

Induction of Antiproliferative Connective Tissue Growth Factor Expression in Wilms' Tumor Cells by Sphingosine-1-Phosphate Receptor 2

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Abstract

Connective tissue growth factor (CTGF), a member of the CCN family of secreted matricellular proteins, regulates fibrosis, angiogenesis, cell proliferation, apoptosis, tumor growth, and metastasis. However, the role of CTGF and its regulation mechanism in Wilms' tumor remains largely unknown. We found that the bioactive lipid sphingosine-1-phosphate (S1P) induced CTGF expression in a concentration- and time-dependent manner in a Wilms' tumor cell line (WiT49), whereas FTY720-phosphate, an S1P analogue that binds all S1P receptors except S1P₂, did not. Further, the specific S1P₂ antagonist JTE-013 completely inhibited S1P-induced CTGF expression, whereas the S1P₁ antagonist VPC44116 did not, indicating that this effect was mediated by S1P₂. This was confirmed by adenoviral transduction of S1P₂ in WiT49 cells, which showed that overexpression of S1P₂ increased the expression of CTGF. Induction of CTGF by S1P was sensitive to ROCK inhibitor Y-27632 and c-Jun NH₂-terminal kinase inhibitor SP600125, suggesting the requirement of RhoA/ROCK and c-Jun NH₂-terminal kinase pathways for S1P-induced CTGF expression. Interestingly, the expression levels of CTGF were decreased in 8 of 10 Wilms' tumor tissues compared with matched normal tissues by quantitative real-time PCR and Western blot analysis. *In vitro*, human recombinant CTGF significantly inhibited the proliferation of WiT49 cells. In addition, overexpression of CTGF resulted in

significant inhibition of WiT49 cell growth. Taken together, these data suggest that CTGF protein induced by S1P₂ might act as a growth inhibitor in Wilms' tumor. (Mol Cancer Res 2008;6(10):1649–56)

Introduction

Connective tissue growth factor (CTGF) is a member of the CCN family, which includes CTGF (also known as CCN2), cysteine-rich 61 (CYR61, also known as CCN1), nephroblastoma overexpressed (NOV, also known as CCN3), and the newly discovered WISP-1/elm1 (CCN4), WISP-2/rCop1 (CCN5), and WISP-3 (CCN6). CCN family members are involved in a variety of biological functions and are thought to be important regulators of tumorigenesis (1, 2). Originally identified as a secreted mitogen from the conditioned medium of human umbilical vein endothelial cells (3), CTGF is widely known to play an important role in different fibrotic diseases (4–6). More recently, CTGF has been implicated as a regulatory protein in several human cancers, including breast cancer (7), pancreatic cancer (8, 9), melanomas (10), and chondrosarcomas (11). Chang et al. (12, 13) found that reduced expression of CTGF was associated with the risk of advanced-stage disease, lymph node metastasis, and shorter survival in lung adenocarcinoma. However, the role of CTGF in Wilms' tumor is not known.

The bioactive lipid sphingosine-1-phosphate (S1P) is the ligand for five G protein-coupled receptors of the endothelial differentiation gene family (S1P₁₋₅). Interaction of S1P with its different receptors results in regulation of diverse cellular functions, as a consequence of activating different downstream signaling pathways. The successful use of a S1P receptor modulator FTY720 in a murine melanoma model (14) and a biospecific anti-S1P antibody in several murine xenograft and allograft models (15) to inhibit tumor progression suggests that the S1P pathway is a promising new therapeutic target in oncology (16). Recently, it has been shown that S1P can regulate CTGF expression in nonmalignant cell types, such as human endothelial cells (17), smooth muscle cells (18), and rat mesangial cells (19). The relationship between S1P and CTGF in human cancers remains unexplored.

Wilms' tumor is the most common malignant renal tumor in children. Although multimodal therapy involving surgery, chemotherapy, and radiation has resulted in high overall cure rates, the significant late effects associated with these treatment modalities highlight the need for alternative therapeutic approaches (20). To date, the mechanisms governing growth

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and metastasis of Wilms' tumor are still largely unknown. Interestingly, Zirn et al. (21) found that CTGF was down-regulated in advanced Wilms' tumors and treatment of Wilms' tumor cell lines with the anticancer agent all-*trans* retinoic acid increased CTGF level by real-time PCR and microarray analysis (22), which suggested that CTGF might act as a negative modulator in Wilms' tumor. In this study, we explored the relationship between S1P and CTGF expression in WiT49 cells (23), a metastatic Wilms' tumor cell line, as well as the role that CTGF might play in Wilms' tumorigenesis.

Results

S1P Induced CTGF Expression and Secretion in WiT49 Cells

Prior reports have shown that S1P induced CTGF expression in various nonmalignant cell types (17-19). To date, it is still unclear whether this effect occurs in human cancers. To address this question, a well-characterized Wilms' tumor cell line, WiT49, was used (23). We found that S1P induced CTGF mRNA expression in a time-dependent manner (Fig. 1A). Western blot analysis further showed that this effect began as early as 0.5 h, reached maximum at 1 to 2 h, and returned to the basal level at 24 h (Fig. 1B). Treatment of WiT49 cells with different concentrations of S1P for 1 h also showed that S1P induced CTGF expression in a concentration-dependent manner by quantitative real-time PCR analysis (Fig. 1C) and Western blot analysis (Fig. 1D). Because CTGF is a secreted protein (24, 25), the conditioned medium of WiT49 cells was collected and detection of CTGF protein was done by Western blot analysis. It showed that S1P increased CTGF secretion time dependently (Fig. 1E). Two detectable bands with molecular weights of about 38 and 42 kDa (Supplementary Fig. S1), one of which might be its *N*-glycosylated form (24, 25), can be seen as early as 0.5 h after S1P treatment (data not shown).

