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Connective Tissue Growth Factor Induces the Proliferation, Migration, and Tube Formation of Vascular Endothelial Cells *In Vitro*, and Angiogenesis *In Vivo*[1]

Tsuyoshi Shimo,*,‡ Tohru Nakanishi,*,† Takashi Nishida,*,†,2 Masahiro Asano,*,2 Manabu Kanyama,§ Takuo Kuboki,§ Takuya Tamatani,‖ Katsunari Tezuka,‖ Motohide Takemura,# Tomohiro Matsumura,‡ and Masaharu Takigawa*,†,3

*Department of Biochemistry and Molecular Dentistry, †Biodental Research Center, ‡Department of Oral and Maxillofacial Surgery II, and §Department of Fixed Prosthodontics, Okayama University Dental School, Okayama 700-8525; ‖Pharmaceutical Frontier Research Laboratories, Japan Tobacco Inc., Yokohama 236-0004; and #Department of Oral Anatomy, Osaka University Faculty of Dentistry, Suita, Osaka 565-0871

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Connective tissue growth factor (CTGF) is a novel cysteine-rich, secreted protein. Recently, we found that inhibition of the endogenous expression of CTGF by its antisense oligonucleotide and antisense RNA suppresses the proliferation and migration of vascular endothelial cells. In the present study, the following observations demonstrated the angiogenic function of CTGF *in vitro* and *in vivo*: (i) purified recombinant CTGF (rCTGF) promoted the adhesion, proliferation and migration of vascular endothelial cells in a dose-dependent manner under serum-free conditions, and these effects were inhibited by anti-CTGF antibodies; (ii) rCTGF markedly induced the tube formation of vascular endothelial cells, and this effect was stronger than that of basic fibroblast growth factor or vascular endothelial growth factor; (iii) application of rCTGF to the chicken chorioallantoic membrane resulted in a gross angiogenic response, and this effect was also inhibited by anti-CTGF antibodies. (iv) rCTGF injected with collagen gel into the backs of mice induced strong angiogenesis *in vivo*. These findings indicate that CTGF is a novel, potent angiogenesis factor which functions in multi-stages in this process.

Key words: angiogenesis, connective tissue growth factor (CTGF), endothelial cells, hypertrophic chondrocytes, neovascularization.

CTGF is a member of an emerging family of extracellular proteins known as the CCN family, which is characterized by significant sequence homology and the conservation of all 38 cysteine residues (*1-3*). This CCN family (Cyr61/Cef10, CTGF/Fisp12, and Nov) comprised three distinct members: (i) murine Cyr61, which was identified as a growth factor-inducible immediate early gene product in mouse fibroblasts (*4*) and its chicken homologue, CEF-10, which represents a phorbol ester repressible v-*src* inducible gene (*5*); (ii) human CTGF and its murine homologue, Fisp 12, whose gene was detected in mouse fibroblasts (*6*); and (*3*) Nov, whose gene was found to be aberrantly expressed in retrovirus-induced avian nephroblastomas (*7*). CTGF is identified as a growth factor in angioendothelial cells that exhibits platelet-derived growth factor (PDGF) (*1*)-like biological and immunological activities, although it exhibits little amino acid sequence homology with either the PDGF A chain or B chain (*1*).

Previously, we cloned a mRNA predominantly expressed in hypertrophic chondrocytes (*8*) from a human chondrosarcoma-derived chondrocyte cell line, HCS-2/8 (*9-14*), by differential display-PCR. Its gene was identical with that of connective tissue growth factor (CTGF) (*1-3*). We also demonstrated the presence of receptors specific for CTGF on the chondrocyte cell line, HCS-2/8 (*15*). These findings suggest that CTGF plays an important role in skeletal development.

Angiogenesis, the development of blood vessels, plays a crucial role in embryogenesis and development (*16*), and has been shown to be regulated by a balance of angiogenic factors and anti-angiogenic factors (*14, 17-21*). A typical example of angiogenesis is observed in the process of endochondral ossification. During this process, cartilage cells first proliferate, mature and produce much extracellular matrix. Then the cells become hypertrophic chondrocytes, and the matrix is mineralized and invaded by

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[2] On leave of absence from the Department of Periodontology and Endodontology II, Okayama University Dental School.

[3] To whom correspondence should be addressed. Tel: +81-86-235-6645, Fax: +81-86-235-6649, E-mail: takigawa@dent.okayama-u.ac.jp

Abbreviations: CTGF, connective tissue growth factor; rCTGF, recombinant CTGF; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; BAE, bovine aorta endothelial; CAM, chorioallantoic membrane; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

capillary sprouts; finally cartilage, an avascular tissue, is replaced by bone, which is highly vascularized. Therefore, it is feasible that CTGF, which is predominantly expressed in hypertrophic chondrocytes, also acts on vascular endothelial cells in bone which is close to the hypertrophic zone of cartilage, but there has been no report on the role of CTGF in angiogenesis. So, we previously investigated the expression of CTGF in endothelial cells, and the effects of its antisense oligonucleotide and antisense RNA on the cells. We found that the expression of CTGF mRNA in proliferating bovine aorta endothelial (BAE) cells was much higher than that in confluent BAE cells, and that the inhibition of endogenous expression of CTGF by its antisense oligonucleotide and antisense RNA suppresses the proliferation and migration of vascular endothelial cells (*22*). However, these findings are only indirect evidence of the angiogenic activity of CTGF. Here we present direct evidence that CTGF is a novel, potent angiogenic factor.

MATERIALS AND METHODS

Materials—DMEM was purchased from Nissui Pharmaceutical (Tokyo). FBS was purchased from Cancera International (Rexclale, Ont., Canada). The plastic dishes and plates were from Sumitomo Bakelite (Tokyo). TetraColor ONE was from Seikagaku Kohgyo (Tokyo). Chemotaxicells lined with polyvinylpyrrolidone filters (pore size: 8-mm-diameter) were purchased from Kurabo (Osaka). Human recombinant basic fibroblast growth factor (bFGF) was obtained from R&D Systems (Minneapolis, MN). Human recombinant vascular endothelial growth factor (VEGF) was from Genzyme (Cambridge, MA). Anti-factor VIII antibodies and anti-laminin antibodies were purchased from DAKO (Carpinteria, CA).

Preparation of Anti-CTGF Antibodies—Anti-CTGF antibodies were 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 homologue 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 Western blotting techniques, we confirmed that the synthetic peptide of CTGF, but not the synthetic peptide of the corresponding region of Cyr61, inhibited the binding of the anti-CTGF antibodies to the 38 kDa CTGF protein.

Cell Culture—BAE cells (kindly supplied by Dr. Katsuhiko Fujii, Teijin Institute for Bio-Medical Research, Teijin Limited, Tokyo) were inoculated at a density of $2\text{-}7\times10^4$/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.

