J. Biochem. 124, 130-140 (1998)

Inhibition of Endogenous Expression of Connective Tissue Growth Factor by Its Antisense Oligonucleotide and Antisense RNA Suppresses Proliferation and Migration of Vascular Endothelial Cells[1]

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Received for publication, February 9, 1998

Previously, we cloned an mRNA predominantly expressed in hypertrophic chondrocytes by differential display-PCR from a human chondrosarcoma-derived chondrocytic cell line (HCS-2/8) that is identical to that of connective tissue growth factor (CTGF). In the present study, we investigated the roles of CTGF in the proliferation and migration of vascular endothelial cells using its antisense oligonucleotide and antisense RNA, because angiogenesis into the hypertrophic zone of cartilage occurs at the final step of endochondral ossification. Immunohistochemical and immunofluorescence techniques revealed that not only hypertrophic chondrocytes but also endothelial cells in the cost-chondral junctions of mouse ribs were stained with an anti-CTGF antibody *in vivo*. Northern blot analysis revealed that CTGF was strongly expressed in chondrocytic cells as well as bovine aorta endothelial (BAE) cells in culture, but not in other types of cells such as osteoblastic cells. Its expression in BAE cells was greater in the growing phase than in the confluent phase. When one-half of a monolayer of a confluent culture of BAE cells had been peeled off, only the cells proliferating and extending into the vacant area were stained with the anti-CTGF antibody. The addition of an antisense oligonucleotide inhibited the proliferation and extension of the BAE cells into the vacant area. The antisense oligonucleotide also inhibited the proliferation of BAE cells in the rapidly proliferating phase. In a Boyden chamber assay, pretreatment with the antisense oligonucleotide markedly inhibited the migration of BAE cells. Furthermore, the abilities to proliferate and migrate of BAE cells, which were stably transfected with expression vectors that generate the antisense RNA of CTGF cDNA, were markedly lower than those of the control. These findings suggest that endogenous CTGF expression is involved in the proliferation and migration of BAE cells.

Key words: angiogenesis, antisense oligonucleotide, antisense RNA, bovine aorta endothelial (BAE) cells, connective tissue growth factor (CTGF).

CTGF is a cysteine-rich polypeptide isolated from angioendothelial cells as a growth factor structurally and functionally related to platelet-derived growth factor (PDGF) (*1*). It exhibits PDGF-like chemotactic and mitogenic activities toward mesenchymal cells and appears to be antigenically related to PDGF, although it exhibits little amino acid sequence homology with either the PDGF A chain or B chain (*1*). It was also found in skin fibroblasts as a repairing growth factor (*2*) and was induced by transforming growth factor-β (TGF-β) (*2, 3*). Its gene belongs to the immediate early gene family (CCN family) which comprises three distinct members (*fisp12*/CTGF, *cyr61*/CEF-10, and Nov), whose encoded proteins are characterized by amino-acid sequence homology (about 60%), the presence of a secretory signal, and complete conservation of 38 cysteine residues (*4, 5*).

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (MT), and grants from the Sanyo Hohso Foundation (MT), the Sagawa Cancer Research Foundation (MT), and the Naito Foundation (MT).

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Abbreviations: RT-PCR, reverse transcriptase-PCR; HCS, human chondrosarcoma-derived chondrocytic cell line; BAE, bovine aorta endothelial; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic factor; αMEM, alpha modification of Eagle's Medium; DMEM, Dulbecco's modified Eagle's Medium; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; TBS, Tris-buffered saline; TBST, TBS-Tween 20; IPAP, Image Processor for Analytical Pathology.

Recently, we cloned an mRNA preferentially expressed in chondrocytes (*6*) from a human chondrosarcoma-derived chondrocyte cell line, HCS-2/8 (*7-12*), by differential display-PCR. Its gene was identical with that of connective tissue growth factor (CTGF) (*1, 2, 4*). We found that CTGF was highly expressed in HCS-2/8 cells and growth cartilage cells, but not expressed in osteoblasts (*6*). The expression was induced by the addition of TGF-β or bone morphogenetic factor-2 (BMP-2) (*6*). *In situ* hybridization revealed that CTGF was selectively expressed in hypertrophic chondrocytes. These findings suggest an important function of CTGF in endochondral ossification (*6*).

In the final step of endochondral ossification, growth cartilage cells actively proliferate, mature, and produce much extracellular matrix. Next, the cells become hypertrophic and the matrix is mineralized. Then, blood vessels invade the hypertrophic zone of cartilage, and finally the cartilage is replaced by bone (*13-15*). Therefore, angiogenesis is a pivotal event in endochondral ossification (*13, 14, 16, 17*).

Angiogenesis is known to be regulated by a balance between angiogenic and anti-angiogenic factors (*18*). We previously reported that cartilage contains anti-angiogenic factors such as CHIAMP (*12, 14, 19-21*), and angiogenic factors such as basic fibroblast growth factor (bFGF) (*22, 23*). These findings led us speculate that CTGF, which is strongly expressed in hypertrophic chondrocytes, is an angiogenic factor. In the present study, we tested this hypothesis and found that CTGF is an autocrine growth factor for endothelial cells *in vitro*. The physiological significance of CTGF in endochondral ossification is also discussed.

MATERIALS AND METHODS

Materials—The alpha modification of Eagle's Medium (αMEM) and Dulbecco's modified Eagle's Medium (DMEM) were purchased from Nissui Pharmaceutical (Tokyo). FBS was purchased from Cancera International (Rexclale, Ont., Canada). Plastic dishes and plates were purchased from Sumitomo Bakelite (Tokyo). Chemotaxicells with polyvinylpyrrolidone filters (pore size, 8-mm-diameter) at the bottom were purchased from Kurabo (Osaka). A Random Primer DNA Labeling Kit, Taq polymerase, and AMV-derived reverse transcriptase were purchased from Takara Shuzo (Tokyo). Restriction enzymes were from New England Biolabs (Beverly, MA). [α-^{32}P]dCTP and [^{3}H]thymidine (925 GBq/mmol) were purchased from Amersham International plc (Aylesbury, UK). Proteinase K, DNase I, Geneticin (G418), and acid guanidinium thiocyanate-phenol reagent were from Gibco BRL (Gaithersburg, MD), Promega (Madison, WI), Sigma (St. Louis, MO), and Nippon Gene (Tokyo), respectively. Polyvinylidene difluoride (PVDF) membranes and goat anti-rabbit IgG antibodies were from Bio-Rad Laboratories (Hercules, CA). Human recombinant basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were purchased from Organon Teknika (West Chester, PA).

Preparation of an Anti-CTGF Antibody—An anti-CTGF antibody was raised in rabbits by immunization with a synthetic peptide of CTGF composed of 20 amino acids, the sequence of which was the same as that of Fisp12 (the mouse homolog of CTGF), but not that of Cyr61. The IgG fraction purified with Mab Trap G II (Pharmacia, Uppsala, Sweden) was used for histological studies and Western blotting. Using the Western blotting technique, we confirmed that the synthetic peptide of CTGF, but not that of the corresponding region of Cyr61, inhibited the binding of the anti-CTGF antibody to the 38 kDa CTGF protein.

