Growth Factors in Bone Matrix

ISOLATION OF MULTIPLE TYPES BY AFFINITY CHROMATOGRAPHY ON HEPARIN-SEPHAROSE*

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The mineralized matrix of osseous tissue harbors abundant mitogenic activity which is extractable by demineralizing solvents. In bovine bone powder free of blood and cartilage contamination, the volume concentration of mitogens is up to 20 times greater than in serum. Growth factor activity in bone extracts was quantitated on quiescent mouse BALB/c3T3 fibroblasts, where [3H]thymidine incorporation for 48 h was stimulated up to 200-fold in a linear, dose-dependent manner. Six distinct bone-derived growth factors (BDGFs) have been resolved and partially purified (up to 44,000-fold) on heparin-Sepharose using NaCl gradient elution. Provisionally named by the NaCl molarity at which they elute, these BDGFs include BDGF-0.45 (25% of total activity). This factor is heat-stable and sensitive to dithiothreitol, and displaces 125I-labeled bovine platelet-derived growth factor in a radioreceptor assay. BDGF-0.45 (~50 ng/g of bone) is closely related or identical to bovine platelet-derived growth factor. BDGF-1.1 (10%) has a PI of 5.2 and shows a 16,600-dalton doublet on sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blots stained with antisera to bovine anticoagulant fibroblast growth factor. Two activities with high heparin affinity resemble cationic forms of fibroblast growth factor. BDGF-1.5 is the dominant factor in fetal membranous bone (50%), but is less abundant in adult bone (20%). BDGF-1.7, a 17,500-18,400-dalton triplet, is virtually absent in fetal bone (7%) but abundant (30%) in adult bone and may be related to cartilage derived growth factor. Two minor activities, BDGF-0.1 (10%) and BDGF-2.0 (7%) have not been characterized. Proliferation of bovine capillary endothelial cells was strongly supported by BDGFs 1.1, 1.5, and 1.7, but not by 0.45. These four purified BDGFs and the crude bone extract were also strongly mitogenic for rat osteoblasts while depressing alkaline phosphatase specific activity by 2–3-fold. Bone exhibits the most complex spectrum of growth factor activities of any tissue yet described. Bone cells and other indigenous cell types must be considered as possible sources of the BDGFs, in addition to sequestration from blood. Mechanisms for unmasking or release of BDGFs from the mineralized matrix resulting in local action on target cells are undoubtedly important for the development and maintenance of bone tissue.

Polypeptide growth factors appear to play an important role in the development and growth of osseous tissue. Bone is unique because of its abundant mineralized extracellular matrix, which may sequester growth factors and modulate their biological action through complex modes of release and presentation to responding cells. Extracellular matrix accounts for about 90% of the total weight of compact bone and is composed of microcrystalline calcium phosphate resembling hydroxyapatite (60%) and fibrillar type I collagen (27%). The remaining 3% consists of minor collagen types and other proteins including osteocalcin, osteonectin, matrix 4-carboxyglutamic acid protein, phosphoproteins, sialoglycoproteins, and glycoproteins, as well as proteoglycans, glycosaminoglycans, and lipids (1). Osteoblasts are principally responsible for biosynthesis of this complex matrix. Additional levels of compositional and biological complexity are imposed by: 1) matrix adsorption of numerous plasma proteins; 2) osteoclastic remodelling of calcified cartilage during enchondral bone development; 3) the capillary network with its associated endothelial cells and basement membranes; and 4) hematopoietic marrow and blood-borne cells including monocytes, the apparent precursors of multinucleated osteoclasts which resorb bone, and other leukocytes which may regulate resorption via monokines and lymphokines (2–4). Polypeptide growth factor effects on bone are necessarily divided into two areas of study: endogenously produced endocrine factors, which act on specific bone target cells, and endogenously produced "local" factors with possible autocrine or paracrine action. Known growth factors of extrasosseous origin have been investigated for their effects on bone in organ culture (5). Fundamental work with fetal rat calvaria showed that epithelial growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-1) generally stimulated cellular proliferation. EGF and FGF depressed formation of the collagenous extracellular matrix, whereas PDGF and IGF-1 (somatomedin-C) stimulated collagen synthesis (5). EGF, PDGF, and transforming growth factors (TGF-α, TGF-β) may also play a role in bone resorption, as they stimulate

*The abbreviations used are: EGF, epidermal growth factor; BDGF, bone-derived growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor; TGF, transforming growth factor; aFGF, anionic FGF; cFGF, cationic FGF; CDGF, cationic cartilage-derived growth factor; SGF, skeletal growth factor; hSGF, human SGF; PBS, phosphate-buffered saline; GFI, growth factor unit; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DME, Dulbecco's modified Eagle's medium; BCE cells, bovine capillary endothelial cells; HGF, hypothalamic growth factor; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; TPCK, t-1-tosylamido-2-phenylethyl chloromethyl ketone.

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calcium release by a prostaglandin-mediated mechanism (6-8). Endogenous or local growth factors of possible relevance include: 1) bone-derived growth factor, an 11-kDa protein produced by cultured fetal rat calvaria (BDGF-II originally, but now called BDGF (5, 10); 2) TGF-β, a 25-kDa protein recently demonstrated from the same organ culture system (9, 10); 3) cartilage-derived factor, an 11-kDa somatomedin-C-like substance (11); 4) cationic cartilage-derived growth factor (CDGF), a 19-kDa FGF-like protein (12); 5) skeletal growth factor (hSGF), with 83-kDa and 9-12-kDa forms isolated from human bone matrix (13, 14); 6) bone morphogenetic protein, an 18.5-kDa bovine protein with bone-inducing activity (15); 7) a bovine osteoinductive factor of 20-22 kDa and a 22-kDa mitogenic factor from rat bone (16); and 8) cartilage-induction factors from bovine bone (17) (26-kDa species cartilage-induction factor A, now recognized to be identical to TGF-β (18), and cartilage-induction factor B), and from chicken bone (19). Other local factors affecting bone cells include interleukin-1 (5, 6), macrophage-derived factors (20, 21), and endothelial cell-derived growth factor (CDGF), and protein hydrolysed by neoplastic cells (23-25). A wide range of assay systems involving different target cells and various bioassays have been used in the study of the above-mentioned factors.

The present report surveys the various growth factors isolated from bovine bone matrix using a nondiscriminatory mitogenic screening method with BALB/c-JT3 mouse fibroblasts in vitro (26). Target cell activity on capillary endothelial cells and osteoblasts is also indicated. Furthermore, the informative technique of chromatography on heparin-Sepharose, permitting classification of growth factors based on their empirically defined affinity (27, 28), has been applied to the isolation and partial purification of several bone-derived growth factors.

