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# Expressions of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase genes are stimulated by recombinant platelet-derived growth factor isomers

(gene activation/protein kinase C/atherosclerotic lesion)

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ABSTRACT **The plausible role that platelet-derived growth factor (PDGF) has in the localized pathophysiological changes that occur in the arterial wall during development of atherosclerotic lesions led us to investigate the influence of recombinant (r)PDGF isomers -AA, -AB, and -BB on the expression of low density lipoprotein receptor (LDL-R) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [(*S*)-mevalonate:NAD$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.88] genes. In addition, we clarified the role of protein kinase C (PKC) in expression of the two genes in human skin fibroblasts and vascular smooth muscle cells. The various rPDGF isoforms are distinct in their ability to activate transcription of both genes: (*i*) Both rPDGF-AA and -BB stimulate transcription of the LDL-R gene; in contrast, rPDGF-BB, but not -AA, activates transcription of the HMG-CoA reductase gene. (*ii*) All recombinant isoforms of PDGF activate transcription of the c-*fos* gene. (*iii*) While rPDGF-dependent transcription of the LDL-R gene occurs independently of PKC, transcription of the HMG-CoA reductase gene appears to involve the action of that enzyme.**

The means by which eukaryotic cells acquire cholesterol from their environment include a receptor-mediated endocytosis of low density lipoprotein (LDL) cholesterol, via the LDL-receptor (LDL-R) pathway, or by synthesis *de novo*, the rate-limiting step that is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [(*S*)-mevalonate:NAD$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.88] (1, 2). Furthermore, a receptor-independent scavenger pathway has been postulated (3).

The differentiation process of cells (for example, macrophages) coincides with transcriptional activation of LDL-R and HMG-CoA reductase genes, which are inherently involved in regulation of their cellular cholesterol metabolism (4). Auwerx *et al.* (4), using the tumor promotor phorbol ester and protein kinase C (PKC) agonist phorbol 12-myristate 13-acetate (PMA) and PKC inhibitors, found that the transcription of both genes is controlled by PKC.

Unlike PMA, platelet-derived growth factor (PDGF) is a physiological mitogen with multiple biological functions, one of which is its role in the development of atherosclerotic lesions (5). Native PDGF stimulated the expression of LDL-R mRNA in fibroblasts (6); however, the effect of the recombinant (r)PDGF isomers on the expression of the LDL-R gene was not known. Moreover, PDGF activated the expression of the protooncogenes c-*myc*, c-*fos*, *JE*, and *KC* in BALB/c mice and 3T3 cells (7); the transcriptions induced by PDGF were stable, as evidenced by a nuclear runoff transcription assay (7). These four PDGF-inducible genes respond differently, however, to at least two intracellular secondary messengers, PKC being but one of them (7). All three dimeric forms of PDGF (PDGF-AA, -AB, and -BB) exist in nature, and they interact with their corresponding receptors (8). The binding of PDGF to its receptors results in autophosphorylation of the receptors and phosphorylation of tyrosine residues of certain cytoplasmic substrates (9, 10). In addition, PDGF stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate and activates PKC (11, 12). To evaluate the effect of the three rPDGF isoforms on inositol phospholipid metabolism and PKC activation, we have found a distinct ability of rPDGF-AA and -BB to stimulate the receptor-dependent signal transduction pathway in vascular smooth muscle cells (VSMCs) (11). However, according to recent investigations these biochemical changes may not be sufficient to account for the mitogenic response of various cell types to PDGF (12).

In view of the unique capabilities of the various isoforms of rPDGF, it was desirable to study their influence on the regulation of expression of LDL-R and HMG-CoA reductase genes. In addition, the role of PKC in induction of both genes was studied in human skin fibroblasts and VSMCs. The isoforms of rPDGF are distinct in their ability to activate the transcription of both genes: (*i*) Both rPDGF-AA and -BB stimulate transcription of the LDL-R gene; in contrast, rPDGF-BB, but not -AA, activates transcription of the HMG-CoA reductase gene. (*ii*) All isoforms of rPDGF activate transcription of the c-*fos* gene. (*iii*) While rPDGF-dependent transcription of the LDL-R gene occurs independently of PKC, transcription of the HMG-CoA reductase gene appears to involve the action of that enzyme.

