Novel intracellular effects of human connective tissue growth factor expressed in Cos-7 cells

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Abstract **To clarify the multiple functionality of connective tissue growth factor (CTGF), we examined the effects of nascent CTGF within the cell by transient expression. In Cos-7 cells, expression of human CTGF induced an altered cell morphology. It was associated with an increased cellular DNA content and loose attachment, indicating the cells were in G2/M phase. Overexpression of CTGF did not induce cell growth, whereas recombinant CTGF efficiently stimulated the proliferation extracellularly. These results indicate that intracellular CTGF may act as an antimitotic agent, thus it should also be noted that nascent CTGF was found to accumulate around the central mitotic machinery.**
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Key words: Connective tissue growth factor; Chondrocyte; Cell cycle arrest

1. Introduction

Connective tissue growth factor (CTGF) was designated based on the initial finding that it had chemotactic and mitogenic activity for fibroblasts [1]. It is a cysteine-rich secretory protein of 36–38 kDa, which is composed of 349 amino acid residues [2,3], and belongs to the CCN family, which consists of *ctgf* (human)/*fisp12* (mouse), *cef10* (chick)/*cyr61* (mouse), *nov* (chicken) and several recently reported genes such as *elm1/wisp-1, ctgf-3/ctgf-L/wisp-2/cop1*, and *wisp-3* [2–5].

The biological function of CTGF has been found to be diverse and complex. In addition to the fact that CTGF is expressed during wound healing [6], which may be regarded as the actual outcome in vivo of previously reported chemotactic and mitogenic activities, novel integrated functional aspects of CTGF were uncovered through our research on chondrocyte growth and differentiation. In seeking regulatory factors involved in many stages in the process of endochondral ossification, we isolated a cDNA which is identical to that of CTGF from a human chondrocytic cell line, HCS-2/8 [7–10], and found it to be one of such cellular factors [11]. Since this discovery, we have obtained evidence that CTGF plays a central role in the growth and differentiation of chondrocytes [12]. Namely, differential expression of CTGF mRNA was observed to follow the course of chondrocyte differentiation.

The highest level of CTGF expression was observed at the hypertrophic stage, whereas relatively lower levels of expression were found in earlier stages [11,12]. Stimulation of proliferation and differentiation of chondrocytes is also conducted by CTGF [12]. Furthermore, CTGF was found to be involved in angiogenesis [14,15], which is required in the last stage of endochondral ossification. Also of note, enhancement of CTGF expression by bone morphogenetic protein 2, as well as transforming growth factor-β was observed in HCS-2/8 cells [11]. As such, we are rediscovering CTGF as a potential factor to promote endochondral ossification in many stages, which may be called 'ecogenin'.

The multiple function of CTGF is thought to comprise a combination of several different functional properties. It is known that secreted CTGF stimulates cell growth and other effects on chondrocytes through its specific surface receptor(s) [12,16]. Nevertheless, no investigation has been presented on the intracellular effect and behavior of CTGF in the expressing cells. Thus, in order to elucidate the complex functionality of CTGF, we examined the putative intracellular functions of CTGF before it is released from the cell via transient expression experiments. In the present study, a novel function of CTGF through an alternative intracellular pathway is indicated.

2. Materials and methods

2.1. Molecular clones
The human CTGF eukaryotic expression plasmid, pcDNA3.1(−)-ctgf was described previously [16]. As a control, the parental plasmid, pcDNA3.1(−) (Invitrogen, Groningen, The Netherlands) was prepared as well. For the expression of a control protein, β-galactosidase, another plasmid called pCH110 (Amersham Pharmacia Biotech, Uppsala, Sweden) was purchased and utilized. The plasmids were purified through Qiagen Tip columns (Qiagen, Hilden, Germany) prior to transfection experiments.

2.2. Cells
A monkey kidney cell line, Cos-7, was maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified air containing 5% CO_2.