**MATERIALS AND METHODS**

**Bone Powder**—Mandibles were dissected from previously frozen heads of 3-9-month fetal calves (Holstein) and cleaned of adherent soft tissue. All possible sources of cartilaginous tissue were removed, including the condylar, angular, and coronoid processes, and the anterior symphysis. Batches of 500 g, wet weight, were passed through a meat grinder (Hobart) and then shredded at 0 °C in an ice/water slurry with a large Waring blender operated at top speed. The bone fragments were then reduced to a fine powder by homogenizing with 6 liters of 0.5 M ammonium EDTA, pH 7.5, with gentle stirring for 24 h at 4 °C. The supernatant extract was recovered after centrifugation at 2,000 x g for 10 min and then centrifuging at 12,000 x g for 20 min and discarding the supernatant. Extraction was accomplished by resuspension in 1260 ml of 0.5 M ammonium EDTA, 0.02 M Tris-HCl, pH 7.5, with gentle stirring for 24 h at 4 °C. The supernatant extract was recovered after centrifugation at 10,000 x g, combined with a 400-ml wash of the pellets, and filtered through Whatman No. 113 paper. Dialysis at 4 °C in 40-mm flat Spectrapor 1 tubing (Spectrum Medical Industries, Los Angeles, CA) against water for 4 days (20 1 x 4 changes) and 0.02 M NH₄HCO₃ for 2 days (20 1 x 4 changes) was followed by lyophilization, yielding 1.88 g of dry powder.

**HCl/Guanidine HCl Extraction**—Dry bone powder (200 g) was washed in 1600 ml of 0.02 M Tris-HCl, pH 7.6, at 4 °C for 20 min and then centrifuged at 2,000 x g for 10 min, and the pellet was resuspended in 1600 ml of water at 0 °C. While stirring on ice, 120 ml of 12.3 M HCl was added dropwise in 5 min and the acidified slurry was mixed for another 45 min, by which time CO₂ evolution had ceased and the pH was stable at 3.3. After centrifugation the supernatant was filtered through a 3-mm pad of ashless cellulose powder (Whatman) supported on No. 113 paper. The extract was dialyzed against water for 4 h, lyophilized, and then centrifuged in two fractions, each of soluble HCl extract and 822 mg of water-insoluble residue. The HCl-extracted bone powder residue was resuspended in 800 ml of 4 M guanidine HCl, 0.05 M Tris-HCl, pH 7.8, and the pH adjusted to 7.5 with NaOH. Extraction proceeded for 24 h at 4 °C and was terminated by centrifugation. The supernatant was washed with 150 ml of water and centrifuged, and the combined supernatants were dialyzed against water at 4 °C for 4 days. During dialysis a white flocculate developed which was lyophilized separately yielding 747 mg of white fluffy water-insoluble guanidine extract. The lyophilized supernatant yielded 489 mg of soluble extract.

**Heparin-Sepharose Chromatography**—At 4 °C, 5 g of heparin-Sepharose (Pharmacia) was washed with 3 M NaCl, 0.01 M Tris-HCl, pH 7.0 ("NT"), and then 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.0 and packed in a siliconized glass column (1.6 x 9.5-cm bed volume) pumped at a flow rate of 28 ml/h (27). Lyophilized bone extracts (0.3-2 g, dry weight) were suspended at 20-30 mg/ml and dialyzed against 0.1 M NT at 4 °C overnight. After centrifugation at 27,000 x g for 20 min, the clear supernatant was weighed (for volume determination) and a small aliquot frozen for growth factor assay. Stability of total growth factor activity at −20 °C was excellent. Collection of 2.8 ml of effluent fractions was initiated at the onset of sample application, and after 1 h, washing with 0.1 M NT for 1 h, a gradient of 0.1-3 M NT was pumped through the column. Typically, the gradient volume was 570 ml, composed of 300 ml of 0.1 M NT and 270 ml of 3 M NT for initial hydrostatic equilibrium in the two-chambered mixing device. Absorbance at 280 nm was monitored with a flow spectrophotometer (Allex). Conductivity was measured at 23 °C (CDM3, Radiometer) on 30-fold aqueous dilutions of 0.2-ml aliquots of every tenth fraction and compared to a standard curve of similarly diluted NT buffer standards for computation of the NaCl gradient. Fractions were stored at 4 °C until assayed for growth factor activity. Uncorrected recovery of applied total growth factor units ranged from 30 to 45%.

Fractions containing BDGF activity were pooled and diluted for recycling through a 1 x 1-cm bed heparin-Sepharose column. BDGF pools eluting at 1.1-2.0 M NaCl were diluted to 0.4 M NaCl with water (final volume 100-400 ml) and pumped through the column at 100 ml/h three to six times to maximize adsorption. After washing with 50 ml of 0.5 M NaCl, the column was eluted three times with 3-ml aliquots of 2.5 M NT, pausing 15 min between each elution. The pooled eluant containing 60-90% of the total activity was dialyzed and concentrated as described below.

**Dialysis and Concentration of BDGF Pools**—Dialysis of BDGF fractions in precoated Spectrapor 2 tubing was limited to 2 h at 4 °C to minimize adsorptive losses. The efficiency was maximized by internal mixing of bags during lateral shaking (2 strokes/s) in partially filled screw-capped bottles. Dialysis medium was changed every 3-4 days and recycling through a 1 liter SBT buffer (0.145 M NaCl, 0.095 M K₂HPO₄-KH₂P0₄, pH 7.2 (PBS) containing 5 μg/ml bovine serum albumin and 0.5 mg/ml DTT) was pumped through the column. Typically, 0.5% Tween-20; 0.5 M NaC1, 0.095  M NaCl with water (final volume 100-400 ml) and pumped through the column at 100 ml/h to three times to maximize adsorption. After washing with 50 ml of 0.5 M NaCl, the column was eluted three times with 3-ml aliquots of 2.5 M NT, pausing 15 min between each elution. The pooled eluant containing 60-90% of total activity was dialyzed and concentrated as described below.

**Inactivation Studies of BDGFs**—Purified pools of individual BDGFs were tested for stability to heat, proteinase, and various reagents as follows. In 1.5-ml microcentrifuge tubes were placed 50 μl of SBT buffer (0.145 M NaCl, 0.095 M KH₂PO₄-K₂HPO₄, pH 7.2 (PBS) containing 5 μg/ml bovine serum albumin and 0.5 mg/ml DTT (TWEEN-20 (Sigma)), 1-15 GFU of BDGF activity, test reagents in PBS, and PBS to a total volume of 100 μl. Treatments included: (a) 10 min at 100 °C; (b) 0.5% Tween-20; (c) 0.1% SDS; (d) 50 μg/ml trypsin (TPCK-treated, Worthington, 216 units/mg) for 100 min at 37 °C and blocked with a 4-fold excess of soybean trypsin inhibitor (Worthington) either before or after the 37 °C incubation period; (e) 4 M guanidine HCl; (f) 5 mM dithiothreitol (DTT); (g) 4 M guanidine HCl plus 5 mM DTT; (h) 0.1 M HCl; (i) 0.1 M KOH; (j) 1 mM p-hydroxymercuriobenzoate (Sigma); (k) control. Treatments a-d were stored in ice until used, and treatments e-j were stored after 90 °C for 23 h in 0.1 M NaCl dialyzed against PBS at 4 °C. Spectrapor 2 dialysis bags (1 x 14 cm, flat) were filled with SBT buffer, emptied completely with the aid of a silicone tubing.
covered hemostat sponge, and then filled with the 100-µl sample, allowing recovery of 88–95 µl after dialysis. Six aliquots (2–30 µl) of each treated or control BDGF sample were assayed for growth factor activity. Treatment blanks lacking BDGFs were prepared for all test conditions and assayed both in the presence and absence of 10 µl of calf serum to verify their lack of effect on cell responsiveness. Linear plots of the data yielded GFU/ml for quantitative comparison of remaining activity.