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 was determined with a hemocytometer by the Trypan blue exclusion method, or by measurement of the absorbance at 450 nm after the addition of TetraColor ONE to the cultures. 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 materials was determined as described (*23, 24*).

Migration Assay—rCTGF-stimulated migration of BAE cells was assayed by the modified Boyden chamber method involving Chemotaxicells lined with polyvinylpyrrolidone filters (pore size: 8 μm-diameter). A cell suspension (0.4 ml) containing 4×10^4 cells was introduced in the Chemotaxicells and then the Chemotaxicells were placed in the wells of a 24-well plate containing 0.8 ml of medium supplemented with rCTGF or rCTGF and anti-CTGF antibodies. After incubation for 4 h at 37°C, the filters of the Chemotaxicells were removed, fixed, stained, and mounted on glass slides. The number of cells that had migrated to the bottom of the filters was determined under a microscope. Five high power fields ($\times$200) were chosen at random.

Cell Adhesion Assay—Immunological plates (96-well) were coated with 5-20 μg/ml rCTGF containing 0.1% BSA in PBS at 4°C, blocked with 6% BSA in PBS for 1 h at room temperature, and then washed with PBS three times. In some experiments anti-CTGF antibodies or the IgG fraction of pre-immune serum was incubated for 15 min at room temperature. BAE cells were plated at 3×10^4/well in serum-free DMEM. After incubation for 1 h, DMEM containing 10% FBS was added to full wells and the plates were placed bottom up for 15 min at room temperature. After discarding the floating cells, TetraColor ONE was added to the cultures, followed by incubation for 1 h at 37°C under 5% CO_2 in air. The attachment efficiency was determined by measurement of extract A_{450}.

Tube Formation Assay—BAE cells were inoculated at a density of 3×10^5/well into 35-mm-diameter dishes with 3.0 ml of DMEM containing 10% FBS. The medium was changed on day 3, and the cells were cultured for 5 days. On day 5, when monolayers had developed, the medium was changed to serum-free DMEM. After 24 h incubation, the factors were added to the cultures at the concentrations indicated and the cells were cultured for 12 h.

Chorioallantoic Membrane (CAM) Assay—Angiogenic activity was assayed on CAM as described by Takigawa *et al.* (*17*). Embryonic CAM were treated on day 10 with 1 μg of rCTGF or bFGF absorbed on sterile Whatman GB/B glass fiber filter disks (6 mm in diameter; Reeve-Angel, Clifton, USA). The disks prepared with 20 μl of factor were placed upside down on windows which had been made in the egg shells on day 8 of incubation. The embryos were killed 5 days later by injection of 10% formalin in PBS. Each CAM was excised, fixed in 10% formalin in PBS, inverted, and then examined under a stereomicroscope. Angiogenesis was determined from the density of capillaries on the glass fiber filters. The response of the CAM was scored qualitatively as follows: +++ (very strong), ++ (strong), + (moderate), ± (slight), or − (no effect). The double blind method was used to determine the angiogenesis activity.

Subcutaneous Implantation of rCTGF—The carrier was Type I collagen derived from bovine skin (Collagen PC-I; Koken, Japan). rCTGF at 10 μg was mixed with 10 mg of type I collagen in a centrifuge tube and then lyophilized. The rCTGF/carrier mixture was pressed into globular pellets immediately before implantation. Pellets containing 10 mg of type I collagen alone were made and lyophilized as a control. The rCTGF/carrier and control pellets were implanted into the dorsal subcutaneous tissues of rats (Wister, male, 5 weeks after birth). The rats (six from each

group) were sacrificed on day 7 as described (*25*).

Histological and Immunohistochemical Analyses—The explants of rCTGF/carrier and control pellets were fixed in 10% paraform-aldehyde in PBS for 24 h, dehydrated in alcohol and then embedded in paraffin. Sections of 5 μm in thickness were then deparaffined with xylene and rehydrated by ethanol treatment. For routine histology, the sections were stained with hematoxylin and eosin. For immunohistochemistry, the sections were incubated in a moist chamber at 4°C overnight with anti-factor VIII antibodies diluted 1:500 with PBS or anti-laminin antibodies diluted 1:500 with PBS. Then, they were stained with a PAP Universal DAKO Stain Kit according to the manufacturer's protocol (DAKO).

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

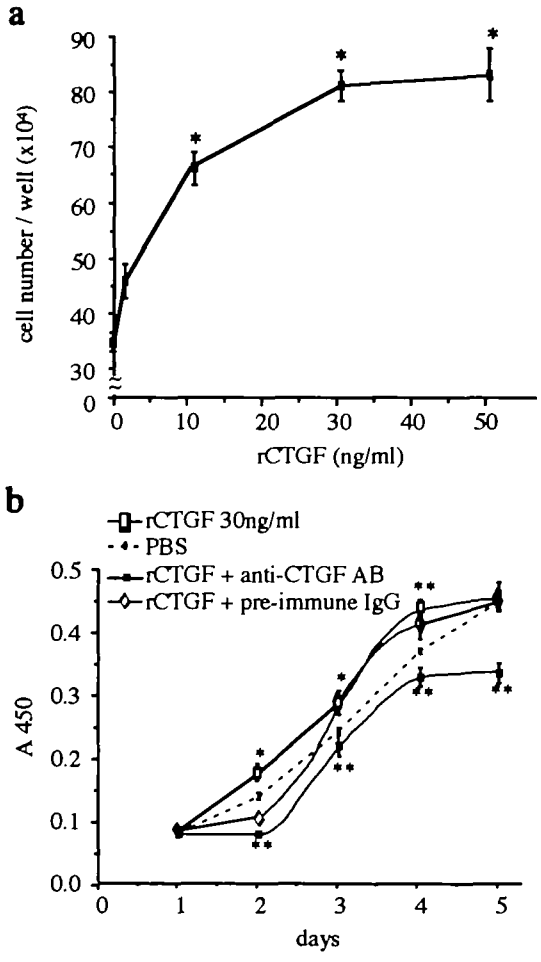

Fig. 1. **Effect of rCTGF on the proliferation of BAE cells *in vitro*.** (a) BAE cells were inoculated at a density of 5×10^4/well into 3.5 cm-diameter dishes and then cultured in serum-free DMEM. After 24 h (on day 1), rCTGF was added to the cultures at the concentrations indicated and the cells were cultured for a further 4 days. The medium was changed on day 3 and rCTGF was added simultaneously. The cell number was determined on day 5. Asterisks indicate significant differences between controls and rCTGF at the significance level of $^*p<0.01$. (b) BAE cells were inoculated at a density of 5×10^3/well into 96-well plates and then cultured in DMEM containing 10% FBS. After 24 h (on day 1), the medium was changed to DMEM containing 0.5% FBS and rCTGF (30 ng/ml), or rCTGF (30 ng/ml) with pre-immune IgG (25 μg/ml) or anti-CTGF antibodies (IgG, 25 μg/ml). On day 3, the medium was changed to DMEM containing 0.5% FBS and the factors. On days 1-5, 10 μl of TetraColor ONE was added to 100 μl of culture. The wells were incubated for 1 h at 37°C under 5% CO_2 in air and then proliferation was quantitated by measurement of the absorbance at 450 nm ($^*p<0.05$, $^{**}p<0.01$).