## METHODS

**PDGF-AA, -AB, and -BB Isomers.** The three isomers were obtained from GIBCO/BRL.

**Measurement of Mitogenicity.** The mitogenic effect was measured by incorporation of [$^3$H]thymidine into VSMCs and fibroblasts according to the protocol of Chesterman *et al.* (13).

**Fibroblasts.** Three different primary fibroblast cell lines derived from human skin by sterile punch biopsies were cultivated in minimal essential medium with Earle's salts (MEM; GIBCO) supplemented with 10% fetal calf serum (Seromed), 1% vitamins (GIBCO), and 8 mM L-glutamine (GIBCO), in 75-ml Falcon tissue culture flasks until confluent (14). Medium was changed every second day; no antibiotics

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Abbreviations: LDL-R, low density lipoprotein receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PKC, protein kinase C; rPDGF, recombinant platelet-derived growth factor; VSMC, vascular smooth muscle cell; PMA, phorbol 12-myristate 13-acetate.

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or antimycotics were added; cells were used between the fourth and eighth passage.

**Isolation and Cultivation of VSMCs.** VSMCs were prepared as described by Ross (15) and Ross and Glomset (16). The samples were examined with electron and fluorescence microscopes to ensure the authenticity of the subcultured cells; immunofluorescent staining of the appropriate marker proteins was performed routinely. Cells were grown under the same conditions and in the same medium as fibroblasts. VSMCs were used between the fourth and eighth passage.

**Cell Stimulation.** To study the induction of genes for LDL-R and HMG-CoA reductase in the cells, the culture medium was replaced 2 days before cell stimulation with the PDGF isoforms, with Q-MEM (MEM supplemented with 1% vitamins and 8 mM L-glutamine), to bring the cells to quietude.

To stimulate the cells, one of the three rPDGF isomers was added (10 ng/ml) to Q-MEM; confluent cell cultures ($1 \times 10^7$ cells) were treated for various time periods (0, 0.5, 1, 2, 4, and 8 hr).

**RNA Isolation and Northern Blot and Dot Blot Analyses.** Quantitation of mRNA was performed at various time intervals as indicated below. RNA was isolated by a guanidinium isothiocyanate method (17) and equal amounts of RNA, determined by $A_{260}$, were denatured at 95°C for 5 min in sample buffer and then size-fractionated by electrophoresis through 1% agarose gels containing 0.02 M morpholinosulfonate, 2% formaldehyde, and 0.2 μg of ethidium bromide per ml (18). Gels were visualized by UV illumination to determine the position of 28S and 18S rRNA bands, to assess integrity of RNA, and to verify that equal amounts of RNA had been loaded into all wells before transfer to nylon membranes (BRL). Transfer of RNA to membranes was accomplished by capillary blotting overnight with 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

Dot blot analysis was performed as described by Costanzi and Gillespie (19). In brief, $10^7$ cells were resuspended in 4 ml of ice-cold 10 mM Tris·HCl, pH 7.0/1 mM EDTA/10 mM vanadylribonucleoside complex; 0.4 ml of 5% Nonidet P-40 was added, and the mixture was incubated on ice for 5 min. The nuclei were pelleted by centrifugation (15,000 × *g* for 2 min) and the supernatant fluid was then extracted with 4.4 ml of buffered phenol/chloroform (1:1) adjusted to pH 5.0. The aqueous phase was separated and mixed with 4.4 ml of chloroform/isoamyl alcohol (24:1). The aqueous phase was mixed with 1.3 ml of 20× SSC and 1.2 ml of 37% formaldehyde and incubated for 15 min at 60°C. The samples were serially diluted into 15× SSC (1×, 2.5×, 5×, 10×, and 50×) and filtered through a prewetted nylon membrane filter with a vacuum dot blot apparatus (Bio-Rad). Each slot was washed twice with 500 μl of 6× SSC and then air dried.