2.3. DNA transfection
A cationic liposome-mediated DNA transfection technique was employed, using a commercially available reagent (LipofectAMINE: Gibco BRL, Rockville, MD, USA). Cos-7 cells were seeded at a density of 2×10^5 per 35 mm diameter tissue culture well 16 h prior to transfection. DNA transfection was carried out following the manufacturer's optimized protocol for Cos-7 cells, using 1 μg of plasmid DNA per sample [17,18]. At 12 or 24 h post transfection, cells were subjected either to metabolic labeling or to immunofluorescence experiments as described below.

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2.4. Metabolic labeling and radioimmunoprecipitation analysis

Twenty-four hours after transfection, cells were starved in D-MEM without methionine containing 5% dialyzed FBS for 1 h at 37°C. Metabolic labeling was performed subsequently by adding 200 μCi of L-[^{35}S]methionine per sample for 2 h at 37°C. After being labeled, the cells were washed with D-MEM for the chase phase of the experiments, and were maintained in 1 ml of growth medium. At 0 or 24 h after chasing, cells were washed with Dulbecco's phosphate-buffered saline (PBS) and harvested in 1 ml of a radioimmunoprecipitation analysis (RIPA) buffer, as described previously [17,19]. The supernatant at the 24 h timepoint was also saved at −20°C after adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 200 μM. Immunoprecipitation was carried out for both cleared cell lysate and tissue culture supernatant with the anti-CTGF rabbit serum [13] and protein G-Sepharose (Amersham Pharmacia Biotech). After being washed six times with the RIPA buffer, a high-salt wash buffer, and a low-salt wash buffer (twice each), as previously described [19], the immunocomplex was dissociated in 1×sodium dodecyl sulfate (SDS) sample buffer containing 2.5% 2-mercaptoethanol, and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Prior to autoradiography, signals were enhanced by fluorography, using a commercially available reagent (Amplify: Amersham Pharmacia Biotech).

2.5. Indirect immunofluorescence analysis

Cells were seeded on a glass coverslip in a 35 mm diameter tissue culture well. On those cells, DNA transfection was performed as described above. Twelve or 24 h after transfection, cells were fixed in 3.5% formaldehyde in PBS, and were permeabilized in 0.1% NP40 in PBS, as described previously [20]. Incubation with antibodies was performed at 37°C for 1 h, followed by an extensive wash with PBS. The anti-CTGF rabbit serum and anti-α-tubulin mouse monoclonal antibody (Sigma, St. Louis, MO, USA) were used as primary antibodies at a 200-fold dilution in 3% bovine serum albumin in PBS. Fluorescein isothiocyanate-conjugated anti-rabbit goat IgG and tetramethylrhodamine isothiocyanate-conjugated anti-mouse goat IgG (Sigma) were used at a dilution of ×100 as secondary antibodies in the same buffer. In the secondary antibody mixture, DAPI was also added at a final concentration of 1 μg/ml to visualize cellular DNA. The mounted cells were viewed under an epifluorescent microscope (Olympus, Tokyo, Japan).

2.6. Cell growth assay

For the evaluation of the effect of recombinant CTGF (rCTGF) on Cos-7 cell proliferation, 10 000 cells were seeded in a well of a 24 well tissue culture plate, in the presence or absence of 50 ng/ml of rCTGF [16]. For the evaluation of the effect of the intracellular expression of CTGF on cell growth, 25 000 cells were seeded at the same scale (24 well scale). DNA transfection for those cells was carried out with 250 ng of plasmid DNA at a quarter scale of that described. Cell counting was performed at 2 days post addition, or post transfection.

