

Apolipoprotein D Inhibits Platelet-Derived Growth Factor-BB–Induced Vascular Smooth Muscle Cell Proliferated by Preventing Translocation of Phosphorylated Extracellular Signal Regulated Kinase 1/2 to the Nucleus

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Objective—Elevated apolipoprotein D (apoD) levels are associated with reduced proliferation of cancer cells. We therefore investigated whether apoD, which occurs free or associated with HDL, suppresses vascular smooth muscle cell (VSMC) proliferation, which is related to the pathobiology of disease.

Methods and Results—Intense immunoreactivity for apoD was observed in human atherosclerotic plaque but not in normal coronary artery. However, an increase in apoD mRNA was seen in quiescent relative to proliferating fetal lamb aortic VSMCs, and in the rat aortic VSMC line (A10), we demonstrated uptake of apoD from serum. Stable transfection of apoD in A10 cells in the absence of serum did not influence VSMC proliferation assessed by [³H]-thymidine incorporation. ApoD, administered at a dose of 100 ng/mL, completely inhibited basal as well as platelet-derived growth factor (PDGF)-BB–induced VSMC proliferation ($P < 0.01$) but had no effect on fibroblast growth factor–induced VSMC proliferation. ApoD did not suppress PDGF-BB or fibroblast growth factor-2–induced phosphorylation of extracellular signal regulated kinase (ERK) 1/2 but selectively inhibited PDGF-BB–mediated ERK1/2 nuclear translocation.

Conclusions—Our data suggest that apoD selectively modulates the proliferative response of VSMC to growth factors by a mechanism related to nuclear translocation of ERK1/2. (*Arterioscler Thromb Vasc Biol.* 2003;23:2172-2177.)

Key Words: apolipoprotein D ■ vascular smooth muscle cells ■ platelet-derived growth factor-BB ■ fibroblast growth factor-2 ■ extracellular signal–regulated kinase phosphorylation and nuclear translocation ■ proliferation

Abnormal proliferation of vascular smooth muscle cells (VSMCs) is a critical component of atherosclerosis and arterial restenosis after angioplasty.¹ The mechanism has been related to a response to injury in which growth factors such as basic fibroblast growth factor (FGF-2) and platelet-derived growth factor (PDGF) are released, stimulating proliferation and migration of VSMCs, leading to the formation of a neointima. Binding of PDGF to its receptor leads to the activation of several cell-signaling pathways associated with both VSMC proliferation and migration, such as those related to mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, phosphatidylinositol 3-kinase (PI3-kinase), and phospholipase C- γ (PLC- γ).²

Many epidemiological studies have demonstrated that elevated levels of LDL and reduced levels of HDL are risk factors in the development of atherosclerosis.³ The apolipoprotein (apo) portion of HDL consists mainly of apoA-I

(70%) and A-II (20%), in addition to A-IV, C, E, J, and D. There is an inverse relationship, both in human subjects and in experimental animals, between apoA-I, apoE, and atherosclerosis.^{3,4} ApoE is induced by growth arrest in human and mouse fibroblasts,⁵ and in rat VSMC, apoE significantly inhibits PDGF-BB–induced VSMC proliferation by partially suppressing MAPK activity and by preventing the increase in cyclin D1, which is necessary for cells to enter the G1 phase of the cell cycle.⁶ ApoJ inhibits basal as well as PDGF-BB–induced cell proliferation.⁷

Apolipoprotein D (apoD) was isolated and partially characterized in 1973 and found to comprise 1% to 2% of the total protein content of HDL.⁸ Approximately 83% of apoD is present in HDL, and more than 50% of this is complexed to apoA-II.⁹ A reduction in the relative content of apoD in HDL rather than serum levels of apoD correlate with an increased incidence of myocardial infarction.¹⁰ The relative roles of free

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apoD and that complexed to HDL in the pathobiology of vascular disease have not been investigated.

