Accelerated Wound Repair, Cell Proliferation, and Collagen Accumulation Are Produced by a Cartilage-derived Growth Factor

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ABSTRACT Cartilage-derived growth factor (CDGF), a cationic polypeptide of ~18,000 mol wt, was prepared from bovine articular cartilage; other sources were bovine and human scapular and costal cartilage. Previous studies have shown that CDGF stimulates the proliferation of cultured mouse fibroblasts as well as chondrocytes and endothelial cells from various sources. In this study, CDGF was shown to stimulate dose-dependently the accumulation of DNA and collagen by rat embryo fibroblasts and a population of fibroblasts derived from granulation tissue. CDGF also stimulated the proliferation of cultured bovine capillary endothelial cells dose-dependently. To evaluate the effects of CDGF in vivo, we implanted polyvinyl alcohol sponges subcutaneously in rats. 6 d postimplantation, sponges were injected with 300 μg of partially purified CDGF, a dose which takes into account the cell numbers in the sponges as compared with cell cultures. CDGF rapidly disappeared from the sponges and only ~10% of the initial dose was present at 4 h. Despite its transient presence, CDGF caused a relative increase in sponge DNA content of 2.6-fold at 48 h and 2.4-fold at 72 h. We repeated the sponge experiment by using 500-ng injections of CDGF purified to near homogeneity by heparin-Sepharose chromatography. Purified CDGF caused significant increases in sponge collagen, protein, and DNA content at 48 and 72 h after a single injection. The effects of CDGF were abolished by heat and unaffected by reduction of disulfide linkages. Morphologically, CDGF did not evoke an inflammatory response, and its effect on proliferating endothelial cells and fibroblasts was, therefore, probably direct. However, increases in DNA content of sponges could not be fully accounted for by increased DNA synthesis, which suggests that recruitment may be an important component of the in vivo response. Taken together, the effects of CDGF on cultured cells and granulation tissue suggest that the sustained presence of CDGF in vivo may greatly enhance its effects upon wound repair.

A number of polypeptide growth factors that stimulate the proliferation of cells in culture have been described in the last decade. These include epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor (5, 6, 17). Many of the growth factors are large polypeptides that have specific receptor-mediated interactions with their target cells, and such interactions are associated with cell proliferation in vitro. By contrast, the efficacy of growth factors in vivo is not nearly so clearly established. This is in part the result of the difficulty of administering growth factors and measuring their cellular response in vivo.

The cartilage-derived growth factor (CDGF)[1] is a cationic polypeptide with a molecular weight of ~18,000 which can

[1] *Abbreviations used in this paper*: BCE, bovine capillary endothelial; CDGF, cartilage-derived growth factor; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TCA, trichloroacetic acid.

be obtained from articular, scapular, and costal cartilage of bovine or human sources (3, 12). CDGF is probably related to a capillary endothelial cell growth factor derived from a rat chondrosarcoma (19). CDGF stimulates the proliferation and migration of cultured fibroblasts, chondrocytes, and endothelial cells (4, 11, 22). In this paper, we compare the effects of CDGF on the proliferation and collagen synthesis of cultured fibroblasts with those observed in vivo. We studied the in vivo effects by examining the organizational response in subcutaneously implanted polyvinyl alcohol sponges in rats. In addition, the proliferation and motility of endothelial cells stimulated by CDGF in culture was compared with the induction of angiogenesis in sponges in vivo.

MATERIALS AND METHODS

Preparation of CDGF Extracellular Matrix: The preparation of bovine articular cartilage from the joints of 1–14-d-old calves has been described previously (3). For the preparation of cartilage extracellular matrix, cartilage was diced finely with a scalpel and resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.5, that contained 0.2% clostridial collagenase (150 U/ml, CLS II, Worthington Biochemical Corp., Freehold, NJ), penicillin (200 U/ml), and streptomycin (200 μg/ml). The cartilage was digested in a capped sterile tube for 16–18 h at 37°C with constant agitation. The digest was passed through a 153-μm nylon sieve (HC3-110, Tetko, Inc., Elmsford, NY) to remove debris and undigested material. Chondrocytes were removed from the filtered digest by sedimentation at 1,000 *g* for 10 min. The supernatant, which was designated as the cartilage matrix digest, was sterilized by filtration through a 0.45-μm filter (Nalgene, Rochester, NY). The cartilage matrix digest contains mostly protein and proteoglycan at a concentration of ~25 mg/ml. For short-term storage (≤1 mo), the cartilage matrix digest was stored frozen at −20°C. For long-term storage, the cartilage matrix digest was dialyzed against three changes of distilled deionized water (Spectrophor tubing, 6,000–8,000 M_r cutoff, Fisher Scientific Co., Pittsburgh, PA), lyophilized, and stored as a powder at −20°C.

Ion Exchange Chromatography: Cartilage matrix digest was partially purified by cation exchange chromatography on Bio-rex 70 (200–400 mesh, Bio-Rad Laboratories, Richmond, CA). Columns (2 × 9 cm, 28 ml bed vol) were equilibrated with 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.0, at a flow rate of ~20 ml/h. Samples were applied to the column in one of two ways. In the first, 50 ml of frozen cartilage matrix digest was thawed and mixed with 50 ml of the equilibration buffer. The sample was then filtered through a 0.45-μm Nalgene filter and applied to the column. Alternatively, 1 g of lyophilized cartilage matrix digest was resuspended in 100 ml of equilibration buffer, filtered with a 0.45-μm Nalgene filter, and applied to the column. After sample loading, the column was washed with 90 ml of equilibration buffer. Bound material was eluted with a gradient of 0.1–1 M NaCl in 0.01 M Tris-HCl, pH 7.0, in 140 ml. 8-ml fractions were collected and monitored for absorbance with a Gilford 2400-S spectrophotometer 280 nm (Gilford Instruments Laboratory, Inc., Oberlin, OH), for conductivity with a radiometer (Rainin Instrument Co., Woburn, MA), and for the ability to stimulate DNA synthesis in BALB/c 3T3 cells. The material from this procedure was designated partially purified CDGF.

Heparin-Sepharose Chromatography: Crude growth factor was prepared by extraction of bovine scapular cartilage with 1 M guanidine chloride as described previously (12). An extract (30 g/700 ml) was resuspended in a column buffer of 0.1 M NaCl and 0.01 M Tris HCl, pH 7.5. The sample was divided in half, and each half was applied at 4°C to a column (160 ml bed vol) of heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at a flow rate of 60 ml/h. The columns were washed first with 0.15 M NaCl and 0.01 M Tris, pH 7.5 (1,300 ml) and then with 0.5 M NaCl and 0.01 M Tris, pH 7.5 (600 ml). Growth factor was eluted from both columns by batch elution with 2.5 M NaCl and 0.01 M Tris, pH 7.5 (1,000 ml). The two 0.5 M–3.0 M NaCl fractions were pooled and dialyzed exhaustively against 0.1 M NaCl and 0.01 M Tris, pH 7.5 (three changes, 48 h, Spectrophor tubing, 6,000–8,000 M_r cutoff, Fisher Scientific Co.). About 400,000 U (in ~2,000 ml) of growth factor activity were recovered from the two columns and applied to another 160-ml heparin-Sepharose column. After a wash with 0.6 M NaCl and 0.01 M Tris, pH 7.5, growth factor was eluted with a 1,500 ml gradient of 0.6–3.0 M NaCl at a flow rate of 50 ml/h. 8-ml fractions were collected and growth factor activity was determined, as measured by testing for the ability of fractions to stimulate DNA synthesis in 3T3 cells. The concentration of NaCl in each fraction of the gradient was determined with a conductivity meter (radiometer). The material from this preparation was designated purified CDGF.

