Induction of Sustained Expression of Proto-oncogene *c-fms* by Platelet-derived Growth Factor, Epidermal Growth Factor, and Basic Fibroblast Growth Factor, and Its Suppression by Interferon-γ and Macrophage Colony-stimulating Factor in Human Aortic Medial Smooth Muscle Cells

Toshimori Inaba, Takanari Gotoda, Kenji Harada, Masako Shimada, Jun-ichi Ohsuga, Shun Ishibashi, Yoshio Yazaki, and Nobuhiro Yamada

The 3rd Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, 113, Japan

Abstract

Vascular medial smooth muscle cells migrate, proliferate and transform to foam cells in the process of atherosclerosis. We have reported that the intimal smooth muscle cells express proto-oncogene *c-fms*, a characteristic gene of monocyte-macrophages, which is not normally expressed in medial smooth muscle cells. In the present study, we demonstrated that combinations of platelet-derived growth factor (PDGF)-BB and either epidermal growth factor (EGF) or fibroblast growth factor (FGF) induced high expression of *c-fms* in normal human medial smooth muscle cells to the level of intimal smooth muscle cells or monocyte-derived macrophages, whereas *c-fms* expression by PDGF-BB alone was $^1/_{10}$ and both EGF and FGF had no independent effect on *c-fms* expression. By contrast, interferon (IFN)-γ and macrophage colony-stimulating factor (M-CSF) suppressed the induction of *c-fms* expression. These results indicate that multiple growth factors and cytokines may play a role in the phenotypic transformation of medial smooth muscle cells to intimal smooth muscle cells in atherosclerotic lesions by altering *c-fms* expression. (*J. Clin. Invest.* 1995. 95:1133–1139.) Key words: *c-fms* • PDGF • EGF • M-CSF • smooth muscle cell • atherosclerosis

Introduction

In atherosclerotic lesions, vascular smooth muscle cells undergo structural and functional changes from a contractile to a synthetic phenotype, in particular, transform into foam cells in the intima by actively taking up lipoprotein cholesterol through receptor-mediated endocytosis (1–3). We have previously demonstrated that vascular smooth muscle cells isolated from the intima of atherosclerotic lesions express, a characteristic gene of monocyte-macrophages, *c-fms* (4), suggesting not only that the mechanism underlying the process of atherosclerosis induces alterations in the regulation of normal *c-fms* gene expression in vascular smooth muscle cells but also that factors inducing *c-fms* gene expression in vascular smooth muscle cells play an important role in the process of atherosclerosis. Cells expressing *c-fms* can respond to macrophage colony-stimulating factor (M-CSF),[1] an 85-kD homodimeric glycoprotein, through binding to specific high-affinity surface receptors encoded by *c-fms* that constitute one member of a family of growth factor receptors with intrinsic tyrosine kinase activity. The rapid phosphorylation at tyrosine initiates a cascade of metabolic and gene regulatory events (5–8). The expression of the *c-fms* gene in vascular smooth muscle cells initiates biological events including cell surface binding of M-CSF, tyrosine-phosphorylation of M-CSF receptor, and an increased rate of cell proliferation in response to M-CSF, and vascular smooth muscle cells expressing *c-fms* transform into foam cells by taking up modified low density lipoprotein (LDL) (4).

In atherosclerotic process, various cytokines and growth factors play important roles in phenotypic changes of vascular smooth muscle cells (3). We have recently demonstrated that platelet-derived growth factor (PDGF)-BB induces *c-fms* expression in medial smooth muscle cells, however, the induced mRNA level of *c-fms* is transient and extremely low as compared to its expression in monocyte-derived macrophages or intimal smooth muscle cells isolated from the intima of atherosclerotic lesions (9). Our observations suggest that other growth factors and cytokines in addition to PDGF-BB are required for stable and high expression of *c-fms* in vivo. In the present study, we investigated factors that are involved in the regulation of *c-fms* expression in normal vascular smooth muscle cells, to define the mechanism underlying the process of atherosclerosis.

Methods

Materials. Recombinant human PDGF-BB homodimer, recombinant human epidermal growth factor (EGF), and recombinant human basic-fibroblast growth factor (FGF) were purchased from Genzyme Co. (Boston, MA). Recombinant human interferon (IFN)-γ, and recombinant human endothelin-1 were obtained from Shionogi Pharmaceutical Co. (Osaka, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. Recombinant human M-CSF was a purified product obtained from Morinaga Milk Industry Co., Ltd. (Tokyo, Japan) (4, 9). Dibutyryl cyclic AMP, insulin, PMA, pertussis toxin, cycloheximide, and staurosporine were obtained from Sigma Chemical Co. (St. Louis, MO). DME was obtained from GIBCO BRL (Gaithersburg, MD). All other chemicals were of analytical grade.

