Regulation of Connective Tissue Growth Factor Gene Expression in Human Skin Fibroblasts and During Wound Repair

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Connective tissue growth factor (CTGF) is a cysteine-rich peptide that exhibits platelet-derived growth factor (PDGF)-like biological and immunological activities. CTGF is a member of a family of peptides that include serum-induced immediate early gene products, a v-src-induced peptide, and a putative avian transforming gene, *nov*. In the present study, we demonstrate that human foreskin fibroblasts produce high levels of CTGF mRNA and protein after activation with transforming growth factor beta (TGF- β) but not other growth factors including PDGF, epidermal growth factor, and basic fibroblast growth factor. Because of the high level selective induction of CTGF by TGF- β , it appears that CTGF is a major autocrine growth factor produced by TGF- β -treated human skin fibroblasts. Cycloheximide did not block the large TGF- β stimulation of CTGF gene expression, indicating that it is directly regulated by TGF- β . Similar regulatory mechanisms appear to function in vivo during wound repair where there is a coordinate expression of TGF- β 1 before CTGF in regenerating tissue, suggesting a cascade process for control of tissue regeneration and repair.

INTRODUCTION

The regeneration of tissue after injury proceeds via an ordered sequence of invasions by specialized cell types that remove debris and bacteria, restore the vascular supply, and replace lost tissue. Transforming growth factor β (TGF- β) appears to be a primary regulatory agent during the regeneration and repair process. TGF- β is contained in high amounts in platelets (Assoian *et al.*, 1983) and is produced by both activated macrophages and neutrophils (Assoian *et al.*, 1987; Grotendorst *et al.*, 1989), all of which are present at the initiation of the repair response. TGF- β has been demonstrated to act as a growth stimulatory factor for mesenchymal cells (Childs *et al.*, 1982; Leof *et al.*, 1986; Yaar *et al.*, 1990) and as a growth inhibitor for endothelial or epithelial cells (Tucker *et al.*, 1984; Heimark *et al.*, 1986; Takehara *et al.*, 1987; Coffey *et al.*, 1988). In addition it can effect the rate of extracellular matrix

production by connective tissue cells (Ignatz and Massague, 1986; Roberts *et al.*, 1986). The growth stimulatory effect of TGF- β in monolayer cultures of mesenchymal cells appears to be mediated via an indirect mechanism of induced automitogens. Leof *et al.* (1986) reported that TGF- β elevated platelet-derived growth factor (PDGF) B chain mRNA levels and induced secretion of PDGF-related peptides in AKR-2B cells. Battegay *et al.* (1990) detected PDGF-AA synthesis induced by TGF- β in smooth muscle cells and found that the TGF- β induced DNA synthesis could be neutralized by antibodies to PDGF-AA. We have found that TGF- β induced delayed DNA synthesis in primary cultures of human foreskin fibroblasts via an autocrine production of PDGF-related peptides (Soma and Grotendorst, 1989). These peptides exhibited properties similar to a novel growth factor we have recently identified that is secreted by human vascular endothelial cells termed connective tissue growth factor (CTGF) (Bradham *et al.*, 1991). Although CTGF exhibits PDGF-like biological

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activities and appears to be antigenically related to PDGF, it has little peptide sequence homology to either the PDGF A or B chain peptides. The peptide sequence of CTGF reveals that it is a member of a family of peptides including the v-src-induced CEF-10 peptide identified in src-transformed chicken embryo fibroblasts (Simmons *et al.*, 1989), cyr61 cloned from mouse BALB/c 3T3 fibroblasts after serum induction (O'Brien *et al.*, 1990), and the serum-induced fisp12 (Ryseck *et al.*, 1991) or TGF- β -induced BIGM2 transcript identified in 3T3 cells (Brunner *et al.*, 1991). CTGF is also related to the newly described *nov* gene product that is overexpressed in nephroblastomas induced by myeloblastosis-associated virus (Joliot *et al.*, 1992).

