1990

Connective Tissue Growth Factor: A Cysteine-Rich Mitogen Secreted by Human Vascular Endothelial Cells

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CONNECTIVE TISSUE GROWTH FACTOR:

A CYSTEINE-RICH MITOGEN SECRETED

BY HUMAN VASCULAR ENDOTHELIAL CELLS

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the in the College of Graduate Studies.

Program in Molecular and Cellular Biology and Pathobiology

1990

Approved by:

Chairman, Advisory Committee

ABSTRACT

Human umbilical vein endothelial (HUVE) cells have been previously reported to express the genes for the A and B chain peptides of the dimeric molecule Platelet-Derived Growth Factor (PDGF) and to secrete PDGF-related factors into culture media. Anti-human 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 approximately 36-38 kd MW and that less than 15% 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 our data indicate is 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 mitogen Connective Tissue Growth Factor (CTGF).

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INTRODUCTION

The peptide growth factors are a diverse group of protein hormones which are involved in the migration, proliferation and differentiation of various cell types. Growth factors act in a paracrine or autocrine fashion by binding to specific cell surface receptors on their target cells and mediate their biological effects either directly by receptor phosphorylation of cytoplasmic proteins or through the activation of cytoplasmic second messengers. The result of these mechanisms includes kinase activation and protein phosphorylation, increase in calcium and other cation concentration, induction of transcription of specific genes and translation of proteins necessary for progression through the cell cycle. (Pardee,1989 for review.) Both growth factors (Waterfield et al.,1983;Doolitle et al.,1983) and growth factor receptors (Downward et al.,1984;Sherr et al.,1985) are related to cellular oncogenes, thereby linking the control of normal growth with pathological growth and neoplasia. Clinically, a thorough understanding of the peptide growth factors will lead to therapies which will augment normal processes such as wound healing and inhibit the pathological effects of these factors in tumorogenesis and cancer metastasis.

The growth factors involved in epithelial and mesenchymal cell development and regeneration work in concert to cause the migration and proliferation of cells in areas needed such as epithelia of the skin and gut or in the healing of a wound.

Studies done in fibroblasts (Stiles et al.,1979) demonstrated that stimulation with at least two types of growth factors are necessary for cells to enter and progress through the cell cycle. Platelet-Derived Growth Factor (PDGF) has been described as a competence factor and binding of PDGF to cell surface receptors of quiescent fibroblasts causes cells to move from the G_0 stage to the G_1 stage of the cell cycle (Stiles et al.,1979). The G_1 phase is a preparatory, biosynthetic stage characterized by protein phosphorylation, calcium ion increases, kinase activation and the induction of the fos (Greenburg and Ziff,1984) and myc (Kelly et al.,1983;Cochran et al,1983) genes, which produce nuclear proteins involved in gene regulation. Cells made competent by the action of PDGF will remain in the G_1 stage and not progress further in the cell cycle unless stimulated by a progression factor such as Epidermal Growth Factor (EGF) or Insulin-like Growth Factor (IGF). These progression factors will cause the fibroblasts to enter the S phase of the cycle in which DNA is duplicated (Leof,et al.,1982;Leof et al.,1983). Cells which enter the S phase will complete the cell cycle by progressing through G_2 and into the mitotic M stage without further external stimulation by growth factors or other agents. Stimulation with a progression factor first and then a competence factor results in cells only entering the G_1 phase of the cycle. Further stimulation with a progression factor is needed to enter the S phase. This

indicates that certain induced proteins and second messengers in the competence stage are necessary for proper responses to progression factors and completion of the cell cycle (Stiles et al.,1979). The work with fibroblasts demonstrates that the regulation of cellular proliferation is a complex process that requires multiple intracellular signals and external stimuli to induce entry and completion of the mitotic cycle. Therefore, the availability of competence and progression factors to target cells in vivo must be optimized in both time and place for tissue regeneration and development to occur. Investigations into the nature of growth factors have determined that there are families of structurally and functionally similar molecules which are expressed differentially by cells and tissues and this multiplicity ensures that the necessary biological activity is present when needed. A brief synopsis of these families of factors involved in connective tissue growth is instrumental in understanding the work we have done with PDGF and growth factors biologically related to PDGF.

Epidermal Growth Factor (EGF) was originally purified from mouse salivary glands (Cohen,1962) and later from human urine (Cohen and Carpenter,1975) and platelets (Oka and Orth,1983). The biologically active molecule is a 53 amino acid peptide containing three internal disulfide bonds (Gregory,1975;Taylor et al.,1972). EGF is a mitogen for epithelial and endothelial cells and fibroblasts. The ligand

binds a 170 kd cell surface receptor which is related to the erb B oncogene (Downward et al.,1984). A related molecule, Transforming Growth Factor alpha (TGF-α) exists in multiple species ranging from 5-20 kd with the smallest form being a 50 amino acid molecule with 30% homology to EGF including conservation of the six cysteine residues (Marquardt et al.,1984). This sequence homology and formation of the three intramolecular disulfide bonds may indicate similar protein tertiary structure and explain binding of both EGF and TGF-α to the same cell surface receptor (Massague,1983). In addition to the EGF and TGF-α peptides, a larger molecule of 27-30 kd has been identified in human platelets which is immunologically related to these two molecules and binds the same receptors (Oka and Orth,1983;Bowen-Pope and Ross,1983).

Transforming Growth Factor beta (TGF-β) is a 25 kd dimeric molecule composed of two identical monomers held together by nine disulfide bonds (Derynck et al.,1985). TGF-β has been identified in various tissues and purified from human platelets (Assoian et al.,1983) and human placenta (Frolich,et al.,1983). TGF-β has been shown to be a mitogen for a variety of mesenchymal cell types in monolayer culture (Shipley et al.,1985; Roberts et al.,1985; Massague,1984), although further investigation has indicated that this may be an indirect effect involving the induction of PDGF related molecules (Leof et al.,1986;Soma and Grotendorst,1989). TGF-β is an inhibitor of endothelial (Baird and Durkin,1986;

Frater-Schroder et al.,1986; Heimark et al.,1986;Muller et al.,1987; Takahara et al.,1987) and epithelial cells (Masui et al.,1986;Tucker et al.,1984) and regulates the differentiation of bronchial epithelial and other cell types (Masui et al 1986). Two different TGF-β monomers (Beta 1 and Beta 2) have been identified and share greater than 70% amino acid sequence homology, including conservation of all cysteine residues (Cheifetz et al.,1987). A third monomer (TGF-Beta 3) has recently been cloned (ten Dijke et al.,1988).

Fibroblast Growth Factor (FGF) was originally identified from extracts of pituitary and brain as an activity which stimulated the growth of 3T3 cells (Armelin,1973; Gospodarowicz,1974). The biological activity was later shown to be due to two proteins, acidic FGF (pI 5.6) (Maciag,et al.,1984;Thomas,et al.,1984) and basic FGF (pI >9.0) (Esch,et al.,1985) which share a 55% amino acid sequence homology (Esch,et al.,1985). A human cDNA clone for acidic FGF (Jaye et al.,1986) has been sequenced as has a cDNA clone for basic FGF (Abraham et al.,1986) and both messages indicate 155 amino acid precursor molecules. The FGF proteins do not have leader peptides and therefore are not thought to be secreted molecules but are released upon tissue damage and cell rupture. These growth factors are mitogenic for cells of mesenchymal origin including fibroblasts, endothelial and smooth muscle cells, myoblasts and osteoblasts. Fibroblast growth factor molecules have been found in most tissues

examined and range in size from 15,000 to 18,000 MW. Higher MW forms have been identified and multiple forms of mRNA transcripts detected, suggesting differential protein processing and mRNA splicing. Several other proteins have been identified which constitute a family of homologous proteins with similar biological activities. The FGF-5 protein is a 267 amino acid molecule with 43% homology to FGF which is mitogenic for both fibroblasts and endothelial cells (Zhan et al.,1988). The gene product of the hst\K-fgf gene is a 206 amino acid protein with similar biological activity which also has 43% amino acid homology to FGF and was isolated from a Karposi's sarcoma (Delli-Bovi et al.,1987; Delli-Bovi et al.,1988). Both the FGF-5 and hst\K-fgf proteins are translated with a signal sequence and are secreted into media from transfected tissue culture cells. A fifth member of this family is the product of the int-2 gene which has a 44% amino acid sequence homology to FGF (Dickson and Peters,1987).

Platelet-Derived Growth Factor (PDGF) has been characterized as a mitoattractant due to its chemotactic effect on fibroblasts and smooth muscle 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). PDGF has not been shown to be mitogenic on epithelial or endothelial cells. PDGF was originally identified (Ross et al.,1974;Kohler and Lipton,1974) and purified (Antoniades et al.,1979;Heldin et al.,1979;Heldin et

al.,1981) from the alpha granules of human platelets. Platelet PDGF is a dimeric molecule which migrates on SDS-polyacrylamide gels at approximately 30 kd. Reduction of dimeric PDGF yields A chain (17 kd) and B chain (14 kd) peptides which share a 60% sequence homology including conservation of seven cysteine residues. The gene for the A chain is located on chromosome 7 (Bonthron,et al.,1988) and the B chain (c-sis) gene on chromosome 22 (Dalla Favera et,al. 1981). The monomeric peptides are not biologically active as a mitogen (Antoniades,1981) or a chemoattractant (Grotendorst et al.,1981). The three possible isoforms of PDGF, the AA and BB homodimers and the AB heterodimer, are found naturally and all are biologically active (Heldin et al.,1986; Betsholtz et al.,1985; Stroobant and Waterfield,1984). Two high affinity cell surface receptors have been identified and are tyrosine kinases (Heldin et al.,1981;Gronwald et al.,1988;Hart et al.,1988;Heldin et al.,1988). Differential binding of the PDGF isoforms to these receptors has been reported with the type alpha receptor binding all three isoforms and type beta binding only the BB and AB dimers (Bowen-Pope et al.,1989). The B chain of PDGF demonstrates sequence homology with the v-sis oncogene and may be involved in cell transformation by an autocrine mechanism (Doolittle et al.,1983;Robbins et al.,1983;Owen et al,1984). Human macrophages activated with bacterial endotoxin secrete a PDGF related activity into culture media which is neutralized by anti-PDGF antibody

(Martinet et al.,1986). Immunoblots of this conditioned media with an anti-PDGF antibody indicate that the biological activity is produced by a 16 kd monomeric molecule antigenically related to PDGF and has been termed Macrophage Derived Growth Factor (MDGF) (Pencev and Grotendorst,1988). Partial amino acid sequence was obtained for this peptide indicates that it is not related to the A or B chain of platelet PDGF but is a product of the Connective Tissue Activating Peptide III gene, which has recently been cloned and sequenced (Wenger,et al.,1989). The biological activity may be due to similar conformation of PDGF dimers and the monomeric MDGF protein and binding of the macrophage ligand to the PDGF cell surface receptor.

The work presented here with PDGF-related proteins secreted by human vascular endothelial cells provides evidence of a group of peptides which produce their biological effects through the PDGF cell surface receptor probably due to conformational similarity of the mature proteins. The brief synopsis of growth factors presented above demonstrates that the peptide growth factors have evolved into families of multiple genes and proteins capable of producing similar biological activities. It seems probable then that there are other genes and proteins related to PDGF which are chemotactic and mitogenic for mesenchymal cells in addition to the presently identified PDGF A and B chain peptides.

Conditioned media from cultures of Human Umbilical Vein

Endothelial (HUVE) cells have been reported to contain factors which compete with platelet PDGF for binding to the PDGF cell surface receptor of fibroblasts and demonstrate PDGF-related biological activity (DiCorletto,1984). HUVE cells express both the A and B chain genes of PDGF (Collins et al.,1985; Collins et al.,1987; Doolitle et al.,1987). The B chain gene of PDGF produces a single transcript in HUVE cells of 3.8 kb (Collins et al.,1985). The HUVE cell PDGF A chain gene is expressed as three mRNA transcripts of 2.8,2.3 and 1.7 kb, with the longest transcript thought to be the only one translated (Bonthron et al.,1988). The HUVE A chain mRNA differs from A chain transcripts derived from neoplastic glioma cell lines by alternative splicing in which exon six of the genomic sequence is removed from the HUVE transcript, resulting in a translated peptide lacking a highly basic 15 amino acid carboxy terminal region found in the glioma peptide (Collins et al.,1987; Tong et al., 1987). Since both the A and B chain genes are expressed, the secreted molecules could be AB heterodimers or AA or BB homodimers. The question we originally asked was what form of PDGF is secreted by human endothelial cells?

We have investigated the composition and nature of the PDGF-related mitoattractant activity secreted by human vascular endothelial cells. PDGF-immunoreactive molecules were purified from HUVE cell conditioned media by anti-PDGF IgG affinity chromatography. Biological assays of these

affinity purified proteins in conjunction with immunoblots using sequence specific anti- A and B chain PDGF antibodies indicate that less than 15% of the PDGF-related molecules contain sequences which are antigenically related to PDGF A or B chain. The major mitoattractant secreted by these cells appears to be a 36 kd molecule which is related to platelet PDGF but is not a product of the PDGF A or B chain gene. Screening of an expression cDNA library made from the mRNA of HUVE cells with anti-PDGF antibody has resulted in the cloning and sequencing of a cDNA which encodes a 38 kd peptide which our data indicates is the primary source of the PDGF-related biological activity secreted by HUVE cells. We have termed this new mitogen Connective Tissue Growth Factor.

RESULTS

Identification and partial purification of PDGF-related mitogenic activity from HUVE cells.

The initial studies of the nature of the Platelet-Derived Growth Factor related proteins secreted by Human Umbilical Vein Endothelial (HUVE) cells were done to determine the relative molecular weight of the molecules produced, the amount of the proteins secreted by these cells, and their specific biological activity.

