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Short communication

Transforming growth factor-β_1 induces apoptosis via connective tissue growth factor in human aortic smooth muscle cells

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Abstract

We examined the possible involvement of connective tissue growth factor (CTGF) in the apoptosis induced by transforming growth factor-β_1 (TGF-β_1) in human aortic vascular smooth muscle cells (HASC). In quiescent HASC, TGF-β_1 induced the mRNA and protein of CTGF. A CTGF antisense oligonucleotide inhibited this induction. TGF-β_1 significantly reduced cell viability and induced DNA fragmentation, and the CTGF antisense oligonucleotide reversed these effects. Moreover, TGF-β_1 activated caspase 3 in HASC, and the CTGF antisense oligonucleotide reduced this activation. These findings show that CTGF plays a key role in the TGF-β_1-induced apoptosis in HASC.

Keywords: CTGF (connective tissue growth factor); TGF-β_1 (transforming growth factor β); Apoptosis; Caspase; Antisense oligonucleotide

1. Introduction

Transforming growth factor-β (TGF-β) is a cytokine produced by many cell types (Border and Ruoslahti, 1992). Increased expression of TGF-β in primary arterial and secondary restenotic lesions suggests that this cytokine has a role as a regulator of arteriosclerosis (Majesky et al., 1991). TGF-β inhibits the proliferation and promotes the migration of vascular smooth muscle cells (Grainger et al., 1993). Although TGF-β was classically reported to arrest growth in the G_1 phase of the cell cycle (Roberts et al., 1985), it has more recently been reported to play an important role as an inducer of apoptosis in a variety of cells (Oberhammer et al., 1993; Hsing et al., 1996; Hughes et al., 1996).

Connective tissue growth factor (CTGF) is expressed in very high levels in atherosclerotic, but not normal, human blood vessels (Oemar et al., 1997). CTGF expression is localized predominantly in non-proliferating cells. Recently, a TGF-β responsive element was found in the CTGF gene (Grotendorst et al., 1996), but the relationship between TGF-β and CTGF in human vascular smooth muscle cells remains to be determined.

In this study, we attempted to clarify whether CTGF plays a key role in TGF-β-induced apoptosis in human vascular smooth muscle cells.

2. Materials and methods

2.1. Cell culture

Human aortic vascular smooth muscle cells (Hishikawa and Lüscher, 1998) were purchased from KURABO (Tokyo, Japan). Cells were cultured in SmBM medium and passages 4–8 were used for the experiments. All experiments were performed after a 48-h incubation in Dulbeco's modified eagle medium (GIBCO BRL) with insulin–transferrin–selenite supplement (Sigma, St. Louis, MO, USA).

2.2. Preparation of antisense, sense and scramble oligonucleotides of CTGF

A CTGF antisense oligonucleotide was constructed with a 16 mer derived from the starting translation site, which contained the initial ATG of the 5′ (sense) and 3′ (antisense) end. The sequences were as follows: the antisense, 5′-TACTGGCGGCGGTCAT-3′ (Shimo et al., 1998); the sense, 5′-ATGACCGCCGCCAGTA-3′ (Shimo et al., 1998); the scramble, 5′-GGTCTAGCTTGCGGAC-3′f.

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2.3. Northern blot analysis

Total RNA was isolated with Trizol reagent (GIBCO BRL) according to the manufacturer's instruction (Hishikawa and Lüscher, 1997). A 0.6-kb cDNA fragment contained within the open reading frame of CTGF was used as probe. The membranes were exposed to Hyperfilm ECL (Amersham, UK) for 1 h at room temperature.

2.4. Western blot analysis

Polyclonal anti-CTGF antibody was a generous gift of Dr. Roel Goldschmeding (Utrecht) (Ito et al., 1998). The primary antibody was used at 1:250 times dilution. To ensure that an equal amount of protein loading was loaded, Coomasie brilliant blue staining was performed before blotting. The membranes were finally visualized by using the ECL kit (Amersham, UK) (Hishikawa and Lüscher, 1997).

2.5. Cell viability assay

Cell viability was assessed by using a MTT kit (Boehringer Mannheim, Germany) according to the manufacturer's instruction.

2.6. 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 kit from Boehringer Mannheim (Germany) according to the manufacturer's instruction.

2.7. 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.