We were interested in determining whether the PDGF-related peptides produced by the TGF- β -treated human skin fibroblasts were CTGF or CTGF related and if the expression of their transcripts was controlled by TGF- β . We also wanted to determine the kinetics of TGF- β and CTGF gene expression during wound repair in an in vivo model for connective tissue regeneration and repair. The results reported below demonstrate that TGF- β induces the expression of the CTGF transcript and protein in human skin fibroblasts. Other growth factors do not mimic this effect suggesting the presence of unique control elements specifically regulated by TGF- β . TGF- β and CTGF transcripts are coordinately expressed during normal wound repair indicating that similar regulatory mechanisms are functioning in vivo. These data support a cascade model for regeneration and repair where paracrine growth factors (initiators) activate an autocrine growth regulatory system in connective tissue cells that would sustain the replication phase of the repair process.

MATERIALS AND METHODS

Cell Culture

Human foreskin fibroblasts were grown from explants of newborn foreskin. NIH/3T3 cells were a gift from S. Aaronson (National Cancer Institute, Bethesda, MD). Both cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in an atmosphere of 10% CO₂ and 90% air. Human foreskin fibroblasts were used in these experiments before the sixth passage. Human umbilical vein endothelial cells were isolated from fresh human umbilical cords by collagenase perfusion (Jaffe, 1987) and maintained in medium 199 with 20% FCS, 0.68 mM L-glutamine, 20 μ g/ml Gentamicin (Quality Biological, Gaithersburg, MD), 90 μ g/ml porcine heparin (Sigma Chemical, St. Louis, MO), and 50 μ g/ml Endothelial Cell Growth Supplement (Sigma).

Growth Factors

TGF β 1 was a gift from Gideon Straussman (Otsuka Pharmaceuticals, Rockville, MD). Recombinant PDGF BB was obtained from Chiron (Emeryville, CA). Purified murine epidermal growth factor (EGF) was purchased from Biomedical Technologies (Stoughton, MA). Purified acidic fibroblast growth factor (FGF) was purchased from Sigma.

Western Blot Analysis

For detection of CTGF with anti-human PDGF IgG, cell lysates were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions on 12% acrylamide gels and transferred to nitrocellulose filters by electroblotting. For detection of PDGF A or PDGF B chain peptides, samples were run under reducing conditions. In all cases the blots were incubated for 1 h in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS) (100 mM NaCl, 50 mM Tris-HCl pH 7.4) with 2% nonfat powdered milk (TBS-milk), before overnight exposure to the specific antibody. For CTGF detection 15 μ g/ml antihuman PDGF IgG diluted in TBS-milk was used as the primary antibody. To detect PDGF A or B chains specific goat anti-PDGF A or B chain peptide antibodies raised to synthetic peptides that correspond to the first 28 amino acids at the amino terminal region of the mature PDGF A or B chain protein respectively were used at 20 μ g/ml in TBS-milk. Filters were washed five times in TBS-milk, 5 min each, and incubated with alkaline-phosphatase-conjugated affinity purified rabbit anti-goat IgG (1:1000 dilution) (Organon Teknika-Cappel, West Chester, PA) in TBS-milk for 90 min. The filters were washed three times with TBS-milk followed by two washes in TBS, and the antigens were detected using a commercial alkaline phosphatase substrate kit (Sigma).

Bacterial Expression of Immunoreactive CTGF Peptide

Clone DB60R32, a full length CTGF cDNA, was subcloned into the EcoRI site of the pET 5 expression vector (Studier *et al.*, 1990) in both sense and antisense orientations. Vectors were transformed into *Escherichia coli* BL21DE3, and cells were grown in M9TB media with ampicillin until an OD₆₀₀ reached 0.7 and Isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM. After a 2-h incubation, the cells were pelleted, lysed, and inclusion bodies were removed as previously described (Hoey, 1990). Cell extracts were used for neutralizing anti-PDGF IgG in western blot analysis.

Message Amplification Phenotyping (Mapping) Reaction and DNA Sequencing

First strand cDNA was synthesized from TGF- β -treated human foreskin fibroblasts total RNA using oligo-dT as a primer as described by Brenner *et al.* (1989). Polymerase chain reaction (PCR) was performed using a pair of primers that corresponded to portions of the CTGF mRNA open reading frame. These were designated HO1 (5'-CGGA-ATTCGACAGTGCCAACCATGACC-3'), GG2(5'-GACCTTCCTGAGAGCG-3'), GG1 (5'-AGCTGCAAGTACCACTGC-3'), and HO2 (5'-CCGAATTCTTAATGTCTCTCACTCTC-3'), respectively. These PCR products were digested with *Pst* I or *Sma* I and run a 2% agarose gel. The amplified PCR fragment from GG1-HO2 was digested with *Pst* I and *Eco*RI and cloned into the *Eco*RI-*Pst* I site of M13 phage vector and sequenced by the dideoxycytosine termination method (Sanger *et al.*, 1977) using the Sequenase kit (United States Biochemical, Cleveland, OH).

