

Connective Tissue Growth Factor: a Cysteine-rich Mitogen Secreted by Human Vascular Endothelial Cells Is Related to the SRC-induced Immediate Early Gene Product CEF-10

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Abstract. Human umbilical vein endothelial (HUVE) cells have been previously reported to express the genes for the A and B chains of PDGF and to secrete PDGF-related factors into culture media. Antihuman PDGF IgG affinity chromatography was used to purify PDGF-related activity from HUVE cell-conditioned media. Immunoblot analysis of the affinity-purified proteins with anti-PDGF IgG and antibodies specific for the A or B chain peptides of PDGF combined with chemotactic and mitogenic assays revealed that the major PDGF immunorelated molecule secreted by HUVE cells is a monomer of ~36–38 kD and that

<10% of the purified biologically active molecules are PDGF A or B chain peptides. Screening of an HUVE cell cDNA library in the expression vector lambda gt11 with the anti-PDGF antibody resulted in the cloning and sequencing of a cDNA with an open reading frame encoding a 38-kD cysteine-rich secreted protein which we show to be the major PDGF-related mitogen secreted by human vascular endothelial cells. The protein has a 45% overall homology to the translation product of the v-src-induced CEF-10 mRNA from chick embryo fibroblasts. We have termed this new mitogen connective tissue growth factor.

THE vascular endothelium, which forms the nonthrombogenic lining of blood vessels, was once considered a relatively inert membrane. Recent studies of endothelial cells have demonstrated that they participate in numerous structural and physiological functions of the circulatory system. Structural proteins including collagens and laminin are secreted into the basement membrane, while procoagulant, anticoagulant, and platelet regulatory proteins and growth regulatory molecules are secreted from the luminal membrane into the circulating blood or into a wound site (Jaffe, 1984, 1987). Cultured endothelial cells have previously been shown to secrete factors that are chemotactic and mitogenic for connective tissue cells. Approximately 30% of this biological activity can be neutralized by antibodies specific for human PDGF (DiCorleto, 1984).

PDGF has been described as a mitoattractant due to its chemotactic effect on connective tissue cells at lower concentrations (0.1–1.0 nM) and its mitogenic effect on these cells at higher concentrations (0.5–5 nM) (Grotendorst and Martin, 1986). Because of the dual biological activity of this molecule, PDGF is believed to be a major factor involved in the normal healing of wounds and pathologically contributing to the lesions of atherosclerosis, fibrotic diseases, and oncogenesis. PDGF was originally identified (Ross et al., 1974; Kohler and Lipton, 1974) and purified (Antoniades et al., 1979; Heldin et al., 1981) from the alpha-

granules of human platelets. Platelet PDGF is a dimeric molecule that migrates on SDS-polyacrylamide gels at ~30 kD. Reduction of interchain disulfide bonds yields A chain (17 kD) and B chain (14 kD) monomers that are not biologically active (Antoniades, 1981; Antoniades et al., 1979; Grotendorst et al., 1982). Conditioned media from cultures of human umbilical vein endothelial (HUVE) cells contain factors that compete with platelet PDGF for binding to the PDGF cell surface receptor of fibroblasts and demonstrate PDGF-related biological activity (DiCorleto, 1984). HUVE cells express both the A and B chain genes of PDGF (Collins et al., 1985; Collins et al., 1987). Since both PDGF gene transcripts are present, the exact nature of the proteins responsible for the PDGF-related mitoattractant activity found in HUVE cell-conditioned media was uncertain. The secreted molecules could be AB heterodimers or AA or BB homodimers. All three isoforms have been purified from natural sources and are biologically active (Heldin et al., 1986; Betsholtz et al., 1986; Stoobant and Waterfield, 1984). Our initial interest was to determine the type of PDGF molecules secreted by vascular endothelial cells. During the course of these studies we have identified a new peptide that appears to be responsible for the PDGF-related mitoattractant activity present in the endothelial cell-conditioned media. We have termed this protein connective tissue growth factor (CTGF).¹

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1. Abbreviations used in this paper: CTGF, connective tissue growth factor; HUVE, human umbilical vein endothelial.

Materials and Methods

Cells

HUVE cells were isolated from fresh human umbilical cords by collagenase perfusion (Jaffe, 1987) and maintained in medium 199 with 20% FCS, 0.6 mM L-glutamine, 20 µg/ml Gentamicin, 90 µg/ml porcine heparin (Sigma Chemical Co., St. Louis, MO), and 50 µg/ml endothelial cell growth supplement (Sigma Chemical Co.). Cells used for media collection were third passage cells. Cells were identified as endothelial cells by their nonoverlapping cobblestone morphology and by positive staining for factor-VIII related antigen. NRK cells were obtained from American Type Culture Collection (Rockville, MD). NIH/3T3 cells were a gift from S. Aaronson (National Cancer Institute, Bethesda, MD), and both cell lines were maintained in DMEM, 10% FCS, 20 µg/ml Gentamicin. Fetal bovine aortic smooth muscle cells were obtained from tissue explants as previously described (Grotendorst et al., 1981) and maintained in DMEM, 10% FCS, 20 µg/ml Gentamicin, and used in assays at second or third passage.

Growth Factors and Antibodies

Human PDGF was purified to homogeneity from platelets as described previously (Grotendorst, 1984). Recombinant AA, BB, and AB chain dimeric PDGF molecules were obtained from Creative Biomolecules (Hopkinton, MA). FGF was obtained from Sigma Chemical Co. Purified PDGF or synthetic peptides containing the amino and carboxyl sequences of the mature PDGF A and B chain molecules were used to raise antibodies in goats. Goats were immunized with 20 µg of purified PDGF or 50 µg of synthetic peptide in Freunds complete adjuvant by multiple intradermal injections. Immune sera were collected 7 d after the fourth rechallenge (in Freunds incomplete adjuvant) and subsequent challenges. The anti-PDGF antibody did not show any cross-reactivity to TGF- β , EGF, or FGF in immunoblot analysis. The antipeptide antibodies were sequence specific and did not cross-react with other synthetic peptide sequences or with recombinant PDGF peptides that did not contain the specific antigenic sequence. This was determined by Western blot and dot blot analysis.

Antibody Affinity Column

Goat anti-human PDGF IgG (150 mg) was covalently bound to 25 ml of Affi-Gel 10 support (Bio-Rad Laboratories, Cambridge, MA) according to the manufacturer's instructions with a final concentration of 6 mg IgG/ml gel. The column was incubated with agitation at 4°C for 18 h with 1 liter of HUVE cell media that had been conditioned for 48 h. The gel was then poured into a column (5 × 1.5 cm), washed with 4 vol of 0.1 N acetic acid made pH 7.5 with ammonium acetate, and the antibody-bound PDGF immunoreactive proteins were eluted with 1 N acetic acid. Peak fractions were determined by biological assays and immunoblotting and the fractions pooled.

