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# Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases

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**Connective tissue growth factor (CTGF) is known to be a potent angiogenic factor. Here we investigated how CTGF and matrix metalloproteinases (MMPs) are involved in the early stage of hypoxia-induced angiogenesis using human breast cancer cell line, MDA231, and vascular endothelial cells. Hypoxic stimulation (5% $O_2$) of MDA231 cells increased their steady-state level of *ctgf* mRNA by ~2-fold within 1.5 h, and the levels remained at a plateau up to 6 h, and then decreased by 12 h as compared with the cells cultured under the normoxic condition. Membrane-type 1 MMP (MT1-MMP) mRNA levels was also increased within a few hours of the exposure to hypoxia. Indeed, ELISA revealed that the CTGF protein/cell in medium conditioned by MDA231 cells exposed to hypoxia was maximally greater at 24 h than in the medium from normoxic cultures and that the secretion rate (supernatant CTGF/cell layer CTGF) increased in a time-dependent manner from 24 to 72 h of hypoxic exposure. Hypoxic induction of CTGF was also confirmed by immunohistochemical analyses. Furthermore, zymogram analysis revealed that the production of active MMP-9 was also induced in MDA231 cells incubated under hypoxic conditions. Finally, we found that recombinant CTGF also increased the expression of a number of metalloproteinases that play a role in the vascular invasive processes and decreased the expression of tissue inhibitors of metalloproteinases by vascular endothelial cells. These findings suggest that hypoxia stimulates MDA231 cells to release CTGF as an angiogenic modulator, which initiates the invasive angiogenesis cascade by modulating the balance of extracellular matrix synthesis and degradation via MMPs secreted by endothelial cells in response to CTGF. This cascade may play critical roles in the hypoxia-induced neovascularization that accompanies tumor invasion *in vivo*.**

## Introduction

The growth of new blood vessels from the pre-existing vascular tree, which is designated as angiogenesis, occurs in situations such as wound and fracture healing, arthritis, cardiovascular and cerebral ischemia and almost every type of cancer (1). Angiogenesis is an invasive process that requires the lysis of the extracellular matrix (ECM), and proliferation and migration of endothelial cells followed by the synthesis of new matrix components (2).

This process is regulated by a balance between positive and negative angiogenic factors, and is disregulated in various diseases, especially in cancers (1,3,4). Therefore, an understanding of the cellular events involved in angiogenesis and their molecular basis is of great therapeutic importance for the treatment of malignancies.

Connective tissue growth factor (CTGF) is a cysteine-rich secretory protein of 36–38 kDa, which is composed of 349 amino acid residues, and its gene belongs to the CCN family, which consists of *cef10/cyr61*, *ctgf/fisp12* and *nov*, as well as several recently reported genes such as *elm1/wisp1*, *ctgf-3/ctgf-L/wisp-2/cop1* and *wisp-3* (5–10). Because of its ability to induce the expression of the ECM molecules, CTGF has been proposed to play an important role in connective tissue cell proliferation and ECM deposition as one of the mediators of transforming growth factor-β (TGF-β) (11). Recently, novel integrated functional aspects of CTGF were uncovered through our research. We found that *ctgf* was highly expressed in human chondrocytic HCS-2/8 cells and hypertrophic chondrocytes in growth cartilage (10,12). Indeed, recombinant CTGF promoted the proliferation and differentiation of chondrocytes (10,13), osteoblasts (10,14) and vascular endothelial cells (10,15) in culture. Furthermore, CTGF has been suggested to be involved in angiogenesis at the last stage of endochondral ossification (15,16). During this stage, CTGF may act on vascular endothelial cells and osteoblasts in bone that is close to the hypertrophic zone of cartilage. Also of note, CTGF was shown to be highly up regulated in certain tumors to play a pivotal role in the control of the proliferation and migration of endothelial cells—the essential components of a new blood vessel (17,18). Therefore, CTGF has been considered to be one of the most important regulators of both physiologic as well as pathologic angiogenesis.

The matrix metalloproteinases (MMPs) comprise a family of extracellular endopeptidases that selectively degrade components of the ECM. There are now known to be at least 20 structurally related members in this family, of which molecules are either secreted (collagenases, gelatinases, stromelysins) or membrane-bound (MT-MMPs). The collagenases, which include interstitial collagenase (MMP-1), possess a unique capacity to cleave native triple helical collagen types I, II and III (19). The stromelysins, which include stromelysin-1 (MMP-3) and matrilysin (MMP-7), have broad substrate specificity and are able to degrade proteoglycans, laminin, fibronectin and the non-helical domains of collagen types IV and IX (20). Two metallogelatinases, MMP-2 (gelatinase A, 72-kDa type-IV collagenase) and MMP-9 (gelatinase B, 92-kDa type-IV collagenase) are thought to play a key role in promoting tumor

**Abbreviations:** BFGF, basis fibroblast growth factor; CTGF, connective tissue growth factor; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cells; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP; TIMP, tissue inhibitors of metalloproteinase; UTR, untranslated region; VEGF, vascular endothelial growth factor.

invasion and tissue remodeling by inducing the proteolysis of several ECM components (21,22). This idea arose from the finding that a number of malignant tumor cells secrete large amounts of type-IV collagen-degrading enzymes (22). In addition, membrane-type 1 metalloproteinase (MT1-MMP) is another such key enzyme among MMPs, and its overexpression seems to have a significantly beneficial effect on tumor growth (23). MT1-MMP has been shown to have an important role in MMP-2 activation on cell membranes. Recent studies indicated that host-derived gelatinase A (MMP-2) binds to cell-surface proteins MT-MMP (24) and integrin $a_v\beta_3$ (25) of tumor or endothelial cells, this interaction may modulate the behavior of these cells. One of the initial steps in the angiogenic process is the degradation of the subendothelial basement membrane and surrounding ECM. This process is mediated by ECM-degrading proteolytic enzymes, including MMPs. Following matrix breakdown, they function to promote angiogenesis by regulating endothelial cell attachment, proliferation, migration and growth, either directly or indirectly through the release of growth factors stored within the ECM (26–30). Such interactions among growth factors and MMPs, all of which are key modulators of angiogenesis and tumor progression, are suspected.