RESULTS

rCTGF Stimulates Directed Proliferation of BAE Cells—To assess the potential angiogenic activity of CTGF directly, we prepared recombinant CTGF (rCTGF) by gene transfer of CTGF cDNA inserted in a mammalian expression vector (pcDNA 3.1) to HeLa cells (*15*).

At first, we evaluated the ability of rCTGF to induce the proliferation of BAE cells. When BAE cells were cultured for 4 days in serum-free medium with various concentrations of rCTGF, the cell number increased dose-dependently, the maximal stimulation being seen at the concentration of 30-50 ng/ml (Fig. 1a). In addition, when 30 ng/ml of rCTGF was added to subconfluent cultures of BAE cells, the incorporation of [^{3}H]thymidine increased to 145% (36,552±2,272 cpm/well *versus* 53,161±970 cpm/well) ($p<0.01$) of that in those treated with PBS after 24 h (data not shown). As shown in Fig. 1b, BAE cells inoculated at a density of 5,000/96-well microwells and cultured with 20 ng/ml rCTGF in medium containing 0.5% FBS from day 1 grew faster than the control ones. When the anti-CTGF antibodies were added with rCTGF, the cells proliferated more slowly than those treated with pre-immune IgG (Fig. 1b), indicating that growth promoting ability is an intrinsic property of CTGF and cannot be attributed to possible contaminants in the protein preparations.

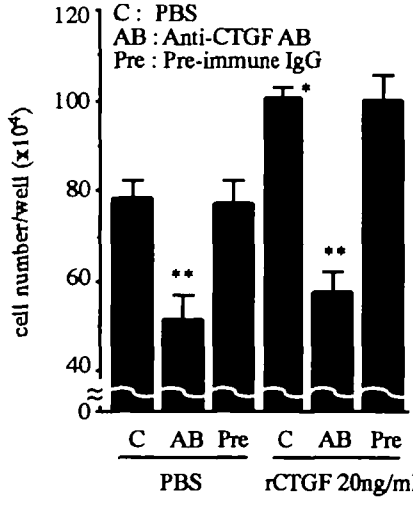

Fig. 2. **Effect of anti-CTGF antibodies on the proliferation of BAE cells induced by rCTGF.** BAE cells were inoculated at a density of 3×10^4/well into 35-mm-diameter dishes and then cultured in DMEM containing 10% FBS. After 24 h (on day 1), the medium was changed to DMEM containing 0.5% FBS and rCTGF with pre-immune IgG (50 μg/ml) or anti-CTGF antibodies (IgG, 50 μg/ml). On day 3, the medium was changed to DMEM containing 0.5% FBS and rCTGF with pre-immune IgG (50 μg/ml) or anti-CTGF antibodies (IgG, 50 μg/ml). On day 5, the number of cells was determined. The values represent mean cell numbers ±SD for five cultures. Asterisks denote statistically significant differences from PBS controls ($^*p<0.01$) or pre-immune IgG controls ($^{**}p<0.01$).

Anti-CTGF Antibodies Inhibited the Proliferation of BAE Cells—The inhibitory effect of the anti-CTGF antibodies was also confirmed by determining the cell number using BAE cultures inoculated with cells at a lower density (Fig. 2). The cell number of rCTGF (20 ng/ml)-treated cultures was 129% ($*p<0.01$) of that of controls after 4 days, when the control cultures were subconfluent (Fig. 2). Anti-CTGF antibodies also inhibited this stimulatory effect to 57% ($**p<0.01$) of the CTGF stimualted culture one (Fig. 2). The anti-CTGF antibodies also inhibited the spontaneous proliferation of BAE cells to 66.4% ($**p<0.01$) of the pre-immune IgG-treated control level (Fig. 2), suggesting the autocrine action of CTGF in BAE cells (*22*).

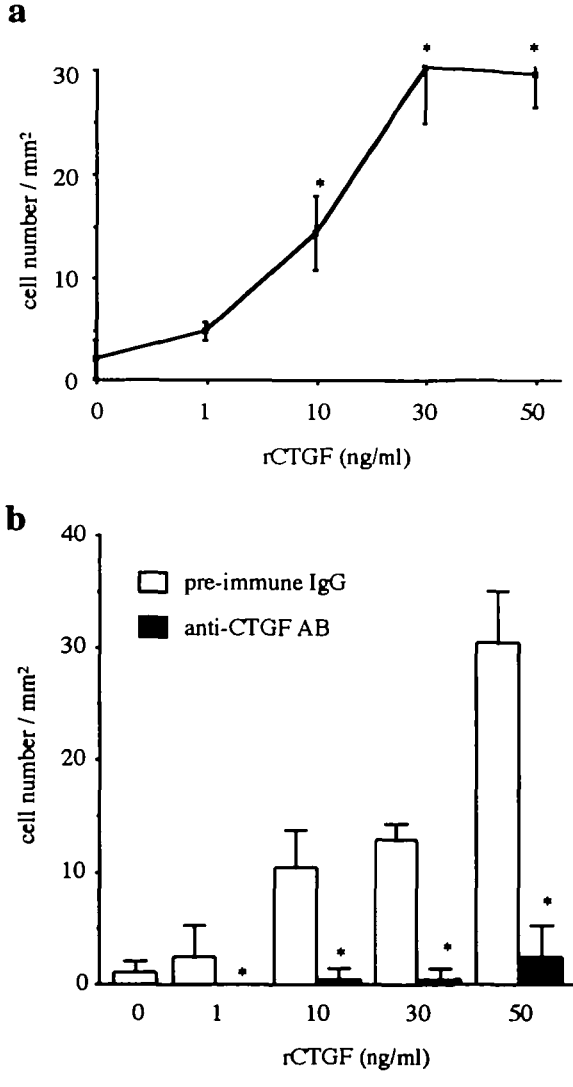

Fig. 3. **Stimulation by rCTGF of the migration of BAE cells and its inhibition by anti-CTGF antibodies.** The migration of cells was measured by the modified Boyden chamber technique, as described under "MATERIALS AND METHODS." To the lower chamber, 800 μl of DMEM containing BSA (0.2 mg/ml) and rCTGF (a), or 800 μl of DMEM containing BSA (0.2 mg/ml), and rCTGF and anti-CTGF antibodies (b) was added. To the upper chamber, cells suspended in 400 μl of DMEM containing BSA (0.2 mg/ml) were added (4×10^4 cells in each well). Asterisks indicate significant differences between pre-immune antibodies and anti-CTGF antibodies at the significance level of $*p<0.01$.