Endothelial cells were obtained by collagenase perfusion from the veins of fresh human umbilical cords and cultured in growth media containing 20% fetal calf serum, basic FGF and heparin. The growth media was removed from confluent cultures, the cells washed and the growth media replaced with serum-free media containing no other additives. Aliquots of this serum-free media were removed periodically and dialyzed against 1 N acetic acid. The proteins were lyophilized, resuspended and run on 12% polyacrylamide electrophoresis gels. The proteins were electroblotted to nitrocellulose and the membranes immunoblotted using anti-PDGF goat IgG as a first antibody and rabbit anti-goat IgG alkaline phosphatase conjugated as a second antibody. The proteins were visualized after incubation with a substrate solution (Fig. 1). The results indicated constitutive secretion of several species of molecules which are immunologically similar to human platelet PDGF but are of higher molecular weight (36-38 kd)

Figure 1.

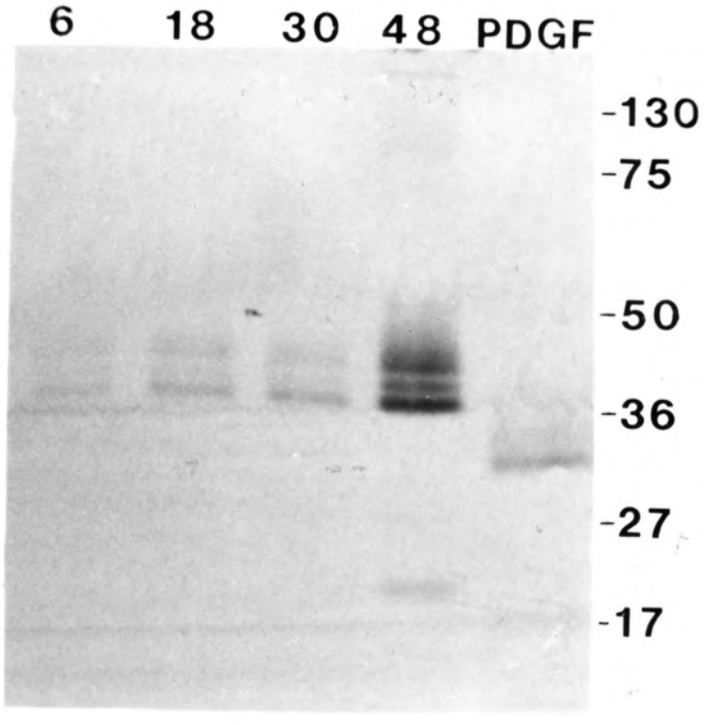


Figure 1. Constitutive secretion of PDGF-immunoreactive factors by HUVE cells.

HUVE cells were grown to confluence in 6 well plates. The growth media was removed, cells 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 anti-human PDGF antibody.

Five nanograms of purified platelet PDGF was run as reference. Positions of MW markers (BioRad) is indicated at right.

than the expected 30-32 kd of platelet PDGF or the PDGF A or B chain homodimers. The concentration of the proteins in the media increases with time, indicating that the proteins are being produced by the cells. The anti-PDGF antibody used in these experiments was made by immunizing a goat with PDGF purified to homogeneity from human platelets (Grotendorst, 1984). The amino acid composition of the antigen was obtained and was consistent with human platelet PDGF and a single band was observed on silver stained polyacrylamide gels. This antibody does not cross-react with other growth factors such as TGF-β, FGF or EGF in immunoblot analysis and is able to detect less than 500 picograms of dimeric PDGF molecules or 10 nanograms of reduced, monomeric A or B chain peptides on immunoblots.

The biological activity of the serum-free conditioned media was analyzed by mitogenic and chemotactic assays. These results indicated biological activity in the conditioned media equivalent to 15 ng/ml of platelet PDGF after 48 hours conditioning time (Fig. 2). The activity is comparable to platelet PDGF in that it is both chemotactic and mitogenic for the fibroblasts used as target cells. Prior incubation of the conditioned media with 60 μg/ml of anti-human PDGF IgG neutralized approximately 30% of the mitogenic and chemotactic activities. This is in agreement with previous reports (DiCorletto,1984). The mitogenic activity remaining after antibody neutralization of the PDGF-related fraction is

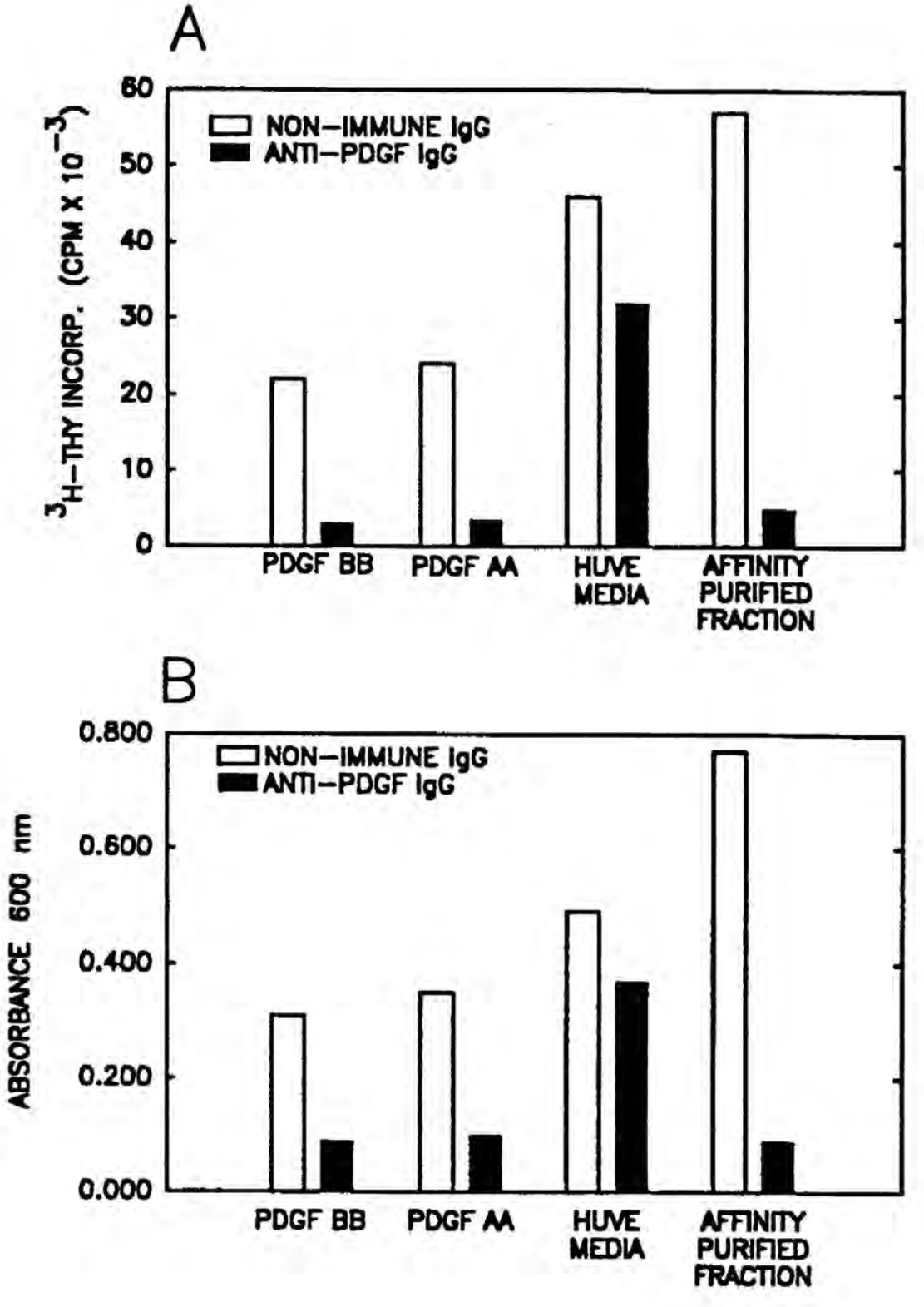

A
60
50
40
30
20
10
0
^{3}H-THY INCORP. (CPM X 10^{-3})
NON-IMMUNE IgG
ANTI-PDGF IgG
PDGF BB
PDGF AA
HUVE MEDIA
AFFINITY PURIFIED FRACTION
B
0.800
0.600
0.400
0.200
0.000
ABSORBANCE 600 nm
NON-IMMUNE IgG
ANTI-PDGF IgG
PDGF BB
PDGF AA
HUVE MEDIA
AFFINITY PURIFIED FRACTION

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 μl of HUVE cell serum-free conditioned media (48 hrs) which was dialyzed against 1 N acetic acid, lyophilized, and resuspended in DMEM before addition to test wells. Affinity purified fraction is 5 μl/ml of combined, concentrated major pool from Affi-Gel 10 affinity column. Anti-PDGF IgG or non-Immune IgG (30 μg/ml) was added to the samples and incubated 18 hrs at 4°C prior to testing in the mitogenic assay. The data points in A and B are the mean of triplicte samples with a standard deviation of less than 5%. The experiments were repeated at least three times with similar results.

B. Chemotactic assays were performed as described using 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 BSA, conditioned for 48 hrs. Affinity purified fraction is 2.5 μl/ml of combined, concentrated major pool from Affi-Gel affinity column. Antibody neutralization is performed as described under A.

probably due to FGF and the remaining chemotactic activity to fibronectin, both of which are secretory products of endothelial cells. In these assays and throughout this project, bacterially produced recombinant PDGF AB, AA and BB dimeric molecules are used as control standards. These molecules offer the advantage of being homogeneously pure, with no chance of contamination by other growth factors present in human platelets (e.g. TGF-β,TGF-α). In biological assays conducted periodically in our lab, these recombinant molecules produce activities identical to the purified naturally occuring isoforms of PDGF.

The presence in the HUVE cell conditioned 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). In similar work previously done, we had shown that the non-transformed BSC-1 monkey kidney epithelial cell line constitutively secretes PDGF-like activity into culture media and that 90% of the activity is neutralized by anti-PDGF IgG. Immunoblots of the dialyzed media proteins using the anti-PDGF antibody indicated a single band which co-migrated on polyacrylamide gels with the PDGF BB homodimer. This was consistent with the production of PDGF B chain mRNA but no A chain mRNA in these cells (Kartha et al.,1988).

In order to obtain greater amounts of the PDGF-like proteins for further analysis, the HUVE cells had to be kept in media containing 20% fetal calf serum, since these cells begin to die after 24 hours 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). 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 anti-human 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 both the higher MW molecules observed in the serum-free conditioned media and immunoreactive proteins in the 30-32 kd range (Fig.3). The major species secreted migrated at 36 kd on acrylamide gels and represented at least 50% of the total immunoreactive protein. The peptides which comigrated with purified platelet PDGF and synthetic A and B chain dimeric standards represented less than 15% of the total immunoreactive protein. Prior incubation of the antibody with purified PDGF blocked antibody binding to all of the molecules, indicating shared antigenic determinants with

Figure 3.

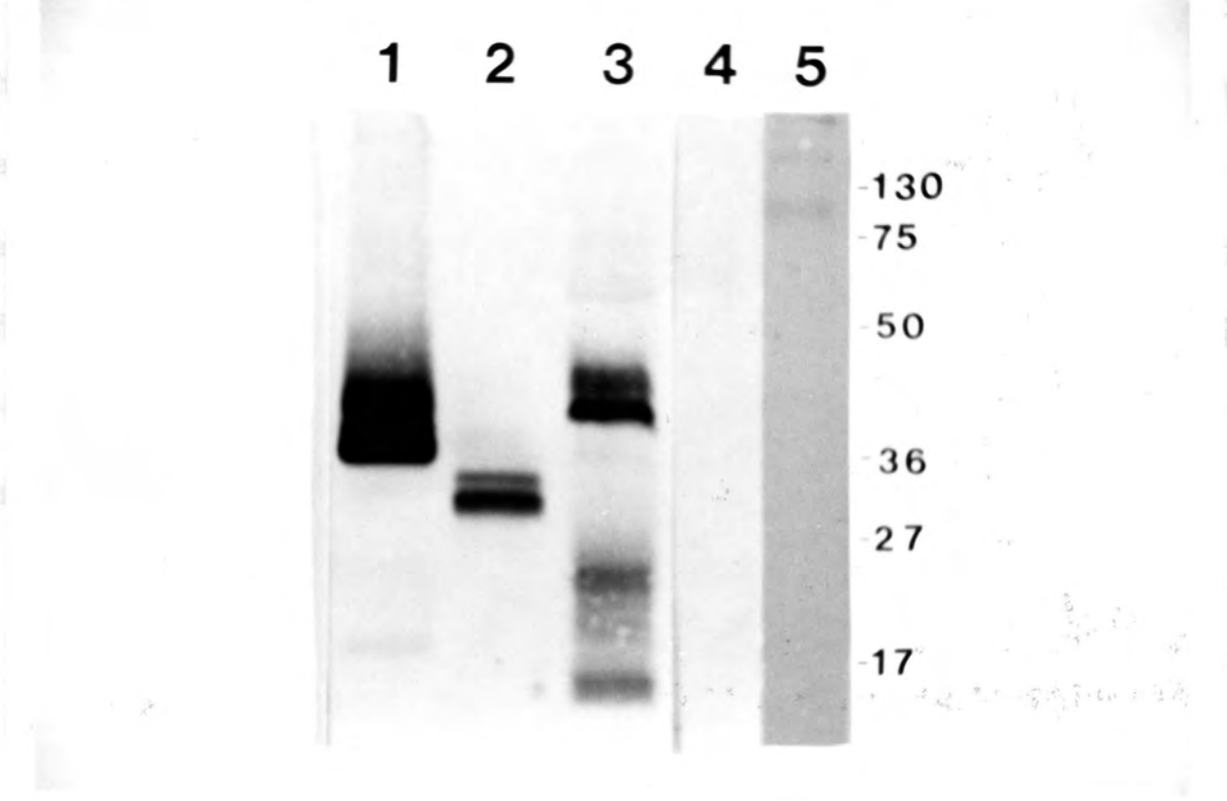


Figure 3. Immunoblot of HUVE cell secreted PDGF-related factors.