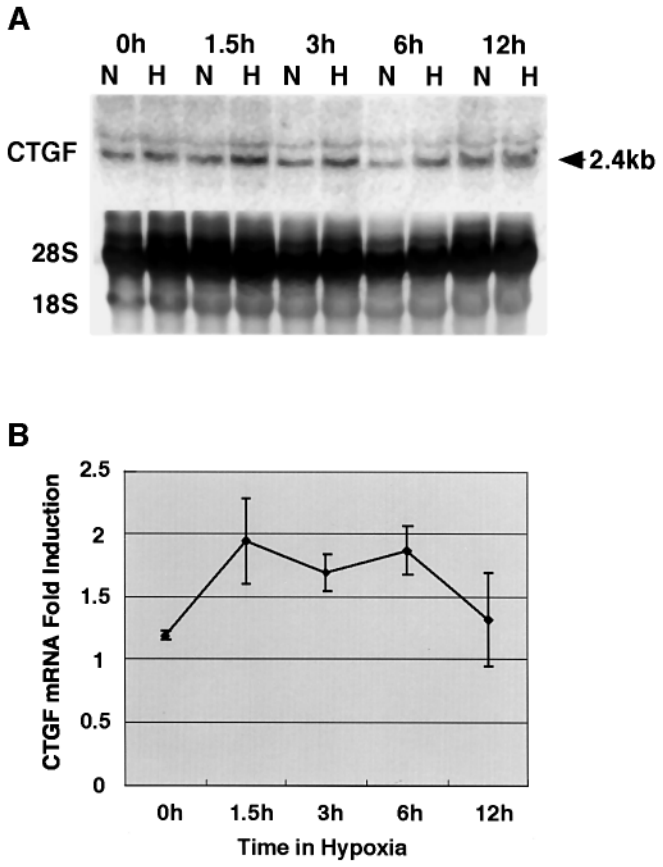



**Fig. 1.** Hypoxia-enhanced CTGF expression in MDA231 cells. (**A**) MDA231 cells were exposed to normoxic (N) or hypoxic (H) conditions for the indicated times. Total RNA was extracted and analyzed by northern blotting for *ctgf* mRNA expression. Hybridization signals for CTGF in the autoradiogram and signals for rRNA in the methylene blue-stained membrane are displayed. (**B**) Quantification of the fold-induction of *ctgf* mRNA signal at the indicated times. Relative fold-induction by hypoxic versus normoxic conditions is comparatively shown. Mean values of the results of two experiments are displayed with error bars (standard deviations).

In the present study, we investigated (i) how CTGF is involved in early stage of hypoxia-induced angiogenesis using human breast cancer cell line, MDA231 and (ii) whether or not MMPs are related to the hypoxia-induced angiogenesis as well as CTGF production. A possible model of the invasion of certain tumors is illustrated herein.

## Materials and methods

*Cell culture and hypoxic condition*

MDA231 (MDA-MB-231) cells derived from a human breast cancer were obtained from the American Type Culture Collection (Rockville, MD). They were cultured at 37°C and 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) derived from human umbilical cords were purchased from Biowhittaker (Walkersville, MD) and used between passages three and seven. These cells were cultured in EBM-2 complete medium (Clonetics, San Diego, CA). Before use in experiments, HUVECs were maintained in an EBM-2 medium without hydrocortisone for at least 24 h. The removal of hydrocortisone was necessary because it inhibits metalloproteinase production. Once passed and plated, endothelial cells grew normally even in the absence of hydrocortisone. Hypoxia experiments were performed for the indicated times in a humidified triple gas model BL-40M incubator (BIO-LABO, Tokyo, Japan) calibrated to deliver 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 37°C.

*Plasmid constructs and DNA transfection*

We had constructed a firefly luciferase-*ctgf* chimeric gene construct, pGL3UTRS, as described by Kubota *et al.* (31). A CTGF promotor-derived firefly luciferase expression plasmid was obtained from Japan Tabacco (Yokohama, Japan) (32). Twenty hours prior to transfection, $2\times10^5$ MDA231 cells were seeded in a 35 mm tissue culture dish. Liposome-mediated DNA transfection was performed with 1 μg of each pGL3 derivative in combination with 0.5 μg of pRL-TK (internal control; Promega, Madison, WI), according to the manufacturer's methodology (LipofectAMINE; Lifetechnologies, Rockville, MD). Twenty-four hours after transfection, they were placed under the normoxic or hypoxic condition for 6 h. The cells were lysed in 500 μl of a passive lysis buffer (Promega), and the cell lysate was directly used in the luciferase assay system, as described below (31).

*Luciferase assay*

The Dual Luciferase System (Promega) was used for the sequential measurement of firefly and *Renilla* luciferase activities with specific substrates of beetle luciferin and coelenterazine, respectively. Quantification of both luciferase activities and calculation of relative ratios were carried out manually with a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA).

