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Effects of Tumor Necrosis Factor-α on the Synthesis of DNA, the Secretion of Matrix Metalloproteinases/Tissue Inhibitors of Metalloproteinases, and the Activity of Invasive Migration in Cultured Vascular Smooth Muscle Cells

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To address the question of whether or not tumor necrosis factor-α (TNF-α) regulates the functions of vascular smooth muscle cells (SMCs), dense or sparse cultures of the cells derived from human aorta were treated with TNF-α or TNF-α neutralizing antibody (TNF-α Ab). The incorporation of [^{3}H]thymidine into the acid-insoluble fraction of SMCs was significantly inhibited by TNF-α, but stimulated by TNF-α Ab only when the cells had a high cell density. TNF-α significantly increased the accumulation of matrix metalloproteinase-1 and -3 (MMT-1 and -3, respectively) in the conditioned medium of dense SMCs, but does not affect that of tissue inhibitors of metalloproteinases-1 (TIMP-1); MMP-9 and TIMP-2 were undetectable. The invasive migration of SMCs determined by a Transwell system was stimulated by neither TNF-α nor TNF-α Ab. Taking these results together, it is suggested that TNF-α regulates DNA and MMP synthesis in dense SMCs but does not affect their invasive migration.

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INTRODUCTION

Atherosclerosis is the principal vascular lesion in the pathogenesis of myocardial and cerebral infarction, which is initiated by vascular endothelial cell damage followed by an intimal hyperplasia of vascular smooth mucle cells (SMCs). When the vascular endothelium is damaged, endothelial cells will migrate to the damaged area to be re-endothelialized. However, if the repair of the endothelium is insufficient, blood cells such as platelets and macrophages accumulate at the damaged site and secrete cytokines/growth factors that stimulate the migration and proliferation of SMCs.[1)]

In atherosclerotic plaques, tumor necrosis factor-α (TNF-α) as well as other cytokines/growth factors are detected.[1)] The main cell type responsible for the secretion is postulated to be macrophages[2)] that are in general present in the plaques with SMCs and T lymphocytes.[3)] TNF-α regulates vascular endothelial cell function, for example, by inhibition of the proliferation,[4)] increase in the procoagulant activity,[5)] and induction of plasminogen activator inhibitor-1 synthesis.[6)] The cytokine also regulates SMC functions, including the synthesis of nitric oxide,[7)] prostaglandin E_2[8)] and platelet-derived growth factor-AA.[9)] Migration and growth of SMCs are crucial events in the pathogenesis of atherosclerosis, however, the role of TNF-α is incompletely understood.

We investigated the effects of TNF-α on the synthesis of extracellular matrix components such as glycosaminoglycans and collagen using a culture system of SMCs. It was found that TNF-α changes the composition of glycosamonoglycans synthesized by cultured SMCs.[10)] Recently, it has been shown that TNF-α inhibits the synthesis of collagens, especially types IV and V, in the SMCs only when their density is high.[11)] These results clearly indicate that TNF-α modulates the property of the extracellular matrix of SMCs.

We hypothesized that TNF-α may regulate the growth and migration of SMCs because the metabolism of the extracellular matrix often influences cell growth.[12)] In the present study, we investigated the effects of TNF-α on the synthesis of DNA, the se-

cretion of metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) that can participate in the cell migration, and the invasive migration activity in cultured human aortic SMCs.

MATERIALS AND METHODS

Materials —— Human aortic SMCs were purchased from Kurabo (Osaka, Japan). RPMI 1640 medium and fetal bovine serum were from Nissui Pharmaceutical (Tokyo, Japan) and Whittaker (Walkersville, MD, U.S.A.), respectively. Bovine serum albumin (BSA) was from Miles (Kankakee, IL, U.S.A.). Tissue culture dishes and plates were obtained from Iwaki (Chiba, Japan). [*Methyl*-^{3}H]Thymidine (2.7 TBq/mmol) was from ICN Biomedicals (Irvine, CA, U.S.A.). Recombinant human TNF-α and TNF-α neutralizing antibody (TNF-α Ab) were purchased from Genzyme (Cambridge, MA, U.S.A.). Recombinant human platelet-derived growth factor (PDGF-BB) was from Oncogene Science (Manhasset, NY, U.S.A.). Transwell was obtained from Costar (Cambridge, MA, U.S.A.). Other reagents were from Nacalai Tesque (Kyoto, Japan).