RNA Isolation and Northern Blotting

Total RNA was isolated from cultured cells by acid guanidinium thiocyanate-phenol-chloroform extraction as reported previously (Chomczynski and Sacchi, 1987). Total RNA was electrophoresed on an agarose/formaldehyde gel and transferred to nitrocellulose. Equivalent amounts of RNA were transferred as judged from staining of the ribosomal RNA present in each sample on the nitrocellulose filter. The CTGF probe was a 2.1-kilobase (kb) *Eco*RI insert of clone DB60R32 in the Bluescript Vector (Bradham *et al.*, 1991). The TGF- β 1 probe was a 1.0-kb *Nar*I fragment of the human TGF- β 1 cDNA kindly provided by Graeme Bell (Howard Hughes Institute, University of Chicago). The myc probe was a 1.8-kb *Eco*RI fragment of the third exon of human c-myc obtained from Oncor (Gaithersburg, MD). The

Figure 1. (A) Western blot analysis of PDGF-related proteins produced by human foreskin fibroblasts. Confluent cultures of human foreskin fibroblasts were incubated with DMEM containing 0.1 mg/ml BSA with 10 ng/ml of TGF β , PDGF-BB, EGF, or FGF for 24 h and cell extracts analyzed for CTGF production by Western blot using an anti-PDGF IgG. (B) Blocking of anti-PDGF immunoreactivity of the TGF- β induced peptides with recombinant CTGF protein. Bacterial extracts containing recombinant CTGF were obtained as described under MATERIALS AND METHODS. Antihuman PDGF IgG was incubated with bacteria protein extracts (250 μ g/ml) from control (antisense CTGF) or CTGF-producing cells for 2 h at 4°C and subsequently used in the Western blots. Cell extracts from control or TGF- β -treated human foreskin fibroblasts were obtained as described above.

fos probe was a 1.0-kb *Pst* I-*Pvu* II fragment of FBJ osteosarcoma virus v-fos obtained from Oncor. Hybridizations were performed using 1×10^6 cpm/ml of these probes labeled with [α - 32 P]dCTP by using a Random Primer DNA Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Autoradiography was performed at -70°C for 24–72 h by using X-ray films and intensifying screens.

Isolation of Wound Tissue and Wound RNA

Stainless steel mesh chambers were implanted in the flanks of 200-g Fisher 344 female rats that had been anesthetized by injection of pentobarbital (50 mg/kg) as described previously (Grotendorst *et al.*, 1985). Six chambers were implanted in each animal in a total of 30 animals. Three animals were killed on days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 after implantation of the chambers. The chambers were removed, and the tissue on the outside of the chambers was carefully and completely removed. The chambers were cut open, and the tissue contained within the chambers was collected. Total RNA was extracted from the tissue obtained from 6 to 18 chambers (pooled from 1–3 animals) by guanidine-isothiocyanate extraction and CsCl centrifugation (Chirgwin *et al.*, 1979). The amount of RNA recovered ranged from 100–300 μ g during the different days of collection. The presence of TGF- β 1 and CTGF transcripts was determined by Northern blot analysis using a cDNA probes as described above.

Nucleotide Sequence Accession Number

The GenBank accession number for the CTGF sequence is M36965.