Cell Culture—Bovine aorta endothelial cells (BAE) (kindly supplied by Dr. Katsuhiko Fujii, Teijin Institute for Bio-Medical Research, Teijin Limited, Tokyo), a human chondrosarcoma cell line (HCS-2/8) (*7-12*), a human osteosarcoma cell line (Saos-2) (*24*), a human squamous cell carcinoma cell line (A431) (*25*), and a human glioblastoma cell line (A7) (*26*) were inoculated at a density of 2-7×10^4 cells/cm^2 into 10-cm-diameter dishes, 6-well plates, or 96-well microwell plates, and then cultured in DMEM supplemented with L-glutamine (2 mM) and 10% heat-inactivated FBS at 37°C under 5% CO_2 in air. Mouse osteoblastic cells (MC3T3-E1) derived from newborn C57BL/6 mouse calvaria (*27, 28*) were inoculated at a density of 1×10^4 cells/cm^2 and cultured in αMEM containing 10% FBS.

Immunoperoxidase Staining and Immunofluorescence Staining of CTGF—Cost-chondral junctions of mouse ribs were fixed with ethanol and acetic acid (99 : 1, v/v) for 2 h at room temperature. Frozen sections (4 μm thick) were prepared and endogenous peroxidase activity was eliminated by incubation with methanol containing 0.3% hydrogen peroxide for 30 min. Then, the sections were reacted with the IgG fraction of the anti-CTGF antibody diluted 1:100 with phosphate-buffered saline (PBS) for 1 h at 37°C. Secondary immunoreactions were performed for 1 h at room temperature with HRP-conjugated anti-rabbit IgG diluted 1:100 with PBS. Immunostaining was visualized by treatment with 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB) containing hydrogen peroxidase for 10 min. Finally, the sections were stained with hematoxylin and then mounted in glycerol:PBS. For indirect immunofluorescence staining, frozen sections were incubated for 45 min at 37°C, first with the anti-CTGF antibody diluted 1:200-500 with PBS, and then with FITC-conjugated anti-rabbit IgG diluted 1:100 with PBS. After washing with PBS, the specimens were mounted and examined by immunofluorescence confocal laser scanning microscopy (LSM-GB 200; Olympus, Tokyo). Control specimens were incubated with diluted normal serum or with secondary antibodies alone, and no positive reactions were observed.

For the BAE culture, the cells were fixed in 10% formalin in PBS for 15 min at 4°C, and then washed with distilled water four times and with PBS three times. The samples were next immersed in methanol containing 0.3% hydrogen peroxide to inactivate endogenous peroxidases. After washing in PBS, they were incubated in PBS containing 10% normal goat serum to block nonspecific protein binding and then reacted with the anti-CTGF antibody diluted 1:100 with PBS for 16 h at 4°C. Next, they were reacted with a secondary antibody and stained with a Vectastain Elite (peroxidase) kit (Vector Laboratories) according to the instruction manual. The color reaction was performed with a freshly prepared 0.1% DAB solution. Tissue sections were counterstained with methylgreen and then mounted with

glass coverslips. Finally, photomicrographs of the BAE cultures were taken directly.

Western Blotting—Cells were dissolved in 1% SDS and then electrophoresed on 12% SDS-polyacrylamide gels. The separated proteins were transferred to a PVDF membrane using a semi-dry blotting apparatus (Multiphor II, Pharmacia Biotech). The membrane was soaked in a Tris-buffered saline (TBS) solution [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl] containing 2% skim milk for 30 min at 37°C, and then incubated in a TBS-Tween 20 (TBST) solution [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.15% Tween 20] containing 2% skim milk and 500-fold diluted anti-rabbit CTGF IgG for 8 h at 25°C. The membrane was then washed with a TBST solution containing 2% skim milk and 2,000-fold diluted alkaline phosphatase conjugated goat anti-rabbit IgG for 90 min at 37°C. Then the membrane was washed with the TBST solution, incubated in an APB solution [0.1 M Tris-HCl (pH 9.5) containing 0.1 M NaCl and 5 mM $MgCl_2$] at 37°C for 5 min, and then stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Determination of the Growth Rate—For determination of cell proliferation, cell layers were washed three times with Ca^{2+}- and Mg^{2+}-free PBS, and then the cells were dispersed with 0.25% trypsin. Then the number of viable cells, as judged on Trypan blue exclusion, was determined with a hemocytometer. For determination of the rate of DNA synthesis, cultures were labeled with [^{3}H]thymidine (4.8 MBq/ml) for 4 h. The radioactivity incorporated into acid-precipitable material was determined as described (*29, 30*).

Northern Blot Analysis—All cells were harvested when they became confluent, and total RNA was isolated from cultured cells with acid guanidinium thiocyanate-phenol reagent according to the method of Chomczynski and Sacchi (*31*). Total RNA was treated with 200 μg/ml of proteinase K in HB buffer [50 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 5 mM EDTA and 0.5% SDS] for 1 h at 37°C at least three times, and then the RNA was treated with DNase I. About 15 μg of total RNA was treated with 2 ml of a gel buffer [0.2 M MOPS, 50 mM sodium acetate, and 5 mM EDTA (pH 7.0)], 3.5 ml of formaldehyde, and 10 ml of formamide for 15 min at 55°C, and then chilled on ice. These samples were applied to a 1% denatured agarose gel and then electrophoresed for about 2 h at 80 V. Then the gel was soaked in 50 mM NaOH, 0.1 M Tris-HCl (pH 7.5), and 20×SSC sequentially, and the processed total RNA in the gel was transferred to a nitrocellulose filter which had been pre-soaked in 2×SSC for about 15 h under osmotic pressure. After the transfer, the nitrocellulose filter was baked for 2 h at 80°C and then soaked in a pre-hybridization solution (5×SSC, 1% SDS, and 1×Denhardt solution) for 2 h at 65°C. Then the filter was soaked in a hybridization solution [0.02 M Tris-HCl (pH 8.0) containing 0.75 M NaCl, 2.5 mM EDTA, 1×Denhardt solution, 1% SDS, 0.5 mg/ml salmon sperm DNA, and 5 μl of radiolabeled probe [a 120 bp fragment of CTGF cDNA described elsewhere (*6*)], labeled with [α-^{32}P]dCTP, using the Random Primer DNA Labeling Kit, for 15-20 h at 65°C, and then washed with 2×SSC for 5 min and 0.2×SSC containing 0.1% SDS for 30 min at 65°C (4 times). The dried filter was exposed to an imaging plate and then analyzed with a Bioimaging Analyzer BAS 2000 (Fuji Film, Tokyo).

Preparation of Sense and Antisense Oligonucleotides of CTGF—Antisense oligonucleotides were constructed with a 16 mer derived from the starting translation domain, which contained the initial ATG of the 5′ (sense) or 3′ (antisense) end. Their sequences were as follows: antisense oligonucleotide, 5′-TACTGGCGGCGGTCAT-3′; sense oligonucleotide, 5′-ATGACCGCCGCCAGTA-3′.