**Growth Factor Assay—**BALB/c/3T3 mouse fibroblasts were subcultured and used for determination of growth factor activity essentially as described by Klagesbrenner et al. (80). Briefly, cells were propagated in DMEM: Dulbecco’s modified Eagle’s medium (GIBCO) containing 4.5 g/liter glucose, 2.2 g/liter NaHCO₃, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% calf serum (Colorado Serum Co., Denver, CO). To prevent diminution of assay sensitivity caused by the outgrowth of spontaneously transformed cells, culture density was never allowed to exceed 75% confluence, and subcultures of 2 × 10⁵ cells/75 flask were established weekly. Stock cultures of BALB/c/3T3 cells were kindly provided by Y. Shing (Children’s Hospital, Boston, MA). Costar 96-well plates were seeded with 2,000 cells/well in 200 µl of DMEM and grown for 7–10 days in a 5% CO₂, 95% air incubator at 37 °C, at which time the confluent, quiescent cells were challenged with various growth factors. Without changing the original plating medium, each well was supplemented with up to 50 µl of test substances and 1 µCi of [³²P]thymidine (6.7 Ci/mmol, New England Nuclear) in 10 µl of PBS. The 16 wells in columns 1 and 12 were not used because of erratic response. After gentle agitation to mix thoroughly, the plates were incubated for 42–48 h. Analysis of acid-insoluble [³²P]thymidine in cell DNA involved the following treatments using 200 µl of reagent/well: medium removal; PBS wash, 20 s; methanol fix, 5 min (twice); water wash, 20 s (4 times); 5% trichloroacetic acid fix, 10 min at 4 °C (twice); water wash, 20 s (4 times); dissolving cell layer in 150 µl of 0.3 M NaOH; and withdrawal and counting by liquid scintillation in 6-ml polyethylene vials containing 2 ml of Insta-Gel (Packard). Fractions from heparin-Sepharose columns were tested in 2–20 µl-aliquots. High NaCl concentration attenuated [³²P]thymidine incorporation in response to the standard serum stimulation (10 µl of NT buffer). 0.5 M Tris-HCl (pH 7.5) in 1/1 NT control, 1 M (40%), 1.5 M (40–60%), but BDGF peak heights were not corrected for this effect. One GFU was defined as half of the maximal response to calf serum as shown in Fig. 1. The unstimulated background was 600–3,000 cpm/well; maximal serum stimulation was 80–200-fold above background, and 1 GFU was 64,000–120,000 cpm.

**Endothelial Cell Growth Factor Assay—**Bovine capillary endothelial (BCE) cells were plated in Costar 24-well plates at 10⁵ cells/0.5 ml DMEM/well following the procedure of Folkman et al. (31). On the following day unattached cells were removed (plating efficiency approx. 80–90%), and fresh DMEM with or without added growth factors. After 72 h at 37 °C, cells were detached with 0.5 ml of 0.25% trypsin, transferred to 9.5 ml of Isoton, and counted with a Coulter counter (Model ZF, Coulter, Hialeah, FL). All factors were assayed at multiple dilutions in duplicate wells. The presence of the 10% calf serum in the DMEM medium is insufficient to stimulate proliferation of the BCE cells, and there is an absolute requirement for additional endothelial cell growth factors (31). In these experiments the positive control was provided by HGF, a CGF-derived growth factor from bovine hypothalamus (28). All factors were simultaneously assayed on BALB/c/3T3 cells to provide quantitation of the GFU added to the BCE cultures.

**Osteoblast Assays—**Osteoblasts were isolated by sequential collagenase digestion of newborn rat calvaria (32). Cells were cultured in Minimal Essential Medium (#410-1500, GIBCO) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). Osteoblastic phenotype was confirmed by the 60–100-fold elevation of cyclic AMP 15 min after challenge with bovine parathyroid hormone (32) and the high endogenous expression of alkaline phosphatase (32–34). At confluence, cells were trypsinized and replated in 24-well plates at 2.5 × 10⁵ cells/well. Cells were confluent within 7 days and at 14 days responded to added BDGF treated to add 10–50 µg BDGF/well) without changing the medium. Wells treated with buffer vehicle alone or with calf serum were used as controls. [³²P]thymidine (5 µCi/well) was added simultaneously to all cultures. After 48-h cells were washed, fixed, and counted as described above for BALB/c/3T3 cells. Alkaline phosphatase was quantitated at pH 9.3 (32, 33) with p-nitrophenyl phosphate (Sigma) as the substrate.

**Gel Electrophoresis—**SDS-PAGE was performed in 0.75-mm thick 15% gels according to Laemmli (35). Silver staining followed a modification of Ref. 36. Protein standards (Bethesda Research Laboratories) were loaded at 100 ng/band/lane. Two-dimensional electrophoresis was done by Protein Databases, Inc., Huntington Station, NY. Isoelectric focusing at 10 °C utilized 0.75-mm thick 1% iso-Gel agarose dabs cast on Gelbond (FMC Corp., Rockland, ME) containing 2.5% (w/v) pH 3.5–10 ampholites (LKB) and run at 300 V constant power (600 V limit) for 90 min. The anode was 0.5 M acetic acid, and the cathode was 1 M NaOH. Lanes containing protein p markers (FMC Corp. and Gallard-Schleisnger, Carle Place, NY) were cut off, fixed in 12.5% trichloroacetic acid at 0 °C, and stained with Amido Schwarz. Lanes containing growth factors were rapidly sliced into 55 strips of 2.7-mm width with a stack of razor blades. Each strip was suspended in 100 µl of SBF buffer in a 1.5-ml tube, and after three freeze-thaw cycles in 18 h, the buffer was assayed for activity.

**Miscellaneous—**Protein was determined either by a modified Lowry procedure (37) with a bovine serum albumin standard using a Varian/Cary 2290 spectrophotometer, or by amino acid analysis after acid hydrolysis. DNA was determined by the method of Vytasek (38) using 3,5-diaminobenzic acid hydrochloride and a calf thymus DNA standard (Sigma), and monitoring the fluorescence with an LS-5 luminescence spectrometer (Perkin-Elmer). MG63 human osteosarcoma cells were obtained from the American Type Culture Collection. All chemicals were reagent grade.