Cells. Human aortic medial smooth muscle cells were explanted from the human aorta by the method of Fischer-Dzoga et al. (9, 10).

Address correspondence to Nobuhiro Yamada, The 3rd Dept. of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, 113, Japan. Phone: 81-3-3815-5411; FAX: 81-3-5802-2955.

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; FGF, fibroblast growth factor; M-CSF, macrophage-stimulating factor.

The cells were passaged three times by trypsinization and seeded in 10 ml of DME containing 10% FCS in 100-mm dishes. Subconfluent cells were cultured with 10 ng/ml PDGF-BB and various growth factors in DME containing 1% FCS for 8 h at 37°C after a 24-h preincubation in serum-free medium. Rabbit medial and intimal smooth muscle cells were cultured and subconfluent cells were used for experiments as described previously (4, 9). Monocytes were isolated from peripheral blood of a normolipidemic healthy donor using the Ficoll-Hypaque gradient method as described previously (11, 12). The cells were used as monocyte-derived macrophages after 9 d of culture (13).

Quantitative analysis of M-CSF receptor mRNA and Northern blot analysis. Total RNA was isolated from cultured smooth muscle cells by the acid-guanidine phenol chloroform method (14). A competitive PCR method was used to measure M-CSF receptor (*c-fms*) mRNA levels as previously described (3, 15, 16). 1 μg of total RNA was reverse-transcribed with random hexamer primers. The DNA fragment of human *c-fms* gene (240 bp) was preparatively amplified with a pair of primers and used in the competitive PCR as a control template. Aliquots of the cDNA products were coamplified with indicated amounts of the control genomic DNA (240 bp). The amplified fragments of cDNA (164 bp) lack an intron.

Blot hybridization studies were performed for *c-fms* mRNA of human smooth muscle cells. 10 μg of total RNA was electrophoretically fractionated on 1% (wt/vol) agarose/2.2 M formaldehyde gel and transferred onto a nylon membrane. The membrane was hybridized with the cDNA probe for human *c-fms* (9). The filters were washed in 2× SSC (1× SSC: 150 mM NaCl, 10 mM sodium citrate) and 0.5% (wt/vol) SDS at room temperature for 15 min twice, then in 0.2× SSC and 0.5% (wt/vol) SDS at 65°C for 15 min, and finally exposed to radiographic films at −80°C.

Ribonuclease protection assay. A 1.2-kb Pst I fragment (2048–3194) of the human *c-fms* cDNA was subcloned into pBluescript II SK (−) (Stratagene Corp., La Jolla, CA), and this plasmid was digested with Bgl II. The digested DNA was transcribed and labeled with [α-^{32}P]UTP (specific activity, 800 Ci/m mol; Amersham Corp., Arlington, IL) using Riboprobe Gemini System according to the method described in the instruction (Promega Corp., Madison, WI). A resultant 354-bp antisense RNA probe was used for ribonuclease protection assay. Ribonuclease protection assay was performed by the method described in the instruction of Ribonuclease protection assay kit (Ambion Inc., Austin, TX). 10 μg of total RNA was hybridized with ~ 20,000 cpm of the RNA probe overnight at 42°C, and then digested with the mixture of RNase A and RNase T1. A specific RNA for human *c-fms* that was protected from digestion, was separated on a 8 M urea sequencing gel.

In vitro transcription assay. The bacterial chloramphenicol acetyltransferase (CAT) and the SV40 enhancer were isolated from pCAT-control plasmid (Promega) as a 1.9-kbp HindIII-SalI fragment. A 550-bp SacI fragment (-606 to -56) containing 5′ non-coding region of *c-fms* genomic DNA (17) was introduced into the pBS-CAT/SV40 plasmid containing a 1.9-kbp HindIII-SalI fragment in pBluescript II KS(−) (18), in a sense orientation immediately upstream of the CAT gene. CAT plasmid and control β-galactosidase plasmid (pSV-β-Gal) (Promega) were introduced into human aortic smooth muscle cells as follows; subconfluent cells, cultured on a 35-mm dish, were cotransfected with lipofectin. Both CAT plasmid (1.5 mg) and pSV-β-Gal (0.5 mg) were mixed with 10 ml of Lipofectin Reagent (GIBCO BRL). After a 15-min incubation with foam lipid-DNA complex at room temperature, the mixture was added to the culture medium of Opti-MEM I Reduced Serum Medium (GIBCO BRL), which was exchanged just before transfection. After 8 h, the culture medium was replaced by DMEM (GIBCO BRL) containing 10% FCS. After 56 h, the medium was supplemented with PDGF-BB (10 ng/ml) and/or EGF (3 ng/ml), and was incubated subsequently for 4 h. The cells were harvested for measurement of CAT (19) and β-galactosidase activity as described by Herbomel et al. (20). In brief, transfected cells were washed three times with phosphate-buffered saline, scraped into 500 μl of the lysis buffer of 40 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl, lysed by shearing through a 25-gauge needle 10 times, and centrifuged at 14,000 *g* for 2 min at 4°C. An aliquot of the supernatant (20 μg) was incubated for 1 h at 37°C in a standard CAT assay in a final volume of 0.15 ml of containing 0.9 nmol of [^{14}C]chloramphenicol (50 nCi) (Amersham) and 0.53 mM butyryl-CoA. All assays were linear with respect to incubation time and concentration of extract protein. The reaction products were chromatographed on Merck 5748 silica gel plates developed in a chloroform/methanol (95:5 vol/vol) solvent system, and finally exposed to radiographic films at −80°C. Then, butyrated products were scraped and counted. Each cell lysate was simultaneously analyzed for CAT enzyme activity and control plasmid β-galactosidase activity. The measured CAT activity was normalized relative to β-galactosidase activity.

