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Module-Specific Antibodies against Human Connective Tissue Growth Factor: Utility for Structural and Functional Analysis of the Factor as Related to Chondrocytes

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Connective tissue growth factor/hypertrophic chondrocyte specific gene product 24 (CTGF/Hcs24/CCN2) shows diverse functions in the process of endochondral ossification. It promotes not only the proliferation and differentiation of chondrocytes and osteoblasts *in vitro*, but also angiogenesis *in vivo*. The *ctgf* gene is a member of the gene family called CCN, and it encodes the characteristic 4-module structure of this family, with the protein containing IGFBP, VWC, TSP and CT modules. We raised several monoclonal antibodies and polyclonal antisera against CTGF, and located the epitopes in the modules by Western blotting. For mapping the epitopes, *Brevibacillus*-produced independent modules were utilized. As a result, at least 1 antibody or antiserum was prepared for the detection of each module in CTGF. Western blotting with these antibodies is expected to be useful for the analysis of CTGF fragmentation. Moreover, we examined the effects of these monoclonal antibodies on the biological functions of CTGF. One out of 3 humanized monoclonal antibodies was found to neutralize efficiently the stimulatory effect of CTGF on chondrocytic cell proliferation. This particular antibody bound to the CT module. In contrast, surprisingly, all of the 3 antibodies recognizing IGFBP, VWC and CT modules stimulated proteoglycan synthesis in chondrocytic cells. Together with previous findings, these results provide insight into the structural-functional relationships of CTGF in executing multiple functions.

Key words: CCN family, chondrocyte, connective tissue growth factor, module, monoclonal antibody.

Abbreviations: IGFBP, insulin-like growth factor binding module; VWC, von Willebrand factor type C module; TSP, thrombospondin type 1 repeat; CT, C-terminal module.

Connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24(CTGF/Hcs24) is a cysteine-rich secretory protein with a molecular mass of 36–38 kDa. It is now classified as a gene product of the second member of the CCN gene family (CCN2) (*1–6*). The acronym CCN stands for the original members of this family, *i.e.*, *Ctgf*, *Cyr61*, and *Nov*. Recently, several other genes, including *ctgf-3*/*ctgf-L*/*wisp-2*/*cop 1*, *wisp-1*/*elm 1*, *and wisp-3*, have been discovered and newly classified as members of this gene family (*7–11*). The CCN family genes, with an exception of *wisp-2*, encode proteins consisting of 4 conserved modules, *i.e.*, insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C module (VWC), thrombospondin type 1 repeat (TSP), and the C terminal module. This characteristic structure is assumed to enable the multiple interactions of these proteins with other molecules, which is critical for them to exert their diverse functions.

As such, CTGF is also known to be a multifunctional growth factor. It acts as a mitogenic and chemotactic cytokine for fibroblasts (*1*, *3–5*, *12*, *13*), which provides a theoretical basis for the involvement of CTGF in the wound healing process and in fibrosis (*4*, *14–20*). In growth plate chondrocytes, CTGF plays a central role in their growth and differentiation. The highest level of CTGF expression is observed in the hypertrophic layers of growth cartilage (*5*, *21*, *22*). Proliferation and proteoglycan synthesis are stimulated in primary cultures of chondrocytes *in vitro*, whereas type X collagen and alkaline phosphatase production are induced at more differentiated stages by exogenously-added CTGF (*5*, *22*, *23*). Interestingly, in articular chondrocytes, CTGF promotes growth and maturation of chondrocytes without promoting them to hypertrophy (*24*). Therefore, CTGF may be a feasible tool for use in regenerating damaged articular cartilage and is being evaluated for possible clinical application. Furthermore, CTGF has been found to promote the proliferation and migration of vascular endothelial cells, which are also required for the final stages of endochondral ossification (*5*, *25*, *26*). The involvement of