S1P-Induced CTGF Expression Was Mediated by S1P₂ Receptor

There are five S1P receptors (S1P₁₋₅), which have been identified to bind specifically to S1P (26). We have analyzed S1P receptor expression in Wilms' tumor specimens and confirmed that S1P₁, S1P₂, S1P₃, among others, seem to be expressed *in vivo* (Supplementary Fig. S2). To elucidate which S1P receptor was responsible for this induction, we used several pharmacologic agonists and antagonists of S1P receptors. The immunosuppressor FTY720 is metabolized *in vivo* by sphingosine kinase activity. Its phosphorylated derivative, FTY720-phosphate (FTY720-P), is a potent agonist of all S1P receptors except S1P₂ (27, 28). In contrast to S1P, stimulation of WiT49 cells with FTY720-P did not increase the CTGF levels (Fig. 2A), suggesting that CTGF expression might be mediated by S1P₂. To substantiate this notion, we used the specific S1P₂ antagonist JTE-013 (29). Preincubation of WiT49 cells with JTE-013 inhibited S1P-induced CTGF expression in a dose-dependent manner (Fig. 2B). In contrast, inhibition of S1P₁ signaling by the specific S1P₁ antagonist VPC44116 (30) or Gi signaling by pertussis toxin did not affect the CTGF expression induced by S1P (Fig. 2B), whereas these treatments effectively inhibited S1P-induced WiT49 cell migration (data not shown). Finally, we overexpressed S1P₂ or S1P₁ in WiT49 cells by adenoviral

transduction. In agreement with our hypothesis, overexpression of S1P₂ into WiT49 cells increased the basal level of CTGF protein (Fig. 2C), most likely due to the autocrine effect of endogenously made S1P (29). In fact, blockade of S1P₂ with JTE-013 in the absence of exogenous S1P significantly inhibited this effect (Supplementary Fig. S3). Taken together, these data indicate that S1P-induced CTGF expression is mediated by S1P₂.

RhoA/ROCK and c-Jun NH₂-Terminal Kinase Pathways Mediated S1P-Induced CTGF Expression, whereas p38 and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase Pathways Were Partially Involved

It has been shown that Rho GTPase RhoA and three mitogen-activated protein kinases, including c-Jun NH₂-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), act as the downstream molecules of S1P₂ (31). Pull-down assay showed that RhoA was activated by S1P in a time-dependent manner. Western blot analysis also showed that JNK, p38, and ERK were rapidly activated on the stimulation by S1P at 5 to 10 min (Fig. 3A). To delineate the downstream signaling pathways of S1P-induced CTGF expression by S1P₂, various pharmacologic inhibitors were used. Interestingly, inhibition of ROCK and JNK signaling pathways with the ROCK inhibitor Y-27632 and the JNK inhibitor SP600125 completely blocked S1P-induced CTGF mRNA expression as did the S1P₂ antagonist JTE-013. Blockade of p38 and ERK pathways with the p38 inhibitor SB203580 and ERK inhibitor U0126 only showed partial inhibition (about 50-65%; Fig. 3B), which suggested that ROCK and JNK might be required for CTGF induction by S1P and that p38 and ERK were only partially involved. Consistent with the results of quantitative real-time PCR, Western blot analysis also showed that CTGF protein levels were regulated similarly as its mRNA (Fig. 3C).

CTGF Expression Was Suppressed in Wilms' Tumor Tissues

Having shown that CTGF could be induced by S1P/S1P₂ pathway in WiT49 cells and based on the findings that S1P, mainly produced from hematopoietic system and vascular endothelium (32-34), is a normal constituent of human plasma and serum (35), we were interested in knowing the expression level of CTGF *in vivo*. Therefore, 10 frozen Wilms' tumor tissues were analyzed. Consistent with Zirn et al.'s findings (21), quantitative real-time PCR showed that CTGF mRNA levels were decreased in 8 of 10 Wilms' tumor tissues when compared with their matched normal tissues (Fig. 4A), and these results were further confirmed by Western blot analysis (Fig. 4B).

