Apolipoprotein E Receptor Binding Versus Heparan Sulfate Proteoglycan Binding in Its Regulation of Smooth Muscle Cell Migration and Proliferation

Debi K. Swertfeger‡ and David Y. Hui§

From the Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0529

Received for publication, March 15, 2001, and in revised form, May 2, 2001
Published, JBC Papers in Press, May 11, 2001, DOI 10.1074/jbc.M102357200

This study showed that synthetic peptides containing either a single copy or tandem repeat of the receptor binding domain sequence of apolipoprotein (apo) E, or a peptide containing its C-terminal heparin binding domain, apoE-(211–243), were all effective inhibitors of platelet-derived growth factor (PDGF)-stimulated smooth muscle cell proliferation. In contrast, only the peptide containing a tandem repeating unit of the receptor binding domain sequence of apoE, apoE-(141–155)m, was capable of inhibiting PDGF-directed smooth muscle cell migration. Peptide containing only a single unit of this sequence, apoE-(141–155), or the apoE-(211–243) peptide were ineffective in inhibiting PDGF-directed smooth muscle cell migration. Additional experiments showed that reductively methylated apoE, which is incapable of receptor binding yet retains its heparin binding capability, was equally effective as apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation. Additionally, the receptor binding domain-specific apoE antibody 1D7 also mitigated the anti-migratory properties of apoE on smooth muscle cells. Finally, pretreatment of cells with heparinase failed to abolish apoE inhibition of smooth muscle cell migration. Taken together, these data documented that apoE inhibition of PDGF-stimulated smooth muscle cell proliferation is mediated by its binding to heparan sulfate proteoglycans, while its inhibition of cell migration is mediated through apoE binding to cell surface receptors.

Research in the past two decades has clearly demonstrated that apolipoprotein (apo) E protects against vascular disease (1). Mice deficient in apoE emphasize the importance of apoE in protection against atherosclerosis. The apoE-deficient mice generated by gene targeting approach have plasma cholesterol levels of 60–85 mg/dl and do not develop atherosclerosis under normal conditions. Atherosclerotic lesions in mice with only one copy of the apoE gene (apoE+/−) were 10 times more severe than lesions in wild type mice that were fed a high fat/high cholesterol diet, despite relative similar cholesterol levels between the two groups of animals (326 versus 238 mg/dl). In these studies, a direct correlation between serum cholesterol levels and atherosclerotic lesion size was not observed. Additionally, transgenic expression of apoE in the arterial wall inhibited atheroma formation and severity without affecting plasma cholesterol level and lipoprotein profile in cholesterol-fed C57BL/6 mice (6). Taken together, the data showing an inverse relationship between apoE level and atherosclerotic lesion size, but a lack of correlation between total cholesterol and atherosclerosis lesion size, have led to the hypothesis that apoE may have direct impact on vascular occlusive diseases in a manner in addition to, and independent of, its property as a cholesterol-transporting apolipoprotein (7).

Recent data from our laboratory have indicated that apoE protection against vascular occlusive disease may be directly related to its modulation of vascular smooth muscle cell response to stimulation. We have shown that apoE inhibits platelet-derived growth factor (PDGF)- or oxidized LDL-induced smooth muscle cell migration and proliferation (8). ApoE inhibition of smooth muscle cell proliferation was shown to be due to cell cycle arrest resulting from the inhibition of growth factor-induced cyclin D1 activation (8). The mechanism of this inhibition appeared to be mediated through inducible nitric-oxide synthase signaling pathways (9). In contrast, apoE inhibition of growth factor-induced smooth muscle cell migration was independent of inducible nitric-oxide synthase activation (9). Thus, it is likely that apoE inhibition of smooth muscle cell migration is mediated through mechanism(s) distinct from the pathways involved with its anti-proliferative effects. The hypothesis that apoE inhibits growth factor-induced smooth muscle cell migration and proliferation via distinct mechanisms was further supported by data showing that low doses (0.1–5 μg/ml) of apoE were capable of inhibiting smooth muscle cell migration, while higher doses (25 and 50 μg/ml) were necessary for apoE inhibition of smooth muscle cell proliferation (9).