**RESULTS**

**Yield of Growth Factors from Bone Powder—**Extraction of fetal calf mandibular bone powder yielded abundant growth factor activity (570 GFU/g of dry bone), particularly under the neutral demineralizing conditions provided by 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5 (Table I). Less than 1% of the total activity is extracted by 0.05 M Tris-HCl buffer alone, with intermediate amounts released by other solvents. Inclusion of 4 M guanidine HCl in the protocol reduces the growth factor yield by 36%, perhaps because of denaturation of certain BDGFs that has been demonstrated below (see Table IV). Following the 0.5 M HCl extraction with a second extraction in 4 M guanidine HCl, a procedure shown by Sampath and Reddi (39) to solubilize osteoinductive activity, results in only minor additional yield of growth factor activity (Table I). Based on high yield and specific activity, the EDTA/Tris procedure was utilized primarily, although HCl extraction was also advantageous because of its more rapid yield of a relatively high specific activity product. In some studies, proteinase inhibitors (29) were included in the EDTA extraction procedure, but without significant improvement of yield. Because of the potential toxicity of the inhibitors in subsequent bioassays, they were not generally utilized.

**Quantitative titration of the growth factor activity in each extract with the quiescent BALB/c/3T3 mouse fibroblast assay (Fig. 1) was performed as shown in Fig. 2. It was necessary to set up 20 separate culture wells containing 20–200 µl aliquots of several dilutions in order to accurately determine the GFU/ml of stock from the linear portion of the curve. The plateau observed above 1.5 GFU is primarily a characteristic of the assay which, by convention, cannot respond to

**Table 1**

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Soluble dry wt</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µM Tris-HCl, pH 7.5</td>
<td>2.0</td>
<td>5</td>
</tr>
<tr>
<td>4 M GdnHCl/Tris</td>
<td>6.9</td>
<td>95</td>
</tr>
<tr>
<td>0.5 M EDTA/Tris</td>
<td>14.7</td>
<td>570</td>
</tr>
<tr>
<td>4 M GdnHCl/Tris</td>
<td>18.7</td>
<td>550</td>
</tr>
<tr>
<td>0.5 M HCl</td>
<td>4.8</td>
<td>118</td>
</tr>
<tr>
<td>0.5 M HCl followed by 4 M GdnHCl/Tris</td>
<td>2.5</td>
<td>32</td>
</tr>
</tbody>
</table>

* Fetal calf mandibular powder.
Materials and Methods

The growth factor activity in bone matrix is displayed in the heparin-Sepharose chromatogram of the EDTA extract of fetal calf mandible, where at least six separate peaks of activity are evident (Fig. 3a). One of these fails to bind to the column and washes through in 0.1 M NaCl during the original sample application. The other peaks elute during the NaCl gradient between 0.47 and 2.0 M NaCl. This result is also typical of adult bovine femur extracted with EDTA (Fig. 3b) or HCl (Fig. 3c), although the relative proportions of individual peaks vary (Table II). The actual elution positions of these growth factor peaks on an individual lot of heparin-Sepharose are exceedingly reliable, showing less than ±5% variation in NaCl molarity from the nominal average positions at 0.45, 1.1, 1.5, 1.7, and 2.0 M. For purposes of simplification and clarity, the individual BDGFs have been provisionally named by their typical elution positions: BDGF-0.45, BDGF-1.1, BDGF-1.5, etc. For the prominent doublet at 1.5–1.7 M NaCl (Fig. 3, b and c), the actual separation between the two peaks was 0.11–0.15 M NaCl, and the first peak was conventionally called BDGF-1.5. Of these various activities, the doublet containing BDGF-1.5 and BDGF-1.7 generally accounted for over half of the total (Table II), with BDGF-0.45 the next most abundant. BDGF-1.1 was a relatively minor peak and required application of at least 15,000 GFU of crude material to the column for detection. The small amount of activity at 1.9–2.4 M NaCl (BDGF-2.0) was particularly difficult to assay because of attenuation of the growth factor response at high osmolarity. BDGF-0.1 eluting with the bulk of protein was observed to be enlarged artifactually if the sample were applied too rapidly for quantitative adsorption of the other BDGFs.

Table II lists the relative activity in each BDGF peak. These values were computed by summation of the product [GFU/ml × ml/fraction] over the entire column profile. In Fig. 3c, where the mitogenic activity was beyond the linear range, reassembly of 2- and 8-μl aliquots of the column fractions provided meaningful data for peak integration. The most interesting variation in the BDGF elution pattern was the dramatic increase in BDGF-1.7 found in adult bovine femur (Fig. 3c) as compared with the relative paucity of this activity in fetal calf mandible (Fig. 3a). Differences stemming from bone maturity and membranous (mandible) versus endochondral (femur) developmental origin probably account for this observation.

Purification of BDGFs—In all the studies with heparin-Sepharose, the elution of bulk bone matrix proteins (A280) was virtually complete by 1.0 M NaCl (Fig. 3). Three of the four principal BDGFs elute beyond this point, where the average A280 of the eluant is ≤0.002. This provides a reasonably high degree of purification for BDGF-0.45 (37-fold) and BDGF-1.1 (480-fold), and phenomenal levels for BDGF-1.5 (up to 12,000-fold) and BDGF-1.7 (2,600-fold) in a single step (Table III). Repetition of this step brings the latter factors, particularly BDGF-1.7, to near homogeneity with an overall yield of 20% (Table III and Fig. 4). Silver-stained SDS-polyacrylamide gels show BDGF-1.5 to contain species of 27.5 and 14 kDa, with a minor component at 18 kDa (Fig. 4a), although activity has not yet been correlated with individual bands. BDGF-1.7 consists of a triplet of polypeptides of 17.5–18.4 kDa (Fig. 4b). In both cases the effectiveness of the second heparin-Sepharose step is apparent. For pure polypeptide growth factors studied in the BALB/c/3T3 assay, the amount more than 2 GFU/well. Secondarily, the cellular response to mitogens may be attenuated at high protein loading. The response was typically linear between 0.2 and 1 GFU. All stocks were thoroughly dialyzed against 0.1 M NaCl prior to assay to eliminate interference from EDTA and other salts, and dilutions were prepared in SBT buffer (see “Materials and Methods”). To verify that the mitogenic stimulation assessed by [3H]thymidine incorporation was truly reflecting DNA synthesis, parallel fluorimetric assays of DNA were done. Fresh 10% calf serum increased DNA by up to 181% compared to untreated quiescent control wells after 48 h. In a dose-dependent fashion, two different EDTA extracts of bone and partially purified heparin-Sepharose fractions BDGF-0.45 and BDGF-1.5 caused increases in total DNA of 62–214% (data not shown).

Heparin-Sepharose Affinity Chromatography of BDGFs—

FIG. 1. Typical standard curve for quantitation of growth factor activity on BALB/c/3T3 mouse fibroblasts. Calf serum and [3H]thymidine were added to microtiter wells containing 20,000 quiescent cells in 200 μl of complete DME culture medium. After 48 h, the trichloroacetic acid-insoluble incorporation into cellular DNA was quantitated as described under “Materials and Methods.” One GFU is defined as 50% of the maximal serum stimulated incorporation of [3H] above the background. Typically, background levels ranged from 600 to 9,000 cpm, and 1 GFU was 64,000–120,000 cpm. A 20-well serum standard curve was run every assay, and each microtiter plate in an assay series was verified to have equivalent background and mitogenic responsiveness by addition of serum to selected wells.