CTGF Inhibited Cell Proliferation in WiT49 Cells

Having shown that significant levels of CTGF were secreted in response to S1P *in vitro*, and that decreased CTGF levels were detected in tumor tissues *in vivo*, we tested what the effect of CTGF was on Wilms' tumor cell proliferation. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done in WiT49 cells using human recombinant CTGF protein. After treatment of WiT49 cells with different concentrations of CTGF for 48 h, we found that CTGF significantly inhibited cell proliferation at 1 μg/mL (22% inhibition) and

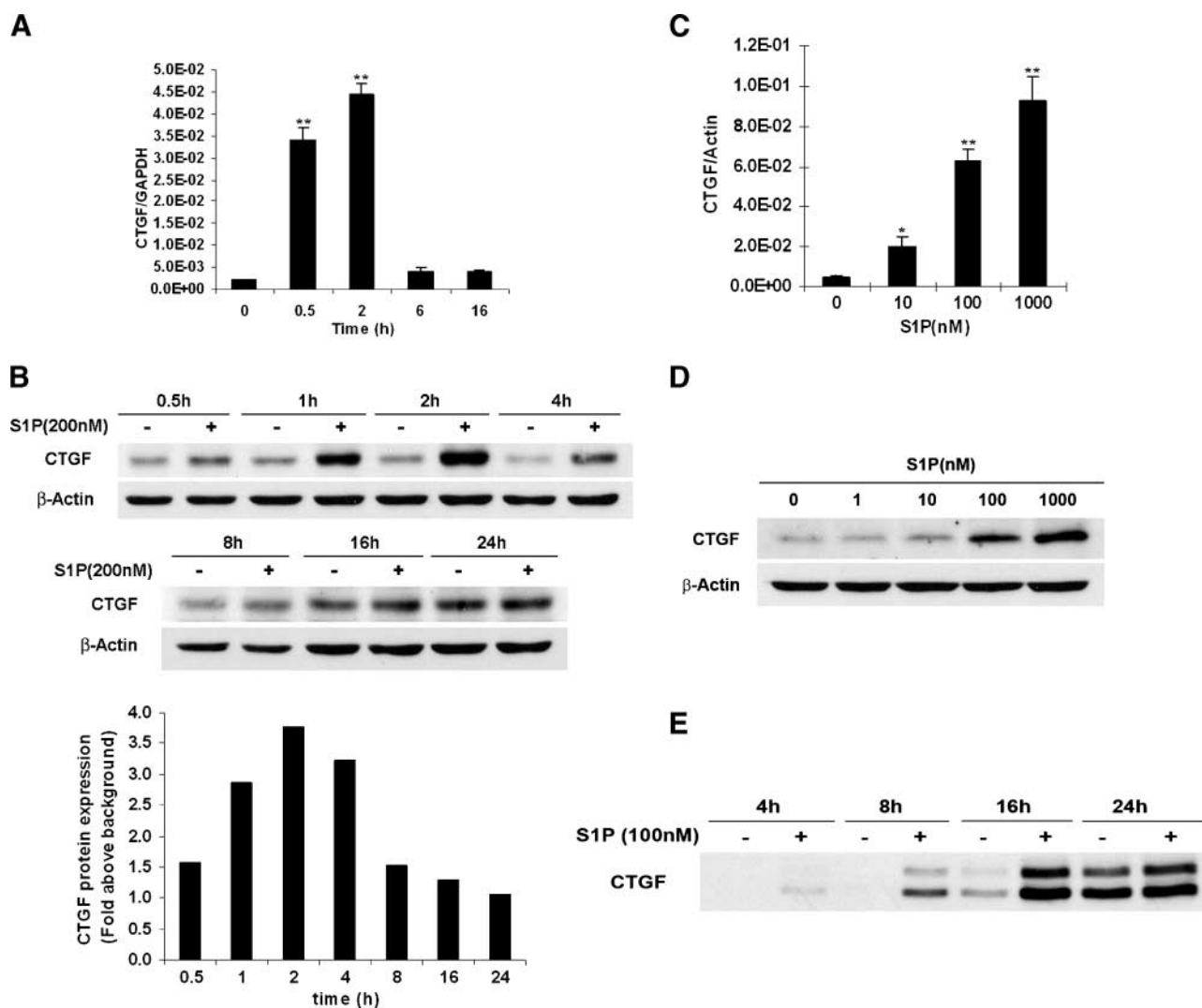


FIGURE 1. S1P induced CTGF expression and secretion in WiT49 cells. **A** and **B.** WiT49 cells were serum starved for 24 h and then treated with S1P for different times before quantitative real-time PCR (**A**, 100 nmol/L S1P) or Western blot analysis (**B**, 200 nmol/L S1P) was done. **, $P < 0.01$ versus control (0 h). Relative ratio in **B** represents the fold induction of CTGF by S1P treatment compared with that in nontreatment control at each different time point. CTGF expression was normalized to the housekeeping gene β -actin. **C** and **D.** WiT49 cells were serum starved for 24 h and then treated with different concentrations of S1P for 1 h before quantitative real-time PCR (**C**) or Western blot analysis (**D**) was done. *, $P < 0.05$; **, $P < 0.01$ versus nontreatment control. **E.** Conditioned media of WiT49 cells were collected and prepared as in Materials and Methods and followed by Western blot analysis.

5 μ g/mL (40% inhibition). However, heat-inactivated CTGF did not have any effect on cell proliferation (Fig. 5A). To confirm that the recombinant CTGF used in our study was biologically active, we checked the active forms of ERK and AKT, which were shown to be the downstream molecules of CTGF signaling (36, 37). Consistent with Yosimichi et al.'s findings (36, 37), 1 μ g/mL CTGF activated ERK and AKT phosphorylation in WiT49 cells (Supplementary Fig. S4).