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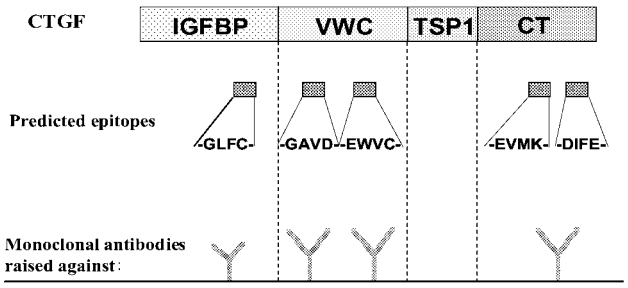

Fig. 1. **Computer-associated prediction of highly immunogenic portions in human CTGF in comparison with the distribution of epitopes of monoclonal antibodies raised against full-length CTGF.** Location and key amino acid sequences of the epitopes predicted by the Rothbard/Taylor algorithm are displayed beneath the schematic representation of the CTGF molecule. The antibodies actually raised are symbolized under the modules containing the epitopes. Sizes of the antibody symbols denote relative titer as determined by Western blotting. Abbreviations: IGFBP, insulin-like growth factor binding module; VWC, von Willebrand factor type C module; TSP, thrombospondin type 1 repeat; CT, C terminal module.

CTGF in tumor angiogenesis has also been suggested, but the roles of CTGF in oncogenesis remain controversial, although the protein is actually expressed in several types of malignancies (*27–29*).

As stated above, such multiple functions of CTGF ought to be enabled by the differential use of the 4 modules interacting with distinct functional counterparts. Therefore, specific antibodies targeted to each module are desirable for analyzing the module structure-function relationship. Toward this objective, we raised and analyzed such antibodies for independent detection of all 4 modules. Moreover, our biological analysis with chondrocytic cells suggests distinct signaling pathways assigned for certain functions conferred by CTGF.

MATERIALS AND METHODS

Antibodies and Antisera—MHCT2, a murine monoclonal anti-CTGF antibody (subclass; IgG1κ) that was previously utilized as the detection antibody in an ELISA system, was prepared as described previously (*30*). The other monoclonal antibodies, JMAb25, 27, and 31, were prepared and humanized based on an established methodology using a recombinant CTGF protein as an immunogen (*31*). These antibodies were purified by POROS Protein affinity column chromatography, followed by filtration through a micropore membrane (pore size: 0.22 μm). The subclass of these 3 antibodies was determined to be human IgG2κ. All of the monoclonal antibodies were kindly prepared and provided by JT Central Pharmaceutical Research Institute (Takatsuki). For control experiments, purified murine normal IgG1κ was also purchased and utilized (Sigma, St Lous, MO, USA)

The 2 polyclonal antisera used, anti-CTGFw and anti-CTGFc, were raised in rabbits using a recombinant CTGF protein and a synthetic peptide, respecitvely, as immunogens. The amino acid sequence of this peptide, RPCEADLEENIKKGKKCIRTPK, corresponds to the junction of the TSP and CT modules (*32*).