Apolipoprotein E binds to all members of the LDL receptor gene family as well as to heparan sulfate proteoglycans (HSPG) on the surface of mammalian cells (10–12). Recent studies from several laboratories showed that ligand binding to LDL receptor gene family members can mediate signal transduction events (13–19). Likewise, ligand binding to heparan sulfate proteoglycans may also induce signal transduction events (20–22). Smooth muscle cells are known to express HSPG as well as many receptors of the LDL receptor gene family, including the

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
LDL receptor, LRP, the VLDL receptor, and the apoE receptor-2 LR8 (10, 11, 23). The goal of the current study is to determine whether apoE inhibition of smooth muscle cell migration and proliferation is mediated through its interaction with cell surface receptors or with HSPG.

**EXPERIMENTAL PROCEDURES**

**Materials—**Type I collagenase, elastase, and heparinase III were obtained from Sigma. Dulbecco’s modified Eagles’ medium (DMEM), fetal bovine serum, and PDGF-BB were purchased from Life Technologies, Inc. [3H]Thymidine was obtained from PerkinElmer Life Sciences. Transwell polycarbonate membrane filters were purchased from Corning Costar Corp. (Cambridge, MA). Antibodies against smooth muscle-specific α-actin were obtained from Sigma.

**Human ApoE and ApoE Peptides—**Human apoE was isolated from fresh plasma by healthy volunteers by the method of Cardin et al. (24). The purity of apoE was assessed by SDS-polyacrylamide gel electrophoresis, and samples containing only a single band with Mr = 34,000 were used. Purified apoE was resuspended in phosphate-buffered saline and added directly to the culture medium without reconstitution with lipids. In selected experiments, lysine residues in apoE were modified by reductive methylation according to the procedure of Weisgraber et al. (25). The reductive methylated apoE was extensively dialyzed against phosphate-buffered saline and stored at 4 °C for no more than 1 week prior to use.

Peptides containing residues 141–155, heretofore designated as apoE-(141–155), or residues 211–243, designated as apoE-(211–243), were synthesized chemically by the Synpep Co. (Hopkinton, MA). The sequence of each peptide was verified by mass spectrometry. The lyophilized peptides were reconstituted in phosphate-buffered saline, dialyzed extensively against the same buffer, aliquoted in small quantities, and stored at −20 °C until use.

**Isolation of Primary Mouse Smooth Muscle Cells—**Mouse aortic smooth muscle cells were isolated using a modification of Mimura’s procedure (26). Briefly, thoracic aortas were dissected from eight mice and the adventitial tissue trimmed away. The aortas were then incubated in Hank’s solution containing 1 mg/ml collagenase and 3.3 units/ml elastase for 30 min at 37 °C. Remaining adventitial tissue was dissected away, and the remaining tissue was incubated in Hank’s solution containing collagenase (1 mg/ml) and elastase (3.3 units/ml) for 1 h at 37 °C. Cell clumps were dissociated by aspiration through a 10-ml pipette. The cell suspension was centrifuged at 150 × g for 5 min at room temperature and resuspended in 10 ml of DMEM containing 10% fetal bovine serum. The isolated cells were characterized as smooth muscle cells based on positive immunohistochemistry staining with anti-α-actin antibodies and by morphological characteristics similar to that observed with human smooth muscle cells. The primary smooth muscle cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells between passage 1 and 5 were used for experiments.