*Collection of conditioned medium and total RNA*

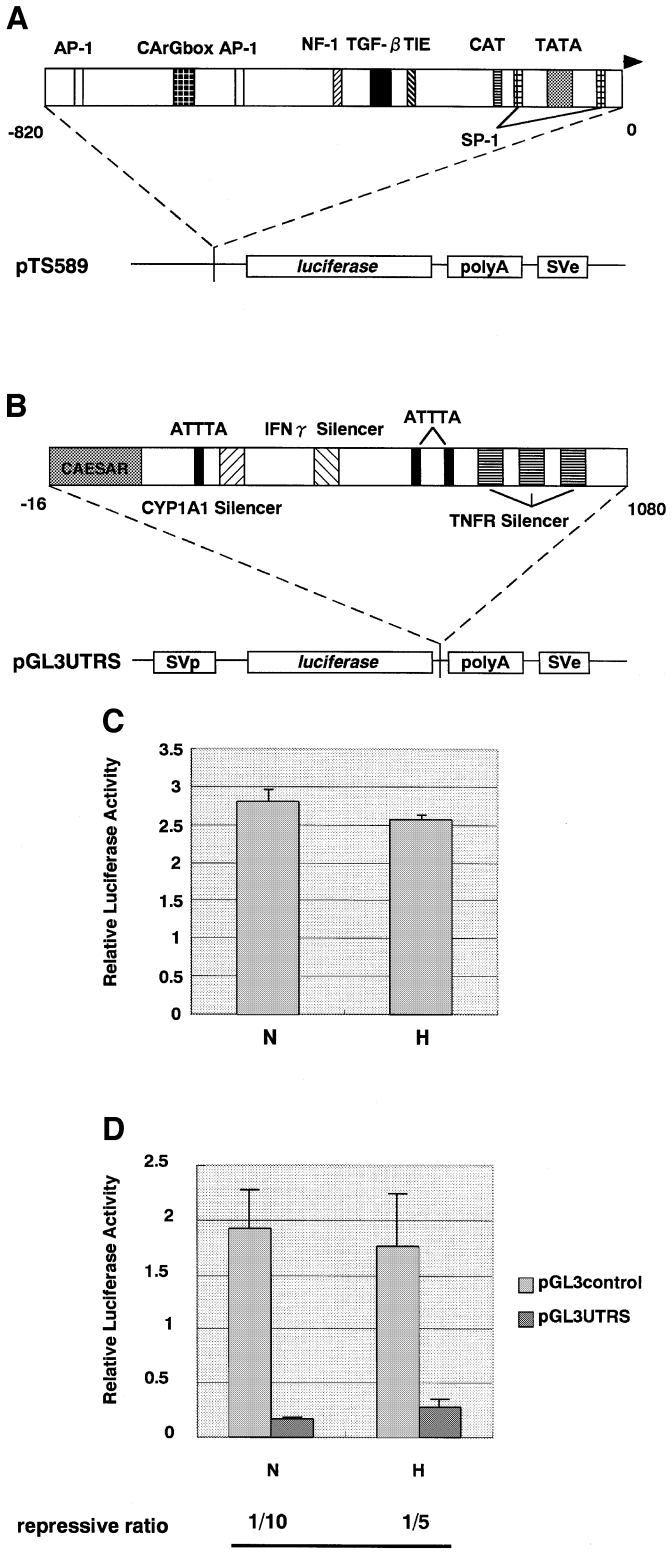
The MDA231 cells were inoculated at a density of $1\times10^6$ cells in 10 cm diameter dishes and cultured for 60 h in DMEM containing 10% FBS. The cells were replenished with fresh medium and further cultured for 24 h. Finally, they were replenished with fresh medium and placed under the normoxic or hypoxic condition for the desired times. The cell culture supernatant and cell layer fraction were harvested for subsequent analysis. Total cellular RNA was harvested as described in the next subsection. HUVECs were inoculated at a density of $8\times10^5$ cells in 10 cm diameter dishes and cultured in EBM-2 complete medium for 48 h. The cells were then maintained in EBM-2 medium without hydrocortisone for 24 h and thereafter placed in a hydrocortisone-free, serum-free EBM-2 medium with or without CTGF (50 ng/ml). Total cellular RNA was harvested at various times.

*Northern blot analysis*

Total cellular RNA was isolated from MDA231 cells by using Trizol reagent (Lifetechnologies), and the obtained RNA was treated with DNase I (Promega) for 30 min at 37°C. Fifty micrograms of total cellular RNA was subjected to electrophoresis on 1% formaldehyde–agarose gel and transferred onto a Hybond-N filter (Amersham Pharmacia Biotech, Aylesbury, UK). Full-length human *ctgf* cDNA was used as a probe. cDNA probes for MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MT1-MMP, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 were obtained by reverse transcriptase–polymerase chain reaction (RT–PCR) amplification. Total RNAs for such preparative RT–PCR were prepared from the following human cultured cells: endothelial cells (HUVEC) for MMP-1, MMP-2, TIMP-1 and TIMP-2; cervical carcinoma cells (HeLa) for MMP-7; melanoma cells (A375) for MMP-3; chondrosarcoma cells (HCS2/8) for MMP-9. Adequate synthetic oligonucleotide pairs were prepared as specific primers: MMP-1, 5′-cgactctagaaacacaagagcaaga-3′ and 5′-aaggttagcttactgtcacacgctt-3′ (product size, 786 bp); MMP-2, 5′-ccac-

gTgagaagcccaTggggcccc-3′ and 5′-gcagccTagccagcagTcggaTTTgaTg-3′ (product size, 486 bp); MMP-3, 5′-acgggctcctggcacacg-3′ and 5′-cgtcccgggtgtagagtc-3′ (product size, 729 bp); MMP-7, 5′-ggtcacctacaggatcgtatcatat-3′ and catc- actgcattaggatcagaggaa-3′ (product size, 373 bp); MMP-9, 5′-acgggctcctggcacacg-3′ and 5′-cgtcccgggtgtagagtc-3′ (product size, 288 bp); MT1-MMP, 5′-tggacacggagaattttgtgctg-3′ and 5′-ggccccgccagaactggccaatg-3′ (product size, 300 bp); TIMP-1, 5′-caattccgacctcgtcatca-3′ and 5′-tcagagccttggaggagct-3′ (product size, 429 bp); TIMP-2, 5′-agatgtagtgatcagggcca-3′ and 5′-aggagccgtcacttctctt-3′ (product size, 479 bp).

Each probe was radiolabeled with [α-$^{32}$P]deoxycytidinetriphosphate (dCTP) by using a random primer DNA labeling kit (Takara Shuzo Co., Tokyo, Japan). Membranes were pre-hybridized in ULTRAhyb solution (Ambion, Austin, TX) at 42°C for 30 min. Hybridization was performed overnight at 42°C with $1\times10^7$ c.p.m./ml of $^{32}$P-labeled probe. The hybridized membrane was washed twice for 15 min each time at 42°C in 2× sodium citrate–sodium chloride (SSC) buffer containing 0.1% sodium dodecyl sulfate (SDS), and twice for 15 min each time at 42°C in 0.1× SSC containing 0.1% SDS. The filter was then exposed to an X-ray film at –70°C.