Cell Culture —— SMCs were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in 100 mm dishes in a humid atmosphere of 5% CO_2 in air until confluence. They were transferred into 6-well culture plates at 1×10^4 cells/cm^2 and cultured for 24 hr ("sparse cultures") or until confluence ("dense cultures").

DNA Synthesis —— The medium containing the sparse and dense SMCs was replaced by 1 ml of RPMI 1640 medium supplemented with 0.1% BSA after washing of the cells with the medium. The cells were incubated at 37°C for 24 hr with TNF-α (0.1, 1 or 10 ng/ml) or TNF-α Ab (100, 200, 500, 750 or 1000 U/ml) and labeled with [^{3}H]thymidine (20 kBq/ml) during the last 3 hr of the incubation. After labeling, the medium was discarded and the cells were gently washed with Ca and Mg-free phosphate-buffered saline. The cells were harvested with a rubber policeman in the presence of Ca and Mg-free phosphate-buffered saline, and cell homogenate was prepared by sonication. Incorporation of the radioactivity into a 5% trichloroacetic acid-insoluble fraction of the cell homogenate was measured by liquid scintillation counting. A portion of the cell homogenate was used for the determination of DNA content by the fluorometric assay[13] to express the incorporated radioactivity as dpm/μg DNA.

Accumulation of MMPs and TIMPs —— Dense cultures of SMCs were prepared in 24-well culture plates and treated with TNF-α (0.1, 1 or 10 ng/ml) for 24 hr in 0.25 ml of RPMI 1640 medium supplemented with 0.1% BSA. After treatment, the conditioned medium was harvested and analyzed for the accumulation of MMP-1, -2, -3 and -9, and TIMP-1 and -2 by a commercially available enzyme immunoassay kit (Fuji Chemical Industries, Takaoka, Japan). The cells were collected by trypsinization and the cell number at the end of the treatment was counted with a particle counter (Sysmex CDA-500). The content of MMPs and TIMPs was expressed as μg/10^6 cells.

Invasive Migration Assay —— Dense cultures of SMCs were prepared in 100 mm dishes and labeled with [^{3}H]thymidine (20 kBq/ml) for 24 hr in RPMI 1640 medium supplemented with 10% fetal bovine serum. After labeling, the cells were harvested by trypsinization and suspended in RPMI 1640 medium supplemented with 0.1% BSA after washing with the medium. The cells were then seeded into the upper Transwell chamber and placed on the lower chamber that contained RPMI 1640 medium supplemented with 0.1% BSA and TNF-α (1 or 10 ng/ml) or TNF-α Ab (500 or 1000 U/ml) or PDGF-BB (10 ng/ml). The Transwell system was incubated at 37°C for 6 hr, and the conditioned medium of the lower chamber was harvested. The lower well and the outside of the bottom of the upper chamber were washed with Ca and Mg-free phosphate-buffered saline; the wash was combined with the conditioned medium from the lower chamber, then the radioactivity was measured by liquid scintillation counting.

Statistical Analysis —— Data were analyzed for statistical significance by ANOVA and Bonferroni's multiple *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

Although Cooper and Beasley reported that mRNA encoding for TNF-α was not detectable in cultured human SMCs,[14] expression of the cytokine is observed in the cells treated with low density lipoprotein[15] or with bacterial lipopolysaccharides.[8] Thus, TNF-α is suggested to regulate the SMC functions in an autocrine fashion under certain conditions. Figure 1 shows the incorporation of [^{3}H]thymidine into the acid-insoluble fraction of