ApoD is present in early passage quiescent but not proliferating human fibroblasts in culture.¹¹ This inverse relationship between apoD and proliferation has also been found in breast¹² and prostate cancer cell lines. We now report the presence of apoD in atherosclerotic plaque and a link between apoD expression and uptake in VSMCs and inhibition of proliferation mediated by PDGF-BB but not FGF-2. The mechanism seems related to suppression of translocation of phospho-ERK1/2 from the cytoplasm to the nucleus, an event that is necessary for the mitogenic effect of growth factors.^{13–15}

Methods

Immunohistochemistry

Immunohistochemistry was performed to determine whether apoD is present in atherosclerotic plaque. Details of the methodology are in the online supplement, available at <http://atvb.ahajournals.org>.

Cell Culture

Rat A10 cells were used from passages 8 to 35. The cells were maintained in DMEM (Cellgro) with 10% FBS (Wisent) and 1% penicillin-streptomycin (Wisent). Primary ovine VSMCs from the descending aorta of fetal sheep were harvested at 100 days of gestation, and explants were performed as previously described.¹⁶ The cells were maintained in Medium 199 containing 10% FBS and 1% antibiotics-antimycotics (Gibco BRL) and were used at passage 2 or 3.

Northern Blotting

Primary ovine aortic VSMCs were incubated in Medium 199 containing 10% FBS or in serum-free differentiation medium containing 0.1 $\mu\text{mol/L}$ insulin, 1 nmol/L selenium, 0.1 mmol/L sodium pyruvate, 200 $\mu\text{mol/L}$ ascorbic acid, and 5 $\mu\text{g/mL}$ transferrin.¹⁷ RNA was extracted from the ovine cells at various time points using the RNeasy extraction protocol (Qiagen). RNA samples were separated on a formaldehyde agarose gel and transferred to a hybrid membrane. The membrane was probed with a partial 238 base pairs (bp), and ovine apoD cDNA was cloned in our laboratory and labeled with γ -[³²P]-dCTP (10⁶ cpm/mL) (Perkin-Elmer Life Sciences). The membrane containing RNA from A10 cells was also hybridized with probes from 2 different species, murine apoD cDNA (950 bp) and human apoD cDNA (809 bp), supplied by Dennis Drayna (Genentech) (to E.R.). Hybridization with GAPDH cDNA (1100 bp) (Clontech) was performed to confirm integrity of RNA and equal RNA loading.

Cell Proliferation Assays

Cell proliferation was measured in both A10 and ovine aortic SMCs using either [³H]-thymidine incorporation or the MTT colorimetric assay (details are available in the online supplement). Cells were seeded at a density of $\approx 1 \times 10^4$ cells/mm² and allowed to attach overnight in DMEM plus 10% FBS. Quiescence was induced by incubation for 48 hours in serum-free media. The cells were then treated with 10% FBS, 10 ng/mL PDGF-BB (Sigma), 5 ng/mL FGF-2 (Sigma), or purified human apoD (E.R., Université du Québec à Montréal) for the indicated time and concentration. [³H]-thymidine (1 $\mu\text{Ci/mL}$) was added to the cells 2 to 8 hours before harvesting, and thymidine incorporation was assayed by liquid scintillation counting or the MTT assay was performed.

Western Immunoblotting

After induction of quiescence in serum-free media, both ovine aortic VSMCs and A10 cells were studied under various conditions. Total cell lysates were harvested and protein samples were separated on

8% to 16% tris-glycine gels by SDS-PAGE and transferred to a PVDF membrane. The gels were stained in Coomassie blue to confirm equal protein loading. Membranes were blotted with the relevant antibodies (ApoD 5G10 1:1500 [University of Ottawa], green fluorescence protein [GFP] 1:1000 [Clontech], phospho-ERK 1/2 1:2000 [Cell Signaling Technology]). After rinsing, the membranes were probed with horseradish peroxidase-conjugated sheep anti-mouse antibody (1:3000 to 1:5000) (Amersham Biosciences) and visualized by enhanced chemiluminescence.

