

Inhibition of Connective Tissue Growth Factor (CTGF/CCN2) Expression Decreases the Survival and Myogenic Differentiation of Human Rhabdomyosarcoma Cells

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ABSTRACT

Connective tissue growth factor (CTGF/CCN2), a cysteine-rich protein of the CCN (*Cyr61*, *CTGF*, *Nov*) family of genes, emerged from a microarray screen of genes expressed by human rhabdomyosarcoma cells. Rhabdomyosarcoma is a soft tissue sarcoma of childhood deriving from skeletal muscle cells. In this study, we investigated the role of CTGF in rhabdomyosarcoma. Human rhabdomyosarcoma cells of the embryonal (RD/12, RD/18, CCA) and the alveolar histotype (RMZ-RC2, SJ-RH4, SJ-RH30), rhabdomyosarcoma tumor specimens, and normal skeletal muscle cells expressed CTGF. To determine the function of CTGF, we treated rhabdomyosarcoma cells with a CTGF antisense oligonucleotide or with a CTGF small interfering RNA (siRNA). Both treatments inhibited rhabdomyosarcoma cell growth, suggesting the existence of a new autocrine loop based on CTGF. CTGF antisense oligonucleotide-mediated growth inhibition was specifically due to a significant increase in apoptosis, whereas cell proliferation was unchanged. CTGF antisense oligonucleotide induced a strong decrease in the level of myogenic differentiation of rhabdomyosarcoma cells, whereas the addition of recombinant CTGF significantly increased the proportion of myosin-positive cells. CTGF emerges as a survival and differentiation factor and could be a new therapeutic target in human rhabdomyosarcoma.

INTRODUCTION

Connective tissue growth factor (CTGF or CCN2) is a cysteine-rich protein originally identified in conditioned medium of human umbilical vein endothelial cells (1). It belongs to the CCN family of genes, which is composed of five other members: *Cyr61* (cysteine-rich protein 61), *Nov* (nephroblastoma overexpressed gene), *WISP* (Wnt-1-induced secreted protein)-1, *WISP*-2, and *WISP*-3 (2, 3). These proteins share an NH₂-terminal secretory signal peptide and four conserved domains with sequence similarities to insulin-like growth factor (IGF)-binding proteins, von Willebrand type C factor, thrombospondin 1, and a cysteine knot characteristic of other growth factors, including platelet-derived growth factor, nerve growth factor, and transforming growth factor (TGF)- β (4).

CTGF is involved in many biological processes such as cell proliferation, survival, migration, differentiation, and angiogenesis and plays a role in osteogenesis, in chondrogenesis, in the development of vasculature, in placentation (5), and in wound healing (6). Moreover, CTGF is expressed in various pathological conditions: fibrosis (7, 8), scleroderma (9), atherosclerosis (10), and renal diseases (11), in which it mainly acts as a TGF- β downstream mediator.

The function of CTGF in human cancer is still unclear (12). Its angiogenic activity suggests a role for CTGF in tumor growth and vascularization (13). CTGF expression has been found in various

tumors of mesenchymal, epithelial, and lymphoid origins (14–24) possibly with different roles in each tumor type. Overexpression of *CTGF* gene in primary breast cancer was associated with an advanced stage of disease (21); however, functional studies in the human breast cancer cell line MCF-7 showed that CTGF promotes apoptosis (25). In cartilaginous tumors and in fibroblast- and endothelial-cell-derived tumors, inverse correlations were reported between malignant phenotype and the level of *CTGF* expression (14, 19).

CTGF emerged from a microarray screen of genes expressed by human rhabdomyosarcoma cells previously conducted in our laboratory (26). Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood. It is committed to the myogenic lineage but cannot complete the myogenic developmental program (27). The role of CTGF in human rhabdomyosarcoma or skeletal muscle cells is presently unknown. In this paper, we investigated the presence of an autocrine loop based on CTGF in human rhabdomyosarcoma and its effects on proliferation, survival, and myogenic differentiation.

MATERIALS AND METHODS

Cell Lines. Six human rhabdomyosarcoma cell lines of the two main histotypes were used: RD/12 and RD/18, two clones of the RD cell line (28), and CCA (29), derived from tumors of the embryonal histotype; and RMZ-RC2 (30), SJ-RH4, and SJ-RH30 (31), derived from tumors of the alveolar histotype. Cells were routinely cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a 7% CO₂ humidified atmosphere at 37°C. Culture media were purchased from Invitrogen (Milan, Italy).

Myogenic Differentiation. RD/18 and RMZ-RC2 can differentiate along the myogenic pathway if cultured in a medium with a low-serum supplement (DMEM plus 2% horse serum; Refs. 26, 30). The level of myogenic differentiation was determined by indirect immunofluorescence on cytocentrifuged samples, fixed with ethanol:acetone (3:7) at –20°C, using the antiembryonic myosin monoclonal antibody BF-G6 (32) as reported previously (30). The percentage of myosin-positive cells was determined in a Leica DM microscope. For each sample, at least 400 cell elements in random fields were analyzed.