**Smooth Muscle Cell Migration Assay—**The migration of mouse vascular smooth muscle cells toward a PDGF-BB gradient was examined according to the procedure of Law et al. (27), as described previously (8, 9). Briefly, smooth muscle cells were made quiescent by incubation with DMEM and 0.4% fetal bovine serum for 48 h. Cells (2 × 10^5/ml) were incubated in solution with or without apoE for 30 min at 37 °C and 0.1-ml aliquots were added to the top chamber of tissue culture-treated Transwell polycarbonate membrane with 8-μm pores in 24-well plates. The lower Transwell compartment contained 0.6 ml of DMEM, 0.4% fetal bovine serum, and 0.2% bovine serum albumin, with or without 10 ng/ml PDGF-BB. After incubating for 4 h at 37 °C, the upper surface of the filters was washed with phosphate-buffered saline. The cells were then fixed with methanol for 10 min at 4 °C followed by hematoxylin staining. The number of cells that migrated to the lower surface of the filters was determined by counting the cells in six high power fields (×400). Data are presented as the percentage of cells that migrated to the lower surface as compared with PDGF treatment alone without apoE. All experiments were performed in triplicate and were repeated at least three times.

**Determination of Smooth Muscle Cell Proliferation—**Smooth muscle cell proliferation was measured based on the incorporation of [3H]thymidine into cellular DNA as described previously (8, 9). Briefly, cells were plated into 96-well plates at a density of 2.5 × 10^5 cells/well and allowed to attach for 24 h at 37 °C. Quiescence was induced by incubating the cells in DMEM containing 0.4% fetal bovine serum for 48 h at 37 °C, after which time the experiment was initiated. Test reagents, such as apoE or apoE peptides, were diluted in DMEM containing 0.4% fetal bovine serum, 0.2% bovine serum albumin, and 10 ng/ml PDGF-BB before adding to the cell culture. Cells incubated in medium without PDGF-BB served as control to determine basal [3H]thymidine incorporation into cellular DNA during the quiescence phase. Five hours prior to the end of the experiment, 1 μCi of [3H]thymidine was added to the culture medium. Cells were harvested onto filters using a cell harvester to determine [3H]thymidine incorporation 24 h after the addition of the test reagents. Radioactivity was quantified by liquid scintillation counting. Data shown are from an individual representative experiment. All experiments were performed with five replicates per experiment, and each experiment was repeated at least three times.

**RESULTS**

Initial experiments compared peptides containing either a single receptor binding domain sequence of apoE or a tandem repeat of the same sequence with a peptide containing the C-terminal heparin binding domain sequence of apoE for their inhibition of PDGF-induced smooth muscle cell migration and proliferation. While none of these peptides were cytotoxic, as determined by lactate dehydrogenase release, the incubation of smooth muscle cells with 3–12 μM concentration of any of these apoE peptides resulted in the suppression of PDGF-stimulated cell proliferation (Fig. 1). The apoE-(211–243) peptide was found to be the most effective in inhibiting smooth muscle cell proliferation, with complete abolishment of [3H]thymidine incorporation into cellular DNA observed at 12 μM. The apoE-(141–155) and apoE-(141–155)2 peptides were less active than the apoE-(211–243) peptide, with ~50% inhibition observed at 12 μM (Fig. 1).

The effect of the apoE peptides on smooth muscle cell migration toward PDGF was dramatically different from that observed for their inhibition of cell proliferation. The peptide with the tandem repeating sequence of the receptor binding domain, apoE-(141–155)2, inhibited PDGF-directed smooth muscle cell migration when used at a concentration greater than 0.75 μM (Fig. 2). Thus, the effectiveness of the apoE-(141–155)2 peptide in inhibiting smooth muscle cell migration was similar to that observed for intact apoE (Fig. 2 and Ref. 9). In contrast, the peptide containing only a single copy of the receptor binding domain of apoE, apoE-(141–155), and the peptide containing
ApoE Inhibition of Cell Migration and Proliferation

importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by taking advantage of previous observations that reductive methylation of apoE abolishes its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).