Biological Assays

Chemotactic activity was determined in the Boyden chamber chemotaxis assay with NIH 3T3 or bovine aortic smooth muscle (BASM) cells as previously described (Grotendorst et al., 1981, 1987). Mitogenic assays were performed using 96-well plates and NRK fibroblasts or NIH 3T3 cells as target cells. The cells are plated in DMEM, 10% FCS, and the NRK cell cultures used 10–14 d after confluence and 3T3 cells made quiescent by incubating for 2 d in serum-free DMEM, 0.2 mg/ml BSA before use. Sample proteins and dilutions of known standards were added to the wells and the plates incubated at 37°C in 10% CO₂, 90% air for 18 h, after which ³H-thymidine at a final concentration of 5 µCi/ml was added and incubated for an additional 2 h. The media was removed, the cells washed, and DNA synthesis determined from the ³H-thymidine incorporation into TCA-precipitable material by scintillation counting.

Gel Electrophoresis and Immunoblotting

Electrophoresis was performed on 12% polyacrylamide gels containing SDS (Laemmli, 1970) unless otherwise stated. Immunoblotting was performed by electroblotting the proteins to a nitrocellulose membrane and incubating the membrane in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl (TBS) with 2% nonfat dry milk at 25°C for 1 h to block nonspecific antibody binding. The blocking solution was removed and the antibody (15 µg/ml) added in TBS containing 0.5% nonfat dry milk and 1 µg/ml sodium azide and in-

cubated overnight at 25°C. The membranes were then washed five times in TBS, 0.5% milk for 10 min each wash and then incubated with alkaline phosphatase-conjugated affinity-purified rabbit anti-goat IgG (KPL, Gaithersburg, MD) at a 1:1,000 dilution in TBS containing 0.5% milk at 25°C for 1 h. The filters were then washed with TBS five times, 10 min each time, and the blot was developed using an alkaline phosphatase substrate solution (0.1 M Tris-HCl, pH 9, 0.25 mg/ml nitro blue tetrazolium, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate).

Receptor Competition Assays

Assays were performed using confluent cultures of NIH 3T3 cells in 24-well plates (Costar Data Packaging Corp., Cambridge, MA) grown in DMEM, 10% FCS, 10 µg/ml Gentamicin. The growth media was removed and the cells were washed twice with serum-free DMEM, 0.2 mg/ml BSA, and the plates placed on ice for 30 min in serum-free DMEM, 0.2 mg/ml BSA. Test samples and controls were made up in serum-free DMEM, 0.2 mg/ml BSA containing 5–10 ng/ml of HUVE affinity-purified proteins and a serial dilution of one of the recombinant PDGF isoforms in a concentration range of 300–16 ng/ml. 1-ml aliquots of the samples were placed into wells of the 24-well plates and incubated on ice on a platform rocker for 2 h. After the incubation period, the cells were washed three times for 10 min each on ice with PBS. The proteins bound to the surface of the cells were eluted with 500 µl of 1 N acetic acid for 10 min. The acetic acid elution samples were lyophilized, resuspended in 5 mM HCl, run on 12% polyacrylamide gels, and immunoblotted to nitrocellulose using the anti-PDGF antibody.

RNA Isolation and Northern Blotting

Total RNA was isolated from cells in monolayer culture cells by the method of Chomczynski and Sacchi (1987). Lyophilized RNA was resuspended in gel loading buffer containing 50% formamide and heated at 95°C for 2 min before loading (20 µg per lane total RNA) onto 2.2 M formaldehyde, 1% agarose gels and run at 50 V. Integrity of RNA was determined by ethidium bromide staining and visualization of 18S and 28S rRNA bands. After electrophoresis the RNA was transferred to nitrocellulose by blotting overnight with 10× SSC. The nitrocellulose was air dried and baked at 80°C for 2 h in a vacuum oven. Hybridization was performed overnight at 46°C with the addition of 5 × 10⁵ CPM per ml of ³²P-labeled probe. Normally, for Northern blots the entire plasmid was labeled and used as a probe. Labeling was done with a random primer labeling kit from Boehringer Mannheim Biochemicals (Indianapolis, IN) according to instructions provided. After hybridization, membranes were washed twice in 2× SSC, 0.1% SDS for 15 min each at room temperature, once for 15 min in 0.1× SSC, 0.1% SDS room temperature, and a final 15-min wash in 0.1× SSC, 0.1% SDS at 46°C. Blots were autoradiographed at -70°C on Kodak X-omat film.

Library Screening, Cloning, and Sequencing

Standard molecular biology techniques were used to subclone and purify the various DNA clones (Sambrook et al., 1989). Clone DB60 was picked from a lambda-gt1 HUVE cell cDNA library by induction of the fusion proteins and screening with anti-PDGF antibody. Plaques picked were rescreened and positive clones replated at low titer and isolated.

The EcoRI insert from clone DB60 was cloned into the M13 phage vector and single-stranded DNA obtained for clones with the insert in opposite orientations. These M13 clones were then sequenced by the dideoxy method (Sanger, 1977) using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) and ³⁵S-dATP (duPont Co., Wilmington, DE). Both strands of DNA for this clone were completely sequenced using primer extension and both GTP and ITP chemistry. The sequencing reactions were done according to the manufacturer's instructions. Aliquots of the sequencing reactions were run on both 6% acrylamide (16 h) and 8% acrylamide (6 h) gels, vacuum dried, and autoradiographed for at least 18 h.

The cDNA fragment from clone DB60 was ³⁵P-CTP labeled and used to rescreen the HUVE cell cDNA lambda gt1 library. Several clones were picked and the largest, the 2,100-bp clone designated DB60R32, was subcloned into Bluescript phagemid. Subclones were made of PstI, KpnI, and EcoRI/KpnI restriction fragments, also in Bluescript. These subclones were sequenced by double-stranded plasmid DNA sequencing techniques using Sequenase as described above. The 1,458-bp EcoRI/KpnI clone containing the open reading frame was subcloned into M13 mp18 and M13 mp19, and both strands of DNA were completely sequenced using single-stranded DNA sequencing techniques with primer extension and both GTP and ITP chemistry.

In Vitro Transcription and Translation

In vitro transcription reactions were done using the 2,100-bp cDNA clone DB60/32 in the Bluescript KS vector. The plasmid was cut with XbaI, which cuts the plasmid once in the multiple cloning site of the vector 3' to the cDNA insert. The T7 promoter site located 5' to the cDNA insert was used for transcription. The in vitro transcriptions were done with a kit supplied with the Bluescript vector by Stratagene Cloning Systems and the manufacturer's instructions were followed.

In vitro translation reactions were done using nuclease-treated rabbit reticulocyte lysate and ^{35}S -cysteine in a cysteine-free amino acid mix for labeling of the peptide. The reactions were done with a kit supplied from Promega Biotech (Madison, WI) and the manufacturer's instructions were followed. The reactions were done in a final volume of 50 μl containing ^{35}S -cysteine 1 mCi/ml (1,200 Ci/mMole, DuPont Co.), and serial dilutions of mRNA from the in vitro transcription reactions in concentrations ranging from 50 to 500 ng per reaction tube. The reactions were incubated at 30°C for 60 min. Aliquots of the reactions were run reduced or nonreduced on 12% polyacrylamide electrophoresis gels, dried, and autoradiographed.