Production of Single CTGF Modules by Brevibacillus choshinensis—For the transformation of bacteria, a series of expression plasmids were initially constructed. The cDNA fragments of the 4 module-encoding regions were prepared by polymerase chain reaction (PCR) amplification with the cDNA of the full-length coding region of *ctgf* as a template, and subcloned into a parental vector, pNC-H3, between unique *Nco*I and *Hin*dIII sites, as shown in Fig. 1A. *Brevibacillus choshinensis* (strain: HPD31) was transformed with one of the module expression plasmids, and cultured for the production and secretion of each protein module, as described previously (*33*). The culture supernatant was collected and sterilized by filtration. The protein of interest in the culture supernatant was confirmed to be an extra signal by Coomasie Brilliant Blue (CBB) staining and immunoblotting with antibody against an oligohistidine tag fused at the C-terminus of each protein after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blotting—The crude bacterial secretion containing each module was denatured in a reducing SDS sample buffer, boiled for 5 min, and electrophoresed in a 15–25% SDS-polyacrylamide gel. The separated proteins were transferred onto an Immobilon (Millipore, Billerica, MA, USA) transfer membrane with a semi-dry blotting apparatus. The membrane was soaked for 60 min at 37°C in 5% skim milk in phosphate-buffered saline supplemented with Tween 20 (PBS-T; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.05% Tween 20) and then incubated in PBS-T solution containing JMAb27 (100-fold diluted) or another anti-CTGF monoclonal antibody or anti serum (1,000-fold diluted) and bovine serum albumn (1%) for 1 h at 37°C. The membrane was washed with PBS-T solution and further incubated with a horseradish peroxide-conjugated secondary antibody against human, mouse, or rabbit IgG for 1 h at 37°C. Positive signals were visualized by an enhanced chemiluminescence method using an experimental kit (Renaissence: DuPont/NEN, Wilmington, DE, USA).

Cell Culture and Evaluation of Proteoglycan Synthesis—Human chondrocytic HCS-2/8 cells (*34–39*) were grown to confluence in 48-well multi-plates containing Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). The cells were then preincubated in serum-free medium for 24 h, and further incubated for 5 h in the same medium containing an antibody (50 μg/ml) against CTGF, with or without recombinant CTGF (rCTGF: 50 ng/ml). Then, [^{35}S]sulfate (37 MBq/ml) dissolved in PBS was added to the culture at a final concentration of 370 kBq/ml, and the incubation was continued for another 17 h. After labeling, the cultures were digested with 1 mg/ml actinase E, and the radioactivity of the material precipitated with cetylpyridinium chloride was measured in a scintillation counter.

MTT Assay—For the evaluation of cell proliferation, HCS-2/8 cells (5×10^3) were seeded into regular D-MEM in each well of 96-well plates (Falcon Laboratories, McLean, VA, USA). After 2 days, the cells were treated with 50 μg /ml of the desired monoclonal antibody and rCTGF (50 ng/ml) for 5 h, and then their proliferation was evaluated by the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma)

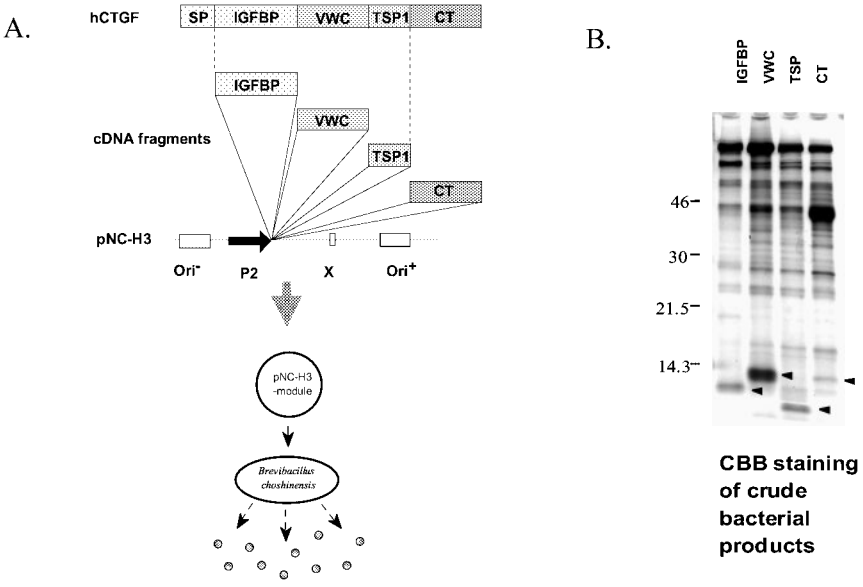

Fig. 2. **A: Strategy for the production of individual modules by *Brevibacillus chosinensis*.** Expression plasmids containing cDNAs encoding the modules were constructed (upper panel), and the secetory production of each module was obtained from each transformant bearing the expression plasmid (lower panel). Abbreviations: P2, CWP promoter P2 region; Ori, origin of replication; X, transcription terminator (*33*). Module abbreviations are the same as those in Fig. 1. Positions of molecular weight standards are shown to the left of the panel. **B: Crude production of each module by *Brevibacillus chosinensis*.** Proteins from the culture supernatants of the bacterial transformants shown in panel A were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue (CBB) staining. Extra bands corresponding to the modules are indicated by arrowheads.