Proteins were run on a 12% polyacrylamide gel electroblotted to a nitrocellulose membrane and the immunoreactive factors visualized with anti-human PDGF IgG as described under Experimental Procedures. Lane 1, 5 μl 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 μl of reduced HUVE affinity purified proteins. Lane 4, 5 μl of HUVE affinity purified HUVE proteins but with anti-human PDGF antibody blocked with 300 ng of PDGF. Lane 5, Control column of 10 μl of anti-PDGF Affi-Gel 10 affinity purified protein fraction from media which was not conditioned by cells.

dimeric platelet PDGF (Fig.3,lane 4). In order to insure that none of the antibody binding molecules detected on immunoblots was derived from fetal calf serum or other additives in the culture media, a new, unused antibody affinity column was made and media which was never conditioned by cells was incubated at 37 C for two days and 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 biological activity was detected (data not shown).

When platelet PDGF or the recombinant dimers were reduced with 100 mM dithiothreitol and boiled for 5 minutes in the presence of SDS, monomeric A chain (17 kd) and B chain (14 kd) peptides were observed on immunoblots. Treating the HUVE molecules in 100 mM DTT sample buffer and boiling for 5 minutes resulted in slower migration of the major portion of the immunoreactive molecules on polyacrylamide gels (Fig. 3). Most of the immunoreactive molecules migrated at 38-39 kd and less intense bands were observed at 25 kd and 14 kd. It is necessary to run at least 10 times as much reduced protein as nonreduced in order to detect the reduced proteins. This is consistent with the affinity of our antibody for monomeric forms of the PDGF A and B chain peptides. Since our antibody was produced against dimeric platelet PDGF, a lower affinity to monomeric forms is not unexpected.

Our preliminary data indicated that HUVE cells constitutively secrete several molecules which share some

antigenic properties with human platelet PDGF and exhibit similar biological activities. Immunoblotting procedures with both non-reduced and reduced affinity purified protein samples indicated that the major immunoreactive protein in the biologically active samples was a monomeric molecule which migrated at approximately 38 kd.

In addition to these peptides which constitute the major portion of the PDGF immunoreactive molecules, species which migrated at higher MW were observed in the first few fractions eluted from the anti-PDGF affinity column. In order to determine which of all these bands on the immunoblots were PDGF-specific, and possibly which were A or B chain related, a protein mixture was made by taking an aliquot of each of the fractions eluted from the anti-PDGF IgG affinity column used to purify these proteins from HUVE cell conditioned media. An immunoblot was made with four aliquots of this mixture and the membrane was cut into four sections and a section incubated with anti-PDGF IgG previously blocked with one of the three isoforms of PDGF and one section with unblocked antibody (Fig. 4). We found that each of the different isoforms of PDGF block binding of the antibody to the major secretory products of the endothelial cells to an equal degree. The higher molecular weight proteins were unaffected by the antibody blocking and are probably due to nonspecific binding of the antibody. The PDGF isoforms also block antibody binding to each other (i.e. AA dimers block binding

Figure 4.

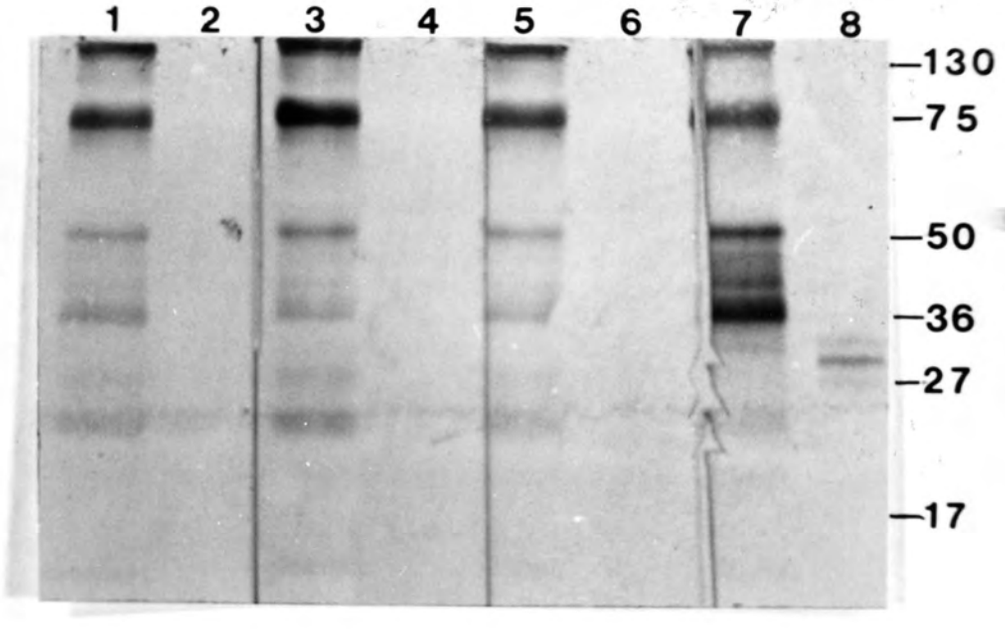


Figure 4. Immunoblot of affinity purified HUVE proteins demonstrating anti-PDGF antibody blocking with the isoforms of PDGF.

Lanes 1 and 2 anti-PDGF antibody previously incubated with recombinant PDGF AA homodimer. Lane 1 contains 1 μl aliquot of HUVE affinity purified proteins. Lane 2 contains 20 nanograms of PDGF AA homodimer.

Lanes 3 and 4, anti-PDGF antibody previously incubated with recombinant PDGF AB heterodimer. Lane 3 contains 1 μl aliquot of HUVE affinity purified proteins. Lane 4 contains 10 nanograms of PDGF AB heterodimer.

Lanes 5 and 6, anti-PDGF antibody previously incubated with recombinant PDGF BB homodimer. Lane 5 contains 1 μl aliquot of HUVE affinity purified proteins. Lane 6 contains 10 nanograms of PDGF BB homodimer.

Lanes 7 and 8, anti-PDGF antibody. Lane 7 contains 1 μl aliquot of HUVE affinity purified proteins. Lane 8 contains 10 nanograms of PDGF AB heterodimer.

to BB dimers). Therefore, the major IgG clones in this polyclonal antibody appear to be against similar epitopes on the PDGF isoforms and the endothelial cell molecules.

From this data alone we were not able to determine which of the peptides in the nonreduced lane corresponded to the 38 kd peptide in the reduced samples, representing the majority of the immunoreactive protein, and which produced the 25 and 14 kd peptides on our immunoblots. In order to analyze this, we labeled cultures of HUVE cells with ^{35}S-cysteine and affinity purified the anti-PDGF immunoreactive peptides from the conditioned media. Equal aliquots of the labeled affinity purified proteins were run both reduced and non-reduced on polyacrylamide gels and electroblotted to nitrocellulose and the membrane processed as for immunoblots using the anti-PDGF antibody. The immunoblots were then autoradiographed (Fig.5). Transmission scanning of the lanes of the autoradiograph determined that the 36 kd band in the nonreduced lane and the 38 kd band in the reduced lane have equivalent intensity. This indicates that the 36 kd molecule is monomeric and shifts to the apparent higher molecular weight due to unfolding of the molecule upon reduction of intramolecular disulfide bonds.

There was still a question as to whether these higher molecular weight PDGF immunorelated proteins could be monomeric precursor forms of PDGF A or B chain peptides which were being secreted by endothelial cells without being processed by proteolytic enzymes to the 30-32 kd size as is

Figure 5.

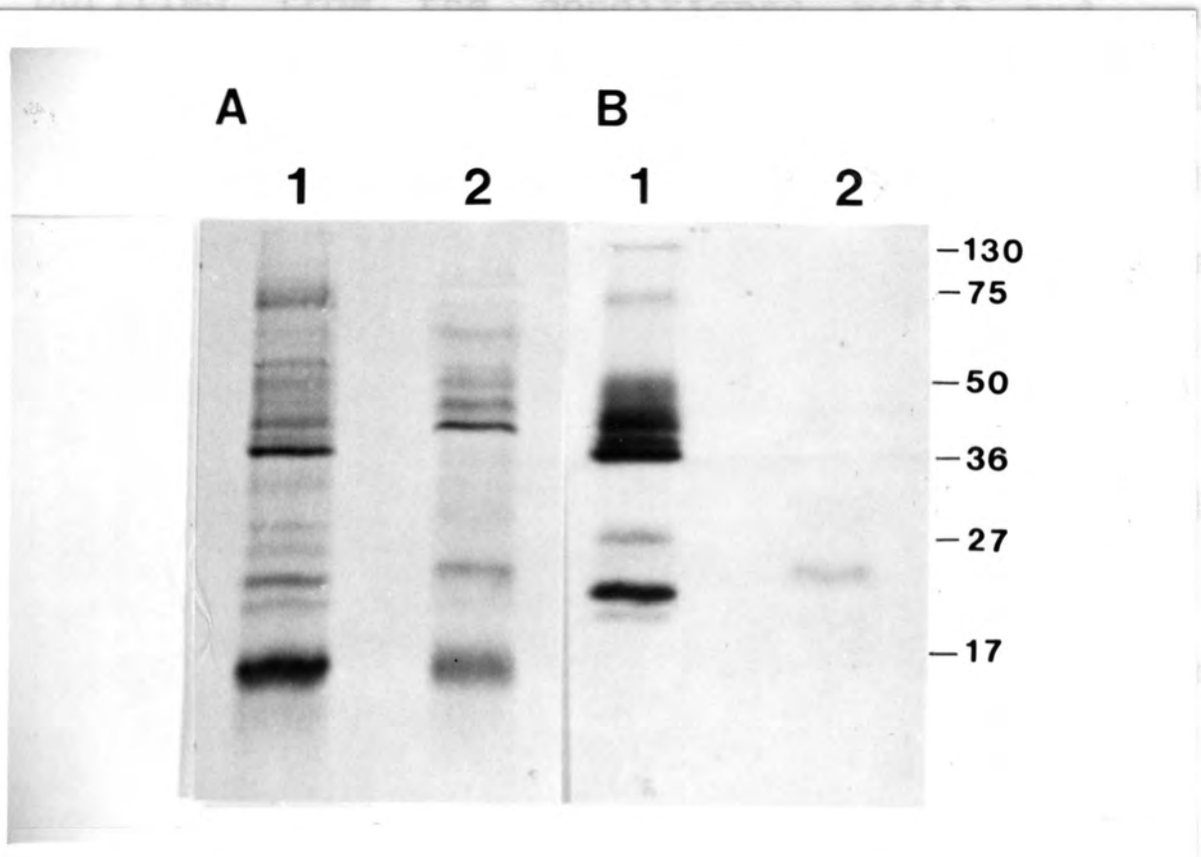


Figure 5. Autoradiograph and immunoblot of ^{35}S-cysteine labeled HUVE cell secreted proteins.

Confluent cultures of HUVE cells were labeled for 22 hours in cysteine-free DMEM containing 250 μCi/ml of ^{35}S-cysteine. The labeled PDGF-immunoreactive proteins were affinity purified from the conditioned media and equal aliquots of the proteins run on 12% polyacrylamide gels. The proteins were electroblotted to nitrocellulose and the membranes processed as for immunoblots. The immunoblots were then autoradiographed.

Side A is autoradiograph of immunoblot shown on Side B.

Lane 1. 10 μl aliquot of labeled affinity purified proteins,nonreduced.

Lane 2. 10 μl aliquot of labeled affinity purified proteins,reduced.

the case for other cell types studied (Kartha et al.,1988). Since the RNA message for both the A and B chains of PDGF is expressed in HUVE cells, we wanted to determine which of the immunorelated molecules were A chain or B chain peptides and which, if any, were not products of the A or B chain genes.

We have produced a series of antibodies in goats to synthetic peptides of 30 amino acid length which represent sequences in the amino and carboxy terminal regions of the processed PDGF A and B chain peptides. These antibodies are specific for the reduced, monomeric A and B chain peptides and do not cross react when tested on immunoblots with the reduced recombinant AA, BB and AB dimers. The peptide antibodies used in these experiments are specific for the amino (residues 79-107) and carboxy (residues 161-189) ends of the mature processed B chain peptide of platelet PDGF and the amino end of the processed A chain molecule (residues 92-119) and the carboxy end of the short (endothelial) form of the A chain molecule (residues 167-193 + DVR; Collins et al.,1987). When 100 nanograms of the affinity purified HUVE cell PDGF-like proteins are reduced and immunoblotted using the anti amino terminal A chain antibody, we detect a single band of peptides at 17 kd (Fig. 6). Comparison with 10 nanograms of reduced recombinant A chain peptides on the same immunoblot indicates that only 10 nanograms of proteins containing the amino terminal sequence are present in 100 nanograms of HUVE cell molecules. Similar results were obtained using the antibody

Figure 6.