3. Results

3.1. Expression of human CTGF in Cos-7 cells by DNA transfection

Utilizing a eukaryotic expression plasmid of human CTGF, in which a *ctgf* cDNA is driven by a cytomegalovirus promoter, we could efficiently express human CTGF in a monkey kidney cell line, Cos-7. Expression of human CTGF was firstly confirmed by metabolic labeling of the transfected cells and subsequent RIPA (Fig. 1). Strong signals that were specifically reactive to the anti-CTGF serum were detected immediately after labeling with L-[^{35}S]methionine. At this time point, two distinct signals were present, which might represent a pro-CTGF with unprocessed signal peptide as well as a commonly recognized 36 kDa processed CTGF. Interestingly, 24 h after labeling, the band with slower migration in SDS–PAGE almost completely disappeared, suggesting its identity as a precursor. Of note, even after 24 h of chase, no CTGF or
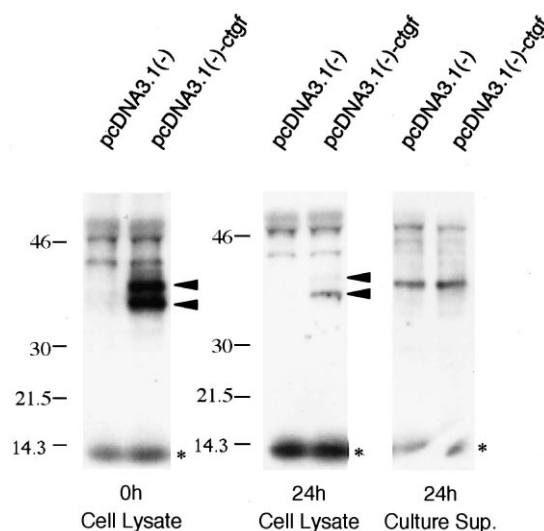

Fig. 1. Transient expression of human CTGF in Cos-7 cells. Cells were transfected with either pcDNA3.1(−), or pcDNA3.1(−)-ctgf, and metabolically labeled with L-[^{35}S]methionine for 2 h. The labeling was followed by a chase period. Immunoprecipitation was performed either with cell lysate or with tissue culture supernatant with an anti-CTGF serum. Positions of molecular weight markers are shown at the left of each panel in kilodaltons. Arrowheads indicate specific signals of human CTGF expression, whereas asterisks denote non-specific signals with an apparent molecular weight of 10 kDa, which are also observed in negative controls.

its related protein was detectable in the culture supernatant of the transfected cells (Fig. 1). Therefore, this system may be useful for the analysis of early cellular events that are caused by intracellular nascent CTGF before secretion. Immunofluorescence analyses revealed that CTGF was found inside the cells from 12 h post transfection with interesting intracellular distribution patterns as described later (Fig. 2).

3.2. High levels of intracellular expression of CTGF give rise to an altered cellular morphology in transfected cells

In CTGF-expressing cells, no evident alteration in morphology was observed 12 h after transfection. Nevertheless, after 24 h, most CTGF-expressing cells displayed an abnormal morphology. These cells were characterized by a loose attachment to the tissue culture chamber surface with marked changes in appearance (Fig. 2). The population with these morphological phenotypes perfectly corresponded to CTGF-positive immunostaining. CTGF-negative cells around the CTGF-positive cells showed no such changes, which indicates that these morphological changes are not transmissible, and thus should not be the consequence of the extracellular CTGF function.