In addition, to further confirm the antiproliferative role of CTGF in WiT49 cells, we transiently transfected WiT49 cells with PCMV-SPORT6-CTGF plasmid. CTGF expression was confirmed by Western blot analysis (data not shown). Compared with vector control transfected cells, WiT49 cells overexpressing CTGF showed significant reduction in their growth rate as measured by MTT assay after day 4 (Fig. 5B). Taken together, these data support an antiproliferative role for CTGF in Wilms' tumor.

Discussion

Although substantial progress has been made in the treatment of Wilms' tumor, the failure of some patients to respond to current treatments along with emerging data about long-term risks of those treatments call for the development of new therapeutic approaches. Bioactive lipids, such as S1P, are increasingly being recognized as therapeutic targets for many diseases, including cancer. Several investigators, including our laboratory, have observed the differential effect of ligand-S1P₁ and S1P₂ interactions. We have previously shown that S1P₂ has an inhibitory effect on glioblastoma cell migration, whereas S1P₁ stimulates migration (38). In the current study, we begin to look beyond S1P-driven cellular migration to explore its regulation of a member of the CCN family of signaling proteins. Our interest in this area was driven by two recent studies, which have identified CTGF as a target protein in Wilms' tumor (21, 22). Using

microarray and real-time PCR analysis of patients from SIOP protocols, Zirn et al. (21) found that diminished expression of CTGF was associated with a higher risk of relapse and disease progression. In another related study, treatment of Wilms' tumor cell lines with the differentiating agent retinoic acid caused an increase in CTGF expression (22), further suggesting that CTGF dysregulation may occur in Wilms' tumor. These findings in conjunction with evidence from experiments done in nonmalignant cells (17-19) that suggest regulation of CTGF by S1P prompted our investigations.

In our study, we used WiT49 cell line as our system model because our previous study showed that in WiT49 cells various S1P receptors were expressed at different levels in a similar expression pattern of Wilms' tumor tissues. Quantitative real-time PCR and Western blot analysis showed that S1P, which is mainly produced from hematopoietic system and vascular endothelium (32-34), and physiologically detectable at concentrations of ~200 nmol/L in human plasma and 500 nmol/L in human serum (35), could induce CTGF expression in WiT49 cells at a concentration as low as 10 nmol/L. It only took 2 h to reach the peak level, indicating that CTGF was an immediate early gene during this process (Fig. 1). Western blot analysis of conditioned medium from WiT49 cells further showed that S1P induced CTGF secretion in a time-dependent manner, with the modified band (42 kDa) greatly enhanced (Fig. 1E; Supplementary Fig. S1). As an exogenous stimulator, the bioactive lipid S1P has various biological functions, which are carried out by interaction with its receptors and subsequent activation of downstream pathways. S1P receptors play important roles in

angiogenesis, vascular development, lymphocyte trafficking, and cancer (38-41), in which usually S1P₁ and S1P₂ display opposite effects. However, little is known about which S1P receptor is responsible for S1P-mediated CTGF induction. To address this question, we used several techniques, including the use of S1P analogue FTY720-P, specific S1P₂ antagonist JTE-013, as well as adenoviral transduction (Fig. 2). Our findings conclusively show that S1P-induced CTGF expression was mediated by S1P₂, not by S1P₁ or other receptors.

S1P₂ mainly couples G_{12/13} protein and activates small Rho GTPase RhoA as well as three mitogen-activated protein kinase signaling pathways (26, 31, 38). In our study, RhoA and three mitogen-activated protein kinases (JNK, p38, and ERK) were found to be rapidly activated by S1P in WiT49 cells. To further investigate the downstream pathways of S1P-induced CTGF expression, different pharmacologic inhibitors were used. The results showed that the ROCK inhibitor Y-27632 and JNK inhibitor SP600125 completely blocked S1P-induced CTGF expression in WiT49 cells, whereas the p38 inhibitor SB203580 and the ERK inhibitor U0126 only partially inhibited this induction (Fig. 3), which suggested that S1P-induced CTGF expression might mainly be mediated by RhoA/ROCK and JNK pathways in WiT49 cells.

CTGF is a multifunctional protein that engages a wide variety of biological processes. On one hand, CTGF has been reported to have angiogenic properties. It can stimulate the adhesion, proliferation, migration, and tube formation of vascular endothelial cells as well as the neovascularization of chicken chorioallantoic membrane, whereas anti-CTGF antibody blocked all these

