Synergistic effects of transforming growth factor-β on the expression of *c-fms*, macrophage colony-stimulating factor receptor gene, in vascular smooth muscle cells

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Abstract Vascular smooth muscle cells (SMC) transform to foam cells in the process of atherosclerosis. We have reported that SMC derived from the intima of atherosclerotic lesions express *c-fms*, macrophage colony-stimulating factor receptor gene, which is not normally expressed in medial SMC. In the present study, we demonstrated that transforming growth factor-β (TGF-β) synergistically induced expression of *c-fms* in the presence of platelet-derived growth factor-BB in human medial SMC, a level comparable to that observed in the intima. The induction of *c-fms* was not inhibited by protein kinase C (PKC) inhibitor, suggesting that TGF-β induces *c-fms* via a PKC-independent pathway. These results suggest that TGF-β plays an important role in the phenotypic change of smooth muscle cells to macrophage-like cells in the process of atherosclerosis.

Key words: Vascular smooth muscle cell; Atherosclerosis; Macrophage colony-stimulating factor; Transforming growth factor-β; Platelet-derived growth factor

1. Introduction

Foam cell formation is the most characteristic event during the process of early atherosclerosis. Foam cells are derived from both monocyte-derived macrophages and smooth muscle cells (SMC). Peripheral monocytes enter the subendothelial space and differentiate to tissue macrophages that actively take up lipoprotein cholesterol through receptor-mediated endocytosis. SMC migrate from the media to the intima of the arterial wall where they proliferate and transform to foam cells [1,2]. We and others have demonstrated that two characteristic genes of macrophages, macrophage colony-stimulating factor (M-CSF) receptor gene, encoded by proto-oncogene *c-fms*, and scavenger receptor gene, are induced on SMC derived from the intima of atherosclerotic lesions [3,4]. We speculate that the factors involved in the atherosclerotic process cause changes in the regulation of *c-fms* gene expression in normal vascular SMC by inducing a phenotypic change to macrophage-like cells. Recently, we reported that platelet-derived growth factor (PDGF)-BB transiently induces gene expression of *c-fms* in normal vascular SMC through a protein kinase C (PKC)-independent pathway [5]; with the addition of epidermal or fibroblase growth factor (EGF or FGF), the gene expression is similar to that in intimal SMC involving PKC activation [6]. In the present study, we found that the combination of PDGF-BB and transforming growth factor-β (TGF-β) induces the stable expression of *c-fms* in normal vascular SMC, to the same extent as in intimal SMC.

2. Materials and methods

2.1. Cells

Human aortic SMC were explanted by the method of Fischer-Dzoga et al. [7]. Cells were passaged three times by exposure to trypsin and seeded in 10 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD) containing 10% fetal bovine serum in 100-mm dishes, and used for experiments at the 4th to 6th passage. Subconfluent cells were cultured in serum-free medium for 24 h before experiments and then medial SMC were cultured with DMEM containing 1% fetal calf serum (FCS) in the presence of both TGF-β and PDGF-BB at 37°C. Protein concentration was determined by the BCA assay (Pierce, Rockford, IL).

2.2. Quantitative analysis of M-CSF receptor mRNA

Human SMC were treated with a combination of TGF-β (1 ng/ml) and PDGF-BB (10 ng/ml) for various times (1–48 h) or with various concentrations (0.5–3 ng/ml) of TGF-β for 8 h in DMEM containing 1% FCS after incubation in serum-free medium for 24 h. After incubation with TGF-β, total RNA was isolated by the acid-guanidine phenol chloroform method [8]. A competitive polymerase chain reaction method was used to measure M-CSF receptor (*c-fms*) mRNA levels as previously described [3].

2.3. RNase protection assay

A 1.2-kb *Pst*I fragment (2048–3194) of the human *c-fms* cDNA was subcloned into pBluescript II SK(−) (Stratagene, La Jolla, CA), and this plasmid was digested with *Bgl*II. A probe specific to human *c-fms* was labeled with [α-^{32}P]UTP (specific activity 800 Ci/mmol; Amersham, Arlington Heights, IL) by the method described in the instruction of the Riboprobe Gemini System (Promega, Madison, WI). 354-bp antisense transcripts were used for the ribonuclease protection assay.