DNA synthesis and uptake of acetylated LDL. Subconfluent cells were used, and medium was replaced with serum-free RPMI 1640 for 48 h before the experiment. After incubating with human PDGF-BB and/or human EGF in RPMI 1640 containing 1% fetal calf serum for 24 h, 1 μCi/ml of [^{3}H]thymidine (1 mCi/ml; NEN Research Products, Boston, MA) was added. Incubation was performed for 4 h, and then the radioactivity in the cells was determined (4).

LDL (d = 1.019–1.063 g/ml) was prepared from human plasma containing 0.1% EDTA, 0.02% sodium azide, and 0.5 mg/ml benzamidine, from fasted normolipemic volunteers, and isolated by sequential ultracentrifugation in a 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA) as described previously (21, 22). LDL was acetylated by repetitive additions of acetic anhydride (13, 23) and lipoproteins were radioiodinated with Na^{125}I using the iodine monochloride method (24, 25). Subconfluent cells were cultured with PDGF-BB and/or EGF in DME containing 1% FCS for 8 h at 37°C after a 24-h preincubation in serum-free medium, and further incubated with PDGF-BB and/or EGF in the presence of 10 μg/ml ^{125}I-acetylated LDL for 5 h at 37°C to study cell association and degradation of ^{125}I-acetylated LDL. Cell association and degradation of ^{125}I-acetylated LDL were determined by the method described previously (13, 26). Nonspecific cell association and degradation were determined by the addition of a 50-fold excess of unlabeled acetylated LDL.

Results

c-fms mRNA analysis. The mRNA of *c-fms* was not detected in human aortic smooth muscle cells using northern blot hybridization with a cDNA probe of *c-fms* as we reported (4, 9). Even when 10 ng/ml PDGF-BB induced the gene expression of *c-fms* in human aortic smooth muscle cells, mRNA was not detected in northern blot hybridization (9). In the presence of both 3 ng/ml EGF and 10 ng/ml PDGF-BB, mRNA of *c-fms* was detected in human aortic smooth muscle cells as a comparable mRNA level to that in human monocyte-derived macrophages (Fig. 1 *A*). However, *c-fms* expression was not induced by these growth factors in human skin fibroblasts (unpublished observation).

To estimate the mRNA level of *c-fms,* we used a sensitive competitive polymerase chain reaction method. In this assay, the amplified fragments of cDNA (164 bp) were shorter than those of genomic DNA (240 bp) due to lack of an intron (Fig. 1 *B*). Since cDNA and genomic DNA fragments can be coamplified with the same set of primers and their sizes are different, coamplification of the two fragments should occur in a concentration-dependent manner. It is possible to quantitate the amount of cDNA present, by titrating an unknown amount of cDNA against a dilution series containing known amounts of the corresponding genomic DNA. For instance, the same amounts of genomic DNA and cDNA fragments were obtained when the concentration of genomic template (240 bp) was 8×10^{-2} pg/μl in the assay for the estimation of *c-fms* mRNA level in