FIG. 2. Titration of total growth factor activity in the EDTA-extractable proteins of calf mandible bone powder. The salt-free lyophilized bone extract was dialyzed at 20 mg/ml against 0.1 M NaCl, centrifuged at 27,000 × g for 15 min, and assayed on quiescent BALB/c/3T3 cells in microtiter wells as described under “Materials and Methods” (see Fig. 1). Protein was determined by a modified Lowry procedure. For this preparation, 30 μg of total protein was equivalent to 1 GFU (see also Table I).
Bone-derived Growth Factors

TABLE I

Distribution of growth factors in bovine bone matrix

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Extract</th>
<th>Heparin-Sepharose elution, [NaCl]</th>
<th>% of total recovered activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal mandible</td>
<td>EDTA</td>
<td>1.48</td>
<td>46 (7)</td>
</tr>
<tr>
<td>Adult femur</td>
<td>EDTA</td>
<td>1.48</td>
<td>46 (7)</td>
</tr>
<tr>
<td>Adult femur</td>
<td>HCl</td>
<td>&lt;1</td>
<td>52 (3)</td>
</tr>
<tr>
<td>Adult femur HCl</td>
<td>GdnHCl</td>
<td>&lt;1</td>
<td>52 (3)</td>
</tr>
<tr>
<td>MG63 osteosarcoma cells</td>
<td>NaCl</td>
<td>&lt;1</td>
<td>52 (3)</td>
</tr>
</tbody>
</table>

*Final pH 3.3 due to buffering of 0.5 M HCl by bone mineral.

TABLE II

Purification of bone-derived growth factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Crude extract</th>
<th>1st heparin column</th>
<th>2nd heparin column</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDGF-0.45</td>
<td>0.028</td>
<td>1.04 (37)</td>
<td>1.04 (37)</td>
</tr>
<tr>
<td>BDGF-1.1</td>
<td>0.012</td>
<td>5.8 (8)</td>
<td>5.8 (8)</td>
</tr>
<tr>
<td>BDGF-1.5</td>
<td>0.018</td>
<td>212 (2,000)</td>
<td>212 (2,000)</td>
</tr>
<tr>
<td>BDGF-1.7</td>
<td>0.029</td>
<td>75 (754)</td>
<td>75 (754)</td>
</tr>
</tbody>
</table>

Yield 35% 20%

described in the legend to Fig. 1, and the 1 µM equivalent to 1 GFU (72,400) is indicated on the ordinate. Recovery of total growth factor activity was 40%. b, heparin-Sepharose affinity chromatography of growth factors extractable by EDTA from adult bovine femur. A total of 905 mg of EDTA extract (44,800 GFU) was applied and eluted with a 570-ml linear NaCl gradient starting at fraction 31 as described for a. Fractions of 2.5 ml were collected and assayed to determine NaCl molarity (C—C) and growth factor activity (C—C). and 1 GFU was 87,500 cpm. c, heparin-Sepharose affinity chromatography of growth factors extractable by HCl from adult bovine femur. A total of 812 mg of HCl extract (19,900 GFU) was applied and eluted with a 570-ml linear NaCl gradient as described for a. Fractions of 2.5 ml were collected and assayed to determine NaCl molarity (C—C) and growth factor activity (C—C), and 1 GFU was 74,000 cpm.

Stability Properties of the Individual BDGFs—In the course of investigating stability of purified BDGFs to various treatments, it became obvious that the nemesis of nonspecific adsorptive loss had to be solved first. The problem of loss was most acutely observed for the purest preparations where the total protein concentration was often below 1 µg/ml. After demonstrating that the nonionic detergent Tween-20 did not interfere with the growth factor assay at up to 0.05% (w/v) final concentration in the culture well, a standard mixture called SBT (see “Materials and Methods”) was developed for virtually quantitative recovery of BDGFs for bioassay after dialysis and dilution. Table IV indicates the properties of of protein corresponding to 1 GFU is typically about 200 pg (27). Thus for the 200 GFU of purified BDGF-1.7 loaded in Fig. 4b, the intensity of the silver-stained triplet band (about 40 ng of protein standard) is within reason. Doublet or triplet bands after electrophoresis of highly purified growth factors on SDS-polyacrylamide gels is a common observation (27, 40, 41). It is estimated that purification of approximately 100,000-fold would be necessary to obtain homogeneous preparations of BDGFs.

![Graph](image)
Bone-derived Growth Factors

FIG. 4. Silver-stained SDS-polyacrylamide electrophoretic gels of growth factor pools isolated from bovine bone matrix. a, purification of BDGF-1.5 by two successive NaCl elutions from heparin-Sepharose; b, purification of BDGF-1.7 by two successive NaCl elutions from heparin-Sepharose. Approximately 200 GFU of each activity was loaded in each lane.

TABLE IV

Stability of bone-derived growth factors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BDGF-0.45</th>
<th>BDGF-1.1</th>
<th>BDGF-1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% activity remaining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM DTT*</td>
<td>21</td>
<td>98</td>
<td>89</td>
</tr>
<tr>
<td>5 mM DTT + 4 M GdnHCl*</td>
<td>24</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>4 M GdnHCl*</td>
<td>106</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>0.1 M HCl, 25 °C 90 min*</td>
<td>95</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Heat, 100 °C, 10 min</td>
<td>93</td>
<td>98</td>
<td>105</td>
</tr>
<tr>
<td>Trypsin, 50 μg/ml, 37 °C, 90 min</td>
<td>93</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin + SBTP, 37 °C, 90 min</td>
<td>105</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>Dialyzed control/diluted control*</td>
<td>1.30</td>
<td>1.18</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* Samples dialyzed after treatment; recovery of activity after dialysis of untreated samples ranged from 68 to 130%.

* SBTI, soybean trypsin inhibitor.

several BDGFs and provides a framework for their differentiation and comparison with known polypeptide growth factors. All of the BDGFs were truly nondialyzable in Spectrapor tubing, having been efficiently retained during extensive dialysis in the initial extract preparation, as well as after heparin-Sepharose chromatography and during the present stability studies. Incubation for 90 min at 37 °C with 50 μg/ml TPCK-trypsin destroyed BDGF-0.45 and BDGF-1.1 completely, whereas BDGF-1.5 was partially resistant. Prior inhibition of the trypsin with soybean trypsin inhibitor sensibly blocked the degradation of growth factor activity. Trypsin-inhibitor blanks had no effect on the mitogenic response to calf serum. Boiling at 100 °C for 10 min destroyed BDGF-1.1 and BDGF-1.5, but had no effect on BDGF-0.45. The remarkable stability of BDGF-0.45 was further reflected by its resistance to 4 M guanidine HCl and 0.1 M HCl, a property not shared by the other two factors. In contrast, BDGF-0.45 is quite sensitive to transient exposure to 5 mM DTT, losing some 80% of its activity. BDGF-1.1 and BDGF-1.5 are stable to DTT, and this sulphydryl compound curiously protects BDGF-1.1 from inactivation by 4 M guanidine HCl. There is no apparent loss of activity in 0.5% Tween-20 or 0.1% SDS (data not shown). Based on the heparin affinity and the stability properties in Table IV, BDGF-0.45 strongly resembles PDGF (27, 41), whereas BDGF-1.1 with its pI of 5.2 (Fig. 5) is analogous to anionic FGF (aFGF) (40). Certain properties of BDGF-1.5 and BDGF-1.7 are common to cationic FGF (cFGF) (28, 42) and to cFGF-like factors isolated from cartilage and tumors (12, 27, 28, 42, 43) (see “Discussion”).