After blotting, the membranes (for both Northern and dot blot) were dried and UV irradiated to fix the RNA. Prehybridization was performed at 65°C overnight [10 mM EDTA/0.5% SDS/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)], followed by hybridization at 65°C in 1 M NaCl/7 mM EDTA/0.1 M Tris·HCl, pH 8.0/5× Denhardt's solution/1.0% SDS/250 μg of tRNA/25 ng of heat-denatured $^{32}$P-labeled cDNA probes per ml (LDL-R, HMG-CoA reductase, HLA-β, and c-*fos*) (18).

The blots were washed in 1 M NaCl/7 mM EDTA/0.1 M Tris·HCl, pH 8.0/0.5% SDS at 25°C for 10 min, followed by a 20-min wash in the same solution at 65°C; washing was repeated four times. Afterwards, the membranes were exposed to Kodak XAR film in a Kodak intensifying screen at −70°C for 24 hr (18). Autoradiograms were analyzed by densitometry (ASBA-analyzing program).

LDL-R mRNA was detected with a 1.9-kilobase (kb) *Bam*HI fragment of the human LDL-R cDNA clone pLDL-R-3 (American Type Culture Collection; ATCC no. 57004). The HMG-CoA reductase gene was detected with a *Bam*HI fragment (1.5 kb) obtained from ATCC (no. 57042). A *Pst* I fragment of the HLA-β gene (ATCC no. 31748) was used as a constitutive control gene. Labeling was performed by multiprime reaction (Amersham; multiprime reaction kit). The c-*fos* gene was characterized with an end-labeled oligonucleotide (British Biotechnology; no. BPR33).

Table 1. Effect of the various rPDGF isoforms on [$^3$H]thymidine incorporation into human skin fibroblasts and VSMCs

| PDGF isoform | $ED_{50}$ in human skin fibroblasts | $ED_{50}$ in vascular smooth muscle cells |
|---|---|---|
| -AA | 23 ng/ml | 28 ng/ml |
| -AB | 5 mg/ml | 10 ng/ml |
| -BB | 3 ng/ml | 3 ng/ml |

Dose–response of [$^3$H]thymidine incorporation in quiescent human skin fibroblasts and human VSMCs after treatment with various concentrations of rPDGF isoforms (0.5–50 ng/ml). PDGF-induced cell proliferation was determined by pulse labeling the cells 20 hr after addition of PDGF isoforms with 0.5 μCi of [$^3$H]thymidine for 4 hr (1 Ci = 37 GBq). The $ED_{50}$ values of all three recombinant PDGF isoforms (-AA, -AB, -BB) are the mean values of three individual determinations.

## RESULTS

**Effects of the Three rPDGF Isomers (-AA, -AB, -BB) on *de Novo* Synthesis of DNA.** The three rPDGF isomers induced a dose-dependent proliferative effect in both cell types—i.e.,

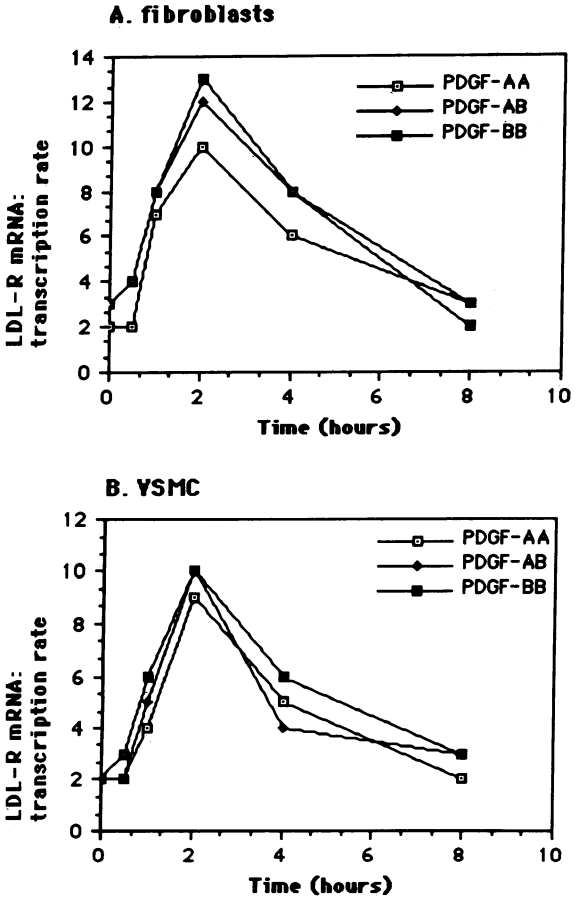


FIG. 1. Time course of the accumulation of LDL-R mRNA in human fibroblasts (*A*) and VSMCs (*B*) after treatment with rPDGF isoforms -AA, -AB, and -BB. RNA was isolated at various times after addition of rPDGF isoforms and was quantified by dot blot hybridization.