SDS PAGE: CDGF was analyzed by SDS PAGE (13). The polyacrylamide gels were developed with silver stain (15). Molecular weight markers were purchased from Bio-Rad Laboratories and from Bethesda Research Laboratories.

Size Exclusion Chromatography: CDGF (14,000 U/2.5 ml), purified by heparin-Sepharose chromatography, was applied to an ACA 54 (LKB Instruments, Inc., Gaithersburg, MD) size-exclusion column (1.6 × 100 cm) equilibrated with 0.5 M NaCl, 0.01 M Tris HCl, pH 7.5. Growth factor was eluted at a flow rate of 6 ml/h, and fractions (2.8 ml) were collected and tested for the ability to stimulate DNA synthesis in 3T3 cells. The column was calibrated with the molecular weight markers serum albumin (M_r 68,000), myoglobin (M_r 17,800), and ribonuclease (M_r 13,700).

Assay of DNA Synthesis in 3T3 Cells: The growth of BALB/c 3T3 cells, the preparation of confluent, quiescent 3T3 cells in 96-well microtiter plates (Costar Corp., Cambridge, MA), and the methods for measuring the incorporation of methyl-[^{3}H]thymidine into DNA have been previously described (11). In brief, a microtiter plate that contained ~2 × 10^4 confluent, quiescent BALB/c 3T3 cells per microtiter well was prepared. The CDGF sample and [^{3}H]thymidine (4 μCi/ml) were added, and the incorporation of [^{3}H]thymidine into DNA was measured after a 36–48-h incubation. In the bioassay, background incorporation was typically 200–1,000 cpm, and maximal incorporation was ~100,000–120,000 cpm. A unit of growth factor activity is defined as the amount of growth factor required to half-maximally stimulate incorporation of [^{3}H]thymidine (~50,000–60,000 cpm) into the DNA of 10^4 3T3 cells.

Assay of Capillary Endothelial Cell Proliferation: Bovine capillary endothelial (BCE) cells were prepared from adrenal glands as described by Folkman et al. (4). BCE cells were grown and maintained in tumor-conditioned medium, which we prepared by incubating mouse sarcoma 180 cultures for 48 h in Dulbecco's modified Eagle's (DME) medium supplemented with 10% calf serum and diluting culture supernatants with an equal volume of fresh DME + 1% calf serum. BCE cell proliferation was measured in 24-well microtiter plates (16-mm diam microtiter wells, Costar Corp.). The tumor-conditioned medium was removed, and the cells were trypsinized, resuspended in fresh DME + 10% calf serum, and plated sparsely into microtiter wells (10,000 cells/well). The next day, the medium (0.5 ml) that contained unattached cells was removed, and the attached cells were fed with fresh DME + 10% calf serum to which growth factor preparations had been added. After a 72-h incubation at 37°C, the cells were detached from the microtiter well with 0.5% trypsin and 0.2% EDTA and counted in a particle counter (model Zf, Coulter Electronics, Inc., Hialeah, FL).

Assay of Cell Proliferation and Collagen Synthesis in CDGF-stimulated Rat Fibroblasts: Confluent cultures of rat embryo fibroblasts (provided by F. O'Neill, Veterans Administration Medical Center, Salt Lake City, UT) were trypsinized and replated at low density (10,000 cells/well) on 24-well Costar culture dishes in DME that contained 10% fetal calf serum (FCS) to compare the effects of CDGF on rat fibroblasts with those observed with 3T3 cells. After overnight incubation to allow attachment, cell layers were rinsed with PBS and re-fed with DME that contained 0.5% FCS and 0–32 μg/ml CDGF. Positive-control wells that contained 5 or 10% FCS were also included. Incubation was continued for 72 h in these conditions, and then these media were removed by aspiration and replaced with labeling medium that contained 0.5% dialyzed FCS, ascorbic acid (50 μg/ml), β-aminoproprionitrile (64 μg/ml), and [^{3}H]proline (translation grade, 20 μCi/mM, New England Nuclear, Boston, MA). After incubation for 3 h, media were harvested, clarified by centrifugation, and precipitated with 10% trichloroacetic acid (TCA). Residual TCA was removed from the 40,000 *g* pellets by successive washes with ethanol and ether. TCA-insoluble proteins were dissolved in 0.05 M NaOH, neutralized, and subjected to digestion with purified bacterial collagenase (Form III, Advance Biofactures, Lynnbrook, NY) by the use of standard methodologies (16) as described below. Cell layers were rinsed with PBS, dissolved in 0.05 M NaOH, and assayed for DNA content by use of a fluorometric assay (8). Data were obtained from duplicate experiments with triplicate test conditions.

In a second set of experiments, fibroblasts were isolated from sponge granulation tissue, prepared as described below, on the sixth day after sponge implantation. Sponges were dissected free of adventitious tissue, washed in PBS containing antibiotics (penicillin, 1,000 U/ml; streptomycin sulfate, 1,000 μg/ml; fungizone, 0.25 μg/ml), minced, and incubated in DME that contained 1 mg/ml collagenase (CLSPA, Worthington Biochemical Corp.) and antibiotics for 3 h at 37°C. Cells were dislodged by repeated aspiration with a Pasteur pipette. Sponge material was allowed to settle, and cells were decanted and collected by centrifugation. The cell pellet was resuspended in DME that contained 10% FCS and seeded onto culture flasks. Secondary cultures, which had the morphology of fibroblasts, were derived by trypsinization at confluence and were then used to seed 24-well dishes with 10^4 cells/well as described above. After exposure to varying concentrations of purified CDGF in 0.5% FCS, cell layers were analyzed for DNA accumulation (9).

Assay of Fibroplasia, Protein Synthesis, and Collagen Proliferation in Sponge Implants In Vivo: Sponge implants were placed beneath the ventral panniculus carnosus of 200-g rats by a standard technique using 9 × 2 mm polyvinyl sponge disks (20). Each animal was implanted with four sterile sponges, and analysis of each experimental condition was performed on three identically treated sponges from individual rats. 6 d after implantation, one set of animals was killed by ether anesthesia and the sponges were removed for determination of base-line conditions. Three sets of experimental conditions were used: (*a*) sponges were injected with 0.1 ml of partially purified CDGF (150 U; 300 μg) or purified CDGF (150 U) in 0.9% saline; (*b*) sponges in the same animal received an injection of 0.9% saline alone; and (*c*) uninjected sponges were maintained in rats that received no CDGF. Experimental animals were sacrificed 0, 48, and 72 h after injection. Implanted sponges were dissected free of loosely adherent fat and muscle and placed into DME that contained antibiotics (penicillin, 1,000 U/ml, and streptomycin, 1,000 μg/ml) and 10% FCS.

For evaluation of collagen and protein synthetic activity, sponges were finely minced and cultivated in a gyrotory water bath at 37°C in 3 ml DME that contained [^{3}H]proline (30 μCi/ml), β-aminoproprionitrile (64 μg/ml), ascorbic acid (50 μg/ml), 1% dialyzed FCS, and a 10-fold lower concentration of antibiotics than described above. At the end of the incubation, the minced tissue was rinsed with ice cold PBS that contained 1 mg/ml unlabeled proline and homogenized in 0.5 M acetic acid that contained pepstatin (1 μg/ml) and *N*-ethyl maleimide (10 mM) to inhibit acid proteases. The homogenate was extracted overnight at 4°C, and aliquots were removed for amino acid analysis and DNA determination. The homogenate was then clarified by centrifugation (18,000 *g*, 4°C, 20 min), and the supernatant was precipitated with 10% TCA for collagenase digestion (16) as described above.