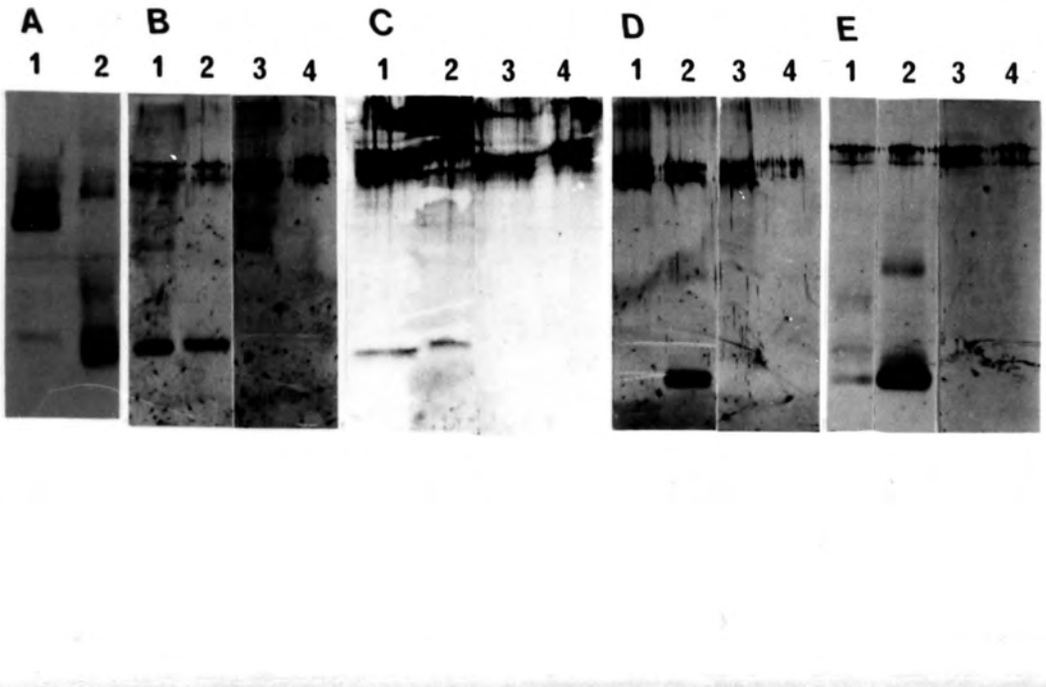


Figure 6. Immunoblot of HUVE cell secreted proteins using A and B chain peptide antibodies.

A. Primary antibody is anti-human PDGF IgG. Lane 1, 10 ng of HUVE affinity purified media proteins, non-reduced. Lane 2, 100 ng of HUVE affinity purified proteins, reduced.

B. Primary antibody is anti amino terminal A chain serum for lanes 1 and 2 and the antibody blocked with the peptide antigen for lanes 3 and 4. Lanes 1 and 3 100 ng of HUVE purified media protein, reduced. Lanes 2 and 4, 10 ng of AA short form homodimer, reduced.

C. Primary antibody is anti carboxy terminal A chain serum for lanes 1 and 2 and the antibody blocked with the peptide antigen for lanes 3 and 4. Lanes 1 and 3, 100 ng of HUVE purified media proteins, reduced. Lanes 2 and 4, 10 ng of AA homodimer, reduced.

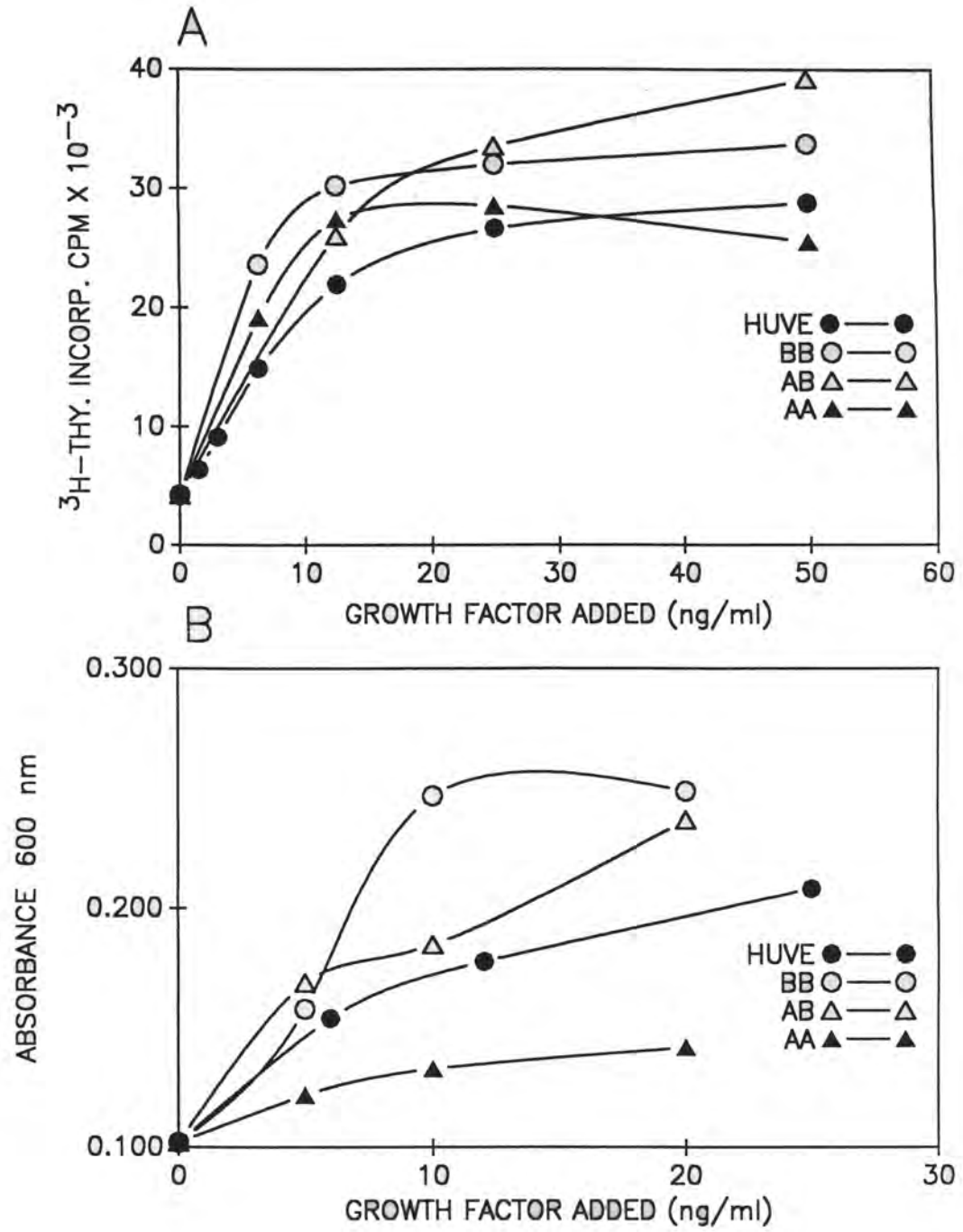
D. Primary antibody is anti amino terminal B chain serum for lanes 1 and 2 and the antibody blocked with the peptide antigen for lanes 3 and 4. Lanes 1 and 3 and 200 ng of HUVE purified media proteins, reduced. Lanes 2 and 4 are 20 ng of BB homodimer, reduced.

E. Primary antibody is anti carboxy terminal B chain serum for lanes 1 and 2 and the antibody blocked with the peptide antigen for lanes 3 and 4. Lanes 1 and 3, 100 ng of HUVE purified media proteins, reduced. Lanes 2 and 4 are 20 ng of BB homodimer, reduced.

specific for the carboxy terminal of the PDGF A chain (Fig. 6). The data from these two antibodies indicated that of the total PDGF immunorelated molecules secreted by HUVE cells, only about 10% contained sequences found in the PDGF A chain amino or carboxy terminal regions. The antibody specific for the 30 amino acid sequence in the amino terminal of the PDGF B chain peptide did not detect any molecules in 200 nanograms of reduced endothelial cell proteins. This antibody is our least sensitive and it may be that we are not able to detect the small amount of PDGF B chain molecules present. The B chain carboxy terminal peptide antibody detects reduced molecules at 25 kd and at 14 kd in 100 nanograms of HUVE cell proteins. Comparison with 20 nanograms of reduced BB homodimer run as a standard on the same blot indicates that about 5% of the endothelial cell PDGF related molecules contain PDGF B chain amino acid sequences. The 14 kd molecule is probably reduced B chain which has been proteolytically processed. The 25 kd molecule may be reduced 50 kd B chain precursor molecule, a small amount of which is found in the conditioned media. These data indicate that approximately 15% of the PDGF immunoreactive molecules secreted by HUVE cells contain sequences of the mature PDGF A and B chain molecules. The remaining 85% of this biologically active protein fraction is not related to the PDGF peptides.

Major chemotactic and mitogenic activity is produced by 36 kd peptide and not PDGF peptides.

In order to determine if the chemotactic and mitogenic activities observed in the partially purified media proteins were the result of the proteins containing the PDGF A and B chain peptides or were the result of molecules which 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. Sufficient quantities of the samples were prepared to perform several mitogenic and chemotactic assays and immunoblots with aliquots of each dilution. 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. 7). As an example, at 20 ng/ml of either the recombinant PDGF isoforms or the HUVE cell molecules, the biological activity elicited from the target cells was at or near maximum, but the immunoblot of equal aliquots of these same samples show that no PDGF A or B chain peptides were detected in the HUVE cell molecule samples. The detection


A
40
30
20
10
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^{3}H-THY. INCORP. CPM X 10^{-3}
0
10
20
30
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50
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GROWTH FACTOR ADDED (ng/ml)
HUVE
BB
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AA
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0.300
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ABSORBANCE 600 nm
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GROWTH FACTOR ADDED (ng/ml)
HUVE
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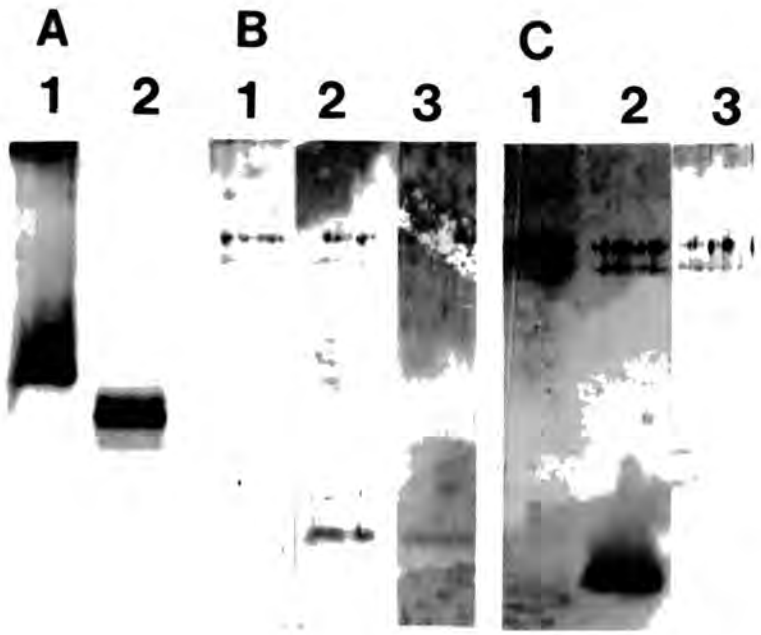
A
1
2
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1
2
3
C
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2
3

Figure 7. Biological assays and immunoblots of serial dilutions of HUVE cell affinity purified media proteins and recombinant PDGF standards.

Mitogenic assay (A) and chemotactic assay (B) performed as described with NIH 3T3 cells. The test samples in both biological assays and the immunoblot are identical aliquots of the same sample. Data points are the mean of three samples and the standard deviation is less than 10%.

Fig. 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 anti carboxy 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.

level on this blot with these peptide antibodies was less than 1.25 nanograms for A chain peptides and less than 2.5 nanograms for B chain peptides. As shown above, we estimate that of the total fraction of anti-PDGF immunoreactive molecules secreted by HUVE cells, less than 15% contain sequences of the PDGF A and B chain gene products. This being the case, the biological activity produced by the test samples in these dilution assays cannot be accounted for by PDGF A or B chain containing molecules. These data strongly indicated that the 36-39 kd PDGF-immunoreactive protein species present in these samples were biologically active.

In order to substantiate the binding of the endothelial cell molecules to the PDGF cell surface receptors, competitive receptor binding assays were performed. Since immunoblots of the affinity purified endothelial cell proteins indicated the presence of multiple PDGF-related 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. Because the isoforms of PDGF and the major PDGF immunorelated proteins 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 hours 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. 8). The data show that the 36 kd immunoreactive peptide bound to the cell surface of NIH 3T3 cells. This binding could 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 cell and that PDGF BB can compete with this binding.

The data which we now had gave strong evidence that the PDGF related proteins from HUVE cells were able to bind the PDGF cell surface receptor of mesenchymal cells and elicit both chemotactic and mitogenic activity equivalent to the isoforms of PDGF. The biological activity was being produced by proteins which did not contain sequences present in the amino or carboxy terminals of the PDGF A or B chain peptides. The presence of three predominant bands on immunoblots and the fact that PDGF A and B chain peptides constituted 15% of the biologically active molecules complicated efforts to purify the biologically active molecules which were not A or B chain related.

Figure 8.

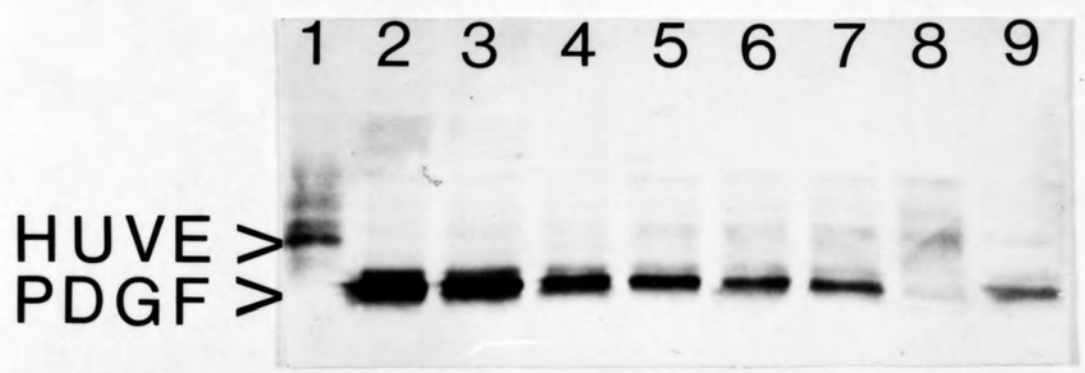

Figure 8. PDGF cell surface receptor binding competition assay with NIH 3T3 cells and HUVE affinity purified proteins competing with recombinant PDGF BB homodimer.

