGF-γ) were originally reported as EGF-independent transforming agents, but at present their status is unclear due to the complexity of the interactions between TGF-α, and TGF-β, and other serum factors in various culture systems (4, 15–18).

Rat calvarial BDGF has been purified by gel-permeation chromatography and reversed-phase HPLC (rpHPLC). Preliminary studies in calvariae suggested that more purified BDGF preparations were less mitogenic than material derived from Sephadex chromatography. Therefore, we questioned whether rpHPLC separated interdependent activities and tried to determine whether our preparations shared biological or biochemical properties with TGF-α, TGF-β, or PDGF. The last factor has special significance, since osteosarcoma cells produce material immunologically cross-reactive with PDGF (19). As a result of this work we have begun to describe some of the growth-stimulating activities of BDGF for nonskeletal tissue-derived fibroblasts.

MATERIALS AND METHODS

Preparation of BDGF. Calvariae from 21-day rat fetuses were cultured in serum-free BGJ$_b$ (GIBCO) medium without albumin and phenol red. Culture medium was collected daily for 4 days, pooled, acidified, and dialyzed (M_r cut-off 3500) against 0.05 M acetic acid. The retentate (CM-BDGF) was lyophilized, redissolved in 1.0 M acetic acid, and filtered through Sephadex G-75; the material that was eluted at M_r 6000–45,000 (Sephadex-BDGF) either was pooled, lyophilized, and stored at −70°C until testing or was fractionated further by rpHPLC on μBondapak C_{18} (Waters Associates). Sephadex-BDGF was applied to the column in 20% methanol/0.025 M acetic acid and eluted with a methanol gradient in 0.1 M acetic acid. Biologically active material was eluted at 50–60% methanol; the active fractions (C_{18}-BDGF)

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Abbreviations: BDGF, bone-derived growth factor; CM, conditioned medium; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; rpHPLC, reversed-phase HPLC. CM-BDGF is acidified and dialyzed calvarial CM. Sephadex-, C_{18}-, and CN-BDGF, respectively, represent BDGF purified by sequential Sephadex G-75 chromatography and μBondapak C_{18} and CN rpHPLC.

*To whom reprint requests should be addressed at Research Laboratory, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105.

were collected, pooled, dried, and rechromatographed by rpHPLC on μBondapak CN. Gradient elution in 0.1% trifluoroacetic acid produced active material at ≈23% acetonitrile (CN-BDGF). When 1.2 μg of CN-BDGF (equivalent to 100 ml of CM) was subjected to $NaDodSO_4$/PAGE in the absence of reducing agents, silver staining showed a band that stained negatively (white), indicating vast protein excess (personal communication, W. Wray, National Institutes of Health), at M_r 11,000; this overloaded gel also revealed two faintly staining bands at M_r 22,000. Relative protein concentrations were determined by the Bradford method (20) with bovine serum albumin as standard.

To assay BDGF for EGF (TGF-α), TGF-β, and PDGF, we tested either crude CM-BDGF, Sephadex-BDGF, or C_{18}-BDGF to study the effects of BDGF on NRK cell replication and anchorage-independent colony formation, we used highly purified CN-BDGF. Since Sephadex-BDGF contained multiple activities, it was tested as a M_r 6000–45,000 pool and as narrower fractions (see legend to Fig. 4) to assay mitogenic and transforming activities.

Cell Culture. Normal rat kidney fibroblasts (NRK, clone 49F) obtained from the American Type Culture Collection (CRL 1579) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (both from GIBCO), penicillin (6 mg/ml) and streptomycin (5 mg/ml); incubation was at 37°C in a 5% CO_2 atmosphere maintained at 88% relative humidity. To study effects on replication, cells were grown to confluence (≈10^5 cells per cm^2) in 2-cm^2 wells and then maintained in DMEM containing 2% newborn calf serum (maintenance medium) for 20 hr prior to factor addition. Cultures were refed with maintenance medium (200 μl) containing the factors of interest, for 23–24 hr.

