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Short communication

Connective tissue growth factor induces apoptosis via caspase 3 in cultured human aortic smooth muscle cells

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Abstract

Connective tissue growth factor (CTGF) stimulates proliferation of fibroblasts and endothelial cells, but nothing is known about its role in smooth muscle cells. In this study, the effects of recombinant human CTGF (r-hCTGF, 0.5–10 μg/ml) on cultured human aortic vascular smooth muscle cells were investigated. r-hCTGF significantly reduced cell viability, increased apoptosis, and augmented caspase 3 activity. Moreover, r-hCTGF-induced apoptosis was significantly inhibited by an antibody to CTGF and a caspase-3 inhibitor, Z-Asp(Ome)-Glu-(Ome)Val-Asp(Ome)-FMK. These results suggest that r-hCTGF activates caspase 3 and induces apoptosis.

Keywords: CTGF (connective tissue growth factor); TGF-β (transforming growth factor β); Apoptosis; Caspase; Atherosclerosis

1. Introduction

Connective tissue growth factor (CTGF) is a novel cysteine-rich, secreted peptide (Oemar and Luscher, 1997). CTGF was originally identified in conditioned medium of human umbilical vein endothelial cells (Bradham et al., 1991) and has been shown to play a role in various human diseases including systemic scleroderma (Igarashi et al., 1995), atherosclerosis (Oemar et al., 1997), and renal diseases (Ito et al., 1998). CTGF family members are the immediate early growth-responsive genes that are thought to regulate cell proliferation, differentiation, embryogenesis, and wound healing. Recombinant CTGF protein stimulates type I collagen and fibronectin production in normal rat kidney (NRK) fibroblasts (Frazier et al., 1996). When injected subcutaneously, recombinant CTGF induces granulation and fibrosis in neonatal NIH Swiss mice (Frazier et al., 1996). In addition, the polypeptide stimulates DNA synthesis in cultured fibroblasts (Oemar and Luscher, 1997).

To clarify the effect of CTGF on smooth muscle cells, we treated cultured human aortic smooth muscle cells with recombinant human CTGF (r-hCTGF) and investigated its effect on cell viability and induction of apoptosis.

2. Materials and methods

2.1. Cell and materials

Human aortic vascular smooth muscle cells were purchased from KURABO (Tokyo, Japan). Cells were cultured in smooth muscle basal medium (SmBM) (Hishikawa and Luscher, 1998) and the cells in passages 4–8 were used for experiments. All experiments were performed after a 48-h incubation in serum-free Dulbecco's modified eagle medium (DMEM) (GIBCO, Grand Island, NY) with insulin–transferrin–selenite supplement (Hishikawa et al., 1994) (Sigma, St. Louis, MO). r-hCTGF and antibody to CTGF was generously provided by Japan Tobacco (Osaka, Japan). An inhibitor of caspase-3, Z-Asp(Ome)-Glu-(Ome)Val-Asp(Ome)-FMK (Z-DEVD-FMK), was obtained from Calbiochem.

2.2. Cell viability assay

Cell viability was assessed with a Cell Proliferation Kit (Boehringer Mannheim, Germany). Briefly, the cells were plated in 96-well plates in a final volume of 100 μl of culture medium. After treatment with r-hCTGF, 10 μl of

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the labeling reagent was added to each well and incubated for 4 h at 37°C. Then, 100 μl of the solubilization solution was added to each well and incubated overnight at 37°C. Optical density was determined at A550–A690 nm, using a micro-plate reader (BIO RAD, CA, USA).

2.3. DNA fragmentation assay

Enrichment of mononucleosomes or oligonucleosomes in human aortic vascular smooth muscle cells was quantitatively determined by sandwich-enzyme immunoassay with a Cell Death Detection ELISA^{PLUS} from Boehringer Mannheim. Briefly, 5×10^4 cells cultured in 24-well plates were treated with r-hCTGF for 48 h. At the end of the incubation, the plates were centrifuged at $200 \times g$ and the medium was aspirated. A solubilizing solution (100 μl) was added to each well and incubated for 10 min. After centrifugation at $200 \times g$, the supernatant of the cell lysate was added to a 96-well microplate precoated with a monoclonal antibody against histone.

2.4. Terminal deoxytransferase-mediated dUTP biotin nick end-labeling (TUNEL) staining

TUNEL staining was performed with a commercially available kit (In Situ Cell Death Detection Kit) from Boehringer Mannheim according to the manufacturer's instructions. To calculate the percentage of TUNEL-positive cells, we counted all cells from four random microscopic fields at 100× magnification.

2.5. Caspase 3-like activity

Caspase 3-like activity was determined with the caspase 3 assay kit (BIOMOL, PA, USA), which detects chromophore *p*-nitroanilide after cleavage from the labeled substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide.