Lane 1 contains 10 nanograms of HUVE affinity purified proteins and lane 9 contains 10 nanograms of recombinant PDGF BB homodimer.

Lanes 2 through 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 hours at 4 C with serum-free DMEM containing 10 ng of affinity purified proteins from HUVE cell conditioned media and varying concentrations of recombinant PDGF BB. The concentration of PDGF in lane 2 is 300 ng; lane 3 is 150 ng; lane 4 is 75 ng; lane 5 is 37.5 ng; lane 6 is 18.75 ng; lane 7 is 9.4 ng and lane 8 has no addition of PDGF.

Cloning and sequencing of the cDNA for Connective Tissue Growth Factor.

In order to further characterize these PDGF related molecules, 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). Clone selection is accomplished by the binding of the antibody to fusion proteins which are part β-galactosidase from the vector and part human endothelial cell protein from the HUVE cDNA inserts. 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 system indicated that two of the clones picked contained fragments of the PDGF B chain cDNA open reading frame sequence. One of these clones was a 1.8 kb fragment similar to one previously isolated by Collins,et al. (1985) using a c-sis cDNA probe. This result established that the library screening process with the anti-PDGF antibody was an effective method for identifying cDNA clones producing fusion proteins containing PDGF related antigens. 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. This clone was designated DB60. In order to demonstrate specificity of the anti-PDGF antibody binding to

the fusion protein produced by clone DB60, the phage was plated on a 100 mm plate and the fusion protein induced by placing two pieces of IPTG soaked nitrocellulose on the plate and processed in the same manner as for the library screening. One filter was incubated with the anti-PDGF antibody and the other was incubated with the anti-PDGF antibody previously blocked by incubation with affinity purified HUVE cell secreted proteins. Antibody binding to the protein produced by the clone DB60 was completely blocked by the affinity purified proteins (Fig.9). This antibody blocking provides evidence that the HUVE cell affinity purified proteins seen on immunoblots are the same as the endothelial cell portion of the fusion protein produced by clone DB60. Clone DB60 was subcloned into Bluescript phagemid (Stratagene). A ^{32}P-labeled probe was made of DB60 and used on a Northern blot of 20 μg of total RNA isolated from HUVE cells (Fig. 10). The blot indicated probe hybridization with an mRNA of 2.4 kilobases, which is a message of sufficient size to produce the proteins in the 38 kd molecular weight range seen on immunoblots of the reduced affinity purified proteins. The DB60 clone was used to rescreen the HUVE cell cDNA lambda gt11 library and the largest clone isolated contained a 2100 base pair insert. A probe made with the 500 bp fragment in clone DB60 hybridized to the Eco RI insert in the new clone, labeled DB60R32, indicating that it was a larger cDNA clone containing sequences homologous to the 500 bp sequence in clone DB60

Figure 9.

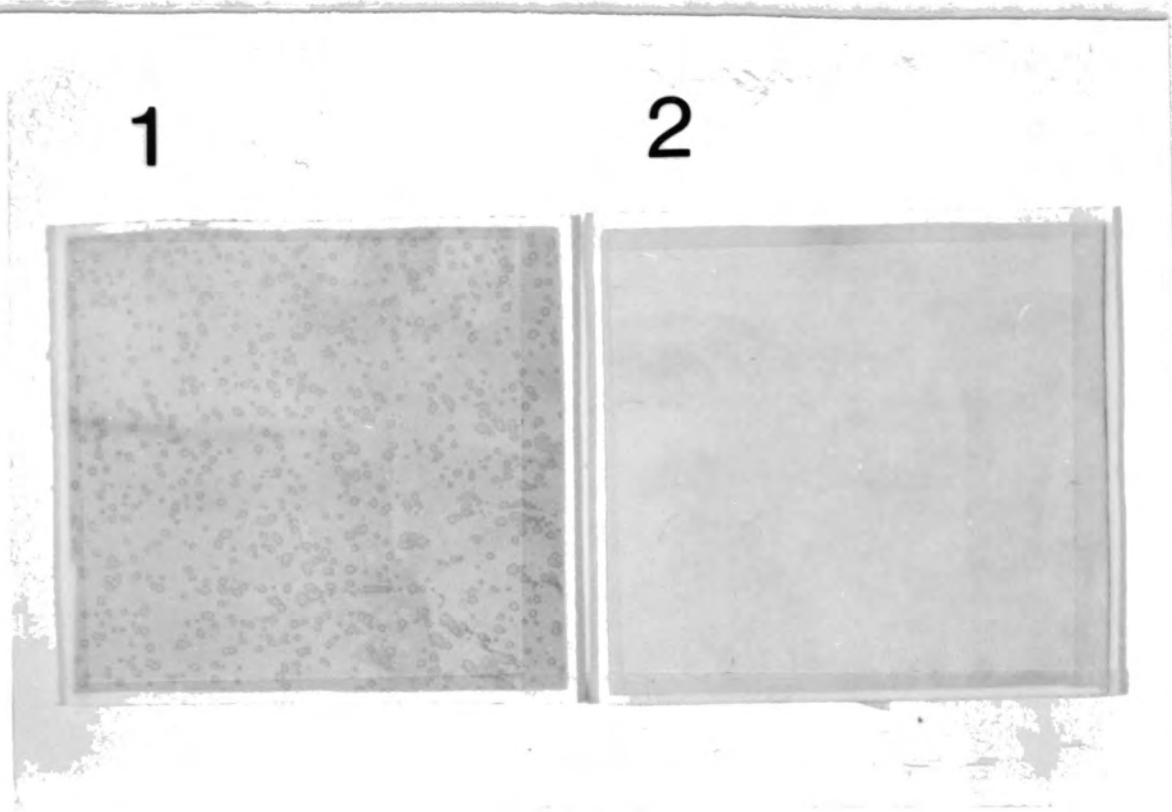

Figure 9. Lambda gt11 plaque lifts demonstrating blocking of anti-PDGF IgG to CTGF/β-galactosidase fusion protein by previous incubation of the antibody with affinity purified CTGF from HUVE cells.

The lambda gt11 clone DB60 which contained the cDNA fragment for CTGF was plated on 100 mm plates in Y1090 bacteria, the plaques allowed to form and the induced fusion protein blotted on nitrocellulose. The blotted protein was incubated with the anti-PDGF antibody as a primary antibody (Side 1) or with the anti-PDGF previously blocked by incubation with 400 ng/ml of affinity purified CTGF proteins from HUVE cell conditioned media (Side 2). The blots were processed as for library screening and incubated with the alkaline phosphatase substrate in the same container simultaneously for the same period of time.

Figure 10.

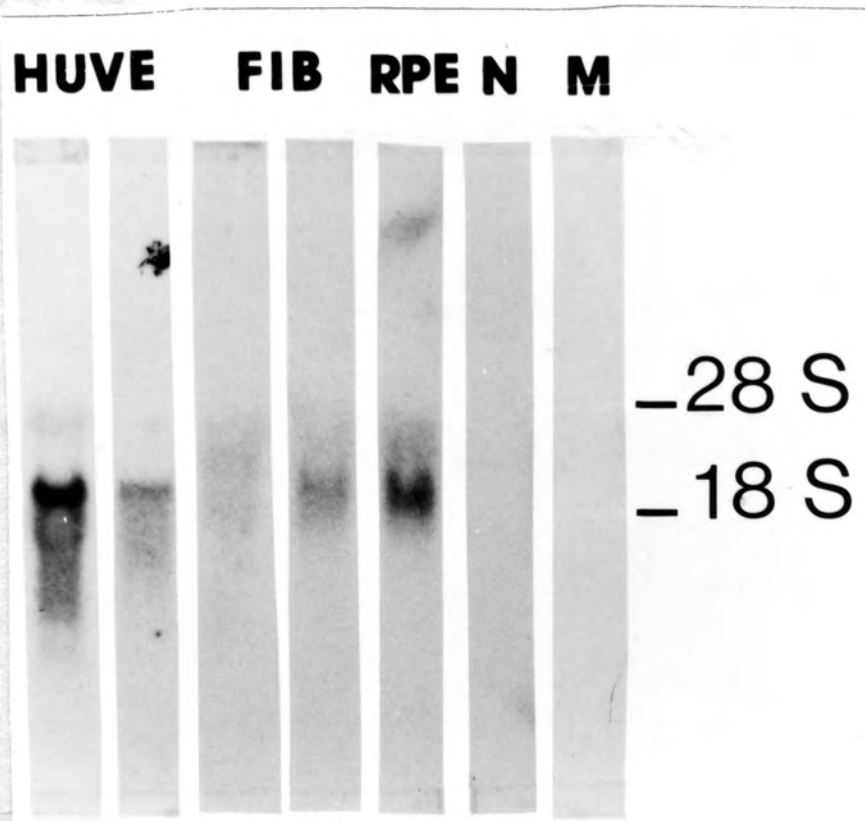

Figure 10. Northern blot of total cellular RNA demonstrating hybridization of CTGF gene probe.

The hybridization probe for first lane was clone DB60,the 500 bp clone selected from the gt11 library. Probe for all other lanes was clone DB60R32,the 2100 bp cDNA of the CTGF gene.

Lane 1. 20 μg of total RNA from HUVE cells.

Lane 2. 20 μg of total RNA from HUVE cells

Lane 3. 20 μg of total RNA from human fibroblasts.

Lane 4. 20 μg of total RNA from human fibroblasts stimulated with TGF-beta for five hours.

Lane 5. 20 μg total RNA from TGF-beta stimulated RPE cells.

Lane 6. 20 μg total RNA from human neutrophils.

Lane 7. 20 μg total RNA from human macrophages.

which was picked with the anti-PDGF antibody (Fig. 11). The 2100 bp clone DB60R32 was also used as a probe in Northerns of total RNA from HUVE cells and hybridized with a single 2.4 kb message (Fig. 10). Work previously done in this lab had shown that primary cultures of human foreskin fibroblasts (Soma and Grotendorst,1989) and human retinal pigment epithelial (RPE) cells (Campochiaro et al.,1989), upon stimulation with TGF-beta, secrete into culture media PDGF-related biological activity. In both cases, the secreted PDGF-related proteins produce a pattern similar to the HUVE peptides on immunoblots using the anti-PDGF antibody with the major protein species in the 36-39 kd MW range. Fibroblasts which were not incubated with TGF-β did not secrete these peptides. Northern blots of 20 μg of total RNA from TGF-beta stimulated fibroblasts (5 hours) and RPE cells (24 hours) using the DB60R32 clone as a probe demonstrated hybridization to a 2.4 kb message. Human fibroblasts which were not treated with TGF-β did not express this mRNA (Fig. 10). Pencev and Grotendorst (1987) had demonstrated the secretion of the 16 kd MDGF peptide by endotoxin stimulated human macrophages and shown this to be the primary protein species producing the PDGF-related biological activity found in the conditioned media from these cells. In order to determine if there was any relationship between MDGF and CTGF or if CTGF were induced in leucocytes, 20 μg of total RNA from stimulated macrophages and neutrophils were run on agarose gels and blotted. Probing

Figure 11.

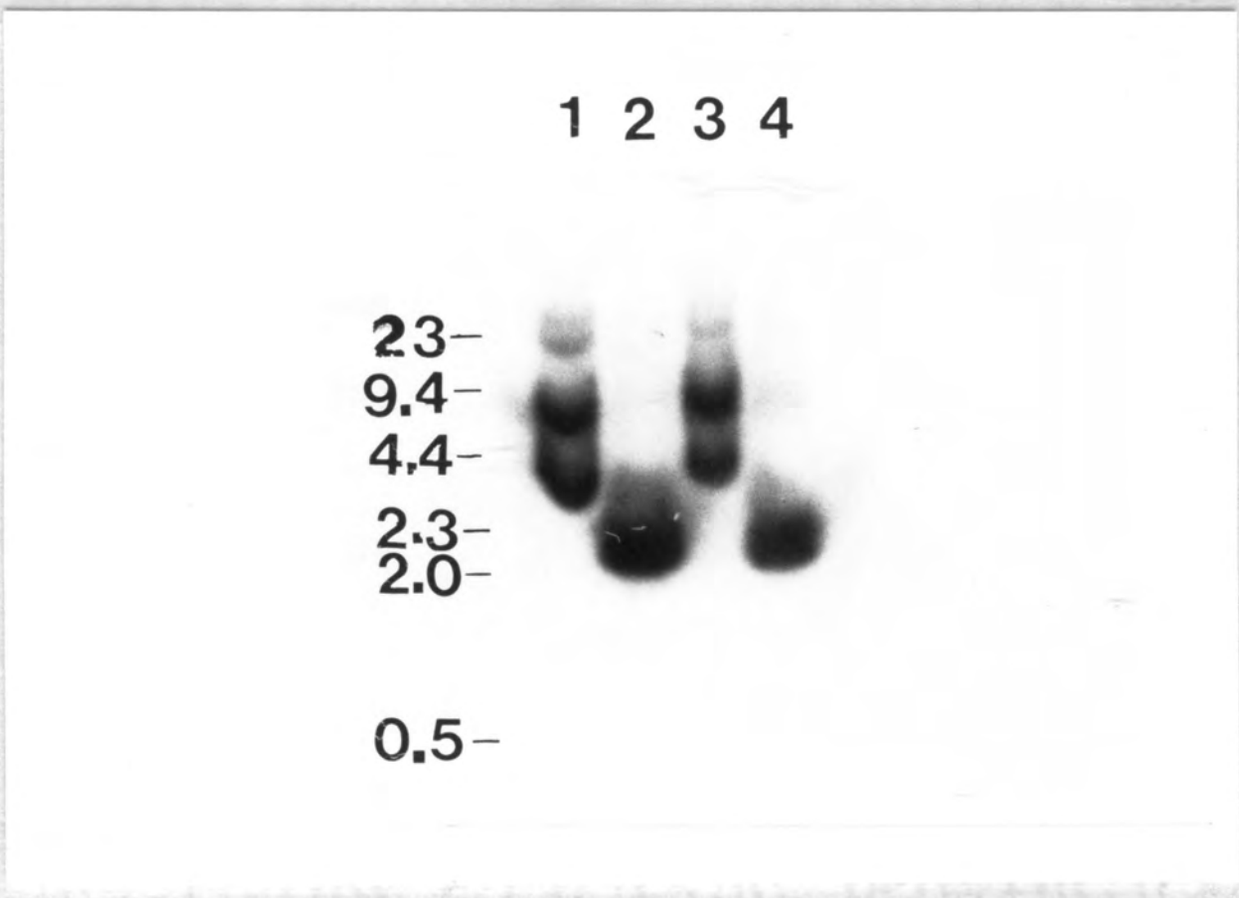

Figure 11. Southern blot showing hybridization of Eco RI fragment of clone DB60 hybridizing to immobilized 2100 bp fragment of clone DB60R32.