rCTGF Stimulates the Migration of BAE Cells and Anti-CTGF Antibodies Inhibit the Migration of BAE Cells—As shown in Fig. 3a, rCTGF also stimulated the migration of BAE cells dose-dependently and the effect was observable at a concentration of 10 ng/ml, a plateau being reached at

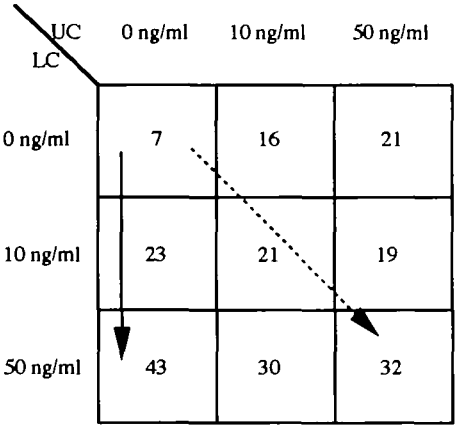

Fig. 4. **Checkerboard analysis of the response to rCTGF of BAE cells.** The factor was added to the upper compartment (UC) and/or lower compartment (LC) at the concentrations indicated. The solid arrow indicates the chemotactic gradient (most cells migrate when the concentration of the stimulant is highest in the LC and lowest in the UC). The dashed arrow indicates the gradient of chemokinesis [most cells migrate when the concentration of the stimulant is highest overall (in the UC or LC)]. The results are the averages for three experiments.

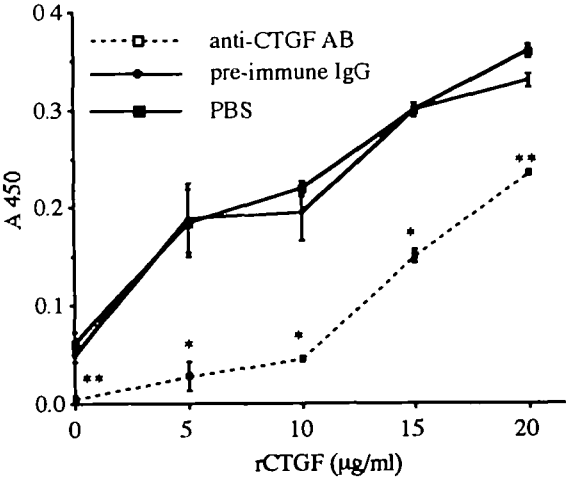

Fig. 5. **Adhesion of BAE cells to rCTGF.** Immunological plates (96-well) were coated with 5-20 μg/ml rCTGF containing 0.1% BSA in PBS at 4°C and then blocked with 6% BSA in PBS for 2 h at 4°C. Then the plates were washed with PBS three times. In some experiments, anti-CTGF antibodies or the IgG fraction of pre-immune serum was incubated for 15 min at room temperature. BAE cells were plated at 3×10^4/well in serum-free DMEM. After incubation for 1 h, serum-free DMEM was added to full wells and the plates were inverted for 15 min. For determination of the attached cell number, TetraColor ONE was added to the wells, followed by incubation for 1 h at 37°C under 5% CO_2 in air. The attachment efficiency was determined by measurement of the absorbance at 450 nm. Asterisks indicate significant differences between pre-immune IgG and anti-CTGF antibodies at the significance level of $*p<0.05$ or $**p<0.01$ respectively. Data in the figure are representative of three experiments yielding similar results.

30 ng/ml. Anti-CTGF antibodies, but not pre-immune antibodies, almost completely inhibited the stimulation of cell migration by rCTGF (Fig. 3b).

rCTGF Stimulates Directed Migration of BAE Cells—To determine whether the effect of rCTGF comprised chemotaxis (directed cell migration) or chemokinesis (random cell movement), we performed checkerboard analysis (Fig. 4). rCTGF stimulated cell migration most effectively when it was placed only in the lower chamber, which is consistent with the directed chemotactic activity of CTGF. Cell migration was also induced by the presence of rCTGF in the upper chamber, and both the upper and lower chambers, but the effects were less than that observed in the presence of rCTGF only in the lower chamber. These findings showed that rCTGF stimulates directed chemotaxis of BAE cells, although it has chemokinesis activity.

rCTGF Stimulates the Adhesion of BAE Cells and Anti-CTGF Antibodies Inhibit the Adhesion of BAE Cells—We next assessed the ability of CTGF to mediate cell attachment. As shown in Fig. 5, many more BAE cells became attached to rCTGF-coated plastic plates than control plates without rCTGF. The effect depended on the concentration

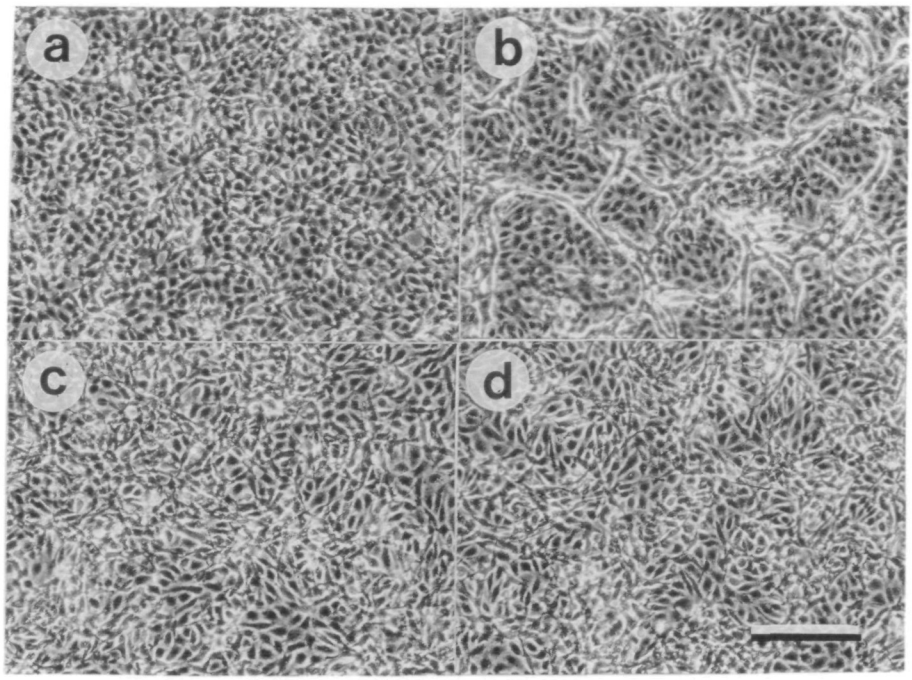

Fig. 6. **Induction of capillary-like tube formation on the addition of rCTGF.** BAE cells were inoculated at a density of 3×10^5/well into 35-mm-diameter dishes with 3.0 ml of DMEM containing 10% FBS. The medium was changed on day 3, and the cells were cultured for 5 days. When the cells became monolayers (day 5), the medium was changed to serum-free DMEM. After 24 h incubation, 50 ng/ml of rCTGF (a), bFGF (b), VEGF (c), or PBS (d) was added to the cultures. After 12 h, photomicrographs of the BAE cells were taken directly. Bar, 20 μm.

