

Apolipoprotein D and Platelet-Derived Growth Factor-BB Synergism Mediates Vascular Smooth Muscle Cell Migration

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Abstract—We identified apolipoprotein (apo)D in a search for proteins upregulated in a posttranscriptional manner similar to fibronectin in motile smooth muscle cells (SMCs). To address the function of apoD in SMCs, we cloned a partial apoD cDNA from ovine aortic (Ao) SMCs using RT-PCR. We documented a 2.5-fold increase in apoD protein but no increase in apoD mRNA in Ao SMCs 48 hours after a multiwound migration assay ($P<0.01$). Confocal microscopy revealed prominent perinuclear and trailing edge expression of apoD in migrating SMCs but not in the confluent monolayer. Stimulation of Ao SMCs with 10 ng/mL platelet-derived growth factor (PDGF)-BB increased apoD protein expression ($P<0.05$). Moreover, PDGF-BB-stimulated migration of human pulmonary artery SMCs was suppressed by knock-down of apoD using RNAi. Stable overexpression of apoD in Ao SMCs cultured in 10% fetal bovine serum promoted random migration by 62% compared with vector-transfected cells ($P<0.01$). Overexpression of apoD or addition of exogenous apoD to a rat aortic SMC line (A10) stimulated their migration in response to a subthreshold dose of PDGF-BB ($P<0.05$). This was unrelated to increased phosphorylation of ERK1/2 or of phospholipase C- γ 1, but correlated with enhanced Rac1 activation. This study shows that apoD can be expressed or taken up by SMCs and can regulate their motility in response to growth factors. (*Circ Res.* 2004;95:179-186.)

Key Words: vascular smooth muscle ■ apolipoprotein D ■ cell migration ■ platelet-derived growth factor ■ Rac1

The ductus arteriosus (DA) is a fetal vessel that develops a neointima in late gestation and closes on constriction after birth. Our previous studies related formation of the DA neointima to heightened vascular smooth muscle cell (SMC) migration, which is linked to increased fibronectin synthesis relative to that in SMCs from the aorta (Ao).¹⁻⁴ Subsequent studies showed that the increase in DA fibronectin synthesis is regulated by enhanced mRNA translation involving an interaction between an RNA binding protein, identified as light chain 3 (LC3) of microtubule-associated proteins 1A and 1B³ with an AU-rich element (ARE) in the 3'-untranslated region (3'UTR) of fibronectin mRNA. An LC3 protein affinity column was then used to identify mRNAs in which enhanced translation might be similarly regulated in motile SMCs. One of the bound transcripts encoded the 3'UTR of apoD (unpublished data, 2004); therefore, the present study was undertaken to establish whether apoD has a role in SMC motility.

ApoD is a 29- to 30-kDa glycoprotein identified in plasma.⁵⁻⁶ It has a lipocalin structure predicting that it

binds small hydrophobic ligands.⁷ Subsequently, apoD was identified as a carrier molecule with high affinity for steroids such as progesterone, as well as arachidonic acid⁸ and metabolites that enhance SMC migration.⁹ ApoD, also a component of HDL, is present in human serum at concentrations of 47 to 155 μ g/mL.¹⁰ It has been localized to pericytes in developing blood vessels¹¹ and is seen in close association with mature blood vessels in a variety of animal tissues,^{12,13} most recently by our group in human atherosclerotic plaques.¹⁴ Induction of apoD occurs in neuronal,¹⁵⁻¹⁸ as well as nonneuronal cells¹⁹ after injuries that act as stimuli for cell migration. ApoD interacts weakly with the long form of the leptin receptor,²⁰ whose ligand, leptin, promotes SMC migration.²¹ Moreover, other lipoproteins such as apoJ²² and other lipocalins are associated with cell migration and are prevalent in tissues where active remodeling is taking place.²³ It was, however, unknown whether apoD is promigratory either alone or in association with motogenic factors such as platelet-derived growth factor (PDGF).²⁴

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We now demonstrate that apoD is associated with SMC migration in fetal lamb DA and aortic (Ao) SMCs and that PDGF-BB can regulate its expression and localization. Moreover, apoD appears necessary for human pulmonary artery (hPA) SMC migration in response to PDGF-BB. We also report in A10 cells, an apoD-null SMC line derived from rat aorta, that uptake of apoD or transfection of cells with a plasmid expressing apoD stimulates cell motility in response to a subthreshold dose of PDGF-BB. This synergism is associated with activation of Rac1²⁵ and appears independent of phosphorylation of MAP kinase²⁶ or of phospholipase (PLC)- γ .²⁷

Materials and Methods

Cell Culture

Fetal Rambouillet lambs (supplied by D. Swartz, Buffalo, NY) were delivered by Caesarian section on day 100 of a 145-day timed gestation. Ductus arteriosus and Ao were removed en bloc, and SMCs cultured from explants as previously described²⁸ (see expanded Materials and Methods section in the online data supplement available at <http://circres.ahajournals.org>). Studies were approved by the Animal Care Committees of the Hospital for Sick Children, Toronto, Canada and Buffalo Children's Hospital in accordance with the guidelines of the American Physiological Society. In some experiments, cells were maintained in differentiation medium (DM) containing 0.1 μ mol/L insulin, 1 nmol/L selenium, 0.1 mmol/L sodium pyruvate, 200 μ mol/L ascorbic acid, and 5 mg/mL transferrin with 0.1% BSA.