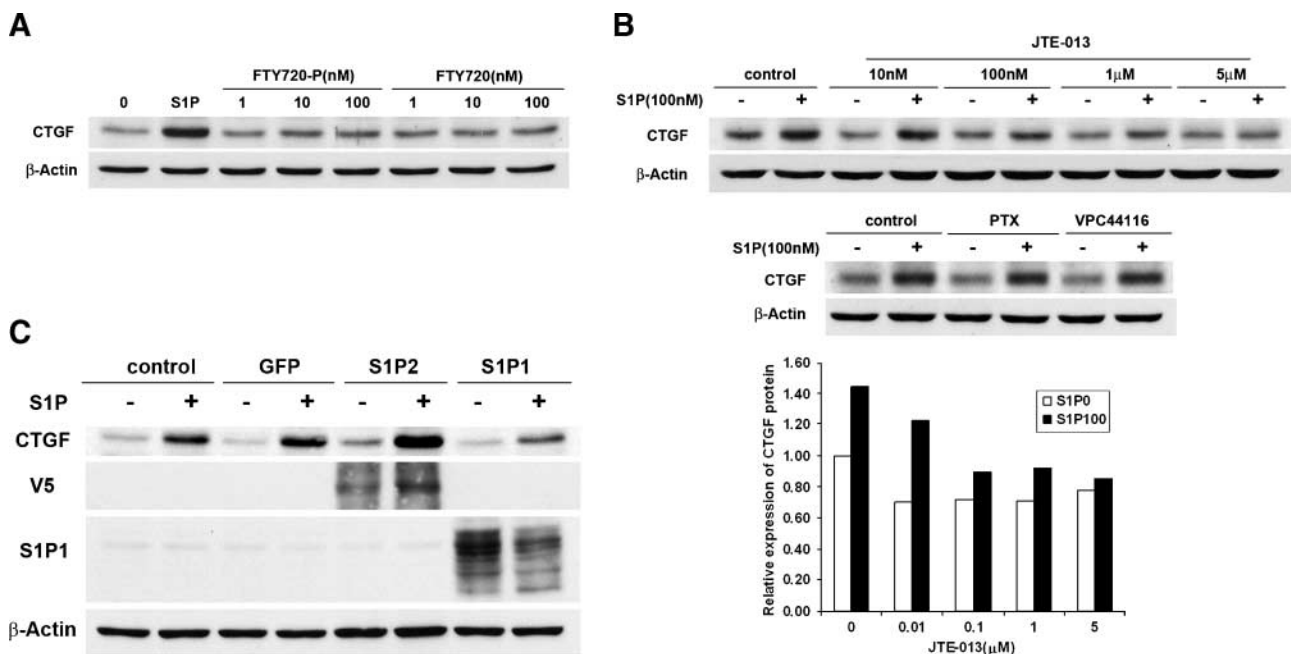


FIGURE 2. S1P-induced CTGF expression was mediated by S1P₂. **A.** WiT49 cells were serum starved for 24 h and then treated with 100 nmol/L S1P or different concentrations of FTY720-P or FTY720 for 1 h before Western blot analysis was done. **B.** WiT49 cells were pretreated with S1P₂ antagonist JTE-013 (10 nmol/L, 100 nmol/L, 1 μmol/L, and 5 μmol/L), S1P₁ antagonist VPC44116 (1 μmol/L), or Gi protein inhibitor pertussis toxin (*PTX*; 400 ng/mL) for 0.5 h after serum starvation and then stimulated with 100 nmol/L S1P for another 1 h before Western blot analysis was done. The relative expression of CTGF protein in cells treated with or without JTE-013 was normalized to that in control cells without JTE-013 or S1P treatment, which was regarded as 1. S1P0, no S1P treatment; S1P100, 100 nmol/L S1P treatment. **C.** WiT49 cells were infected with adenovirus overexpressing S1P₂, S1P₁, or green fluorescent protein (*GFP*) with multiplicity of infection of 100. After 16 to 24 h, cells were serum starved for 24 h and then stimulated with 100 nmol/L S1P for another 2 h before Western blot analysis was done.