RESULTS

The PDGF-related Protein Produced by Human Foreskin Fibroblasts Is Identical to CTGF

We recently described a novel PDGF-related polypeptide secreted by human umbilical vein endothelial cells

termed CTGF (Bradham *et al.*, 1991). Previously, several groups have shown that TGF- β stimulates the growth of fibroblastic cell types via an autocrine production of PDGF-related peptides (Leof *et al.*, 1986; Soma and Grotendorst, 1989; Battagay *et al.*, 1990). Our studies with human foreskin fibroblasts indicated that TGF- β stimulated the synthesis and secretion of peptides with molecular weights (38 kDa) and biological and immunological activities similar to CTGF. Also, the TGF- β -induced DNA synthesis could be specifically blocked by our anti-human PDGF IgG suggesting that it was acting as an autocrine growth factor. Therefore, we wanted to determine whether the PDGF-related peptides secreted by human foreskin fibroblasts were in fact related to CTGF. Confluent human foreskin fibroblasts were treated with TGF- β , PDGF, EGF, or FGF at 10 ng/ml, a concentration that induced a maximal mitogenic response, for 24 h. Cells were harvested, lysed, and analyzed by Western transfer blotting using anti-human PDGF IgG. As shown in Figure 1A, only TGF- β -treated cells expressed high levels of a 38-kDa PDGF-related protein, whereas PDGF, EGF, or FGF did not induce the production of this peptide. The antibody binding to this peptide was completely blocked when the antihuman PDGF IgG was incubated with recombinant CTGF polypeptide produced in the bacterial pET system (Figure 1B), demonstrating the immunological crossreactivity of the PDGF-related peptide produced by the human foreskin fibroblasts and CTGF. By using antibodies specific for the PDGF A or B chain peptides, we could not detect any PDGF A or B chain peptides produced by the cells in response to TGF- β in our Western blot assay (Figure 2). These results confirm our previous studies and indicate that CTGF is functioning as an autocrine mediator of DNA synthesis in TGF- β -treated human skin fibroblasts.

Figure 2. Western blot of control and TGF- β -treated fibroblast cell extracts and TGF- β activated cell conditioned media for PDGF A and B chain peptides. Cell extracts from control (Control) and TGF- β -treated (10 ng/ml for 24 h) fibroblasts cultures (TGF- β cell) were prepared as under Figure 1 and analyzed for PDGF A (A) or PDGF B (B) peptides using chain specific antisera. In addition the PDGF-related peptides were isolated by immunoabsorption with an antihuman PDGF IgG affinity matrix from media conditioned by the TGF- β -treated cells (TGF- β media). Recombinant PDGF A (5 ng) or PDGF B (5 ng) were run for reference standards. No detectable PDGF A or B chain peptide was present in either the cell layer or media from either the control or TGF- β treated cells.

Figure 3. Expression of CTGF transcripts in various cell types involved in wound repair. Total cellular RNA (10 μ g) was isolated, electrophoresed in a 1.5% agarose gel, transferred onto nitrocellulose filters, probed with 32 P-labeled DB60R32 fragment of the CTGF cDNA, and processed as described under MATERIALS AND METHODS. RNA samples were isolated from: HUVE, human umbilical vein endothelial cells; CON-FIB, human foreskin fibroblasts (control); TGF- β -FIB, human foreskin fibroblasts (treated with 10 ng/ml of TGF- β for 4 h); NEU, human peripheral neutrophil activated with F-met-leu-phe (100 ng/ml for 6 h); MAC, human peripheral blood monocytes activated with LPS (10 μ g/ml for 6 h).

We next wanted to examine the expression of the CTGF transcript in the cultured fibroblasts. Total RNA prepared from control and TGF- β -treated human fibroblasts and human umbilical vein endothelial cells as a positive control were analyzed for the presence of a CTGF-related transcript. As seen in Figure 3, we found a 2.4-kb CTGF-related transcript strongly induced by TGF- β in the fibroblasts that ran similarly to the authentic CTGF transcript in human umbilical vein endothelial cells. In contrast only very low levels of CTGF transcripts were detected in total RNA isolated from control human skin fibroblasts. Additionally, the cell types that produce large amounts of TGF- β during the wound repair process, such as activated human peripheral blood neutrophils or macrophages, did not contain

CTGF transcripts. These studies suggest that the CTGF gene is strongly induced by TGF- β and that its expression is restricted to endothelial cells and fibroblastic cells during wound repair.