Expression of CTGF. Total RNA was extracted from cultured cells and from surgical specimens by Tri-Zol reagent (Invitrogen, Milan, Italy). One μ g of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase in the presence of oligo-dT and dNTP (Invitrogen). cDNA (1 μ l) was amplified using TaqPlatinum DNA polymerase in a final volume of 25 μ l with 1 mM MgCl₂ and dNTP (0.2 μ M each) and specific primer pairs (0.5 μ M each) for CTGF (direct, 5'-GCATCCGTAATCCCAAAATCTC-3'; reverse, 5'-ATGTCTCTACTCTCTGGCTTC-3') and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Clontech, Palo Alto, CA). The amplification was carried out as follows: 95°C for 30 s, 60°C for 30 s, 72°C for 1 min (20–25 cycles for *GAPDH* and 25–30 cycles for *CTGF*). The amplification products (452 bp for *GAPDH* and 304 bp for *CTGF*) were visualized on ethidium bromide-stained agarose gels.

CTGF expression was also analyzed by quantitative real-time PCR using an ABI Prism 5700 sequence detection system (Applied Biosystems, Milan, Italy). Real-time PCR was performed using Taqman Universal PCR Master Mix Reagents (Applied Biosystems). *CTGF* primers and probes were designed using Primer Express software version 2.0: direct, 5'-AGCCGCTGTG-CATGGT-3'; reverse, 5'-GGGAGTACGGATGCACTTTTGTG-3'; and MGB-probe 5'-FAM-CCTTGCGAAGCTGAC-minor groove binder/nonfluorescent quencher 3'. *GAPDH* was used as an endogenous reference gene (TaqMan

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Human GAPDH Control Reagents; Applied Biosystems). Primers and probes were used at a concentration of 100 nM. The quantity of *GAPDH* and *CTGF* in each sample was calculated using the threshold cycle number interpolated to a standard curve obtained with a serial dilution of a *CTGF*-expressing sample. The level of *CTGF* expression was then normalized to the *GAPDH* one and related to a sample chosen as a calibrator.

Treatment with Antisense Oligonucleotides and siRNA. The *CTGF* antisense and the control scramble oligonucleotides were purchased from Biog-nostik GmbH, Gottingen, Germany (human *CTGF* antisense kit). The *CTGF* small interfering RNA (siRNA) was purchased from Dharmacon Research, Lafayette, CO. The 21-nucleotide *CTGF* siRNA sequence, designed according to the Dharmacon Research's website instructions, consisted of a 19-nucleotide duplex region and a 2-deoxythymidine overhang at each 3'-terminus and targeted the coding region 360–380 relative to the first nucleotide of the start codon of *CTGF* mRNA (GenBank accession no. NM_001901).

Cells were seeded in 24-well plates ($40\text{--}100 \times 10^3$ cells/well) in DMEM plus 10% fetal bovine serum. The following day (day 1), *CTGF* antisense or scramble oligonucleotides or *CTGF* siRNA were added to cultures at a concentration of $0.25 \mu\text{M}$ in $250 \mu\text{l}$ of DMEM without antibiotics and serum in the presence of 0.8% Oligofectamine, a reagent that increases the transfection efficiency of oligonucleotides (Invitrogen, Milan, Italy). After 4 h, cells were shifted to DMEM plus 2% horse serum supplemented with antisense oligonucleotides or siRNAs at a concentration of $0.25 \mu\text{M}$. Medium with oligonucleotides or siRNAs was renewed after 72 h (day 4). Cells were harvested and counted after 24, 48, 72, and 144 h of treatment. The number of viable cells was determined by trypan blue dye exclusion.

Treatment with r-hCTGF. Human recombinant CTGF (r-hCTGF) was provided by FibroGen, San Francisco, CA. RD/18 and RMZ-RC2 cells were treated with 0.05 and $0.5 \mu\text{g/ml}$ of r-hCTGF during culture in DMEM + 2% horse serum. The treatment was renewed every 72 h. Cells were harvested and counted after 4, 7, and 10 days of culture, and cytocentrifuged slides were prepared to evaluate the level of myogenic differentiation.

Evaluation of Apoptosis. Cells were harvested without discarding cells in the supernatant, and apoptosis was evaluated both by Hoechst 33342 staining (Merck, Milan, Italy) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as reported (33). After Hoechst staining, the percentage of apoptotic cells was determined by morphological examination of the nuclei: cells with typical fragmented or condensed nuclei were considered apoptotic (34). The TUNEL assay was performed according to the manufacturer's instructions (*In situ* cell death detection kit; Fluorescein, Roche Diagnostics GmbH, Mannheim, Germany). Briefly, trypsinized and floating cells were harvested, fixed with a paraformaldehyde solution (4% in PBS, pH 7.4), permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) and stained by terminal deoxynucleotidyl transferase with fluorescein-labeled nucleotides. The percentage of fluorescent cells was determined by count of at least 400 elements for each sample.