Relative collagen synthesis was quantified as the amount of radioactivity released into TCA-soluble material after limited digestion with highly purified bacterial collagenase. Each sample was corrected for nonspecific release of radioactivity in blanks incubated without enzyme. Percentage collagen synthesis was corrected for the over-representation of amino acids in the protein (16). Collagen content was determined by amino acid analysis of hydrolyzed (18 h, 110°C) sponge under the assumption that the average collagen molecule contained 10% hydroxyproline residues. DNA content of sponges was measured in homogenates by use of the fluorometric assay described above (8).

Assay of DNA Synthesis in Granulation Tissue: Implanted sponges (four per animal) were injected on day 4 with 150 U of partially purified CDGF (n = 2), saline, or nothing. Rats were injected intraperitoneally with methyl-[^{3}H]thymidine (250 μCi) 6 h before they were killed. Animals were sampled 6, 24, 48, and 72 h after the initial CDGF injection. For the determination of the proportion of newly synthesized DNA in granulation tissue in vivo, labeled sponges were rinsed, homogenized in 0.1 M NaOH, and extracted overnight at 4°C. Aliquots were taken for fluorimetric determination of DNA content, TCA-precipitable radioactivity, and TCA-soluble disintegrations per minute. Total uptake per sponge, uptake per milligram wet weight, incorporation per sponge, and incorporation per microgram DNA were determined. To correct for animal variability, all data were expressed as the ratios of CDGF-injected to matched control values.

Measurement of the Disappearance of CDGF from Sponge Implants: ^{125}I-CDGF (10 μg) was prepared with Bolton-Hunter reagent (4,000 Ci/mmol, New England Nuclear) according to manufacturer's instructions, and had an initial specific activity of 3 × 10^5 cpm/μg. The rate of disappearance of CDGF when injected into implanted sponge granulomas was measured by injection of 0.1 ml ^{125}I-CDGF (3 × 10^3 cpm) into 6-d-old sponge implants (two per animal), and removal of four sponges immediately and at 0.5, 4, 24, 48 and 72 h. Sponges were dissolved in 1 ml NCS (Amersham Corp., Arlington Heights, IL), and radioactivity was quantitated by liquid scintillation counting.

Histological Examination of Sponge Granulomas: At least two sponge implants from each time period and experimental condition were dissected free of adherent tissue and bisected perpendicularly. Half of each sponge was fixed in formalin and processed for light microscopy. The other half was fixed in Karnovsky's fixative (10), embedded in Spurr resin, thick sectioned (1 μm), and stained with toluidine blue.

RESULTS AND DISCUSSION

Isolation and Purification of CDGF Activity

Fig. 1 shows isolation of partially purified CDGF by use of Bio-rex 70 resin. With this procedure, it was possible to isolate a protein fraction that had strong positive effects on DNA synthesis in 3T3 cells, and to separate this material from low molecular weight proteins with few or no such effects. However, SDS PAGE indicated that several polypeptide species were present (data not shown). As measured by absorbance at 280 nm, the protein that co-eluted with CDGF represents a minor fraction of the total protein in the cartilage matrix preparation.

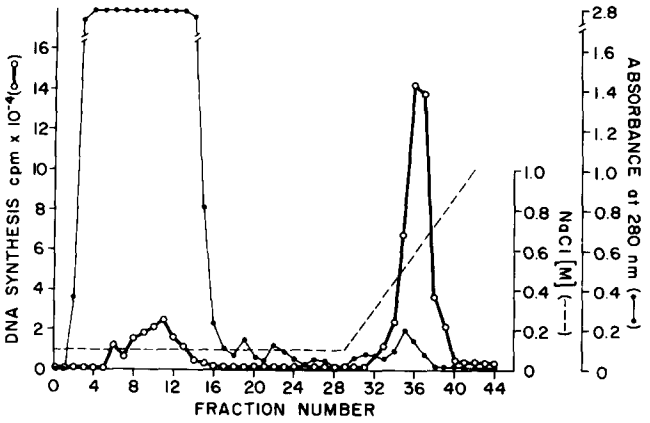

FIGURE 1 Cation exchange chromatography of CDGF from bovine articular cartilage. Cartilage matrix, ~1.25 g, was prepared by collagenase digestion. The sample was diluted with an equal volume of equilibration buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.0) and applied to a 2 × 9 cm column of Bio-rex 70. The column was washed with equilibration buffer. Growth factor was eluted from the column with a gradient of 0.1 to 1.0 M NaCl in equilibration buffer. Activity was measured by the ability of fractions to stimulate DNA synthesis in BALB/c 3T3 cells.

To demonstrate that a unique fraction of cartilage matrix was responsible for biological activity, we used heparin-Sepharose chromatography to purify CDGF. A growth factor from rat chondrosarcoma was recently isolated by this procedure (19).

When extracts of bovine cartilage were applied to a column of heparin-Sepharose, growth factor bound tightly to the column and was eluted with 1.6–1.8 M NaCl. (Sullivan, R., and M. Klagsbrun, manuscript in preparation.) A high degree of purification was obtained by use of two cycles of heparin-Sepharose chromatography. In the first cycle, crude extract was applied to the column and after a wash with 0.5 M NaCl, CDGF was eluted with 2.5 M NaCl. Much more than 99% of the contaminating material was removed (data not shown). This preparation of CDGF was applied to a second heparin-Sepharose column and, after binding, was eluted with a NaCl gradient as shown in Fig. 2. SDS PAGE analysis of the highly purified CDGF preparation is shown in Fig. 3 (lane *1*). Only a few bands are present. The predominant band is a doublet with molecular weights of ~18,000–19,000. The molecular weight of the doublet corresponded closely with the molecular weight of CDGF activity found after size-exclusion chromatography (Fig. 4). It is possible that both bands in the doublet are forms of CDGF. Doublets have been found after the purification of platelet-derived growth factor (1) and fibroblast growth factor (6), and it has been suggested that these are due to processing, proteolytic cleavage that occurs during purification, or different primary structure. Highly purified CDGF stimulated half-maximal DNA synthesis (1 U of activity) at concentrations of ~10–15 ng/ml (Fig. 5). CDGF stimulated not only 3T3 cell proliferation but chondrocyte (11) proliferation as well.

The experiments described below indicate that both types of CDGF preparations elicited similar biological responses.

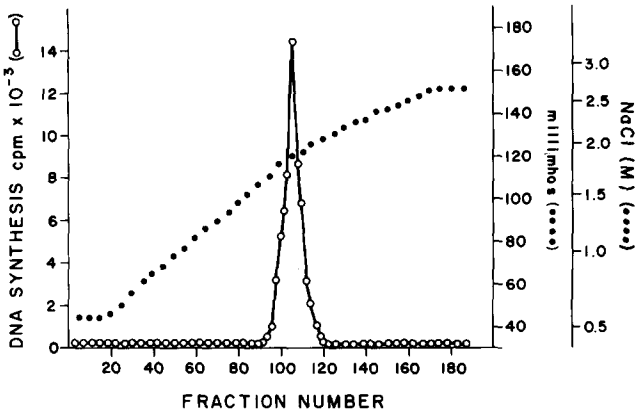

FIGURE 2 Affinity chromatography of CDGF on heparin-Sepharose. About 30 g crude bovine scapular cartilage extract was applied to a column of heparin-Sepharose. After washes in 0.15 and 0.5 M NaCl, CDGF was eluted by batch with 2.5 M NaCl (not shown). About 400,000 U CDGF activity was applied to another column of heparin-Sepharose. After a wash with 0.15 M NaCl, CDGF was eluted with a gradient of 0.6 to 3.0 M NaCl. Fractions were collected and tested for the ability of samples to stimulate DNA synthesis in 3T3 cells. o, [^{3}H]thymidine incorporation into DNA. ●●●●●, conductivity or molarity of NaCl.