Reverse Transcriptase-PCR (RT-PCR)—About 20 μg of isolated total RNA was treated with 0.8 U of DNase I at 37°C for 30 min. The total RNA was reverse transcribed to cDNA using an oligo dT 16 primer with AMV-derived reverse transcriptase for 30 min at 42°C. Then the cDNA was amplified with two primers, named DD24 and DD24R2 (*6*), with Taq polymerase in the presence of [α-^{32}P]dCTP. The amplification conditions were as follows: 95°C (1 min)-57°C (1 min)-72°C (2 min) for 30 cycles. The PCR products were applied on acrylamide gels (6%) and then electrophoresed. Then the gels were exposed to imaging plates and then analyzed with a Bioimaging Analyzer BAS2000.

Plasmid Construction—The eukaryotic expression vector (pRc/CMV) was constructed as follows: a CTGF cDNA fragment containing the entire coding region was placed downstream of the CMV promoter element in the antisense orientation. The plasmid was constructed as follows: CTGF cDNA fragments corresponding to the 1,050 bp covering from the AUG start codon to the UGA stop codon were subcloned into the pRc/CMV expression vector from the pUC18 vector at the *Xba*I-*Hin*dIII site.

Stable Transformation—BAE cells (5×10^6) were transfected with 10 μg recombinant plasmid by electroporation using a Gene Pulser (0.3 kV, 960 μF). Two days later, the cells were diluted tenfold and cultured thereafter in DMEM containing 0.4 mg/ml of geneticin (G418). The surviving clones were trypsinized and expanded individually. Mock cells were similarly transfected with a control vector (pRc/CMV) without the insert.

Proliferation and Migration Assays—To assess the ability of BAE and BAE-derived cells to proliferate and migrate, cells were inoculated at a density of 1×10^5 cells/35-mm-diameter dish and grown to confluence. Then one half of the monolayer was peeled off with a cell scraper, washed with PBS, and then cultured for an appropriate period, as described previously (*32*). Photographs of the cells were taken and the distance that the cells migrated was determined with an Image Processor for Analytical Pathology (IPAP) (Sumika Technos, Osaka).

Migration Assay—bFGF-stimulated migration of BAE cells was assayed by a modified Boyden chamber method (*33*) using Chemotaxicells with polyvinylpyrrolidone filters (pore size, 8-μm-diameter) at the bottom. A cell suspension (0.4 ml) containing 4×10^4 cells was introduced into Chemotaxicells and then the Chemotaxicells were placed in the wells of a 24-well plate that contained 0.8 ml of medium supplemented with 10 ng/ml of bFGF. After incubation for 4 h at 37°C, the filters in the Chemotaxicells were removed, fixed, stained, and then mounted on glass slides. The numbers of cells that migrated to the bottoms of the filters were determined under a microscope. Five high power fields (×200) were chosen at random.

Statistical Analysis—Data were analyzed by means of Student's *t* test. All experiments were repeated at least twice with similar results.

RESULTS

Expression of CTGF in Endothelial Cells In Vivo and In Vitro—Previously, we showed that CTGF mRNA was strongly expressed in hypertrophic chondrocytes in costal cartilage and the vertebral column of mouse embryos (E17). In order to clarify the role of CTGF in endochondral ossification, we first investigated the expression of CTGF in cost-chondral junctions of newborn mouse ribs by means of immunohistochemical and immunofluorescence techniques. As shown in Fig. 1, in accordance with the expression pattern of CTGF mRNA reported previously (*6*), chondrocytes in the hypertrophic zone, but not in the proliferating and resting zones, were strongly stained with an anti-CTGF antibody with both techniques. However, more intense staining was observed in endothelial cells in bone. Immunofluoresence staining for CTGF also revealed that the most intense signal was in endothelial cells invading cartilage from bone (Fig. 1C).

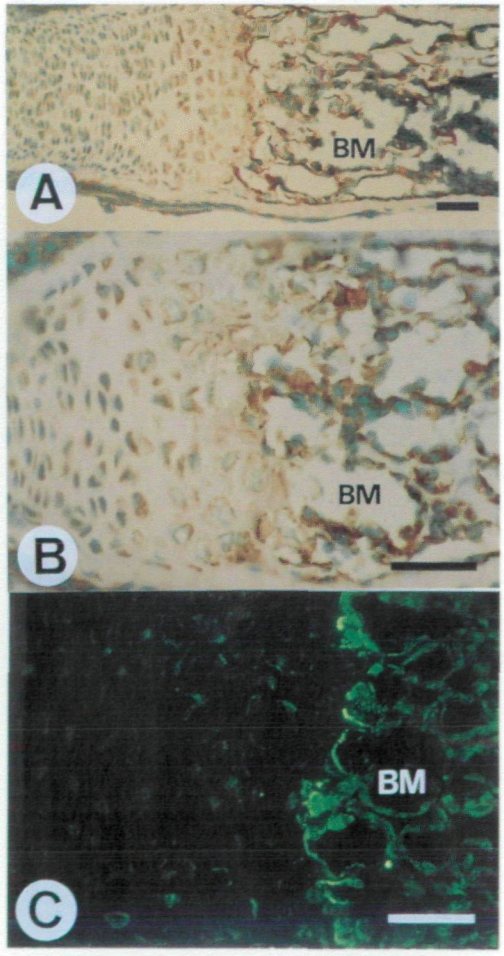

Fig. 1. **Immunoreactive localization of CTGF in cost-chondral junctions of ribs of newborn mice on day 2 after birth.** Frozen sections were reacted with an anti-CTGF antibody, and then visualized by immunoperoxidase (A and B) and immunofluorescence (C) staining. Immunoreactivity was observed in hypertrophic chondrocytes and endothelial cells in bone close to cartilage (A-C). In particular, the endothelial cells invading cartilage were strongly positive. BM, bone marrow. Bar, 50 μm.

Expression of the CTGF Transcript in Endothelial Cells—The expression level of CTGF mRNA was at first analyzed by RT-PCR using specific primers located in sequence tag no. 24 (*6*). Specific PCR products were detected in BAE cells and HCS-2/8 cells, but little or no products were detected in ostoblastic or osteosarcoma cells (data not shown). The expression level of CTGF was also analyzed by Northern blotting using CTGF cDNA as a probe. As shown in Fig. 2A, CTGF mRNA was strongly expressed in BAE, and was also expressed in HCS-2/8 cells, but little or no products were detected in Saos-2, MC3T3-E1, A431 and A7 cells.

Figure 2B shows the results of Northern blotting of CTGF mRNA in proliferating and confluent BAE cells. The expression of CTGF mRNA in proliferating BAE cells was 4.3 times higher than that in confluent BAE cells (Fig. 2C), suggesting that CTGF mRNA was expressed to a greater extent in the growth phase than in the quiescent phase.