Cell Proliferation. Cell proliferation was measured by bromodeoxyuridine (BrdUrd) incorporation as reported previously (35). Briefly, after 24 or 48 h of treatment with the antisense oligonucleotide, cells were incubated with BrdUrd ($10 \mu\text{M}$; Sigma, St. Louis, MO) for 2 h at 37°C and were harvested, counted, and fixed in 70% ethanol; DNA was denatured with 2 N HCl. Cells in S phase, labeled by BrdUrd, were detected by indirect immunofluorescence using an anti-BrdUrd mouse monoclonal antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) followed by FITC-conjugated antimouse immunoglobulins. At least 400 elements were counted for each sample.

Western Blot. Conditioned media (72 h) of treated and untreated cells were collected at the 7th day of culture. A protease inhibitor (aprotinin, $2 \mu\text{g/ml}$; Sigma, Milan, Italy) was added and media were centrifuged at 2000 g at 4°C for 20 min to remove cellular debris. Fifteen μl of each supernatant were separated on a 12% polyacrylamide gel (Ready Gel; Bio-Rad, Milan, Italy) under reducing conditions and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Equal amounts of protein loading were confirmed by Ponceau S solution staining (Sigma). The membrane was then blocked with 5% nonfat dry milk/PBS with 0.1% Tween 20 for 1 h and was incubated overnight at 4°C with $0.5 \mu\text{g/ml}$ anti-CTGF goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation of membranes with a horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature, the presence of CTGF

proteins was detected using a colorimetric reaction (Opti-4CN Substrate kit; Bio-Rad).

RESULTS

Expression of *CTGF* in Human Rhabdomyosarcoma. The expression of *CTGF* was studied by reverse transcription-PCR in six human rhabdomyosarcoma cell lines of the embryonal (RD/12, RD/18, and CCA) or alveolar (RMZ-RC2, SJ-RH4, and SJ-RH30) histotype, in rhabdomyosarcoma tumor specimens of both histotypes, and in normal skeletal muscle. All of the cell lines tested, all of the rhabdomyosarcoma tumor samples, and all of the skeletal muscle tissue abundantly expressed *CTGF* with an amplification product clearly evident after 30 cycles in all samples (Fig. 1). Real-time PCR analysis showed differences in the level of expression among the six cell lines, but no relation with histotype of origin was found because the lowest expressor (RD/18) and the highest expressor (CCA) shared the embryonal histotype (Table 1).

A *CTGF* Autocrine Loop in Human Rhabdomyosarcoma Cells. A *CTGF*-specific receptor has not yet been cloned. Thus, to investigate the role of *CTGF* in human rhabdomyosarcoma, cell lines were treated with a *CTGF* antisense oligonucleotide or with a *CTGF* siRNA. siRNAs silence gene expression in a sequence-specific post-transcriptional way; therefore, they constitute a useful strategy for identifying gene function (36, 37).

Treatment with the antisense oligonucleotide caused an 85% decrease in the level of *CTGF* mRNA, judged by quantitative real-time PCR (Fig. 2A). Moreover, *CTGF* protein level was also decreased by antisense treatment. Conditioned media of untreated Oligofectamine and scramble oligonucleotide-treated cells contained several *CTGF* isoforms and fragments (M_r 38,000, 34,000, 30,000, and 20,000). All of the isoforms and fragments were almost absent in antisense oligonucleotide-treated cell-conditioned medium (Fig. 2B).

The *CTGF* antisense oligonucleotide strongly inhibited the growth of rhabdomyosarcoma cells of both embryonal and alveolar histotypes (Fig. 3): RD/12 cell growth was reduced by 90%, RD/18 and RMZ-RC2 cell growth by 70% after 7 days of culture in comparison with untreated cells. The treatment with *CTGF* siRNA also inhibited the