3.3. The CTGF-induced altered morphology is associated with increased cellular DNA content

The loosely attached CTGF-expressing Cos-7 cells reminded us of cells that are heading for mitosis. DAPI staining showed that a major population of the CTGF-expressing cells did actually contain increased amounts of nuclear DNA at the time point of 24 h post transfection, which is precisely accompanied by the observed morphological changes (Fig. 3). Of note, overexpression of an unrelated protein, bacterial β-galactosidase, yielded no such effects, providing no link between
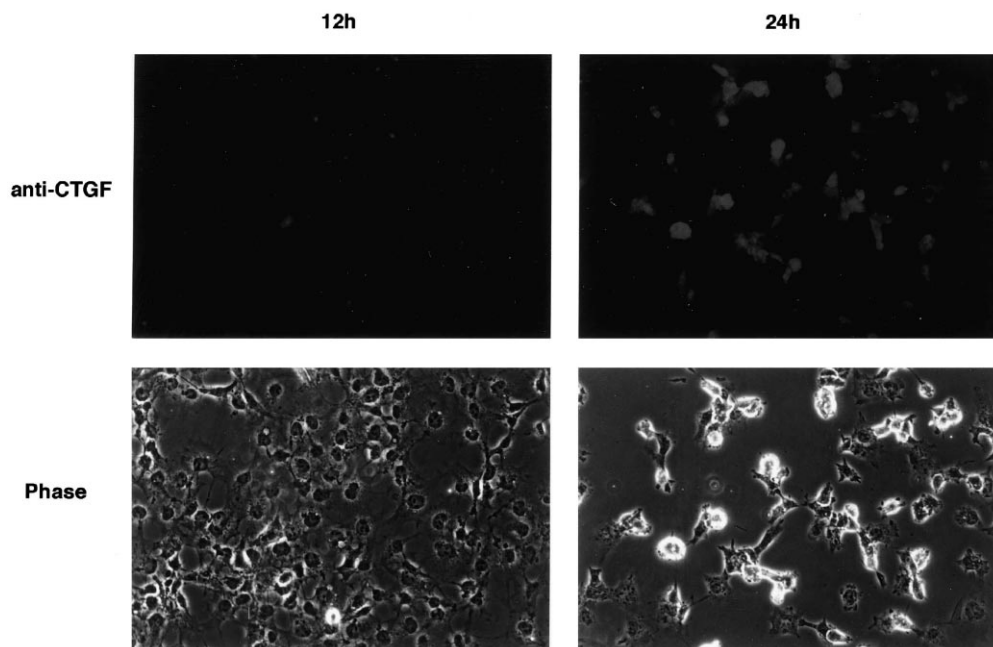
Fig. 2. Indirect immunofluorescent detection of transiently expressed CTGF, which is accompanied by morphological alteration at 24 h post transfection. Cells were transfected with pcDNA3.1(−)-ctgf and viewed at the indicated time points after transfection. The same fields were viewed by epifluorescent (top) and phase contrast (bottom) microscopy. At 24 h post transfection, the CTGF-expressing cell population clearly corresponds to the population with altered morphology, while no change is observed in the cells without CTGF expression. Photomicrographs were taken at a magnification of 165×.

the overexpression process itself and the increase in DNA content with morphological alteration. Interestingly, the typical alignment of mitotic chromosomes, which can be found in the control cells, is apparently absent in the CTGF-expressing cells. Thus, it can be concluded that the observed morphological alteration and increased cellular DNA content were caused by intracellular CTGF. Also, the increased DNA content without mitotic chromosomes indicates that those cells should be in late G2 phase or at the G2/M boundary in the cell cycle.

3.4. No mitogenic effect of intracellular CTGF expression

An increased DNA content and loose attachment merely indicate that cells are in the G2/M phase, whether intracellular CTGF promotes the cells to enter, or arrests them in G2/M phase. In addition, CTGF at a concentration under the detection threshold of RIPA might exert a receptor-mediated function. To address these concerns, we evaluated the mitogenic effects of transient CTGF expression. No positive effects on cell proliferation were observed even 48 h after transfection (90.6% of the controls), while extracellular human recombinant CTGF which was directly added to the tissue culture medium efficiently stimulated the proliferation of Cos-7 cells within 48 h (153% of the controls). As such, the CTGF-expressing cells are likely to be arrested in, rather than rushed to, G2/M phase, and producing no biologically significant extracellular CTGF within 48 h after transfection.

3.5. Intracellular distribution of nascent CTGF

As is seen in Fig. 2, transiently expressed CTGF was not distributed evenly and diffusely in the transfected cells at 12 h post transfection. There seems to be a particular spot of accumulation for nascent CTGF. As such, we performed double staining studies with antibodies against cellular proteins.