Previous studies have shown that both the receptor binding domain and the domain between residues 211 and 243 are capable of heparin binding (28, 29). Moreover, the receptor binding properties of apoE require multiple copies of the receptor binding domain (30, 31). Thus, the differential effects of the apoE peptides on PDGF-stimulated smooth muscle cell proliferation and migration suggest that apoE inhibition of smooth muscle cell migration may be mediated by its interaction with cell surface receptors, while its inhibition of cell proliferation requires its interaction with HSPG. This hypothesis was examined by taking advantage of previous observations that reductive methylation of lysine residues in apoE abolished its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The effect of apoE peptides on PDGF-directed smooth muscle cell proliferation was determined as the percent of cells that migrated toward PDGF-BB in the absence of apoE. The data represent mean ± S.E. from three samples in a single representative experiment. Each experiment was repeated at least three times with similar results.

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).

Previous studies have shown that both the receptor binding domain and the domain between residues 211 and 243 are capable of heparin binding (28, 29). Moreover, the receptor binding properties of apoE require multiple copies of the receptor binding domain (30, 31). Thus, the differential effects of the apoE peptides on PDGF-stimulated smooth muscle cell proliferation and migration suggest that apoE inhibition of smooth muscle cell migration may be mediated by its interaction with cell surface receptors, while its inhibition of cell proliferation requires its interaction with HSPG. This hypothesis was examined by taking advantage of previous observations that reductive methylation of lysine residues in apoE abolished its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).

Previous studies have shown that both the receptor binding domain and the domain between residues 211 and 243 are capable of heparin binding (28, 29). Moreover, the receptor binding properties of apoE require multiple copies of the receptor binding domain (30, 31). Thus, the differential effects of the apoE peptides on PDGF-stimulated smooth muscle cell proliferation and migration suggest that apoE inhibition of smooth muscle cell migration may be mediated by its interaction with cell surface receptors, while its inhibition of cell proliferation requires its interaction with HSPG. This hypothesis was examined by taking advantage of previous observations that reductive methylation of lysine residues in apoE abolished its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).

Previous studies have shown that both the receptor binding domain and the domain between residues 211 and 243 are capable of heparin binding (28, 29). Moreover, the receptor binding properties of apoE require multiple copies of the receptor binding domain (30, 31). Thus, the differential effects of the apoE peptides on PDGF-stimulated smooth muscle cell proliferation and migration suggest that apoE inhibition of smooth muscle cell migration may be mediated by its interaction with cell surface receptors, while its inhibition of cell proliferation requires its interaction with HSPG. This hypothesis was examined by taking advantage of previous observations that reductive methylation of lysine residues in apoE abolished its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).

Previous studies have shown that both the receptor binding domain and the domain between residues 211 and 243 are capable of heparin binding (28, 29). Moreover, the receptor binding properties of apoE require multiple copies of the receptor binding domain (30, 31). Thus, the differential effects of the apoE peptides on PDGF-stimulated smooth muscle cell proliferation and migration suggest that apoE inhibition of smooth muscle cell migration may be mediated by its interaction with cell surface receptors, while its inhibition of cell proliferation requires its interaction with HSPG. This hypothesis was examined by taking advantage of previous observations that reductive methylation of lysine residues in apoE abolished its ability to bind receptors (25, 32), without interfering with its heparin binding properties (25, 33). In the current experiments, reductively methylated apoE was found to be equally effective as native apoE in inhibiting PDGF-stimulated smooth muscle cell proliferation, with complete inhibition observed at 1.5 μM (Table I). In contrast to the results observed on apoE inhibition of cell proliferation, reductive methylation of apoE abolished its ability to inhibit smooth muscle cell migration toward PDGF (Fig. 4).