We next wanted to analyze the sequence of the CTGF transcript induced by TGF- β treatment of human foreskin fibroblasts to determine if it was identical to the endothelial product or was the product of a closely related gene. Initially, we used several pairs of oligonucleotide primers to amplify regions of the putative CTGF mRNA after first strand cDNA synthesis by reverse transcriptase using oligo-dT as a primer (Figure 4A). For each pair of primers, the PCR products were the same size as predicted from the original CTGF cDNA sequence, and all amplified fragments exhibited the predicted patterns after digestion with several restriction endonucleases (Figure 4B). Also, all amplified inserts hybridized strongly with a CTGF cDNA probe indicating a specific amplification of the CTGF mRNA. One of the amplified fragments that represented the 3' region of the open reading frame (bases 484–1202) was digested with *Pst* I and *Eco*RI, cloned into M13, and sequenced. Comparison of sequence data between the CTGF cDNA and PCR product indicated it was identical to the CTGF mRNA. These data indicate that TGF- β -treated human foreskin fibroblasts express the same CTGF mRNA as that isolated from human umbilical vein endothelial cells demonstrating that the PDGF-related polypeptide secreted by the TGF- β human foreskin fibroblasts is identical to the CTGF peptide produced by human umbilical vein endothelial cells.

Regulation of CTGF Gene Expression in Human Skin Fibroblasts

The CTGF transcript is related to two immediate early genes, *cyr61* (O'Brien *et al.*, 1990) and *fisp12* (Ryseck *et al.*, 1991) (possibly the murine CTGF homologue), identified in 3T3 cells after serum stimulation. It appears that the *fisp12* is the murine homologue of CTGF. Ad-

Figure 4. (A) Map of CTGF cDNA indicating the locations of primers used for PCR amplification. Single-stranded cDNA made from TGF- β -treated human foreskin fibroblasts RNA was used as the template in the PCR mapping analysis. (B) Restriction endonuclease analysis of PCR products. The PCR products shown in (A) were run on 0.8% agarose gel. The primers, restriction endonuclease used for digestion, and predicted size of the fragments are as follows: lane 1, HO1-GG2, not digested, 364; lane 2, HO1-GG2, *Pst* I digested, 265, 99; lane 3, GG1-HO2, not digested, 719; lane 4, GG1-HO2, *Pst* I digested, 390, 329; lane 5, GG1-HO2, *Sma* I digested, 415, 187, 117.

Figure 5. Comparison of CTGF induction by TGF- β , PDGF, EGF, and FGF in human foreskin fibroblasts. The levels of CTGF, c-fos, and c-myc transcripts in human foreskin fibroblasts after activation by either TGF- β , PDGF-BB, EGF, or FGF was compared. Confluent human foreskin fibroblasts were incubated in serum free media with 10 ng/ml of TGF- β , PDGF-BB, EGF, or FGF. At the indicated times, total cellular RNA was isolated and nitrocellulose filters were prepared as described under MATERIALS AND METHODS. Each lane represents the level of transcript in 10 μ g of total RNA that was hybridized to a 32 P-labeled CTGF, c-fos, or c-myc cDNA probe.

ditionally, it has been shown that the *cyr61* transcript is induced by a variety of growth factors including PDGF and FGF as well as phorbol esters. We wanted to compare the regulation of the CTGF gene with other immediate early genes such as c-fos and c-myc in response to growth factor stimulation of the fibroblasts. Confluent cultures of human foreskin fibroblasts were treated with 10 ng/ml of TGF- β , PDGF, EGF, or FGF. At the indicated times after growth factor addition, total cellular RNA was extracted and analyzed by Northern blot (Figure 5).

The growth factor-induced expression of the CTGF gene was markedly different from that of c-fos or c-myc. TGF- β stimulated a detectable increase in the CTGF gene transcript within 30 min after addition that increased to a very high level within 4 h and remained at high levels for 24 h after the addition of growth factor. In contrast, PDGF, EGF, and FGF stimulated a relatively small and transient increase in CTGF transcript that peaked 1–2 h after addition of growth factor and returned to basal levels by 4–6 h. Fibroblasts treated with PDGF, EGF, and FGF showed a rapid and transient induction of the 2.2-kb c-fos transcript with the maximal level 60 min after the addition of growth factor. PDGF, EGF, and FGF also induced high levels of c-myc gene expression with EGF stimulating the highest and most prolonged elevation of c-myc gene expression. The c-myc transcripts were observed 30 min after stimulation and increased to maximal levels at 4–6 h and then decreased to basal levels by 24 h after stimulation. These patterns of expression are consistent with previous reports (Paulsson *et al.*, 1987a; Yaar *et al.*, 1990). In con-