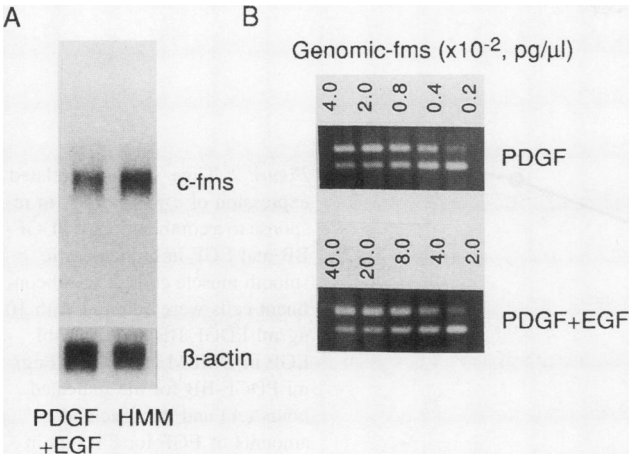

Figure 1. Northern blot hybridization analysis (*A*) and quantitative analysis (*B*) of M-CSF receptor mRNAs isolated from human aortic medial smooth muscle cells. Human aortic medial smooth muscle cells were explanted from the human aorta. Subconfluent cells were cultured with 10 ng/ml human PDGF-BB and 3 ng/ml human EGF in DMEM containing 1% FCS for 8 h after a 24 h preincubation in serum-free medium. Thereafter, 10 μg of total RNA was applied to Northern blot hybridization with the cDNA probe for human *c-fms*. A competitive polymerase chain reaction method was used to measure M-CSF receptor (*c-fms*) mRNA levels. 1 μg of total RNAs isolated from the cells was reverse-transcribed with random hexamer primers. Aliquots of the cDNA products were coamplified with indicated amounts of the control genomic DNA (240 bp). mRNA levels were estimated by titrating an unknown amount of cDNA (164 bp) against a dilution series containing known amounts of the corresponding genomic DNA.

human aortic smooth muscle cells in the presence of 10 ng/ml PDGF-BB and 3 ng/ml EGF (Fig. 1 *B*).

In assays of human aortic smooth muscle cells in the presence of PDGF-BB only, the mRNA levels were estimated at 8 $\times$ 10^{-3} pg/μl genomic DNA (240 bp). These results indicate that the transcription of *c-fms* in human aortic smooth muscle cells was increased 10-fold in the presence of both PDGF-BB and EGF compared to PDGF-BB alone and that the induced mRNA level was comparable to those of human monocyte-derived macrophages and rabbit intimal smooth muscle cells (8 $\times$ 10^{-2} versus 17.5 $\times$ 10^{-2} and 10 $\times$ 10^{-2} pg/μl, respectively, Table I). In agreement with these results, ribonuclease protection assay demonstrated that mRNA level of *c-fms* in human medial smooth muscle cells in the presence of both PDGF-BB and EGF was comparable to that in human monocyte-derived macrophages (data not shown).

We have previously reported that PDGF-AA, interleukin (IL)-1β, IL-3, tumor necrosis factor-α, insulin, endothelin-1, interferon-γ, noradrenalin, transforming growth factor-β, phorbol ester, and dibutyryl cyclic AMP do not induce *c-fms* expression (9). In the present study, we further found that 3 ng/ml EGF, 80 ng/ml IL-2, 10 ng/ml IL-4, 25 ng/ml IL-6, and 3 ng/ml basic FGF did not induce *c-fms* expression in human aortic medial smooth muscle cells using a sensitive competitive PCR method.

We observed that 10 ng/ml PDGF-BB and 3 ng/ml EGF induced the expression of *c-fms* 8 to 12 h after their addition into the culture medium (Fig. 2 *A*), and gene expression was enhanced 10^3 to 10^4-fold in response to both PDGF-BB and EGF in human aortic smooth muscle cells. This enhanced expression of *c-fms* was sustained at least 48 h after exposure to both PDGF-BB and EGF as compared to that by PDGF-BB alone. The effect of PDGF and EGF on *c-fms* expression was dose-dependent on concentrations of EGF (Fig. 2 *B*). The amounts of RNA/cell were not significantly different among untreated cells, cells treated with PDGF alone, and cells treated with PDGF and EGF. In addition to EGF, 3 ng/ml basic-FGF and 100 nM PMA enhanced the effect of PDGF-BB on *c-fms* expression (Table II), whereas insulin, endothelin-1, and dibutyryl cyclic AMP had no additional effect on *c-fms* expression induced by PDGF-BB (Tables II and III). Pretreatment of aortic smooth muscle cells with PMA for 24 h or staurosporine suppressed *c-fms* expression in the presence of both PDGF-BB and EGF to half of that in controls (Table III). As shown in Table III, pertussis toxin did not influence it, suggesting that pertussis toxin-sensitive G protein was not involved in the enhanced expression of *c-fms*.

Table I. mRNA Expression of c-fms in Vascular Smooth Muscle Cells

	mRNA level (10^{-2} pg/μl)	Fold
Human monocyte-derived macrophage	17.5	1.0
Human aortic medial SMC	0.002	1.1 $\times$ 10^{-4}
Rabbit aortic medial SMC	0.001	0.6 $\times$ 10^{-4}
Rabbit aortic intimal SMC	10.0	0.6

Rabbit intimal smooth muscle cells (SMC) were isolated from intima of balloon-injured aorta (4), and human monocyte-derived macrophages, human and rabbit aortic medial SMC were cultured as described in Methods. 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).