2.8. Statistics

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

3. Results

As shown in Fig. 1, TGF-β_1 (5 ng/ml for 48 h) strongly induced CTGF mRNA (Fig. 1A) and protein (Fig. 1B) in quiescent human aortic vascular smooth muscle cells. This induction, however, was inhibited by co-treatment with the CTGF antisense oligonucleotide (20 μg/ml) (Fig. 1A and B).

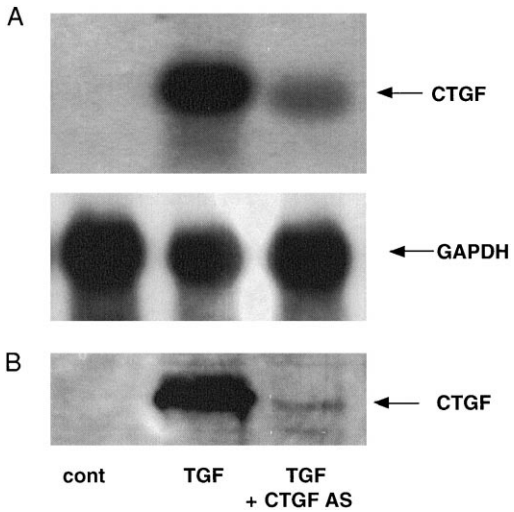

Fig. 1. (A) Northern blot of CTGF mRNA induced by TGF-β_1. After 48 h under serum-free conditions, the cells were incubated with TGF-β_1 (5 ng/ml) in the absence and presence of the CTGF antisense oligonucleotide (20 μg/ml) for 48 h. The lower panel shows the blot of GAPDH. TGF represents TGF-β_1 (5 ng/ml), CTGF AS, the CTGF antisense oligonucleotide (20 μg/ml). (B) Western blot of CTGF protein induced by TGF-β_1. After 48 h under serum-free conditions, the cells were incubated with TGF-β_1 (5 ng/ml) in the absence and presence of the CTGF antisense oligonucleotide (20 μg/ml) for 48 h. In each lane, 20 μg of protein was loaded. TGF represents TGF-β_1 (5 ng/ml) and CTGF AS, the CTGF antisense oligonucleotide (20 μg/ml).

Next we tested whether TGF-β_1 caused cell death in HASC *via* CTGF, using the CTGF antisense oligonucleotide. Although the CTGF antisense oligonucleotide alone (20 μg/ml) had no effect, TGF-β_1 (5 ng/ml) significantly reduced cell viability and this effect was reversed by treatment with CTGF antisense oligonucleotide (10–20 μg/ml) (Fig. 2A). CTGF sense (20 μg/ml) and scramble (20 μg/ml) oligonucleotides had no effect on TGF-β_1-induced cell death (Fig. 2A). Moreover, as in the case with the cell viability, the CTGF antisense oligonucleotide alone (20 μg/ml) had no effect on DNA fragmentation (Fig. 2B). TGF-β_1 significantly increased DNA fragmentation, but this effect was completely reversed by treatment with the CTGF antisense oligonucleotide (10–20 μg/ml), not with the sense (20 μg/ml) or the scramble (20 μg/ml) oligonucleotides (Fig. 2B).

Caspases are crucial components of cell death pathways. Among the caspases identified, caspase-3 is the most important, because it is commonly activated by numerous death signals, cleaves a variety of important cellular proteins and is reported to cause DNA fragmentation (Janicke et al., 1998). The CTGF antisense oligonucleotide alone had no effect on caspase 3-like activity, but TGF-β_1 significantly activated it (Fig. 2C). The CTGF antisense oligonucleotide significantly reduced the TGF-β_1-induced