Bacterial expression of immunoreactive CTGF peptide was accomplished by subcloning clone DB60R32 into the EcoRI site of the pET 5 expression vector (Studier et al., 1990) in both sense and inverse orientations (as determined by restriction enzyme digest analysis). Cultures of cells were grown in M9 media to an OD 600 of 0.7 and the media made 0.4 mM IPTG and incubation continued for 2 h. The cells were pelleted, lysed, inclusion bodies removed by centrifugation, and aliquots of the pellet extracts run on 12% polyacrylamide gels and immunoblotted using the anti-PDGF antibody.

For expression in *Xenopus* oocytes, mature *X. laevis* females were obtained from Nasco (Fort Atkinson, WI) and maintained at room temperature. Frogs were anesthetized by hypothermia and the ovarian tissue was surgically removed. Ovarian tissue was minced and digested the 0.2% collagenase (type II; Sigma Chemical Co.) in OR-2 without calcium (Wallace et al. 1973) for 2-3 h. Unblemished stage VI oocytes (Dumont, 1972), 1.3-mm diameter, were then carefully selected and microinjected.

Stage VI oocytes (5-10 at a time) were placed on a hollowed plexiglass platform and drained of excess OR-2 solution. Approximately 50 nl of sample containing 10 ng of RNA was injected into the animal pole just above the oocyte equator using a Leitz system microinjector. After injection, oocytes were returned to OR-2 buffer with 0.1% BSA and incubated for 24 h at 25°C. Viable oocytes were then pooled and extracted by homogenization in 100-mm NaCl, 10 mm Tris, pH 7.5, with ten strokes of a Dounce homogenizer (20 μl /oocyte). The homogenate was then mixed with an equal volume of freon to remove pigment and lipid and centrifuged at 10,000 rpm for 30 s to separate the phases. The top aqueous phase was removed and tested for chemotactic activity using NIH 3T3 cells as described above.

Results

Identification and Partial Purification of PDGF-immunorelated Mitogen from HUVE Cells

Initial studies of the PDGF-related growth factors secreted by HUVE cells were done by removing the serum-containing growth media from confluent cultures of cells and replacing it with serum-free media. Aliquots of this media were removed periodically and the proteins immunoblotted using an antibody specific for human platelet PDGF (Fig. 1). This antibody does not cross-react with any other known growth factors and is able to detect <500 pg of dimeric PDGF or 10 ng of reduced, monomeric A or B chain peptide on immunoblots. The results indicated constitutive secretion of several species of molecules which are immunologically similar to platelet PDGF but are of higher relative molecular mass (36-39 kD) than the expected 30-32 kD molecular mass of platelet PDGF or A chain or B chain homodimers. Chemoattractant and mitogenic assays performed with this serum-free conditioned media indicated the total biological activity present was equivalent to 15 ng/ml of platelet PDGF after a 48-h conditioning period (Fig. 2). Incubation of the media

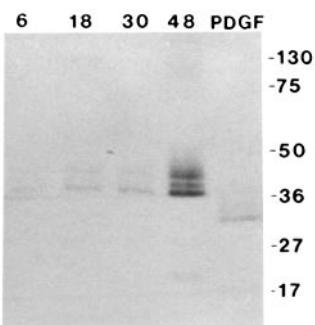


Figure 1. Constitutive secretion of PDGF-immunoreactive factors by HUVE cells. HUVE cells were grown to confluence in 6-well plates. The growth media was removed, cells were washed with PBS, and 1 ml of serum-free media was added to each well. The media was removed after conditioning for the period of time indicated (hours), dialyzed against 1 N acetic acid, and lyophilized. The samples were then run on 12% PAGE, electroblotted to nitrocellulose, and visualized with the antihuman PDGF antibody. 5 ng of purified platelet PDGF was run as reference. Positions of mol wt markers (Bio-Rad Laboratories) are indicated at right.

with 30 $\mu\text{g}/\text{ml}$ of anti-human PDGF IgG neutralized ~20-30% of the mitogenic activity and similar amount of the chemotactic activity. This is in agreement with previous reports (DiCorleto, 1984).

The presence in HUVE culture media of several species of PDGF-immunoreactive molecules was unexpected, particularly molecules of higher molecular weight than those of the A and B chain dimeric molecules anticipated to be produced and secreted by endothelial cells (Collins et al., 1987; Sitaras et al., 1987). To obtain greater amounts of the PDGF-like proteins for further analysis, the HUVE cells had to be kept in media containing 20% FCS, as the cells begin to die after 24 h in serum-free or low serum media. The PDGF-immunoreactive proteins were partially purified from the serum-containing media by use of an antibody affinity column made with the anti-human PDGF IgG and an Affi-Gel 10 support (BioRad Laboratories). When aliquots of the partially purified proteins were assayed for chemotactic and mitogenic activity, all biological activity could be neutralized by prior incubation of the proteins with the antihuman PDGF antibody (Fig. 2). This indicated that the only biologically active molecules present in the partially purified media proteins were PDGF-immunorelated molecules. Aliquots of the partially purified proteins were immunoblotted using the same anti-PDGF antibody and the data indicated the presence of the higher molecular weight molecules observed in the serum-free conditioned media (Fig. 3). The major species secreted migrates on polyacrylamide gels at 36 kD and comprises at least 50% of the total immunoreactive protein purified from conditioned media. The immunoreactive species migrating at 37 and 39 kD constitute most of the remaining immunoreactive protein. A similar pattern is seen with proteins labeled with ^{35}S -Cysteine and affinity purified with the anti-PDGF IgG immunoaffinity column (data not shown). Less than 15% of the total affinity-purified proteins comigrate with purified platelet PDGF or recombinant PDGF isoforms. Prior incubation of the antibody with purified PDGF (300 ng PDGF/2 μg IgG) blocked antibody binding to all of the molecules, indicating shared antigenic determinants with dimeric platelet PDGF (Fig. 3, lane 4). Interestingly, when the antibody was blocked with recombinant AA, BB, or AB dimers, antibody binding to the HUVE-secreted proteins was inhibited equally by all three