Wounding Assays

A multiwound culture model was used to induce cell migration. Confluent monolayers of ovine Ao, DA, and a rat aortic SMC line (A10) grown on cover slips were wounded with a multitooth scratch comb with 1-mm teeth. Ovine SMCs were cultured in M199/10% fetal bovine serum (FBS) and A10 cells in DMEM/10% FBS for 12, 24, or 48 hours. Preparation of whole cell lysates and cytosolic fractions is described in the online data supplement.

Immunocytochemistry

Ductus arteriosus and Ao SMCs at passage 2 were plated on 2-chamber slides at a density of 1.5×10^5 cells/well. To induce migration, half the cells were scraped with a rubber policeman, and the remainder grown for 12, 24, or 48 hours in 10% FBS. For the PDGF-BB studies, subconfluent Ao SMCs were starved in DM for 48 hours and then treated with 10 ng/mL PDGF-BB for 4 hours. At each specified time point, cells were fixed with 100% ice-cold methanol at -20°C and dried at RT. After blocking in 1% BSA/PBS for 30 minutes at 37°C , cells were probed with a monoclonal mouse anti-human apoD antibody (B29) (1:25) for 1 hour. Cells were then washed and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50) (Jackson ImmunoResearch Laboratories) and counterstained with (300 μ mol/L) 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) nuclear stain. Confocal microscopy (Zeiss LSM 510 confocal microscope) was performed on 0.35- μ m sections of cells using $63\times$ magnification, and representative sections were acquired. Slow, intermediate, and fast migrating cells were classified as being 1, 2, and 3 microscopic fields away from the wound edge. Intensity of apoD was assessed semi-quantitatively in 10 migrating cells in each field.

Chemokinetic Migration Assays

To quantify the effect on rates of migration of apoD overexpression in Ao SMCs, cells were tracked in M199/10%FBS or M199/0.1% BSA using time-lapse video microscopy. As a control, Ao SMCs transfected with pAP2 vector were used. Locomotion was monitored for 6 hours using a $\times 10$ objective attached to a Zeiss Axiovert 100 inverted microscope equipped with Hoffman Modulation contrast

optical filters and a 37°C heated stage. Images were captured with a CCD video camera module attached to a controller. Motility was assessed with Northern Eclipse software. Migration speed was determined as the sum of hourly distances divided by the total time.

ApoD Expression in Response to PDGF-BB

Aortic SMCs at passage 2 were grown to 60% to 80% confluence, and then starved in defined media for 48 hours to deplete cells of apoD. The time course of PDGF-BB-mediated apoD induction was determined by treating the cells with 10 ng/mL PDGF-BB for 4, 24, or 48 hours and assessing apoD expression by western immunoblot. The optimal dose of PDGF-BB required to induce apoD expression was determined by treating the cells for 4 hours at 1, 5, 10, and 50 ng/mL PDGF-BB and measuring apoD by Western immunoblot.

ApoD siRNA SMARTpool Transfection

Human pulmonary artery smooth muscle cells (hPASMCs) (Cascade Biologics) were seeded at a density of 3.25×10^5 cells in T25 flasks. Transfections were performed using a custom SMARTpool of short interfering RNA oligonucleotides (siRNAs) targeted against apoD mRNA (Dharmacon). As controls, luciferase RNAi as well as

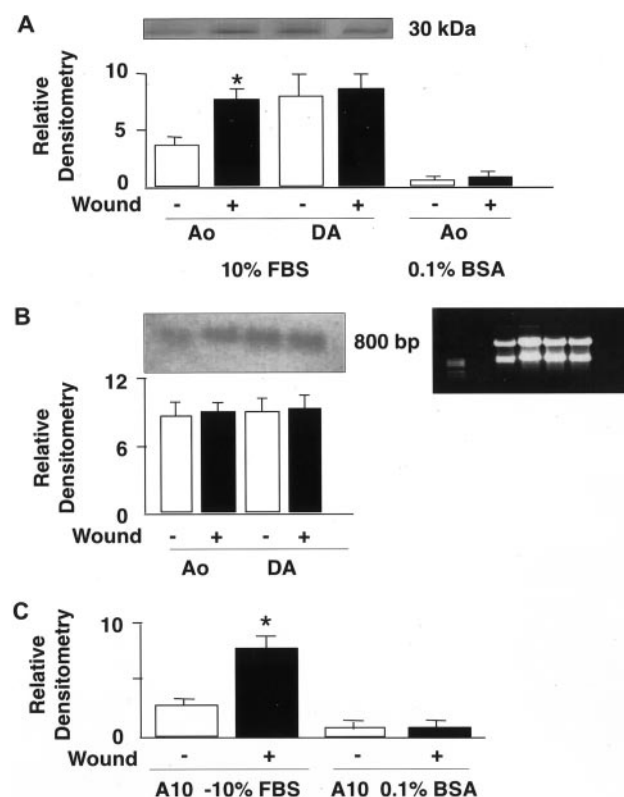


Figure 1. ApoD protein and mRNA levels in response to wounding of SMCs. **A**, Representative Western immunoblot of apoD protein monitored 48 hours after wounding of Ao and DA SMCs in 10% FBS and in serum free media (0.1% BSA). Graph depicts densitometric analysis with bars representing mean \pm SEM of 3 experiments. * $P<0.05$ vs nonwounded. **B**, Representative Northern blots of ovine Ao SMCs wounded or nonwounded for 48 hours in the presence of 10% FBS using the [32 P]-dCTP labeled ovine apoD cDNA and GAPDH as a control. Densitometric analysis is depicted with bars representing mean \pm SEM of 3 experiments. On the RHS, ethidium bromide stained 28S and 18S ribosomal RNA on an agarose gel indicates RNA loading and quality. **C**, Densitometric analysis of apoD protein by Western immunoblot in wounded vs nonwounded A10 cells in 10% FBS and in serum-free media (0.1% BSA). Bars represent mean \pm SEM of 3 experiments. * $P<0.05$ vs nonwounded.