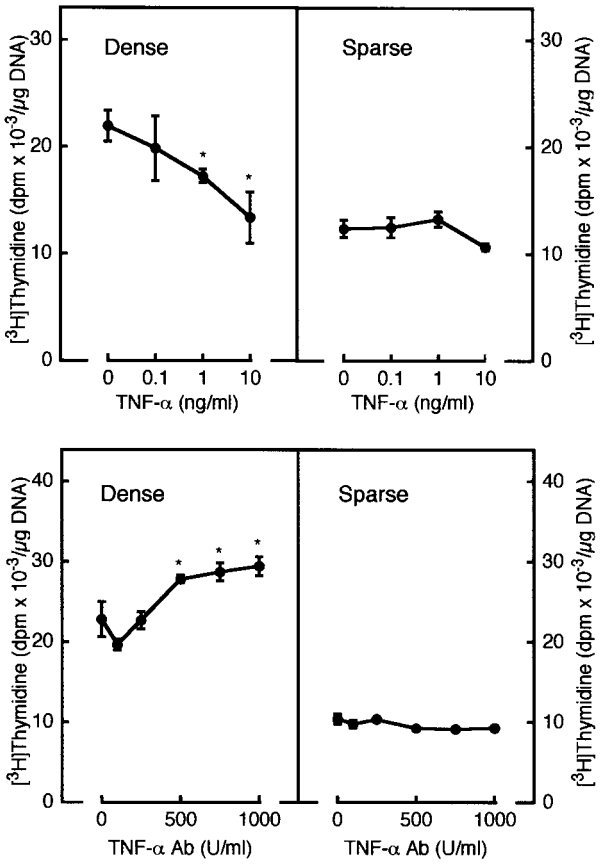

Fig. 1. Incorporation of [^{3}H]Thymidine into the Acid-insoluble Fraction of Dense and Sparse SMCs after Exposure to TNF-α or TNF-α Ab

Dense and sparse cultures of SMCs were incubated at 37°C for 24 hr with TNF-α (0.1, 1 or 10 ng/ml) or TNF-α Ab (100, 200, 500, 750 or 1000 U/ml) and labeled with [^{3}H]thymidine during the last 3 hr of the incubation. Values are means ± S.E. of five samples. *Significantly different from the corresponding control, $p < 0.05$.

dense and sparse SMCs after exposure to TNF-α or TNF-α Ab. The [^{3}H]thymidine incorporation in the dense cells was significantly inhibited by TNF-α in a dose–dependent manner, but that in the sparse cells was unaffected by the cytokine. On the other hand, TNF-α Ab significantly increased the [^{3}H]thymidine incorporation in a dose–dependent manner in the dense cells but not in the sparse cells. These results indicate that not only exogenous but also endogenous TNF-α suppresses the DNA synthesis of SMCs only at a high cell density. We showed that TNF-α suppresses the synthesis of collagen in SMCs at a high cell density.[11] Thus, it is suggested that TNF-α may be a modulator of SMC function depending on the cell density. Although the mechanism is unclear, cell density–dependent regulation of vascular cell functions has also been shown in the proteoglycan synthesis of endothelial cells[16] and in the growth of SMCs[17] after exposure to transforming growth factor-β.

Table 1 shows the effect of TNF-α on the accumulation of MMPs and TIMPs in the conditioned medium of dense cultures of SMCs. The accumulation of MMP-2 and TIMP-1 was unaffected by TNF-α; MMP-9 and TIMP-2 were undetectable. In contrast, the accumulation of MMP-1 and -3 was significantly increased by the cytokine in a dose–dependent manner. This suggests that TNF-α selectively stimulates the synthesis of MMP-1 and -3 in the regulation of MMP/TIMP system in dense SMCs. Since TNF-α stimulates the expression of MMP-1, -3 and -9 in vascular endothelial cells[18] and induces angiogenesis,[19] we considered that the cytokine can stimulate the migration of SMCs. Figure 2 shows the effect of TNF-α and TNF-α Ab on the invasive migration activity of SMCs in the Transwell system. It was shown that neither TNF-α nor TNF-α Ab stimulated the invasive migration, although PDGF, as a positive control,[20] stimulated it. Thus, it is suggested that an increase in the secretion of MMP-1 and -3 by TNF-α may be involved in the turnover of extracellular matrix metabolism, but does not enhance the invasive migration activity in SMCs.