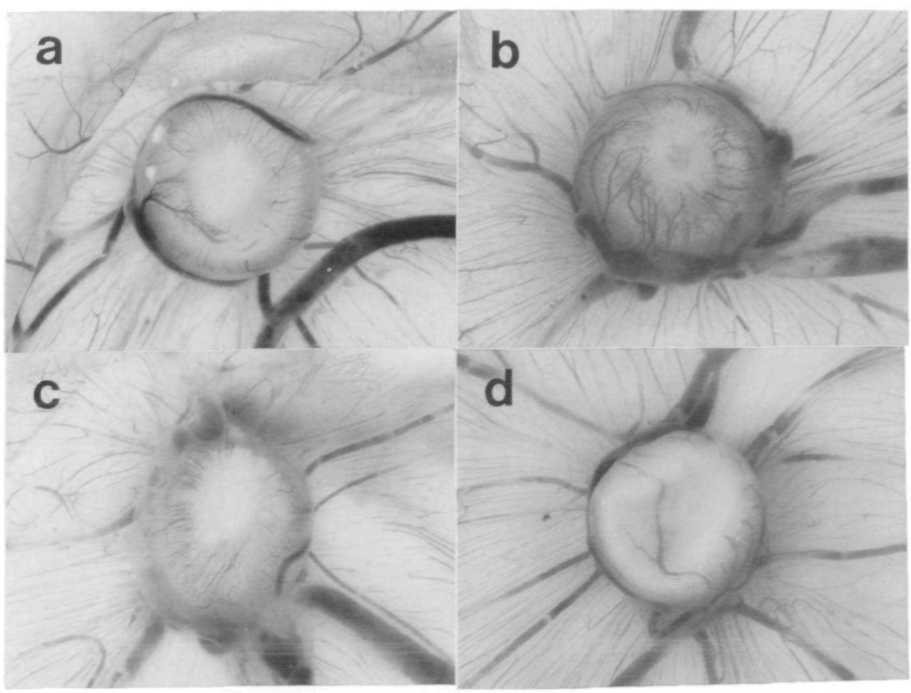

Fig. 7. **Representative results of chorioallantoic membrane (CAM) assays.** Embryonic CAM were treated on day 10 with rCTGF or bFGF absorbed on sterile glass fiber filter disks. The disks prepared using 20 μl of factor were placed upside down on windows which had been made in the egg shells on day 8 of incubation. The embryos were killed 5 days later by injection of 10% formalin in PBS. The CAM were excised, fixed in 10% formalin in PBS, inverted, and then examined under a stereomicroscope. Neovascularization occurred in chorioallantoic membranes at 5 days after the application of glass fiber disks containing 1 μg of rCTGF (a), 2 μg of rCTGF (b), 1 μg of bFGF (c), or PBS as a control (d).

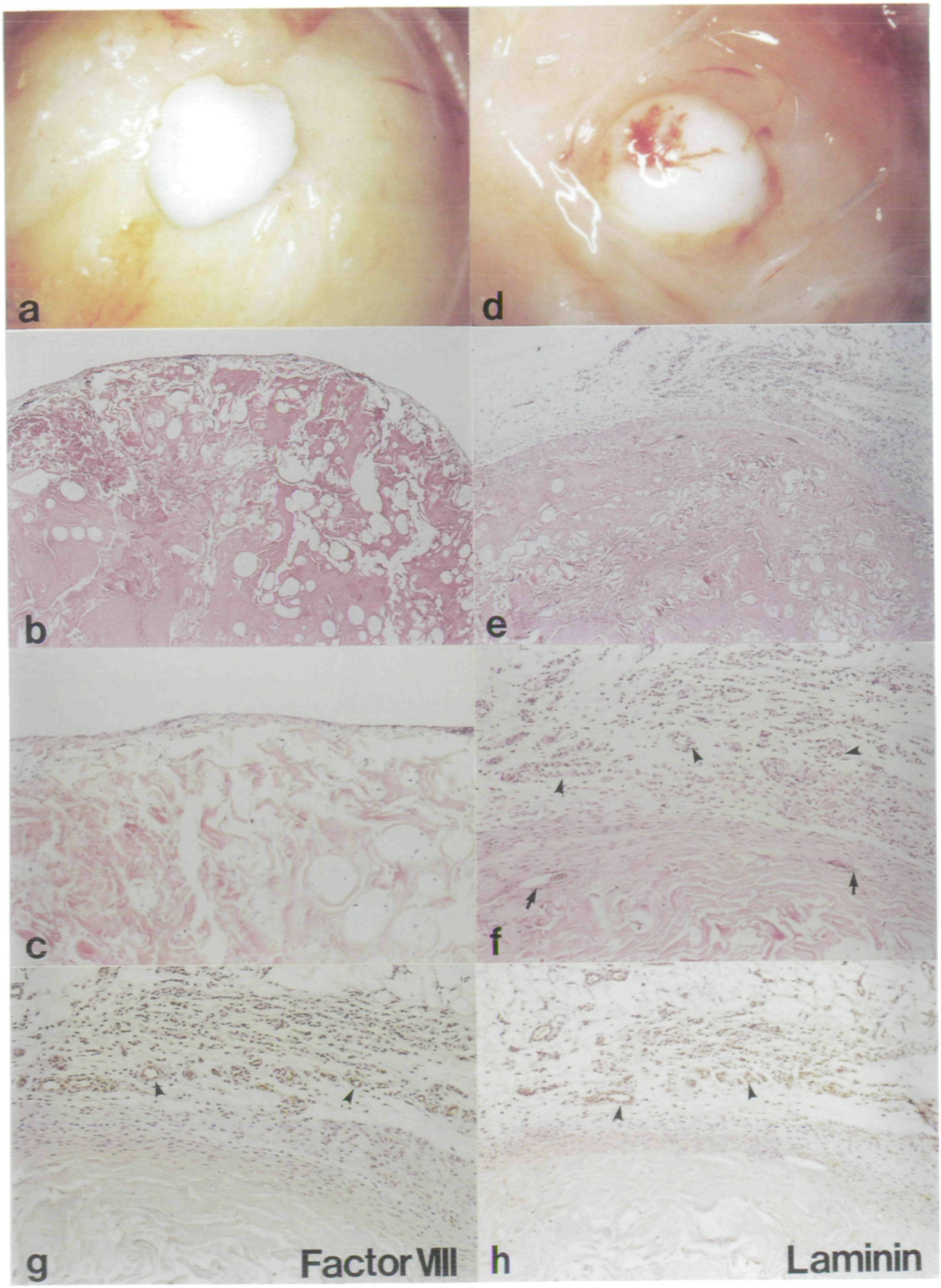

Fig. 8.