Among them, an antibody to visualize α-tubulin provided distinctive findings (Fig. 4). In interphase cells, a single centrosome is observed adjacent to the nucleus, which is accompanied by radial expansion of microtubule fibers. In this cell, CTGF is found at the central portion of the radial expansion, which involves the cell centrosome – a microtubule organization center (MTOC). The observed localization of nascent CTGF suggests a possible interaction of CTGF and the cen-
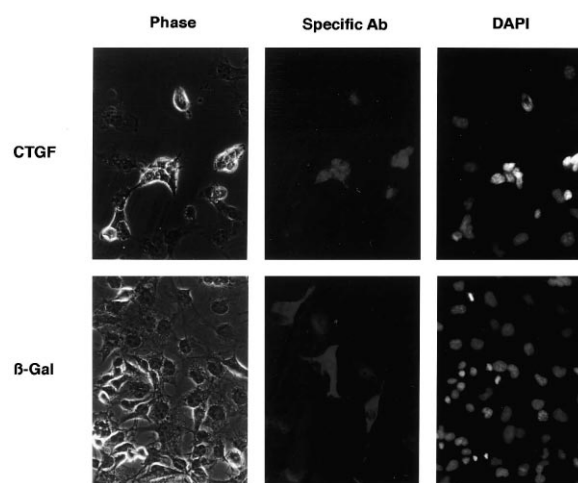

Fig. 3. The loose-attaching morphology induced by CTGF is accompanied by increased cellular DNA content. Cos-7 cells were transfected with either pcDNA3.1(−)-ctgf or pCH110 (a β-galactosidase expression plasmid as a negative control), and stained with specific antibodies and DAPI. The same fields were viewed under a phase contrast (left) and epifluorescent (middle and right) microscope. Note that the DNA increase in naturally dividing cells is independent of β-galactosidase expression. Magnification: 330×.
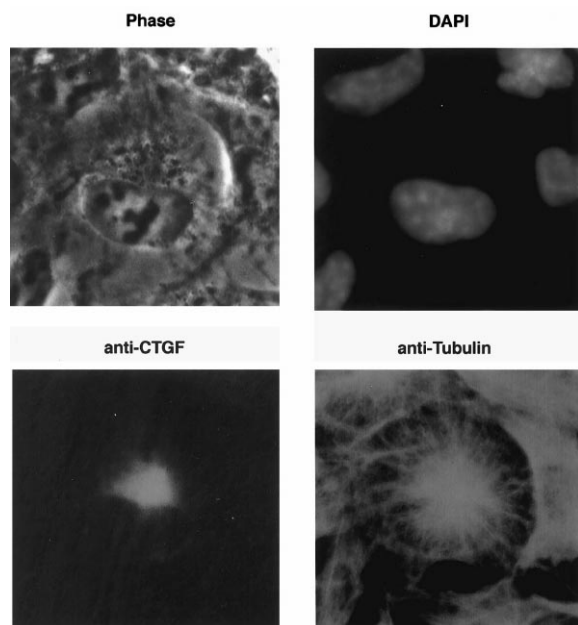
Phase

DAPI

anti-CTGF

anti-Tubulin

Fig. 4. Intracellular localization of nascent CTGF 12 h after transfection. Enlarged images of a CTGF-expressing interphase cell to examine the localization site of nascent CTGF. Indirect immunofluorescence to visualize the distribution of α-tubulin was added. The distinctive shape of the nucleoli (Phase) and lack of increase in DNA content (DAPI) with a single centrosome (Phase/anti-Tubulin) indicate that the cell is in interphase. CTGF is shown to be accumulated around the radial center of microtubule fibers (anti-CTGF/anti-Tubulin), where the centrosome is located.

tral mitotic machinery in exerting the observed effect of intracellular CTGF.

4. Discussion

As suggested by its name, CTGF is an intercellular mediator of mitogenic and chemotactic signals in fibroblasts and endothelial cells [1]. However, the fact that CTGF acts as a multiple mediator of chondrocyte growth and differentiation prompted us to seek putative alternative pathways by which different functions are mediated. Here, the present data indicated such an alternative pathway in addition to its classical, receptor-mediated functioning route. Surprisingly, while working as a mitotic factor outside the cell, CTGF was found to lead the cells to cell cycle arrest before being secreted. A preliminary basis for the functional aspect of CTGF as a cell cycle regulator was provided by its possible interaction with a central part of the cell division apparatus. Additionally, this is the first report describing a single protein with apparently opposing extracellular and intracellular effects on mitosis.