*Enzyme-linked immunosorbent assay*

Enzyme-linked immunosorbent assay (ELISA) was carried out as described elsewhere (33). Briefly, a sandwich ELISA system with two different anti-human CTGF monoclonal antibodies (MHCT1 and MHCT2; mouse IgG1) was developed. ELISA plates (Greiner, Frickenhausen, Germany) were coated for 1 h with MHCT1 monoclonal antibody at 10 μg/ml in phosphate-buffered saline (PBS) at room temperature. Next, equivalent amounts of samples were added to the wells, and the plate was incubated for 1 h. After the plate had been washed with 0.05% Tween 20 in PBS (PBS-T), biotinylated MHCT2 monoclonal antibody at 2 μg/ml in 1% bovine serum albumin (BSA)–PBS-T was added, and incubation was carried out for 1 h. After the addition of streptavidin–β-galactosidase (Lifetechnologies) and its specific substrate, the fluorescence intensity of the wells was determined at 460 nm (excitation 360 nm) by a Cytoflor 2300 system (Millipore, MA).

*Immunofluorescence analysis*

After growth on glass coverslips, MDA231 cells were incubated for 24 h under the normoxic or hypoxic condition. Then the cells were washed with PBS and fixed 20 min with PBS containing 3.5% formaldehyde, and then permeabilized in 0.1% NP-40 in PBS. After the fixed cells had been washed three times with PBS, they were incubated for 1 h with an anti-CTGF serum (1/100) in 3% BSA–PBS. Then the cells were washed three times with PBS and thereafter reacted with an FITC-conjugated anti-rabbit IgG serum in 3% BSA–PBS for 1 h. The cells on the coverslips were finally washed three times with PBS, and the coverslips were mounted on glass slides and viewed under an epifluorescence microscope (Olympus, Tokyo, Japan).

*Gelatin zymography*

MDA231 cells were cultured as described under subsection 'Collection of conditioned medium and total RNA', and then the conditioned medium was harvested and lypophilized. Equivalent amounts of samples were dissolved in a Tris–HCl buffer containing 30% glycerol, 7.7% SDS and 0.3% bromophenol blue at pH 6.8. The samples were subjected to SDS–PAGE using a 10% gel containing 0.1% gelatin. After electrophoresis, the gel was soaked in 50 mM Tris–HCl (pH 7.5) containing 2.5% Triton X-100 at room temperature with gentle shaking for 2 h, and then incubated for 72 h in 50 mM Tris–HCl (pH 7.5) containing 5 mM $CaCl_2$ and 0.2 M NaCI at 37°C. The gels were then stained with Coomassie Brilliant Blue.

*Sequence analysis software*

To predict putative *cis*-acting elements in the *ctgf* 3′-untranslated region (UTR) and promoter, we used GENETYX ver. 8.0 analysis software (Software Development Co., Tokyo, Japan).

**Fig. 2.** Molecular constructs for the evaluation of the (**A**) promoter activity and (**B**) repressive effect of the 3′-UTR in the CTGF gene. Experimentally proven and putative *cis*-elements are illustrated. Abbreviations: SVp, SV40 promoter; SVe, SV40 enhancer; poly A, SV40 polyadenylation signal; luciferase, firefly luciferase gene; CAESAR, *cis*-acting element of structure-anchored repression (36). Numbers indicate nucleotide positions on the sense strands counted from the transcription initiation site on the genomic DNA (A), or the junction between the coding region and 3′-UTR on the cDNA (B). (**C**) Effects of hypoxia on the CTGF promoter activity MDA231 cells were co-transfected with 1 μg of the CTGF promoter-luciferase reporter plasmid (pTS589) and 0.5 μg of *Renilla*-luciferase reporter plasmid (pRL-TK, internal control), and then allowed to recover for 24 h after transfection. After recovery, the transfected cells were exposed to hypoxia or maintained in normoxia for 6 h, and the cells were then assayed for luciferase activity. Mean values of the results of four experiments are displayed with error bars (standard deviations). (**D**) Repressive effect of *ctgf* 3′-UTR under hypoxic conditions. The whole 3′-UTR was fused to luciferase cDNA in pGL3-control (Promega) (B). The resultant plasmid, pGL3UTRS, or pGL3-control and pRL-TK were used to co-transfect MDA231 cells, which were then placed under the normoxic (N) or hypoxic (H) condition for 6 h. Mean values of the results of four experiments are displayed with error bars (standard deviations). Repressive ratio by the *ctgf* 3′-UTR under normoxic or hypoxic conditions is described at the bottom of the panel.

## Results

*Hypoxia increases steady-state ctgf mRNA in MDA231 cells*

The human breast cancer cell line MDA231 was selected for study of the regulation of the CTGF expression by hypoxia, because MDA231 cells were found to secrete CTGF under hypoxic conditions (17). Here we found that hypoxic treatment of MDA231 cells along a time course of 12 h resulted in significant CTGF mRNA enhancement between 1.5 and 6 h (Figure 1A). Hypoxic enhancement of steady-state *ctgf* mRNA in MDA231 cells was maximally upregulated at 1.5 h, with the level being ~2-fold higher than that under normoxia (Figure 1B). Such a transient enhancement may reflect its role in an early stage of angiogenesis rather than in later events, such as adaptive response or neovascularization. These findings suggest that CTGF may be one of the key modulators in initiating the angiogenic cascade in MDA231 cells.