The ribonuclease protection assay was performed by the method described in the instructions for the Ribonuclease protection assay kit (Ambion Inc., Austin, TX). 25 μg of total RNA was hybridized with approximately 20000 cpm of probe overnight at 42°C, and then was digested with a mixture of RNase A and RNase T1. Protected fragments were separated on 5% acrylamide/8 M urea gels.

3. Results

mRNA of *c-fms* was not detected and was not induced by 10 ng/ml PDGF-BB in human aortic medial SMC using the RNase protection assay with a riboprobe of *c-fms*, whereas mRNA of *c-fms* was detected in human monocyte-derived macrophages. In the presence of both 10 ng/ml PDGF-BB and 1 ng/ml TGF-β, mRNA of *c-fms* was obviously induced in human SMC (Fig. 1A).

To estimate the mRNA level of *c-fms*, we used a sensitive competitive polymerase chain reaction method. In assays of

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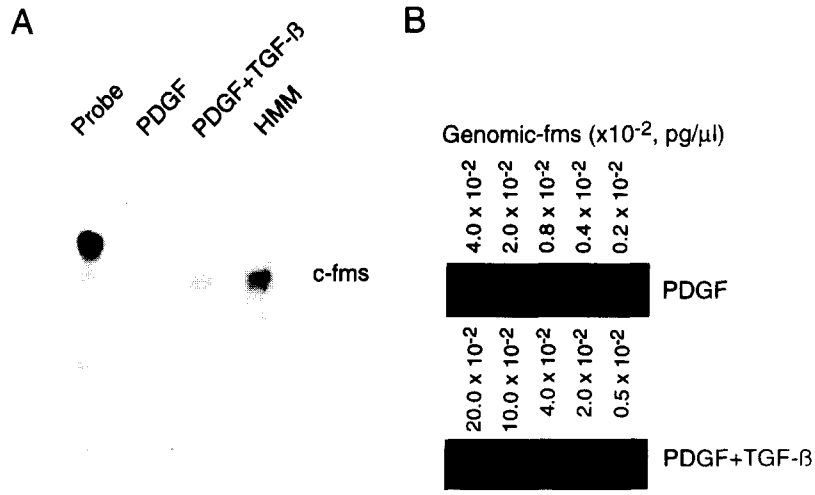

Fig. 1. Ribonuclease protection assay (A) and quantitative analysis (B) of M-CSF receptor mRNAs isolated from vascular SMC. A: Subconfluent SMC were cultured with 1 ng/ml human TGF-β or 10 ng/ml human PDGF-BB. Human monocyte-derived macrophages (HMM) were cultured as described [3]. 25 μg of total RNA was applied to the ribonuclease protection assay. B: 1 μg of total RNAs isolated from the cells were reverse-transcribed with random hexamer primers. Aliquots of the cDNA products were co-amplified with indicated amounts of the control genomic DNA. mRNA levels were estimated by titrating an unknown amount of cDNA against a dilution series containing known amounts of the corresponding genomic DNA.

aortic medial SMC in the presence of both 10 ng/ml PDGF-BB and 1 ng/ml TGF-β, the mRNA level was estimated at 4.0×10^{-2} pg/μl genomic DNA, whereas they were 0.8×10^{-2} pg/μl genomic DNA in the presence of PDGF-BB alone and 0.002×10^{-2} pg/μl genomic DNA at non-stimulated conditions (the absence of growth factors). These results indicate that the transcription of *c-fms* in human medial SMC was increased 2000-fold in response to both PDGF-BB and TGF-β and the addition of TGF-β stimulated mRNA expression of *c-fms* 5-fold as compared with PDGF-BB alone (Fig. 1B, Table 1). We have recently reported that 5 ng/ml heparin binding EGF-like growth factor (HB-EGF) induces the mRNA of *c-fms* to the level of 7.0×10^{-2} pg/μl genomic DNA which is comparable with that of human monocyte-derived macrophages [9].