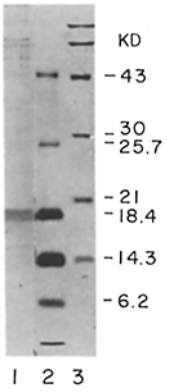

FIGURE 3 SDS PAGE of CDGF. The peak of CDGF activity shown in Fig. 2 (~700 U, 200 ng) was analyzed by SDS PAGE. The polyacrylamide gel slab was stained with a silver stain. Lane *1*, CDGF; lane *2*, molecular weight markers (Bethesda Research Laboratories); lane *3*, molecular weight markers (Bio-Rad Laboratories). KD, kilodaltons.

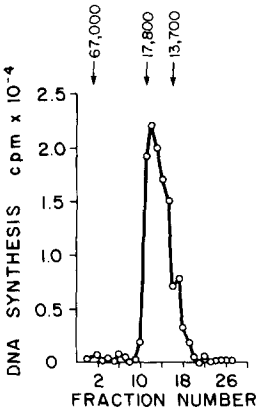

FIGURE 4 Size-exclusion chromatography of CDGF. CDGF (14,000 U) purified by chromatography on heparin-Sepharose as described in Fig. 2 was applied to a size-exclusion column of ACA 54. Fractions were collected and tested for the ability to stimulate DNA synthesis in 3T3 cells. Molecular weight markers were serum albumin (*67,000*), myoglobin (*17,800*), and ribonuclease (*13,700*).

Effects of CDGF on Cell Proliferation and Collagen Synthesis in Rat Fibroblasts

The continuous presence of 0.5–32 μg/ml of partially purified CDGF stimulated serum-starved fibroblasts to proliferate, as measured by DNA production (data not shown). The effect at the lowest dose tested was less than that seen with 0.5% FCS, but the effect at the higher doses exceeded that produced by 5% FCS in stimulating mitosis. Highly purified CDGF prepared by heparin affinity chromatography was tested on fibroblasts derived from granulation tissue on day 6 after implantation (Fig. 6).

Partially purified CDGF stimulated collagen synthesis, expressed as the percentage of total protein synthesis, in rat embryo fibroblasts in a dose-dependent manner (Fig. 7). At a concentration of 1 μg/ml per 10^4 cells, the percent collagen synthesis was increased at least threefold over serum-starved controls. At 4 μg/ml per 10^4 cells, percentage collagen synthesis was almost 50% greater than that observed with 5% FCS. Similar increases in percentage collagen were observed in the cell layer; however, this response reached a maximum at 16 μg/ml of CDGF (not shown).

The absolute rate of collagen synthesis was not determined in these experiments. Since noncollagen protein synthesis may have been decreased, it was not proven that CDGF exerted a specific effect on collagen biosynthesis. In other fibroblast cultures, the level of type I collagen mRNA is known to vary as a function of the rate of proliferation (20), so that the relative increase in collagen synthesis may be secondary to the mitogenic activity of CDGF.

When these positive in vitro effects of CDGF on cell proliferation and collagen synthesis are compared with the in vivo results, it should be recognized that these effects resulted from continuous exposure of the fibroblasts to CDGF. By contrast, in the in vivo studies described below, the exposure of maturing granulation tissue to CDGF was transient rather than continuous.

Effects of CDGF on BCE Cell Proliferation

Fig. 8 shows the positive dose response of BCE cells to partially purified CDGF. The doses used extended to levels more than 10-fold higher than those used in the rat embryo fibroblast studies. Under conditions of continuous exposure, the positive effect on BCE cell proliferation was obviously more pronounced in the range of 5–20 μg/ml than at higher doses. Migration of endothelial cells was previously shown to be stimulated by partially purified CDGF (22).

Effects of CDGF on Sponge Implants

Preliminary experiments showed that the maximal effects on DNA content of a single injection of partially purified CDGF into 6-d-old sponge implants was obtained more than 24 h after the injection (data not shown). Thus, sampling at 48 and 72 h was used. Dosage was based on the results of cell

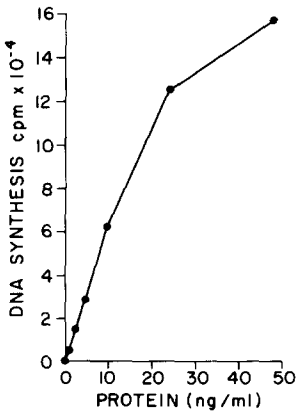

FIGURE 5 Dose-dependent stimulation of DNA synthesis by purified CDGF. CDGF was tested at various concentrations for the ability to stimulate DNA synthesis in 3T3 cells.

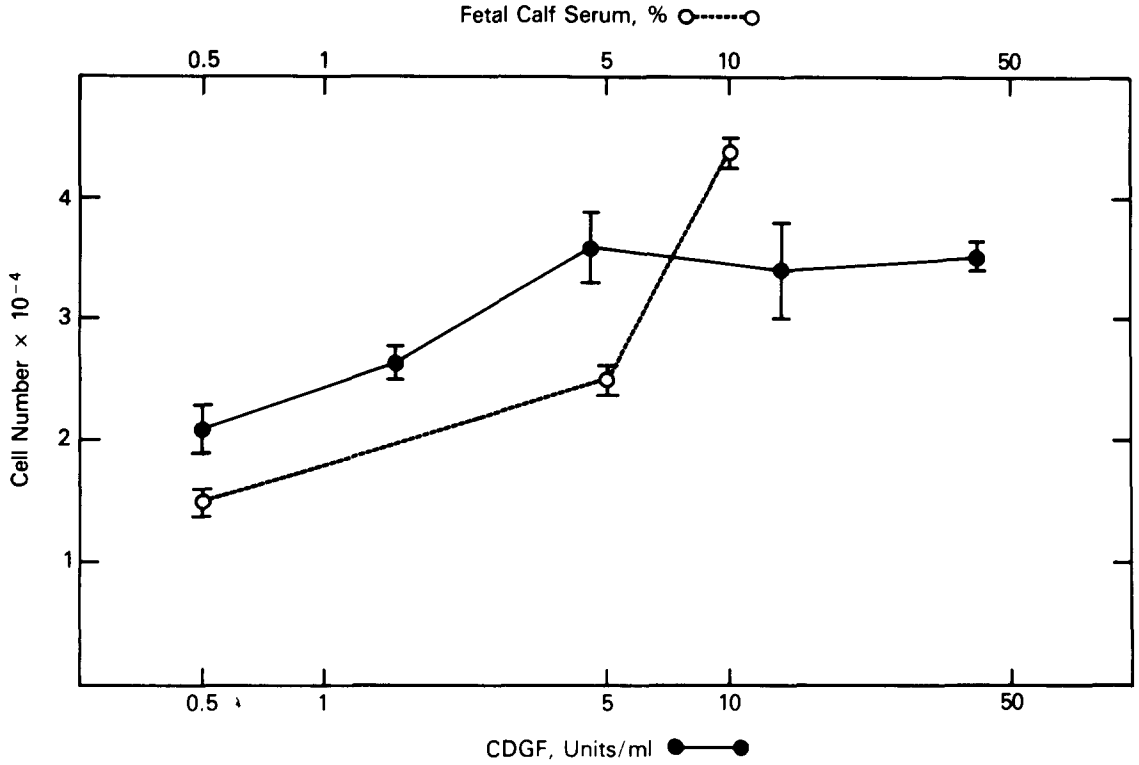

FIGURE 6 Mitogenic effects of purified CDGF on granulation tissue fibroblasts in vitro. Cells from secondary passage were plated at 10^4 cells per culture well and exposed for 3 d to either FCS or purified CDGF. Data are expressed as mean ± SEM.