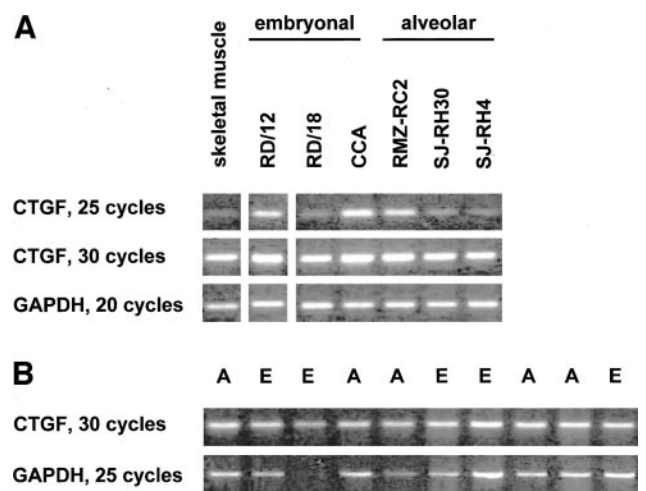


Fig. 1. Expression of connective tissue growth factor (*CTGF*) in human rhabdomyosarcoma. *A*, expression of *CTGF* mRNA in normal skeletal muscle cells and in human rhabdomyosarcoma cell lines of the embryonal (RD/12, RD/18, CCA) and the alveolar (RMZ-RC2, SJ-RH4, SJ-RH30) histotype by reverse transcription-PCR (RT-PCR; 25–30 cycles). *B*, expression of *CTGF* mRNA in human rhabdomyosarcoma tumor specimens of the embryonal (*E*) and the alveolar (*A*) histotype by RT-PCR (30 cycles). The amplification of *GAPDH* (20–25 cycles) was carried out to check that a comparable amount of cDNA was analyzed.

Table 1 Expression of connective tissue growth factor (CTGF) in human rhabdomyosarcoma cell lines by quantitative real-time PCR

Cell line	Histotype	Relative CTGF expression ^a
RD/18	Embryonal	1.0
RD/12	Embryonal	12.8
CCA	Embryonal	41.3
RMZ-RC2	Alveolar	4.3
SJ-RH4	Alveolar	1.3
SJ-RH30	Alveolar	2.8

^a The level of CTGF expression of each rhabdomyosarcoma cell line was normalized over that of the lowest expressor.

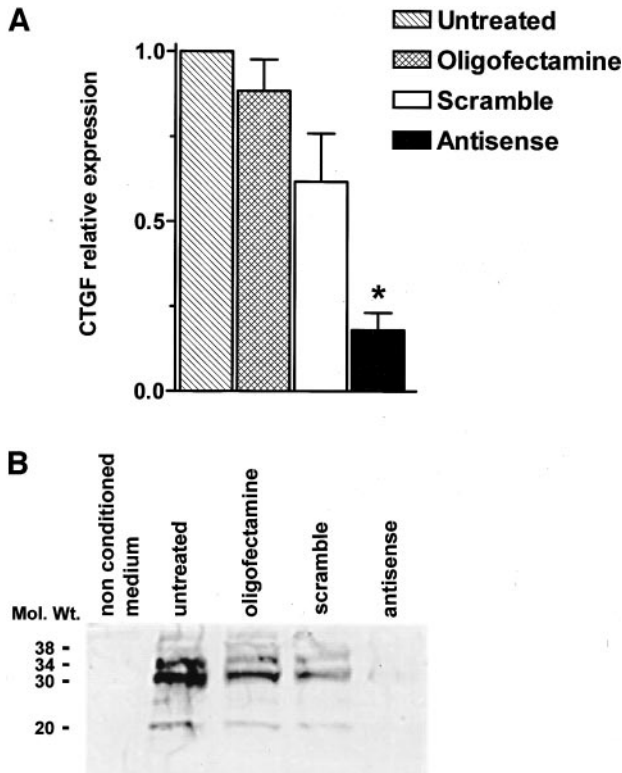


Fig. 2. Effects of connective tissue growth factor (CTGF) antisense oligonucleotide on CTGF expression and production. **A**, quantitative real-time PCR analysis of the CTGF expression level in RD/12 cells after 144-h treatment normalized over untreated cells. The results were compared by the Student's *t* test (*, $P < 0.05$ or lower in all comparisons). ■, untreated cells; ▨, Oligofectamine-treated cells; □, scramble oligonucleotide-treated cells; ■, antisense oligonucleotide-treated cells. **B**, Western blot analysis of indicated RD/12 conditioned media, collected at the 7th day of culture, using an anti-CTGF polyclonal antibody. The differences in CTGF expression levels were confirmed after densitometric analyses of band intensities and normalization to cell yield. Molecular sizes (*Mol. Wt.*) were determined with TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) referring to precision plus protein standards (Bio-Rad, Milan, Italy) run. *Mol. Wt.*, M_r in thousands.

growth of RD/12 cells, but to a lower extent: RD/12 cell growth was reduced by 60% after 7 days of culture compared with untreated cells (Fig. 3). These data indicate the presence of a CTGF autocrine/paracrine loop that contributes to rhabdomyosarcoma cell growth.

Proliferation and Survival. Cell growth can be sustained either by stimulation of proliferative rate and/or by inhibition of apoptosis. To investigate the role of autocrine CTGF in these processes, the effects of CTGF antisense oligonucleotide on proliferation and survival were studied. Treatment with the antisense oligonucleotide did not modify the proliferative index of RD/12 and RD/18 cells as determined by BrdUrd staining (Fig. 4), and, consistent with this result, exogenously added r-hCTGF did not affect rhabdomyosarcoma cell growth (data not shown). In contrast, treatment with CTGF antisense oligonucleotide significantly increased the percentage of apoptotic cells (Fig. 5).