Cell Counts. At the end of culturing the cells were washed with cold isotonic buffer (146 mM NaCl/11 mM dextrose/35 mM Tris·HCl, pH 7.4), and a single-cell suspension was prepared. Cell numbers were determined by counting in a fixed-volume hemacytometer.

DNA Determination. An aliquot of suspended cells was sequentially precipitated with cold 5% trichloroacetic acid and ethanol containing 10 mM potassium acetate. DNA was collected by centrifugation and measured according to the fluorometric method of Kissane and Robbins (21).

[^{3}H]Thymidine Incorporation. For determination of DNA-synthesis rates, cells were incubated with [*methyl*-^{3}H]thymidine (ICN, 60–80 Ci/mmol, 5 μCi/ml; 1 Ci = 37 GBq) for 2 hr at the end of treatment. Incorporation was terminated by washing with cold isotonic buffer and lysis in 0.4 M NaOH/0.4% $NaDodSO_4$. The lysate was combined with two water washes of the culture well, and the sample was acidified with trichloroacetic acid (5% final concentration) at 4°C. Cold-acid-precipitable material was collected by centrifugation and solubilized by heating in 0.2 M NaOH. An aliquot was used to measure acid-precipitable [^{3}H]thymidine incorporation.

EGF Radioligand–Receptor Assay. Inhibition of ^{125}I-labeled EGF binding to NRK cells by BDGF was examined by methods adapted from others (22, 23). Confluent 2-cm^2 cultures were held in maintenance medium for 24 hr, washed with cold isotonic buffer, and refed with serum-free DMEM containing 0.1% (wt/vol) bovine serum albumin, 50 mM Hepes (pH 6.8), and ^{125}I-labeled EGF (New England Nuclear) at 30 ng/ml (this concentration was saturating for NRK cells when assayed under identical conditions); some cultures also received nonradioactive EGF (Collaborative Research, Waltham, MA) or BDGF. After incubation for 2 hr at 4°C, the medium was aspirated, and the cells were washed with cold isotonic buffer. Cells were lysed with 0.2 M NaOH and the lysates were collected and combined with three washes of the culture wells. The entire sample was used to determine cell-associated radioactivity.

The radioligand–receptor assay was repeated using A431 membranes, which are enriched for EGF receptor and therefore provide more sensitive substrate (24). The assay was performed according to directions provided by the manufacturer of the assay kit (Biomedical Technologies, Norwood, MA).

Soft-Agar Transformation Assay. Anchorage-independent growth was assayed by methods developed by Todaro and others (9–11). Culture wells (2-cm^2) were partially filled with DMEM containing 10% newborn calf serum and 0.6% noble agar (Difco). The underlay was allowed to solidify while a single-cell suspension of NRK-49F cells was prepared. An aliquot of cells was diluted with concentrated stock solutions of agar, medium, and test factor(s) so that the final concentrations were 6×10^4 cells per ml, 0.25% agar, and 10% serum; factor levels are detailed in the text. One hundred microliters of the final suspension was pipetted over the underlay, and the cultures were incubated without further addition for 7–14 days under normal conditions. Transformation was assessed in unfixed and unstained cultures by use of a light microscope. Colonies, counted in 9–24 random 3.1-mm^2 microscopic fields from three or four replicate culture wells, were categorized as small (≥100 μm in diameter) or large (≥200 um in diameter).

PDGF Assay. Anti-PDGF antibody neutralization experiments were carried out with polyclonal antiserum prepared in rabbits against PDGF and kindly provided by C.-H. Heldin (Uppsala Universitets, Sweden). PDGF (Biomedical Technologies) or BDGF was added to NRK cells in the presence and absence of antibody and examined for effects on [^{3}H]-thymidine incorporation.

Statistical Methods. Data are expressed as means and SEM. Statistical differences were assessed by Student's *t* test.