The 2100 bp Eco RI insert from the HUVE cDNA lambda gt11 expression library was picked by hybridization to clone DB60,the clone originally picked with anti-PDGF antibody. The insert was subcloned into Bluescript phagemids KS and SK. Lanes 1 and 2 are aliquots of Bluescript KS/DB60R32 DNA uncut (lane 1) and cut with Eco RI (lane 2). Lanes 3 and 4 are aliquots of Bluescript SK/DB60R32 DNA uncut (lane 3) and cut (lane 4) with Eco RI. The DNA was run on a 1% agarose gel,transferred to nitrocellulose membrane and probed with the 500 bp Eco RI insert from clone DB60. This demonstrated that the inserts of cDNA contained regions of homologous sequence.

the blots with DB60R32 indicated that the CTGF message is not expressed or is not expressed in abundance to be detected on total RNA blots as would be expected from the high concentrations of active peptides found in conditioned media (Fig. 10).

In order to determine the size of the translation product of the DB60R32 cDNA clone, the 2100 bp insert was cloned into the Bluescript vector and transcribed in vitro using the T7 polymerase promoter and the mRNA transcript was translated in vitro using a rabbit reticulocyte lysate method with ^{35}S-cysteine for labeling. Aliquots of the translation reaction were run reduced with 50 mM DTT on 12% polyacrylamide gels (Fig. 12). The data indicate a protein product which migrates at an apparent molecular weight of 38 kd and co-migrates with the reduced affinity purified proteins from the HUVE cell conditioned media.

The 2100 bp insert of clone DB60R32 was sequenced initially by subcloning of Pst I and Kpn I restriction fragments into Bluescript and using double stranded dideoxy methods (Fig. 13). This determined an open reading frame of 1076 base pairs. An Eco RI/Kpn I 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 analog ITP. The cDNA nucleotide sequence

Figure 12.

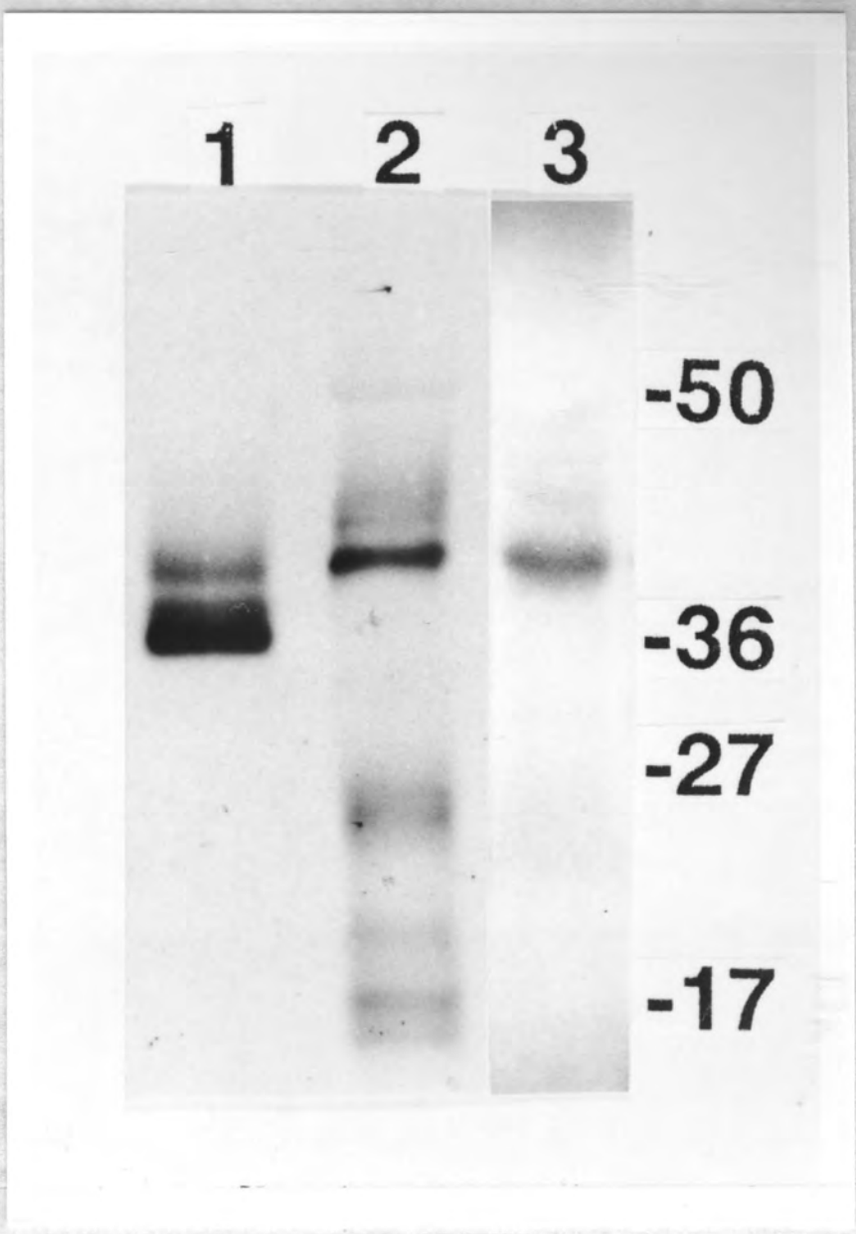

Figure 12. In vitro translation product of cDNA clone DB60R32.

Lanes 1 and 2 are an immunoblot using the anti-human 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 μl of a 50 μl rabbit reticulocyte in vitro translation reaction which incorporated 1 μg of RNA transcript from an in vitro transcription reaction using the 2100 bp clone DB60R32 in Bluescript phagemid. Autoradiograph was exposed for 24 hours.

CLONE ORIENTATION AND SEQUENCING MAP FOR CONNECTIVE TISSUE GROWTH FACTOR cDNA'S

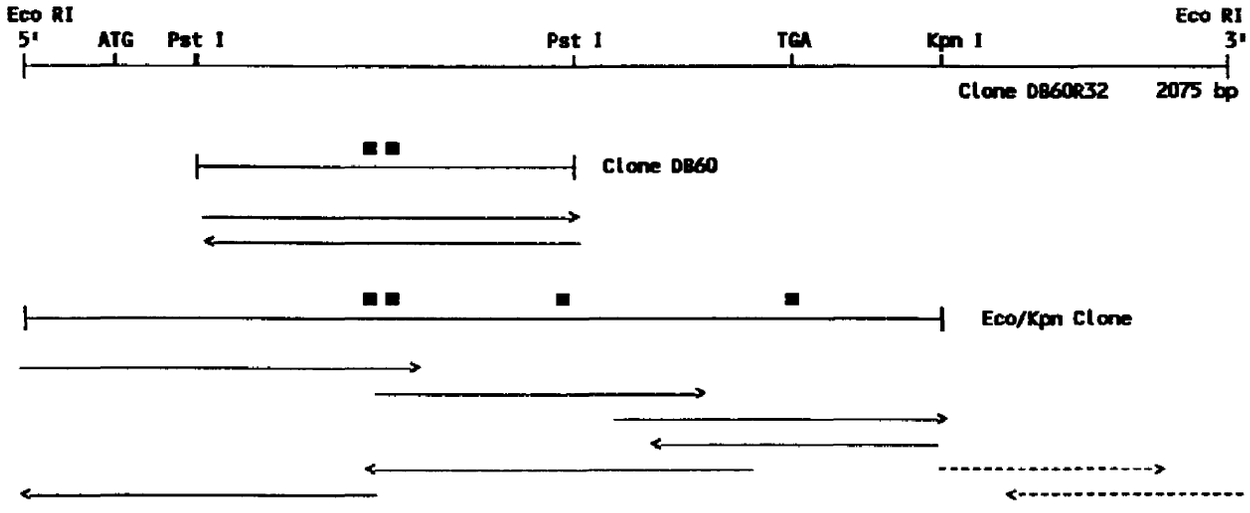

Figure 13. Sequence map of CTGF cDNA clone.

Top line represents the 2075 bp DB60R32 clone containing open reading frame of CTGF. Restriction enzyme sites used in subcloning are indicated as are the ATG initiation site and the TGA termination codon. The position of clone DB60, picked from the HUVE cDNA library with anti-PDGF antibody, is indicated. Solid squares indicate locations of primers used in sequencing. Solid arrows indicate reads of single stranded sequencing and broken arrows indicates region in 3' end for which only double stranded sequencing data is available.

indicates an open reading frame of 1047 bp which encodes a 38,000 MW protein (Fig.14). This protein has a 45% overall homology with the translation product of the v-src-inducible chicken embryo fibroblast CEF-10 mRNA (Simmons et al.,1989) (Fig. 15).

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CCCGGCCGACAGCCCCGAGACGACAGCCCGGCGCGTCCCGGTCCCCACCTCCGACCACCGCCAGCGCTCCAGGCCCCGCGCTCCCCGCTCGCCGCCACC      99

GCGCCCTCCGCTCCGCCCGCAGTGCCAACC  ATG ACC GCC GCC AGT ATG GGC CCC GTC CGC GTC GCC TTC GTG GTC CTC CTC     180
                                Met Thr Ala Ala Ser Met Gly Pro Val Arg Val Ala Phe Val Val Leu Leu      17

GCC CTC TGC AGC CGG CCG GCC GTC GGC CAG AAC TGC AGC GGG CCG TGC CGG TGC CCG GAC GAG CCG GCG CCG CGC     255
Ala Leu Cys Ser Arg Pro Ala Val Gly Gln Asn Cys Ser Gly Pro Cys Arg Cys Pro Asp Glu Pro Ala Pro Arg      42

TGC CCG GCG GGC GTG AGC CTC GTG CTG GAC GGC TGC GGC TGC TGC CGC GTC TGC GCC AAG CAG CTG GGC GAG CTG     330
Cys Pro Ala Gly Val Ser Leu Val Leu Asp Gly Cys Gly Cys Cys Arg Val Cys Ala Lys Gln Leu Gly Glu Leu      67

TGC ACC GAG CGC GAC CCC TGC GAC CCG CAC AAG GGC CTC TTC TGT GAC TTC GGC TCC CCG GCC AAC CGC AAG ATC     405
Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu Phe Cys Asp Phe Gly Ser Pro Ala Asn Arg Lys Ile      92

GGC GTG TGC ACC GCC AAA GAT GGT GCT CCC TGC ATC TTC GGT GGT ACG GTG TAC CGC AGC GGA GAG TCC TTC CAG     480
Gly Val Cys Thr Ala Lys Asp Gly Ala Pro Cys Ile Phe Gly Gly Thr Val Tyr Arg Ser Gly Glu Ser Phe Gln     117

AGC AGC TGC AAG TAC CAG TGC ACG TGC CTG GAC GGG GCG GTG GGC TGC ATG CCC CTG TGC AGC ATG GAC GTT CGT     555
Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp Gly Ala Val Gly Cys Met Pro Leu Cys Ser Met Asp Val Arg     149

CTG CCC AGC CCT GAC TGC CCC TTC CCG AGG AGG GTC AAG CTG CCC GGG AAA TGC TGC GAG GAG TGG GTG TGT GAC     630
Leu Pro Ser Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys Cys Glu Glu Trp Val Cys Asp     167

GAG CCC AAG GAC CAA ACC GTG GTT GGG CCT GCC CTC GCG GCT TAC CGA CTG GAA GAC ACG TTT GGC CCA GAC CCA     705
Glu Pro Lys Asp Gln Thr Val Val Gly Pro Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro     192

ACT ATG ATT AGA GCC AAC TGC CTG GTC CAG ACC ACA GAG TGG AGC GCC TGT TCC AAG ACC TGT GGG ATG GGC ATC     780
Thr Met Ile Arg Ala Asn Cys Leu Val Gln Thr Thr Glu Trp Ser Ala Cys Ser Lys Thr Cys Gly Met Gly Ile     217

TCC ACC CGG GTT ACC AAT GAC AAC GCC TCC TGC AGG CTA GAG AAG CAG AGC CGC CTG TGC ATG GTC AGG CCT TGC     855
Ser Thr Arg Val Thr Asn Asp Asn Ala Ser Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg Pro Cys     242

GAA GCT GAC CTG GAA GAG AAC ATT AAG AAG GGC AAA AAG TGC ATC CGT ACT CCC AAA ATC TCC AAG CCT ATC AAG     930
Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys Ile Arg Thr Pro Lys Ile Ser Lys Pro Ile Lys     267

TTT GAG CTT TCT GGC TGC ACC AGC ATG AAG ACA TAC CGA GCT AAA TTC TGT GGA GTA TGT ACC GAC GGC CGA TGC    1005
Phe Glu Leu Ser Gly Cys Thr Ser Met Lys Thr Tyr Arg Ala Lys Phe Cys Gly Val Cys Thr Asp Gly Arg Cys     292

TGC ACC CCC CAC AGA ACC ACC ACC CTG CCG GTG GAG TTC AAG TGC CCT GAC GGC GAG GTC ATG AAG AAG AAC ATG    1080
Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu Phe Lys Cys Pro Asp Gly Glu Val Met Lys Lys Asn Met     317

ATG TTC ATC AAG ACC TGT GCC TGC CAT TAC AAC TGT CCC GGA GAC AAT GAC ATC TTT GAA TCG CTG TAC TAC AGG    1155
Met Phe Ile Lys Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe Glu Ser Leu Tyr Tyr Arg     342

AAG ATG TAC GGA GAC ATG GCA TGA  AGCCAGAGAGTGAGAGACATTAACTCATTAGACTGGAACTTGAACTGATTCACATCTCATTTTTCC    1245
Lys Met Tyr Gly Asp Met Ala Term                                                                       349

GTAAAAATGATTTCAGTAGCACAAGTTATTTAAATCTGTTTTTCTAACTGGGGGAAAAGATTCCCACCCAATTCAAAACATTGTGCCATGTCAAACAAA    1344
TAGTCTATCTTCCCCAGACACTGGTTTGAAGAATGTTAAGACTTGACAGTGGAACTACATTAGTACACAGCACCAGAATGTATATTAAGGTGTGGCTTT    1443
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TTGTTTGTGCCTTTTTATTTTTGTTTTTAATGCTTTGATATTTCAATGTTAGCCTCAATTTCTGAACACCATAGGTAGAATGTAAAGCTTGTCTGATCG    1938
TTCAAAGCATGAAATGGATACTTATATGGAAATTCTGCTCAGATAGAATGACAGTCCGTCAAAACAGATTGTTTGCAAAGGGGAGGCATCAGTGTCTTG    2037
GCAGGCTGATTTCTAGGTAGGAAATGTGGTAGCTCACG*                                                                2075
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Figure 14. Nucleotide sequence and amino acid translation of Connective Tissue Growth Factor 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 ATTTA sites which are underlined. The Genbank accession number for this CTGF cDNA sequence is M36965.