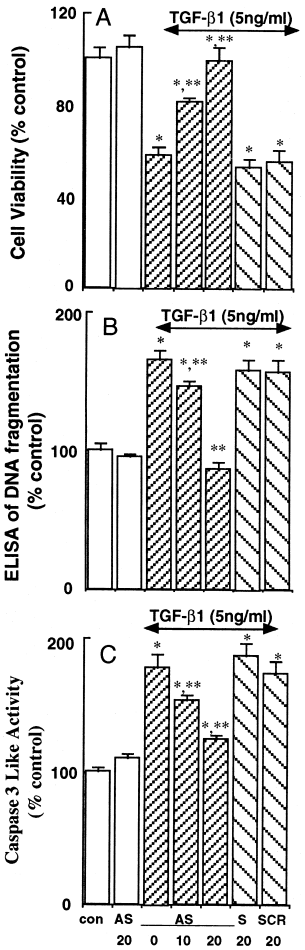

Fig. 2. (A) Effect of TGF-β_1 and CTGF antisense oligonucleotide on cell viability. Cells were incubated with or without TGF-β_1 (5 ng/ml) for 48 h in the absence and presence of CTGF antisense, sense and scramble oligonucleotide; con represents no treatment. AS, S and SCR represent antisense, sense and scramble, respectively. Values are means ± S.E.M. ($n = 8$). (B) Effect of TGF-β_1 and CTGF antisense oligonucleotide on DNA fragmentation. Experimental conditions and the abbreviations are the same with those in the panel A. Values are means ± S.E.M. ($n = 8$). (C) Effect of TGF-β_1 and CTGF antisense oligonucleotide on caspase 3-like activity. Experimental conditions and the abbreviations are the same with those in the panel A. Values are means ± S.E.M. ($n = 6$). $^*P < 0.05$ compared to con. $^{**}P < 0.05$ compared to TGF-β_1 alone.

activation of caspase 3-like activity, but the sense and scramble oligonucleotides had no effect (Fig. 2C).

4. Discussion

In the present study, we demonstrated that TGF-β induces apoptosis via CTGF in quiescent cultured human aortic vascular smooth muscle cells. Apoptosis plays an essential role in atherosclerosis (Haunstetter and Izumo, 1998), and TGF-β induces apoptosis in cells such as endothelial cells (Tsukada et al., 1995). Moreover, TGF-β appears to be most active in lipid-rich atherosclerotic lesions, and also limits smooth muscle cell proliferation in fatty lesions (Bobik et al., 1999). These results strongly suggest that TGF-β may induce apoptosis in vascular smooth muscle cells, but the mechanisms underlying apoptosis are poorly understood. CTGF is a good candidate as effector molecule in TGF-β-induced apoptosis, but no specific inhibitors of CTGF are available. Thus we used the CTGF antisense oligonucleotide to clarify the interaction between TGF-β and CTGF. In the present study, the CTGF antisense oligonucleotide dose dependently reversed TGF-β_1-induced apoptosis. Control experiments with the sense and scramble oligonucleotides showed that these oligonucleotides had no effect on TGF-β_1-induced CTGF induction and apoptosis, suggesting that there was a specific effect of CTGF antisense oligonucleotide in TGF-β_1-treated cells. Moreover we have recently reported that the overexpression of CTGF gene induces apoptosis in human aortic vascular smooth muscle cells (Hishikawa et al., in press). Taken together, TGF-β_1-induced apoptosis is, at least in part, mediated through CTGF in human aortic vascular smooth muscle cells.

Although TGF-β_1 not only promotes but also inhibits the proliferation of vascular smooth muscle cells (Grainger et al., 1993), only lesion growth was reported in previous studies using TGF-β_1 protein and gene delivery (Majesky et al., 1991; Nabel et al., 1993; Schulick et al., 1998). Overexpression of TGF-β_1 in the arterial vascular wall promotes intimal proliferation, but its withdrawal is associated with regression of vascular lesions accompanied by apoptosis in vascular smooth muscle cells (Schulick et al., 1998). The mechanism of these two divergent actions of TGF-β_1 is still unclarified, but our results may help us to understand this issue. During the period of TGF-β_1 overexpression, the possible direct effect on growth of TGF-β_1 may dominate the induction of apoptosis by CTGF. In the TGF-β_1 withdrawal phase, CTGF, which has been induced by TGF-β_1, may work alone and induce apoptosis in the lesions. In fact, CTGF is expressed at very high levels in atherosclerotic but not proliferative lesions (Oemar et al., 1997).

In summary, our data suggest an intermediate role for CTGF in the pathogenesis of vascular diseases in which TGF-β_1 plays a key role. CTGF is a new potential target for a therapeutic intervention for atherosclerosis.

References

Bobik, A., Agrotis, A., Kanellakis, P., Dilley, R., Krushinsky, A., Smirnov, V., Tararak, E., Condron, M., Kostolias, G., 1999. Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. Circulation 99, 2883–2891.