RESULTS

Cell Replication and DNA Synthesis. EGF was used to establish the mitogenic response of NRK cells. At 1–100 ng/ml, EGF increased cell number in density-dependent growth-arrested cells by 50–100% and DNA content by 60–110%. EGF also increased [^{3}H]thymidine incorporation into acid-insoluble material; this effect was dose-dependent and was maximal (7- to 8-fold increase) at 10–30 ng/ml (Fig. 1).

CN-BDGF was a potent mitogen for NRK cells. At low concentrations (20–120 ng/ml), it increased cell number 40%, whereas at 800 ng/ml, an increase of 70% was detected. BDGF increased DNA content by 20% at 5–50 ng/ml, and at 500 ng/ml a 50% increase was observed. BDGF also produced a marked increase in [^{3}H]thymidine incorporation into acid-insoluble material. The effect was detected at a concentration as low as 3 ng/ml (15–25% the estimated concentration present in calvarial CM, based on CN-BDGF containing 12–20 ng of protein/ml of CM), was linear up to 30–100 ng/ml, and appeared to saturate at about 1 μg/ml (Fig. 2).

EGF/TGF-α Content. Since highly purified BDGF appeared to retain less, or less persistent, mitogenic activity than found in cruder preparations (data not shown), we questioned whether synergistic factors such as those necessary for anchorage-independent growth were separated by rpHPLC. Neither Sephadex- nor μBondapak C_{18}-purified BDGF could compete with ^{125}I-labeled EGF for binding to NRK cells by radioligand–receptor assay, even at concentrations that were maximally stimulatory to replication (Table 1). We repeated this experiment with A431 cell membrane preparations, which are sensitive to as little as 25 pg of added EGF per ml; we found, by extrapolation, <2 pg of EGF or

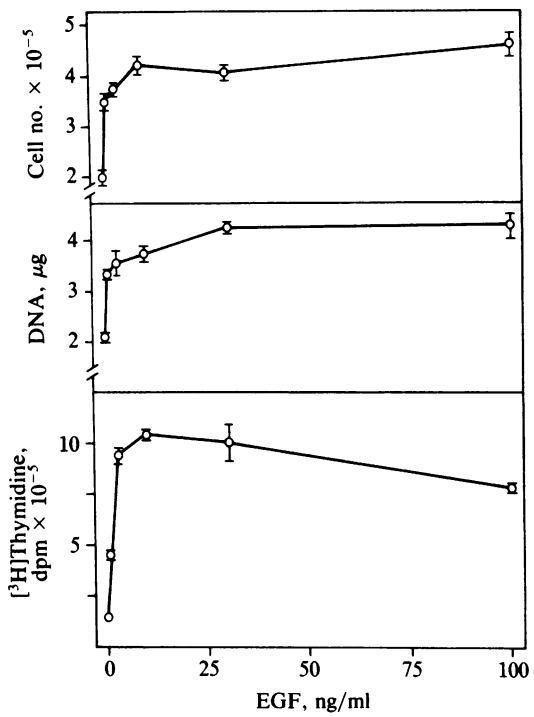

FIG. 1. Effect of EGF on NRK-49F cell replication. Cell number, DNA content, and [³H]thymidine incorporation were measured in separate experiments. Data represent means ± SEM of four replicate culture wells.

TGF-α per ml of unchromatographed crude CM (Table 1) but as much as 1–2 ng/ml in fetal rat serum (not shown).