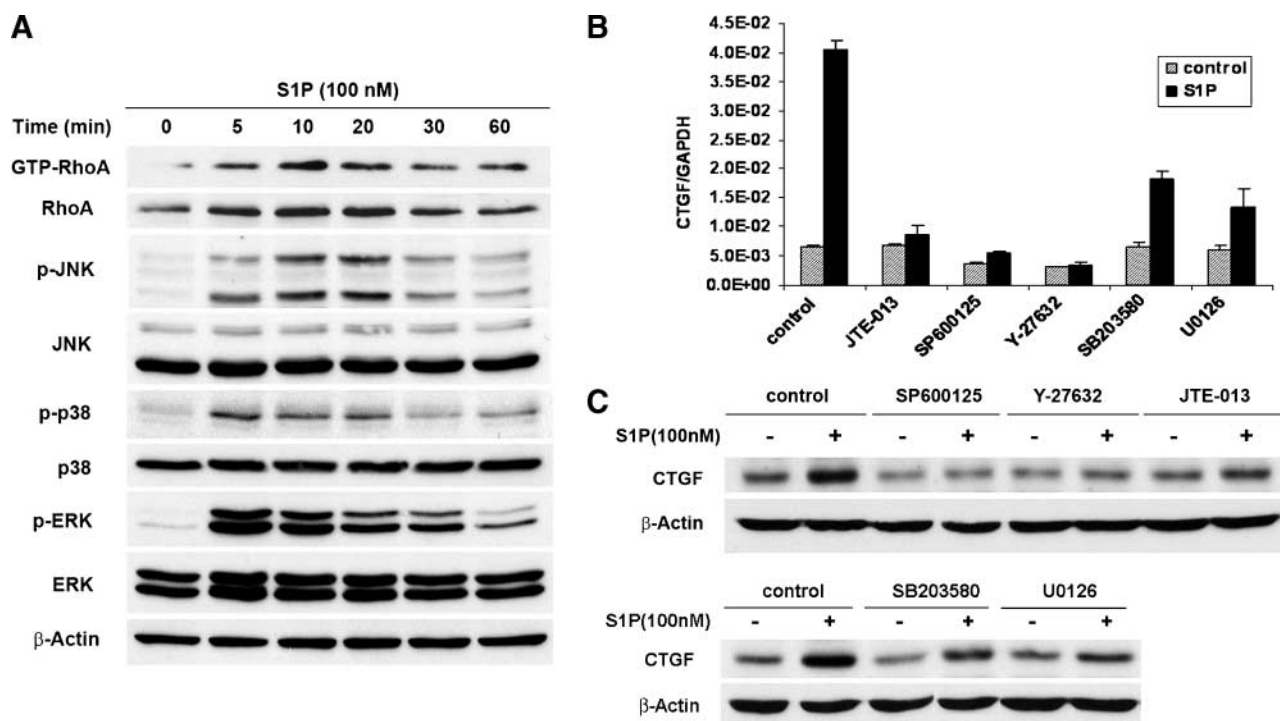


FIGURE 3. S1P-induced CTGF expression was mainly mediated by RhoA/ROCK and JNK pathways. **A.** WiT49 cells were serum starved for 24 h and then treated with 100 nmol/L S1P for different times before pull-down assay and Western blot analysis were done. The blot was stripped and reprobed with β -actin antibody to confirm equivalent loading of lanes. **B** and **C.** WiT49 cells were pretreated with various pharmacologic inhibitors (20 μ mol/L SP600125, 10 μ mol/L Y-27632, 1 μ mol/L JTE-013, 20 μ mol/L SB203580, and 10 μ mol/L U0126) for 0.5 h after serum starvation for 24 h and then stimulated with 100 nmol/L S1P for another 1 h before quantitative real-time PCR (**B**) and Western blot analysis (**C**) were done.

effects (42). On the other hand, CTGF has been regarded as a tumor suppressor. It can induce apoptosis in human breast cancer cells (43) and suppress cell proliferation in non-small cell lung cancer cells (44) and human oral squamous cell carcinoma-derived cells (45). Moreover, recent studies showed that lower CTGF expression was negatively associated with survival and mortality in lung adenocarcinoma and colorectal cancer (13, 46). Further study in lung adenocarcinoma has shown that inhibition of these effects was likely achieved by promoting hypoxia-inducible factor-1 α protein degradation and thus vascular endothelial growth factor inhibition (12). Therefore, the biological function of CTGF seems to be cell type specific and it is probably due to the complex contextual interactions within a specific tumor milieu. For example, recently, integrins were reported to be cell surface receptors of CTGF and a novel integrin α 5 β 1 binding site was thereafter found in module 4 of CTGF (47, 48). However, we cannot exclude that some other surface receptors, growth factors, or cytokines may exist to interact with CTGF and thus promote or inhibit tumor growth (1, 49).

Interestingly, in our study, we found that CTGF was decreased in 80% (8 of 10) of Wilms' tumor tissues obtained from patients enrolled in Children's Oncology Group studies when compared with matched normal tissues (Fig. 4). This prompted us to investigate the role of CTGF in Wilms' tumor cell proliferation. MTT assay showed that human recombinant CTGF produced in *Escherichia coli*, at the concentration of 1 and 5 μ g/mL, had an inhibitory effect on WiT49 cell proliferation (Fig. 5A). Overexpression of CTGF in WiT49

cells by plasmid transfection further confirmed this result (Fig. 5B). However, the role of CTGF in Wilms' tumor may extend beyond an antiproliferative effect and this role needs to be further investigated. In addition, although S1P could induce CTGF expression in Wilms' tumor via S1P₂, we do not propose a simple causal relationship between S1P₂ and CTGF in Wilms' tumorigenesis. In fact, evaluation of S1P₂ in a limited number of Wilms' tumor specimens and normal tissues suggested that S1P₂ mRNA levels were higher in tumor tissues (Supplementary Fig. S5). We concur with accumulating data suggesting that the role of S1P signaling in tumorigenesis is complex and is likely dependent on many factors, such as the level of S1P, differential expression of S1P receptors, resultant effect of downstream effectors, and the specific tumor micro-environments. Our findings do however extend the biological relevance of S1P₂ beyond that of solely an inhibitor of migration and invasion and suggest that its antitumorigenic effects include activation of potential tumor suppressors, such as CTGF.

In summary, our results reveal that S1P can induce CTGF expression in human cancer cells. This is the first time that this induction is mediated by S1P₂, which further extends our knowledge of the biological functions of S1P₂ beyond that of inhibition of cellular migration. Moreover, based on the lower levels of CTGF expressed in untreated Wilms' tumor patients and the inhibitory effect of CTGF on Wilms' tumor cell proliferation, CTGF protein induced by S1P/S1P₂ might act as a tumor suppressor in Wilms' tumor.