Production of BDGF-like Factors by MG63 Human Osteosarcoma—Several reports that PDGF-like factors were produced in bone organ culture (5, 25) or by U2-OS osteosarcoma cells expressing the v-sis oncogene (44, 50) prompted the investigation of MG63 human osteosarcoma for growth factor synthesis. This cell line has osteoblastic properties but does not express the v-sis oncogene (44), and thus might not be expected to make a PDGF-like substance. Heparin-Sepharose chromatography of 34,000 GFU extracted with 1 M NaCl from the cell layer of confluent MG63 cells showed no BDGF-0.45, but there was abundant activity eluting between 1.1 and 1.5 M NaCl (Table II). Secretion of additional growth factors cannot be ruled out, since the growth medium containing 10% fetal calf serum was not studied. Thus the osteoblast-like cell remains a likely candidate for biosynthesis of at least several of the polypeptide growth factors found in bone matrix.

Endothelial Cell Growth Factor Activity of BDGFs—Stimulation of BCE cell proliferation in the system established by Folkman et al. (31) was tested with similar results in two separate experiments, one of which is shown in Fig. 6. BDGF-1.1, BDGF-1.5, and BDGF-1.7 were potent mitogens for capillary endothelial cells, whereas BDGF-0.45 was inactive. The dose-response curves shown in Fig. 6 for the three active BDGFs are substantially similar to HGF (28), which is the normal positive control in this assay. The lack of effect of BDGF-0.45 at levels as high as 20 GFU/well further confirms the resemblance of this factor to PDGF, which also has no activity on BCE cells (27). Morphologically, the 72-h response of BCE cells to HGF is characterized by increased order in the culture, with local whorls of aligned confluent cells. The three positive BDGFs also clearly elicited this effect.

BDGF Effects on Osteoblasts—Quiescent, confluent cultures of rat osteoblasts exposed for 48 h to crude bone extracts, to isolated BDGF peaks from heparin-Sepharose, or to fresh 5% serum showed enhanced incorporation of [3H]thymidine (4–6-fold) and increased DNA content (1.5–2-fold) relative to buffer controls. The concentration at which the maximal mitogenic effect of each BDGF component was attained varied from 0.4 to 20 GFU/ml, with BDGF-0.45 and BDGF-1.1

FIG. 5. Isoelectric focusing of BDGF-1.1. Forty GFU of BDGF-1.1 was applied to an agarose slab isofocusing gel. After running, lanes were immediately sliced, eluted, and assayed on BALB/c3T3 cells (see “Materials and Methods”). Similarly prepared blank lanes which contained no sample showed no stimulation of [3H]thymidine incorporation, nor were they inhibitory to standard serum stimulation. The pH gradient (●—●) was calculated from positions of pI standard proteins run on the same plate and stained.
were diluted in 0.5 ml of DME complete medium and added to wells initially seeded with 10,000 BCE cells in 24-well Costarplates, following the method of Folkman et al. (31). After 72 h, wells were photographed, and the cells were trypsinized and suspended in 10 ml of isotic saline for Coulter counting. Standard error of the mean for four separate counts on duplicate wells is indicated by the vertical bars. HGF, (28, 31) served as the positive control.

**Table V**

*Mitogenic effects of BDGFs on various target cell types*

Mitogenic stimulation was tested over a dose range of 0-50 GFU/ml. The + indicates significant mitogenesis relative to vehicle-treated controls; – indicates no difference relative to controls. Target cells tested were mouse BALB/c/3T3 fibroblasts, BCE cells, and osteoblasts isolated from newborn rat calvaria.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Fibroblasts</th>
<th>Endothelial cells</th>
<th>Osteoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDGF crude</td>
<td>+</td>
<td>ND*</td>
<td>+</td>
</tr>
<tr>
<td>BDGF-0.1</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BDGF-0.45</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>BDGF-1.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDGF-1.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDGF-1.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDGF-2.0</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND*, experiment not done.

**DISCUSSION**

Bone is unique among tissues in the variety of polypeptide growth factors which it harbors. The total activity of 570 GFU/g obtained by EDTA extraction (Table I) is divided among five or six independent activities separable by heparin-Sepharose affinity chromatography (Fig. 3, Table II). In contrast, cartilage (12) and brain (28, 42) exhibit a very simple growth factor pattern on heparin-Sepharose, with only one or two principal peaks of activity. Although the exposure of a single factor to proteolytic degradation, or aggregation of a factor with different "carrier" proteins, could theoretically account for multiple peaks in bone, this is unlikely for several reasons. First, inclusion of proteolytic inhibitors during the extraction of bone powder failed to alter the observed pattern of growth factor activities. Second, the properties of each of the separate BDGFs with regard to stability (Table IV), target cell action (Fig. 6; Table V), and other molecular properties are clearly distinct.

This study was designed to survey in a quantitative fashion the growth factors of bone matrix. An underlying assumption was that the bioassay with quiescent BALB/c/3T3 fibroblasts would be nondiscriminatory in its response to various BDGFs resolved by heparin-Sepharose. This bioassay is widely used, and is known to respond in a quantifiable, dose-dependent manner to EGF, PDGF, CDGF, aFGF, cFGF, anionic retinoid-derived growth factor, chondrosarcoma-derived growth factor, HGF, and IGF-1 (12, 27, 28, 30, 41, 42, 46), which covers a wide spectrum of competence and progression factors (47). It is indeed possible that bone matrix contains factors in addition to the six BDGFs of Table II. Screening of extracts and heparin-Sepharose chromatograms with other specific bioassays would answer this question. Other factors could also have been overlooked because of the requirement for a second, synergistic factor separated during heparin-Sepharose chromatography. The presence of 10% "spent" calf serum in the BALB/c/3T3 assay provides some degree of complementation of single isolated factors with their synergistic requirements. However, TGF-β, now recognized to be abundant in bone (17, 18) and to be produced in bone organ culture (9), was not detected by routine screening in this study because BALB/c/3T3 cells do not respond to TGF-β except weakly in the presence of 1 ng/ml of EGF (data not shown).