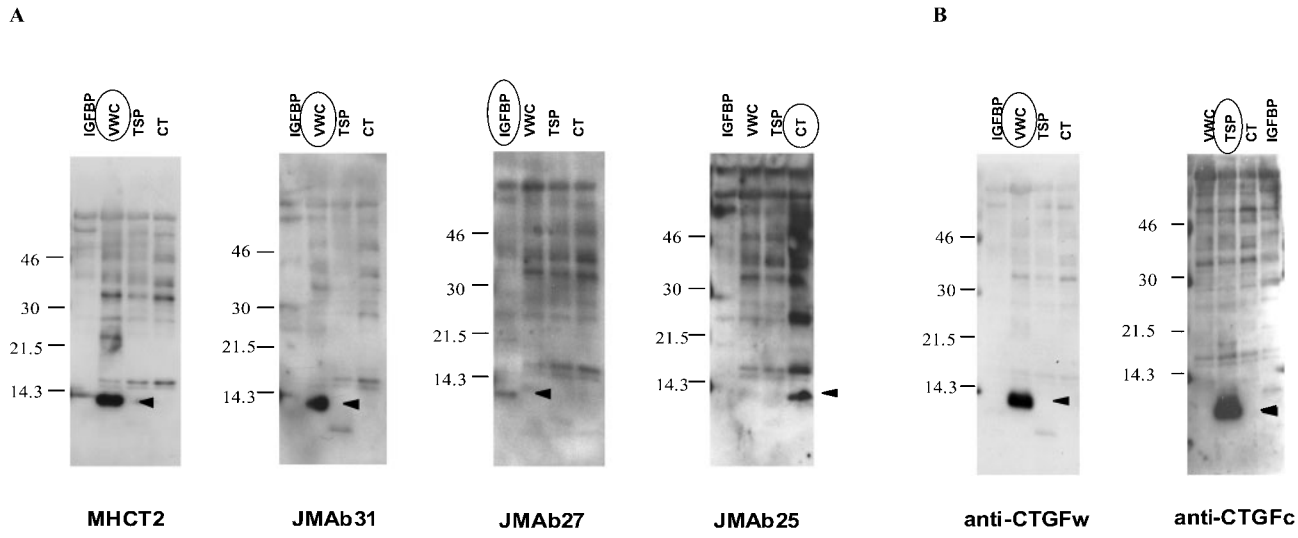

Fig. 3. **Determination of the epitope distribution of the anti-CTGF antibodies among the 4 modules.** A: Western blotting analysis of the module-containing proteins shown in Fig. 2B by 4 monoclonal antibodies. The recognized modules are circled in each panel and the specific signal is indicated by an arrowhead. A few other antibodies that showed no reactivity to either module were also raised and supposed to recognize the tertiary structure of CTGF (data not shown) B: Western blotting analysis with 2 polyclonal antisera. Note that the order of the samples in the last panel is different from that of the others. Positions of molecular weight standards are shown at the left.

assay, as previously described (*29*). Briefly, 10 μl of 0.5% MTT in distilled water was added to 100 μl of medium in each well. After incubation for 4 h at 37°C, the medium was removed; and the cells were lysed in 0.04 M HCl 2-propanol. Then, the absorbance of the lysate was measured at a wavelength of 570 nm (excitation: 630 nm).