trast, we could not detect a TGF- β stimulation of c-fos transcripts at any of the time points examined. Furthermore, TGF- β was the least effective inducer of c-myc transcripts. Studies on the human *cyr 61*-related transcript revealed a pattern similar to that of c-myc, rather than CTGF, because it exhibited a transient increase after all growth factors with TGF- β being the least effective. Similar data were obtained when we used NIH/3T3 cells to examine the growth factor inducibility of the CTGF (*fisp12*) mRNA, indicating that this pattern of inducibility is not unique to human skin fibroblasts (Figure 6). These results suggest the presence of specific TGF- β control elements regulating CTGF gene expression.

To determine whether the CTGF gene was directly controlled by TGF- β or was secondarily induced by another TGF- β -regulated gene product, we examined the effect of cycloheximide on the TGF- β induction of CTGF gene expression in human foreskin fibroblasts. Cells were treated with 20 μ g/ml of cycloheximide for 2 h before the addition of 10 ng/ml of TGF- β for 4 h. For comparative purposes c-myc induction was examined in parallel cultures of fibroblasts treated with PDGF-BB after cycloheximide treatment. A superinduction of the CTGF mRNA as well as c-myc mRNA was observed in the presence of cycloheximide (Figure 7). These data demonstrate that CTGF mRNA induction by TGF- β does not require de novo protein synthesis, indicating that the increase in CTGF mRNA is directly regulated by TGF- β .

Expression of TGF- β 1 and CTGF During Wound Repair

Because the CTGF protein appears to function as an autocrine growth stimulator for connective tissue cells and its production is regulated by TGF- β , we wanted to examine the expression of both TGF- β and CTGF during wound repair. We investigated the levels of TGF- β and CTGF transcripts in a wound repair model developed by Schilling *et al.* (1959) that consists of a stainless steel mesh chamber implanted subcutaneously in rats. The chamber induces a wound repair response that exhibits the classic phases of inflammation, connective

Figure 6. CTGF mRNA induction in mouse NIH/3T3 cells. Confluent NIH/3T3 cells were incubated in serum free media with 10 ng/ml of TGF- β , PDGF-BB, EGF, or FGF for 4 h, and Northern blot analysis was performed as described under MATERIALS AND METHODS. Each lane contained 20 μ g of total RNA.

Figure 7. Effect of cycloheximide on the regulation of CTGF and c-myc mRNA in human foreskin fibroblasts. Confluent human foreskin fibroblasts were treated with 20 μ g/ml of cycloheximide (CHX) for 2 h, then 10 ng/ml of PDGF-BB or TGF- β was added and incubated an additional 4 h. Northern blot analysis was performed as described under MATERIALS AND METHODS.

tissue infiltration, neovascularization, and matrix deposition (Schilling *et al.*, 1959; Hunt *et al.*, 1967; and Grotendorst *et al.*, 1985). Chambers were implanted in the dorsal flanks of 200 g female Fisher 344 rats, and RNA was isolated at various time points from the tissue that formed within the wound chambers. Total RNA (20 μ g) from wound tissue, normal rat skin, rat peritoneal macrophages (TGF- β 1 positive), and TGF- β -treated human skin fibroblasts (CTGF positive) were analyzed by Northern blot hybridization with either a TGF- β 1 or CTGF cDNA probe. These experiments demonstrated the presence of TGF- β 1 and CTGF transcripts in the wound tissue but not in uninjured skin (Figure 8). The TGF- β 1 transcript was present at the earliest times assayed with it highest level on day 3 followed by a rapid decrease to very low levels by day 9. Histological analysis of day 3 tissue reveals large numbers of leukocytes (Grotendorst *et al.*, 1985) that produce TGF- β 1 (Assoian *et al.*, 1987; Grotendorst *et al.*, 1989). Interestingly, the CTGF transcript exhibits a very different pattern of expression with a peak on day 9 that coincides with the initial ingrowth of granulation tissue composed of connective tissue cells and vascular endothelial cells both of which express the CTGF mRNA in culture (Bradham *et al.*, 1981). Both of these transcripts return to basal levels by the termination of the repair process. These data indicate that the expression of CTGF in wound tissue is preceded by TGF- β 1 as predicted by the in vitro cell culture experiments.