Materials and Methods

Materials

DMEM was purchased from Sigma. Fetal bovine serum was purchased from Hyclone. Nutrient mixture F-12 and penicillin-streptomycin were purchased from Life Technologies. S1P was purchased from Biomol and JTE-013 was from Tocris Bioscience. Human recombinant CTGF was purchased from BioVendor. FTY720-P and FTY720 were kindly provided by Dr. Volker Brinkmann (Novartis, Basel, Switzerland) and VPC44116 was obtained from Dr. Kevin R. Lynch. Pertussis toxin, U0126, SB203580, SP600125, and Y-27632 were purchased from Calbiochem. Primary antibodies for CTGF, RhoA, JNK, p38, and β -actin were from Santa Cruz Biotechnology. Antibodies for phosphorylated JNK, phosphorylated p38, phosphorylated AKT, phosphorylated ERK, and ERK were from Cell Signaling Technology and V5 antibody was from Invitrogen. S1P₁ (E49) monoclonal antibody was developed and characterized as described previously (50). Fresh-frozen Wilms' tumor specimens and their matched normal tissues, which are those pieces of kidney obtained near the tumors and verified by pathologic evaluation, were obtained through collaboration with the Children's Oncology Group Biopathology Center.

Cell Culture, Adenoviral Transduction, and Plasmid Transfection

The WiT49 cell line, derived from a primary lung metastasis of an aggressive Wilms' tumor (23), was cultured in 1:1 DMEM/nutrient mixture F-12, 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. For adenoviral transduction, cells were infected with adenovirus containing green fluorescent protein, S1P₁, or S1P₂ for 16 to 24 h (multiplicity of infection, 100) before Western blot analysis was done. For

plasmid transfection, WiT49 cells were transfected with either plasmid PCMV-SPORT6-CTGF (Open Biosystems) or vector control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol before MTT assay was done.

Conditioned Medium Collection

WiT49 cells were plated in 60-mm cell culture dishes (Nunc) at a density of 1×10^6 per dish. After attachment, they were serum starved for 24 h and then treated with or without 100 nmol/L S1P in 3 mL/dish of serum-free DMEM for different times. The conditioned medium was collected, spin down at $1,000 \times g$ for 5 min at 4°C, and then concentrated by Amicon Ultra-4 10K centrifugal filter device (Millipore) at $7,000 \times g$ for 20 min at room temperature.

Quantitative Real-time PCR

Total RNA was isolated from WiT49 cells treated with S1P under different conditions or Wilms' tumor tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol and cDNA was made as previously described (38). Primers were designed using Primer Express 2.0 (Applied Biosystems) according to the software guidelines. Sequences were as follows: *CTGF* gene, 5'-CAGAGTGGAGCGCCTGTTC-3' (forward) and 5'-CTGCAGGAGCGTTGTCAT-3' (reverse); *SIP₁* gene, 5'-GCAGCAGCAAGATGCGAAG-3' (forward) and 5'-CGATGAGTGATCCAGGCTTTT-3' (reverse); *SIP₂* gene, 5'-GGCC-TAGCCAGTTCTGAAAGC-3' (forward) and 5'-GCGTTCC-AGCGTCTCCTT-3' (reverse); *SIP₃* gene, 5'-CTGGTGAC-CATCGTGATCCTC-3' (forward) and 5'-ACGCTCACCACA-ATCACCAC-3' (reverse); *GAPDH* gene, 5'-TGCACCACCA-ACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGT-CATGAG-3' (reverse); and β -actin gene, 5'-GACAGGATGCA-

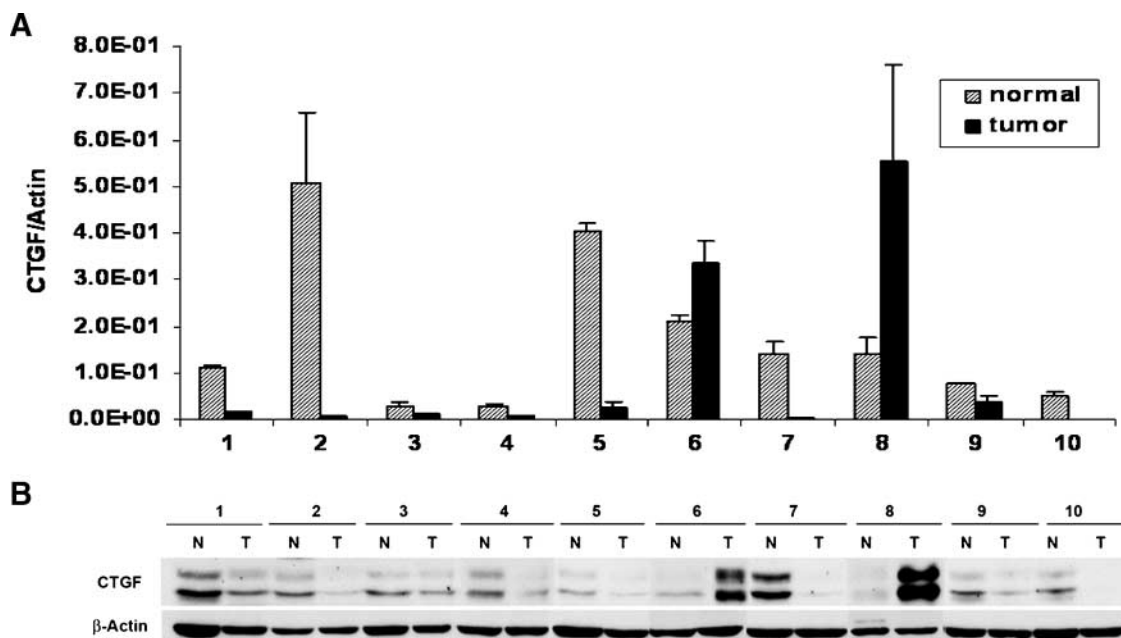


FIGURE 4. CTGF expression was decreased in Wilms' tumor tissues compared with matched normal tissues. **A.** Quantitative real-time PCR for CTGF mRNA expression in 10 Wilms' tumor tissues and their matched normal tissues. CTGF expression was normalized to the expression of the housekeeping gene β -actin. Columns, mean; bars, SD. **B.** Western blot analysis for CTGF protein in these corresponding tissues. Every small piece of tissue (~100 mg) was homogenized in 1 mL radioimmunoprecipitation assay buffer before Western blot analysis was done. N, normal tissue; T, Wilms' tumor.