Heparin-Sepharose affinity chromatography is a powerful tool for analysis of growth factor activity profiles in bone matrix (Fig. 3). Overall recovery of the total applied crude growth factor activity ranged from 30 to 45%. Several significant corrections elevate this value. First is the attenuation of the bioassay by the high NaCl concentrations present in the largest BDGF peaks. Second is the adsorptive loss incurred by contact of fractions with low total protein concentration with the column, tubing, and fraction collection tubes. In a pilot study, addition of 0.1 volume of 2 × SBT buffer to each tube prior to collection significantly enhanced the recovered activity of BDGF-1.1, BDGF-1.5, and BDGF-1.7, although this is not advisable if protein purification is the motive. In combination, the corrected overall recovery from heparin-Sepharose can be improved to 60-90%, and this argues against the possibility that significant amounts of BDGFs have escaped detection or been subjected to synergistic losses. Heparin-Sepharose thus stands as a reliable medium for resolving and purifying many different growth factor activities (12, 27, 28, 42, 43), in addition to providing valuable information for growth factor classification (28, 42). Application to characterize pathological alteration of growth factor profiles caused by nutritional, metabolic, or hereditary disorders appears very promising.

The volume concentration of total growth factor activity in bone is at least 1,000 GFU/ml (from Table I assuming an average density of 1.9 g/ml for bone powder). This is about 100 times higher than the levels required to cause maximal mitogenic stimulation of BALB/c/3T3 fibroblasts, capillary endothelial cells (Fig. 6), and osteoblasts (Table V). Obviously the matrix must play an important role in sequestering and masking these factors, since the cells of bone are not continuously proliferating. Although the growth factor activity in bone appears high, especially in contrast with the 50-100 GFU/ml (principally PDGF) for calf serum (Fig. 1), it is modest in comparison with the 20,000 GFU of aFGF/g of bovine brain, wet weight (40), and the 30,000 GFU of hepatoma growth factor activity/g of packed human hepatoma cells (43).

Extraction of BDGFs requires desmineralization (Table I), but this is not adequate evidence to establish the mineralized extracellular matrix as the precise locus of the BDGFs. In fact, some or all of these activities could be associated with the cells (osteoblasts, osteocytes, osteoclasts, mesenchymal...
cells, capillary endothelial cells, monocytes/macrophages, and less abundant types) normally present in compact bone of membranous or endochondral origin. Marrow elements, cartilage, and bone matrix are excluded by the extraction procedures (see Materials and Methods), and the pre-extraction washes of the finely ground bone powders (<150 μm) with water and hypotonic buffer thoroughly removed trapped blood and many cytosolic components without repressing appreciable growth factor activity (Table I). Ultimate access to the factors, whether associated with cells, extracellular organic matrix, or hydroxyapatite, requires dissolution of the bulk matrix by demineralization. Several laboratories have used 4 M guanidine HCl extraction subsequent to demineralization with 0.5 M HCl to obtain osteoinductive (15, 39) or chondroinductive (17, 19) factors from bone. We have not found this extraction protocol to yield more than 26% of the total BDGF activity obtainable by 0.5 M EDTA alone (Table I). In addition, heparin-Sepharose profiles of the 4 M guanidine HCl extract of HCl-demineralized bone revealed no new peaks of mitogenic activity (Table II). Intrinsic sensitivity of certain BDGFs to 4 M guanidine HCl and 0.1 M HCl (Table IV) partially explains this reduced yield. In contrast to the 0.1 M HCl, pH 1, conditions in Table IV, it is important that the 0.5 M HCl extraction of BDGFs was actually at pH 3.3 due to the buffering effect of bone mineral. This accounts for the presence of acid-labile BDGFs in 0.5 M HCl extracts (Table II). Importantly, Sampath and Reddi (39, 48) have reported that the osteoinductive activity of rat bone is also present in HCl and EDTA extracts, raising the interesting possibility that among the BDGFs resolved by heparin-Sepharose is one with osteoinductive activity.

Tentative Classification of the Bone-derived Growth Factors

Information from the present study warrants the following classification of bovine BDGFs.

**BDGF-0.45**—BDGF-0.45 elutes coincident with authentic human PDGF from heparin-Sepharose, and it shares properties of stability to boiling, low pH, 4 M guanidine HCl, and inactivation by DTt and trypsin with authentic PDGF (41). Identically to PDGF, BDGF-0.45 is a potent mitogen for BALB/c 3T3 fibroblasts and rat osteoblasts but is inactive on bovine capillary endothelial cells. BDGF-0.45 displaces bovine 125I-PDGF in a fibroblast radioreceptor assay (performed by E. Raines and R. Ross, U. of Washington, Seattle (41, 49)) at levels consistent with its GFU concentration from parallel mitogenic assays. It is closely related or identical to bovine PDGF and present at a level of about 50 ng/g of dry bone. Recent antibody studies showed no PDGF biosynthesis by cultured rat calvaria (9), so the PDGF-like activity in bone may be of platelet (41) or macrophage (21) origin. Subtle differences in structure are possible between BDGF-0.45, the heterodimer PDGF, and homodimers of A and B chains or sia gene products which also compete with PDGF for cellular receptors (50).

**BDGF-1.1**—BDGF-1.1 elutes from heparin-Sepharose coincident with anionic bovine retinal FGF (aFGF, 40) and anionic retina-derived growth factor (46). Immunohistological staining displays BDGF-1.1 on SDS-PAGE Western blots as a 15.6-kDa doublet using the specific antibody to bovine aFGF (provided by K. Thomas and M. Rios-Candelore, Merck Institute, Rahway, NJ) at antigen levels consistent with the applied GFU. BDGF-1.1 is inactivated by boiling, low pH, 4 M guanidine HCl and trypsin, but is stable to DTt and curiously spared by DTt from inactivation by 4 M guanidine HCl. Partially purified BDGF-1.1 contains no detectable PDGF activity in the radioreceptor assay (49). Agarose isoelectric focusing reveals a single activity band at PI 5.2 (Fig. 5) which is active on fibroblasts, osteoblasts, and endothelial cells. Because all of these properties are consistent with those reported for aFGF (40) and anionic retina-derived growth factor (46), BDGF-1.1 is concluded to be identical to aFGF and is present at 0.1–0.5 ng/g of dry bone (based on immunohistochemical estimates) to 12 ng/g of bone (assuming 5 GFU/ng of protein, 27).

**BDGF-1.5**—BDGF-1.5 is the most abundant activity in fetal membranous bone and is present in adult endochondral bone also. Inactivated by boiling, low pH, 4 M guanidine HCl, and relatively slowly by trypsin, but stable to DTt, BDGF-1.5 in its 44,000-fold present state of purification contains two protein species of 14 (basic pi) and 27 kDa (acidic pi) by silver-stained two-dimensional SDS-PAGE, as well as a trace of material at 18 kDa (Fig. 4a), which could be contamination from BDGF-1.7. There is no cross-reactivity in the PDGF radioreceptor assay (49). Active on fibroblasts, osteoblasts, and endothelial cells, BDGF-1.5 elutes from heparin-Sepharose coincident with the position of cFGF (42) and is probably a cFGF-like activity.

**BDGF-1.7**—BDGF-1.7 elutes from heparin-Sepharose in a position similar to the 19 kDa, 9.5 CDGF (12, 42). An abundant activity in adult endochondral bone but not in fetal membranous bone (Table II), BDGF-1.7 has been purified to near homogeneity (Table III), with silver-stained SDS-PAGE indicating a triplet of 17.5–18.4 kDa (Fig. 4b). Active on fibroblasts, osteoblasts, and endothelial cells (Fig. 6), BDGF-1.7 shows no PDGF activity; it is possibly a CDGF-like protein (and thus also cFGF-like).