Immunocytochemistry

Cells were seeded at a density of 3×10^4 cells per well on 4-chamber cell slides, quiescence was induced as described above, and cells were treated with PDGF-BB (10 ng/mL) or FGF-2 (5 ng/mL) either alone or in the presence of exogenous apoD (100 ng/mL) for 2 and 4 hours. Cells were then fixed by 4% paraformaldehyde in PBS for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100 in PBS. After preincubation in blocking solution containing 1% albumin, slides were incubated with a polyclonal rabbit anti-phospho-ERK1/2 1:250 (Cell Signaling Technology) or the mouse monoclonal anti-human apoD 2B9¹⁸ 1:25 overnight at 4°C. Secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (1:200 and 1:50, respectively, Jackson Research Laboratories) were applied for 1 hour. Secondary antibodies alone were used as a control. Cell nuclei were stained with 1:1000 diluted DAPI (Sigma) in PBS. The slides were then examined using a confocal microscope (Zeiss LSM 510 Confocal Microscope) or a deconvolution microscope (Leica DMRA2 with a Leica CTRMIC controller).

Stable Transfection of A10 Cell

The human apoD sequence was isolated from a recombinant pBlue-script SK plasmid using EcoRI and BamHI. This was subsequently inserted into the multiple cloning site of the AP2 plasmid that also contains the sequence encoding GFP. The plasmid AP2 was then transfected using Lipofectamine according to manufacturer's instructions into the 293GPG human kidney retroviral packaging cell line. 293GPG cells transfected with GFP alone were used as a negative control. Beginning 3 days after transfection, viral supernatant was collected daily for 1 week and frozen at -80°C . A10 cells were plated at 4×10^4 cells per well and allowed to adhere. Media were removed and replaced with 293GPG supernatants containing retrovirus in the presence of 6 $\mu\text{g/mL}$ polybrene daily for 3 days. Cells were then sorted for GFP fluorescence by flow cytometry after 7 days of growth. Cells positive for GFP were then plated and maintained in DMEM plus 10% FBS. After incubation in serum-free media for 48 hours to deplete the cells of apoD from serum, western immunoblots were performed to confirm endogenous apoD and GFP protein expression.

Growth Curves

Cells were plated in DMEM plus 10% FBS and allowed to attach overnight. They were then washed and incubated with serum-free media. On selected days, cells were trypsinized and counted using a hemocytometer. Cell viability was determined by adding trypan blue (0.4%) to the cells before counting.

Statistical Analyses

A 1-way ANOVA was used to detect differences between groups of 3 or more. A level of $P < 0.05$ was considered statistically significant. Post hoc comparison of individual groups was performed using Fisher's test. Data are represented as mean \pm SEM. The number of experiments performed is indicated in the figure legends.

Results

ApoD Is Detected in Atheroma But Not in Normal Coronary Artery

See the online supplement and the online Figure.

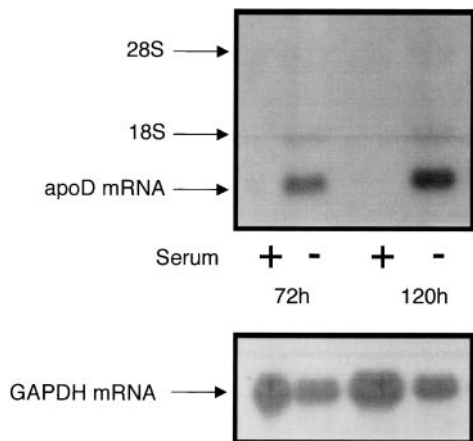


Figure 1. ApoD mRNA in proliferating and quiescent VSMCs. Top, Northern blot analysis of total RNA extracted from primary ovine VSMCs incubated for 72 or 120 hours in the presence of 10% FBS or in serum-free differentiation medium (DM). Bottom, The membrane was stripped and hybridized with γ - ^{32}P -dCTP-labeled GAPDH to demonstrate similar RNA loading conditions.