fibroblasts and VSMC—and the rank order of potency was PDGF-BB > -AB > -AA (Table 1). Thus, the $ED_{50}$ values required for stimulation of *de novo* synthesis of DNA are well within the comparable ranges of the dissociation constants for binding of the PDGF isomers to specific cell-surface receptors (20).

**Effects of rPDGF-AA, -BB, and -AB on Induction of Transcription of the LDL-R Gene.** A marked and transient induction of the transcription of the mRNA of the LDL-R gene followed the addition of the various rPDGF isomers; however, the kinetics of mRNA transcription were not different for the three isomers in fibroblasts (Fig. 1*A*) and VSMCs (Fig. 1*B*) when compared with the effect of native PDGF as reported by Mazzone *et al.* (6). The LDL-R transcription of mRNA began to increase 60 min after addition of rPDGF isomers (10 ng/ml) and reached a maximum at 2 hr; it then declined after another 4 hr.

**Effects of rPDGF-AA, -BB, and -AB on Induction of Transcription of the Genes for HMG-CoA Reductase and c-*fos*.** Although rPDGF-AA, -AB, and -BB stimulated transcription of the LDL-R gene, HMG-CoA reductase gene transcription was not induced by rPDGF-AA (0.5–50 ng/ml). In contrast, rPDGF-AB and -BB induced transcription of the HMG-CoA reductase gene (Fig. 2). Note the time course for HMG-CoA reductase mRNA transcription in response to rPDGF-BB (10 ng/ml), which reached its maximum 4 hr after addition of the isomer. The effectiveness of PDGF-BB (and -AB) was ≈10-fold higher than the basal level of mRNA when studied in both cell types; this effect was nearly the same as that of PMA.

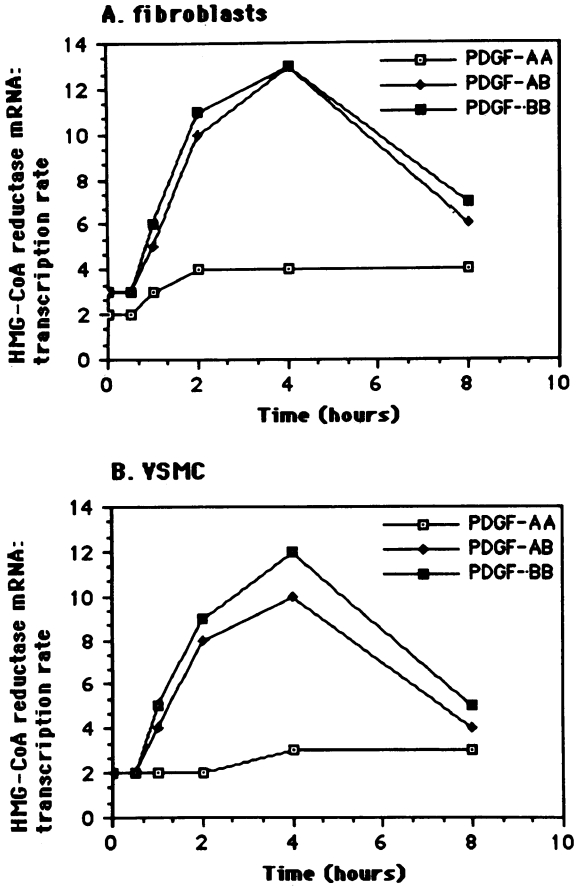


FIG. 2. Time course of the accumulation of HMG-CoA reductase mRNA in human fibroblasts (*A*) and VSMCs (*B*) after treatment with rPDGF isoforms -AA, -AB, and -BB. RNA was isolated at various times after addition of rPDGF isoforms and was quantified by dot blot hybridization.