cyclophilin RNAi were used (Dharmacon), all in concentrations of 100 nmol/L of siRNA complexed with 5 μ L of Lipofectamine 2000 (Invitrogen) in 500 μ L of Opti-MEM I (Invitrogen). Knockdown of ApoD was assessed at 24 and 48 hours for both mRNA by TaqMan Q-PCR and protein by Western immunoblotting.

Boyden Chamber Assays

Boyden chambers (fibronectin-coated PET track-etched membranes with 8- μ m pore size) (Falcon) were used for the migration assays. The transfected hPASMCs (3×10^4 cells) were added to the upper chamber, and the lower chamber was filled with 750 μ L of DMEM containing 10 ng/mL PDGF-BB. The chambers were incubated for 6 hours at 37°C, 5% CO₂. Cells at the top of the filter were then scraped off with a Q-tip to remove nonmigrating cells, and the remaining cells that migrated to the bottom of the filter were fixed and stained with Diff-Quik (Baxter Scientific Products). The cells were counted in 6 separate fields in each experiment using a light microscope (40 \times objective) and average values were obtained. In other experiments, apoD-transfected A10 SMCs were grown to 60% to 80% confluence and starved in defined media for 72 hours. PDGF-BB (1, 2, 5, and 10 ng/mL) was plated in the bottom chamber and then 2.5×10^4 A10 cells were added to each upper Boyden chamber, and migration was assessed after a 5-hour incubation. To

determine the influence of apoD uptake on SMC migration, untransfected cells were grown to 60% to 80% confluence, and starved in defined media for 72 hours. Then, 10, 100, or 1000 ng/mL human apoD purified from breast fluid from patients with gross cystic disease (supplied by E.R.) were added for 18 hours, and the cells harvested for Boyden chamber assays or for Western immunoblotting to confirm uptake of apoD.

Intracellular Signaling Mechanism

PDGF-BB and ERK1/2, p38, and PLC γ -1

To assess signaling pathways that might be influenced by the interaction between PDGF-BB and apoD, A10 SMCs were pretreated with 100 ng/mL human apoD for 18 hours and then with 1 and 10 ng/mL PDGF-BB for 5, 10, and 30 minutes. Expression of pERK1/2, p38, or PLC γ -1 was assessed by Western immunoblot as detailed in the online data supplement.

Rac 1 Activity Assay

To assay the activity of Rac1, the SMC lysate was affinity purified using a fragment of p21-activated kinase 1 (PAK1) expressed as a fusion protein with glutathione S-transferase (GST).²⁹ (see online data supplement). Affinity purified proteins were separated by 15%

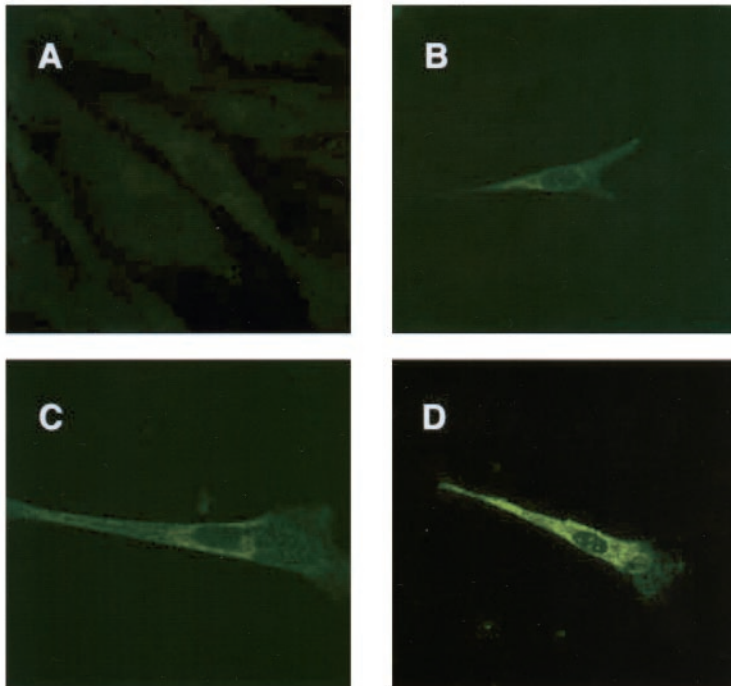
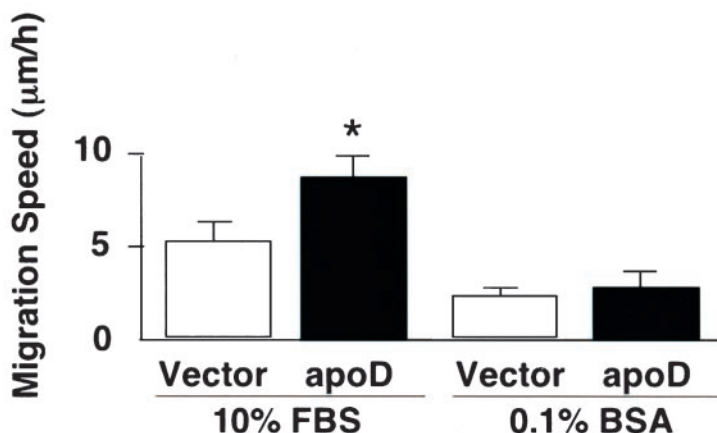