COMPARISON OF CTGF AND CEF-10 PREDICTED PEPTIDE SEQUENCES

Figure 15. Comparison of amino acid sequences of the translated cDNA for Connective Tissue Growth Factor 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 aligned cysteine residues are indicated with an asterisk.

DISCUSSION

The data presented here indicate that the major connective tissue mitoattractant secreted by HUVE cells is a 38 kd monomeric molecule which is antigenically related to the dimeric platelet PDGF and competes with PDGF for binding to the surface of NIH 3T3 cells, but is not a product of the PDGF A or B chain genes. Our data indicate 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 of the total chemotactic and mitogenic activity secreted by these cells. 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 (less than 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 evident that there are some common antigenic determinants shared among these proteins. The anti-PDGF antibody has high affinity to the nonreduced forms of the PDGF isomers and the CTGF molecule and ten-fold less affinity to the reduced forms of these peptides. The PDGF A and B chain monomers are not biologically active peptides. This suggests that there is a similar shape and structure between the dimeric PDGF isomers

and the CTGF molecule and that this conformational similarity results in the formation of common antigenic epitopes and the receptor binding sites of these molecules.

The sequence of the cDNA for CTGF indicates an open reading frame of 1047 nucleotides with an initiation site at 130 and a TGA termination site at position 1177 (Fig. 14). The ATG codon at position 130 and another at position 145 fit the concensus 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 and a 49% homology with the CEF-10 cDNA. If a putative alternative splicing region is deleted, the nucleotide homology to the CEF-10 mRNA increases to 59%. The 3' region contains three copies of the pentanucleotide sequence ATTTA shown to be involved in mRNA destabilization and frequently found in growth factor and oncogene cDNA 3' regions (Shaw and Kamen,1986).

The CTGF open reading frame encodes a 349 amino acid peptide which contains 39 cysteine residues, indicating a protein of complex structure with multiple intramolecular disulfide bonds. This may explain the shift in mobility observed on polyacrylamide gels following reduction of the molecule with dithiothreitol. 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. The region of the protein between residue 32 (proline) and 208 (alanine) is the sequence of the CTGF peptide included in the fusion protein produced by clone DB60 in lambda gt11 and bound the anti-PDGF antibody in the library screening process. There is a 45% overall sequence homology between the CTFG 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. 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 discrepency could be due to differences incurred during evolution of chicken and human genes. Significantly,this region is bordered by areas of very high homology (85% identity) between the two molecules and 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. The PDGF A chain gene exibits alternative splicing with the transcript produced in glioma cell line containing a highly basic exon of 18 amino acids that is absent in the A chain

transcript produced by endothelial cells (Collins et al.,1987). The Vascular Endothelial Growth Factor (VEGF) is a 46 kd dimeric protein with mitogenic specificity for vascular endothelial cells and having a 20% amino acid homology to the PDGF A and B chain genes (Leung et al.,1989; Keck et al.,1989). Internal alternative splicing near the carboxy terminal adds 44 amino acids to the monomer (Tischer et al.,1989). The biological significance of both of these splicing events has not yet been determined.

Our data demonstrate that vascular endothelial cells grown in monolayer in vitro with 20% fetal calf serum present in the media constitutively secrete the CTGF peptides. The retinal pigment epithelial cells also constitutively secrete this molecule, but TGF-β stimulation increases production of the protein. Human dermal fibroblasts only secrete the CTGF peptide and express the mRNA after stimulation with TGF-β (10 ng/ml in serum-free media). There is no PDGF-related biological activity in the conditioned media of cells which have not been stimulated with TGF-β. The fibroblasts were also shown to express the PDGF A chain mRNA upon TGF-β incubation, but immunoblots of the affinity purified proteins from conditioned media using the PDGF A or B chain specific anti-sera indicated very little production of A chain peptide in relation to the CTGF molecules. The fact that the affinity purified proteins from the conditioned media from primary cultures of these three cell types have similar biological

activity, produce the same pattern of peptides on immunoblots with an anti-PDGF antibody and express a single 2.4 kb transcript on Northern blots with the clone DB60R32 as a probe, but only under conditions in which the proteins are secreted, argues strongly that the proteins are products of the CTGF gene.

By using subtractive and differential screening, Simmons et al. (1989) cloned the CEF-10 mRNA which was one of 12 identified cDNA sequences transcribed from mRNA's that were induced soon after the production of the src phosphoprotein $pp60^{v\text{-}src}$ in chicken embryo fibroblasts transfected with a temperature sensitive vector containing a src cDNA insert. The CEF-10 mRNA was induced in non-transfected CEF cells when the serum concentration in the culture media was increased from 0.5% to 10%. The src protein and serum induce the expression of the "immediate early genes" necessary for the G_0-G_1 transition in the cell cycle (Rollins and Stiles,1989 for review). One major group of these genes consist of intranuclear DNA-binding proteins including fos, myc and jun which are essential for cell cycle regulation. The other major group of genes induced by serum and src produce secretory proteins with cytokine characteristics. One of these is the JE 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 interluken-2 (Rollins et al.,1988). Another is the murine 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 src and serum inducible mRNA cloned from CEF cells (Bedard et al.,1987; Sugano et al.,1987). It is significant that other secreted proteins induced by src and serum in this system have cytokine and growth factor activities which is consistent with our findings for the CTGF protein.

The PDGF molecule is the predominant identified competence growth factor in serum. The data presented here indicate that the CTGF molecule has biological activity similar to PDGF and binds the PDGF cell surface receptor. CTGF may be another competence factor which 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. The in vitro conditions of endothelial cells growing in monolayer may resemble those during angiogenesis. Secretion of CTGF during angiogenesis could facilitate the growth of smooth muscle cells and fibroblasts, thereby forming a matrix and substrate for the continued growth of the endothelial cells and neovascularization. The secretion of CTGF by both endothelial cells and TGF-β stimulated fibroblasts could be important in wound healing and tissue repair. Immediately after formation

of a wound, platelets present at the site would secrete PDGF and TGF-β. The PDGF would be mitogenic for the fibroblasts and smooth muscle cells present and set up a chemotactic gradient to bring more of these cells to the wound site. PDGF is quickly cleared from wound fluid so the CTGF peptide secreted by endothelial cells and TGF-β stimulated fibroblasts would continue to serve as a competence mitogen for the proliferation of connective tissue cells. The CTGF protein could also play a role in atherosclerosis. PDGF mRNA has been detected in arterial tissues (Barrett and Benditt,1987) and the message appears to be elevated in atherosclerotic lesions (Barrett and Benditt,1988). The secretion of CTGF peptides by endothelial cells would stimulate the chemotaxis and growth of smooth muscle and fibroblasts at the plaque site, thereby aggrivating 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. Experiments to determine the transforming potential of the CTGF cDNA are underway.

Currently in the lab, work is underway to determine more information concerning the biochemistry and biological significance of CTGF. In order to examine genomic structure, fragments of the cDNA are being used as a probe to screen human genomic libraries. These genomic clones will enable us to examine the 5' end of the gene for transcription regulatory

sites and to examine the intron/exon structure of the gene. We are particularly interested in alternative splicing mechanisms which may be involved in the expression of this gene, the tissues and cell types which express alternate transcripts, and the differences in biological function of these proteins. The CTGF cDNA will also be used to probe RNA from various cell types and tissues to determine expression of the transcripts. Internal primers for PCR reactions will be made to examine and clone possible splicing alternatives from cDNA of the different cell types used. The cDNA for CTGF will also be used to probe wound chamber RNA for expression of this gene during various stages of wound healing. It will also be interesting to probe embryonic tissue and samples from pathological conditions such as atherosclerotic tissue and solid tumors for the expression of CTGF to determine the involvement of this mitogen in development and disease. The oncogenic potential of CTGF will be investigated by transformation of mesenchymal cells with the CTGF cDNA in expression vectors. Concurrently with these experiments with the cDNA and gene of CTGF, we will also be examining the expression and secretion of the CTGF protein with antibodies specific to synthetic peptides representing various segments of the molecule. Furthermore, in order to conduct biological studies with pure CTGF, we will produce recombinant CTGF by recombinant methods in a bacterial or eucaryotic expression system. The goal of these investigations is a thorough

understanding of the CTGF protein, the gene which encodes it and the biological functions of CTGF in normal and pathological tissue growth.

EXPERIMENTAL PROCEEDURES

CELLS

Human umbilical vein endothelial (HUVE) cells were obtained from human umbilical cords by a modification of Jaffe (1973). Cords were obtained from local hospital delivery rooms and placed into a sterile cup containing sterile Tris buffered saline (50 mM Tris;BioRad;100 mM sodium chloride,Fisher;pH 7.4) and could be stored for up to 12 hours after birth at 4 C before use. Only normal,healthy cords were obtained and only sections without clamp marks were used to collect cells to avoid smooth muscle cell contamination of cultures. Cords were removed from the container under a vertical flow hood and placed in a sterile tray with enough Modified Tyrodes Solution (MTS) (140 mM sodium chloride;3 mM potassium chloride,Fisher;0.3 mM sodium phosphate monobasic,Fisher;5 mM dextrose,Fisher) to wet cord and wash off coagulated blood. A piece of sterile Tygone tubing was placed into one end of the vein,clamped with a plastic cable tie and the cable tie secured with suture to the tubing. A 60 ml syringe was connected to the tubing by way of a valve and approximately 30 mls of MTS was forced through the vein to remove blood clots and cells. A short piece of Tygone tubing is then inserted into the other end of the vein and secured with cable tie and suture. Approximately 15 mls of a sterile 0.2% solution of Collagenase type IV (Worthington Biochemicals) in MTS was put into the vein by the syringe and

the other end of the vein closed off using a hemostat on the tubing. The cord was placed into a 37 C water bath for 20 minutes. After incubation in the water bath,the cord was removed and briefly massaged with fingers to loosen cells. Approximately 30 mls of MTS was then forced through the vein with the syringe and the cells collected in a sterile 50 ml conical tube. The cells are pelleted by centrifugation at 400 g for 10 minutes and resuspended in 5 mls of culture media (Media 199,Sigma;20% fetal calf serum,HyClone;50 ug/ml Endothelial Cell Growth Supplement,Sigma (a prep. of basic Fibroblast Growth Factor);90 ug/ml heparin,Sigma;10 ug/ml Gentamicin,Quality Biological). The cells were then placed in a 25 ml tissue culture flask (Costar) which was previously coated with sterile 2% gelatin solution (Sigma). The cells were grown at 37 C in an atmosphere of 95% air,5% CO_2. After reaching confluence,the cells were trypsinized (0.1% trypsin,Sigma;0.1% EDTA,Sigma in phosphate buffered saline (PBS) (120 mM sodium chloride,2.7 mM potassium chloride in 10 mM phosphate buffer,pH 7.4,premixed from Sigma) and passaged into a 75 ml culture flask. One or two more passages of the cells were made at a 1:3 split. Conditioned media was collected from the second and third passage cells. Media was changed every other day. Cells were identified as endothelial cells by their non-overlapping cobblestone morphology and by positive immunohistochemical staining for Factor-VIII related antigen (anti-Factor VIII antibody from Dako Corp.).

Normal Rat Kidney (NRK) fibroblasts were obtained from American Type Culture and NIH 3T3 cells were a gift from S.Aaronson (NCI,Bethesda,MD),and both cell lines were maintained in Dulbeco's Modified Eagles Medium (DMEM),(Sigma),10% fetal calf serum,20 ug/ml Gentamicin. Fetal bovine aortic smooth muscle cells were obtained from tissue explants (Grotendorst et al.,1981),maintained in DMEM,10% fetal calf serum,20 ug/ml Gentamicin and used in assays at second or third passage. Foreskin fibroblasts were grown from explants of newborn human foreskins and cultured in DMEM,10% fetal calf serum,10 ug/ml Gentamicin. Cells were used no later then the tenth 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). Porcine TGF-β was obtained from R and D Systems,Inc. Minn. MN. FGF was obtained from Sigma.