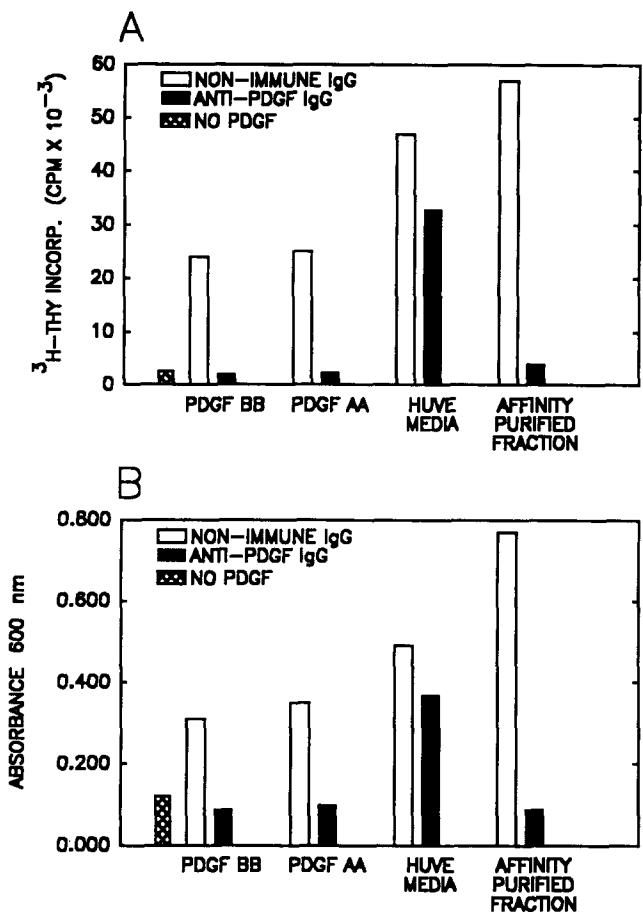


Figure 2. Chemotactic and mitogenic assays of HUVE cell-conditioned media and affinity-purified PDGF immunoreactive factors. (A) Mitogenic assay performed as described using NRK cells as target cells. PDGF BB is 5 ng/ml. PDGF AA is 10 ng/ml. HUVE media is 250 μ l of HUVE cell serum-free conditioned media (48 h) which was dialyzed against 1 N acetic acid, lyophilized, and resuspended in DMEM before addition to test wells. Affinity-purified fraction is 5 μ l/ml of combined, concentrated major pool from Affi-Gel 10 affinity column. Anti-PDGF IgG or nonimmune IgG (30 μ g/ml) was added to the samples and incubated 18 h at 4°C before testing in the mitogenic assay. Data points in A and B represent the mean of triplicate samples and the standard deviation is <5%. The experiments were repeated at least three times with similar results. (B) Chemotactic assays were performed as described under Materials and Methods using NIH 3T3 cells as target cells. PDGF BB is 5 ng/ml. PDGF AA is 10 ng/ml. HUVE media is serum-free DMEM, 0.2 mg/ml conditioned for 48 h. Affinity-purified fraction is 2.5 μ l/ml of combined, concentrated major pool from Affi-Gel affinity column. Antibody neutralization is performed as described under A.

dimeric forms, suggesting that the antibody recognizes common epitopes present on all three PDGF dimers and the HUVE-secreted molecules (our unpublished observations). To insure that none of the antibody binding molecules detected on Western blots were derived from FCS or other additives in the culture media, a new, unused antibody affinity column was made and media not conditioned by cells was processed exactly as the conditioned media. No PDGF-immunoreactive molecules were detected in the fractions from this column by immunoblot (Fig. 3, lane 5) and no

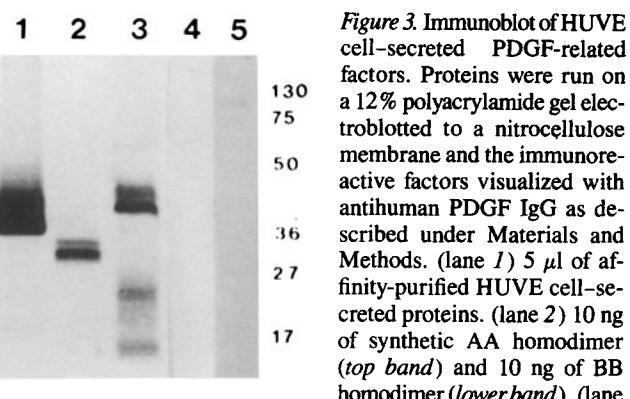


Figure 3. Immunoblot of HUVE cell-secreted PDGF-related factors. Proteins were run on a 12% polyacrylamide gel electroblotted to a nitrocellulose membrane and the immunoreactive factors visualized with antihuman PDGF IgG as described under Materials and Methods. (lane 1) 5 μ l of affinity-purified HUVE cell-secreted proteins. (lane 2) 10 ng of synthetic AA homodimer (*top band*) and 10 ng of BB homodimer (*lower band*). (lane 3) 50 μ l of reduced HUVE affinity-purified proteins. (lane 4) 5 μ l of HUVE affinity-purified HUVE proteins but with antihuman PDGF antibody blocked with 300 ng of PDGF. (lane 5) Control column of 10 μ l of anti-PDGF Affi-Gel 10 affinity-purified protein fraction from media which was not conditioned by cells.

biological activity was detected (data not shown). When platelet PDGF or the recombinant dimers are reduced with 100 mM DTT, monomeric A chain (17 kD) and B chain (14 kD) peptides are observed on immunoblots. Treating the HUVE molecules in a 100-mM DTT sample buffer results in slower migration of the major immunoreactive peptides on polyacrylamide gels (Fig. 3, lane 3). Most of the immunoreactive molecules migrate at 38–39 kD and less intense bands are observed at 25 and 14 kD. It is necessary to run at least 10 times as much reduced protein as nonreduced in order to detect the reduced molecules. This is consistent with the affinity of our antibody for monomeric forms of the PDGF A and B chain peptides. These data indicated that the major species in the PDGF-related affinity-purified proteins from conditioned media of HUVE cells was monomeric peptide which migrates on acrylamide gels at an apparent molecular mass of 36 kD nonreduced and 38 kD when reduced.

Major Chemotactic and Mitogenic Activity Is Produced by 36-kD Peptide and Not PDGF Peptides

To determine if the chemotactic and mitogenic activities observed in the partially purified media proteins were from molecules containing the PDGF A and B chain peptides or were the products of molecules that do not contain these sequences, biological assays were performed with serial dilutions of the affinity-purified media proteins and serial dilutions of recombinant PDGF AA and BB homodimers and the AB heterodimer. (Fig. 4). Sufficient quantities of the samples were prepared to perform the mitogenic and chemotactic assays and the immunoblots with aliquots of each dilution sample. The mitogenic activity of the HUVE affinity-purified factors observed was comparable to the activity elicited by all three recombinant PDGF dimers. The chemotactic activity was comparable to the AB heterodimer, producing less response than the BB homodimer and greater response than the AA homodimer. When the biological activity of the samples was compared with immunoblots of equivalent amounts of the same samples, no A chain nor B chain molecules were detected in the test samples (Fig. 4). These data demonstrate the major biological activity present in the anti-PDGF affin-