Figure 2. ApoD is induced in migrating Ao SMCs in response to wounding. Representative confocal microscopic images (67 \times magnification) of Ao SMCs wounded for 48 hours in the presence of serum, fixed and stained using a primary antibody against the native form of apoD and a FITC-conjugated goat anti-mouse IgG secondary antibody. A, Nonwounded cells in the confluent monolayer 48 hours after wounding. B, Minimal perinuclear staining was seen in cells at the wound edge. C, Representative cell that migrated two microscopic fields from the edge with intense perinuclear staining extending to the rear of the cell. D, Cell that migrated three microscopic fields from the wound edge. Bottom, Random locomotion of apoD and vector-transfected (Tx) Ao SMCs in 10% FBS tracked by video microscopy over a period of 6 hours. Bars represent mean \pm SEM of 3 experiments. * $P < 0.01$.



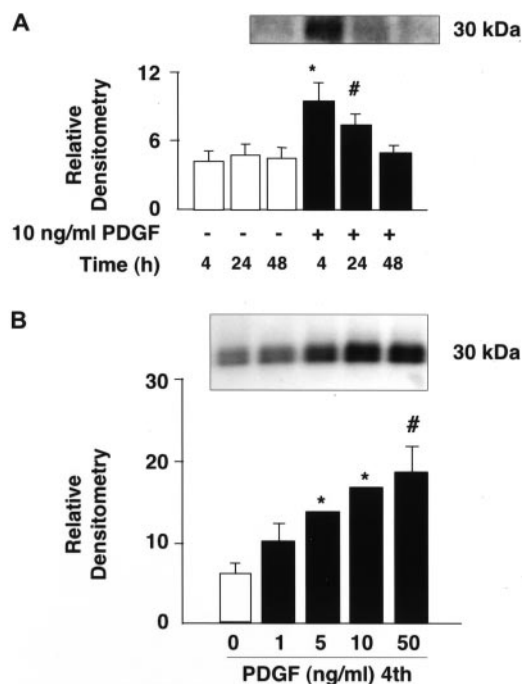


Figure 3. PDGF-BB and apoD expression in Ao SMCs. A, Quiescent Ao SMCs were treated with PDGF-BB (10 ng/mL) and apoD protein was monitored by Western immunoblot 4 to 48 hours later. Bars represent mean \pm SEM of 3 experiments. * P <0.01 vs untreated control. B, ApoD was monitored in ovine Ao SMCs by Western immunoblot in responses to PDGF-BB at concentrations 5 to 50 ng/mL. Bars represent mean \pm SEM of 3 experiments. * P <0.05 vs unstimulated control.

SDS-PAGE, transferred to a nitrocellulose membrane, immunoblotted with a Rac1 monoclonal antibody (Transduction Laboratories), and detected with anti-mouse peroxidase-conjugated IgG secondary antibody and enhanced chemiluminescence (ECL) detection reagents (Boehringer Mannheim). Rac1 activity was expressed relative to total cellular Rac1, ascertained by parallel immunoblotting of cell lysate that was not subjected to affinity purification.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to detect differences between groups of three or more. A significance level of P <0.05 was considered statistically significant. Post hoc comparison of individual groups was performed using Fischer's test of multiple comparisons. Data are represented as mean \pm SEM, and the number of independent experiments is given in the Figure legends.

Details of cloning ovine apoD cDNA, Northern and Western immunoblot analyses, retroviral-mediated transfection of ApoD, and Rac1 assay are found in the online data supplement.

Results

Cloning Ovine apoD

A 238-bp ovine cDNA fragment was generated by RT-PCR as described in the Materials and Methods. This fragment was 86% identical at the nucleotide level and 80% identical at the amino acid level to human apoD cDNA (online Figure S1 in the online data supplement).

Apolipoprotein D After Wounding of Ao SMCs and A10 SMCs

In the presence of serum, scratch-wounding induced a 2- to 3-fold increase in apoD protein in lysates of Ao SMCs as

assessed by Western immunoblot. The concentration of apoD was similar to that observed in nonwounded DA SMCs. No further increase in apoD was seen with wounding of DA SMCs (Figure 1A). In the absence of serum (in 0.1% BSA), the amount of apoD in the cell lysates was reduced and not influenced by wounding (Figure 1A). This suggests that serum is required for increased apoD synthesis or stability in response to wounding of Ao cells or that apoD, which is a component of FBS, is being taken up by the cells in response to wounding. The higher levels of apoD in DA cells imply heightened synthesis by a transcriptional or posttranscriptional mechanism, greater stability of the protein, or greater uptake that is not influenced by wounding. To further address whether induction of synthesis may explain the differences in wounded and nonwounded DA and Ao cells, we monitored apoD mRNA levels by Northern blot. ApoD steady-state mRNA levels were similar and not significantly changed after wounding in the presence of serum in Ao and DA SMCs (Figure 1B).