We observed that the combination of PDGF-BB and TGF-β induced the expression of *c-fms* at 8–12 h after addition to the culture medium (Fig. 2A), and the gene expression was enhanced 2000-fold in response to these growth factors in aortic medial SMC. This enhanced expression of *c-fms* was sustained at least 48 h after exposure to the growth factors. The effect of this combination on the *c-fms* expression was dependent on the concentration of TGF-β and the maximal effect was obtained by 1 ng/ml of TGF-β (Fig. 2B). The amount of total RNA/cell was not significantly different between untreated cells and growth factor-treated cells. Pretreatment with protein kinase C inhibitors, staurosporine (50 nM) and calphostin C (10 nM), had no significant effect on *c-fms* expression in the presence of these growth factors (Fig. 3).

4. Discussion

mRNA of M-CSF receptor encoded by *c-fms* was not detected in normal vascular smooth muscle cells by the protection assay, and we found that the mRNA expression in the sensitive assay using a competitive polymerase chain reaction was extremely low in normal vascular smooth muscle cells compared to human monocyte-derived macrophages. The M-CSF receptor and PDGF-β receptor are generally expressed in a mutually exclusive manner, i.e. the M-CSF receptor is expressed primarily on monocyte-macrophages [10] and the PDGF receptor gene is expressed on mesenchymal cells [11]. We have recently focused on mechanisms involved in the phenotypic change of vascular SMC to phagocytic cells which is characterized by expression of the M-CSF receptor encoded by *c-fms*. We have demonstrated that multiple growth factors and cytokines regulate *c-fms* expression in vascular SMC which do not normally express this gene [3,5,6,9] and that transcription factor PU.1 plays an essential role in the phenotypic conversion of vascular smooth muscle cells to

Table 1
mRNA expression of *c-fms* in vascular smooth muscle cells

	mRNA level ($\times10^{-2}$, pg/μl)	fold
Human aortic medial SMC	0.002	1.0
Human monocyte-derived macrophage	17.5	8.75×10^{3}
Human aortic medial SMC in the presence of		
10 ng/ml PDGF-BB	0.8	4.0×10^{2}
10 ng/ml PDGF-BB +1 ng/ml TGF-β	4.0	2.0×10^{3}
5 ng/ml HB-EGF	7.0	3.5×10^{3}

Subconfluent human aortic medial smooth muscle cells were cultured with the indicated factors in DMEM containing 1% FCS for 8 h after a 24-h pre-incubation in serum-free medium. Human monocyte-derived macrophages, human aortic medial SMC were cultured as described [3]. Total RNA was isolated from cells and levels of mRNA expression were estimated by a competitive polymerase chain reaction method and expressed as genomic *c-fms* concentrations (10^{-2} pg/μl).