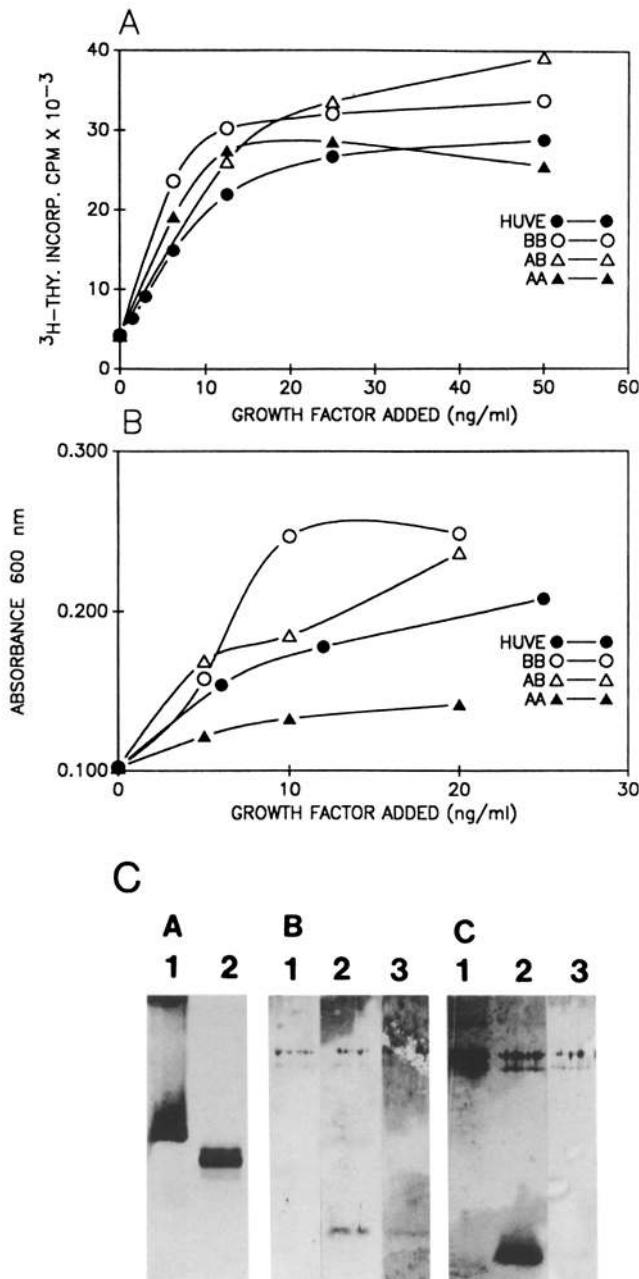


Figure 4. Biological assays and immunoblots of serial dilutions of HUVE cell affinity-purified media proteins and recombinant PDGF standards. (A and B) Filled circle is HUVE cell affinity-purified media proteins; open circle is BB homodimer; open triangle is AB heterodimer; closed triangle is AA homodimer. Mito- genic assay (A) and chemotactic assay (B) were performed as described with NIH 3T3 cells. The test samples in both biological assays and the immunoblot are equal aliquots of the same dilution sample. Data points represent the mean of triplicate samples with SD < 10%. (C) (immunoblot A) Primary antibody is anti-human PDGF IgG. (lane 1) 20 ng HUVE purified media proteins. (lane 2) 20 ng AB heterodimer. (immunoblot B) Primary antibody is anti-amino terminal A chain serum. (lane 1) 20 ng of HUVE-purified media proteins, reduced. (lane 2) 20 ng AA homodimer, reduced. (lane 3) 1.25 ng AA homodimer, reduced. (immunoblot C) Primary antibody is anticarboxy terminal B chain serum. (lane 1) 20 ng HUVE-purified media proteins, reduced. (lane 2) 20 ng BB homodimer, reduced. (lane 3) 2.5 ng BB homodimer, reduced.

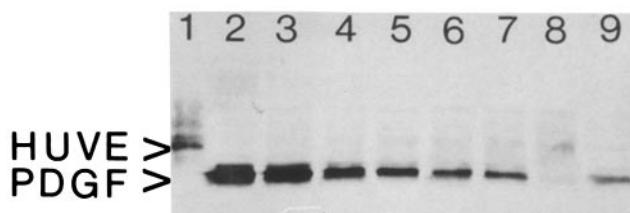


Figure 5. PDGF cell surface receptor-binding competition assay with NIH 3T3 cells and HUVE affinity-purified proteins competing with recombinant PDGF BB homodimer. Lane 1 contains 10 ng of HUVE affinity-purified proteins and lane 9 contains 1.0 ng of recombinant PDGF BB homodimer. Lanes 2–8 are proteins dissociated in one well of a 24-well plate from the cell surface of NIH 3T3 cells with acetic acid. The cells in each well were incubated for 2 h at 4°C with serum-free DMEM containing 10 ng of affinity-purified protein from HUVE cell-conditioned media and varying concentrations of recombinant PDGF BB. The concentration of PDGF in lane 2 is 300 ng; lane 3, 150 ng; lane 4, 75 ng; lane 5, 37.5 ng; lane 6, 18.75 ng; lane 7, 9.4 ng; and lane 8 contains no addition of PDGF.

ity-purified fraction cannot be accounted for by PDGF A or B chain-containing molecules and imply that the major PDGF-immunoreactive protein species present in these samples (the 36-kD peptide) is biologically active and does not contain amino acid sequences found in the amino and carboxy termini of the PDGF A or B chain peptides.

To substantiate the binding of the endothelial cell molecules to the PDGF cell surface receptors, competitive receptor binding assays were performed. Because immunoblots of the affinity-purified HUVE cell-secreted proteins indicated the presence of multiple PDGF immunoreactive molecules, ^{125}I -labeled PDGF competition assays could not be used since this would not indicate which molecules in this mixture were competing for binding of the labeled PDGF for the receptors on the target cells. Since the isoforms of PDGF and the major PDGF immunorelated protein secreted by HUVE cells are of different molecular weights, we were able to demonstrate receptor binding competition on immunoblots. Direct binding of the anti-PDGF immunoreactive peptides to NIH 3T3 cells was demonstrated by incubating monolayers of the 3T3 fibroblasts with the anti-PDGF affinity-purified proteins (10 ng/ml) for 2 h at 4°C. Bound peptides were released by washing of the cell layer with 1 N acetic acid and quantitated by immunoblot analysis using anti-PDGF IgG (Fig. 5). The data show that the 36-kD immunoreactive peptide binds to cell surface of NIH 3T3 cells. This binding can be competed by increasing concentrations of recombinant PDGF BB added to the binding media. These data suggest that the CTGF peptide binds to specific cell surface receptors on NIH 3T3 cells and that PDGF BB can compete with this binding. Whether CTGF binds to a certain class of PDGF receptors or whether there is some cross reactivity of PDGF BB with CTGF receptors that are distinct from PDGF receptors is not clear from these results and will require a more in-depth study.