The percentage of RD/12 and RMZ-RC2 apoptotic cells determined by morphological evaluation of the nuclei increased five times, and the percentage of RD/18 apoptotic cells increased three times after a 24-h treatment with the antisense oligonucleotide. After 48 h, the percentage of RD/12 apoptotic cells increased 10 times, and that of RD/18 and RMZ-RC2 cells increased 4 times (Fig. 5, A, C, and D). The effects of the antisense oligonucleotide on RD/12 cell survival was also investigated by TUNEL assay (Fig. 5B). TUNEL assay confirmed the results obtained by morphological examination of the nuclei but indicated an even greater effect. Therefore, the inhibition of rhabdomyosarcoma cell growth by interrupting the CTGF autocrine loop was specifically due to a decrease in cell survival.

Myogenic Differentiation. Autocrine growth factors frequently control the balance between cell growth and differentiation in rhabdomyosarcoma. To investigate this aspect, we analyzed the effects of CTGF antisense oligonucleotide and exogenously added human recombinant CTGF on the proportion of rhabdomyosarcoma cells expressing embryonic myosin. RD/12 is characterized by a strong block in myosin expression that was not modified by treatment with the

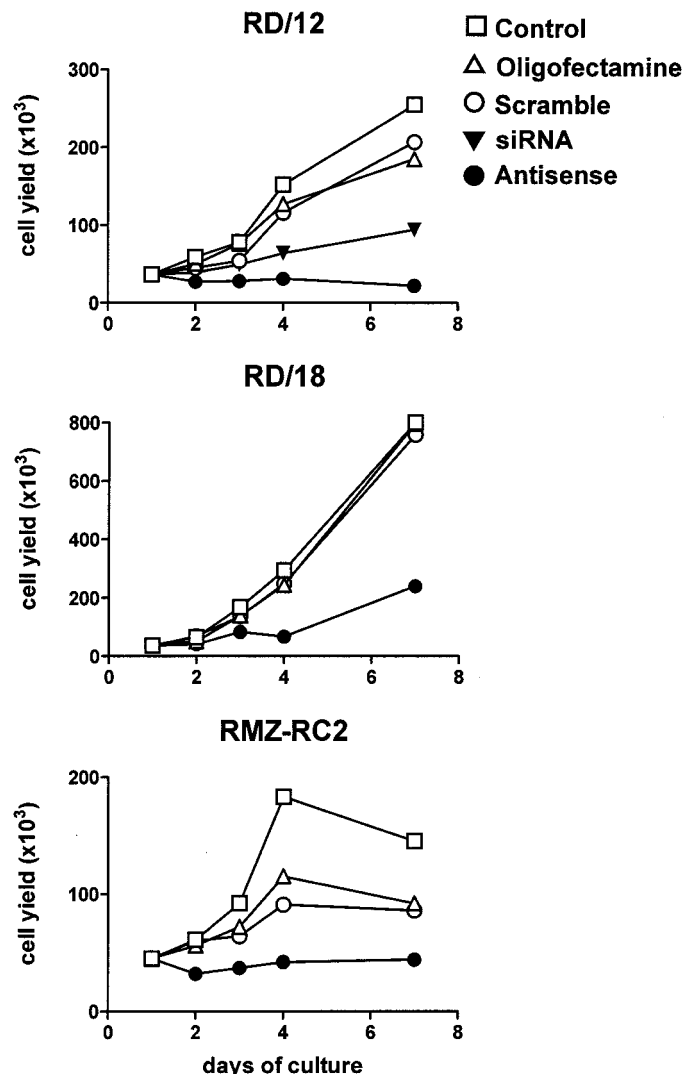


Fig. 3. Effects on rhabdomyosarcoma cell growth of connective tissue growth factor (CTGF) silencing by antisense oligonucleotide (*Antisense*) and small interfering RNA (*siRNA*). Rhabdomyosarcoma cells of the embryonal (RD/12 and RD/18) and the alveolar (RMZ-RC2) histotype were treated with CTGF antisense oligonucleotide (●) or CTGF *siRNA* (only RD/12 cells; ▼) at 0.25 μ M concentration, in the presence of Oligofectamine. Controls included untreated cells (□), cells treated with Oligofectamine alone (△), or with scramble oligonucleotide (○). Data from a representative experiment of three are shown.