Western immunoblot analysis revealed that similar to primary ovine Ao SMCs, there was a 2- to 3-fold increase in apoD in wounded versus nonwounded A10 SMCs in 10% FBS (Figure 1C), whereas in 0.1% BSA, apoD levels were very low and no increase occurred after wounding. Because we failed to detect an apoD mRNA hybridization signal using either murine or human apoD cDNA probes (88% and 78% homologous to rat apoD, respectively; unpublished observations, 2004), we speculated that A10 SMCs might take apoD up directly from serum.

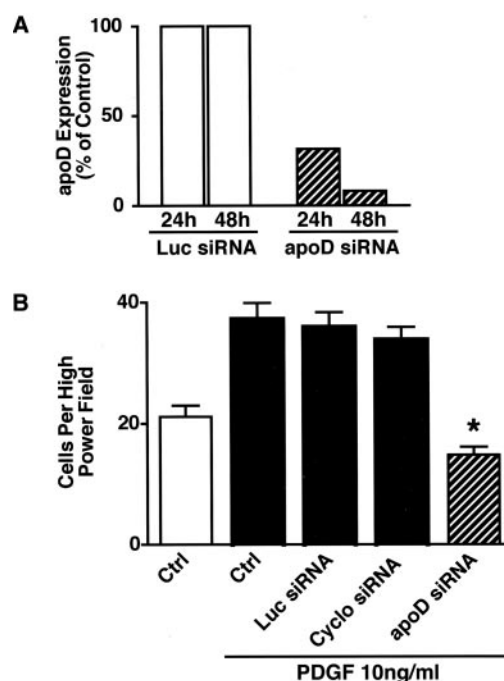


Figure 4. PDGF-BB induced migration and apoD RNAi in hPASCs. A, Representative experiment showing relative levels of apoD mRNA transcripts monitored by quantitative PCR 24 and 48 hours after apoD siRNA or luciferase (Luc) siRNA. B, Migration assays in response to PDGF-BB in cells transfected with apoD siRNA, luciferase (Luc) siRNA, or cyclophilin (Cyclo) siRNA. Bars represent mean \pm SEM of 6 experiments. * P <0.05 for apoD siRNA vs all other conditions.

ApoD Localization in Migrating Ao SMCs in Response to Wounding

We next investigated where apoD might be localized in migrating versus quiescent cells. Confocal microscopy revealed that in Ao SMCs associated with the confluent monolayer or located close to the wound edge, there was minimal perinuclear staining of apoD (Figure 2A and 2B). However, with increasing distance of migration from the wound edge, immunostaining for apoD was more intense in the perinuclear region and extending toward the rear of the cell, but was relatively absent in the leading lamellipodia (Figure 2C and 2D).

ApoD Overexpressing Ao SMCs and Chemokinesis

We next investigated whether apoD overexpression was sufficient to induce chemokinesis. Retroviral induction of stable transfection of apoD in fetal lamb Ao SMCs increased random locomotion by 62% in the presence, but not in the absence of serum as assessed by time-lapse video microscopy (Figure 2, bottom).

PDGF-BB Induction of apoD Expression in Ao SMCs

To exclude the possibility that a prochemokinetic or promigratory serum factor might further elevate apoD levels, we measured apoD protein in response to PDGF-BB at different time points after a 48-hour starvation period. A dose of 10 ng/mL PDGF-BB increased apoD at 4 hours in Ao SMCs and values returned to basal levels at 48 hours as judged by Western immunoblotting of cytoplasmic extracts (Figure 3A). We also observed a dose-dependent increase in apoD in response to PDGF-BB (1 to 50 ng/mL) at 4 hours (Figure 3B).

ApoD siRNA and PDGF-BB Mediated hPASC Migration

To determine whether induction of elevated apoD was necessary for the promigratory response to PDGF-BB, we transfected hPASCs with apoD siRNA and produced a 75% and 90% knockdown of apoD mRNA transcripts by Q-PCR at 24 and 48 hours, respectively, relative to control conditions with either luciferase siRNA or cyclophilin siRNA (Figure 4A); no detectable apoD was observed on Western immunoblotting at 48 hour in cells transfected with apoDsi, whereas strong bands were seen under control conditions (not shown). Migration in Boyden chamber assays in response to PDGF (10 ng/mL) was suppressed in cells transfected with apoD siRNA below levels of untransfected control cells, and control cells transfected with luciferase siRNA or cyclophilin siRNA (Figure 4B).

In A10 cells, an increase in apoD was not observed in response to PDGF-BB (data not shown). Thus, it seemed that A10 cells might be useful in determining how PDGF-BB interacts with apoD in inducing motility, independent of influencing endogenous apoD levels. We confirmed apoD overexpression in A10 transfected SMCs by Western immunoblotting and determined the subthreshold (1 ng/mL), submaximal (2 ng/mL), and maximal (10 ng/mL) doses of PDGF-BB that would induce migration (Figure 5A). ApoD-overexpressing A10 SMCs migrated faster at a subthreshold dose of 1 ng/mL

PDGF-BB, but not at 2, 5, and 10 ng/mL PDGF-BB, compared with vector-transfected cells (Figure 5B).