The transcription of c-*fos* mRNA was similar for all three isoforms of rPDGF in VSMCs and fibroblasts, and it reached the maximum within 30 min (Fig. 3).

In addition, HLA mRNA was constitutively transcribed consistently in all experiments. Furthermore, the increases in steady-state levels of transcribed mRNA of the LDL-R, HMG-CoA reductase, and c-*fos* genes were abolished by treating the cells with actinomycin D (5 μg/ml). This confirmed that the changes induced by addition of the rPDGF isomers occurred at the level of mRNA synthesis, which is in agreement with Mazzone *et al.* (6), who obtained similar results with native PDGF.

**Effect of Polymyxin B on Induction of mRNAs for LDL-R, HMG-CoA Reductase, and c-*fos* by rPDGF-AA and -BB.** If the effects of rPDGF isomers on gene induction are channeled through PKC, then the rPDGF isomer-dependent changes should be abolished by polymyxin B, a PKC inhibitor. While the transcriptional response of the LDL-R gene is induced by the three rPDGF isomers and the PCK agonist PMA, the addition of polymyxin B did not affect the transcriptional change (Fig. 4). In contrast, the gene activation induced by rPDGF-BB, with respect to the HMG-CoA reductase gene, was found to be inhibited by polymyxin B (Fig. 5). Moreover, it also inhibited expression of the c-*fos* gene in both cell types when stimulated by either the rPDGF isomers or PMA (Fig. 6).

## DISCUSSION

The different recombinant PDGF isoforms, at concentrations that occur under physiological conditions, have unique abilities to regulate the transcription of mRNA representing the

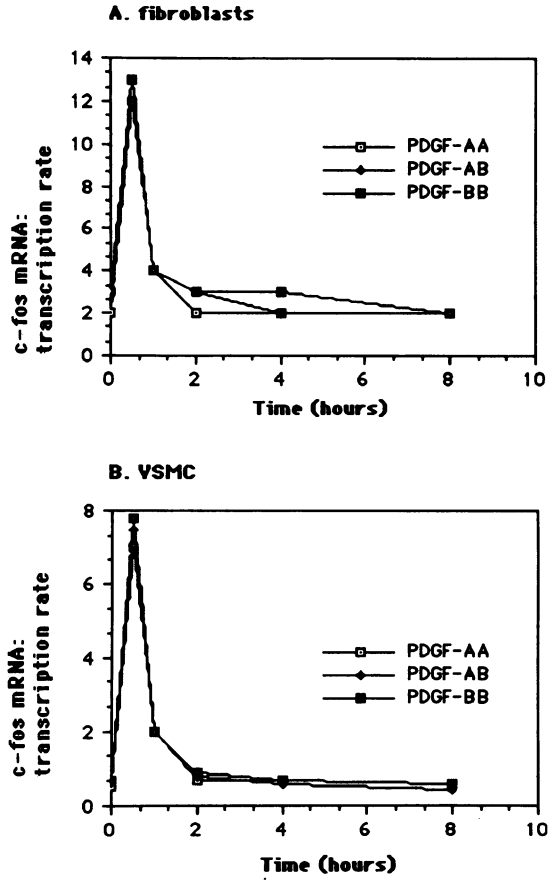


FIG. 3. Time course of accumulation of c-*fos* mRNA in human fibroblasts (*A*) and VSMCs (*B*) after treatment with rPDGF isoforms -AA, -AB, and -BB. RNA was isolated at various times after addition of rPDGF isoforms and was quantified by dot blot hybridization.

genes for LDL-R and HMG-CoA reductase. In addition, all recombinant isoforms promote induction of transcription of the c-*fos* gene, an early response gene. While the signal leading to increased LDL-R mRNA was independent of PKC, the transcription of HMG-CoA reductase and c-*fos* genes apparently is channeled through PKC.