CTGF antisense oligonucleotide (Fig. 6), and by recombinant CTGF (data not shown). The defect in differentiation of RD/18 and RMZ-RC2 is at the level of myosin expression. All cells express myogenic transcription factors such as MyoD and Myf4 as well as muscle-specific intermediate filaments (desmin) but only a minority of cells is capable of myosin expression. The silencing of CTGF expression by the antisense oligonucleotide significantly decreased the myogenic differentiation of RD/18 and RMZ-RC2 cells (more than 50%; Fig. 6). Although exogenously added r-hCTGF did not modify RD/18 and

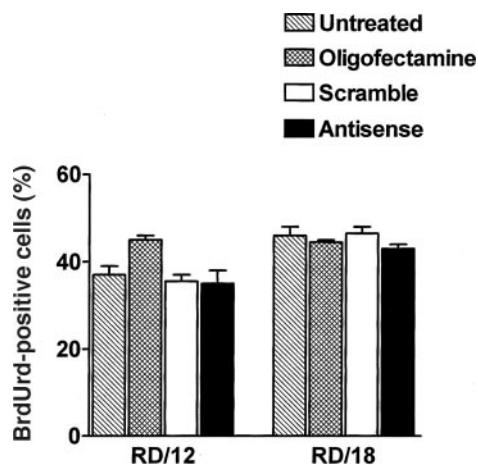


Fig. 4. Effects of connective tissue growth factor (CTGF) antisense oligonucleotide on rhabdomyosarcoma cell proliferation. Each bar, the percentage of BrdUrd-positive cells after 24-h (RD/12) and 48-h (RD/18) treatment. ▨, untreated cells; ▩, Oligofectamine-treated cells; □, scramble oligonucleotide-treated cells; ■, antisense oligonucleotide-treated cells.

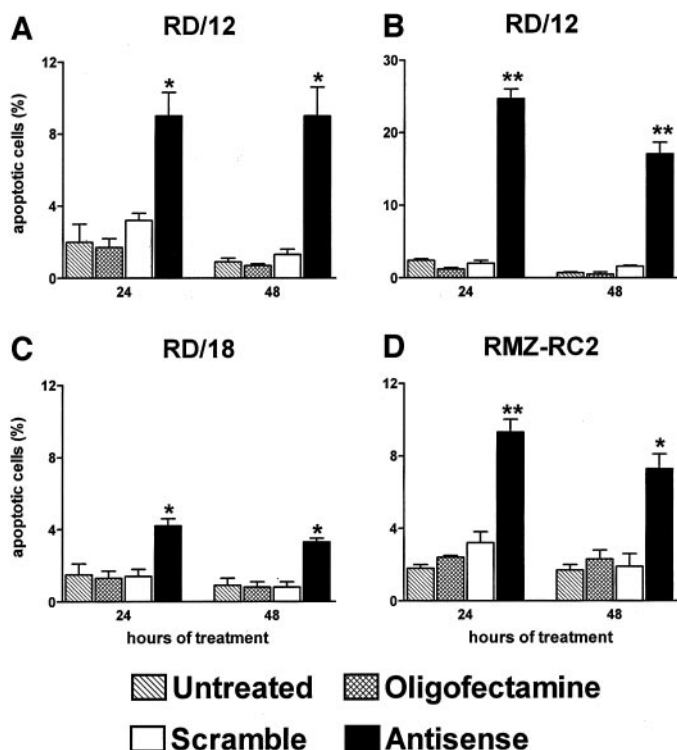


Fig. 5. Effects of connective tissue growth factor (CTGF) antisense oligonucleotide on rhabdomyosarcoma cell apoptosis. A, C, D, each bar, the percentage of apoptotic cells determined by a morphological examination of the nuclei. B, each bar, the proportion of RD/12 apoptotic cells determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The results were compared by the Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). ▨, untreated cells; ▩, Oligofectamine-treated cells; □, scramble oligonucleotide-treated cells; ■, antisense oligonucleotide-treated cells.