**BDGF-0.1**—BDGF-0.1 has not been characterized; it possibly resembles EGF or IGF-1 by its lack of binding to heparin-Sepharose at 0.1 M NaCl.

**BDGF-2.0**—BDGF-2.0 is a minor activity as yet uncharacterized; the affinity for heparin-Sepharose is higher than for any other growth factor yet reported.

In addition to the possibility of local production by various bone cell populations, the BDGFs indicated above and other factors mentioned previously may be sequestered from circulating blood after originating in extraosseous sources such as platelets and brain. Pathologically derived factors, such as those from tumors, could also lodge in the bone matrix. In a sense, the origin of the BDGFs is a moot point, since it has no bearing on their presence in the matrix or their potential regulatory actions on bone cells.

Target Cells for BDGFs and the Relevance to Bone Cell Biology

Many polypeptide growth factors appear to exert a mitogenic effect on differentiated osteoblasts, including BDGF-0.45, 1.1, 1.5, and 1.7 (Table V), cultured rat calvaria factors BDGF and TGF-β (5, 9, 10), SGF (13, 14, 51), EGF, cFGF, PDGF, IGF-1, and cartilage-derived factor (5), macrophage-derived factor (20), endochondral cell-derived growth factor (22), IL-1 (4), and a prostatic tumor factor (24). Most of the BDGFs are generally active on mesodermally derived cells (Table V), but several factors have apparent target-cell selectivity for stimulation of fibroblasts (F), chondrocytes (C), osteoblasts (O), and capillary endothelial cells (E) including: BDGF-0.45 (F and O, but not E (this study)); hSGF (O and C, but not F (13, 14, 51)); endochondral cell-derived growth factor (O, but not F or C (22)); macrophage-derived factor (O and C, but not F (20)); and prostatic tumor factor (O, but not F (24)). Although mitogenesis is often the focus of interest in growth factors, of equal importance is the potential hormonal action by which specific differentiated functions of non-proliferating...
cell populations may be regulated. Growth factor enhancement of osteoblastic functions includes collagen and non-collagen protein synthesis (BDGF (5, 6), hSGF (51), IGF-1 (5)), alkaline proteinase (bSGF (51), rat osteosarcoma differentiation factor (25)), and prostaglandin-dependent bone resorption mediated by osteoblasts (EGF (6)). Osteoclast-activating factor (IL-1) and a related lymphotacin (4, 5), as well as BDGFs in the present study, are found to decrease alkaline proteinase activity, as should be expected from the relationship of this enzyme to osteoblast culture density and proliferation rate (32, 34). Growth factors may also regulate osteoblastic collagenase (52) similar to their elevation of this enzyme in fibroblasts (53, 54).

Of particular relevance to bone may be the enhanced protein kinase activity (55) and the intracellular Ca2+ mobilization (56) accompanying growth factor stimulation of cells. Such stimulation could provide for the special needs of mineralizing tissues involving calcium phosphate mineral deposition and the prolific biosynthesis and secretion of phosphorlated proteins into the extracellular matrix.

**Growth Factors in the Extracellular Matrix and the Local Control of Bone Formation**

The exquisite balance between osteoclastic resorption and osteoblastic formation of bone is currently understood in terms of local regulation or “coupling” (57), in which both the extracellular matrix (58) and local growth factors (5, 13, 20) are believed to play a central role. Access of resident osteoclasts and recruited monocytes to the bone surface destined for resorption is apparently modulated by osteoblasts (69). Within the locus of biochemical signals emanating from an established osteoclastic resorption site, local differentiation and proliferation of osteoblasts is promoted and new bone is formed. Capillary ingrowth involving endothelial cell proliferation in haversian bone also is coupled to resorption by the osteoclastic “cutting cone.” Candidates for providing these signals include growth factors, the collagenous extracellular matrix (61), and the calcium phosphate mineral phase itself.

Osteoclastic resorption can solubilize polypeptide growth factors from bone matrix, and an example of SFG release by osteoclasts has been provided (62). If other growth factors are liberated from matrix or exposed without being proteolytically damaged, it is unknown whether the factors act on target cells in a soluble form or are presented in an adsorbed state on hydroxyapatite. In distinction to endothecial, paracrine, and autocrine routes for growth factor stimulation, cellular interaction with matrix-adsorbed factors could be called “matricrine.” The affinity of growth factors for hydroxyapatite is supported by the BDGF extraction data (Table I) and the many growth factor purification schemes involving hydroxyapatite chromatography (13, 15). Pure powdered calcium phosphate is mitogenic for fibroblasts, rivaling polypeptide growth factors in its potency (47, 63). This curious activity apparently involves direct interaction of the plasma membrane with crystalline mineral surfaces, mimicking the effect of a competence factor and promoting transition of cells from G0 to G1 (47). Osteoblasts in bone are generally restricted from direct contact with the calcium phosphate (hydroxyapatite) mineral phase by a layer of protein-rich non-mineralized osteoid. Direct mineral contact resulting in mitogenic stimulation could occur where 1) osteoblasts migrate into freshly denuded resorption lacunae or 2) osteoid is degraded by osteoblastic collagenase (52). Osteoblast-mediated exposure of hydroxyapatite has also been suggested as a prerequisite for osteoclastic degradation (60, 64).

The extracellular matrix apparently plays a critical role in controlling the distribution and presentation of growth factors. First, the matrix provides a temporally extended storage depot for local messages awaiting decipherment by cells which arrive on the scene long after the producing cell has departed or ceased to function. During bone remodelling, growth factors such as CDF or CDGF produced in the endochondral stage of osteogenesis could be zonally concentrated and redistributed in bone matrix, only to be released at a later stage. Second, the regulation of extracellular matrix biosynthesis by growth factors may actually allow modulation by a feedback loop involving adsorption of the factors to new matrix. The well-characterized systemic responses of the skeleton to endocrine signals cannot explain the complex patterns of bone growth and remodelling which are exquisitely sensitive to local physical stresses (Wolff's law). In a dense, labyrinthine, mineralized tissue such as bone, with many barriers to the free diffusion of circulating hormones, it is provocative to suggest that local responses may be regulated by the reservoir of specific growth factors in the matrix.

**Acknowledgments**—We thank Drs. K. Thomas, M. Rios-Candelore, E. Raines, and J. Sasse for performing assays of BDGFs with other specific growth factor antisera and assay systems, and Dr. Y. Shing for BALB/c/3T3 cells. Drs. E. Canalis, M. Centrella, A. H. Reddi, and T. K. Sampath kindly provided manuscripts prior to publication. K. Butterfield, K. Fried, G. Hintsch, and S. Smith provided expert technical assistance.

**REFERENCES**

21. Shimokado, K., Raines, E. W., Madtes, D. K., Barrett, T. B.,
Bone-derived Growth Factors

64. Chambers, T. J., and Fuller, K. (1985) Calcif. Tissue Int. 37, 162a