For determination of whether or not CTGF is expressed in rapidly proliferating endothelial cells, half a quiescent monolayer of BAE cells was peeled off with a cell scraper

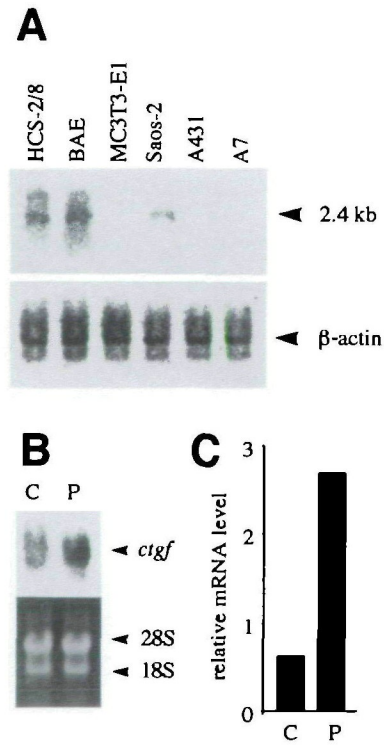

Fig. 2. **Northern blot analysis of the expression of CTGF mRNA in various cell lines (A) and in BAE cells in sparse and confluent cultures (B and C).** About 15 μg of total RNA prepared from confluent cultures of HCS-2/8, BAE, MC3T3-E1, Saos-2, A431, and A7 cells was used. β-Actin was used to determine the relative loading of the lanes. The arrows indicate the positions of mRNA of CTGF and β-actin, respectively. The cells were inoculated at a density of 3×10^5 cells/10-cm-diameter dish with 10 ml of DMEM containing 10% FBS. Cells were harvested on day 3 when they were actively proliferating (lane P), and on day 6 when they had reached confluence (lane C). 18S ribosomal RNA was used to determine the relative loading of the lanes. (C) The amount of mRNA obtained in B was determined densitometrically, and the amount of CTGF mRNA was normalized as to the amount of 18S ribosomal RNA. The ordinate shows the mRNA ratio (CTGF/18S).

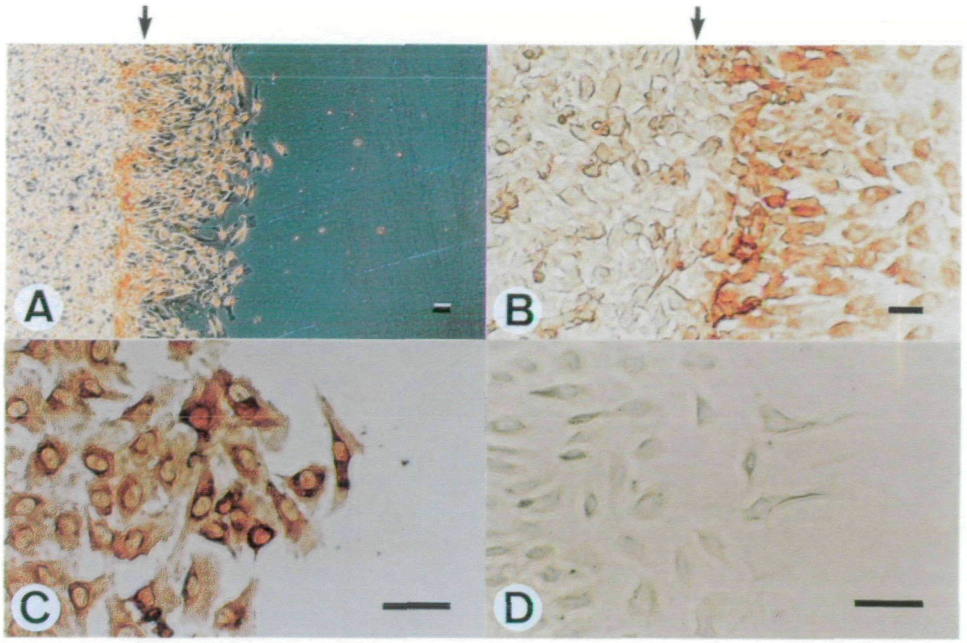

Fig. 3. **Immunohistochemical staining of quiescent BAE cells and BAE cells stimulated to proliferate and migrate.** BAE cells were inoculated at a density of 3×10^5 cells/35-mm dish and then cultured in DMEM containing 10% FBS. When the cells became confluent, one-half of the monolayer was peeled off with a cell scraper at the positions indicated by arrows. After washing with PBS, the remaining halves of the cell layers were cultured in the medium for 12 h. The cell layers were then fixed, stained with an anti-CTGF antibody (A-C) or the IgG fraction of preimmune serum (D), and photographed. CTGF was specifically detected in BAE cells that migrated into the vacant area (A and B). Intense staining was observed in the cytoplasm (C). No staining was observed with the preimmune IgG (D). Bar, 50 μm.

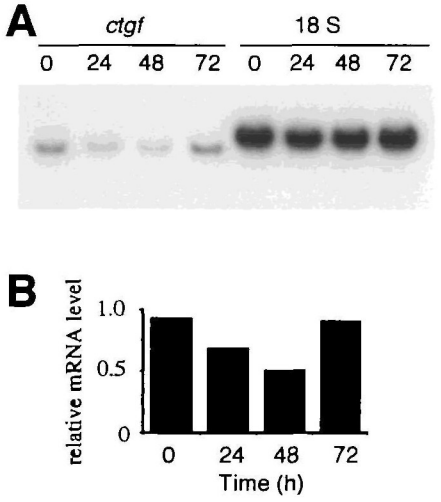

Fig. 4. **Effects of antisense oligonucleotides on the expression of CTGF mRNA in BAE cells.** (A) The expression level of CTGF mRNA in BAE cells analyzed by RT-PCR. BAE cells were inoculated at a density of 3×10^5 cells/well in 6-cm-diameter dishes with DMEM containing 10% FBS. On day 2, 5 μM of antisense oligonucleotides was added. The cells were harvested at the indicated times after the addition of 5 μM of antisense oligonucleotides. (B) The amount of CTGF-specific PCR products was normalized as to the amount of PCR products of 18S ribosomal RNA, as described in the legend to Fig. 2. The ordinate shows the ratio of PCR products (CTGF/18S$\times$4).

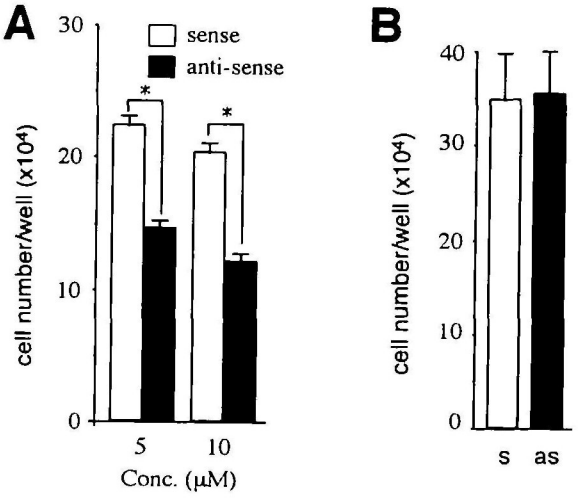

Fig. 5. **Effects of antisense oligonucleotides on the proliferation of BAE cells and MC3T3-E1 cells.** (A) BAE cells were inoculated at a density of 5×10^4/well in 6-well plates and then cultured in DMEM containing 10% FBS. After 24 h (on day 1), antisense or sense oligonucleotides were added to the cultures at the concentrations indicated and then the cells were cultured for a further 4 days. The medium was changed on day 3, the oligonucleotides being added simultaneously. The cell numbers were determined on day 5. The columns and bars are the averages and SD for triplicate cultures. $^*p<0.01$. (B) MC3T3-E1 cells were inoculated at a density of 3.0×10^4/well in 6-well plates and then cultured in αMEM containing 10% FBS. After 24 h, 5 μM of antisense or sense oligonucleotides was added to the cultures and the cells were cultured for a further 4 days. The medium was changed on day 3, the oligonucleotides being added simultaneously. The numbers of cells were determined on day 5. The columns and bars are the averages and SD for triplicate cultures.

and the remainder cultured for 12 h. As shown in Fig. 3, A-C, immunohistochemical staining revealed that CTGF was specifically distributed in BAE cells migrating into vacant areas. A particularly intense signal was observed in the cytoplasm (Fig. 3C). On the other hand, almost no signal was observed in the monolayers of BAE cells.