*CTGF gene enhancement by hypoxia in MDA231 cells is not due to the activation of the CTGF promoter, but to liberation from repressive regulation by the ctgf 3′-UTR*

As shown in Figure 2A, in the first 820 bp of the CTGF promoter sequence, a cluster of different putative binding sites for transcription factors, including hypoxia-inducible AP-1, exists (34,35). However, we therein failed to find hypoxia-response elements (HRE) that mediate mostly the transcriptional response to cellular hypoxia. Additionally, Figure 2B shows several putative negative regulatory elements, including RNA destabilizers (ATTTA) and silencers involved in the 3′-UTR, as well as CAESAR, which was proven experimentally to be a post-transcriptional structured RNA regulatory element (36). To elucidate whether the CTGF promoter region could mediate transcriptional responses to cellular hypoxia or not, the activity of the CTGF promoter under hypoxic conditions was first evaluated with a corresponding reporter plasmid. In this plasmid, the CTGF promoter sequence from –820 to 0 (transcription initiation site) was fused to the firefly luciferase coding sequence. MDA231 cells were co-transfected with this reporter plasmid, pTS589 and the pRL-TK plasmid, which constitutively expresses *Renilla* luciferase under the control of herpes simplex virus thymidine kinase (HSV-TK) promoter and was used to correct for variable transfection efficiency. As the effects of hypoxia on CTGF transcription was relatively early (⩽6 h; Figure 1B), the promoter activity was analyzed within such a time scale. After having been exposed to hypoxia for 6 h, the transfected cells were assayed for luciferase activities. As shown in Figure 2C, reporter gene expression in response to hypoxia was somewhat decreased rather than increased compared with that in response to normoxia. This result demonstrates that the activation of the CTGF promoter was not the cause of the CTGF gene enhancement by hypoxia. Similar results were obtained under serum-deprived conditions as well (data not shown).

Next we comparatively analyzed the repressive regulation by *ctgf* 3′-UTR under normoxic and hypoxic conditions. Previously, we constructed a plasmid that expressed a chimeric luciferase-*ctgf* 3′-UTR mRNA and named it pGL3UTRS (31). The pGL3UTRS, or its parental pGL3 control was used to transfect into MDA231 cells, and the cells were then placed under the normoxic or hypoxic condition for 6 h. *ctgf* 3′-UTR showed significant and reproducible differences in the repressive effect on luciferase gene expression between the two conditions (see Figure 2D). The repressive potential under hypoxic conditions diminished by 2-fold as compared with that in the cells exposed to normoxia. According to the findings obtained above, the increased steady-state level of *ctgf* mRNA in hypoxia-treated MDA231 cells was mostly a result of the liberation from repressive regulation by CTGF 3′-UTR.

*Hypoxia increases the production of CTGF protein in MDA231 cells*

To evaluate whether hypoxic MDA231 cells produced a greater amount of CTGF protein, we first performed a sandwich ELISA to quantify CTGF in the culture medium and cell lysate. The MDA231 cells were cultured as described under subsection ‘Collection of conditioned medium and total RNA’, and then the cell culture supernatant and cell layer fraction were harvested.

As shown in Figure 3A, the CTGF levels (per cell) in supernatants from cultures of MDA231 cells exposed to 24 h of hypoxia were significantly increased compared with those for normoxic MDA231 cells. After 24 h, the difference in CTGF level between hypoxic and normoxic conditions gradually decreased in a time-dependent manner. However, the secretion rate (supernatant CTGF/cell layer CTGF) increased in a time-dependent manner (Figure 3B) under the hypoxic condition, maintaining a high level of free CTGF even after prolonged hypoxic stress. The data were standardized as CTGF secreted/cell, which might appear to represent reduced CTGF production along the time course. However, because of active cell proliferation under both normoxic and hypoxic conditions, total CTGF production actually increased in a time-dependent manner. Finally, we performed immunohistochemical staining of MDA231 cells after 24 h of hypoxia, and found cell-associated CTGF to be more abundant than that under normoxia (Figure 3C). The CTGF-specific signals were distributed abundantly in the cytoplasm with stronger signal intensity in hypoxic cells than in the normoxic cells. Negative controls prepared with pre-immune rabbit serum (PI) showed faint background staining.

*Hypoxia increases mt1-mmp mRNA in MDA231 cells*

Among MMPs, MT1-MMP is also thought to be a potent regulator for neovascularization (23). Thus, we proceeded to determine whether MT1-MMP expression could be up regulated in MDA231 cells in response to hypoxic culture conditions, or not. As shown in Figure 4, *mt1-mmp* mRNA levels displayed a similar tendency toward increase during a few hours of hypoxic exposure as found for the *ctgf* mRNA level. It is worthy of note that the enhanced *mt1-mmp* gene expression sustained until 12 h of hypoxic exposure, unlike the case for *ctgf*. These findings suggest that MT1-MMP is also one of the key modulators to induce angiogenic phenotype during angiogenesis.

*Hypoxia induces MMP-9 secretion from MDA231 cells*

As MT1-MMP has been shown to play an important role in MMP-2 activation on cell membranes, we anticipated that the production of active MMP-2 might be increased in hypoxic MDA231 cells in comparison with that in cells under normoxia. To examine the validity of our hypothesis, we carried out gelatin zymography. As a result, we observed predominant proteolytic bands migrating at molecular weights that were consistent with their identification as MMP-9 species. Of importance, levels of the proteolytic activities were remarkably greater in hypoxic than in normoxic MDA231 cell samples taken at 48 and 72 h (Figure 5). However, MMP-2 activity was not detected in MDA231 cells, regardless of the culture condition.