Cloning Expression and Sequencing of the cDNA for CTGF

To further characterize these PDGF-related molecules, we

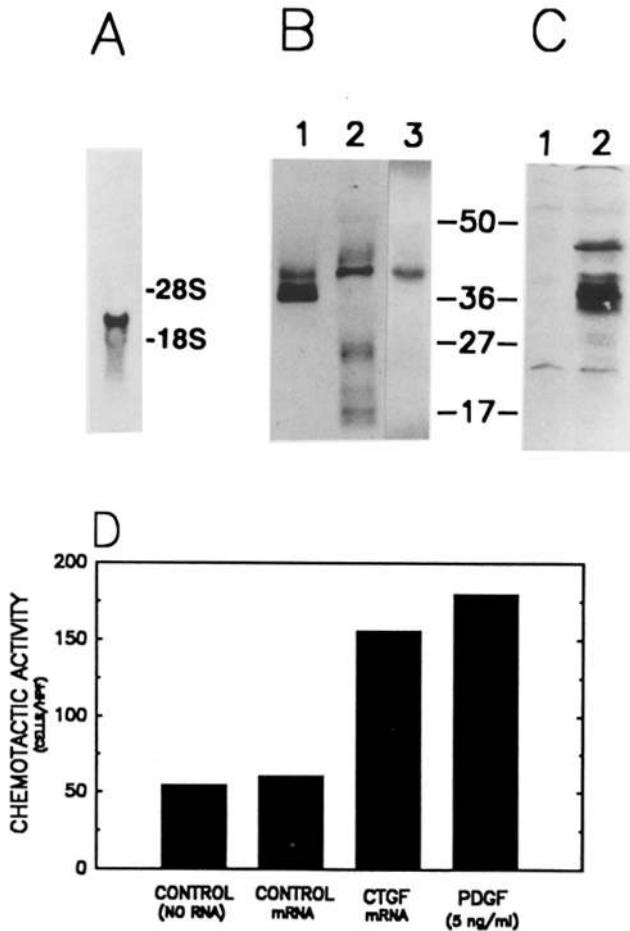


Figure 6. Northern blot of CTGF transcript in HUVE total RNA and in vitro translation product of cDNA clone DB60R32 and immunoreactive peptide from prokaryotic expression. (A) 20 μ g of total RNA from cultured HUVE cells was run on a 1.5% agarose gel, transferred to nitrocellulose, and probed with 32 P-labeled DB60 clone. Autoradiograph indicates hybridization to a 2.4-kb transcript. (B) Lanes 1 and 2 are an immunoblot using the antihuman PDGF antibody with (1) 10 ng nonreduced and (2) 100 ng reduced of HUVE cell affinity-purified proteins from conditioned media. Lane 3 is an autoradiograph of 4 μ l of a 50- μ l rabbit reticulocyte in vitro translation reaction which incorporated 1 μ g of RNA transcript from an in vitro transcription reaction using the 2,100-bp clone DB60R32 in Bluescript phagemid. Autoradiograph was exposed for 24 h. (C) Clone DB60R32 was cloned into the EcoRI site of pET5 prokaryotic expression vector in both sense and inverse orientations. Protein expression was induced by IPTG for 2 h and 20- μ l aliquots of cell pellet extract were run on 12% polyacrylamide gels and immunoblotted using the anti-PDGF antibody. Lane 1 is the antisense control and lane 2 is the sense strand peptide produced by clone DB60R32. (D) Capped mRNA was prepared by in vitro transcription of the DB60R32 clone in Bluescript phagemid after restriction with XbaI. Oocytes were microinjected with buffer (control, no RNA) or 10 μ g of either control or CTGF mRNA. Total oocyte protein was extracted after 24 h. Chemotaxis assays were performed as described under Materials and Methods with NIH 3T3 cells using 25 μ g of oocyte total protein in each sample. PDGF BB (5 ng/ml) positive control is shown for comparison. The results represent the average of triplicate samples with a variation of <10%. These studies were repeated twice.

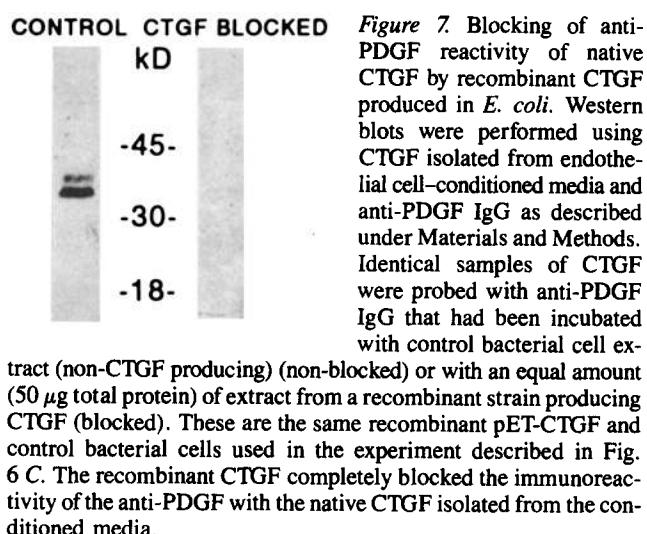


Figure 7. Blocking of anti-PDGF reactivity of native CTGF by recombinant CTGF produced in *E. coli*. Western blots were performed using CTGF isolated from endothelial cell-conditioned media and anti-PDGF IgG as described under Materials and Methods. Identical samples of CTGF were probed with anti-PDGF IgG that had been incubated with control bacterial cell extract (non-CTGF producing) (non-blocked) or with an equal amount (50 μ g total protein) of extract from a recombinant strain producing CTGF (blocked). These are the same recombinant pET-CTGF and control bacterial cells used in the experiment described in Fig. 6C. The recombinant CTGF completely blocked the immunoreactivity of the anti-PDGF with the native CTGF isolated from the conditioned media.

first attempted to obtain sufficient quantities of the CTGF protein for amino acid sequencing. However, the low concentrations of CTGF in the conditioned media of HUVE cell cultures and the costly and time consuming techniques involved in obtaining and culturing these cells made protein purification to homogeneity and amino acid sequencing impractical. Therefore, we used the anti-PDGF antibody to screen an HUVE cell cDNA library made in the expression vector lambda gt11 (a gift from T. Collins, Harvard). Over 500,000 recombinant clones were screened. Several clones which gave strong signals with the anti-PDGF antibody in the screening process were purified and subcloned into the M13 phage vector and partial sequence data obtained by single stranded DNA sequencing. A search of the GenBank DNA sequence data base indicated that two of the clones picked contained fragments of the PDGF B chain cDNA open reading frame sequence. One of these clones was similar to a 1.8-kb insert previously isolated by Collins et al. (1985) using a c-sis cDNA probe. A third clone of 500 bp was completely sequenced and no match was found in a homology search of all nucleotide and amino acid sequences in GenBank (CEF 10 sequence was not available at that time). This clone was designated DB60. Anti-PDGF antibody binding to the fusion protein produced by the clone DB60 was completely blocked by the affinity-purified proteins (not shown). A 32 P-labeled probe was made of DB60 and used on a Northern blot of 20 μ g of total RNA isolated from HUVE cells (Fig. 6A). The blot indicated probe hybridization with an mRNA of 2.4 kb, which is a message of sufficient size to produce the proteins in the 38-kD molecular mass range seen on the immunoblots of the affinity-purified proteins. The DB60 clone was used to rescreen the HUVE cell cDNA lambda gt11 library and the largest clone isolated contained a 2,100-bp insert designated DB60R32. A probe made with the 2,100-bp EcoRI insert of clone DB60R32 also hybridized with a single 2.4-kb message in a Northern blot of total RNA from HUVE cells (not shown). To determine the size of the peptide encoded in the open reading frame of DB60R32, the 2,100-bp insert was cloned into the Bluescript vector and transcribed in vitro using T7 polymerase and the mRNA transcript translated in vitro using a rabbit