Computer-Associated Prediction of Putative Epitopes—The amino acid sequence of human CTGF was analyzed by a computer program (Genetyx-Mac 8.0, Software Development, Tokyo), through an algorithm to predict the Rothbard/Taylor peptide patterns.

RESULTS

Computer-Associated Prediction of the Distribution of Epitopes among the 4 Modules in CTGF—Prior to mapping the epitopes, we predicted the results by computer analysis. Based on a well-established algorithm, several epitopes were predicted in the IGFBP, VWC and CT modules (Fig. 1). In particular, VWC and CT were assumed to be highly immunogenic. In constrast, no putative epitope was predicted in TSP, suggesting the least possibility for an anti-TSP monoclonal antibody by CTGF-immunization. Therefore, in advance, we synthesized an oligopeptide corresponding to the C-terminal end of the TSP module and used it to immunize a rabbit. Characterization of this antiserum was carried out in parallel with the other antibodies, as described later on.

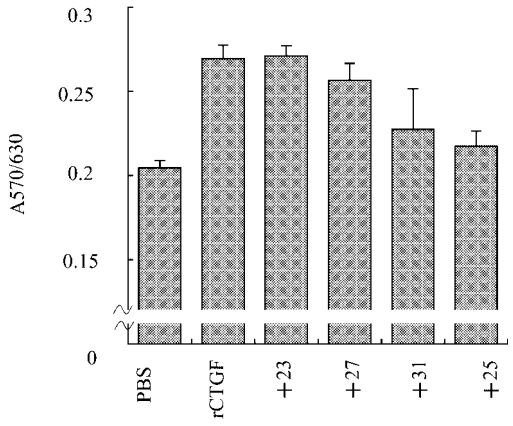

Fig. 4. **Neutralizing effects of the humanized monoclonal antibodies against the proliferation of HCS-2/8 cells stimulated by CTGF.** Cell growth was evaluated by the MTT assay. All columns except PBS represent results in the presence of CTGF. The column marked "23" indicates the experiment with non-specific control IgG (against KLH). The other 3 columns labeled "+27," "+31," and "+25" denote the results with JMAb27, JMAb31, and JMAb25, respectively.

Expression and Production of Individual CTGF Modules by Brevibacillus choshinensis—In order to locate the antibody epitopes against CTGF, we prepared crude bacterial culture supernatants, each containing an individual module of human CTGF. First, we prepared 4 plasmid constructs containing cDNAs encoding the 4 modules for the secretory expression of the individual modules by *Brevibacillus choshinensis* (Fig. 2). After transformation with each plasmid separately, the bacteria were grown, and the proteins in the culture supernatant were analyzed by SDS-PAGE and CBB staining. As shown in Fig. 2A, extra protein signals were detected at the expected apparent molecular weight in the gels. The identity of each signal as a recombinant protein was confirmed by Western blotting to detect the histidine tag added to each protein (data not shown).

Epitope Mapping of Monoclonal Antibodies and Antisera against CTGF—We carried out Western blot analysis to determine the modules that included epitopes for the antibodies and antisera (Fig. 3). We found that a single monoclonal antibody (JMAb27) reacted with the N-terminal module, IGFBP, and that 2 monoclonal antibodies (JMAb31, MHCT2) recognized the VWC module. Interestingly, the polyclonal antiserum (anti-CTGFw) obtained by immunization with full-length CTGF reacted quite strongly only with the VWC module, which indicates the strong immunogenicity of this particular module. As predicted by the computer, no monoclonal antibody against the TSP module was found; however, the polyclonal antiserum against the synthetic peptide corresponding to the C-terminal end of the TSP module efficiently and specifically reacted with this module. Finally, we were able to obtain a monoclonal antibody (JMAb25) having its epitope in the CT module.