Soft Agar Colony-Inducing Activity. To assess whether the effects of crude or purified BDGF were due to EGF-dependent or EGF-independent TGFs, we cultured NRK cells in soft agar with each BDGF preparation, in the presence and in the absence of EGF. For controls, we tested

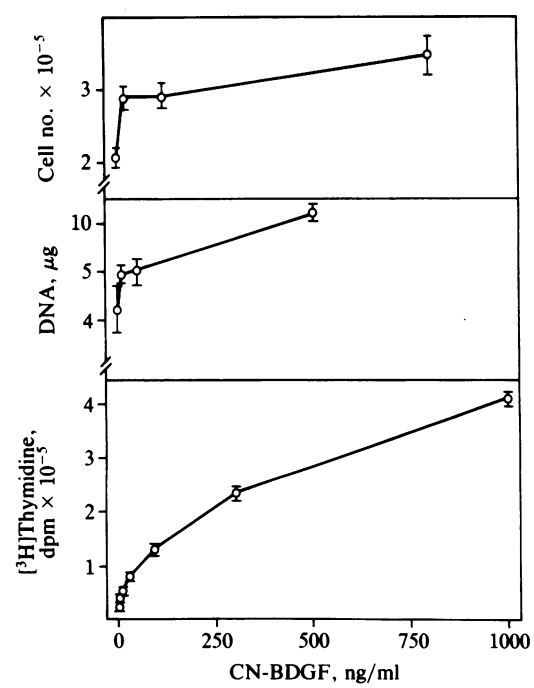

FIG. 2. Effect of μBondapak CN-purified BDGF on NRK-49F cell replication. Cell number, DNA content, and [³H]thymidine incorporation were measured in separate experiments. Data represent means ± SEM of four replicate culture wells.

Table 1. Competition between ^{125}I-labeled EGF and EGF or BDGF for binding to receptors on NRK-49F cells and A431 cell membrane preparations

Addition		^{125}I-labeled EGF
Factor	Concentration, ng/ml	bound, cpm
*Experiment 1. NRK-49F cells**		
None	—	2,306 ± 140
EGF	10	1,567
	20	1,359
	120	1,027
Sephadex-BDGF	200	2,352 ± 118
	2000	2,416 ± 193
	20,000	2,167 ± 290
C_{18}-BDGF	100	2,695 ± 192
	1000	2,407 ± 54
	10,000	2,304 ± 248
Experiment 2. A431 cell membranes†*		
None		18,335
EGF	0.25	15,264
	0.5	14,170
	1	12,857
	5	7,451
	10	5,699
	50	3,137
	20,000	2,043
CM-BDGF	250 × 10³	17,876
	500 × 10³	17,896
	1000 × 10³	18,470

No BDGF concentration tested gave values that differed significantly from the control (no addition) value.

*^{125}I-labeled EGF (30 ng/ml, 158 nCi/ng) was incubated in the absence (n = 4) or presence of EGF (n = 2) or BDGF (n = 3); values are means or means ± SEM. Unconcentrated Sephadex-BDGF contained 1.5 μg of protein/ml, whereas unconcentrated C_{18}-BDGF contained 0.74 μg of protein/ml.

†^{125}I-labeled EGF (159 nCi/ml) was incubated in the absence or presence of EGF or BDGF; values are the means of duplicate determinations, which differed from each other by <10%. Unconcentrated CM-BDGF contained 25 μg of protein/ml.

EGF and TGF-β (Biomedical Technologies). TGF-β alone did not induce anchorage-independent growth, whereas EGF primarily supported small-colony formation; TGF-β and EGF together induced a 2-fold increase in large-colony formation over EGF alone. Highly purified CN-BDGF up to 500 ng/ml did not induce anchorage-independent growth and did not alter the effect of EGF on colony formation (Table 2). This indicated that the mitogenic effect of BDGF was not due to a TGF but did not eliminate whether cruder preparations contained TGF-like factors. Sephadex-BDGF by itself did not induce large- or small-colony formation, indicating absence of TGF-α or EGF-independent TGFs, but when it was assayed in the presence of EGF, TGF-β activity was detected. Soft agar colony inducement with EGF at 2 ng/ml (data not shown) or 20 ng/ml was seen with Sephadex-BDGF concentration as low as 0.25 μg protein/ml (17% the estimated calvarial CM level of 1.5 μg of protein/ml); the effect was maximal at a concentration equal to that in calvarial CM (Table 2 and Fig. 3). This activity had an estimated M_r of 24,000 on Sephadex G-75 chromatography and peaked independently of BDGF by this method (Fig. 4) and by μBondapak C_{18} HPLC (data not shown).