DISCUSSION

In this report, we demonstrate that the major PDGF-related peptide induced by TGF- β in primary cultures of human skin fibroblasts is CTGF. The CTGF cDNA was originally cloned by screening λ gt11 cDNA library prepared from RNA isolated from human umbilical vein endothelial cells with an antihuman PDGF polyclonal

IgG (Bradham *et al.*, 1991). This protein exhibits biological activities similar to that of PDGF because it is both chemotactic and mitogenic for connective tissue cell types such as fibroblasts and smooth muscle cells. CTGF protein production and secretion is selectively induced by TGF- β , and other growth factors such as PDGF, EGF, and FGF did not induce detectable levels of the peptide in either the cell layer or in the media (Soma and Grotendorst, 1989). TGF- β regulates the production of CTGF at the level of the CTGF mRNA and induces a prolonged high level expression of CTGF transcripts in agreement with our previous studies demonstrating that the CTGF peptide continues to accumulate in the media during the first 24 h after TGF- β exposure. TGF- β -induced DNA synthesis in the human skin fibroblasts with kinetics of S-phase entry that were delayed compared with those of other mitogens, suggesting the involvement of an autocrine growth factor (Soma and Grotendorst, 1989). Because this activity could be blocked by our anti-PDGF IgG it appears that CTGF is inducing the mitogenic response. We could not detect any PDGF A or B chain peptides in this same media using an immunological assay that could detect as little as 0.5 ng/ml PDGF, indicating that CTGF is the principal autocrine growth stimulator in the TGF- β treated fibroblasts.

Studies in several laboratories have indicated that the mitogenic action of TGF- β appears to be dependent on the autocrine production of PDGF-related peptides. In AKR-2B cells TGF- β induces the expression of the PDGF B chain gene (Leof *et al.*, 1986), whereas in fibroblasts the PDGF A chain gene is induced (Paulsson *et al.*, 1987b; Soma and Grotendorst, 1989). The PDGF A chain gene is also induced in vascular smooth muscle cells treated with TGF- β (Battagay *et al.*, 1990; Majack *et al.*, 1990). However, the level of PDGF A chain transcript present in our cells is much lower than the level of CTGF mRNA, and we have not been able to detect

Figure 8. Expression of TGF- β and CTGF transcripts during connective tissue regeneration and repair in vivo. Total RNA was prepared from the wound tissue isolated from the Schilling chambers and analyzed by Northern blots for TGF- β 1 and CTGF transcripts as described under MATERIALS AND METHODS. TGF- β positive control RNA was isolated from rat peritoneal macrophages (MAC). CTGF positive control RNA was isolated from TGF- β -treated fibroblasts. RNA from uninjured skin was isolated from normal rats (SKIN). Chamber RNA was isolated at the indicated day after implantation of the Schilling chamber.

PDGF A chain peptides in our TGF- β -treated fibroblasts. Thus, it is possible that different types of fibroblasts may use different growth factors as autocrine stimulators in response to TGF- β .

CTGF mRNA expression is superinduced by TGF- β in the presence of cycloheximide, indicating that de novo protein synthesis is not required for CTGF gene induction and that the CTGF gene is an immediate early gene. Immediate early genes are induced by serum or growth factors and are believed to play important roles in the control of cell proliferation (Cochran *et al.*, 1983; Lau and Nathans, 1987). One category of these genes includes c-fos and c-myc, whose products are nuclear proteins binding to DNA and regulating the transcription of other genes controlling for cell growth and replication (Kelley *et al.*, 1983; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984). The other group encodes secreted peptides with cytokine characteristics such as the KC/gro or JE genes (Cochran *et al.*, 1983; Rollins *et al.*, 1988; Oquendo *et al.*, 1989). The CTGF gene belongs to the latter category because its protein product is secreted and possesses chemotactic and mitogenic activities for connective tissue cells. These gene products may function as paracrine or autocrine cytokines to synchronize cell behavior during the response to injury.