The importance of the receptor binding domain in mediating the anti-migratory properties of apoE was further tested by determining the effect of the receptor binding domain-specific apoE antibody 1D7 (34, 35) on PDGF-induced smooth muscle cell migration. Results showed that the 1D7 antibody alleviated the apoE inhibition of smooth muscle cell migration toward PDGF (Fig. 5). Importantly, 1D7 at similar concentrations did not alleviate apoE inhibition of smooth muscle cell proliferation (data not shown).

Additional experiments were performed to further test the hypothesis that apoE inhibition of smooth muscle cell migration is independent of HSPG binding. In these experiments, only the C-terminal heparin binding domain sequence, apoE-(211–243), were ineffective in inhibiting PDGF-directed smooth muscle cell migration even when higher concentrations were used (Fig. 3).
The interactive role of apoE and HSPG in the modulation of cell functions has been proposed previously. Obunike et al. showed that endogenously expressed apoE enhances cellular proteoglycan synthesis. Exogenously added apoE also yielded a 2-fold induction of proteoglycan synthesis and secretion by smooth muscle cells (38, 39). The latter study further demonstrated a correlation between apoE inhibition of cell proliferation and its induction of HSPG synthesis. Anti-perlecan antibody was also shown to completely abrogate the anti-proliferative effect of apoE (38). Based on these observations, these investigators proposed that apoE, like other anti-proliferative agents such as transforming growth factor-β (40) and heparin (41), inhibits smooth muscle cell proliferation by promoting the synthesis and secretion of perlecan (38). However, the mechanism by which apoE stimulates perlecan synthesis and the cell surface molecules responsible for these apoE effects have not been determined. The current study showed that apoE inhibits smooth muscle cell proliferation via binding to cell surface HSPG. Thus, it is likely that apoE-HSPG interaction induces intracellular signals required for activation of perlecan synthesis and its subsequent inhibition of cell proliferation. The observation that reductively methylated apoE, which does not bind to receptors but remains active in HSPG binding, was capable of inhibiting smooth muscle cell proliferation is supportive of this conclusion.

In contrast to their effects on cell proliferation, peptides containing either a single copy of the receptor binding domain or the distal heparin binding domain of apoE did not inhibit PDGF-directed smooth muscle cell migration. Only the peptide containing a tandem repeating sequence of the receptor binding domain of apoE displayed anti-migratory effects. In fact, the apoE(141–155) peptide was equally as active as native apoE in inhibiting PDGF-directed smooth muscle cell migration. Moreover, our results also showed that reductive methylation of apoE, which abolished its receptor, but not its heparin, binding activities (25, 33), also abolished the anti-migratory, effects of apoE. These results, during observations that reductively methylated apoE, which does not bind to receptors but remains active in HSPG binding, was capable of inhibiting smooth muscle cell proliferation is supportive of this conclusion.

In contrast to their effects on cell proliferation, peptides containing either a single copy of the receptor binding domain or the distal heparin binding domain of apoE did not inhibit PDGF-directed smooth muscle cell migration. Only the peptide containing a tandem repeating sequence of the receptor binding domain of apoE displayed anti-migratory effects. In fact, the apoE(141–155) peptide was equally as active as native apoE in inhibiting PDGF-directed smooth muscle cell migration. Moreover, our results also showed that reductive methylation of apoE, which abolished its receptor, but not its heparin, binding activities (25, 33), also abolished the anti-migratory, but not the anti-proliferative, effects of apoE. These results, during observations that reductively methylated apoE, which does not bind to receptors but remains active in HSPG binding, was capable of inhibiting smooth muscle cell proliferation is supportive of this conclusion.

Fig. 4. Effects of reductive methylation on the ability of apoE to inhibit PDGF-BB-directed smooth muscle cell migration. Quiescent smooth muscle cells were incubated with apoE (closed bars) or reductively methylated apoE (open bars) for 30 min at 37 °C before adding to the top chamber of Transwell membranes in 24-well dishes at a density of 2 × 10^4 cells/well. Cells that migrated toward the lower chamber of the Transwells, which contained basal medium with PDGF-BB (10 ng/ml), were determined after a 4-h incubation period. Basal migration was determined in the absence of PDGF-BB. Maximum stimulation was determined by incubating cells in the absence of apoE pretreatment. Data represent the mean ± S.E. of triplicate samples from a representative experiment. The experiment was repeated at least three times with similar results.