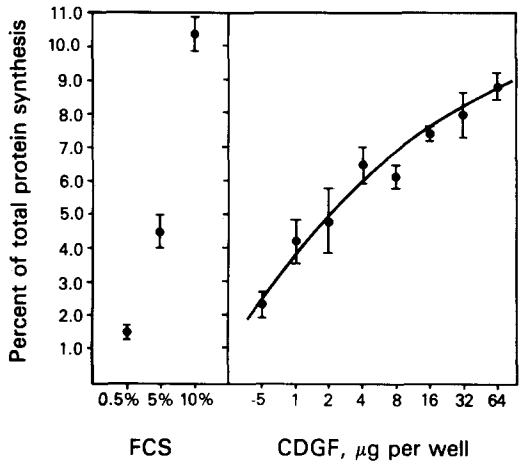

FIGURE 7 Collagen synthesis as a percentage of total protein synthesis in rat embryo fibroblasts exposed to partially purified CDGF and FCS. Data are means ± SE for triplicate determinations in two sets of experiments. The effect of CDGF at concentrations of 2 μg/ml or greater exceeds that of 5% FCS.

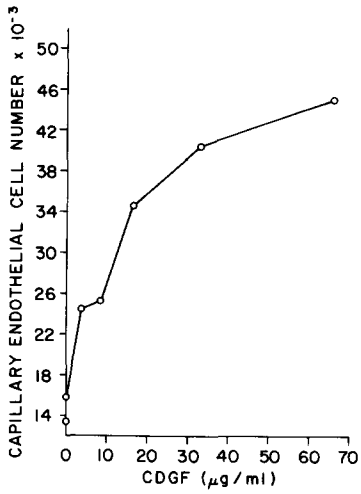

FIGURE 8 Stimulation of capillary endothelial cell proliferation by partially purified CDGF. CDGF was resuspended in DME supplemented with 10% calf serum and added at various concentrations to bovine capillary endothelial cells. After 72 h, the cells were detached with trypsin-EDTA and counted in a Coulter counter.

culture studies on 3T3 cells and rat fibroblasts described above. Each injected sponge received ~0.5 μg CDGF (1 U) per 10,000 cells present within it on day 6 after implantation.

The transient nature of the stimulus provided by injected CDGF is illustrated in Fig. 9, which shows that ^{125}I-CDGF rapidly disappeared from its injection site, the interior of the sponge. By 4 h postinjection, only ~10% of the initial radioactivity of ^{125}I-CDGF could be recovered from the injection site. Thus, the exposure of the granulation tissue to the factor was transient, in contrast to the continuously exposed fibroblasts, 3T3 cells, and endothelial cells in culture. Continuous release of CDGF in vivo may be accomplished by using microencapsulation, incorporation into ethylene vinyl acetate polymer pellets (14), or microdiffusion pumps.

BIOCHEMICAL EFFECTS: Partially purified CDGF accelerated the development of granulation tissue at the site of injection. At 48 and 72 h, the DNA content of test sponges was increased more than twofold over uninjected control sponges (Table I). The slightly increased DNA content of sponges that were injected with saline as compared with uninjected sponges probably reflected a nonspecific, mechan-

ical effect of the injection. Collagen content showed a transient increase, but relative collagen synthesis was not affected. DNA content did not increase between 48 and 72 h in test sponges, which suggested that a maximum effect on the mitosis and recruitment of fibroblasts, the principal cellular constituent of the granulomas, was reached by 48 h.

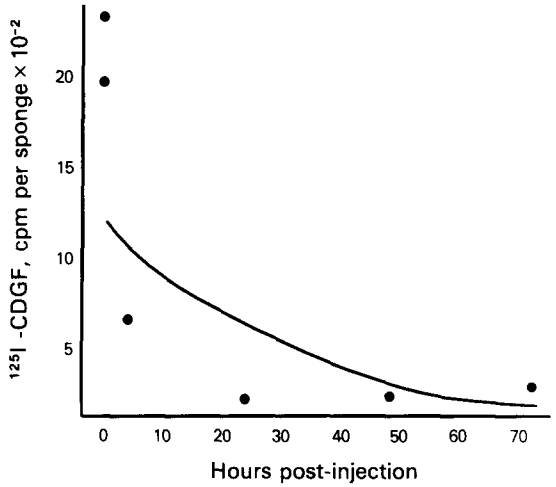

FIGURE 9 Disappearance of ^{125}I-CDGF from the injection site within granulation tissue containing sponges. At 4 h and later, <10% injected CDGF is recoverable from the sponge. Regression line was computer generated.

To rule out the effects of contaminating activities, a second experiment was performed with purified CDGF (150 U/sponge). Fig. 10 illustrates the stimulation of DNA, protein, and collagen accumulation in CDGF-injected sponges as compared with saline-injected controls. As discussed below, the increases in cell number (DNA) and protein content were unlikely to be the result of transient inflammatory response, since these effects were maximal 3 d after injection and not accompanied by any histologic evidence of an inflammatory

TABLE I

Effects of Partially Purified CDGF on Granulation Tissue

Time*	Injection‡	DNA§	Collagen synthesis‖	Collagen content¶
0	None	32.3 ± 2.5	5.7 ± 0.7	492 ± 80
48	None	44.5 ± 4.5	11.1 ± 0.9	491 ± 38
48	Saline	57.4 ± 14.1	8.4 ± 0.9	468 ± 103
48	CDGF	113.9 ± 6.5	6.3 ± 0.7	643 ± 136
72	None	44.3 ± 4.5	6.4 ± 1.3	1,187 ± 220
72	Saline	60.6 ± 8.3	6.3 ± 1.7	887 ± 118
72	CDGF	107 ± 7.7	5.4 ± 0.7	757 ± 103

* Sponges were implanted 6 d before experiment. Time is expressed in hours after initiation of experiment.
‡ Sponges were uninjected (*None*), injected with 100 μl of sterile saline (*Saline*), or injected with 150 U (300 μg) of partially purified CDGF (*CDGF*).
§ Expressed as micrograms per sponge.
‖ Expressed as percent of total protein synthesis.
¶ Expressed as micrograms per sponge as based on hydroxyproline content.