ApoD mRNA in Quiescent, But Not in Proliferating Primary Ovine VSMC

Northern blot analysis performed on primary ovine aortic VSMCs demonstrated that the apoD mRNA transcript is not observed in cells proliferating in the presence of 10% FBS, but a strong hybridization signal is present in cells in which quiescence is induced by incubation in serum-free differentiation medium (Figure 1).

In A10 VSMCs, which are nondifferentiated compared with primary cells and resemble neointimal cells,¹⁹ the apoD mRNA transcript was not seen using the mouse cDNA probe, which possesses an 88% identity to rat apoD cDNA, or using the human cDNA probe, which has 78% identity. Membrane hybridization with GAPDH confirmed RNA integrity in the membrane (data not shown). The relationship between proliferation and uptake of apoD protein level was therefore investigated using Western immunoblotting and immunohistochemistry. Induction of A10 cell proliferation was assessed at various time points after the addition of DMEM plus 10% FBS compared with serum-free DMEM by [^3H]-thymidine incorporation and by an increase in the number of cells in DNA synthesis (S) phase, as determined by flow cytometry. Consistent with other reports in the literature,²⁰ cells stimulated by addition of 10% FBS demonstrated a greater than 2-fold elevation in [^3H]-thymidine incorporation at 18 hours, whereas no change in [^3H]-thymidine incorporation was seen after 18 hours of incubation in serum-free DMEM (data not shown). In addition, 63% of A10 cells were in S phase after 18 hours of treatment with 10% FBS compared with 11% of cells maintained in serum-free media. Western immunoblots on total A10 cell lysates revealed an elevation of apoD protein levels 8 hours after the addition of serum that continued to rise at 18 and 24 hours (Figure 2A). Immunocytochemistry revealed a prominent punctate distribution of apoD throughout the cytoplasm, as assessed 8 to 24 hours after serum stimulation, whereas staining was barely detectable in cells incubated in serum-free media (Figure 2B).

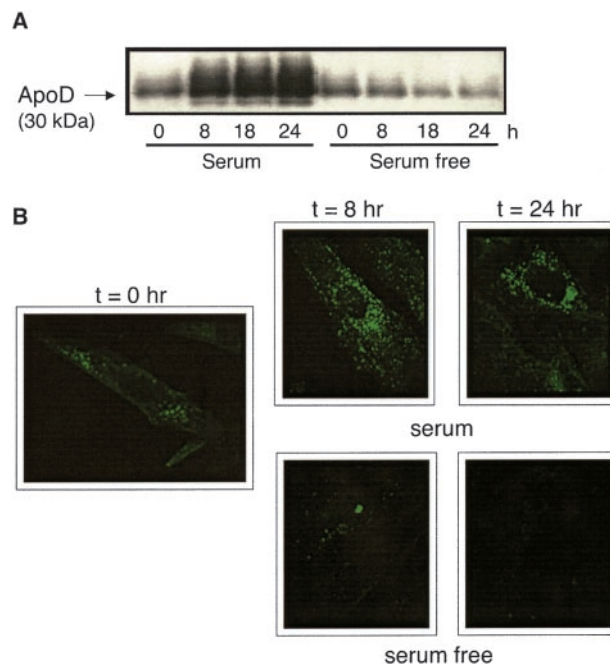


Figure 2. ApoD protein in proliferating and quiescent A10 cells. A, Western immunoblot analysis for apoD protein on total cell lysates representative of 2 separate experiments with similar results. B, Images were taken of A10 cells using a mouse anti-human apoD 2B9 and FITC-conjugated goat anti-mouse IgG at $\times 63$ magnification using confocal microscopy. Diffuse punctate cytoplasmic staining was observed in cells stimulated with serum.