In contrast, *c-fms* mRNA levels in human aortic smooth muscle cells were only 8 $\times$ 10^{-4} pg/μl in the presence of a combination with 10 ng/ml PDGF-BB and 50 units/ml IFN-γ and 1.6 $\times$ 10^{-3} pg/μl in the presence of a combination with 10 ng/ml PDGF-BB and 50 ng/ml M-CSF (Table II). These results indicated the mRNA levels of *c-fms* in the presence of IFN-γ and M-CSF were $^1/_{10}$ and $^1/_5$ that of PDGF-BB alone, respectively (8 $\times$ 10^{-4} and 1.6 $\times$ 10^{-3} versus 8 $\times$ 10^{-3} pg/μl, respectively). The addition of EGF reduced the inhibitory effect of M-CSF on mRNA expression of *c-fms* as compared to in the absence of EGF (Table III).

To study the effect of protein synthesis inhibition on *c-fms* expression by IFN-γ, subconfluent vascular smooth muscle cells were preincubated with 50 μg/ml cycloheximide for 15 min before the experiment and further incubated with 50 μg/ml cycloheximide in the presence of both 10 ng/ml PDGF-BB and 50 units/ml IFN-γ in DMEM containing 1% FCS for 8 h after a 24-h preincubation in serum-free medium. 50 μg/ml cycloheximide did not influence the inhibitory effect of IFN-γ on *c-fms* expression.

In vitro transcription study. A 0.55-kb SacI fragment (-606 to -56) containing 5′ non-coding region of c-fms genomic DNA was introduced immediately upstream from the CAT gene (Fig. 3 *A*). Since the 0.55-kb SacI fragment was reported to have no enhancer activity (17), an exogenous viral enhancer, the SV40 core enhancer, was introduced into CAT plasmid. The promoter constructs were transiently transfected into human aortic smooth

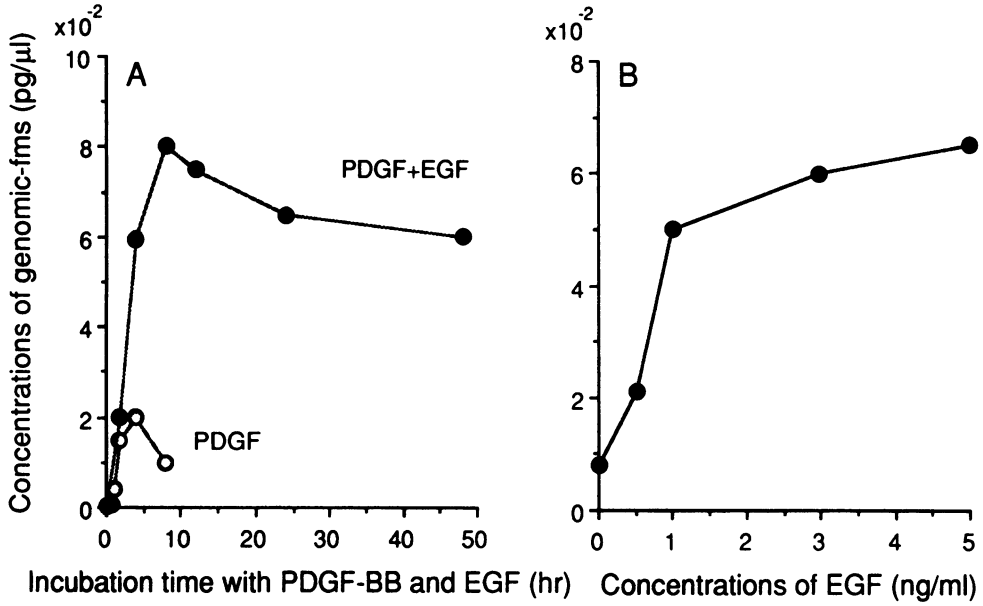

Figure 2. Time- and dose-related expression of *c-fms* mRNA in response to a combination of PDGF-BB and EGF in human aortic smooth muscle cells. The subconfluent cells were cultured with 10 ng/ml PDGF-BB and 3 ng/ml EGF in DMEM and with 10 ng/ml PDGF-BB for the indicated hours (*A*) and the specified amounts of EGF for 8 h (*B*) in DMEM containing 1% FCS at 37°C after a 24 h preincubation in serum-free medium. Each value was estimated by a competitive polymerase chain reaction method as described in Methods.

muscle cells. These cells showed high CAT activity in the presence of 10 ng/ml PDGF-BB (Fig. 3, *B* and *C*). Furthermore, in the presence of both 10 ng/ml PDGF-BB and 3 ng/ml EGF, CAT activity was enhanced twofold as compared to PDGF-BB alone. The EGF effect on CAT activity was less than that on *c-fms* expression demonstrated as mRNA levels, because a full-length of *c-fms* gene may be required to see the full effect of EGF on *c-fms* expression.