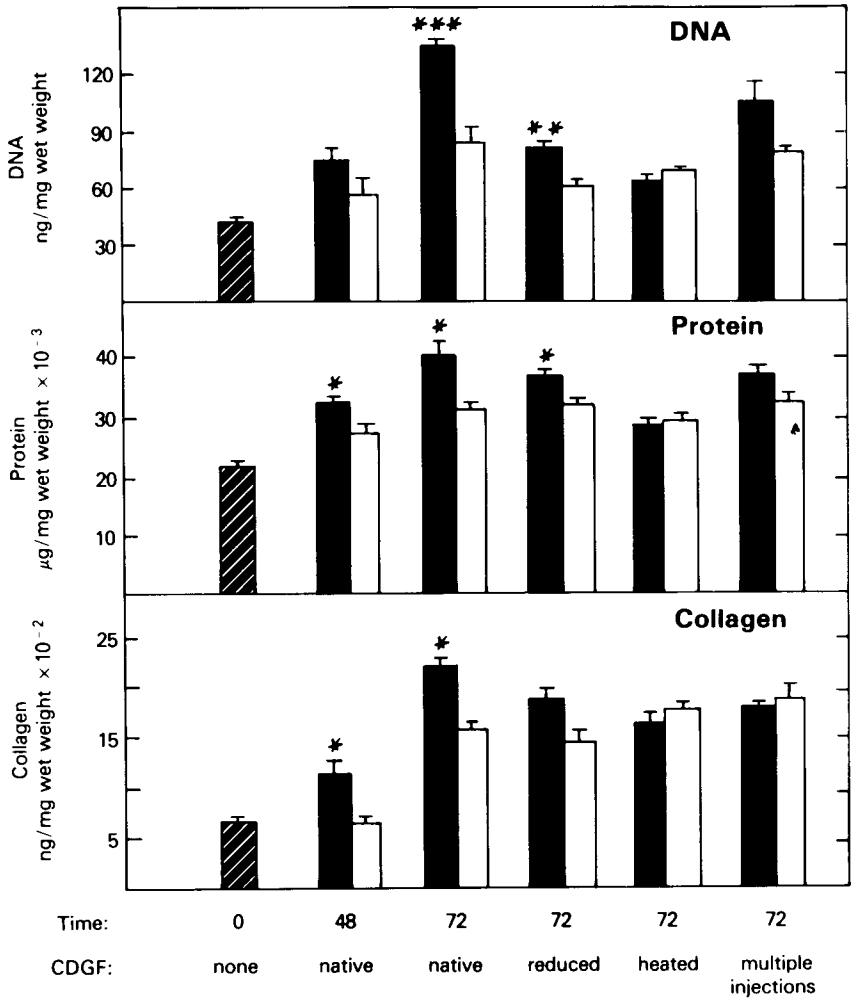

FIGURE 10 Biochemical effects of purified CDGF on sponge granulation tissue. Sponges were injected with 100 μl of test solution on day 6 after implantation and removed immediately (time 0), or 48 (time 48), or 72 h (time 72) after injection. Sponges were analyzed for DNA, total protein, or total collagen content, expressed per milligram wet weight, as described in the text. CDGF-derived results are shown in dark bars as either untreated (*native*), reduced (5 mM dithiothreitol), heat denatured (*heated*), or multiply injected. Error bars represent SEM, and statistical significance (t-test) is shown by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). ▨, base line; ■, CDGF; □, saline.

infiltrate. Repeated daily injections of CDGF were less effective in causing the increase in granulation tissue cellularity.

To help exclude the possibility that CDGF was a homologue of other, well-known growth factors, we used reduction (5 mM dithiothreitol) and heat denaturation (98°C, 10 min) as controls. Whereas the presence of dithiothreitol (Fig. 10) did reduce the accumulation of DNA in both control and treated sponges, reduced CDGF was still an effective stimulus when compared with 0.5 mM dithiothreitol in saline. Heat denaturation abolished the activity of CDGF. Taken together, these results showed that CDGF did not resemble platelet-derived growth factor, which is sensitive to reduction (1) and is heat stable (2). However, like CDGF (3), fibroblast growth factor is resistant to reduction and is heat labile (2, 5). A detailed structural comparison of pure CDGF and fibroblast growth factor will be necessary to ascertain whether there is a relationship between these polypeptides.

The first set of experiments with partially purified CDGF showed only a moderate and transient increase in collagen content, which was not attributable to an increased relative synthesis (Table I). However, collagen accumulation was significantly accelerated at 48 and 72 h by a single injection of purified CDGF (Fig. 10). The relationship between these results obtained in vivo and our findings in vitro of a CDGF-induced increase in relative collagen synthesis (Fig. 7) have several potential explanations: (*a*) the transient presence of CDGF in the sponges as opposed to its continuous presence in cell culture; (*b*) the nature of the target cells; (*c*) the differences in cell density and arrangement; and (*d*) the possible production of collagenases by the invasion of capillaries, fibroblasts, and other cells of granulation tissue.

More prolonged exposure of granulation tissue to CDGF by a method less traumatizing than injection might elicit data more concordant with those illustrated for rat embryo fibroblasts. However, the mechanism of growth factor action may be to increase the number of collagen-producing cells by proliferation or recruitment. Granulation tissue is populated by several cell types, including capillary endothelium, which is specifically stimulated to grow and migrate in vitro by CDGF (22). The production of collagenase is reported to be stimulated in capillary endothelial cells by tumor-derived mitogens (7). Thus, the aspects of tissue remodeling and cell-cell interaction may be important differences between the mode of CDGF action in vivo and in vitro.

MORPHOLOGIC EFFECTS: The morphological effects of CDGF illustrated in Figs. 11–13 included an increased vascular response to CDGF. This was most pronounced at the periphery of the sponge and in the immediately adjacent connective tissue. Capillaries and precapillary arterioles in CDGF-injected sponges were both more numerous and more widely dilated than in controls at these sites at 48 and 72 h (Fig. 12). The extent of organization in CDGF-injected sponges exceeded that in controls at 48 and 72 h. By 72 h, CDGF sponges were infiltrated more than 75% with connective tissue, whereas controls were infiltrated less than 50% (Fig. 11, *a*, *b*), and the granulation tissue deep within CDGF sponges contained more numerous capillaries than did the corresponding connective tissue in controls (Fig. 11 *c*).

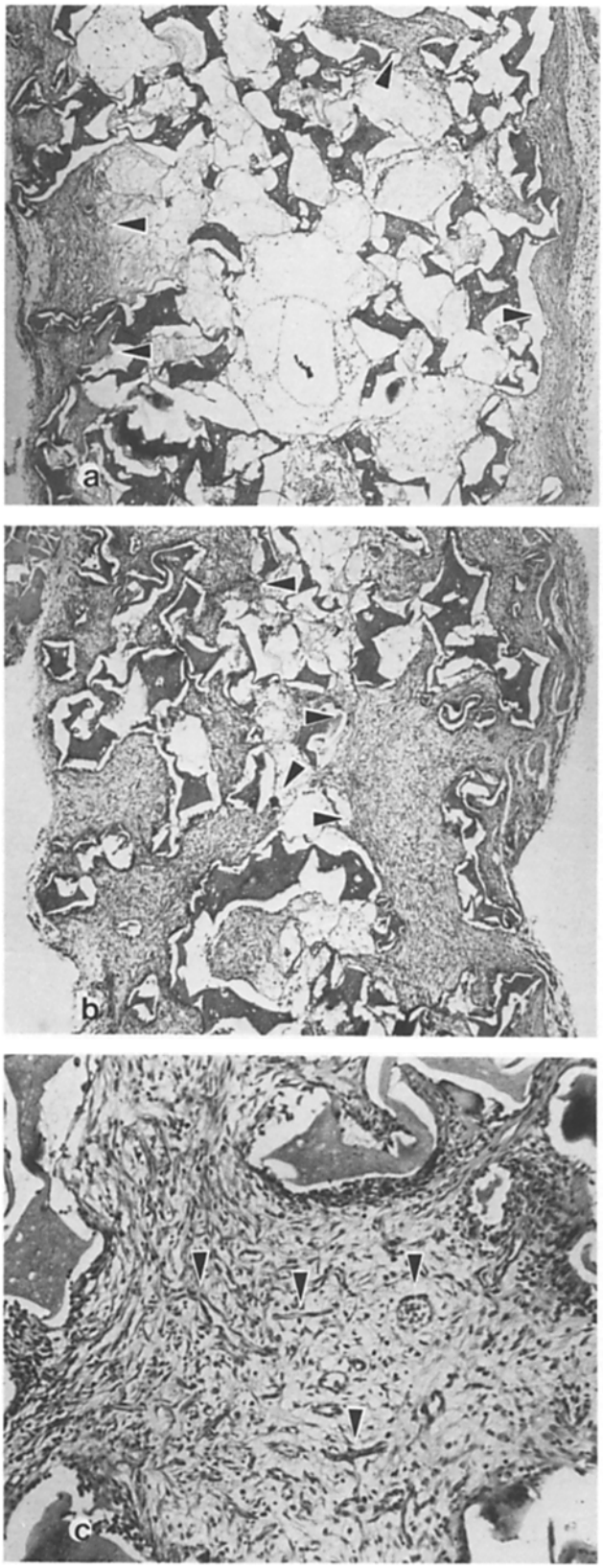

FIGURE 11 Organization of granulation tissue. Transverse sections of sponge implants 72 h postinjection, dorsal surface left, are shown. (*a*) Local control. Granulation tissue has penetrated to the level of arrowheads. Fibrin and small numbers of erythrocytes are present in the center of the sponge. (*b*) CDGF-injected sponge. Connective tissue has extended to zone marked by arrowheads and occupies all except a small central zone. Hematoxylin–eosin stain was used. × 40. (*c*) CDGF-injected sponge showing more numerous and conspicuous small capillaries than in controls (arrowheads). Hematoxylin–eosin stain was used. × 100.