PDGF Content. Addition of polyclonal anti-PDGF antibodies to NRK cells inhibited PDGF-stimulated replication but did not reduce the mitogenic effect produced by an intermediate dose of BDGF (Fig. 5). Since BDGF was tested at the half-maximally effective concentration, any growth promotion due to contaminating PDGF should have been detected at nonsaturating factor levels.

Table 2. Anchorage-independent growth of NRK-49F cells in the presence of EGF, TGF-β, and BDGF alone or in combination

Addition		% transformed*		% large colonies†	
Factor	Concentration, ng/ml	−EGF	+EGF	−EGF	+EGF
None		0	14	0	7
TGF-β	10	0	13	0	16‡
Sephadex-	250	0	17	0	19‡
BDGF	2500	0	23†	0	28‡
	25,000	0	25†	0	35‡
CN-BDGF	5	0	10	0	8
	50	0	12	0	9
	500	0	11	0	6

NRK cells were cultured in the absence or presence of EGF (20 ng/ml) and the indicated concentrations of TGF-β or BDGF. Sephadex-BDGF contained 1.5 μg of protein/ml and produced maximal [^{3}H]thymidine incorporation at 20 μg/ml; CN-BDGF contained 12 ng of protein/ml and produced maximal [^{3}H]thymidine incorporation at 1 μg/ml. Data are from three or more random microscopic fields in each of three replicate cultures; each field averaged 90 plated cells.

*Total number of colonies/total number of cells plated.

†Number of large colonies/total number of colonies.

‡Significantly different ($P < 0.05$) from control (no addition).

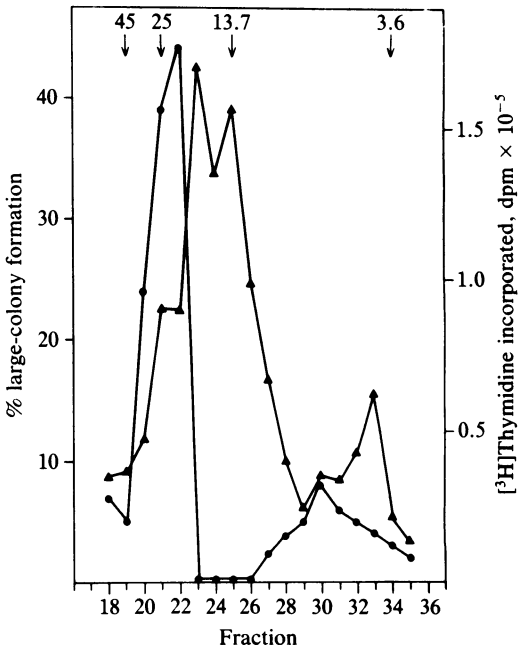

FIG. 4. EGF-dependent soft-agar colony formation (●) and DNA synthesis (▲) in NRK-49F cells induced by Sephadex G-75 chromatography-derived fractions of fetal rat calvarial CM. Numbers above arrows indicate $M_r \times 10^{-3}$. Data represent averages of three replicate cultures.

DISCUSSION

Interest in growth factor-mediated modulation of cell proliferation and differential gene expression has expanded since the purification of a variety of these molecules from diverse tissue sources (1, 2, 6, 25–32). Fetal rat calvariae release autocrine factors (BDGF) in culture and respond to these and to factors from other tissues by specific and characteristic changes in macromolecular synthesis. Some factors enhance total calvarial DNA synthesis in conjunction with either increased or decreased synthesis of bone-cell-specific proteins (33). Therefore, certain factors may affect undifferentiated or non-bone-specific cells, while others may act on calvarial osteoblasts alone or in addition to other cell types also present. Since the calvariae used for assay are metabolically competent, it is difficult to assess whether the agent being assayed has acted alone or in addition to or in synergy with BDGF that the calvariae generate during the treatment period. For these reasons, and to compare BDGF with factors purified in other laboratories, we have begun to characterize the biological and biochemical effects of BDGF on nonskeletal tissue-derived fibroblasts.