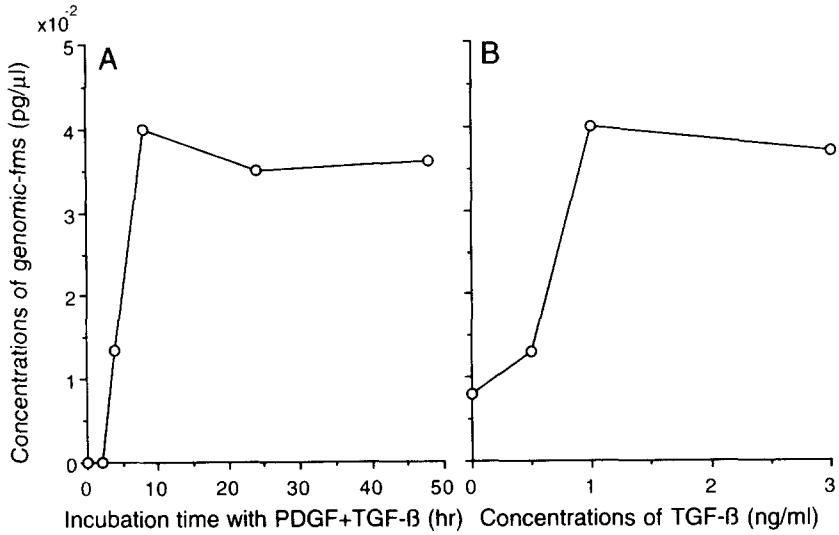

Fig. 2. Time- and dose-related expression of *c-fms* mRNA in response to TGF-β in human aortic smooth muscle cells. The subconfluent cells were cultured with 1 ng/ml human TGF-β and 10 ng/ml human PDGF-BB for the indicated times (A) and with the specified amounts of TGF-β and 10 ng/ml human PDGF for 8 h (B). Each value was estimated by a competitive polymerase chain reaction method.

macrophage-like cells by mediating the induction of *c-fms* [12]. PDGF-BB induces *c-fms* expression in the absence of other growth factors and cytokines. Although this induction of *c-fms* is transient and low [5], we have recently reported that induction of *c-fms* by HB-EGF alone is stable and as high as the expression in intimal SMC isolated from atherosclerotic lesions [9]. Neither TGF-β, EGF nor FGF alone induces *c-fms* expression. In the presence of PDGF-BB, EGF and FGF strongly induce expression of *c-fms*, to the level seen in intimal SMC [6]. The present study further demonstrates that TGF-β induces the stable expression of *c-fms* in the presence of PDGF-BB in medial SMC, a level comparable to that seen in intimal SMC.

We have reported that *c-fms* expression induced by PDGF-BB is mediated through PKC-independent pathways and then augmented by EGF and FGF through pathways including protein kinase C activation [5]. PDGF-BB acts initially to render cells competent to respond to progression factors [13] and then factors such as EGF and FGF stimulate the signal induced by the PDGF-BB homodimer. On the other hand, HB-EGF induces high and stable *c-fms* expression by consecutively stimulating the signals of both PKC-dependent and -independent pathways [9]. The potency of the combination of TGF-β and PDGF-BB in *c-fms* expression is approximately 5–7 times greater than that of PDGF-BB homodimer alone and is close to that of HB-EGF alone. Since the action of TGF-β is not completely inhibited by PKC inhibitors, a major pathway for *c-fms* expression in response to TGF-β may not be PKC activation; the action of TGF-β is different from those of EGF, FGF, and HB-EGF. These results suggest that PKC activation is not essential for stable and high *c-fms* expression and that growth factors secreted by cells in atherosclerotic lesions may induce *c-fms* expression via different pathways. Other investigators have recently demonstrated that TGF-β synergistically stimulates scavenger receptor activity together with other growth factors such as PDGF-BB in SMC [14], suggesting that TGF-β contributes to the phenotypic change of SMC to macrophage-like cells by inducing both *c-fms* and scavenger receptor genes in atherosclerotic lesions.

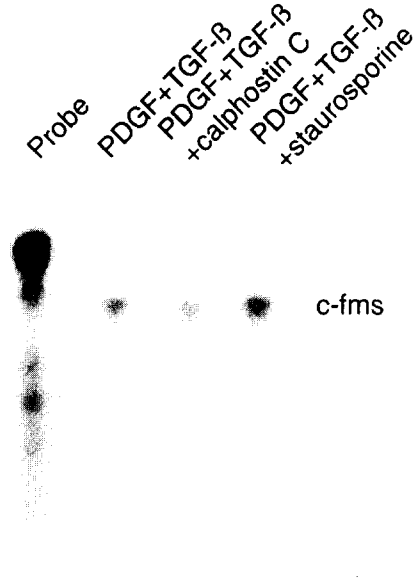

Fig. 3. Ribonuclease protection assay of M-CSF receptor mRNAs isolated from vascular SMC. Subconfluent cells were cultured with 1 ng/ml human TGF-β in the presence or absence of protein kinase C inhibitors (50 nM staurosporine and 10 nM calphostin C) in DMEM containing 1% FCS for 8 h after a 24-h pre-incubation in serum-free medium. Thereafter, 25 μg of total RNA was applied to ribonuclease protection assay with the RNA probe for human *c-fms*.

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