Migration of SMCs from the media into the intima, followed by the proliferation and the synthesis of extracellular matrix components such as collagen, is a crucial event in the pathogenesis of atherosclerosis.[1] Inhibition of the synthesis of DNA and

Table 1. Effect of TNF-α on the Secretion of MMP-1, -2, -3 and -9, and TIMP-1 and -2 in Cultured Vascular Smooth Muscle Cells

	MMP-1 (μg/10^6 cells)	MMP-2 (μg/10^6 cells)	MMP-3 (μg/10^6 cells)	MMP-9 (μg/10^6 cells)	TIMP-1 (μg/10^6 cells)	TIMP-2 (μg/10^6 cells)
Control	142.0± 9.5	20.25±0.70	12.30±0.41	< 0.003	5.022±0.408	< 0.02
0.1 ng/ml TNF-α	136.3±11.2	17.78±1.53	10.88±1.27	< 0.003	5.060±0.564	< 0.02
1 ng/ml TNF-α	199.9± 5.7**	20.70±1.14	20.29±0.86**	< 0.003	5.209±0.267	< 0.02
10 ng/ml TNF-α	206.7± 8.8**	24.64±1.07	20.06±2.72*	< 0.003	5.847±0.014	< 0.02

Dense cultures of human aortic smooth muscle cells were incubated at 37°C for 24 hr with TNF-α (0.1, 1 or 10 ng/ml). Values are means ± S.E. of four samples. Significantly different from the corresponding control, *$p < 0.05$; **$p < 0.01$.

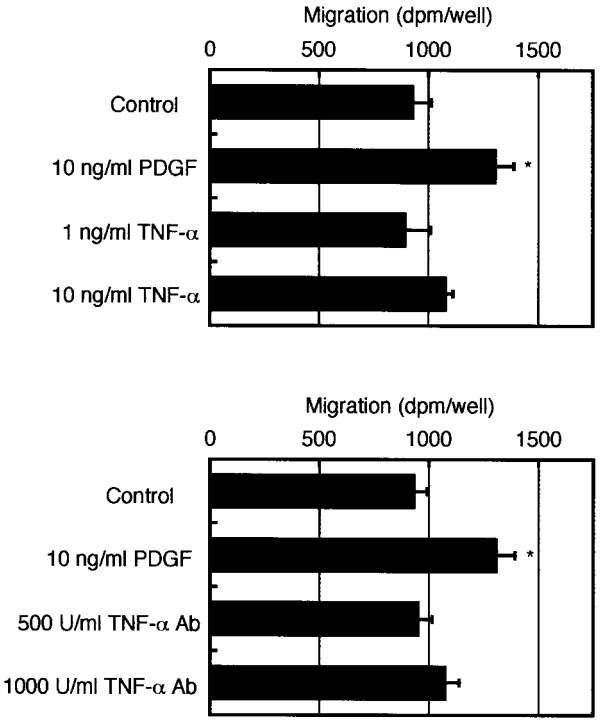

Fig. 2. Effect of PDGF-BB, TNF-α and TNF-α Ab on the Invasive Migration Activity of SMCs

[^{3}H]Thymidine-labeled SMCs were seeded into the upper chamber of Transwell and placed on the lower chamber that contained TNF-α (1 or 10 ng/ml) or TNF-α Ab (500 or 1000 U/ml) or PDGF-BB (10 ng/ml). The Transwell system was incubated at 37°C for 6 hr and the radioactivity of the migrated cells into the lower chamber was counted. Values are means ± S.E. of four samples. *Significantly different from the corresponding control, $p < 0.05$.

collagens[11] in SMCs at a high cell density indicates that TNF-α may play a protective role in the progression of the lesion after the SMC density has become high in atherosclerotic plaques. On the other hand, TNF-α is known to be cytotoxic to vascular endothelial cells,[4,21] and is postulated to contribute to the initiation of atherosclerosis. Taken together, TNF-α may be a promotive factor in the early stage and a suppressive factor in the late stage of atherosclerosis.

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