Overexpression of ApoD and Proliferation in A10 Cells

A10 cells, which do not seem to express apoD mRNA, were transfected with the full-length human apoD cDNA. Western immunoblot analysis was performed on A10 cell lysates to confirm transfection of apoD. Positively transfected cells were selected for GFP fluorescence by flow cytometry and passaged several times to expand the cell number. Untransfected and vector-transfected cells (containing only GFP) exhibit trace amounts of apoD protein in serum-free medium, but 2 different groups of apoD-transfected cells, apoD-1 and apoD-2, demonstrated a 1.9-fold and 3-fold increase in apoD level, respectively (Figure 3A). In these cells, however, growth curves were similar to those in vector-transfected cells (Figure 3B). Attempts were then made to determine whether the expression of endogenous apoD was able to inhibit the stimulation of proliferation induced by growth factors. However, neither apoD nor vector-transfected cells responded by proliferation to 10 ng/mL of PDGF-BB or to 10 or 25 ng/mL of FGF-2.

Exogenous ApoD and A10 Cell Proliferation

Purified apoD protein was then applied to quiescent A10 cells for 18 hours, at which point [^3H]-thymidine incorporation was determined. Significant reproducible inhibition of [^3H]-thymidine incorporation was seen at apoD concentrations of 100 and 1000 ng/mL compared with serum-free media alone ($P < 0.05$; data not shown). In the presence of PDGF-BB (10 ng/mL), apoD was able to inhibit A10 cell proliferation at

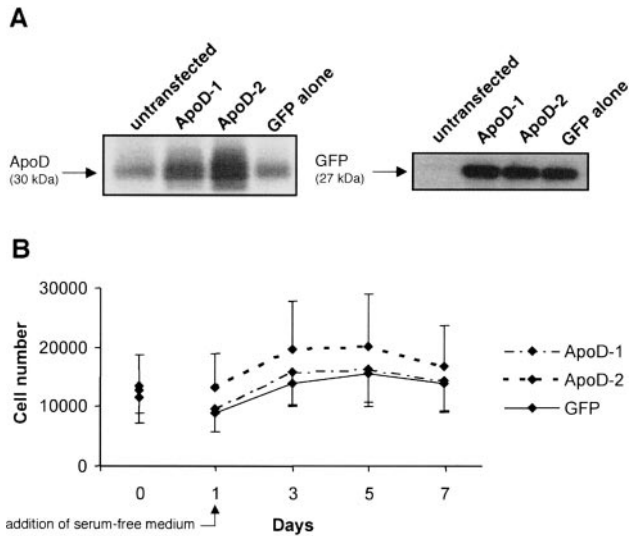


Figure 3. Transfection of ApoD in A10 cells and proliferation. A, Western immunoblots were performed on cell lysates from transfected A10 cells using mouse anti-human apoD and anti-GFP. One group transfected with apoD (apoD-1) has a 1.9-fold greater amount of apoD protein and the other group (apoD-2) has 3 times the amount of apoD protein. B, ApoD- and vector-transfected cells were plated at equal density, and cell counts from triplicate wells demonstrate no significant differences in the rate of growth.

concentrations of 100 and 1000 ng/mL ($P < 0.05$ for each comparison; Figure 4A). Trypan blue exclusion was performed on A10 cells incubated in the presence of apoD in serum-free media, and no cytotoxic effect was seen. We also incubated primary ovine aortic VSMCs with 100 ng/mL of apoD, as described above, and added either PDGF (10 ng/mL) or FGF-2 (5 ng/mL), but only with PDGF-BB did we observe that apoD inhibited proliferation as judged by MTT assay (Figure 4B).