Administration of apoD also stimulated migration in response to a subthreshold dose of PDGF-BB (Figure 6A). In the absence of PDGF-BB, there was no migration regardless of the amount of apoD added. At a low dose of PDGF-BB (1 ng/mL), addition of 100 or 1000 ng/mL apoD promoted migration (Figure 6B). At higher doses of PDGF-BB (5 and 10 ng/mL), a lower dose of 10 ng/mL apoD stimulated migration, indicating a dose response synergistic relationship (Figure 6C and 6D).

ApoD and PDGF Activation of ERK 1/2, PLC γ -1, and Rac1

In different cell types, PDGF-BB-directed chemotaxis has been related to stimulation of activity of MAP kinases (ERK 1/2 and p38), PLC- γ 1, and Rac1.^{25,27,30} Application of a dose of apoD (100 ng/mL), which stimulated migration in the presence of a subthreshold dose of PDGF-BB (1 ng/mL) did not, however, influence phosphorylation of ERK 1/2 levels (P44/P42) (Figure 7A) or of PLC- γ 1. There was greater induction of phosphorylated PLC- γ 1 with 10 ng/mL when compared with 1 ng/mL PDGF-BB±apoD (100 ng/mL) (Figure 7B). We could not relate apoD potentiation of migration to the induction of p38 MAPK because we found no activated p38 in the presence of PDGF-BB, apoD, or both, whereas unphosphorylated p38 was unchanged (data not shown). Rac1 activation was increased with 10 ng/mL or with

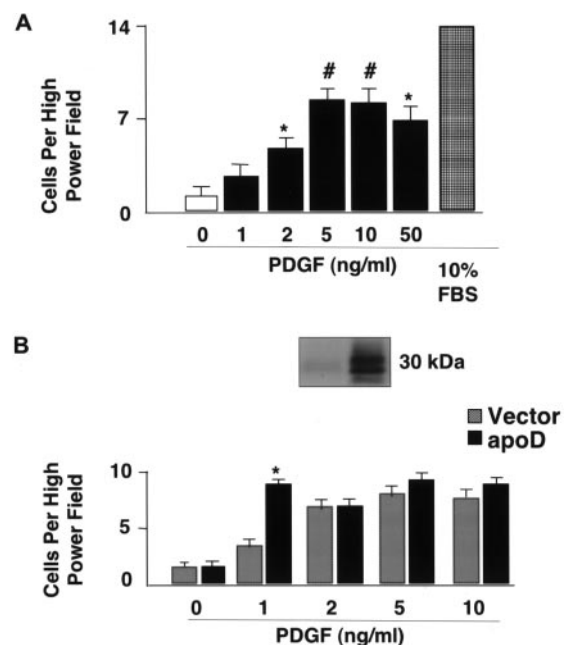


Figure 5. Directional migration of A10 SMCs in response to PDGF-BB and apoD. A, A10 SMCs in Boyden chamber migration assays with the indicated concentrations of PDGF-BB. 10% FBS used as a positive control. Bars represent mean±SEM of 4 experiments. * P <0.05 vs untreated control; # P <0.05 vs 2 ng/mL. B, top, Representative apoD Western immunoblots comparing vector and apoD transfected levels. Bottom, Graphic depiction of Boyden chamber migration assays using apoD-transfected A10 SMCs and different doses of PDGF-BB. Bars represent mean±SEM of 4 experiments. * P <0.05 vs control.

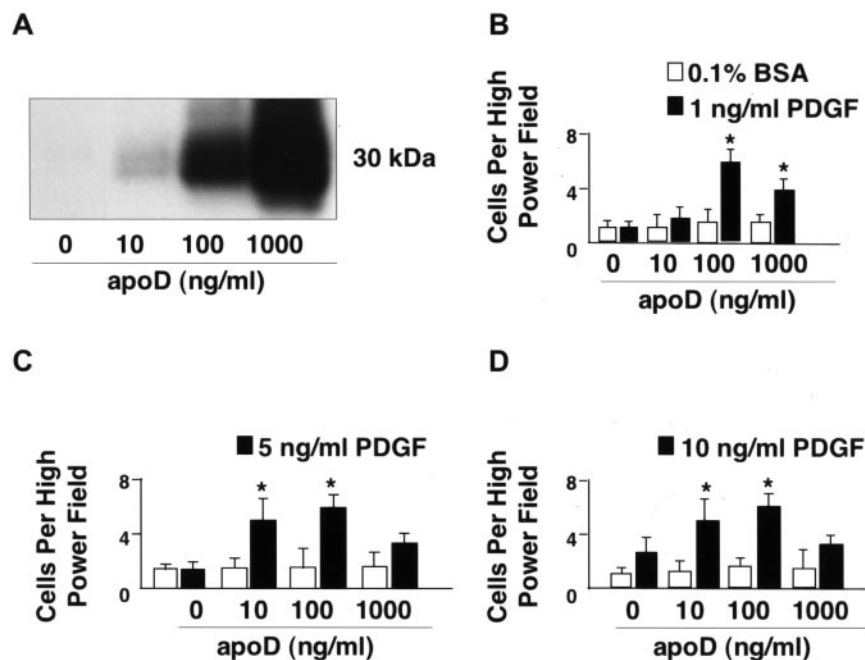


Figure 6. Uptake of apoD by A10 SMCs. A10 SMCs were starved in serum-free media for 72 hours to deplete apoD, then incubated for 18 hours with the indicated concentrations of human recombinant apoD. A, Western immunoblot analysis of total cell lysates. B, A10 SMC migration in response to a sub-threshold dose of 1 ng/mL PDGF with 100 and 1000 ng/mL exogenous apoD. At 5 ng/mL PDGF-BB (C) and at 10 ng/mL PDGF-BB (D), A10 SMC migration with 10 and 100 ng/mL apoD. For B and C, bars represent mean \pm SEM of 4 experiments. * P <0.05 compared with untreated cells at 10 and 100 ng/mL apoD.