of rCTGF in the coating solution. Furthermore, cell adhesion mediated by up to 20 μg/ml of rCTGF was inhibited by anti-CTGF antibodies, indicating that the adhesion is an intrinsic activity of rCTGF.

rCTGF Stimulates the Tube Formation of BAE Cells—Tube formation is the result of a process of dynamic remodeling of vascular systems. When rCTGF was added to a monolayer of BAE cells, the attached endothelial cells extended cell processes, formed cell-cell contacts, and established a branched network comprising elongated, bipolar cells (Fig. 6b). On the other hand, when bFGF and VEGF were added, BAE cells extended cell processes and overlapped each other, but no obvious network was formed (Fig. 6, c and d).

rCTGF Induces Neovascularization and Anti-CTGF Antibodies Blocked the Angiogenesis in Chorioallantoic Membranes—The above *in vitro* findings allowed us to investigate the angiogenic activity of rCTGF *in vivo*. Firstly, we carried out a chorioallantoic membrane (CAM) assay. When glass fiber disks containing rCTGF and bFGF were placed on CAM of chick embryos, these factors induced the formation of small vessels radically directed toward the center of the membrane (Fig. 7, a, b, and c). As a control, PBS showed no effect on the vascular pattern (Fig. 7d). The effect of rCTGF was dose-dependent, and significant at 1 and 2 μg (Table I; $p=0.05$ at 0.5 μg, $p<0.001$ at 1 and 2 μg). Furthermore, the effect of rCTGF was almost the same as that of bFGF (Fig. 7 and Table I). Pre-incubation of rCTGF with anti-CTGF antibodies before absorption to glass fiber filter disks abolished neovascularization entirely (Table I; $p=0.016$).

rCTGF in Collagen Pellets Induces Neovascularization in the Backs of Rats—Next, we injected rCTGF in a collagen pellet into the backs of mice. As shown in Fig. 8, strong angiogenesis into the collagen pellets containing 10 μg of rCTGF had occurred after 1 week. Microscopy revealed many vessels which were immunostained with antibodies against factor VIII (Fig. 8g) and laminin (Fig. 8h), markers of endothelial cells and the basement membrane, respectively. On the other hand, no vessel was observed in the collagen pellets in the control groups.

TABLE I. **The effect of rCTGF on neovascularization in chorioallantoic membranes.**

Test substances	No. of eggs used	No. of eggs with angiogenesis activity[a]				
		−	±	+	++	+++
PBS	7	3	3	1	0	0
rCTGF (0.5 μg)	3	0	3	0	0	0
rCTGF (1 μg)	6	0	0	2	4	0
rCTGF (2 μg)	11	0	0	0	5	6
bFGF (1 μg)	4	0	0	0	3	1
bFGF (2 μg)	7	0	0	0	3	4
rCTGF (1 μg) +anti-CTGF AB (200 μg)	7	2	3	2	0	0
rCTGF (1 μg) +pre-immune IgG (200 μg)	5	0	0	3	2	0

[a]The scoring of angiogenesis is described in the text.

Fig. 8. **Histological and immunohistochemical analyses of type I collagen/rCTGF implants harvested at 1 week.** (a) The explant of a control type I collagen pellet. (b) Low-power magnification of a hematoxylin-eosin stained section of a control pellet. (c) High-power magnification of hematoxylin-eosin staining of a control pellet. (d) The explant of a type I collagen/rCTGF pellet. (e) Low-power magnification of a hematoxylin-eosin stained section of a type I collagen/rCTGF pellet. (f) High-power magnification of hematoxylin-eosin staining of endothelial cells (arrow heads) and blood vessels (arrows) around a type I collagen/rCTGF pellet. (g) Immunohistochemical staining demonstrating positive factor VIII. (h) Immunohistochemical staining demonstrating positive laminin. Original magnification: 10× for (b and e), and 50× for (c, f, g, and h).

DISCUSSION

In the process of angiogenesis, endothelial cells first migrate from the parent vessel and proliferate along a gradient of a chemoattractant toward angiogenic signals, resulting in the formation of nascent capillary sprouts (*26-28*) and tubes with a new basement membrane (*29*). Cellular attachment is also important in this process (*30*). Although CTGF was found in conditioned medium of human umbilical vein endothelial cells (HUVEC) (*1*), there has been no report on the effect of CTGF on angiogenesis *in vivo* and only one report on the effect of CTGF on cultured endothelial cells. That was Kireeva *et al.*'s report showing that Fisp12, the mouse homolog of human CTGF, promoted HUVEC adhesion and enhanced bFGF-induced DNA synthesis (*31*). In the present study, we confirmed that rCTGF promoted the adhesion (Fig. 5) of endothelial cells using BAE cells. However, although Kireeva *et al.* (*31*) reported that Fisp12 has no detectable mitogenic activity without bFGF, we found that rCTGF stimulated the proliferation of BAE cells in medium without serum or growth factors (Fig. 1), suggesting a direct effect of CTGF on the proliferation of endothelial cells. However, BAE cells are known to produce much more bFGF than HUVEC do (*32*), so the growth stimulatory effect on the addition of rCTGF alone might comprise a potentiating effect on the autocrine growth stimulatory action of endogenous bFGF. Further investigation is needed to clarify the cause of this discrepancy.

In addition to cell proliferation and cell adhesion, we found for the first time that rCTGF promoted not only the migration but also the chemotaxis of endothelial cells (Figs. 3 and 4). The stimulatory effects of rCTGF on the proliferation, adhesion and migration of endothelial cells were inhibited by anti-CTGF antibodies, indicating that these effects are indeed due to CTGF itself, and not to any contaminants in the rCTGF preparation. Moreover, we showed that rCTGF, but not bFGF or VEGF, induced capillary-like tube formation by BAE on plastic dishes without any matrix component (Fig. 6). Some investigators reported that bFGF and VEGF induce tube formation by endothelial cells on/in collagen gels (*33-36*), but others failed to induce tube formation in collagen or a fibrin matrix (*37, 38*). So far, there has been no explanation for this discrepancy but it might be due to differences in culture conditions or the different origins of endothelial cells (*33-38*). Anyway, our results clearly demonstrate that rCTGF has strong capillary-like tube forming activity. All these findings indicate that CTGF is a strong angiogenesis factor *in vitro*.