ApoD and PDGF Activation of MAPK ERK1/2

To investigate the mechanism by which apoD inhibits PDGF-BB-induced proliferation, the effect of apoD on the phosphorylation of ERK1/2 was examined. After confirming that apoD is taken up by A10 cells minutes after exogenous

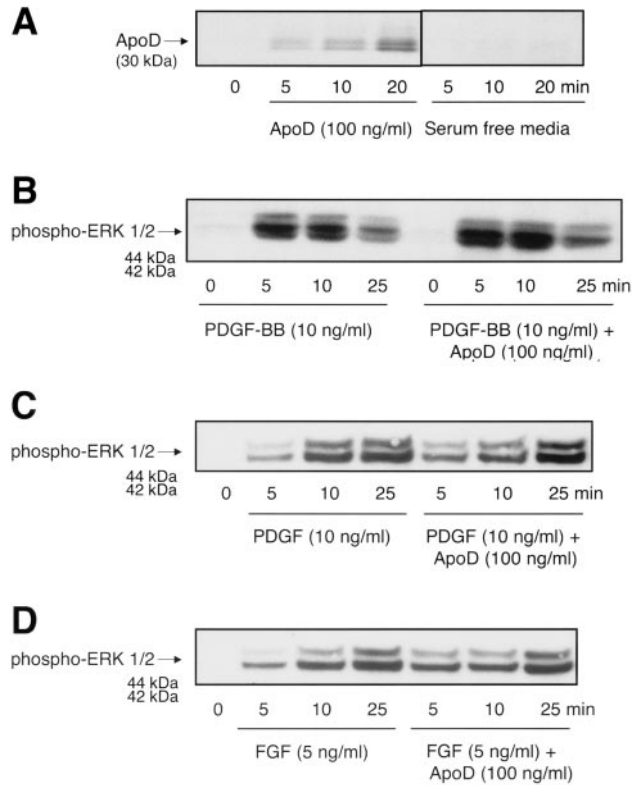


Figure 5. Phosphorylation of ERK1/2 by PDGF in presence of ApoD. A, ApoD (100 ng/mL) was applied to A10 cells after 48 hours of incubation. Western immunoblotting of cell lysates confirmed apoD protein in A10 cell lysates. B, Western immunoblotting for phospho-ERK1/2 (p42/44) demonstrates an increase in cells treated with PDGF-BB. C, Similar results with PDGF-BB±apoD are seen with primary ovine aortic smooth muscle cells. D, After treatment with FGF-2±apoD, there is similar induction of ERK phosphorylation. Immunoblots in A and B are representative of 4 separate experiments; immunoblots in C and D are representative of 2 separate experiments, all with similar results.

application (Figure 5A), quiescent A10 cells were treated with 10 ng/mL of PDGF-BB alone or with 10 ng/mL of PDGF-BB and 100 ng/mL of apoD. PDGF-BB alone results in a large increase in ERK 1/2 phosphorylation at 5 and 10 minutes compared with baseline, which begins to decrease by