All of the recombinant PDGF isomers transiently increased the transcription of LDL-R mRNA in both fibroblasts and VSMCs. This agreed with previous reports showing that native PDGF induced LDL-R gene transcription (6). Auwerx *et al.* (4) reported a direct correlation between PMA-dependent induction of LDL-R transcription and PKC activation (using cells of the THP-1 human monocytic leukemia line). Our results indicate that all PDGF isoforms induce transcription of the LDL-R gene, which is not PKC dependent. The effect of a phorbol ester on a tumor cell line cannot readily be compared, however, to the effect of PDGF isoforms on the normal cell types that were used in our studies. Our data are in agreement with those of Coughlin *et al.* (21), who suggested a PKC-dependent and -independent pathway for transduction of the PDGF signals on gene expression. In this context, a variety of isoforms of PKC with potential impact on gene activation has been entertained (22); moreover, phosphorylation site motifs that are specific for PKC have been described (23). Interestingly, rPDGF-BB has a distinct ability to promote transcription of the HMG-CoA reductase gene, an aspect that has not been demonstrated previously. In contrast to the lack of the effect of rPDGF-AA, rPDGF-BB (and -AB) requires PKC for transcription of the HMG-CoA reductase gene. Similarly, transcription of the c-*fos* gene by all rPDGF isoforms is apparently channeled through PKC. This observation agrees with Hall and Stiles (7), who reported an association between induction of the c-*fos* gene and differential phosphorylation of specific intracellular proteins in response to native PDGF. It is possible, therefore, that induction of HMG-CoA reductase and c-*fos* genes involves the PKC-dependent phosphorylation of specific proteins, which leads to induction of transcription.

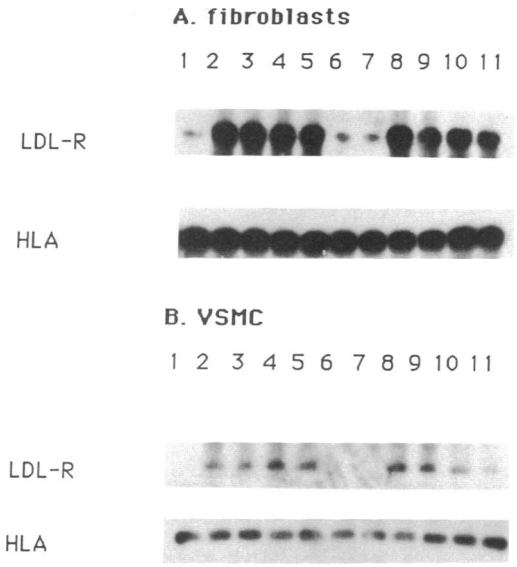


FIG. 4. Electrophoretic analysis of LDL-R mRNA in human skin fibroblasts (*A*) and VSMCs (*B*). Lanes: 2–5, quiescent cells were grown for 2 hr in the presence of 200 nM PMA (lanes 2), rPDGF-AA (lanes 3), rPDGF-AB (lanes 4), or rPDGF-BB (lanes 5); 7–11, quiescent cells were preincubated with polymyxin B (10 $\mu$M) for 30 min (lanes 7) and were then challenged with PDGF-AA (lanes 8), -AB (lanes 9), -BB (lanes 10), or PMA (lanes 11). Untreated cells were used as controls (lanes 1 and 6). Ten micrograms of total RNA was used in each lane. HLA-$\beta$ gene transcription was constitutive.