The intracellular function to affect the cell cycle might provide new insights into how CTGF is able to accomplish such complex missions as observed in wound healing and endochondral ossification. CTGF exerts a variety of effects to potentiate either growth or differentiation in chondrocytes. In the course of differentiation, gene expression of CTGF should be duly regulated by *cis*- and *trans*-regulatory factors [21,22]. By such regulations, marked induction of CTGF gene expression is restricted to particular stages of cell differentiation, as is seen in the hypertrophic stage in chondrocytes, and in certain pathological conditions. Otherwise, the expression level

of CTGF is maintained at relatively low levels [6,12]. Not only the gene regulation, but the secretion and activation mechanisms of CTGF are difficult to describe comprehensively. In the present study, no detectable extra CTGF in the culture supernatant (Fig. 1) and no mitogenic activity were observed during the first 2 days of the posttransfection period. Several ideas can account for the negative findings for active secretion. One possibility is that Cos-7 cells might be defective for CTGF processing and secretion. Another likely interpretation is that it may take several days for CTGF to be secreted as a soluble form in the medium. Indeed, a previous report has shown that, in human foreskin fibroblasts, most of the CTGF remained cell-associated at least for 5 days [23]. Also, it is still possible that a processed form that cannot be detected by our antibody may have been secreted, which is however ignorable, since no CTGF activity was detected via bioassay.

At present, it is not exactly clear at which point in the G2/M phase the CTGF-expressing Cos-7 cells are arrested, or how CTGF causes the cell cycle arrest. However, since most of the affected cells are characterized by loose attachment, increased DNA content without chromosome alignment on the metaphase plate, and no sign of nuclear division, it is assumed that they are arrested at a certain stage between late G2 and early M phases. In this context, the initial accumulation of CTGF around the MTOC (Fig. 4) may be noteworthy. If CTGF has direct interactions with microtubules, or other centrosomal components, one possible mechanism for the observed cell cycle arrest is that in those cells, the mitotic process might be disrupted by spindle dysfunction, and they may not pass the spindle damage/assembly checkpoint [24]. However, the possible interaction of CTGF and mitosis promoting factor [25] and other factors to modify the phosphorylation signaling for passing through mitotic checkpoints cannot be disregarded [26,27]. The putative effect of CTGF on the recently reported p38 kinase function which is thought to be involved in the spindle damage/assembly checkpoint must be considered as well [28]. Analyses to clarify the intracellular CTGF-mediated cell cycle arrest are currently ongoing, together with mutational analyses of CTGF to map the functional domain which is responsible for this alternative function.

The antimitotic effect of nascent CTGF seems to be contradictory to the classical mitogenic effect of CTGF. However, it might still be a critical part of the CTGF functionality. As generally recognized, cell differentiation is usually accompanied by restricted cell growth. Therefore, in certain differentiated cells, a very high level of CTGF expression could be an intracellular signal of terminal differentiation in the expressing cells to halt their cell growth, while leading the secretion of CTGF to stimulate the growth of other cell populations, which is actually represented by the behavior of hypertrophic chondrocytes in growth cartilage [11]. Namely, highest CTGF expression is observed only in terminally differentiated hypertrophic chondrocytes that present few CTGF-specific receptor molecules, whereas mature chondrocytes in the prehypertrophic stage display many functional receptors (Nishida et al., unpublished data). As such, the behavior of intracellular CTGF and its relationship to the differentiated phenotypes in hypertrophic chondrocytes are to be dissected. In this regard, it is also of note that apoptosis was induced by CTGF expression in a particular breast cancer cell line [29]. We have

already confirmed the intracellular accumulation of CTGF in hypertrophic chondrocytes in cartilage (data not shown). Further investigation is currently in progress.

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