Overexpression of Connective Tissue Growth Factor Gene Induces Apoptosis in Human Aortic Smooth Muscle Cells

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Background—Connective tissue growth factor (CTGF) is expressed at very high levels particularly in the shoulder of human atherosclerotic lesions but not in normal blood vessels. Thus, CTGF may be important in the regulation of vascular smooth muscle cell function in atherosclerosis, but its precise role remains elusive.

Methods and Results—Full-length CTGF cDNA driven by a cytomegalovirus promoter was transiently transfected into cultured human aortic smooth muscle cells (HASCs). Northern and Western analysis demonstrated that CTGF was overexpressed in these cells 48 hours after transfection. The effects of CTGF overexpression on cell proliferation were evaluated by [3H]thymidine uptake and cell count in quiescent HASCs or those stimulated with platelet-derived growth factor (PDGF). Although mock transfection showed no effect, CTGF overexpression significantly inhibited cell proliferation in cells stimulated by PDGF. Moreover, CTGF overexpression, but not mock transfection, significantly increased apoptosis as assessed by DNA fragmentation associated with histone, TdT-mediated dUTP biotin nick end-labeling, and appearance of hypodiploid cells by flow cytometry.

Conclusions—Our results for the first time demonstrate that CTGF can also act as a growth inhibitor in human aortic smooth muscle cells at least in part by inducing apoptosis. This may be important for the formation and composition of lesions and plaque stability in atherosclerosis. (Circulation. 1999;100:2108-2112.)

Key Words: atherosclerosis ■ apoptosis ■ growth substances

Connective tissue growth factor (CTGF) is a cysteine-rich peptide that was originally identified as a growth factor secreted by endothelial cells.1 CTGF is mitogenic to NRK cells2 and chemotactic to NIH 3T3 cells.3 In addition, CTGF can induce connective tissue cell proliferation and extracellular matrix synthesis.4 We recently cloned human CTGF from human aorta by a differential cloning strategy and found that CTGF is expressed at very high levels in atherosclerotic but not in normal human blood vessels.5 CTGF expression was localized predominantly in areas with extracellular matrix accumulation, and especially along the shoulder of fibrous caps.5 Interestingly, in human carotid arteries, CTGF-expressing cells are nonproliferating cells, suggesting that CTGF does not stimulate smooth muscle cell proliferation.4

In this study, we examined whether CTGF could stimulate smooth muscle cell proliferation and/or apoptosis. For this purpose, we transiently overexpressed CTGF gene in human aortic smooth muscle cells using plasmid vector containing full-length CTGF cDNA driven by a strong cytomegalovirus (CMV) promoter and examined its effect on proliferation and apoptosis.

Methods

Cell Culture
Human aortic smooth muscle cells (HASCs) were purchased from Clonetics.6 HASCs were cultured in SmBM medium (Clonetics), and cells from passages 4 to 8 from 2 different isolations were used for experiments. All experiments were performed after 48 hours of incubation in serum-free medium with selenite-insulin-transferrin (Sigma).6

Plasmids
To overexpress human CTGF gene in human aortic smooth muscle cells, we constructed a plasmid vector (pCMV-CTGF) driven by CMV promoter. In pCMV-CTGF, the human CTGF cDNA was ligated downstream of the CMV promoter combined with a 5'-untranslated region and intron sequences from the CMV immediate-early gene. In this plasmid, CTGF gene was followed by the bovine growth hormone polyadenylation signal. pCMV vector alone (empty vector) served as control. Transient transfection was performed by use of Superfect (Qiagen) according to the manufacturer’s protocol. Transfection efficiency was evaluated by green fluorescence protein plasmid with fluorescence microscopy. The average transfection efficiency with 1 μg of DNA was 35%.

Northern Blot Analysis
Total RNA was isolated by Trizol reagent (Gibco BRL) according to the manufacturer’s instructions.7 Total RNA (20 μg) was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred to a nylon membrane (Highbond-N, Amersham). Blots were hybridized in QuickHyb (Stratagen) with 32P-labeled cDNA probes prepared by random-prime labeling. A 0.6-kb cDNA fragment contained within the open reading frame of CTGF was used for probe.8 Membranes were exposed to Kodak Bio Max x-ray film at −70°C.
for 2 hours. Visualized blots were analyzed by the public-domain NIH image 1.60 program.

**Western Blot Analysis**

Polyclonal anti-CTGF antibody was a generous gift from Dr Roel Goldschmeding (Dept of Pathology, University of Utrecht, Utrecht, Netherlands). The primary antibody was used at 1:250 dilution. Cell lysates (20 μg) were subjected to 12.5% single percentages gel (Ready Gel, Bio Rad), transferred to polyvinylidine difluoride membranes (Bio Rad), and incubated with anti-CTGF antibody for 1 hour as previously described. Equal amounts of protein loading were confirmed by Coomassie brilliant blue staining before blotting. The membranes were finally visualized by the ECL kit (Amersham). Visualized blots were analyzed by the public-domain NIH image 1.60 program.