Border, W.A., Ruoslahti, E., 1992. Transforming growth factor-beta in disease: the dark side of tissue repair. J. Clin. Invest. 90, 1–7.

Grainger, D.J., Kirschenlohr, H.L., Metcalfe, J.C., Weissberg, P.L., Wade, D.P., Lawn, R.M., 1993. Proliferation of human smooth muscle cells promoted by lipoprotein(a). Science 260, 1655–1658.

Grotendorst, G.R., Okochi, H., Hayashi, N., 1996. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ. 7, 469–480.

Haunstetter, A., Izumo, S., 1998. Apoptosis: basic mechanism and implications for cardiovascular disease. Circ. Res. 82, 1111–1129.

Hishikawa, K., Lüscher, T.F., 1997. Pulsatile stretch stimulates superoxide production in human aortic endothelial cells. Circulation 96, 3610–3816.

Hishikawa, K., Lüscher, T.F., 1998. Felodipine inhibits free-radical production by cytokines and glucose in human smooth muscle cells. Hypertension 32, 1011–1015.

Hishikawa, K., Oemar, B., Felix, F.C., Nakaki, T., Fujii, T., Lüscher, T.F., Overexpression of connective tissue growth factor gene induces apoptosis in human aortic smooth muscle cells. Circulation, in press.

Hsing, A.Y., Kadomatsu, K., Bonham, M.J., Danielpour, D., 1996. Regulation of apoptosis induced by transforming growth factor-beta1 in nontumorigenic rat prostatic epithelial cell lines. Cancer Res. 56, 5146–5149.

Hughes, D.E., Dai, A., Tiffee, J.C., Li, H.H., Mundy, G.R., Boyce, B.F., 1996. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. Nat. Med. 2, 1132–1136.

Ito, Y., Aten, J., Bende, R.J., Oemar, B.S., Rabelink, T.J., Weening, J.J., Goldschmeding, R., 1998. Expression of connective tissue growth factor in human renal fibrosis. Kidney Int. 53, 853–861.

Janicke, R.U., Sprengart, M.L., Wati, M.R., Porter, A.G., 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. 273, 9357–9360.

Majesky, M.W., Lindner, V., Twardzik, D.R., Schwartz, S.M., Reidy, M.A., 1991. Production of transforming growth factor beta 1 during repair of arterial injury. J. Clin. Invest. 88, 904–910.

Nabel, E.G., Shum, L., Pompili, V.J., Yang, Z.Y., San, H., Shu, H.B., Liptay, S., Gold, L., Gordon, D., Derynck, R., Nabel, G.J., 1993. Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. Proc. Natl. Acad. Sci. USA 90, 10759–10763.

Oberhammer, F., Bursch, W., Tiefenbacher, R., Froschl, G., Pavelka, M., Purchio, T., Schulte-Hermann, R., 1993. Apoptosis is induced by transforming growth factor-beta 1 within 5 hours in regressing liver without significant fragmentation of the DNA. Hepatology 18, 1238–1246.

Oemar, B.S., Werner, A., Garnier, J.M., Do, D.D., Godoy, N., Nauck, M., Marz, W., Rupp, J., Pech, M., Lüscher, T.F., 1997. Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. Circulation 95, 831–839.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F., Sporn, M.B., 1985. Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA 82, 119–123.

Schulick, A.H., Taylor, A.J., Zuo, W., Qiu, C.B., Dong, G., Woodward, R.N., Agah, R., Roberts, A.B., Virmani, R., Dichek, D.A., 1998. Overexpression of transforming growth factor beta1 in arterial endothelium causes hyperplasia, apoptosis, and cartilaginous metaplasia. Proc. Natl. Acad. Sci. USA 95, 6983–6988.

Shimo, T., Nakanishi, T., Kimura, Y., Nishida, T., Ishizeki, K., Matsumura, T., Takigawa, M., 1998. Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisense RNA suppresses proliferation and migration of vascular endothelial cells. J. Biochem. (Tokyo) 124, 130–140.

Tsukada, T., Eguchi, K., Migita, K., Kawabe, Y., Kawakami, A., Matsuoka, N., Takashima, H., Mizokami, A., Nagataki, S., 1995. Transforming growth factor beta 1 induces apoptotic cell death in cultured human umbilical vein endothelial cells with down-regulated expression of bcl-2. Biochem. Biophys. Res. Commun. 210, 1076–1082.