In addition to with the *in vitro* assay, we also demonstrated that rCTGF induced angiogenesis *in vivo* using the CAM assay (Fig. 7), and the rat subcutaneous implantation model (Fig. 8). Anti-CTGF antibodies blocked the *in vivo* angiogenesis induced by rCTGF, indicating that this is also due to rCTGF itself, not contaminants in the rCTGF preparation

(Table I). From these *in vivo* and *in vitro* findings, we conclude that CTGF is a novel, strong angiogenic factor capable of inducing principal aspects of the angiogenic process *in vitro* and *in vivo*.

CTGF is a member of an emerging family of extracellular proteins known as the CCN family (*1-3*). Lau and his colleagues (*4, 39*) reported that CYR61, a product of a growth factor-inducible immediate early gene in mouse fibroblasts, which is a member of the CCN family and exhibits 50% amino acid identity with CTGF stimulated the adhesion and directed migration of HUVEC in culture through an $\alpha v\beta 3$ integrin-dependent pathway. Therefore, it would be interesting to determine the roles of integrin $\alpha v\beta 3$ in the adhesion and chemotaxis of endothelial cells to CTGF.

Recently, we demonstrated the presence of receptors specific for CTGF on a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8, by means of a binding assay and a cross-linking study involving ^{125}I-CTGF-labeled recombinant CTGF (*15*). In our preliminary cross-linking study with BAE cells, we also found the formation of a ^{125}I-CTGF-receptor complex with a similar apparent molecular weight to that of HCS-2/8 (unpublished data). Therefore, it is feasible that CTGF promotes the adhesion and chemotaxis of endothelial cells through integrin $\alpha v\beta 3$, because of the structural similarity of CTGF and CYR61, and stimulates the proliferation of endothelial cells through such specific receptors.

No expression of CTGF has been observed in normal blood vessels *in vivo* (*40, 41*), or in quiescent endothelial cells *in vitro*, but we previously demonstrated that the factor is markedly overexpressed in migrating and proliferating endothelial cells *in vitro* (*22*). Moreover, we reported that immunostaining with anti-CTGF antibodies was observed not only in the hypertrophic region of cartilage but also in the endothelial cells invading cartilage from bone in the cost-chondral junctions of newborn mouse ribs, and the endothelial cells were stained more intensely than the hypertrophic chondrocytes (*22*). On the other hand, *in situ* hybridization using the cost-chondral junctions of mice of the same age revealed that the mRNA expression of CTGF was higher in hypertrophic chondrocytes than in the endothelical cells invading cartilage from bone, although the endothelical cells expressed CTGF mRNA (unpublished data). The vasculature is normally quiescent but neovascularization occurs during growth and development, and the newborn mouse rib is a rapidly growing tissue. Therefore, it is feasible that CTGF produced by hypertrophic chondrocytes is released toward endothelial cells in the cost-chondral junctions and acts as a paracrine angiogenesis factor, and then the stimulated endothelial cells produce CTGF as an autocrine angiogenesis factor like bFGF (*42*). In other words, CTGF detected in/on endothelial cells might be the sum of CTGF produced by the hypertrophic chondrocytes and the endothelical cells. Through this mechanism, hypertrophic chondrocyte-derived CTGF may induce angiogenesis as a prelude to the replacement of cartilage with bone. From this point of view, the finding that CTGF is an angiogenesis inducer provides a novel insight into its biological functions in growth and development.

In the adult, endothelial cells are almost in a quiescent stage, but angiogenesis occurs during the processes of wound healing and tumor development (*17*). So, the biochemical finding that the expression of CTGF is induced in granulation tissues during wound healing (*3, 40, 41*) may also suggest a role for CTGF in neovascularization during wound repair. Moreover, the finding that CYR61 promotes angiogenesis and tumor growth (*43*) may also suggest a similar role of CTGF in tumor angiogenesis. Futhermore, some CD31-positive endothelial cells in atherosclerotic plaque vessels, but not normal arteries, express high levels of CTGF mRNA and protein (*44*). Therefore, CTGF and a related protein like CYR61 may also play possible roles in the pathological angiogenesis.

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REFERENCES

1. Bradham, D.M., Igarashi, A., Potter, R.L., and Grotendorst, G.R. (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J. Cell Biol.* **114**, 1285-1294
2. Bork, P. (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* **327**, 125-130
3. Igarashi, A., Okochi, H., Bradham, D.M., and Grotendorst, G.R. (1993) Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol. Biol. Cell.* **4**, 637-645
4. O'Brien, T.P., Yang, G.P., Sanders, L., and Lau, L.F. (1990) Expression of *cyr61*, a growth factor-inducible immediate-early gene. *Mol. Cell. Biol.* **10**, 3569-3577
5. Simmons, D.L., Levy, D.B., Yannoni, Y., and Erikson, R.L. (1989) Identification of a phorbol ester-repressible v-*src*-inducible gene. *Proc. Natl. Acad. Sci. USA* **86**, 1178-1182
6. Ryseck, R.-P., Macdonald-Bravo, H., Matteri, M.-G., and Bravo, R. (1991) Structure, mapping, and expression of *fisp-12*, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ.* **2**, 225-233
7. Joliot, V., Martinerie, C., Dambrine, G., Plassiart, G., Brisac, M., Crochet, J., and Perbal, B. (1992) Proviral rearrangements and overexpression of a new cellular gene (*nov*) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol. Cell. Biol.* **12**, 10-21
8. Nakanishi, T., Kimura, Y., Tamura, T., Ichikawa, H., Yamaai, Y., Sugimoto, T., and Takigawa, M. (1997) Cloning of mRNA preferentially expressed in chondrocyte by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. *Biochem. Biophys. Res. Commun.* **234**, 206-210
9. Takigawa, M., Tajima, K., Pan, H.-O., Enomoto, M., Kinoshita, A., Suzuki, F., Takano, Y., and Mori, Y. (1989) Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. *Cancer Res.* **49**, 3996-4002
10. Takigawa, M., Pan, H.-O., Kinoshita, A., Tajima, K., and Takano, Y. (1991) Establishment from a human chondrosarcoma of a new immortal cell line with high tumorigenicity *in vivo*, which is able to form proteoglycan-rich cartilage-like nodules and to respond to insulin *in vitro*. *Int. J. Cancer* **48**, 717-725
11. Enomoto, M. and Takigawa, M. (1992) Regulation of tumor derived and immortalized chondrocytes in *Biological Regulation of the Chondrocytes* (M. Adolphe, ed.) pp. 321-338, CRC Press, Boca Raton
12. Zhu, J.D., Pan, H.-O., Suzuki, F., and Takigawa, M. (1994) Proto-oncogene expression in a human chondrosarcoma cell line: HCS-2/8. *Jpn. J. Cancer Res.* **85**, 364-371
13. Tuckwell, D.S., Ayad, S., Grant, M.E., Takigawa, M., and