Effects of the Humanized Monoclonal Antibodies on CTGF-Stimulated Cell Proliferation of Chondrocytic HCS-2/8 Cells—In order to estimate which module of CTGF is crucially involved in transmitting the proliferation signal, we evaluated the effects of the monoclonal antibodies against specific modules on CTGF-stimulated cell proliferation. As shown in Fig. 4, the addition of CTGF significantly stimulated the proliferation of HCS-2/8 cells as measured by the MTT assay. This effect was efficiently inhibited by the addition of excess JMAb25 against the CT module. However, the other antibodies against IGFBP and VWC were less inhititory. Control IgG against keyhole limpet hemocyanin (KLH), JMAb23, had no effect, indicating the specificity of these immunoreactions. Therefore, the CT module-mediated molecular interaction is highly crucial for transducing the mitogenic signal of CTGF.

Stimulation of Proteoglycan Synthesis in Chondrocytic HCS-2/8 Cells—Active proteoglycan synthesis is a typical phenotype of differentiated chondrocytes, and is known to be promoted by CTGF. To clarify which module is critically involved in the molecular interaction that results in the enhancement of proteoglycan synthesis by CTGF, we examined the effects of the humanized monoclonal antibodies on the incorporation of [^{35}S]sulfate into CTGF-treated HCS-2/8 cells, expecting neutralization. However, surprisingly, none of the added antibodies inhibited the stimulatory effect of CTGF (Fig. 5A). In fact, the antibodies rather showed additive effects on the overall proteoglycan synthesis stimulated by CTGF. Moreover, each antibody alone was capable of enhancing proteoglycan synthesis even in the absence of exogenously-added CTGF. It should be noted that, under the experimental conditions used, HCS-2/8 cells themselves constitutively produce significant levels of CTGF. The limited effects of the antibodies on proteoglycan synthe-

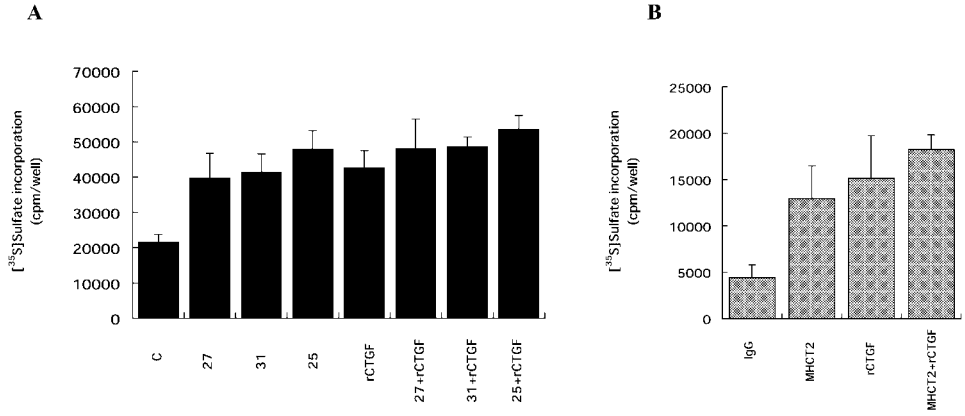

Fig. 5. **A. Stimulation of proteoglycan synthesis by 3 humanized monoclonal anti-CTGF antibodies.** HCS-2/8 cells were prepared and proteoglycan synthesis was determined as described in "MATERIALS AND METHODS." All of the control (C) experiments were performed in the presence of the same amount of control IgG against (KLH). **B. The same evaluation with a murine monoclonal anti-CTGF, MHCT2, where murine normal IgGκ was utilized as a control (C).** Values represent means ± SD for 6 independent samples.