Effects of CTGF Antisense Oligonucleotides on BAE Cells—To analyze the function of CTGF in BAE cells, we next investigated the effects of bovine and human antisense oligonucleotides on the proliferation and migration of BAE cells in culture. To confirm the effectiveness of the antisense oligonucleotides, we first quantitated their effects on the amount of CTGF mRNA by RT-PCR. As shown in Fig.

4, 5 μM of the human antisense oligonucleotide decreased the expression level of CTGF mRNA in BAE cells 24 to 48 h after the addition. The inhibition was transient, the control level being recovered after 72 h (Fig. 4). We obtained almost the same data using bovine antisense oligonucleotide, but the human antisense oligonucleotide was more effective than the bovine one.

When the antisense oligonucleotides were added to cultures of proliferating BAE cells, the increase in cell number was markedly inhibited. The cell number in 5 or 10 μM antisense oligonucleotide-treated cultures of BAE cells after 4 days of treatment from day 1 of culture was 65 and 60%, respectively, of that in sense oligonucleotide-treated controls (Fig. 5A). In addition, when 5 and 10 μM of an antisense oligonucleotide were added to subconfluent cultures of BAE cells, [^{3}H]thymidine incorporation into the DNA was inhibited to 24% (9,248$\pm$721 *versus* 2,233$\pm$713 cpm/well) and 21% (7,321$\pm$703 *versus* 1,519$\pm$216 cpm/well) of that in the case of treatment with sense oligonucleotides, respectively, after 24 h. We obtained almost the same data with the bovine antisense oligonucleotide. In contrast, the antisense oligonucleotides failed to inhibit the proliferation of mouse osteoblastic cells, MC3T3-E1, that did not express CTGF mRNA (Fig. 5B).

To determine whether or not the antisense oligonucleotides only act on rapidly proliferating endothelial cells, we peeled off one-half of a quiescent monolayer of BAE cells and investigated the effect of each antisense oligonucleotide on BAE stimulated to proliferate and migrate into the vacant area. When the antisense oligonucleotides were added just after the scraping, there was marked decreases in proliferation and the distance that the endothelial cells migrated after 12 and 24 h, compared with in sense-treated cells (Fig. 6). When analyzed by IPAP, the distance that the endothelial cells migrated after 12 and 24 h was 76 and 67%, respectively, of that in the control. Sense oligonucleotides had little effect on the cells. Moreover, each antisense oligonucleotide had no effect on the morphology of BAE cells in the remaining half of the monolayer.

To confirm that antisense oligonucleotides inhibit the migration of BAE, we investigated the effects of CTGF antisense oligonucleotides on bFGF-stimulated migration of BAE cells (Fig. 7) by means of a modified Boyden chamber method using Chemotaxicells. BAE cells were pretreated with 10 μM sense or antisense oligonucleotides for 24 h and then introduced into Chemotaxicells. The cells migrating to the bottoms of the filters were counted after 4 h. As shown in Fig. 7, the antisense oligonucleotides inhibited the migration of BAE cells to 65% the level in controls pretreated with sense oligonucleotides.

Effect of Endogenous Inhibition of CTGF on BAE Cells Caused by the Expression of Antisense RNA—CTGF antisense RNA-expression vectors were constructed from a

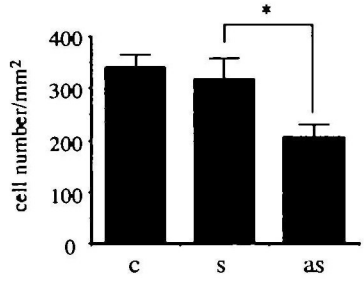

Fig. 7. **Effects of antisense oligonucleotides on bFGF-stimulated migration of BAE cells.** The migration of cells was measured by means of the modified Boyden chamber technique, as described under "MATERIALS AND METHODS." To the lower chambers, 800 μl of DMEM containing 10% FBS and 10 ng/ml bFGF was added. Cells (4×10^4 cells) that had been pretreated with 10 μM of antisense or sense oligonucleotides for 24 h were suspended in 400 μl of DMEM containing 10% FBS and then added to the upper chambers. The columns and bars are the averages and SD for triplicate cultures. *$p <$ 0.01.

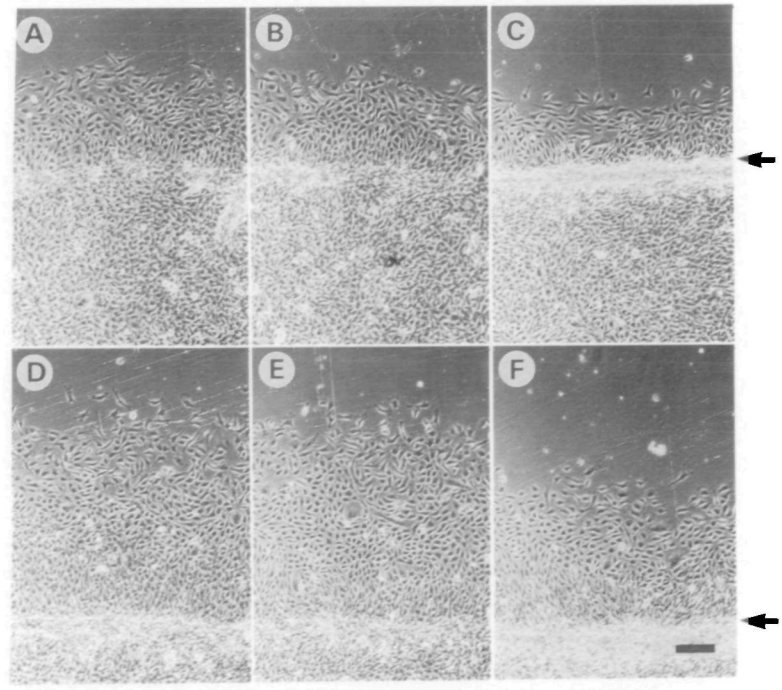

Fig. 6. **Effects of antisense oligonucleotides on quiescent BAE cells and BAE cells stimulated to migrate and proliferate.** When the cells became confluent, one-half of each cell layer was peeled off with a cell scraper, and the remaining halves were then cultured further in the absence (A and D) or presence of the antisense (C and F) or sense (B and E) oligonucleotides for 12 h (A-C) or 24 h (D-F). The arrows indicate the original edge of the wound. Bar, 150 μm.