We have found that BDGF stimulated NRK cell replication. Saturating levels of BDGF produced a maximal increase of 70% in cell number and 50% in DNA content. [^{3}H]Thymidine incorporation into DNA was the most sensitive indicator: BDGF at 3 ng/ml was effective, and maximal concentrations increased labeling 40-fold. The effect on

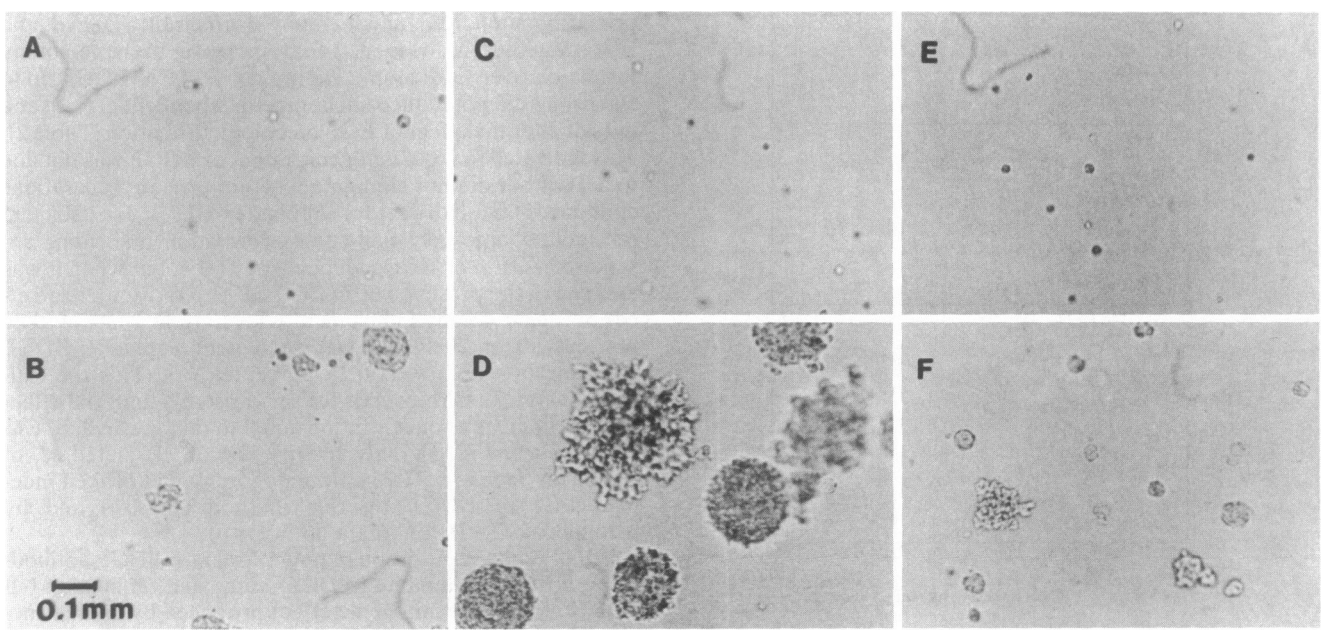

FIG. 3. EGF-dependent soft-agar colony formation induced by fetal rat calvarial BDGF and TGF. Anchorage-independent growth was assayed as described in *Materials and Methods*. (*A*) Control. (*B*) EGF (20 ng/ml). (*C*) Calvarial TGF (6.5× CM concentration). (*D*) EGF plus calvarial TGF. (*E*) CN-BDGF (40× CM concentration). (*F*) EGF plus CN-BDGF.