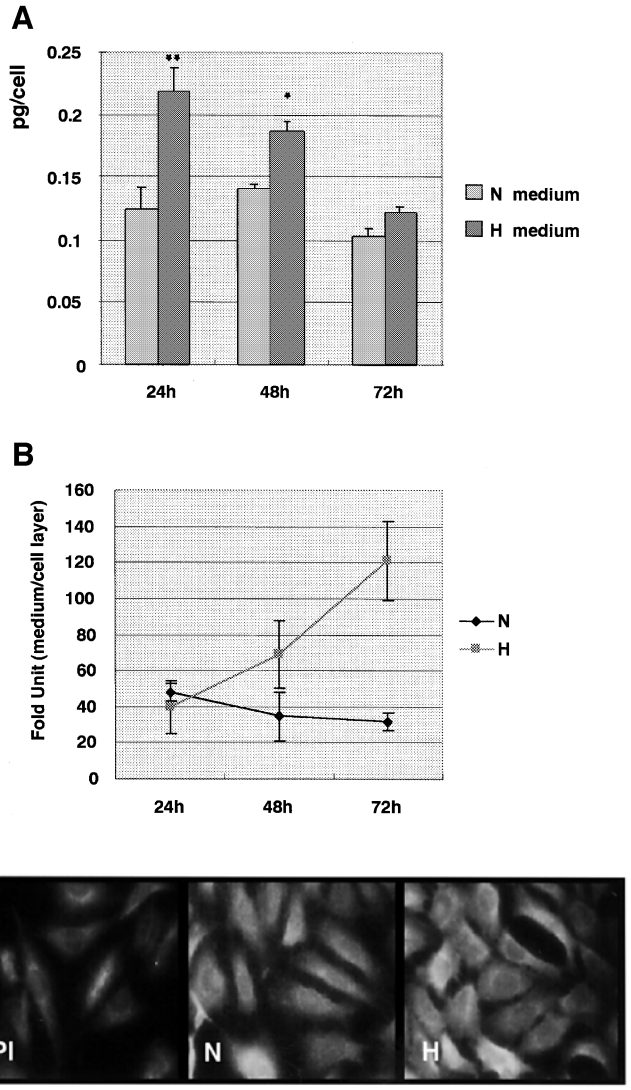



**Fig. 3.** Hypoxia-increased CTGF production by MDA231 cells. (**A**) Hypoxia-increased secretion of CTGF. The MDA231 cells were inoculated at a density of $1\times10^6$ cells in 10 cm diameter dishes. After 84 h, the medium was replaced with fresh medium, and the cells were placed under normoxic (N) or hypoxic (H) condition for the indicated times, and then the cell culture supernatant and cell layer fraction were harvested. Each cell culture supernatant was examined for CTGF content by the sandwich ELISA system (33). CTGF production is represented by the production from a single cell. Asterisks indicate significant differences from normoxia ($*P < 0.05$, $**P < 0.01$). Results are presented as the means ± SD for duplicates. (**B**) Secretion rates. Secretion rates stand for relative values of secreted/cell-associated CTGF as measured by the ELISA. The secretion rate increased in a time-dependent manner under the hypoxic condition. Results are presented as the means±SD for duplicates. (**C**) Hypoxic MDA231 cells accumulate CTGF protein within 24 h. MDA231 cells were maintained in a normoxic environment (N) or exposed to hypoxia (H) for 24 h. Cell-associated CTGF protein was detected using a rabbit anti-CTGF peptide polyclonal serum. Normoxic MDA231 cells were faintly immunopositive. Increased intracellular CTGF protein was evident at 24 h of hypoxia. Specificity of CTGF signals was confirmed by use of a pre-immune serum from the same rabbit used to produce the primary antibody (PI), which serum gave faint background signals.

### *CTGF increases the expression of a number of MMPs but not TIMP-1 and TIMP-2 mRNAs in HUVECs*

Hypoxic enhancement of CTGF production in MDA231 cells was demonstrated clearly. Upon angiogenesis, the release of endothelial cells from their basement membrane and migration through the surrounding ECM may also require the action of MMPs. Thus, we next examined the paracrine role of CTGF

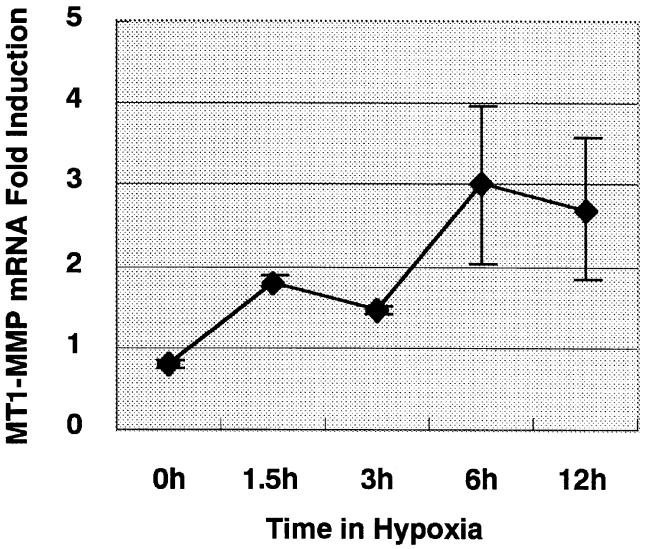



**Fig. 4.** Hypoxia-enhanced *mt1-mmp* expression in MDA231 cells. MDA231 cells were exposed to the normoxic or hypoxic condition for the indicated times. Relative fold-induction by hypoxic versus normoxic conditions is comparatively shown. Mean values of the results of two experiments are displayed with error bars (standard deviations). The *mt1-mmp* mRNA levels displayed a similar increase as seen for *ctgf* mRNA within a few hours of exposure to hypoxia, but the increased expression was sustained longer.

in regulating the level of MMPs and TIMPs from vascular endothelial cells *in vitro*. According to a previous finding, the maximal mitogenic effect on endothelial cells occurred at a dose of 50 ng/ml CTGF (15). Therefore, this concentration was chosen for our experiments. As shown in Figure 6A, CTGF at this dose increased the steady-state mRNA levels of MMP-2, -3, -9 and MT1-MMP mRNA over a 24 h incubation. Significant increases in the transcript levels were seen within 6 h after the addition of CTGF. Transcripts encoding MMP-1 and -7 were also enhanced modestly, but significantly. In contrast, CTGF at a dose of 50 ng/ml reduced the expression of TIMP-2 and, albeit modestly TIMP-1 transcripts within 3 h, but did not affect either mRNA level with prolonged incubation for 24 h compared with the effect of PBS on their expression (Figure 6B). Coupled with the increase in MMP levels, the decrease in TIMP levels might lead to increased proteolysis of the ECM, thus promoting vascular endothelial cell migration.