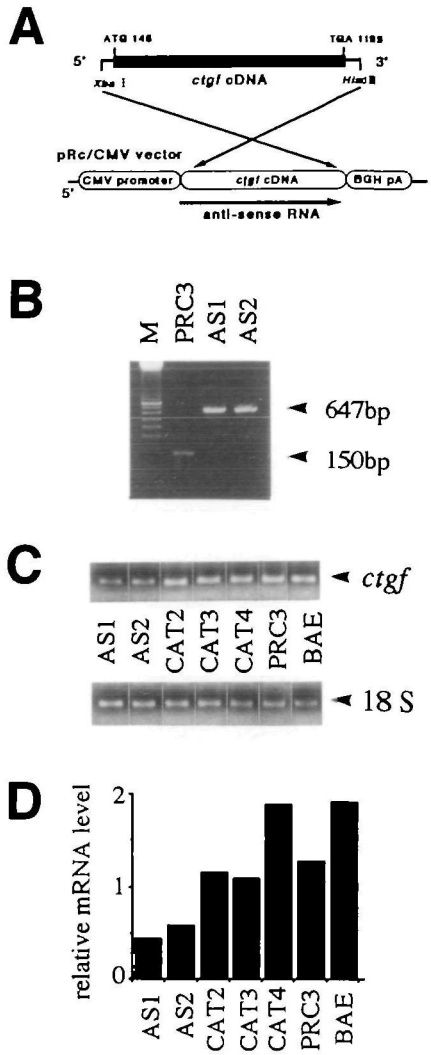

Fig. 8. **Construction of a CTGF antisense RNA expression vector and expression of antisense RNA and reduced expression of CTGF mRNA in two BAE clones (AS1 and AS2) successfully transfected with the vector.** (A) CTGF cDNA fragments corresponding to the 1,050-bp region covering the AUG start codon and UGA stop codon were subcloned into the pRc/CMV expression vector from the pUC18 vector at the *Xba*I-*Hin*dIII site. (B) Total RNA was isolated from BAE cells transfected with the control vector, pRc/CMV (PRC3) or CTGF antisense RNA expression vector (AS1 and AS2), and aliquots of 0.5 μg of DNase I-treated total RNA were subjected to RT-PCR analysis (35 cycles) with vector primers. The predicted lengths of PCR products were about 150 bp for the control vector and about 647 bp for the CTGF antisense RNA expression vector. (C) RT-PCR analysis of the expression of CTGF mRNA in various BAE clones transfected with the CTGF antisense RNA expression vector (AS1 and AS2), the pRc/CMV vector alone (PRC3), and the CAT expression vector, pRc/CMV CAT (CAT2-4), and the parental cells (BAE). The cells were inoculated at a density of 3×10^5 cells/well in 6-cm-diameter dishes with 4 ml of DMEM containing 10% FBS. The cells were harvested three days after the inoculation. About 0.5 μg of DNase I-treated total RNA was subjected to RT-PCR using CTGF primers contained in the 3'-terminal region of CTGF cDNA. (D) The amount of PCR products obtained in C was determined densitometrically and the amount of CTGF cDNA products was normalized as to the amount of PCR products derived from 18S ribosomal RNA. The ordinate is the ratio of PCR products (CTGF/18S).

cloned fragment of CTGF cDNA and placed in the antisense orientation downstream of the CMV promoter (Fig. 8A). Transfection of BAE cells with this expression vector and selection with Geneticin for 20 days produced two clones, named AS1 and AS2. As shown in Fig. 8B, RT-PCR involving primers designed for CTGF and the vector confirmed that these BAE clones were correctly transfected with the antisense RNA expression vectors.

We next compared the amounts of CTGF mRNA in the two BAE clones with those in a BAE clone transfected with the vector alone (PRC3), and BAE clones transfected with CAT expression vectors that were isolated as controls (Fig. 8C). The level of CTGF mRNA in antisense CTGF RNA transfected cells (AS1 and AS2) was 34 and 46% of that in cells transfected with the vector alone (PRC3), and 23-40 and 30-53% of that in cells transfected with CAT genes (CAT2-4), respectively (Fig. 8D).

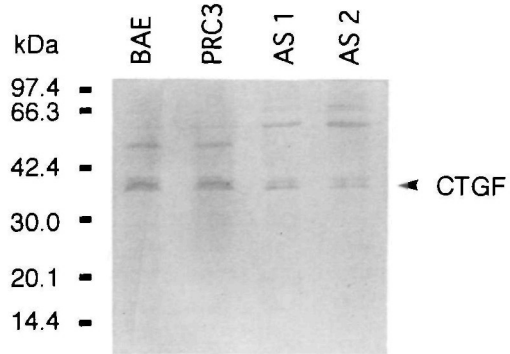

Fig. 9. **Immunoblotting of cytoplasmic CTGF produced by parental BAE cells (BAE), the BAE clone transfected with the vector alone (PRC3), and the BAE clones stably transfected with CTGF antisense RNA expression vectors (AS1 and AS2).** Cytoplasmic preparations from various transformants (20 μg protein) were subjected to Western blot analysis using an anti-CTGF polyclonal antibody, as described under "MATERIALS AND METHODS."

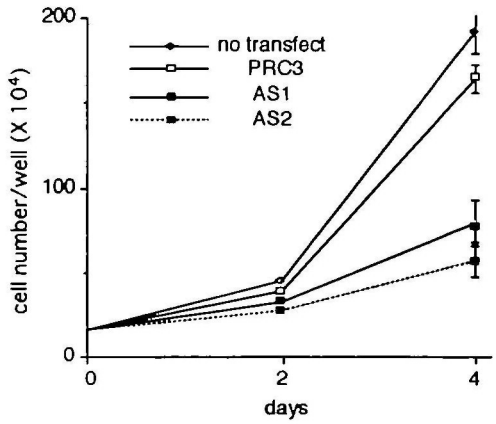

Fig. 10. **Comparison of the growth rates of the BAE clones stably transfected with CTGF antisense RNA expression vectors (AS1 and AS2) and the BAE clone transfected with the vector alone (PRC3).** For calculation of the cell number, BAE cells were inoculated at a density of 1×10^5/well in 6-well plates, cultured in DMEM containing 10% FBS, and counted on days 2 and 4.