Fig. 5. Antibody inhibition of apoE effects on PDGF-directed smooth muscle cell migration. Serum-starved mouse smooth muscle cells were preincubated in the presence or absence of apoE and its receptor binding domain-specific antibody 1D7 for 30 min at 37 °C prior to addition to the top chamber of Transwell membranes in 24-well plates at a density of 2 × 10^4 cells/well. Cells were allowed to migrate toward the PDGF-BB (10 ng/ml) in the lower chamber for 4 h at 37 °C. Cells that migrated to the lower surface of the membrane were counted and expressed as the percent of cells that migrated toward PDGF in the lower chamber in the absence of apoE or heparinase pretreatment. Data represent the mean ± S.E. of triplicate determinations in two separate experiments.

The interactive role of apoE and HSPG in the modulation of cell functions has been proposed previously. Obunike et al. showed that endogenously expressed apoE enhances cellular proteoglycan synthesis. Exogenously added apoE also yielded a 2-fold induction of proteoglycan synthesis and secretion by smooth muscle cells (38, 39). The latter study further demonstrated a correlation between apoE inhibition of cell proliferation and its induction of HSPG synthesis. Anti-perlecan antibody was also shown to completely abrogate the anti-proliferative effect of apoE (38). Based on these observations, these investigators proposed that apoE, like other anti-proliferative agents such as transforming growth factor-β (40) and heparin (41), inhibits smooth muscle cell proliferation by promoting the synthesis and secretion of perlecan (38). However, the mechanism by which apoE stimulates perlecan synthesis and the cell surface molecules responsible for these apoE effects have not been determined. The current study showed that apoE inhibits smooth muscle cell proliferation via binding to cell surface HSPG. Thus, it is likely that apoE-HSPG interaction induces intracellular signals required for activation of perlecan synthesis and its subsequent inhibition of cell proliferation. The observation that reductively methylated apoE, which does not bind to receptors but remains active in HSPG binding, was capable of inhibiting smooth muscle cell proliferation is supportive of this conclusion.

In contrast to their effects on cell proliferation, peptides containing either a single copy of the receptor binding domain or the distal heparin binding domain of apoE did not inhibit PDGF-directed smooth muscle cell migration. Only the peptide containing a tandem repeating sequence of the receptor binding domain of apoE displayed anti-migratory effects. In fact, the apoE(141–155) peptide was equally as active as native apoE in inhibiting PDGF-directed smooth muscle cell migration. Moreover, our results also showed that reductive methylation of apoE, which abolished its receptor, but not its heparin, binding activities (25, 33), also abolished the anti-migratory, but not the anti-proliferative, effects of apoE. These results,
taken together with observations showing the receptor domain-specific apoE antibody abolished the anti-migratory properties of apoE, indicated that apoE inhibition of smooth muscle migration is mediated via its binding to cell surface receptors and is independent of HSPG interaction.

The mechanism by which apoE interaction with receptors results in inhibition of cell migration remains unknown at this time. However, there is increasing evidence to suggest that apoE interaction with LDL receptor gene family member proteins may lead to signal transduction events that can modulate cell functions. For example, apoE binding to apoE receptor-2 (LR8) on platelets has been shown to induce nitric oxide synthesis and inhibit agonist-induced platelet aggregation (42, 43). Interestingly, LR8 contains a Src homology 3 (SH3) consensus sequence, as well as domains that can serve as targets for cGMP and cAMP-dependent protein kinases. Thus, it is possible that apoE inhibits platelet aggregation and induction of nitric oxide synthesis through LR8-mediated signal transduction events. This receptor is also present in the brain and has been shown to be important for reelin signaling and activation of the c-Jun N-terminal kinase-signaling pathway (14, 15, 44). Likewise, both the VLDL receptor and LR8 have been shown to interact with cytosolic adaptor and scaffold proteins and modulate the transmission of extracellular signals to activate intracellular tyrosine kinases (16). Since LR8, VLDL receptor, and LR8 are all present on the surface of smooth muscle cells, it is possible that apoE inhibition of smooth muscle cell migration is mediated through signaling events subsequent to apoE binding to one or more of these receptors. The identification of the receptor responsible for mediating apoE inhibition of smooth muscle cell migration will contribute to our understanding in this regard.