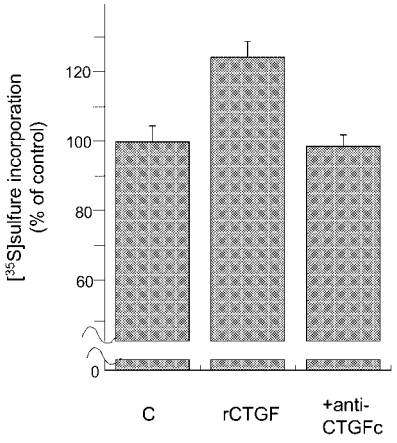

Fig. 6. **Neutralizing effect of the anti-TSP serum (anti-CTGFc) against proteoglycan synthesis by HCS-2/8 cells stimulated by CTGF.** The control experiment (C) was carried out with the addition of the same amount of preimmunized serum from the same rabbit used for the preparation of anti-CTGFc.

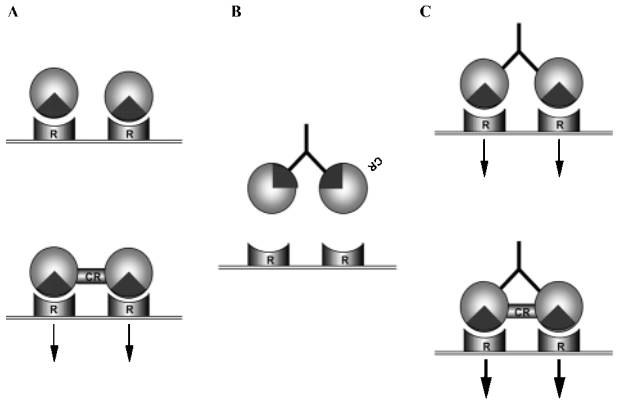

Fig. 7. **Schematic representations of the possible modes of receptor-ligand interaction of CTGF and hypothetical actions of anti-CTGF IgG.** A: Co-receptor-associated signal transmission by CTGF, in which bridging of the receptor-ligand complex is required for the efficient activation of intracellular signaling. B: Neutralizing effect of an antibody through interference with the ligand-receptor interaction. C: Enhancing effect of an anti-CTGF antibody on signal transduction by co-receptor-like bridging of ligand-receptor complexes. Abbreviations: R, CTGF receptor; CR, Co-receptor. Spheres represent CTGF molecules with the ligand module indicated in solid black. The Y-shaped objects indicate anti-CTGF IgG molecules.

sis in the presence of exogenous CTGF can be ascribed to the fact that the proteoglycan synthesis level was already maximally enhanced at this dose by CTGF alone. Therefore, the effects of the antibodies appeared far smaller, as they were evaluated under conditions of saturation. Since all of the control experiments were performed using the same amount of mouse IgG against KLH as used for the monoclonal antibodies (all of the "C" columns), the observed effects were proven to be specific. Similar evaluation was also carried out with the murine monoclonal antibody against VWC, (MHCT2), providing results comparable to the humanized antibodies (Fig. 5B).

The above findings that antibodies against IGFBP, VWC and CT resulted in the uniform stimulation of proteoglycan synthesis of chondrocytic cells prompted us to confirm a previous finding obtained with an antiserum (*22*). As shown in Fig. 6, similar experiments using anti-TSP serum revealed neutralization instead of stimulation of the proteoglycan synthesis enhanced by CTGF, as reported previously (*22*). In such bioassays, the basal levels (of sulfate incorporation) depend highly on the condition of the cells, a factor that is hard to control among independent sets of experiment. Therefore, the basal levels often vary unavoidably among series of experiments (Figs. 5 and 6). Taken together, the TSP-CT junction area in the CTGF molecule plays a pivotal role in mediating the CTGF signal for chondrocytic maturation. The mechanism of the antibody-mediated stimulation of the mature chondrocytic phenotype is discussed later on.

DISCUSSION

In the initial part of this study, we prepared several antibodies and antisera for the specific detection of independent modules of human CTGF. As a result, we established a Western blotting system for the independent detection of all 4 of the modules. The multiple functionality of CTGF is considered to be enabled by the differential use of these 4 modules according to local biological conditions; hence these antibodies should be quite useful for analyzing the structure-function relationships of CTGF. The differential roles of these modules are also suggested by the fact that processed fragments containing fewer modules are observed under particular pathological conditions (*40*). In this context, the utility of such antibodies in clinical diagnosis may also be expected. With this goal in mind, we are currently establishing ELISA systems for the specific quantification of such CTGF fragments.