DNA synthesis and scavenger receptor activity. To characterize the effects of PDGF-BB and EGF on cellular functions, we studied [^{3}H]thymidine incorporation into DNA. As shown in Table IV, 10 ng/ml PDGF-BB, 3 ng/ml EGF and both stimulated the [^{3}H]thymidine incorporation into DNA by 2.6-, 1.5-, and 4.7-fold, respectively, whereas IFN-γ suppressed the effect of PDGF-BB.

Furthermore, we estimated cell association and degradation of acetylated LDL in human medial smooth muscle cells. As shown in Fig. 4, acetylated LDL was degraded by medial smooth muscle cells in response to 10 ng/ml PDGF-BB and 3 ng/ml EGF in the presence of 10 μg/ml acetylated LDL (Fig. 4), and a combination of PDGF-BB and EGF enhanced cell association and degradation of acetylated LDL as compared to either PDGF-BB or EGF alone. Whereas no cell association and degradation of acetylated LDL was demonstrated in the absence of those growth factors as we previously reported (4).

Table II. Effects of Various Factors on mRNA Expression of c-fms in the Presence of PDGF-BB in Human Aortic Medial Smooth Muscle Cells

	mRNA level	Fold*
No addition	0.8	1.0
EGF (3 ng/ml)	8.0	10.0
Basic-FGF (3 ng/ml)	4.0	5.0
Insulin (100 nM)	0.8	1.0
Endothelin 1 (100 nM)	0.8	1.0
PMA (100 nM)	12.0	15.0
Dibutyryl cyclic AMP (1 mM)	0.8	1.0
IFN-γ (50 units/ml)	0.08	0.1
M-CSF (50 ng/ml)	0.16	0.2

Subconfluent human aortic medial smooth muscle cells were cultured with 10 ng/ml PDGF-BB and the indicated factors in DMEM containing 1% FCS for 8 h after a 24 h preincubation in serum-free medium. Thereafter, total RNA was isolated 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). * As compared to no addition.

Table III. Effects of Various Treatments in the Presence of Both PDGF-BB and EGF on mRNA Expression of c-fms in Human Aortic Medial Smooth Muscle Cells

	mRNA level	Fold¶
	10^{-2} pg/μl	
PDGF-BB (10 ng/ml) and EGF (3 ng/ml)		
No treatment	8.0	1.0
PMA preincubation*	4.0	0.5
Staurosporine (50 nM)‡	4.0	0.5
Pertussis toxin (10 ng/ml)§	8.0	1.0
M-CSF (50 ng/ml)‖	6.0	0.75

Subconfluent human aortic medial smooth muscle cells were cultured with both 10 ng/ml PDGF-BB and 3 ng/ml EGF in DMEM containing 1% FCS for 8 h after a 24 h preincubation in serum-free medium. * Cells were preincubated with 100 nM PMA for 24 h before the experiment. ‡ Cells were preincubated with 50 nM staurosporine for 30 min before the experiment and further incubated with 50 nM staurosporine for 2 h. § Cells were preincubated with 10 ng/ml pertussis toxin for 24 h before the experiment and further incubated with 10 ng/ml pertussis toxin for 8 h. ‖ Cells were cultured with 50 ng/ml M-CSF in addition to 10 ng/ml PDGF-BB and 3 ng/ml EGF 8 h after a 24 h preincubation in serum-free medium. Thereafter, total RNA was isolated 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). ¶ As compared to no treatment.