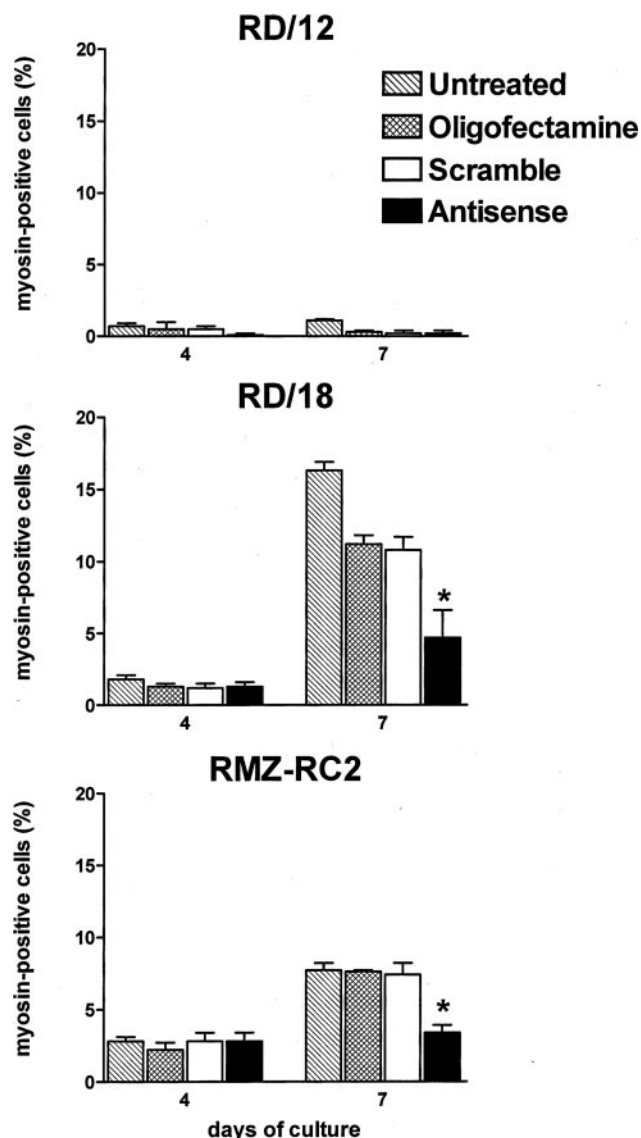


Fig. 6. Effects of connective tissue growth factor (CTGF) antisense oligonucleotide on rhabdomyosarcoma cell myogenic differentiation. Each bar, the percentage of embryonic myosin-positive cells determined after 4 and 7 days of culture in a low-serum supplemented medium. The results were compared by the Student's *t* test (*, $P < 0.05$). ▨, untreated cells; ▩, Oligofectamine-treated cells; □, scramble oligonucleotide-treated cells; ■, antisense oligonucleotide-treated cells.

RMZ-RC2 cell growth, the percentage of myosin-positive cells was significantly increased (Fig. 7).

DISCUSSION

CTGF expression was found in human rhabdomyosarcoma cell lines and in embryonal and alveolar rhabdomyosarcoma tumor specimens. Silencing of CTGF expression triggered apoptosis and inhibited myogenic differentiation, whereas exogenously added r-hCTGF increased myogenic differentiation. Thus, CTGF is a new autocrine survival and differentiation factor for human rhabdomyosarcoma cells.

CTGF enters into the network of growth factors that regulate rhabdomyosarcoma growth and myogenic differentiation. It is known that IGF-II, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF)/TGF- α autocrine loops promote the growth of rhabdomyosarcoma cells (38–40). TGF- β autocrine loop regulates both their growth and myogenic differentiation in a concentration-

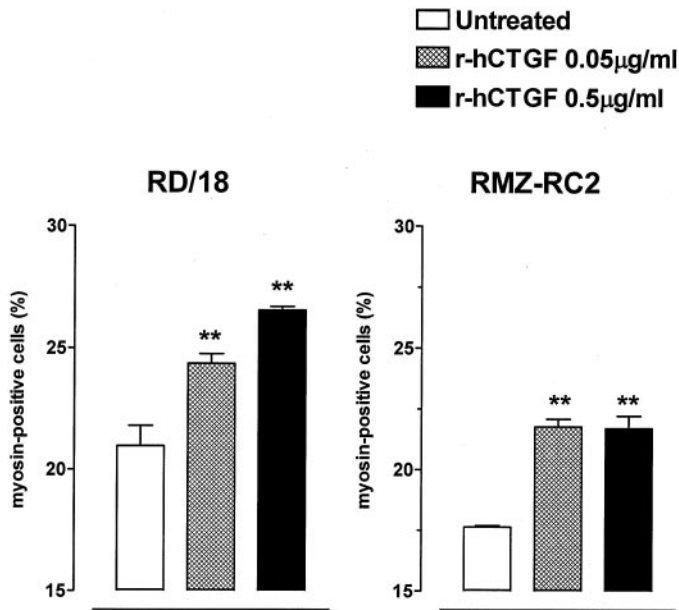


Fig. 7. Effects of exogenously added recombinant human connective tissue growth factor (*r-hCTGF*) on rhabdomyosarcoma cell myogenic differentiation. RD/18 and RMZ-RC2 cells were cultured in DMEM + 2% horse serum in the presence of 0.05 µg/ml *r-hCTGF* (▨) and 0.5 µg/ml *r-hCTGF* (■). Untreated cells were used as controls (□). Each bar, the percentage of myosin-positive cells after 10 days of culture. The results were compared by the Student's *t* test (**, $P < 0.01$ or lower in all comparisons).

dependent way (41), and autocrine nerve growth factor sustains their survival (33). In addition to its direct activities on rhabdomyosarcoma cells, CTGF could interact with TGF- β signaling and with IGF-I and -II actions because it contains an IGF-binding protein domain, and it has been shown that CTGF and IGF-I may act cooperatively (42). *CTGF* promoter contains a TGF- β response element (43), and TGF- β stimulates CTGF expression in connective tissue cells (44, 45). Moreover, CTGF plays a role in various forms of tissue fibrosis and in wound healing as a TGF- β downstream mediator. On the other hand, it has recently been discovered that CTGF itself can strengthen TGF- β 1 signaling (46). Rhabdomyosarcoma cells express TGF- β (41). TGF- β may induce CTGF expression that in turn might promote TGF- β effects or might have CTGF-specific effects.