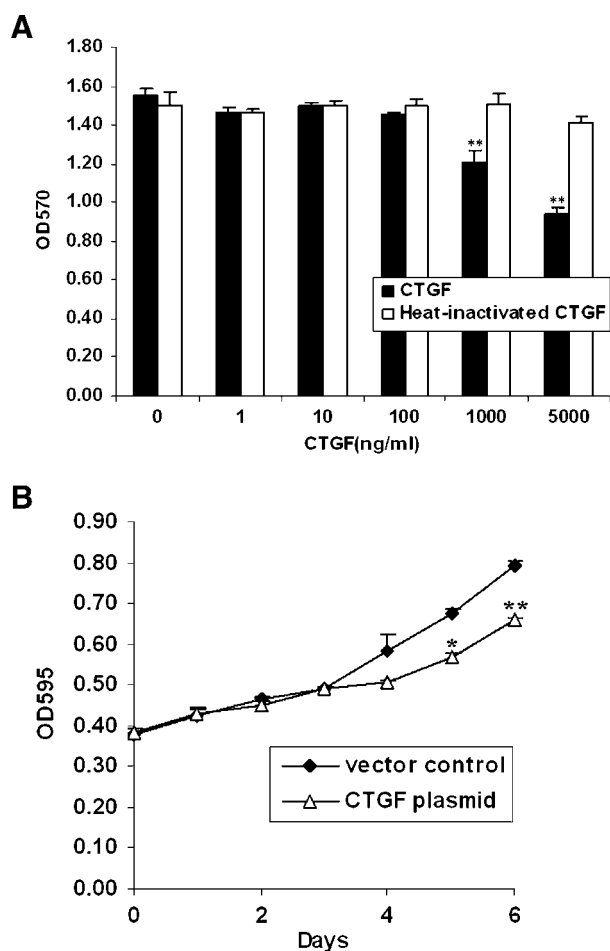


FIGURE 5. CTGF inhibited cell proliferation in WiT49 cells. **A.** WiT49 cells were plated to 96-well plates. After attachment, they were serum starved for 24 h and then incubated with different concentrations of human recombinant CTGF or heat-inactivated CTGF by being boiled for 10 min for an additional 48 h before the MTT assay was conducted. Columns, mean of triplicates; bars, SD. **, $P < 0.01$ versus without CTGF. **B.** WiT49 cells were transfected with either CTGF plasmid or vector control and cultured for indicated days followed by MTT assay. Growth rates were compared between the CTGF plasmid and vector control transfected cells. *, $P < 0.05$; **, $P < 0.01$ versus vector control.

GAAGGAGATTACT-3' (forward) and 5'-TGATCCACATCT-GCTGGAAGGT-3' (reverse). Real-time PCR was done using SYBR Green I DNA binding dye technology on an ABI Prism 7900 HT Sequence Detection System (PE Applied Biosystems). Results were expressed relative to the internal control gene *β-actin* or *GAPDH*.

Western Blot Analysis

WiT49 cells were treated with S1P under different conditions after serum starvation for 24 h. Then, they were washed with ice-cold PBS and homogenized in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 10 mmol/L MgCl₂, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1× protease inhibitor mixture]. Samples were centrifuged at 14,000 × *g* for 20 min at 4°C, and protein concentrations of supernatants

were determined by bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein were separated on 10% SDS-PAGE and blotted to nitrocellulose membranes. The membranes were incubated with the indicated primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized by exposure to X-ray film using Pierce ECL Western Blotting Substrate according to the manufacturer's instructions.

RhoA-GTP Pull-Down Assay

WiT49 cells were treated with 100 nmol/L S1P for the indicated time after serum starvation for 24 h. Then, they were lysed with radioimmunoprecipitation assay buffer. Measurement of RhoA-GTP activity was done using the Rhotekin-RBD protein glutathione *S*-transferase beads (Cytoskeleton) following the manufacturer's instructions. Total and activated RhoA protein was detected by Western blot analysis as described above.

MTT Assay

Proliferation of WiT49 cells treated with human recombinant CTGF or transfected with either CTGF plasmid or control vector was determined by the MTT assay. Briefly, after treatment, cells were incubated at 37°C for 2 h in the presence of MTT (Sigma-Aldrich Corp.), and absorbance was measured according to the manufacturer's instructions.

Statistical Analysis

All experiments on cells were done at least twice on separate occasions. The data are presented as mean ± SD from a representative experiment. The statistical significance of differences between two groups was determined by Student's *t* test using Microsoft Excel software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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