**Cell Proliferation**

Cell number was determined by Coulter counter (Coulter Electronics). DNA synthesis was evaluated by [3H]thymidine incorporation. Forty-eight hours after transfection, HASCs were pulsed with [3H]thymidine for 4 hours and counted with a β-counter (LKB Wallac, MBV AG).

**DNA Fragmentation**

Fragmented DNA associated with histone was detected by use of a cell death detection ELISA kit (Boehringer Mannheim). TdT-mediated dUTP biotin nick end-labeling (TUNEL) was performed with a cell death detection kit from Takara Biomedicals according to the manufacturer’s instructions. To calculate the percentage of TUNEL-positive cells, all cells from 4 random microscopic fields at a magnification of ×100 were counted.

For flow cytometric analysis, both floating and trypsinized adherent cells were collected, washed with PBS, and fixed in 70% ethanol. After fixation, cells were washed with PBS and stained with propidium iodide for 20 minutes under subdued light. Stained cells were analyzed by FACScalibur (Becton Dickinson), and DNA content was analyzed by Modfit software.

**Caspase 3–Like Activity**

The caspase 3–like activity was determined by the caspase 3 assay kit (Biomol), which detects chromophore p-nitroanilide after cleavage from the labeled substrate ac-DEVD-p-nitroanilide.

**Statistics**

Values are mean±SEM from 4 to 6 experiments. Statistical evaluation of the data was performed by use of ANOVA followed by Fisher’s test. A value of *P*<0.05 was considered significant.

**Results**

**Overexpression of CTGF Gene**

To determine whether CTGF gene is indeed overexpressed in HASCs after transfection with pCMV-CTGF, we performed Northern blot analysis 48 hours after transient transfection of the cells (Figure 1A). Cells stimulated with transforming growth factor (TGF)–β were used as a positive control. TGF–β significantly increased a band of 2.4 kb corresponding to CTGF mRNA expression (band I) after 48 hours of stimulation (Figure 1, A and B, top; lane 1). Unstimulated cells (Figure 1A, lane 2), mock transfection (lane 3), and pCMV-CTGF transfection (lane 4) showed minimal endogenous expression of CTGF mRNA (band I), but no significant changes (Figure 1B, top; lanes 2, 3, and 4). As expected, in addition to the 2.4-kb endogenous CTGF mRNA band (I), high-level expression of 1.1-kb CTGF mRNA species (band II) was detected in pCMV-CTGF–transfected HASCs (Figure 1, A and B, bottom; lane 4).

Overexpression of CTGF protein was also confirmed by Western blot analysis in HASCs 48 hours after transfection (Figure 2, A and B). TGF–β–treated HASCs were used as a positive control (Figure 2, A and B; lane 1). Untransfected cells (lane 2) and mock-transfected cells (lane 3) showed minimal CTGF protein expression, which did not differ significantly from untransfected cells. In contrast, pCMV-CTGF–transfected cells (lane 4) significantly increased CTGF protein compared with untransfected cells. In addition, small amounts of CTGF protein were also detected in conditioned medium of pCMV-CTGF–transfected cells, but not in mock-transfected or untransfected HASCs (data not shown).

**CTGF Overexpression on HASC Proliferation**

In the absence of PDGF, CTGF overexpression showed no significant effect on cell proliferation, as assessed by cell number and [3H]thymidine incorporation (Figure 3, A and B). PDGF (5 ng/mL) significantly increased cell number and [3H]thymidine incorporation, but this increase was significantly suppressed by CTGF overexpression (Figure 3, A and B; *P*<0.05). The difference in cell number between mock and CTGF overexpression was still significant at 72 hours. To
clarify whether the growth inhibition by CTGF overexpression is limited to proliferative responses induced by PDGF, we also examined the effects of CTGF overexpression in HASCs stimulated by basic fibroblast growth factor (bFGF; 10 ng/mL). bFGF, which does not induce CTGF, significantly increased [3H]thymidine incorporation in HASCs, and this increase was also significantly suppressed by CTGF overexpression (bFGF with pCMV versus bFGF with pCMV-CTGF, 253 ± 11 versus 104 ± 16 cpm/well; P < 0.05).

Figure 2. Expression of CTGF protein in HASCs. A, Representative Western blot of CTGF protein. Lane 1, Stimulated by TGF-β (2 ng/mL; 48 hours); lane 2, serum-free control; lane 3, transient transfection with pCMV control vector alone; lane 4, transient transfection with pCMV-CTGF. B, Densitometric analysis (n=4). *P < 0.05 vs lane 2.

CTGF Overexpression and DNA Fragmentation

To clarify the mechanism of growth inhibition by CTGF overexpression, we examined the histone-associated DNA fragmentation by ELISA and TUNEL methods. CTGF overexpression significantly increased histone-associated DNA fragmentation in pCMV-CTGF–transfected cells in both the absence and presence of PDGF (Figure 4A; P < 0.05). In contrast, little or no histone-associated DNA fragmentation was detected in mock-transfected cells. CTGF overexpression also significantly increased TUNEL-positive cells in both the absence and presence of PDGF fragmentation (Figure 4B; P < 0.05), but mock transfection showed no effect.