1 ng/mL PDGF-BB+apoD (100 ng/mL) compared with 1 ng/mL PDGF-BB alone (Figure 8), suggesting that cooperative activation of this pathway might account for the apoD-PDGF synergism-inducing motility.

Discussion

This study focused on the novel observations that apoD, a major component of HDL, is produced and can also be taken up by vascular SMCs and positively influences their migratory behavior. We showed that apoD is present in high concentrations in motile cells such as DA SMCs, and increases in Ao SMCs after stimulation of migration by wounding in association with growth factors such as PDGF-BB. In hPASMCs, apoD is necessary for PDGF-BB-mediated migration. In A10 cells, apoD works in concert with PDGF-BB to stimulate SMC motility by a mechanism that could involve activation of Rac1.

Ovine DA SMCs, which show increased migration in culture in association with DA intimal cushion formation,¹⁻³ have higher levels of apoD protein, but not mRNA levels, when compared with cells from the Ao. ApoD protein levels also increase in Ao SMCs in response to wounding, but steady-state mRNA levels are similar.³¹ The discrepancy between high protein and unchanged mRNA levels could be related to heightened mRNA translation after transcription of apoD or to greater stability of the protein. Increased efficiency of translation of apoD mRNA is a possibility because LC3 binds to the 3'UTR of apoD mRNA, and because binding of LC3 to the 3'UTR of fibronectin mRNA increases fibronectin mRNA translation and SMC motility.³ The increase in apoD protein with wounding is also observed in neurons in response to injury.^{17,18} Interestingly, another apolipoprotein, apoJ, is induced in medial and neointimal SMCs in response to balloon injury of rabbit aorta, and stimulates SMC migration and proliferation.²² A similar role

was postulated for another lipocalin, extracellular fatty-acid binding protein.²³

In data not shown, we confirmed increased immunostaining for apoD in fetal lamb DA versus Ao vascular tissues, and intense apoD immunostaining is observed in atherosclerotic plaques relative to normal coronary artery tissues.¹⁴ In some studies, we used A10 cells, a SMC line derived from rat thoracic aorta. A10 cells resemble neointimal cells³² in that they are less differentiated and more migratory than primary SMCs,³³ and the signaling mechanisms involved are relatively well characterized in these cells.³⁴ We could not detect apoD mRNA or apoD protein induction in A10 cells, perhaps because of their less differentiated phenotype, consistent with studies in which poorly differentiated carcinomas express low levels of apoD.³⁵

We showed that PDGF-BB, which is released by wounding²⁴ and is a potent inducer of SMC motility,³⁶ is associated with an increase in apoD protein in ovine SMCs. The transient nature of this effect is consistent with the short half-life of PDGF-BB.³⁷ It was of interest then, that we were able to link PDGF-BB-mediated synthesis or stability of apoD in primary hPASMCs to motility, by showing that knock-down of apoD with RNAi suppressed PDGF-BB-mediated motility.

The apparent absence of endogenous apoD made A10 cells useful in assessing the effect on migration of either exogenous apoD or "endogenous" apoD produced after transfection of the cDNA. Transfection of apoD allowed the A10 SMCs to migrate in response to a subthreshold dose of PDGF.³⁸ Because apoD is prevalent in very high concentrations in the plasma, a physiologically relevant question, especially under conditions when there is endothelial injury, is whether adding apoD to the A10 cells in combination with PDGF-BB would have a similar effect to transfecting apoD. Indeed, by titrating exogenous apoD, we were able to show a dose-dependent synergism with a subthreshold dose of PDGF-BB. It is of interest that apoD binds arachidonic acid, because arachidonic acid pretreatment of