## Discussion

Unlike normal cells, tumor cells are often forced into hypoxic conditions (37), which usually trigger angiogenesis for further tumor progression through activated angiogenic molecules. Normally, angiogenesis is regulated by a balance between positive and negative angiogenic factors and is derailed in various diseases, especially cancers (1–3). Therefore, understanding the molecular regulation for the cellular events involved in hypoxic angiogenesis would be expected to shed light on tumor progression mechanisms. Toward this objective, we designed and carried out a series of *in vitro* experiments on hypoxia using tumor cell lines to mimic the condition of the tumor growth *in vivo*.

CTGF is a novel, potent angiogenic factor (15,16,38). According to our recent research (10), purified rCTGF promoted the adhesion, proliferation and migration of the vascular endothelial cells in a dose-dependent manner, and the effects were inhibited efficiently by anti-CTGF antibody. rCTGF also

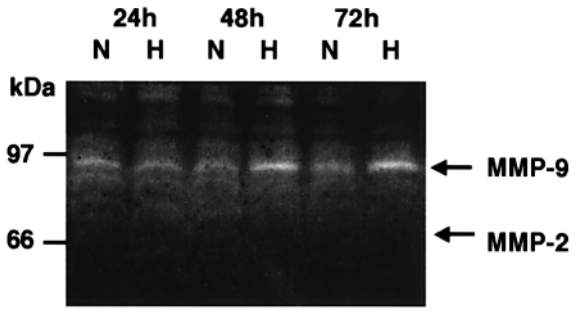



**Fig. 5.** Increased MMP-9 activity from hypoxic MDA231 cells in comparison with that of cells cultured under normoxia. Gelatin zymography revealed predominant proteolytic bands migrating at molecular weights indicating them to be MMP-9 species. Levels of the proteolytic activity were greater in the conditioned medium from hypoxic MDA231 cells than in that from normoxic cells. MMP-2 activity was not detected under hypoxic or normoxic condition.

induced the tube formation of vascular endothelial cells. Moreover, application of rCTGF to the chicken chorioallantoic membrane CAM resulted in a prominent angiogenic response (15). Here, our present study demonstrated that hypoxia caused a significant increase in *ctgf* mRNA expression in MDA 231 cells. The steady-state *ctgf* mRNA expression was maximally up regulated within 1.5 h by hypoxia, becoming ~2-fold higher than that in cells cultured in the normoxic state. These findings suggest CTGF to be one of the key modulators in MDA231 cells to turn on the angiogenic phenotype at an early stage of angiogenesis.

Analogous to CTGF, VEGF is also an important mediator of hypoxic angiogenesis (39). Hypoxia increases VEGF expression by stabilizing *vegf* mRNAs and by activating gene transcription in certain cell lines (40,41). Hypoxia-inducible factor 1 (HIF-1) (42), a basic helix–loop–helix heterodimer consisting of HIF-1α and the aryl hydrocarbon nuclear translocator (43) is activated by hypoxia and binds to specific HRE in the *vegf* gene (41,44). Although hypoxia certainly conferred a higher steady-state *ctgf* mRNA level in MDA231 cells, we failed to find HRE sequences in the CTGF promoter. Consistent with these findings, no response of the CTGF promoter to hypoxia was demonstrated in our luciferase reporter assays. On the other hand, liberation from the repression by the CTGF 3′-UTR was observed under hypoxic conditions. It was shown that the change in the repressive potential between normoxic and hypoxic conditions precisely corresponded to the hypoxia-enhanced increase of *ctgf* mRNA levels (~2-fold) observed by Northern blot analysis. Of note, the 3′-UTRs of a variety of genes has been shown to contain a number of elements that are critical for determining the mRNA stability (45). Thus, although we cannot rule out that other transcriptional regulatory elements may exist in other *ctgf* gene segments, we assume that hypoxia-enhanced *ctgf* expression may be mostly due to the 3′-UTR-mediated increase of mRNA stability in MDA231 cells. Therefore, it is of our interest to search for certain *cis*-acting elements involved in this hypoxic control of gene expression.

The findings in this study demonstrate that MDA231 cells under the hypoxic condition are capable of expressing *mt1-mmp* mRNA and secreting MMP-9. These MMPs function to promote angiogenesis by regulating endothelial cell attachment, proliferation, migration and growth, either directly or indirectly by the release of growth factors such as CTGF, VEGF and BFGF stored within the ECM (26–30). To the contrary, recent reports have demonstrated that the synthesis of many MMPs