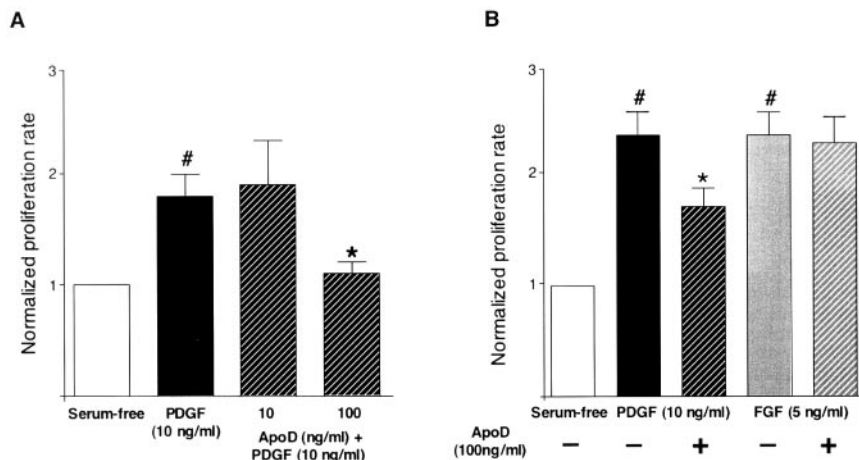


Figure 4. Exogenous ApoD and A10 VSMC proliferation. A, [³H]-thymidine incorporation in A10 cells was significantly elevated in the presence of 10 ng/mL PDGF-BB (# $P < 0.05$) compared with serum-free media alone. Addition of 100 ng/mL of apoD to 10 ng/mL of PDGF-BB results in significant inhibition of PDGF-BB-induced cell [³H]-thymidine incorporation back to baseline (* $P < 0.05$), whereas 10 ng/mL of apoD has no effect. Each bar represents the mean ratio of counts per minute (cpm)±SEM in each condition compared with control of 3 separate experiments. B, MTT assay was carried out under the conditions described above in ovine aortic smooth muscle cells, with similar results after PDGF-BB±apoD, but no change in assessment of cell proliferation is observed when apoD is added to FGF-2.

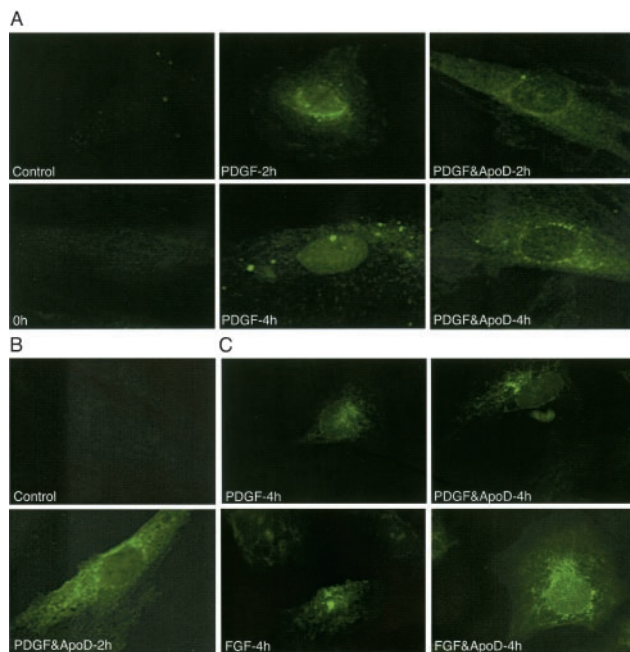


Figure 6. ApoD and nuclear translocation of phospho-ERK 1/2 in A10 VSMCs. A, Immunofluorescent labeling of anti-phospho-ERK1/2 in A10 cells demonstrates the absence of phospho-ERK1/2 at time zero, before the addition of PDGF-BB. PDGF-BB (10 ng/mL) induces nuclear translocation of phospho-ERK1/2 visualized at 2 and 4 hours after treatment. The prevention of nuclear translocation of phospho-ERK1/2 by 100 ng/mL of apoD is demonstrated. B, Immunostaining with anti-apoD in the set of experiments where cells are treated with PDGF-BB and exogenous apoD reveals apoD in a punctate distribution in the perinuclear region. Controls for phospho-ERK1/2 and apoD using secondary antibodies alone show no fluorescence. C, Primary ovine aortic smooth muscle cells demonstrate similar nuclear translocation of ERK1/2 after PDGF-BB that is inhibited by apoD. In contrast, no inhibition of translocation is apparent after FGF-2 stimulation. Original magnification $\times 63$ using confocal microscopy for A and B and deconvolution microscopy for C and D. The presence of cells in these fields was confirmed by nuclear staining (DAPI) (not shown).

25 minutes after stimulation. When quiescent A10 cells are treated with an inhibitory dose (100 ng/mL) of apoD in the presence of 10 ng/mL of PDGF-BB, no reduction in the phosphorylation of ERK1/2 is seen by western immunoblotting (Figure 5B). We also confirmed similar induction of phosphorylation of ERK1/2 after PDGF-BB as well as FGF-2 stimulation of primary aortic VSMCs with a slightly different time course (Figures 5C and 5D).