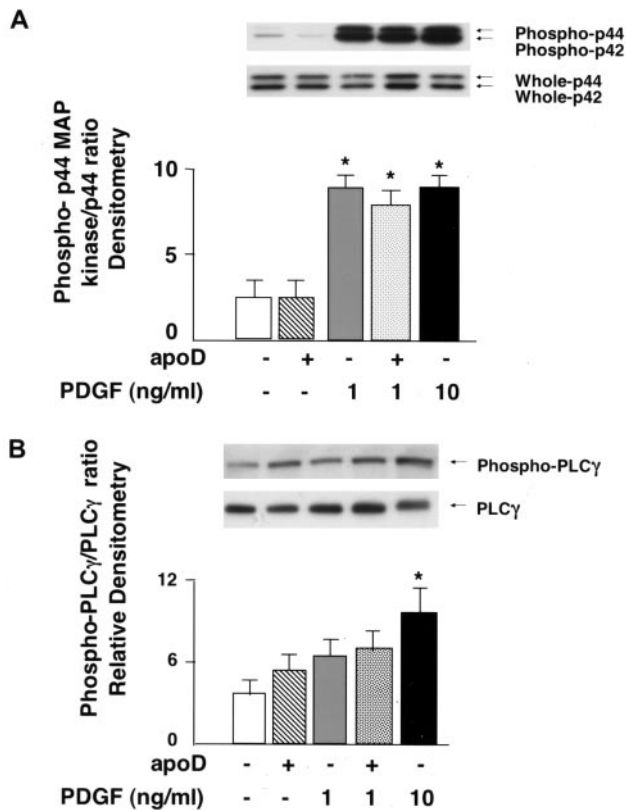


Figure 7. Phosphorylation of ERK 1/2, PLC- γ 1 with PDGF, and apoD. A, Representative Western immunoblots of pERK1/2 (top) and total Erk 1/2 (bottom). A10 cells were pretreated with apoD and then PDGF-BB was added at the concentrations described. B, A10 cells treated as described, and Western immunoblotting performed using a PLC- γ 1 antibody. Bars represent mean \pm SEM from 5 experiments. * P < 0.05 vs control.

SMCs enhances FBS- and PDGF-BB-induced migration.⁹ PDGF-BB also mediates release of arachidonic acid.³⁹

The synergism between apoD and PDGF-BB in inducing SMC motility could not be related to phosphorylation of p38, ERK 1/2,⁴⁰ or PLC- γ .⁴¹ PLC- γ 1 overexpression leads to an enhanced chemotactic response to PDGF-BB in endothelial cells, phosphorylation of p38 was demonstrated in migrating porcine aortic endothelial cells,⁴² and ERK 1/2 is phosphorylated in response to PDGF-induced chemotaxis in human mesangial cells.⁴³ In colonic epithelial cells, leptin promotes invasiveness via rho- and rac-dependent signaling pathways,⁴⁴ and overexpression of wild-type Rac in endothelial cells led to increased motility in response to PDGF-BB.⁴⁵ We did in fact show that activation of Rac1 could be achieved with maximal doses of PDGF-BB, as well as with subthreshold PDGF in combination with apoD. Immunolocalization indicated that apoD is present in the perinuclear region and extending toward the rear end of migrating cells, whereas Rac1 activity is associated with extension of lamellipodia.⁴⁶ It is possible that apoD is important in translocating a protein that directly influences Rac1 activity. ApoD could also influence retraction of the rear of the cell previously associated with cytoskeletal rearrangement⁴⁷ related to regulatory molecules such as rhoA, calcineurin, and tyrosine kinases.⁴⁸ In addition, apoD is associated with protease activity,⁴⁹ required in breaking cell-matrix attachments in motile cells. This novel functional relationship between apoD and SMC motility, may be relevant to developmental as well as disease processes.

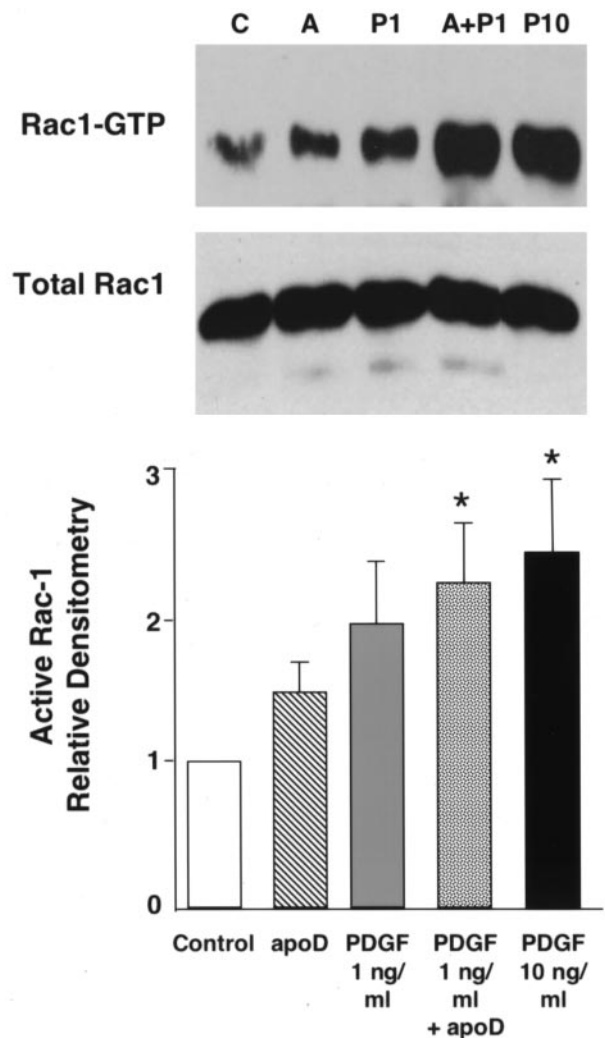


Figure 8. Rac1 activation with PDGF and apoD. Representative immunoblots of affinity-precipitated active, GTP-bound Rac1 (top blot), total cellular Rac1 (bottom blot), and relative densitometric analyses on the bottom under conditions described in Figure 6. Bars represent mean \pm SEM from 6 experiments. * P < 0.05 vs untreated cells. P1=PDGF-BB 1 ng/mL; P10=PDGF 10 ng/mL; A=apoD 100 ng/mL.

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