2.6. Statistics

Statistical evaluation of the data was performed by analysis of variance followed by Fisher's test. $P < 0.05$ was considered significant.

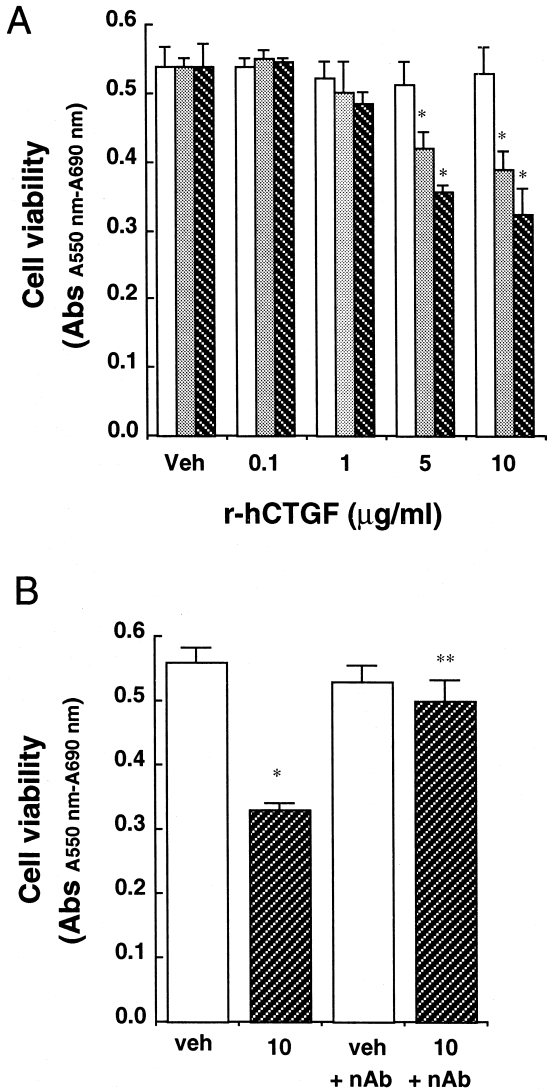

Fig. 1. (A) Effect of r-hCTGF on cell viability. After incubation with serum-free DMEM for 48 h, cells were incubated with r-hCTGF for the indicated periods. Medium with or without r-hCTGF was changed every 24 h. Open bars mean 24 h treatment, hatched bars 48 h and closed bars 72 h. Veh means the vehicle (glycine buffer, final 5 μM) alone. Cell viability is shown as percentage of control (no treatment) at each time point. $^*P < 0.05$ vs. Veh at each time point ($n = 8$–12). (B) Effect of CTGF neutralizing antibody (nAb) on the reduction of cell viability produced by r-hCTGF (10 μg/ml). $^*P < 0.05$ vs. Veh ($n = 8$–12). $^{**}P < 0.05$ vs. r-hCTGF without the antibody.

3. Results

3.1. Effects of r-hCTGF on cell viability

After a 48-h incubation in serum-free medium, human aortic vascular smooth muscle cells were treated with r-hCTGF for 24–72 h. At 1 μg/ml, r-hCTGF showed no effect until 72 h (Fig. 1). At 5 and 10 μg/ml, r-hCTGF significantly reduced cell viability in a time-dependent manner (Fig. 1A).

To clarify the specificity of the induction of apoptosis by r-hCTGF, antibody to r-hCTGF was used. Pre-treatment with antibody (20 μg/ml) alone had no effect (Fig. 1B). However, the antibody significantly attenuated the reduction of cell viability caused by r-hCTGF (10 μg/ml) (Fig. 1B).

3.2. Effects of r-hCTGF on apoptosis

To clarify the mechanism of the reduced cell viability induced by r-hCTGF, we examined apoptosis by TUNEL staining and DNA fragmentation. After a 48-h incubation, r-hCTGF significantly increased TUNEL-positive cells and

DNA fragmentation in a dose-dependent manner (Fig. 2A and B). Moreover, r-hCTGF significantly increased caspase 3 activity, which causes DNA fragmentation (Janicke et al., 1998), in a dose-dependent manner (Fig. 2C).

To clarify the causal relationship of the activation of caspase 3 and the induction of apoptosis by r-hCTGF, the cells were pre-treated with an inhibitor of caspase-3, Z-DEVD-FMK, before r-hCTGF (10 μg/ml) treatment. Pre-treatment with Z-DEVD-FMK (30 μM) significantly reduced r-hCTGF-induced apoptosis and caspase 3 activation (Fig. 2A–C).