Regardless of the exact mechanisms involved, our data documented that the anti-proliferative and anti-migratory effects of apoE are distinct, requiring different concentrations of apoE (9), and are mediated through its interaction with HSPG and cell surface receptors, respectively. A concentration-dependent effect of apoE on cell functions has been reported previously in studies examining its role on regulation of androgen synthesis by the ovary. Dyer and Curtiss (45) showed that low concentrations of apoE promoted androgen synthesis, while high concentrations of apoE were inhibitory. Subsequent studies revealed that the tandem repeat peptide apoE(141–155) mimicked the effect of intact apoE, but the single-copy peptide encompassing apoE residues 129–162 did not stimulate androgen production but rather inhibited androgen synthesis at high concentrations (46). These authors suggested that apoE stimulation of androgen production may be mediated through apoE binding to members of the LDL receptor gene family (46) and that inhibition of androgen synthesis at high apoE concentrations is mediated by a mechanism independent of any LDL receptor-related proteins. Although the exact mechanism by which apoE modulates androgen production in ovaries has not been identified, it is possible that this cell regulatory function of apoE is similar to that observed here, i.e., through processes mediated by distinct cell signaling mechanisms as a consequence of apoE interaction with receptors and HSPG (8, 9).

In summary, this study adds to the expanding literature documenting a direct role of apoE interaction with receptors and HSPG in signal transduction and modulation of cell functions. The current study also documented that apoE interaction with receptors and HSPG modulates distinct cell functions, i.e., inhibition of cell migration and proliferation, possibly through triggering of different signal transduction pathways. Thus, apoE may protect against vascular occlusive diseases by multiple mechanisms, including reverse cholesterol transport and inhibition of smooth muscle cell migration and proliferation. We have previously shown that the apoE level in circulation can directly influence neointimal hyperplasia in response to vascular injury, a process that is independent of lipid transport (47). Accordingly, designing therapeutic agents that can increase the apoE level in circulation may be beneficial in combating vascular disease via multiple mechanisms.
ApoE Inhibition of Cell Migration and Proliferation

40. Iozzo, R. V., Pillarisetti, J., Sharma, B., Murdoch, A. D., Danielson, K. G., Uitto, J., and Mauviel, A. (1997) J. Biol. Chem. 272, 5219–5228
41. Weiser, M. C., Belknap, J. K., Grieshaber, S. S., Kinsella, M. G., and Majack, R. A. (1996) Matrix Biol. 15, 331–340
42. Riddell, D. R., Graham, A., and Owen, J. S. (1997) J. Biol. Chem. 272, 89–95
43. Riddell, D. R., Vinogradov, D. V., Stannard, A. K., Chadwick, N., and Owen, J. S. (1999) J. Lipid Res. 40, 1925–1930
44. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimph, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999) Cell 97, 689–701
45. Dyer, C. A., and Curtiss, L. K. (1988) J. Biol. Chem. 263, 10965–10973
46. Zerbinatti, C. V., and Dyer, C. A. (1999) Biol. Reprod. 61, 665–672
47. Zhu, B., Kuhel, D. G., Witte, D. P., and Hui, D. Y. (2000) Am. J. Pathol. 157, 1839–1848