All of the currently identified growth factor-regulated immediate early mRNAs exhibit a relatively short term induction period (minutes to 2–6 h), whereas CTGF mRNA remains at high levels 24–48 h after induction by TGF- β . Furthermore, only TGF- β stimulated a high and prolonged induction of the CTGF gene in the human fibroblasts. To our knowledge this is the first report of an immediate early gene with protracted kinetics of expression and preferential induction by a single type of growth factor. In this regard CTGF is unique even when compared with other members of the same gene family. The *cyr61* transcript is induced by serum, phorbol esters, PDGF, and FGF in mouse BALB/c 3T3 fibroblasts (O'Brien *et al.*, 1990), and the related *v-src*-induced CEF-10 mRNA identified in chicken embryo fibroblasts is also induced by serum and phorbol esters (Simmons *et al.*, 1989). We find that the human homologue of *cyr61* is induced by PDGF, EGF, and FGF but not by TGF- β (Okochi and Grotendorst, unpublished data), whereas the mouse homologue of CTGF (*fisp-12*/BIGM2) (Brunner *et al.*, 1991; Ryseck *et al.*, 1991) is strongly induced by TGF- β but not PDGF, EGF, and FGF (Figure 5). These findings indicate that TGF- β induces a very different pattern of immediate early gene expression than PDGF, EGF, or FGF.

Studies on the promoter elements of the *fisp-12* gene indicated a lack of any consensus serum response element (SRE) sequences in a region 800 bp 5' upstream of the *fisp-12* gene transcription start site (Ryseck *et al.*, 1991). This does not rule out the possibility that the SREs are located further upstream as in the *cyr61* gene

where the SRE is 2 kb upstream from the transcription start site (Latinkic *et al.*, 1991). However, the lack of such an element is consistent with our data and may explain the relatively low level of inducibility by PDGF, EGF, and FGF. These data also suggest that there are unique control elements regulated by TGF- β that determine CTGF gene expression. Because the TGF- β receptor appears to be a serine/threonine kinase (Mathews and Vale, 1991) rather than a tyrosine kinase like the EGF, FGF, and PDGF receptors (Williams, 1989; Ullrich and Schlessinger, 1990), it is likely that a very different set of secondary signals are produced in TGF- β -activated cells than those activated by a growth factor whose receptor has a tyrosine kinase cytoplasmic domain. The differential induction of the immediate early genes examined in this report is most likely a reflection of these differences in second messages and may be the basis for some of the unique biological actions of TGF- β in growth regulation.

TGF- β appears to function as a primary regulatory agent during wound repair because it is released by platelets during the initial blood clotting reaction and can be produced by both neutrophils and macrophages that are major cellular constituents of the early regenerating wound tissue. It is well recognized that addition of TGF- β to wound sites can accelerate the formation of new connective tissue (Sporn *et al.*, 1983; Roberts *et al.*, 1986; Mustoe *et al.*, 1987). Furthermore, in a growth factor deficient healing-impaired animal model we developed based on adriamycin treatment, which lowers the circulating levels of platelet and leukocytes (Lawrence *et al.*, 1986a), a single addition of TGF- β with PDGF and EGF on the day of surgery was sufficient to restore the repair process (Lawrence *et al.*, 1986b). Addition of PDGF, TGF- β , or EGF alone did not stimulate a large increase in the amount of new connective tissue over the control nontreated wounds indicating that these factors functioned synergistically with each other. This finding is consistent with the fact that all three of these types of growth factors are released as a set by platelets and macrophages during the acute phase of the repair response. Because a single treatment was all that was necessary to reverse the healing deficit and the retention time of the peptides at the wound site is brief (1–2 d), it appears that these agents function as initiators of the repair process.

In our wound chamber model, which is the same as that used in the above described studies, we find that the rat homologue of the TGF- β 1 message is present in abundant levels during the initial days of the repair process, corresponding to the presence of leukocytes at the wound site as expected. In contrast the CTGF transcript does not appear until later days when granulation tissue composed of connective tissue cells and new blood vessels begins to form within the chambers. These studies suggest a coordinate regulation of TGF- β and CTGF in wound tissue and support a cascade model

where initiators of the process stimulate the production of other factors that sustain the regeneration of the lost tissue. Future studies directed at the regulation of expression of the CTGF gene family and the structure and function of their respective products will help us develop a clearer understanding of the role of these peptides during tissue regeneration and repair.

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