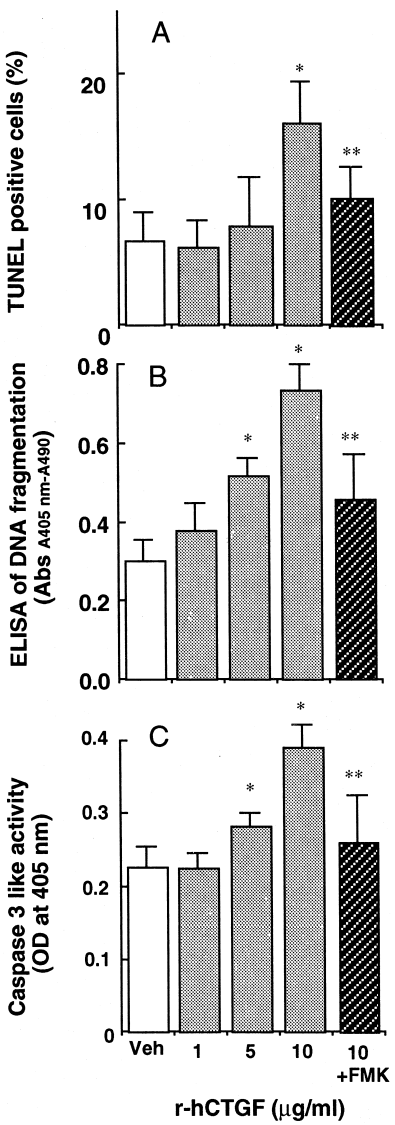

Fig. 2. (A) Effect of r-hCTGF on TUNEL staining. (B) Effect of r-hCTGF on histone-associated DNA fragmentation. (C) Effect of r-hCTGF on caspase 3-like activity. In all experiments in this figure, cells were incubated with serum-free DMEM for 48 h and then incubated with r-hCTGF for another 48 h. A caspase 3 inhibitor (FMK, 30 μM) was added 1 h before r-hCTGF. Medium with or without r-hCTGF and FMK was changed every 24 h. Veh means vehicle alone (glycine buffer, final 5 μM). $^{*}P < 0.05$ vs. Veh ($n = 8–12$). $^{**}P < 0.05$ vs. r-hCTGF (10 μg/ml) ($n = 8–12$).

4. Discussion

Our results demonstrate the pro-apoptotic effect of r-hCTGF in HASC. CTGF is expressed at very high levels in atherosclerotic but not in normal human blood vessels (Oemar et al., 1997). CTGF expression is localized predominantly in areas with extracellular matrix accumulation, and specially along the shoulder of fibrous caps (Oemar et al., 1997). Interestingly, in human carotid arteries, CTGF-expressing cells are non-proliferating (staining negative for proliferating cell nuclear antigen) cells, suggesting that CTGF does not stimulate smooth muscle cell proliferation (Oemar et al., 1997). Our results are compatible with these results and may explain, at least in part, the reason why CTGF-expressing cells are non-proliferating cells.

In human specimens from atherosclerotic lesions of native coronary vessels and saphenous vein grafts, widespread apoptosis was detectable by TUNEL staining (Isner et al., 1995; Haunstetter and Izumo, 1998). Moreover, it is common for atherectomy specimens from restenotic lesions to show evidence of apoptosis (Haunstetter and Izumo, 1998). Potential roles of p53-dependent, Fas-dependent and oxidized low-density lipoprotein-mediated pathways in apoptosis have been described (Haunstetter and Izumo, 1998). Here, we propose a new CTGF-mediated pathway. Loss of smooth muscle cells caused by apoptosis in the fibrous cap of atherosclerotic lesions predisposes the lesions to plaque instability and may increase the risk of unstable angina pectoris and acute myocardial infarction. Taken together, CTGF may play a pivotal role in controlling the stability of atherosclerotic lesions.

Different from our results, CTGF was found to stimulate proliferation in fibroblasts (Frazier et al., 1996) and endothelial cells (Shimo et al., 1998). Recently, Babic et al. (1999) reported that mouse CTGF promotes endothelial cell survival under conditions that normally induce apoptosis. This apparent discrepancy may be explained by differences in species and experimental design (antisense oligonucleotide and recombinant protein; purified mouse CTGF protein and human CTGF protein). In this study, to clarify the role of CTGF in human disease, we chose human aortic smooth muscle cells and r-hCTGF. The CTGF receptor has not been cloned yet, and there may be some differences in receptor distribution and subtypes among species and cells.

5. Conclusion

Recombinant human CTGF induces apoptosis in human aortic vascular smooth muscle cells by activating caspase 3. Our results suggest a new therapeutic approach to cardiovascular diseases by modulation of the stability of atherosclerotic lesions with CTGF.

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