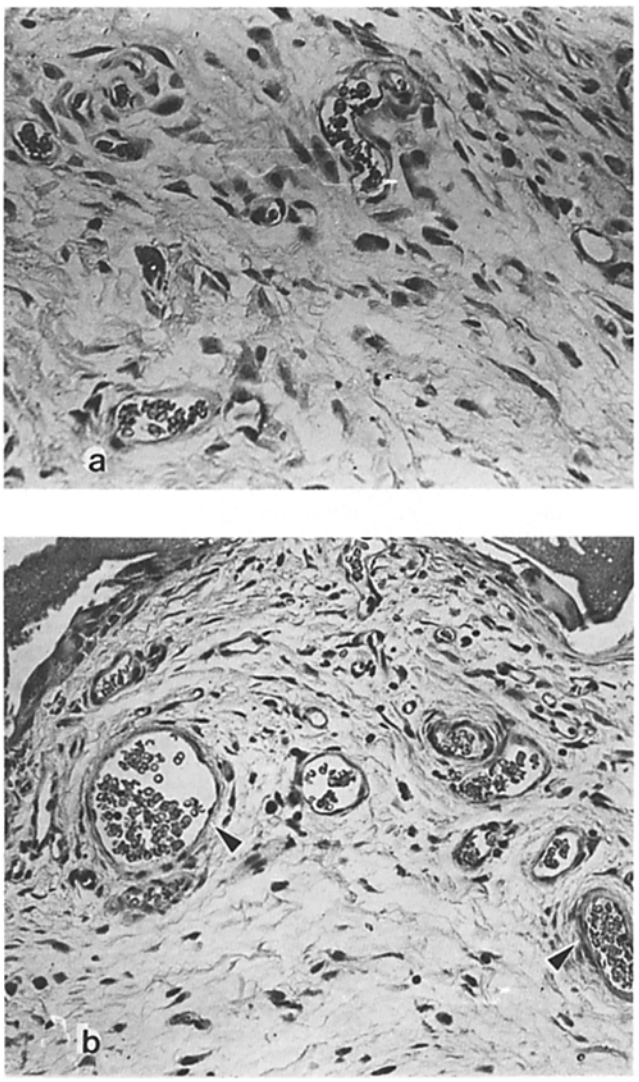

FIGURE 12 Morphology of sponge granulation tissue. Granulation tissue adjacent to ventral surface of sponges, 72 h postinjection. (*a*) Local control. (*b*) CDGF-injected sponge. Note that there are capillaries and marked dilatation of capillaries in *b* (arrowheads). Hematoxylin-eosin stain was used. × 400.

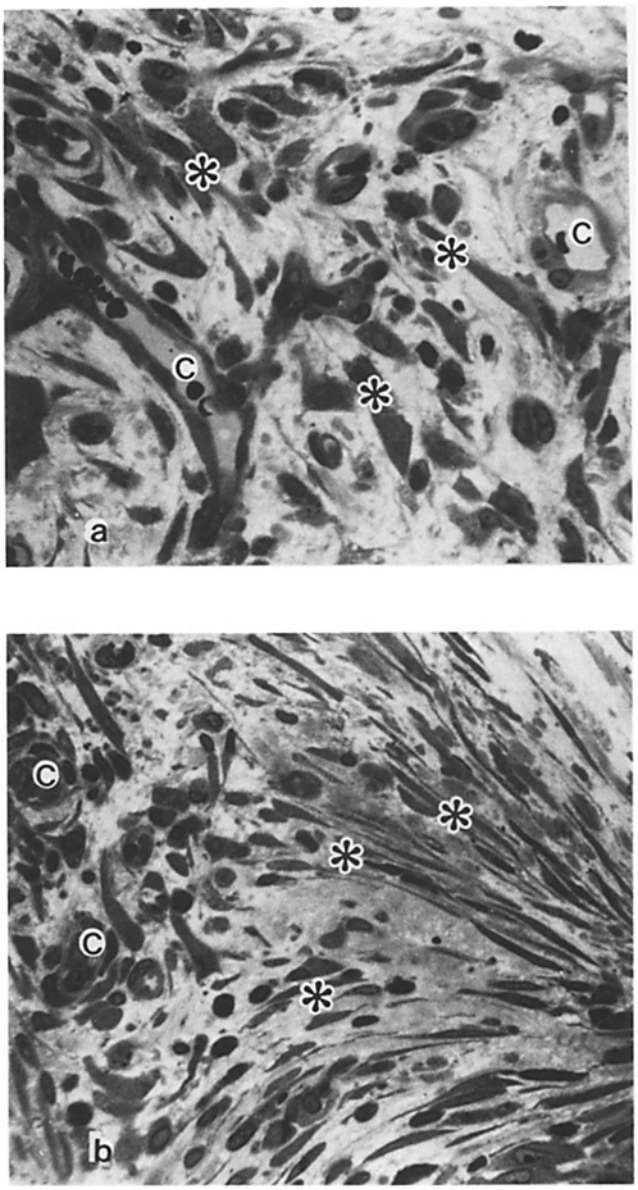

FIGURE 13 Cellular constituents of granulation tissue within sponges. Tissue was removed and fixed 72 h postinjection. (*a* and *b*) 1-μM sections of control (*a*) and CDGF injected sponge (*b*). Note the absence of an inflammatory response. Capillaries (C) and fibroblasts (*) are principal constituents. Fibroblasts are more elongated and more densely packed in CDGF than in control sponges. Toluidine blue was the stain used. × 600.

Although CDGF accelerated the organization of the sponges at 48 and 72 h, it did not incite an inflammatory response; the cellular constituents of both CDGF-injected and control sponges consisted almost exclusively of fibroblasts and capillaries; and only occasional monocytes and plasma cells could be found (Fig. 13, *a* and *b*). Multinucleated giant cells were usually present adjacent to the sponge matrix by 72 h in all sponges. The greater degree of organization of CDGF sponges than of controls at 48 and 72 h confirms the observed increase in DNA, protein, and collagen content.

The morphological response to highly purified CDGF was not different from that seen with the less characterized material. CDGF-induced neovascularization and organization of the sponges was most pronounced in 72-h and multiple-injection sponges. Its effects were less pronounced and about equal in 48-h and dithiothreitol-injected sponges. Again, no CDGF-induced inflammatory response was noted.

Recruitment versus Proliferation

Whereas CDGF had clear-cut mitogenic properties in vitro, the increased content of cells and matrix in granulation tissue could have been the result of other response mechanisms. Another cationic polypeptide growth factor, platelet-derived growth factor, is known to have potent chemoattractant properties (2, 18). To evaluate the relative contribution of ingression and local proliferation of cells, we determined the extent of total uptake, total incorporation, and thymidine incorporation per microgram DNA at several intervals after injection of CDGF on day 4.