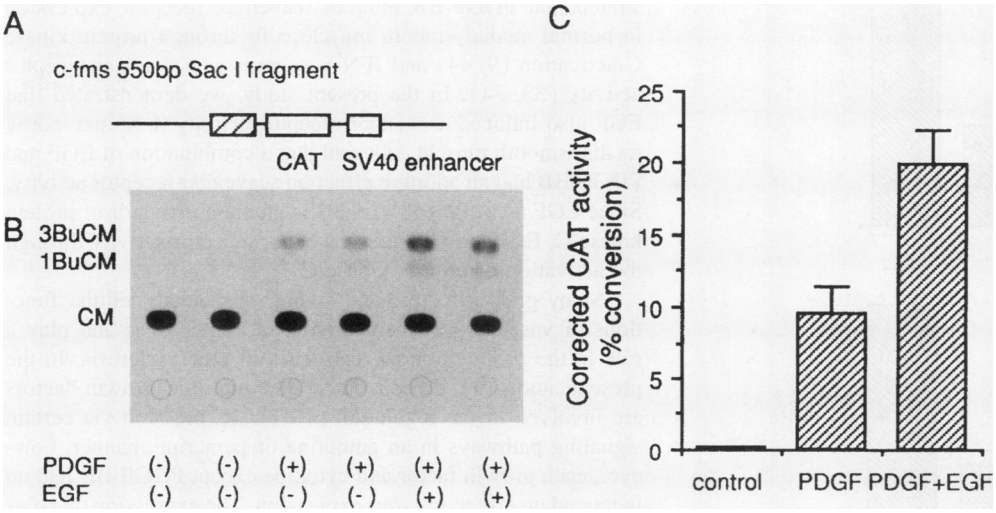

Figure 3. In vitro transcription assay. A 550-bp SacI fragment (-606 to -56) containing 5′ noncoding region of *c-fms* genomic DNA was introduced into the pBS-CAT/SV40 plasmid in a sense orientation immediately upstream of the CAT gene. CAT plasmid and control β-galactosidase plasmid (pSV-β-Gal) were introduced into subconfluent human aortic smooth muscle cells with lipofectin on a 35-mm dish. Transfected cells were cultured with PDGF-BB (10 ng/ml) and/or EGF (3 ng/ml) for 4 h and then cells were harvested for measurement of CAT and β-galactosidase activity. The measured CAT activity was normalized relative to β-galactosidase activity. The results for each CAT plasmid tested represent the mean±SD for three assays.

Discussion

Various cytokines and growth factors secreted by vascular cell components including endothelial cells, monocyte-macrophages, smooth muscle cells, and lymphocytes, are presumed to play important roles in the phenotypic change of vascular smooth muscle cells in an autocrine or paracrine manner (3). Among these factors, the present study demonstrated that a combination of PDGF-BB and either EGF or FGF induced *c-fms* expression in vascular medial smooth muscle cells which do not normally express *c-fms* (4), causing *c-fms* expression to become equivalent to those of human monocyte-derived macrophages and rabbit intimal smooth muscle cells isolated from atherosclerotic lesions, whereas *c-fms* expression by PDGF-BB alone was transient and low, and both EGF and FGF had no independent effect on *c-fms* expression. A combination of PDGF-BB and EGF enhanced not only *c-fms* expression but also thymidine incorporation and scavenger receptor activity. It has been suggested that PDGF-BB acts initially to render cells competent to respond to progression factors (27, 28). Progression factors such as EGF and FGF not only stimulated the signal induced by PDGF-BB but also functioned to keep *c-fms* expression high, resulting in the acceleration of phenotypic transformation of medial smooth muscle cells to intimal smooth muscle cells.

In addition, PMA, which is an activator of protein kinase C, enhanced the effect of PDGF-BB. This indicates that stimulative effects of either EGF or FGF in combination with PDGF-BB on *c-fms* expression may result from protein kinase C activation. Previous studies have shown that prolonged exposure of cells to PMA makes cells refractory to PMA, because protein kinase C activity in PMA-treated cells is down-regulated (29–31). Pretreatment of aortic smooth muscle cells with PMA or staurosporine (protein kinase C inhibitors) suppressed *c-fms* expression in the presence of both PDGF-BB and EGF to only half of that in controls (Table I). These data suggest that the effect of EGF in combination with PDGF-BB can not only be attributed to protein kinase C activation. We have reported that the *c-fms* expression by PDGF-BB alone is not mediated through protein kinase C activation because PMA alone did not induce *c-fms* expression and H7 and staurosporine demonstrated no effects on *c-fms* expression by PDGF-BB (9). Therefore, *c-fms* expression is induced initially by PDGF-BB–specific pathways independent of protein kinase C and then augmented by EGF and FGF through both protein kinase C-dependent and -independent pathways. Furthermore, additions of either dibutyryl cyclic AMP to PDGF-BB (Table II) or pertussis toxin to a combination of PDGF-BB and EGF (Table III) had no effects on *c-fms* expression, indicating that neither cAMP dependent pathway nor pertussis toxin sensitive G protein were involved in sustained and high expression of *c-fms* in the presence of PDGF-BB.

We demonstrated that IFN-γ and M-CSF inhibited the in-

Table IV. Effects of PDGF-BB, EGF, and IFN-γ on Cell Growth in Human Aortic Medial Smooth Muscle Cells

	[^{3}H]Thymidine uptake	%
	dpm/mg cell protein	
No addition	18355±1380	100
10 ng/ml PDGF-BB	48068±569	262
3 ng/ml EGF	26686±1149	145
50 units/ml IFN-γ	13696±1761	75
10 ng/ml PDGF-BB + 3 ng/ml EGF	85308±7583	465
10 ng/ml PDGF-BB + 50 units/ml IFN-γ	27998±2139	153

Smooth muscle cells were seeded at a density of 10^5 cells/ml and medium was replaced with serum-free RPMI 1640 for 48 h before the experiment. After incubating with human PDGF-BB, human EGF, and/or human IFN-γ in RPMI 1640 containing 1% fetal calf serum for 24 h, [^{3}H]thymidine was added, and incubation was performed for 4 h. The radioactivity in the cells was then determined. Each value represents the mean±SD of triplicate wells.