Although immunization to obtain monoclonal antibodies was carried out with a full-length recombinant CTGF protein, 2 out of 5 antibodies were raised against the VWC module. Furthermore, the immunization of a rabbit with CTGF also resulted in the formation of polyclonal IgG, the majority of which recognized VWC. These findings indicate the strong antigenicity of this particular module and are consistent with the results obtained by computer-associated prediction (Fig. 1).

For exerting multiple functions, CTGF is assumed to undergo multiple ligand-receptor interactions using different modules for different functions. Indeed, it was earlier revealed that the enhancement of proteoglycan synthesis by CTGF was mediated by a p38 mitogen-activated protein kinase (MAPK) transduction cascade, whereas a p44/42 MARK pathway was used for the mitogenic signal of CTGF (*41*). Thus, different receptors and ligands may be assigned for transmitting these 2 independent signals. If so, different inhibitory profiles by module-specific antibodies might be expected against these 2 signaling pathways. Consistent with such an assumption, the mitogenic signal of CTGF was most efficiently blocked by the antibody against the CT module, whereas the signal for enhancing chondrocytic phenotypes was inhibited only by the antiserum against a TSP-related peptide. Of note, in early studies on CTGF, a C-terminal fragment with an apparent molecular mass of 10–13 kDa has been reported to be capable of stimulating the proliferation and migration of fibroblasts (*42*, *43*). These findings taken together

indicate that the CT module may contain a ligand that initiates the mitogenic signal transduction by CTGF. Similarly, a crucial interface for initiating the signal transduction to enhance proteoglycan synthesis is assumed to be around the TSP module.

Apart from the inhibitory efficacy, proteoglycan synthesis by HCS-2/8 cells is remarkably enhanced by the addition of any of the monoclonal antibodies against IGFBP, VWC, or CT. These unexpected results can be accounted for by the hypothesis presented below as illustrated in Fig. 7.

It is known that certain growth factors require co-receptor molecules that enable efficient signal transduction through receptor-ligand complexes by bridging a few ligand or receptor molecules. In fact, CTGF itself was recently found to require heparan sulfate proteoglycans to display a certain function (*44*). In that report, direct binding of CTGF with a heparan sulfate proteoglycan is also described. Since the anti-CTGF antibody also binds directly to the CTGF molecule and may form a multimolecular complex, it is likely to act as a proteoglycan-like co-receptor to enhance CTGF signal transduction. In our experiments, even when only a monoclonal antibody was added to HCS-2/8 cells, it had strong effects. These findings are supported by the fact that these cells are constantly producing significant levels of CTGF (*43*), which may provide enough background for the antibody to act as a co-receptor. In contrast, no significant inhibitory effect of the antibodies was observed on cell proliferation in the absence of exogenous CTGF (data not shown). This result was expected, since the epitope involved in mitogenic inhibition may be inaccessible if the ligand-receptor complex has already formed, and it may require stronger affinity for the antibodies to dissociate pre-existing CTGF-receptor complex in the extracellular matrix than to act as a co-receptor. From a clinical point of view, the utility of these humanized antibodies in the regeneration of cartilage may be expected. Owing to immunocomplex formation, direct application of antibodies may pose significant risks. However, they may be safe tools for *ex-vivo* cartilage regeneration. As such, the effects of these humanized monoclonal antibodies on primary chondrocyte cultures are currently being evaluated.

In addition to those analyzed in this study, CTGF has several other different functions. By using these antibodies, we are further analyzing the structural and functional relevance of CTGF in chondrocyte hypertrophy, developmental angiogenesis, and tumor angiogenesis. The accumulation of such data will provide comprehensive findings concerning the multiple functionality of CTGF.

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