After PDGF-BB stimulation, phosphorylated ERK1/2 is translocated from the cytoplasm to the nucleus. Concomitant treatment of A10 cells with 100 ng/mL of apoD, however, impedes this translocation, as assessed both at 2 and 4 hours after treatment (Figure 6A). At the 2-hour time point, the distribution of apoD was confirmed to be punctate and perinuclear (Figure 6B), as in Figure 2B. Similar apoD-mediated inhibition of ERK1/2 nuclear translocation after PDGF-BB stimulation was observed in primary ovine aortic VSMCs. However, inhibition of nuclear translocation of ERK1/2 was not seen after FGF-2 stimulation (Figure 6C).

Discussion

We demonstrate for the first time the presence of apoD in atheromatous plaque but not in normal coronary arteries, providing a rationale for a modulatory effect of apoD on VSMCs in disease. ApoD exists in the plasma both free and in a form associated with HDL. Our studies were designed to assess the effects of free apoD, because the purification procedure is likely to delipidate apoD. ApoD that is delipidated or purified from breast cyst fluid is similar in its biological effects (unpublished studies from ER laboratory, 2002–2003). It would be of interest to assess whether apoD bound to HDL is still transported into the cell and whether under those circumstances it associates with apoA-II. Our findings in primary ovine aortic VSMCs are consistent with observations in cancer cell lines,¹⁴ which demonstrate high apoD mRNA levels in quiescent cells and absence of apoD mRNA in proliferating cells. Other studies from our laboratory, however, indicate that apoD synergizes with PDGF in facilitating VSMC migration (unpublished data).

We used both primary and A10 cells, a VSMC cell line derived from rat thoracic aorta that are nondifferentiated and resemble neointimal cells in atherosclerosis and restenosis.²¹ A10 cells may not synthesize apoD mRNA, because they are nondifferentiated compared with primary VSMCs. This is consistent with studies showing that poorly differentiated carcinomas express low levels of apoD.²² The absence of apoD mRNA in A10 cells made them an excellent model in which to investigate the effect of transfected apoD as well as exogenous apoD on VSMC proliferation. Transfection of the human apoD cDNA was achieved using a retroviral vector system, which has a higher transfection efficiency compared with other methods and results in stable expression of the protein of interest.²³ No change in the growth rate of A10 cells was seen after apoD transfection compared with vector transfection (GFP alone) at baseline, and there was no proliferative response after PDGF-BB or FGF-2 stimulation. This lack of responsiveness may have reflected late passage number after transfection. However, uptake of exogenous apoD in earlier passage A10 cells did inhibit basal as well as PDGF-BB-induced VSMC proliferation. In addition to VSMCs, apoD seems to be produced by fibroblasts and cells in close association with blood vessels,^{24–26} such as pericytes,²⁷ and may also regulate their proliferation.

Whereas ApoE inhibits PDGF-stimulated MAPK activity, apoD did not suppress phosphorylation but only nuclear translocation of phospho-ERK1/2. Interestingly, prevention of translocation, but not phosphorylation of ERK1/2, has recently been described in VSMCs treated with felodipine, a Ca^{2+} antagonist, belonging to a class of drugs widely used to treat hypertension and angina.²⁸ It is intriguing that after FGF-2 stimulation, apoD did not interfere with nuclear translocation of ERK1/2. This would support the notion that different mechanisms regulating ERK1/2 nuclear translocation could be used after growth factor receptor interaction. For example, PDGF-BB-mediated but not FGF-2-mediated effects of ERK1/2 require PI3-kinase,²⁹ which is known to be regulated by apolipoproteins.³⁰ Other mechanisms specifically implicated in nuclear translocation that could be differentially induced in response to growth factors and potentially

influenced by apoD include suppression of Crm-1, a nuclear export protein,¹⁷ activation of rac³¹ or protein kinase A,^{32,33} and the presence of the inactive mitogen kinase phosphatase 3, which functions as an ERK docking molecule.³⁴

In summary, we report novel findings suggesting that intracellular accumulation of apoD is able to selectively inhibit PDGF-BB-induced VSMC proliferation by a mechanism associated with impaired translocation of phospho-ERK1/2 to the nucleus. This function of apoD may modulate the pathobiology of vascular disease.

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