A

B

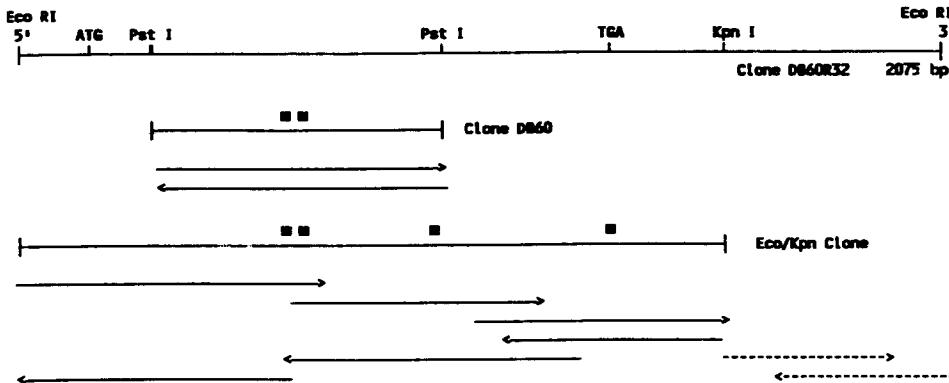


Figure 8. (A) Nucleotide sequence and amino acid translation of CTGF cDNA from human vascular endothelial cells. The open reading frame for the CTGF protein extends from the ATG initiation site at nucleotide 130 to the TGA site at 1177. The possible glycosylation sites at asparagines 28 and 225 are underlined. The putative alternative splicing region is boxed. The 3' region contains three ATTAA sites which are underlined. (B) Sequencing map of CTGF cDNA clone. The top line represents the 2,075-bp DB60R32 clone containing the open reading frame of CTGF. Restriction enzyme sites used in subcloning are indicated as are the ATG initiation site and the TGA termination codon. Squares indicate locations of primers used in sequencing. Solid arrows indicate single-stranded DNA sequencing data and broken arrows indicate area in 3' region sequenced by double-stranded DNA methods.

CTGF -	MTAASMGPVRAFVVLLALC-SRPAVGQNCGCPCCRDPDEAPRCPAGVSLVLDGCCCRV	59
CEF-10 -	MGSAGARPALAAALL-CLARIALGSPCPAVQCPC-AAPQCAPGVGLVPDGCGCCKV	55
CTGF -	CAKQLGEELCTERDPCDPHKGFLCDFGSPANRKIGVCTAK-DGAPCIFGGTVYRSGESFQS	118
CEF-10 -	CAKQLNEDCSRTQPCDHTKGLECNFGASPAATNGICRAQSEGRPEYNNSKIYQNGESFQP	115
CTGF -	SCKYQCTCLDGAVGCMPLCSMDVRLPSPDPCPFPRRVKLPGKCEEWVCDEPKD-----	171
CEF-10 -	NCKHQCTCIDGAVGCIPLCQELS LPNLGCPS PRLVKVPGQCCEEWCDES KDALEELEG	175
CTGF -	-----QTVVGPALAAYRLED TFGPDPTMIRAN---CLVQTTEWSACS	210
CEF-10 -	NFSKEFGLDASEGELTRNNELIAIVKGGLKMLPVGSE PQSRAFENPKCIVQTT SWSQCS	235
CTGF -	KTCGMG1STRVTNDNASCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKISKPIKFEL	270
CEF-10 -	KTCGTG1STRVTNDNPDCKLKETRICEVRPCGQPSYASLKKGGKKCTKTKSPSPVRF TY	295
CTGF -	SGCTSMKTYRAKFCGVCTDGRCCCTPHRTTLPVEFKCPDGEVMKNNMMFIKTCACHYNCP	330
CEF-10 -	AGCSSVKKYRPKCYCGSCVDGRCCCTPQQRTV KIRFR CDDGETFTKS VMMI QSCR CNYNC P	355
CTGF -	GDN DIFESLVYRKMYG DMA	349
CEF-10 -	HANEAYP--FYRLVNNDIHKF RD	376

Figure 9. Comparison of amino acid sequences of the translated cDNA for CTGF and the CEF-10 mRNA translation product. The translated cDNA for human CTGF and avian CEF-10 have a 45% overall homology and a 52% homology if the putative alternative splicing region is deleted. This region is between amino acids 171 (aspartic acid) and 199 (cysteine) in the CTGF sequence. Homologous regions are shaded and the aligned cysteines are marked with an asterisk.

reticulocyte lysate and 35 S-cysteine. Aliquots of the translation reaction were reduced with 50 mM DTT and run on 12% polyacrylamide gels. The data indicate a protein product which migrates at an apparent molecular mass of 38 kD and comigrates with the reduced affinity-purified proteins observed on immunoblots (Fig. 6 B). The clone DB60R32 was subcloned into the EcoRI site of the prokaryotic expression vector pET 5 in both orientations and transfected into *E. coli* strain HMS174. The CTGF protein was induced by IPTG and aliquots of the cell lysate were immunoblotted (Fig. 6 C). The protein produced by clone DB60R32 in the sense orientation produced anti-PDGf immunoreactive peptides in the 36–39 kD molecular mass range, while the anti-sense control produced no immunoreactive peptides. The recombinant peptides produced in the *E. coli* system completely blocked the anti-PDGf reaction with the CTGF peptides present in conditioned media (Fig. 7).

Bioassays of extracts of the recombinant bacterial cells that were producing the CTGF peptide were negative. We then evaluated several eukaryotic expression systems, including microinjection of *Xenopus* oocytes. Injection of *Xenopus* oocytes with 10 ng of RNA preparations derived by in vitro transcription of the DB60R32 clone resulted in the production of a fibroblast chemotactic activity (Fig. 6 D). Control injected cells did not produce this activity. These results indicate that the open reading frame of the DB60R32 clone encodes a protein with chemotactic activity for fibroblastic cells as does CTGF.

The 2,100-bp insert of clone DB60R32 was sequenced initially by subcloning of Pst I and KpnI restriction fragments into Bluescript and using double-stranded dideoxy methods. This determined an open reading frame of 1,047 base pairs and oriented the DB60 insert to the larger cDNA (Fig. 8). An EcoRI/KpnI fragment containing the entire open reading frame was inserted into M13 mp18 and M13 mp19, and both strands of the DNA were sequenced with single-stranded dideoxy methods by primer extension using both GTP and the GTP analogue ITP. The cDNA nucleotide sequence of the open reading frame encoded a 38,000 mol wt protein,

confirming our cell-free translation results and matching the size of the immunopurified peptides (Fig. 8). A new search of the GenBank data base revealed that this cDNA had a 50% nucleotide sequence homology with CEF-10 mRNA, one of the immediate early genes induced in v-src-transformed chicken embryo fibroblasts (Simons et al., 1989) (Fig. 9).