The results of this experiment are displayed in Fig. 14 as the ratios of experimental to control values at each time point. Thus, a ratio for incorporation per microgram DNA less than 1 signifies dilution of the total DNA pool of the sponge with unlabeled DNA. Although thymidine uptake, uptake per wet weight, and total incorporation per sponge were all somewhat elevated above control values, the key parameter of incorpo-

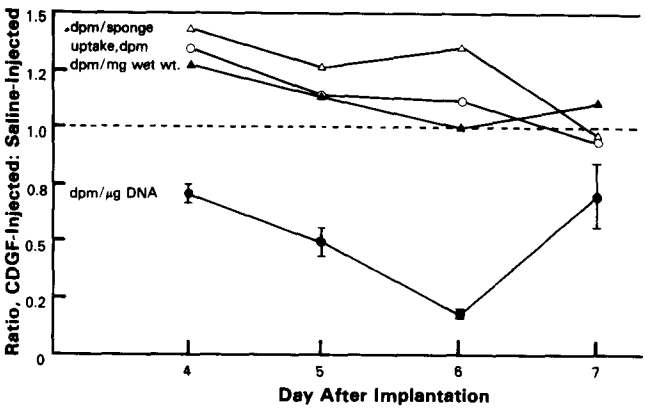

FIGURE 14 Effect of CDGF on in vivo recruitment and proliferation. Data are expressed as the ratio of experimental to control (saline-injected) value. Identity with controls produced a value of 1.0. CDGF or saline was injected on day 4. For each determination, 250 μCi methyl-[^{3}H]thymidine was injected intraperitoneally 6 h before the indicated time to evaluate DNA synthesis. Total thymidine uptake (○), thymidine uptake per milligram wet weight (▲), and total thymidine incorporation per sponge (△) were somewhat elevated above controls, more so at early time points. However, incorporation per microgram DNA (●) was consistently less than control values, particularly on day 6, when actual DNA content was highest in CDGF-injected sponges (data not shown, see Fig. 10).

ration per microgram DNA (*dpm/μg DNA*, Fig. 14) fell below control values on all four days of the experiment, particularly day 6, when relative sponge DNA content was much higher than in controls (data not shown, see Fig. 10). This result suggests that although CDGF has potent mitogenic properties in vitro, these are masked by recruitment of new mesenchymal cells to the site of injection in vivo. Unlike the growth-limiting conditions used to assess the effects of CDGF in cell culture, granulation tissues may be proliferating at maximal rates in the absence of growth factor.

The morphological observations supported the positive mitogenic effect of CDGF on fibroblasts and capillary endothelial cells, but CDGF appeared to be involved in local recruitment of cells to sites of wound repair. It is interesting that CDGF elicited effects similar to those produced by cartilage powder incorporated into sponge implants (21). CDGF exerted two principal effects on sponge morphology: increased vascularity without increased inflammation, and the more complete organization and infiltration by fibroblasts of the sponge interior. These findings agree with the positive effect of CDGF on capillary endothelial cell motility in vitro (22). The observed lack of an inflammatory response to CDGF further supports the concept of a direct effect on granulation tissue rather than an effect mediated through inflammatory cells. Because of the short retention yet potent capacity of CDGF to organize connective tissue, it is likely that a slowly released version of CDGF can be used as a wound healing accelerator or vulnerary agent.

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Note Added in Proof: Recent results indicate that CDGF has potent fibroblast chemotactic activity (Senior, R. M., and J. M. Davidson, unpublished results). Studies that use brain extracts (Buntrock, P., K. D. Jentzsch, and G. Heder, 1982, *Exp. Pathol.*, 26:46–53; Buntrock, P., K. D. Jentzsch, and G. Heder, 1982, *Exp. Pathol.*, 26:62–67; Buntrock, P., M. Buntrock, I. Marx, D. Kranz, K. D. Jentzsch, and G. Heder, 1984, *Exp. Pathol.*, 26:247–254) suggest similar properties for another growth factor.

REFERENCES

1. Antoniades, M. N., C. D. Scher, and C. D. Stiles. 1979. Purification of the human platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 76:1809–1813.
2. Antoniades, M. N., and A. J. Owen. 1982. Growth factors and regulation of cell growth. *Annu. Rev. Med.* 33:445–463.
3. Azizkhan, J. C., and M. Klagsbrun. 1980. Chondrocytes contain a growth factor that is localized in the nucleus and is associated with chromatin. *Proc. Natl. Acad. Sci. USA.* 77:2762–2766.
4. Folkman, J., C. C. Haudenschild, and B. R. Zetter. 1979. Long term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA.* 76:5217–5222.
5. Gospodarowicz, D., and J. S. Moran. 1976. Growth factors in mammalian cell cultures. *Annu. Rev. Biochem.* 45:531–538.
6. Gospodarowicz, D., G. Greenberg, H. Bialecki, and B. R. Zetter. 1978. Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. *In Vitro.* 14:85–117.
7. Gross, J. L., D. Moscatelii, E. A. Jaffe, and D. B. Rifkin. 1982. Plasminogen activator and collagenase production by cultured capillary endothelial cells. *J. Cell Biol.* 95:974–981.
8. Hinegardner, R. T. 1971. An improved fluorimetric assay for DNA. *Anal. Biochem.* 39:197–201.
9. Johnson-Wint, B., and S. Hollis. A rapid *in situ* deoxyribonucleic acid assay for determining cell number in culture and tissue. 1982. *Anal. Biochem.* 122:338–344.
10. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27:137A.
11. Klagsbrun, M., R. Langer, R. Levenson, S. Smith, and C. Lillihei. 1977. The stimulation of DNA synthesis and cell division in chondrocytes and 3T3 cells by a factor isolated from cartilage. *Exp. Cell Res.* 105:99–108.
12. Klagsbrun, M., and S. Smith. 1980. Purification of a cartilage-derived growth factor. *J. Biol. Chem.* 255:10859–10866.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680–685.
14. Murray, J. B., L. Brown, R. Langer, and M. Klagsbrun. 1983. Microsustained release system for epidermal growth factor. *In Vitro.* 19:743–748.
15. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361–365.
16. Peterkofsky, B., M. Chojkier, and M. Bateman. 1982. Determination of collagen synthesis in cell and culture systems. *In* Immunochemistry of the Extracellular Matrix. Vol. II. H. Furthmayr, editor. CRC Press, Inc., Boca Raton, Florida. 19–47.
17. Ross, R., and A. Vogel. 1978. The platelet-derived growth factor. *Cell.* 14:203–210.
18. Seppä, H., Grotendorst, G., Seppä, S., Schiffmann, E., and Martin, G. R. 1982. Platelet-derived growth factor is chemotactic for fibroblasts. *J. Cell Biol.* 92:584–588.
19. Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science (Wash. DC).* 223:1296–1299.
20. Tolstoshev, P., R. A. Berg, S. I. Rennard, K. H. Bradley, B. C. Trapnell, and R. G. Crystal. 1981. Procollagen production and procollagen mRNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth. *J. Biol. Chem.* 256:3135–3140.
21. Woodward, S. C., and J. B. Herrmann. 1968. Stimulation of fibroplasia in rats by bovine cartilage powder. *Arch. Surg.* 96:189–199.
22. Zetter, B. R., J. C. Azizkhan, D. Brouty-Boye, J. Folkman, C. C. Handenschild, M. Klagsbrun, R. Potash, and C. J. Scheiner. 1981. Normal and tumor-derived factors that modulate endothelial cell growth and migration. *In* Plasma and Cellular Modulatory Proteins. D. Bing and R. A. Rosenbaum, editors. Center for Blood Research, Boston. 59–73.