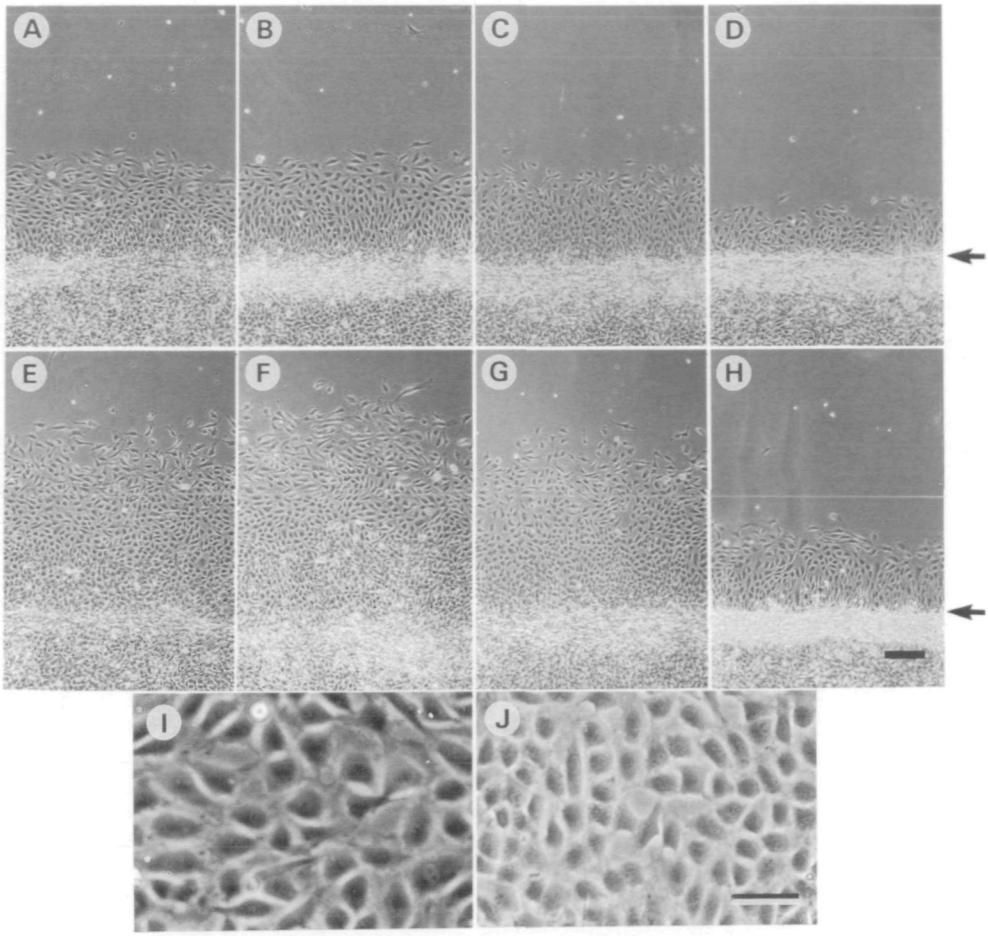

Fig. 11. **Effects of CTGF endogenous inhibition on cell proliferation, migration, and morphology of BAE cells.** Confluent monolayers of endothelial cells were wounded with a cell scraper as described under "MATERIALS AND METHODS." The cells were then further cultured in DMEM containing 10% FBS for 12 h (A-D) and 24 h (E-H). (A and E) BAE with no treatment. (B and F) BAE cells transfected with the control vector, pRc/CMV. (C and G) AS1. (D and H) AS2. (I) A high power view of F. (J) A high power view of G. The arrows indicate the original edge of the wound. Bar, 150 μm. The magnification of A-G is the same as that of H. The magnification of I is the same as that of J.

We also determined the amount of the CTGF protein by Western blotting. As shown in Fig. 9, CTGF production detected by the anti-CTGF antibody was also decreased in AS1 and AS2 transfectants. These results showed that antisense CTGF RNA expressed in transfected cells (AS1 and AS2) inhibited the expression of CTGF mRNA and decreased the CTGF protein level.

Under these conditions, we next analyzed the effects of endogenous inhibition on the proliferation, migration and morphology of BAE cells. When BAE cells were seeded at a density of 1×10^5/well into 6-well plates, the cells proliferated rapidly and reached a density of 1.8×10^6 cells/well. The growth rate of BAE cells transfected with PRC3 was almost the same as that of control BAE cells (Fig. 10). In contrast, BAE cells transfected with antisense CTGF proliferated very slowly. The cell numbers of clone AS1 and AS2 on day 4 were 46 and 33% of those of PRC3 transfected controls (Fig. 10).

When half a monolayer of a confluent culture of AS1 or AS2 had been peeled off, there was marked decreases in proliferation and the distance that the cells migrated into the vacant area after 24 h, compared with in PRC3-transfected cells (Fig. 11). When analyzed by IPAP, the average distance that the antisense CTGF RNA-expressing cells migrated after 12 and 24 h was 72 and 60%, respectively, of that in a control. In the above experiment, no significant difference was observed in the morphology between PRC3-transfected cells and the control cells, but migrating AS1 and AS2 cells were a little smaller in size than the migrating PRC3-transfected cells (Fig. 11, I and J). Moreover, the endogenous inhibition of the expression of CTGF altered the cell shape, leading to a slightly rounded appearance (Fig. 11, I and J).

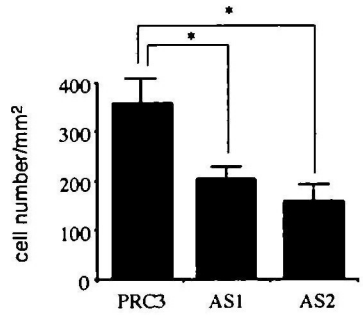

Fig. 12. **Comparison of bFGF-stimulated migration of the BAE cells stably transfected with CTGF antisense RNA expression vectors (AS1 and AS2) and with the vector alone (PRC3).** The migration of cells was measured by means of the modified Boyden chamber technique, as described under "MATERIALS AND METHODS." To the lower chambers, 800 μl of DMEM containing 10% FBS and 10 ng/ml bFGF was added. To the upper chambers, cells suspended in 400 μl of DMEM containing 10% FBS were added (4×10^4 cells in each well). The columns and bars are averages and SD for triplicate cultures. $^*p<0.01$.

The ability of BAE cells expressing antisense RNA of CTGF to migrate was also determined by means of a modified Boyden chamber assay. As shown in Fig. 12, the

bFGF-stimulated migration of AS1 and AS2 cells amounted to 57 and 44% of that of PRC3-transfected cells. Moreover, the number of AS1 cells that migrated to the bottoms of the filters of Chemotaxicells in the absence of bFGF was 61.5% that of PRC3-transfected cells (data not shown).

DISCUSSION

In the present study, we demonstrated the following: (i) immunoreactivity to CTGF in endothelial cells migrating into cartilage from bone *in vivo*, and actively proliferating and migrating endothelial cells *in vitro*, (ii) inhibition by CTGF antisense oligonucleotides of the proliferation and migration of endothelial cells *in vitro*, and (iii) a reduced abilities to proliferate and migrate of endothelial cells expressing CTGF antisense RNA.