Purified PDGF or synthetic peptides 30 amino acids in length containing the amino and carboxy 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 seven days after the fourth rechallange (in Freunds incomplete adjuvant) and subsequent rechallanges. The anti-PDGF antibody did not show any cross-reactivity to TGF-β,EGF,or FGF in immunoblot analysis. The anti-peptide antibodies were sequence specific and did not cross-react with other synthetic peptide sequences or with recombinant PDGF peptides which did not contain the specific antigenic sequence. This was determined by immunoblot and dot blot analysis.

ANTIBODY AFFINITY COLUMN

Goat anti-human PDGF IgG (150 mg) was covalently bound to 25 mls of Affi-Gel 10 support (BioRad) according to the manufacturers instructions with a final concentration of 6 mg IgG/ml gel. The column was incubated with agitation at 4°C for 18 hours with 1 liter of HUVE cell media which had been conditioned for 48 hours. The gel was then poured into a column (5 x 1.5 cm), washed with four volumes of 0.1 N acetic acid made pH 7.5 with ammonium acetate, and the antibody-bound PDGF immunoreactive proteins eluted with 1 N acetic acid. Peak fractions were determined by biological assays and immuunoblotting and the fractions pooled.

CELL LABELING AND AUTORADIOGRAPHY

Confluent cultures of HUVE cells in 75 cm^2 flasks were labeled by incubating the cells for 22 hours in cysteine-free

DMEM containing 250 μCi/ml of ^{35}S-cysteine (Amersham), 20% FCS (previously dialyzed in cysteine-free DMEM), 40 μg/ml Endothelial Cell Growth Supplement,Sigma,90 μg/ml heparin and gentamicin. The ^{35}S-labeled PDGF-immunoreactive proteins were affinity purified from the media with the Affi-Gel 10/anti-PDGF column and aliquots of the proteins run on 12% polyacrylamide gels. As a control, an identical column was prepared with Affi-Gel 10 and non-immune goat IgG. The proteins were electroblotted to nitrocellulose and processed as for Western blotting using the anti-human PDGF IgG as a primary antibody. The nitrocellulose membranes were then autoradiographed on Kodak film, and the amount of 36 kD peptide determined using a Hoeffer densitometer and Gelscan software (Hoeffer Scientific).

BIOLOGICAL ASSAYS

Chemotactic activity was determined in the Boyden chamber chemotaxis assay with bovine aortic smooth muscle (BASM) cells or NIH 3T3 cells as previously described (Grotendorst et al.,1981,1987). Briefly,the chemotactic samples to be tested were made up in serum-free DMEM,0.2% bovine serum albumin (BSA). Anti-PDGF IgG,if added to neutralize activity,was added at 30 μg/ml and incubated at 4 C overnight. Approximately 250 μl of test sample was put into the bottom chamber of a Modified Boyden Chamber and a Nuclepore filter (8 μm pore size) which was previously coated with collagen

type I,was placed over the sample and the top chamber which holds the target cells was secured. The target cells were prepared by trypsinization for no more than two minutes to prevent PDGF receptor down regulation. The cells were suspended in DMEM,2 mg/ml BSA,pelleted by centrifugation,and resuspended in DMEM,0.2 mg/ml BSA at a concentration of 3 X 10^5 cells per milliliter. Human fibronectin was added at a concentration of 5 mg/ml. Aliquots of approximately 810 μl of cells was added to the top chamber and the chambers incubated for four hours in a humidified incubator at 37 C in an atmosphere of 90% air,10% CO_2. After incubation,the filters were removed and the cell nuclei stained with Diff-Quik (American Scientific Products) fixative and staining solutions. The top layer of cells which did not migrate into the pores of the filter was scraped off with a rubber policeman. The cells which migrated into the pores of the filter due to the action of the chemoattractant were either counted or quantitated by placing the filters into the wells of a 96 well plate,extracting the dye with dilute HCl,and reading the absorbance at 600 nanometers with an ELISA reader.

Mitogenic assays were performed using 96 well plates and normal rat kidney (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 days after confluence and 3T3 cells quiesed for 2 days in serum-free DMEM, 0.2 mg/ml BSA before use. Sample proteins and dilutions of known standards are

added to the wells and the plates incubated at 37°C in 10% CO_2, 90% air for 18 hours, after which ^{3}H-thymidine (Amersham) at a final concentration of 5 μCi/ml was added and incubated for an additional 2 hours. The media was removed and the cells washed with cold PBS. The cells were then washed five times with a cold solution of trichloroacetic acid to precipitate the macromolecules. The macromolecules were removed from the wells in a solution of 0.1 N NaOH,0.1% sodium dodecylsulfate (SDS) (BioRad) and DNA synthesis determined from the ^{3}H-thymidine incorporation into the newly formed DNA by scintillation counting.

RECEPTOR COMPETITION ASSAYS

Assays were performed using confluent cultures of NIH 3T3 cells in 24 well plates (Costar) grown in DMEM,10% fetal calf serum,10 μg/ml Gentamicin. The growth media was removed and the cells washed twice with serum-free DMEM,0.2 mg/ml BSA and the plates placed on ice for 30 minutes 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 ng/ml to 16 ng/ml. One milliliter aliquots of the samples were placed into wells of the 24 well plates and incubated on ice on a platform rocker for two hours. After the incubation period,the cells were washed three times for 10 minutes each

on ice with PBS. The proteins bound to the surface of the cells were eluted with 500 ul of 1 N acetic acid for 10 minutes. 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.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Electrophoresis was performed on 12% polyacrylamide (National Diagnostics,Manville,NJ) separating gels with 4% stacking gels containing SDS (Laemmli,1970) unless otherwise stated. The gels were run on mini-gel apparatus ("Mighty-Small" Hoeffer Scientific). Immunoblotting was performed by electroblotting the proteins to a nitrocellulose membrane (Schleicher and Schuell,45 um pore size). Electroblotting was done using the "Multiphor II" blotting apparatus (BRL) in a buffer of 0.025 M Tris,0.192 M glycine,20% methanol,pH 8.3. The membrane was incubated in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl (TBS) with 5% non-fat dry milk (Carnation) at 25°C for 1 hour to block non-specific antibody binding. The blocking solution was removed and the antibody (15 μg/ml) added in TBS containing 0.5 % non-fat dry milk and 1 μg/ml sodium azide and incubated overnight at 25°C. The membranes were then washed 5 times in TBS, 0.5% milk for 10 minutes each wash and then incubated with alkaline phosphatase conjugated affinity purified rabbit anti-goat IgG (Organon Teknika/Cappel,

Malvern,PA) at a 1:1000 dilution in TBS containing 0.5% milk at 25°C for 1 hour. The filters were then washed with TBS five times, 10 minutes each time, and the blot developed using an alkaline phosphatase substrate solution (0.1 M Tris-HCl, pH 9, 0.25 mg/ml nitro blue tetrazolium,Sigma; 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate,Sigma).

RNA ISOLATION AND NORTHERN BLOTTING

Total RNA was isolated from tissue culture cells by the method of Chomczynski and Sacchi (1987). Cells in monolayer were lysed in the culture flask in a solution of 4 M guanidinium thiocyanate,Boehringer Mannhiem;25 mM sodium citrate,pH 7.0,Sigma;0.5% sarcosyl,Sigma;0.1 M 2-mercaptoethanol,BioRad. To the lysed cells solution,1/10 vol. of 2 M sodium acetate,pH 4,equal vol. phenol,1/5 vol. of chloroform/isoamyl alcohol were added sequentially with mixing. The solution was centrifuged at 10,000 g for 20 minutes at 4 C and the RNA in the aqueous phase was precipitated with an equal volume of isopropanol at -20 C for 1 hour. The RNA was pelleted by centrifugation and the pellet resuspended in the above guanidinium thiocyanate solution and again precipitated with isopropanol.

Lyophilized RNA was resuspended in gel loading buffer of 50% formamide,1X MOPS (20 mM MOPS Boehringer Mannheim,5 mM sodium acetate,1 mM EDTA,pH 8.0),6.7% formaldehyde,6% glycerol,bromophenol blue and heated at 95 C for two minutes

before loading (20 μg per lane total RNA) onto 2.2 M formaldehyde,1% agarose gels and run at 50 volts. Integrity of RNA was determined by ethidium bromide staining and visualization of 18S and 28S rRNA bands. After the run,gel was soaked in 10 X SSC (1X,0.15 M NaCl,0.015 M sodium citrate) for 40 min. total with one solution change. RNA was transfered to nitrocellulose by blotting overnight with 10 X SSC. The nitrocellulose was air dried and baked at 80 C for 2 hours in a vacuum oven. The membrane was prehybridized for 30 min. at 46 C in a solution of 50% deionized formamide,0.9 M NaCl,50 mM sodium phosphate pH 7.0,5 mM EDTA,2X Denhardts solution,0.1% SDS,75 μg/ml tRNA,100 μg/ml depurinated salmon sperm DNA,5 μg/ml poly-A RNA (all Sigma). Hybridization is overnight at 46 C in the same solution with the addition of 5 X 10^5 CPM per ml of ^{32}P-labeled probe. Normally for Northern blots the entire plasmid was labeled and used as a probe. Labeling was done with a random prime labeling kit from Boheringer Mannheim according to instructions provided. After hybridization,membranes were washed twice in 2X SSC,0.1% SDS for 15 minutes each at room temperature,once for 15 minutes in 0.1X SSC,0.1% SDS,room temp. and a final 15 minute wash in 0.1X SSC,0.1% SDS at 46 C. Blots were autoradiographed at -70 C on Kodak X-omat film.

DNA CLONING,SEQUENCING AND LIBRARY SCREENING

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-gt11 HUVE cell cDNA library by induction of the fusion proteins and screening with anti-PDGF antibody. Approximately 2 x 10^5 phage were incubated with 600 μl of an overnight culture of Y1090 bacteria at 37 C for 20 minutes. The phage infected bacteria were plated in 0.6% top agarose (Boehringer Mannheim) onto 1.5% bottom agarose (LB medium) plates (150 mm diameter, Fisher) and placed in a 42 C incubator until plaques were visible (approx. 6 hrs). Nitrocellulose circles (45 μm pore size,Schleicher and Schuell) previously soaked in 10 mM IPTG (5'-3',Inc.) and dried were placed on top of the plaques on the agarose plates and the plates incubated at 37 C for 2 hours. This process induced production of the beta-galactosidase/human cDNA fusion protein by the phage. The nitrocellulose membranes were then removed from the plates,the plates placed at 4 C,and the membranes placed in a blocking solution of Tris-buffered saline (TBS) (50 mM Tris,100 mM NaCl,pH 7.4) and 5% nonfat dry milk and incubated with agitation at room temperature for 1 hour. The blocking solution was removed and anti-PDGF IgG (previously incubated overnight with a Y1090 cell lysate in TBS to prevent nonspecific antibody binding to bacterial proteins) at a concentration of 3 μg/ml in TBS with 0.5% nonfat dry milk was

added in sufficient volume to cover the membranes was added and the containers placed on a rocker at 4 C for at least 12 hours. The membranes were washed with TBS,0.5% milk five times for 15 min. each time. The secondary antibody,rabbit anti goat IgG,alkaline phosphatase conjugated at a concentration of 1 μg/ml in TBS,0.5% milk was added and incubated at 4 C for 1 hour with agitation. The membranes were again washed with TBS,0.5% milk five times for 15 min. each. Alkaline phosphatase substrate solution (0.1 M Tris,pH 9.0,0.25 mg/ml nitro blue tetrazoleum,0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) was added and incubated at room temp. The solution was changed several times and a fresh solution usually left on overnight. The membranes were dried and positive clones (as indicated by circles of precipitate from the alkaline phosphatase substrate solution) picked by matching the membranes with the plaques on the agarose plates. Plaques picked were rescreened and positive clones replated at low titer and isolated.

The EcoR I 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 (U. S. Biochemical) and ^{35}S-dATP. 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 manufacturors

instructions. Aliquots of the sequencing reactions were run on both 6% acrylamide (16 hours) and 8% acrylamide (6 hours) gels,vacuum dried and autoradiographed for at least 18 hours.

The cDNA fragment from clone DB60 was ^{32}P-CTP labeled and used to rescreen the HUVE cell cDNA lambda gt11 library. Approximately 100,000 recombinants were plated and rescreened by standard methods (Sambrook et al.,1989). The 2100 bp clone designated DB60R32 was subcloned into Bluescript phagemid. Subclones were made of Pst I,Kpn I,and Eco RI/Kpn I restriction fragments also in Bluescript. These subclones were sequenced by double stranded plasmid DNA sequencing techniques using Sequenase as described above. The 1458 bp Eco RI/Kpn I 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 ASSAYS

In vitro transcription reactions were done using the 2100 bp cDNA clone DB60R32 in the Bluescript KS vector. The plasmid was cut with Xho I 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 and

the manufacturors instructions were followed. The reactions were done in a final volume of 25 μl in a buffer of 40 mM Tris,pH 8.0.8 mM $MgCl_2$,2 mM spermidine and 50 mM NaCl. In addition,the reactions contained 500 uM rATP,rUTP,rCTP;5 μM rGTP;500 μM 7meGpppG cap;30 μM DTT;1 μg Proteinase K treated DB60R32,in Bluescript;1 U RNase inhibitor and 10 U of T7 polymerase. The reactions were incubated for 1 hour at 37 C. The reaction mix was treated with RNase-free DNase for 15 min. at 37 C, phenol:chloroform extracted and the RNA precipitated with ethanol.

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 and the manufacturors instructions were followed. The reactions were done in a final volume of 50 μl and included 35 μl of the supplied rabbit reticulocyte lysate,20 μM amino acid mix (minus cysteine),4 U RNase inhibitor,^{35}S-cysteine 1 mCi/ml(1200 Ci/mMole,DuPont),and serial dilutions of mRNA from the in vitro transcription reactions in concentrations ranging from 100 to 1000 nanograms per reaction tube. The mRNA samples were heat treated at 67 C for 10 minutes and cooled on ice immediately before use. The reactions were incubated at 30 C for 60 minutes. Aliquots of the reactions were run reduced on 12% polyacrylamide electrophoresis gels, dried and autoradiograhped.

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