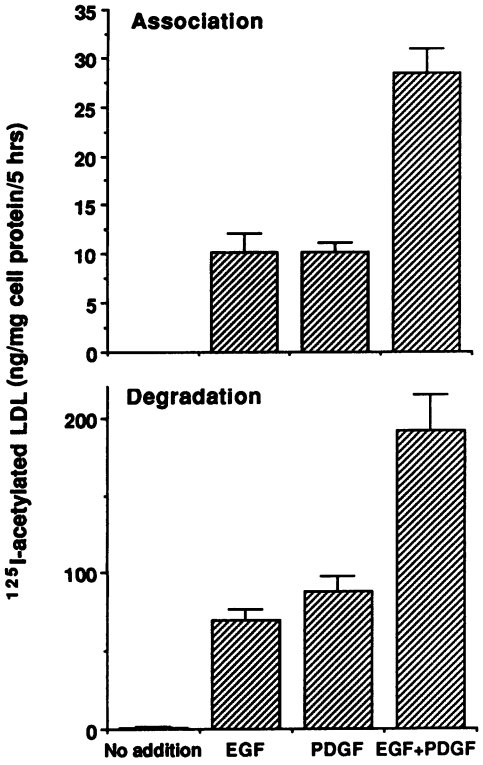

Figure 4. Specific cell association and degradation of ^{125}I-acetylated LDL. Subconfluent medial smooth muscle cells were cultured with 10 ng/ml PDGF-BB and/or 3 ng/ml EGF for 8 h after a 24 h preincubation in serum-free medium, and further incubated with PDGF-BB and/or EGF in the presence of 10 μg/ml ^{125}I-acetylated LDL for 5 h at 37°C. Then cell association and degradation of ^{125}I-labeled lipoproteins were determined. Nonspecific cell association and degradation were determined by the addition of a 50-fold excess of unlabeled lipoproteins and were subtracted from total cell association and degradation. Each value represents the mean of triplicate wells.

duction of *c-fms* gene expression by PDGF-BB in human vascular medial smooth muscle cells. IFN-γ and M-CSF have been reported to have beneficial effects on reducing the rate of atherogenesis in vitro (12, 32–34) as well as in vivo, in which interferon γ prevents the progression of atherosclerosis in cholesterol-fed rabbits (35) and M-CSF prevents it in Watanabe heritable hyperlipidemic rabbits (36, 37). Therefore, the inhibition of *c-fms* expression in vascular smooth muscle cells by IFN-γ and M-CSF appears to be related to the inhibitory process of atherosclerosis, whereas PDGF-BB, EGF, and FGF may be related to the progression of atherosclerosis. M-CSF is known to down-regulate *c-fms* expression at the level of transcription in monocytes (38). Recent reports have characterized the signaling pathway of IFN-γ that involves cytoplasmic proteins such as p91 as key factors mediating growth factor signals to the nucleus (39–42). The activities of these proteins, which reside in the cytoplasm as inactive forms, are resistant to protein synthesis inhibition by cycloheximide (43). We observed that a suppressive effect of IFN-γ on *c-fms* induction was not affected by cycloheximide. Therefore, our result suggests that these cytoplasmic proteins may be involved in signaling pathways of IFN-γ for *c-fms* expression.

We recently have demonstrated gene expression of scavenger receptor as well as *c-fms* in vascular smooth muscle cells isolated from atherosclerotic lesions, and proposed that these gene expressions may be related to the phenotypic conversion of vascular smooth muscle cells to foam cells in atheromatous lesions (4, 9, 44–47). Furthermore, recent studies have demonstrated that PDGF-BB induces scavenger receptor expression in normal medial smooth muscle cells through protein kinase C activation (9, 44) and IFN-γ suppresses scavenger receptor activity (33, 34). In the present study, we demonstrated that EGF also induced scavenger receptor activity in human aortic medial smooth muscle cells and that a combination of EGF and PDGF-BB had an additive effect on scavenger receptor activity. Since EGF as well as PDGF-BB is a potent activator of protein kinase C, EGF may induce scavenger receptor activity through the activation of protein kinase C.

Many growth factors and cytokines regulate cellular functions of vascular wall to maintain its homeostasis and play a role in the progression or regression of atherosclerosis. In the present study, we demonstrated that multiple growth factors are involved in the regulation of *c-fms* expression via certain signaling pathways in an autocrine or paracrine manner, however, each growth factor and cytokine except PDGF-BB had no independent effect on *c-fms* expression. The expression of *c-fms* may explain characteristic aspects of transformation of vascular smooth muscle cells to phagocytic phenotype. Since gene expression of *c-fms* in smooth muscle cells coincides with the phenotypic conversion of medial smooth muscle cells to intimal smooth muscle cells, *c-fms* induction by different growth factors in vascular smooth muscle cells may serve as a good model to study complex interactions of growth factors and cytokines in the arterial wall.

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