Firstly, we specifically detected CTGF in the hypertrophic regions of cartilage and endothelial cells invading cartilage from bone in the cost-chondral junctions of newborn mouse ribs by means of immunohistochemical and immunofluorescence techniques (Fig. 1). The detection of CTGF in the hypertrophic regions was consistent with our previous finding that CTGF mRNA was intensely expressed in the hypertrophic region of the costal cartilage and vertebral columns of mouse embryos (E17) (*6*). While a previous study (*6*) involving *in situ* hybridization using costal cartilage and vertebral columns of mouse embryos (E17) had revealed no signal of CTGF mRNA in blood vessels in connective tissue around cartilage, in the present study, the most intense staining was in endothelial cells located at cost-chondral junctions (Fig. 1). CTGF is a secreted protein originally identified and purified from human umbilical vein endothelial (HUVEC) cells (*1*). This suggests that endothelial cells produce CTGF, but that the production is dependent on the type or state of the endothelial cells. The findings that the CTGF mRNA level in proliferating BAE cells was higher than that in quiescent BAE cells (Fig. 2B), and that only BAE cells migrating into vacant areas after one-half of monolayers had been peeled off, and not quiescent cells, were stained with an anti-CTGF antibody (Fig. 3) clearly showed that only endothelial cells migrating and proliferating produce CTGF. In this regard, it is noteworthy that CTGF is produced by cultured human skin fibroblasts only after stimulation with TGF-β (*2*). It was reported that the TGF-β stimulation of anchorage-independent growth of NRK fibroblasts is dependent on events induced *via* the synergistic action of CTGF-dependent and CTGF-independent signaling pathways (*34*). Moreover, CTGF mRNA is expressed in skin fibroblasts in the sclerotic lesions from patients with systemic sclerosis (*3*), and CTGF mRNA and protein are expressed in atherosclerotic lesions in a human carotid artery (*35*). These findings suggest that CTGF is produced by several types of cells, but that its production is normally suppressed and induced by various stimuli.

In spite of the intense immuno-staining with the anti-CTGF antibody, we observed only weak CTGF mRNA signals in endothelial cells and osteoblastic cells in bone on *in situ* hybridization in young mice (our unpublished data). The discrepancy between the protein and mRNA levels suggests that in cost-chondral junctions of rapidly growing mouse ribs, CTGF is mainly produced by hypertrophic chondrocytes, and then released onto the endothelial cell surface.

The analysis involving antisense oligonucleotides revealed the importance of CTGF in the proliferation and migration of BAE cells. We used the oligonucleotides derived from several different regions of CTGF. One oligonucleotide, which contains the initial ATG at the 5′-end of the sense nucleotide sequence, was effective. At first, we confirmed that the antisense oligonucleotides decreased the level of expression of CTGF mRNA (Fig. 4), because it was reported that antisense oligonucleotides inhibit the formation of mRNA from hn RNA by forming hybrids with the hn RNA following degradation by RNase H (*36*). Under these conditions, DNA synthesis, proliferation (Fig. 5A), and migration of BAE cells (Figs. 6 and 7) were inhibited, suggesting that CTGF plays important roles in these physiological phenomena. On the other hand, the antisense oligonucleotides had no effect on proliferating cultures of MC3T3-E1 cells (Fig. 5B), indicating the specific effect of the antisense oligonucleotides on cells expressing mRNA of CTGF.

We further confirmed the function of CTGF in BAE cells by transfection with expression vectors that generate an antisense RNA (*37-39*). Previous studies showed that endogenous expression of FGF-1 or FGF-2 can effectively modulate the proliferation of prostate cancer cells (*40*). The proliferation, migration and bFGF-stimulated migration of BAE cells transfected with a CTGF antisense RNA expression vector of CTGF were markedly decreased because of continuous inhibition of the expression of mRNA of CTGF. Moreover, the endogenous inhibition of the expression of CTGF altered the cell shape, leading to a slightly rounded appearance (Fig. 11). Kireeva *et al.* (*41*) reported that Fisp12, the mouse homolog of CTGF, is an ECM-associated signaling molecule. These findings suggest that CTGF acts as an adhesion molecule in endothelial cells controlling the proliferation and migration of BAE cells.

Recently, it was found that the *cyr61*, which is an immediate early gene, encodes a protein exhibiting biochemical similarities to Wnt-1 and some growth factors, and sequence homology to a growth factor and a proto-oncoprotein (*5, 42, 43*). CTGF and Cyr61 are closely related, exhibiting 45% sequence identity and complete conservation of all 38 cysteines (*4*). Cyr61 is expressed not only in mouse 3T3 fibroblasts but also in developing mouse cartilaginous elements and placental tissues (*5*), while Fisp12, the mouse homolog of CTGF, is not expressed in cartilage (*41*). This is not consistent with our previous finding (*6*). The purified Cyr61 protein promotes the attachment and spread of endothelial cells, and enhances the effect of bFGF on the DNA synthesis of fibroblasts and vascular endothelial cells, but exhibits no mitogenic activity toward DNA synthesis by fibroblasts and vascular endothelial cells (*44*). Fisp12 also enhances the stimulatory effect of bFGF on DNA synthesis in endothelial cells in culture but has no detectable mitogenic activity (*41*). In the present study, inhibition of CTGF mRNA expression decreased the proliferation of BAE cells in the presence of 10% FBS, and the bFGF-stimulated and non-stimulated BAE migration. Our preliminary studies involving recombinant CTGF revealed that CTGF stimulated the proliferation and migration of cultured endothelial cells in the absence of serum and/or bFGF (unpublished). Further

investigations are needed to clarify the localization and the mechanism of action of CTGF.

The angiogenic factor involved in the invasion by endothelial cells into hypertrophic cartilage in the final stage of endochondral ossification has yet to be identified (*17, 45*). Although bFGF, an angiogenic factor, is present in cartilage, its localization is not defined in the hypertrophic zone (*23*). Carlevaro *et al.* (*46*) suggested that transferin, which is produced by hypertrophic chondrocytes, is one candidate. Jingushi *et al.* (*47*) reported that the gene expression of TGF-β is highest in hypertrophic chondrocytes during endochondral ossification. TGF-β upregulates the expression of CTGF in fibroblasts (*34*) and chondrocytes (*6*). This is consistent with the finding that CTGF expression is greatest in hypertrophic chondrocytes in cartilage (*6*). However, TGF-β is known to inhibit the proliferation of endothelial cells (*48, 49*). Therefore, it is feasible that TGF-β stimulates the production of CTGF by hypertrophic chondrocytes, and that CTGF acts as an important paracrine factor for the proliferation and migration of endothelial cells in angiogenesis in the final stage of endochondral ossification. Although further investigations should be performed to determine whether or not CTGF is an angiogenesis factor, the present study clearly showed that endogenous CTGF expression is involved in the proliferation and migration of endothelial cells in culture.

We thank Dr. Katsuhiko Fujii (Teijin Institute for Bio-Medical Research, Teijin Limited, Tokyo) for providing the BAE cells. We also thank Dr. Akira Yamaguchi (Showa University Dental School) for providing Saos-2, Drs. Yuji Arai and Takumi Ikeda (Kyoto Prefectural University of Medicine), Drs. Tomoyuki Watanabe and Yasunori Katsura (Sumika Technos Co., Ltd.), and Miss Takako Hattori for the technical assistance, Dr. Kojiro Takahashi for the useful discussions, and Mrs. Etsuko Fujisawa for the secretarial assistance.

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