Discussion

The data presented here indicate that the major connective tissue mitoattractant secreted by HUVE cells is a 38-kD monomeric molecule which appears to be antigenically and functionally related to PDGF, but is not a product of the PDGF A or B chain genes. We find that HUVE cells also secrete both PDGF A and B chain molecules, but at much lower concentrations than the 38-kD monomer and these peptides contribute only a minor fraction (<10%) of the total PDGF-related chemotactic and mitogenic activity secreted by these cells under the conditions tested. In the biological assays performed with the affinity-purified proteins from HUVE media, biological activity equivalent to 20 ng/ml of PDGF was obtained at low concentrations of the CTGF molecule where no detectable (<2 ng) PDGF A or B chain molecules were present. All biological activity could be removed by prior incubation of these samples with the anti-PDGf antibody. Because all three isomeric forms of the PDGF dimers blocked antibody binding to the CTGF molecule, it is suggested that there are some common antigenic determinants shared among these proteins although there is little if any peptide sequence homology. The anti-PDGf antibody has high affinity to the nonreduced forms of the PDGF isomers and the CTGF molecule and tenfold less affinity to the reduced forms of these peptides which lack biologically activity. This suggests that there are regions of shared tertiary structure between the PDGF isomers and the CTGF molecule, resulting in common antigenic epitopes and possible receptor binding sites of these molecules.

The sequence of the cDNA for CTGF indicates an open reading frame of 1,047 nucleotides with an initiation site at

position 130 and a TGA termination site at position 1,177 which encodes a peptide of 349 amino acids (Fig. 8). The ATG codon at position 130 and another at position 145 fit the consensus sequence for strong translation initiation sites (Kozak, 1984) and it is assumed that the first ATG is the predominant initiation site. There is a 40% sequence homology between the CTGF cDNA and the cDNA for both the A and B chains of PDGF, suggesting a possible common ancestral gene. The 3' region contains three copies of the pentanucleotide sequence ATTAA shown to be involved in mRNA destabilization and frequently found in cytokine and oncogene mRNA 3' regions (Shaw and Kamen, 1986).

The CTGF open reading frame encodes a peptide that contains 39 cysteine residues, indicating a protein of complex structure with multiple intramolecular disulfide bonds. This may explain the shift to slower mobility observed on polyacrylamide gels after reduction of the molecule with DTT. The amino terminal of the peptide contains a hydrophobic signal sequence indicative of a secreted protein, and there are two N-linked glycosylation sites at asparagine residues 28 and 225 in the amino acid sequence. There is a 45% overall sequence homology between the CTGF peptide and the protein encoded by the CEF-10 mRNA transcript and the homology rises to 52% when a putative alternative splicing region is deleted. All 39 cysteine residues in each peptide can be aligned with few gaps in the sequences (Fig. 9). The region between amino acid residues 171 (aspartic acid) and 199 (cysteine) in the CTGF peptide has no significant homology to the corresponding region in the CEF-10 sequence (amino acids 168–224) and is 28 amino acids shorter. This discrepancy could be due to differences incurred during evolution of the chicken and human genes. However, because this region is bordered by areas of very high homology between the two molecules (>85% identity) it may indicate alternative splicing mechanisms in the expression of this gene. The codons for lysine at residue 170 and aspartic acid at residue 171 together form the AAG/G sequence consistent with a 3' exon/5' exon junction. Alternative splicing is found in other growth factor transcripts such as the PDGF A chain (Collins et al., 1987) and vascular endothelial growth factor (Leung et al., 1989; Keck et al., 1989; Tischer et al., 1989). The biological significance of either of these splicing events has not yet been determined.

Simmons et al. (1989) cloned the CEF-10 mRNA, which was one of 12 identified cDNA sequences transcribed from mRNAs that were induced soon after the production of the src phosphoprotein pp60^{v-src} in chicken embryo fibroblasts. The CEF-10 mRNA was induced in nontransfected CEF cells by serum. Both the src protein and serum induce the expression of the "immediate early genes" many of which are necessary for the G₀–G₁ transition in the cell cycle (for review see Rollins and Stiles, 1989). One major group of these genes consists of intranuclear DNA-binding proteins including fos, myc, and jun, which are essential for cell cycle regulation. Another major group of genes induced by serum and src encodes secretory proteins with cytokine characteristics such as the JC gene (Cochran et al., 1983), which has a cDNA sequence with significant homology to the cytokines macrophage colony stimulating factor (M-CSF), alpha interferon and interleukin-2 (Rollins et al., 1988), or the KC gene (Cochran et al., 1983) which is homologous to the gro gene in humans (Oquendo et al., 1989; Anisowicz et al., 1987).

The gro protein product is related to CEF-4, another of the 12 src and serum-inducible mRNAs cloned from CEF cells (Bedard et al., 1987). We have found that the CTGF gene is rapidly induced by serum in human skin fibroblasts and that cycloheximide treatment does not block this induction (Igarashi, A., and G. R. Grotendorst, manuscript submitted for publication). Thus, the CTGF gene is an immediate early gene which appears to encode a secreted peptide with cytokine activity.

The data presented here suggest that the CTGF molecule has biological activity similar to PDGF and may bind one of the PDGF cell surface receptors. CTGF could function in many biological processes involving the growth of connective tissue. The fact that we find CTGF secreted by vascular endothelial cells indicates that the peptide could be present in serum. The conditions under which the protein would be secreted in vivo are not yet known and we are currently pursuing studies on the regulation of gene expression and protein secretion by cultured endothelial cells. Secretion of CTGF during angiogenesis would facilitate the growth of smooth muscle cells and fibroblasts so that it may play an important role in the control of blood vessel formation during development and wound repair. CTGF could also play a role in atherosclerosis where it could function to recruit smooth muscle cells from the medial layer of the vessel wall into the intima. Other investigators have detected PDGF transcripts in arterial tissues (Barrett and Benditt, 1987), and the PDGF mRNA levels appear to be elevated in atherosclerotic lesions (Barrett and Benditt, 1988). The secretion of CTGF peptides by endothelial cells would also stimulate the chemotaxis and growth of smooth muscle and fibroblasts at the plaque site, thereby aggravating vessel blockage. Whether CTGF production is required for transformation by the src oncogene remains to be determined, but it is interesting that CTGF could function as an autocrine growth factor for src transformed fibroblasts. Direct experiments to determine the transforming potential of this cDNA are currently underway. We are also in the process of constructing vectors for eukaryotic expression systems in order to obtain recombinant CTGF for further analysis. Future experiments using CTGF-specific antibodies and nucleotide probes should help to determine the role of CTGF during the normal biological and pathological processes which involve connective tissue formation.

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