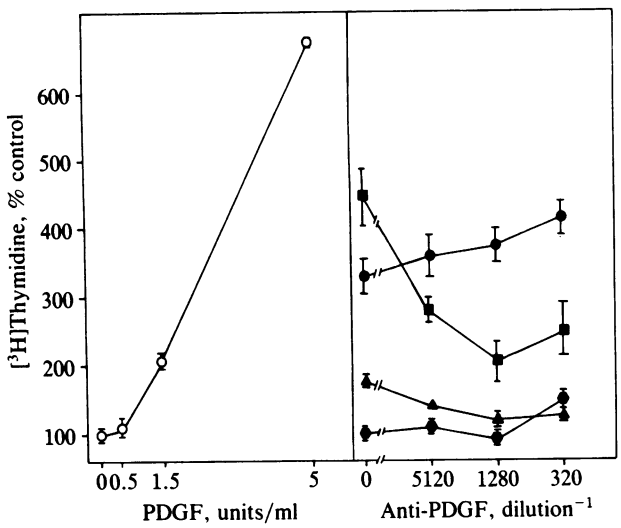

FIG. 5. Effect of anti-PDGF antiserum on PDGF- and BDGF-induced mitogenesis in NRK-49F cells. (*Left*) PDGF dose–response curve. (*Right*) Anti-PDGF neutralization studies; cultures were tested in the absence or presence of various concentrations of anti-PDGF antiserum and either no factors (●), PDGF (1.6 units/ml, ▲), PDGF (2.8 units/ml, ■), or C_{18}-BDGF (5 μg/ml, ●). Data are from two experiments, both using the same lots of reagents.

thymidine incorporation was disproportionately larger than the effect on cell number and DNA content. This discrepancy, also observed with EGF, is not surprising, since the assay uses growth-arrested cells in medium with reduced serum content. It is unlikely that the cells are synthesizing much DNA prior to treatment; thus, stimulation of a small number of cells into active DNA synthesis would give a significant increase in [^{3}H]thymidine incorporation in the absence of appreciable increases in cell number or DNA content.

Maximally stimulatory concentrations of crude or purified BDGF did not displace saturating amounts of ^{125}I-labeled EGF from its receptor on NRK cells or A431 membranes, indicating that the mitogenic activity was not due to EGF or TGF-α. Although calvarial CM contained β-type transforming activity, this did not copurify with BDGF on Sephadex chromatography or rpHPLC. Therefore, fetal rat calvarial CM contains at least two growth-stimulating activities for nonskeletal fibroblasts, and these activities appear to have independent functions.

We do not know the cellular source of bone-culture-derived BDGF or TGF-β. These factors are unlikely to be serum contaminants, since CM contains no insulin or EGF, both of which are present in fetal rat serum. While we cannot exclude BDGF or TGF-β production by non-bone cells trapped in the organ following resection, we do not suspect a platelet source. Crude (unpublished observation) and one-step HPLC-purified BDGF did not contain significant amounts of PDGF, as determined by antibody neutralization studies (reported here), radioimmunoassay and radioligand–receptor assay (C.-H. Heldin, Uppsala Universitets, Uppsala, Sweden), or fibroblast migration assay [G. Grotendorst (National Institutes of Health, Bethesda, MD), personal communication]. Also, the estimated molecular weight and isoelectric point of BDGF and PDGF are different (34). BDGF contained no immunoreactive insulin-like growth factor I or II [P. Nissley (National Institutes of Health, Bethesda, MD) and R. Hintz (Stanford, CA), personal communications] and is unlike fibroblast growth factor, which inhibits calvarial collagen synthesis (35).

The relationship between BDGF and skeletal growth factor (SGF) from human and chick bone is unclear, since SGF does not seem to stimulate fibroblast division (36, 37), but this discrepancy may be due to different assay protocols. Bone morphogenetic protein from bovine bone does stimulate NRK cell growth but does not increase calvarial collagen synthesis (38).

In conclusion, BDGF stimulates fibroblast replication but has no transforming activity for NRK cells. Although fetal rat calvariae release TGF-β, this factor is independent of BDGF.

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