Flow Cytometric Analysis of DNA Content

To evaluate apoptosis, we stained cells with propidium iodide and analyzed DNA content by flow cytometric analysis. Computer analysis (Modfit) revealed the G0/G1 to be ~100, the G2/M ~200, and apoptotic cells ~50 (Figure 5). Both in the absence and in the presence of PDGF, mock transfection showed no effect on the apoptotic peak (Figure 5, B and E), but CTGF overexpression increased the apoptotic peak (Figure 5, C and F).

CTGF Overexpression on Caspase 3 Activity

DNA fragmentation is strongly associated with caspase 3 activation. Therefore, we investigated the activation of caspase 3 by CTGF overexpression. Mock transfection showed no effect on caspase 3 activity compared with control, but CTGF overexpression significantly increased caspase 3 activity in both the absence and presence of PDGF (Figure 6).
This study demonstrates for the first time that in human aortic vascular smooth muscle cells, overexpression of human CTGF inhibits proliferation induced by growth factors such as PDGF or bFGF by a mechanism involving apoptosis. Unlike in animal models, proliferation of smooth muscle cells occurs infrequently and at low levels in human atherosclerotic lesions. In vivo studies using direct gene transfer of TGF-β, a protein that induces CTGF expression, into the vessel wall also revealed that TGF-β has little effect on cell proliferation but induces procollagen synthesis. The stimulatory effects of TGF-β on CTGF expression involves a TGF-β-responsive element in the CTGF gene. These findings are in line with our results, which suggest a role of CTGF as a growth inhibitor and inducer of apoptosis in HASCs.

Although the growth-inhibitory effects of CTGF overexpression in HASCs were convincing in our study, the precise mechanism of action remains to be determined. Because CTGF is able to compete with PDGF for binding to the PDGF cell-surface receptor on fibroblasts, it is possible that CTGF inhibited proliferation by competing with PDGF as a ligand at the PDGF receptor. In our study, however, CTGF inhibited not only DNA synthesis induced by PDGF but also that stimulated by bFGF. Hence, competition of CTGF with PDGF at the receptor level is an unlikely explanation for the growth-inhibitory effects of CTGF overexpression in HASCs. In contrast to our results, previous studies in fibroblasts and endothelial cells reported proliferative effects of CTGF. This apparent discrepancy may be related to differences in the experimental conditions (ie, recombinant protein and antisense oligonucleotide). Furthermore, although the CTGF receptor(s) has not been cloned yet, it is likely that differences in receptor distribution and receptor subtypes as well as signal transduction mechanisms may exist among different cell types. We would therefore like to suggest that the effects of CTGF may differ among different cell types and that the inhibitory effect of TGF-β on cell growth in HASCs is at least in part mediated by CTGF induction.

Apoptosis is an important process for development and morphogenesis of organs and tissues. Cells of cardiovascular organs can undergo apoptosis, as do those of other tissues. Recent studies of human vascular lesions have demonstrated that apoptosis is a prominent feature of both atherosclerosis and restenosis. Degeneration of smooth muscle cells in the fibrous cap of atherosclerotic lesions is also an important aspect of plaque rupture, and many plaque cells are in a process of apoptosis. The fact that in human atherosclerotic lesions CTGF expression is localized especially along the shoulder of fibrous caps, together with the results of the present study, strongly suggests a new role of CTGF in atherosclerosis. Indeed, apoptosis of HASCs in the shoulder regions, where plaque rupture is likely to occur, may destabilize plaques and contribute to the complications of the atherosclerotic process (ie, unstable angina, infarction).

To confirm apoptosis, we examined DNA fragmentation by histone-associated DNA fragmentation using ELISA, the TUNEL method, and flow cytometry, which basically all revealed similar results. In addition, we used DNA laddering with green staining, but the difference was much more striking in experiments using the above-mentioned methods. To further demonstrate the occurrence of apoptosis, we also measured caspase 3–like activity in HASCs overexpressing CTGF. Caspase 3 is a key enzyme to induce DNA fragmentation via the caspase cascade. Oxidized LDL induces...
apoptosis in endothelial cells by activating this enzyme.\textsuperscript{20} In fact, TGF-\(\beta\), which strongly induces CTGF expression, induces apoptosis by activating caspase 3.\textsuperscript{21,22} Taken together, our results therefore suggest that CTGF activates caspase 3 and induces apoptosis in HASCs very much as TGF-\(\beta\) does in many types of cells.\textsuperscript{22–26} At this point, it remains unclear whether apoptosis occurred only in CTGF-overexpressing cells or whether bystander effects also occurred. We used immunocytochemistry for CTGF and the TUNEL method simultaneously, but unfortunately, currently available antibodies are not yet suitable to clarify this aspect.

In conclusion, our results for the first time demonstrate that CTGF, a novel cysteine-rich protein that is markedly overexpressed in human atherosclerosis, inhibits cell proliferation and induces apoptosis in HASCs. CTGF may play an important role in the pathogenesis of atherosclerosis and vascular remodeling by inducing apoptosis of vascular smooth muscle cells.

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