The inhibition of rhabdomyosarcoma cell growth induced by *CTGF* antisense oligonucleotide was specifically due to a significant increase in the percentage of apoptotic cells, whereas the percentage of cells in S phase of the cell cycle was not modified. CTGF can have opposing activities on cells, depending on cell type. CTGF stimulates the proliferation of fibroblast (47), hepatic stellate (48), osteoblastic (49), chondrocytic (50, 51), and vascular endothelial cells (52, 53). It induces apoptosis in the MCF-7 breast cancer cell line (25) and in human aortic smooth muscle cells (54, 55), and it sustains the survival of endothelial cells (56). Our results indicate that, for rhabdomyosarcoma cells, CTGF is a survival factor.

Autocrine growth and survival factors may be involved in the defective myogenic differentiation of rhabdomyosarcoma. Proliferation, survival, and differentiation are strongly linked in the myogenic developmental program. During myogenesis, myoblasts withdraw from the cell cycle, acquire an apoptosis-resistant phenotype, and then express myosin and fuse into myotubes (57). Our data suggest that CTGF is involved in the differentiation process of rhabdomyosarcoma cells: *CTGF* antisense oligonucleotide treatment decreased the myogenic differentiation of RD/18 and RMZ-RC2 cells, and the addition of *r-hCTGF* to culture media increased them. RD/12 cells express CTGF at high levels and are characterized by a functional CTGF

autocrine loop, as demonstrated by the inhibition of growth induced by antisense oligonucleotide, but they are incapable of terminal myogenic differentiation. It is plausible that RD/12 cells retain a defect in the myogenic developmental signaling pathway, not yet identified, that blocks differentiation, even in the presence of differentiation factors such as CTGF. In that case, CTGF may control the survival of RD/12 cells but might not perform its effects on differentiation. Otherwise, RD/12 cells may lack a CTGF receptor that regulates differentiation. The high level of endogenous CTGF found in RD/12 cells may be a feedback consequence of the inability to differentiate.

A CTGF-specific receptor has not yet been identified. A putative specific M_r 280,000 ligand-receptor complex has been found on osteoblastic (49) and chondrocytic cells (58). CTGF can also bind platelet-derived growth factor receptor (1), α_2 macroglobulin receptor (59), integrins $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_M\beta_2$ (56, 60, 61) and probably ErbB4 receptor (62). It is plausible that specific CTGF receptors are involved in CTGF autocrine loop, because RD/12 and RD/18 cells expressed neither platelet-derived growth factor receptor α and β^5 nor ErbB4 receptor by cytofluorometric analysis (63).

Some information about CTGF mode of action in rhabdomyosarcoma could be drawn from CTGF signaling pathways. In chondrocytes, CTGF promotes differentiation through a p38 mitogen-activated protein kinase and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (51). Activation of p38 mitogen-activated protein kinase signaling may also be involved in rhabdomyosarcoma differentiation because it is essential for myoblasts terminal differentiation (64).

On the other hand, CTGF effects on differentiation may be a consequence of its strong effects on survival. In the myogenic pathway, survival factors are necessary for the acquisition of myoblast apoptosis-resistant phenotype and, as a consequence, for differentiation. In addition, they can activate signaling molecules, such as the serine-threonine protein kinase Akt, that regulate both survival and myogenic differentiation (65, 66). CTGF might promote the acquisition of the apoptosis-resistant phenotype, thereafter sustaining differentiation. In this case, CTGF actions might be similar to those of IGF-II in skeletal myoblasts (67). CTGF might also activate signaling molecules common to survival and myogenic differentiation pathways leading to an increased differentiation.

The expression of other members of the CCN family has been found in rhabdomyosarcoma or skeletal muscle cells pointing out the importance of these cysteine-rich proteins in this tumor. CYR61 is expressed in human primary myoblasts and is down-regulated in the embryonal rhabdomyosarcoma cell line RD (68). Nov is highly expressed in fusing myoblasts and myotubes (3). In addition, it is expressed in rhabdomyosarcoma cell lines and tumor specimens, and its expression correlates positively with the level of myogenic differentiation (69). Our results suggest that CTGF promotes the survival and differentiation of rhabdomyosarcoma cells, and we can suppose a similar role in skeletal muscle cells. Therefore, CCN proteins emerge as key regulators of the whole musculoskeletal system controlling the behavior of chondrocytes, osteoblasts, and smooth and skeletal muscle cells.

CTGF may be a useful therapeutic target in human rhabdomyosarcoma. Interfering with the CTGF autocrine loop by use of CTGF antagonists (neutralizing antibodies, small blocking peptides, antisense oligonucleotides, siRNAs) could induce rhabdomyosarcoma cell death and decrease tumor angiogenesis, but additional studies are needed to determine whether CTGF can also control the malignancy of rhabdomyosarcoma cells.

⁵ Unpublished data.

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