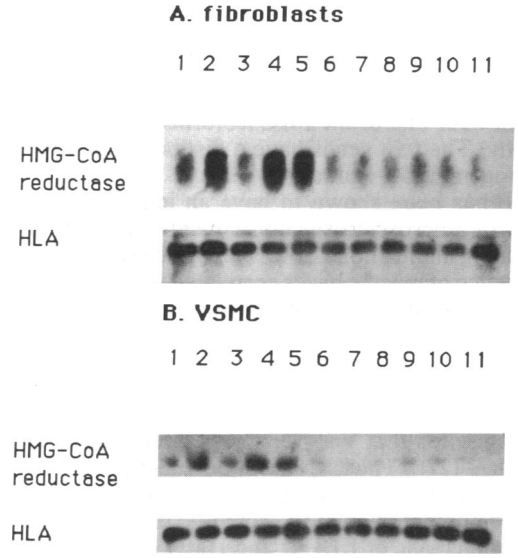


FIG. 5. Electrophoretic analysis of HMG-CoA reductase mRNA in human skin fibroblasts (*A*) and VSMCs (*B*). Lanes: 2–5, quiescent cells were grown for 2 hr in the presence of 200 nM PMA (lanes 2), rPDGF-AA (lanes 3), rPDGF-AB (lanes 4), or rPDGF-BB (lanes 5); 7–11, quiescent cells were preincubated with polymyxin B (10 $\mu$M) for 30 min (lanes 7) and were then challenged with PDGF-AA (lanes 8), -AB (lanes 9), -BB (lanes 10), or PMA (lanes 11). Untreated cells were used as controls (lanes 1 and 6). Ten micrograms of total RNA was used in each lane. HLA-$\beta$ gene transcription was constitutive.

These changes, however, may not be required for induction of the LDL-R gene.

The underlying events for the distinct ability of the rPDGF isoforms to induce the transcription of both genes involving PKC may be one or more of the following: (*i*) A unique intracellular protein that is induced in response to PDGF could elicit the transcription of specific genes (24). (*ii*) One of the various PKC isoforms (23) could transduce a rPDGF-BB

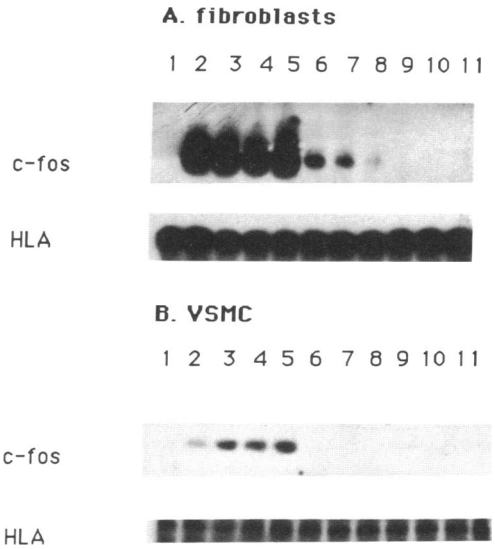


FIG. 6. Electrophoretic analysis of c-*fos* mRNA in human skin fibroblasts (*A*) and VSMCs (*B*). Lanes: 2–5, quiescent cells were grown for 2 hr in the presence of 200 nM PMA (lanes 2), rPDGF-AA (lanes 3), rPDGF-AB (lanes 4), or rPDGF-BB (lanes 5); 7–11, quiescent cells were preincubated with polymyxin B (10 $\mu$M) for 30 min (lanes 7) and were then challenged with PDGF-AA (lanes 8), -AB (lanes 9), -BB (lanes 10), or PMA (lanes 11). Untreated cells were used as controls (lanes 1 and 6). Ten micrograms of total RNA was used in each lane. HLA-$\beta$ gene transcription was constitutive.

second signal leading to specific expression of the HMG-CoA reductase gene. (*iii*) Certain substances—e.g., interleukin 2—act by stimulation of PKC without breaking down inositol phospholipids (25). Thus, rPDGF-BB may be used directly as a substrate for one of the PKC isoforms leading to a second signal. (*iv*) The two genes have a distinct responsiveness to the various rPDGF isoforms.

PDGF has been shown to have an important role in the etiology of atherosclerosis; consequently, our findings have far-reaching implications. It was reported previously (26) that the presence of macrophages correlated with increased levels of PDGF-BB in atherosclerotic lesions; PDGF-BB was found within macrophages in all stages of atherosclerotic lesion development in humans and in nonhuman primates (26). Similarly, *in situ* hybridization experiments for PDGF mRNA, in a series of fibrotic lesions of human atherosclerosis, suggested that VSMCs are the predominant source of PDGF-AA (27). Thus, the unique function of the PDGF isomers may be that their presence exacerbates formation and development of an atherosclerotic lesion. Moreover, the transient increase of LDL-R and HMG-CoA reductase gene transcription induced by PDGF may occur when increased concentrations of cholesterol are required. This may be an explanation for the subsequent decline of the transcriptional events after challenge with PDGF. Further study is necessary to ascertain the role of the various PDGF isomers in gene activation.

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