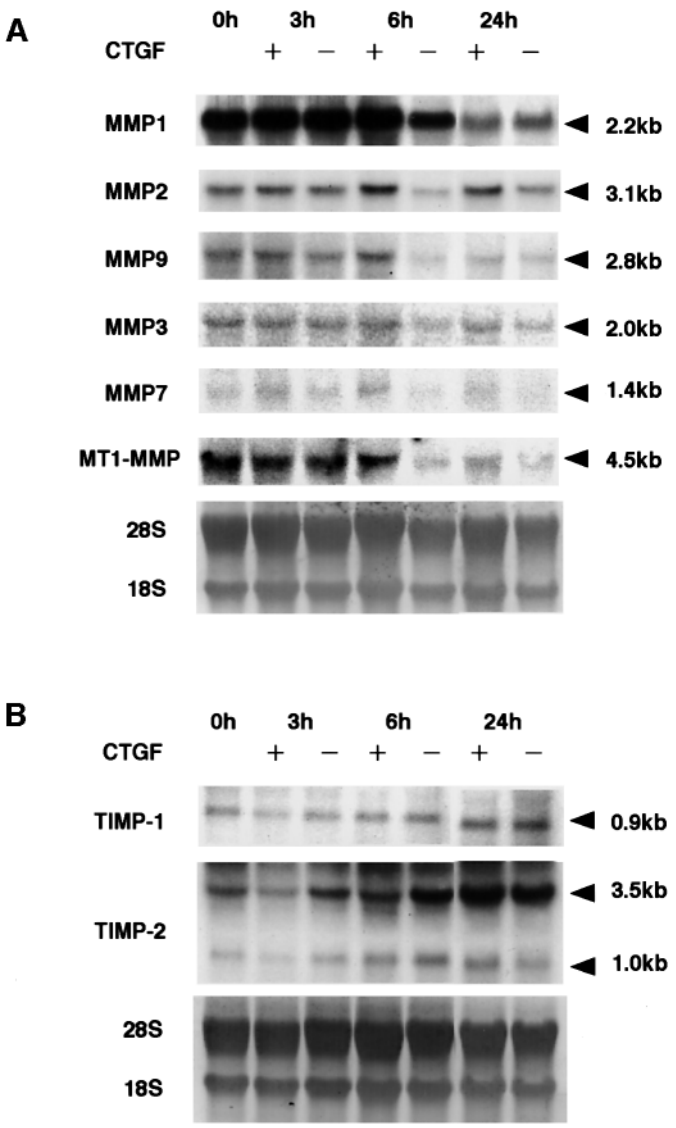



**Fig. 6.** Northern blot assays showing the levels of MMP and TIMP mRNAs in HUVECs treated with CTGF. (**A**) Northern blots of *mmp-1*, *mmp-2*, *mmp-3*, *mmp-7*, *mmp-9* and *mt1-mmp* mRNAs (20 μg/lane) extracted from HUVECs cultured in the absence (–, PBS-treated control cells) or presence of CTGF (+, 50 ng/ml) for the indicated time periods are shown. Sizes of transcripts (indicated by arrowheads) are shown at the right. (**B**) Northern blots of *timp-1* and *timp-2* mRNAs (20 μg/lane) extracted from HUVECs cultured in the absence (–, PBS-treated) or presence of CTGF (+, 50 ng/ml) for the indicated time periods are shown. Arrowheads indicate the transcripts, with their sizes shown at the right.

was regulated by vascular growth factors, cytokines and hormones (27,46). In the present study, CTGF protein production from MDA231 cells exposed to the hypoxic condition was maximally up regulated at 24 h as evaluated by ELISA. Whereas the production of MMP-9 was induced under hypoxia for 48 h and 72 h, but not for 24 h. Given that there was a time difference in protein induction between CTGF and MMP-9 under the hypoxic condition, we hypothesized that autocrine CTGF action may be a crucial pathway to induce MMP-9 expression. However, when MDA231 cells were cultured with various concentrations of purified rCTGF (maximal concentration of 100 ng/ml), up regulation of MMP-9 activity was not clearly observed by gelatin zymography (data not shown). Therefore, other mechanisms are thought to be involved in the

observed MMP-9 induction by hypoxia. With respect to the induction of one MMP by another MMP, MT1-MMP on cell membranes initiates the activation cascade by activating MMP-2, which in turn may be able to convert pro MMP-9 to its active form. The rapid and sustained enhancement of *mt1-mmp* gene expression may account for the later induction of MMP-9. Also, increased release of CTGF after 48 h of hypoxic exposure (when its mRNA induction had already ceased) may be ascribed to the matricrine action of MMP-9 to release CTGF from ECM.

Next, we examined a possible paracrine action in which hypoxia stimulates the release of tumor-derived angiogenic factors that stimulate endothelial cells to migrate toward tumor nests and to degrade basement membranes and surrounding ECM proteins. It has been reported that cultured vascular endothelial cells constitutively express MMP-1, MMP-2 and MT1-MMP (47–49) and are induced to express MMP-9 and MMP-3 in response to inflammatory cytokines and a tumor promoter (47,50). In addition, Chen *et al.* (51) showed that CTGF could upregulate MMP expression (MMP-1, MMP-3) in primary human skin fibroblasts. Here, our studies demonstrate for the first time that rCTGF acts on HUVECs to provoke the expression of a number of metalloproteinases that play a role in the invasive processes for neovascularization. The MMP-2 or MT1-MMP gene does not contain any TPA-response element or Ets binding site, and their gene expression is not thought to be increased by cytokine treatment (52,53). Interestingly, we confirmed the upregulation of not only MMP-1, MMP-3 and MMP-9, but also MMP-2 and MT1-MMP genes in response to CTGF treatment. Thus, CTGF-triggered upregulation of the expression of these matrix-degrading enzymes may be a result of transcriptional activation mediated by a network of transcription factors shared by these MMPs. Also, stromelysins (MMP-3, MMP-7) have been reported to be effective endogenous activators of other members of the MMP family (i.e. proMMP-9, proMMP-1) (54,55). It is assumed that CTGF may regulate the pro-enzyme activation at the upstream of the cascade.

MMP activity is tightly regulated by the action of the TIMPs, specifically and physiologically (56). TIMPs inhibit MMP activities through their non-covalent binding to the active forms of MMPs at molar equivalence. In our present study, treatment of vascular endothelial cells with CTGF decreased the levels of TIMP-1 and TIMP-2 within 3 h compared with those in PBS-treated control cells. Such decreases, coupled with the significant increases in the MMP transcripts within 6 h after the addition of CTGF, suggest that CTGF can critically tilt the balance of proteinases and their respective endogenous inhibitors towards increased proteolysis of the ECM, which eventually promotes vascular endothelial cell migration.

Although the precise molecular mechanisms of the sequential induction of CTGF, MMPs and TIMPs remains unclear, these may act synergistically to control the balance between ECM deposition and degradation during angiogenesis. The interplay of these molecules, especially CTGF and MMPs, appears to be very complex. Further investigations are required to obtain a comprehensive view on this point. Also of note, we reported previously that purified rCTGF promoted the adhesion, proliferation and migration of the vascular endothelial cells in a dose-dependent manner (15). Thus, it will be of interest to investigate whether or not the angiogenic proteins secreted from hypoxic cells are sufficient to drive mitogenesis, including adhesion, proliferation and migration.

In conclusion, based on the data provided here we propose that hypoxia stimulates MDA231 cells to release CTGF as an early angiogenic modulator, which promotes the cascade for angiogenesis in the hypoxic environment by modulating the balance of ECM protein synthesis and degradation to shift toward the latter by up regulating MMP levels and down regulating TIMP ones.

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