Humphries, M.J. (1994) Conformation dependence of integrin-type II collagen binding. Inability of collagen peptides to support $\alpha_2\beta_1$ binding, and mediation of adhesion to denatured collagen by a novel $\alpha_5\beta_1$-fibronectin bridge. *J. Cell Sci.* **107**, 993-1005
14. Ohba, Y., Goto, Y., Kimura, Y., Suzuki, F., Hisa, T., Takahashi, K., and Takigawa, M. (1995) Purification of an angiogenesis inhibitor from culture medium conditioned by a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8. *Biochim. Biophys. Acta* **1245**, 1-8
15. Nishida, T., Nakanishi, T., Shimo, T., Asano, M., Hattori, T., Tamatani, T., Tezuka, K., and Takigawa, M. (1998) Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). *Biochem. Biophys. Res. Commun.* **247**, 905-909
16. Sariola, H. (1985) Interspecies chimeras: an experimental approach for studies on embryonic angiogenesis. *Med. Biol.* **63**, 43-65
17. Takigawa, M., Enomoto, M., Nishida, Y., Pan, H.O., Kinoshita, A., and Suzuki, F. (1990) Tumor angiogenesis and polyamines: α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits B16 melanoma-induced angiogenesis *in vivo* and the proliferation of vascular endothelial cells *in vitro*. *Cancer Res.* **50**, 4131-4138
18. Takigawa, M., Nishida, Y., Suzuki, F., Kishi, J., Yamashita, K., and Hayakawa, T. (1990) Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP and TIMP-2). *Biochem. Biophys. Res. Commun.* **171**, 1264-1271
19. Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W., and Heledin, C.-H. (1989) Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature* **338**, 557-562
20. Rifkin, D.B. and Moscatelli, D. (1989) Recent development in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* **109**, 1-6
21. Leung, D.W., Cachianes, G., Kuang, W.-J., Goeddel, D.V., and Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306-1309
22. Shimo, T., Nakanishi, T., Kimura, K., Nishida, T., Ishizeki, K., Matsumura, T., and Takigawa, M. (1998) Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisense RNA suppress proliferation and migration of vascular endothelial cells. *J. Biochem.* **124**, 130-140
23. Enomoto, M., Pan, H.O., Kinoshita, A., Yutani, Y., Suzuki, F., and Takigawa, M. (1990) Effects of tumor necrosis factor a on proliferation and expression of differentiated phenotypes in rabbit costal chondrocytes in culture. *Calcif. Tissue Int.* **47**, 145-151
24. Takigawa, M., Takano, T., and Suzuki, F. (1981) Effects of parathyroid hormone and cyclic AMP analogues on the activity of ornithine decarboxylase and expression of the differentiated phenotype of chondrocytes in culture. *J. Cell. Physiol.* **106**, 259-268
25. Kuboki, Y., Saito, T., Murata, M., Takita, H., Mizuno, M., Inoue, M., Nagai, N., and Poole, A.R. (1995) Two distinctive BMP-carriers induce zonal chondrogenesis and membranous ossification, respectively; geometrical factors of matrics for cell-differentiation. *Connect. Tissue Res.* **32**, 219-226
26. Folkman, J. and D'Amore, P.A. (1996) Blood bessel formation: What is its molecular basis? *Cell* **87**, 1153-1155
27. Hanahan, D. and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364
28. Risau, W. (1997) Mechanisms of angiogenesis. *Nature* **386**, 671-674
29. Folkman, J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? *Cancer Res.* **46**, 467-473
30. Ingber, D.E. and Folkman, J. (1989) Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J. Cell Biol.* **109**, 317-330
31. Kireeva, M.L., Latinkic, B.V., Kolesnikova, T.V., Chen, C.C., Yang, G.P., Abler, A.S., and Lau, L.F. (1997) Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. *Exp. Cell Res.* **233**, 63-77
32. Hannan, R., Kourembanas, S., Flanders, K., Rogelj, S., Roberts, A., Faller, D., and Klagsbrun, M. (1988) Endothelial cells synthesize basic fibroblast growth factor and transforming growth factor beta. *Growth Factors* **1**, 7-17
33. Wakabayashi, Y., Shono, T., Isono, M., Hori, S., Matsushima, K., Ono, M., and Kuwano, M. (1995) Dual pathways of tubular morphogenesis of vascular endothelial cells by human glioma cells: vascular endothelial growth factor/basic fibroblast growth factor and interleukin-8. *Jpn. J. Cancer Res.* **86**, 1189-1197
34. Jimi, S., Ito, K., Kohno, K., Ono, M., Kuwano, M., Itagaki, Y., and Ishikawa, H. (1995) Modulation by bovine angiogenin of tubular morphogenesis and expression of plasminogen activator in bovine endothelial cells. *Biochem. Biophys. Res. Commun.* **211**, 476-483
35. Ment, L., Stewart, W., Fronc, R., Seashore, C., Mahooti, S., Scaramuzzino, D., and Madri, J. (1997) Vascular endothelial growth factor mediates reactive angiogenesis in the postnatal developing brain. *Brain Res. Dev. Brain Res.* **100**, 52-61
36. Tan, Y. (1998) Basic fibroblast growth factor-mediated lymphangiogenesis of lymphatic endothelial cells isolated from dog thoracic ducts: effects of heparin. *Jpn. J. Physiol.* **48**, 133-141
37. Koolwijk, P., van Erck, M.G.M., de Vree, W.J.A., Vermeer, M.A., Weich, H.A., Hanemaaijer, R., and van Hinsbergh, V.W.M. (1996) Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J. Cell Biol.* **132**, 1177-1188
38. Ilan, N., Mahooti, S., and Madri, J. (1998) Distinct signal transduction pathways are utilized during the tube formation and survival phases of *in vitro* angiogenesis. *J. Cell Sci. Dec.* **18**, 3621-3631
39. Kireeva, M.L., Lam, S.C.-T., and Lau, L.F. (1998) Adhesion of human umbilical vein endothelial cells to the immediate-early gene product cyr61 is mediated through integrin $\alpha_v\beta_3$. *J. Biol. Chem.* **273**, 3090-3096
40. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Grotendorst, G.R., and Takehara, K. (1995) Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J. Invest. Dermatol.* **105**, 280-284
41. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G.R., and Takehara, K. (1996) Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J. Invest. Dermatol.* **106**, 729-733
42. Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* **325**, 257-259
43. Babic, A.M., Kireeva, M., Kolesnikova, T.V., and Lau, L.F. (1998) CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc. Natl. Acad. Sci. USA* **95**, 6355-6360
44. Oemar, B.S., Werner, A., Garnier, J.-M., Do, D.D., Godoy, N., Nauck, M., März, W., Rupp, J., Pech, M., and Lüscher, T.F. (1997) Human connective tisssue growth factor is expressed in advanced atherosclerotic lesions. *Circulation* **95**, 831-839