Distinct Apolipoprotein E Isoform Preference for Inhibition of Smooth Muscle Cell Migration and Proliferation†

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ABSTRACT: The current study compared the effectiveness of the various human apolipoprotein E (apoE) isoforms in inhibiting platelet-derived growth factor- (PDGF-) stimulated smooth muscle cell proliferation and migration. The incubation of primary mouse aortic smooth muscle cells with apoE3 resulted in dose-dependent inhibition of smooth muscle cells stimulated by 10 ng/mL PDGF. Greater than 50% inhibition of smooth muscle cell proliferation was observed at 15 μg/mL of human apoE3. Human apoE2 was less effective, requiring a higher concentration to achieve inhibition comparable to that of apoE3. Human apoE4 was the least effective of the apoE isoforms with no significant inhibition of cell proliferation observed at concentrations up to 15 μg/mL. Interestingly, apoE inhibition of PDGF-directed smooth muscle cell migration did not show preference for any apoE isoforms. Human apoE2, apoE3, and apoE4 were equally effective in inhibiting smooth muscle cell migration toward PDGF. These results are consistent with previous data showing that apoE inhibition of smooth muscle cell proliferation is mediated through its binding to heparan sulfate proteoglycans, whereas its inhibition of cell migration is mediated via binding to the low-density lipoprotein receptor related protein. The low efficiency of apoE4 to inhibit smooth muscle cell proliferation also suggested another mechanism to explain the association between the apolipoprotein ϵ4 allele with increased risk of coronary artery disease.

Apolipoprotein E (apoE),[1] a 34 kDa protein associated with triglyceride-rich lipoproteins and HDL, has long been recognized as a major determinant of individual susceptibility to coronary artery disease (*1*, *2*). The most direct mechanism by which apoE influences atherosclerosis is its ability to reduce plasma cholesterol level by mediating the hepatic clearance of remnant lipoproteins and cholesterol-enriched HDL (*3*). This process is initiated by apoE binding to receptors and heparan sulfate proteoglycans on liver cell surface (*3*). Recent evidence suggested that apoE may also protect against coronary occlusive diseases by mechanisms independent of its ability to modulate plasma lipid levels (*4*). The latter hypothesis was suggested initially by observations that transgenic expression of apoE in the arterial wall significantly inhibited atheroma formation without affecting plasma cholesterol level and lipoprotein profile in cholesterol-fed mice (*5*). Subsequent studies showed that low levels of apoE secreted by the adrenal gland can also inhibit atherosclerosis without correcting for hypercholesterolemia in apoE-deficient mice (*6*).

The precise mechanism by which apoE can suppress atheroma formation without reducing plasma cholesterol level is still under investigation. However, our studies with a mechanically induced endothelial denudation model suggested that one mechanism is related to a direct apoE effect on the vessel wall. In this arterial injury model, apoE was shown to inhibit smooth muscle cell migration from the media to the intima and their subsequent proliferation in response to growth factors present in the serum (*7*). ApoE inhibition of smooth muscle cell migration is mediated by signaling pathways conferred as a consequence of its binding to LRP (*8*−*10*). In contrast, apoE inhibition of smooth muscle cell proliferation appears to be mediated through signaling pathways resulting from apoE interaction with heparan sulfate proteoglycans (*8*, *9*, *11*, *12*).

The human apoE gene is polymorphic with three common alleles encoding the different isoforms, apoE2, E3, and E4, each differing in primary structure at two amino acid residues (*13*−*15*). The most common isoform, apoE3, contains a cysteine at residue 112 and an arginine at residue 158, whereas the apoE2 and apoE4 variants contain cysteines and arginines at both positions, respectively (*15*). This difference in structure results in the apoE isoform-specific difference in lipoprotein metabolism and risk for premature atherosclerosis (*16*, *17*). Whether there is also an apoE isoform preference in suppression of smooth muscle cell migration and proliferation remains unknown. Interestingly, a recent study showed that, after adjustment for all risk factors including plasma lipid levels, subjects with the apoE4 isotype remain at a significantly higher risk for coronary artery disease (*2*). These latest findings suggested that the apoE

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[1] Abbreviations: apoE, apolipoprotein E; PDGF, platelet-derived growth factor; HDL, high-density lipoproteins; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium.

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genotype may also influence other parameters of coronary occlusive diseases in addition to its impact on cholesterol transport. The current study was undertaken to examine the effectiveness of various human apoE isoforms on growth factor-induced smooth muscle cell migration and proliferation.

EXPERIMENTAL PROCEDURES

Recombinant ApoE. Recombinant human apoE2, apoE3, and apoE4 were obtained from bacteria harboring the respective pET32 plasmids encoding the mature forms of the apoE isoforms as a fusion protein with thioredoxin separated by a His tag domain (*18*). The fusion proteins were isolated by affinity chromatography on His·Bind resin (Novagen) and then dialyzed against 20 mM ammonium bicarbonate. The mature forms of apoE2, apoE3, and apoE4 with a Gly-Ser dipeptide addition at the amino terminus were obtained by thrombin digestion of the fusion proteins after their reconstitution in dimyristoylphosphatidylcholine vesicles as described (*18*). The mature apoE isoforms were delipidated and lyophilized until use. Purified apoE isoforms were resuspended in phosphate-buffered saline and used immediately without reconstitution with lipids.

Smooth Muscle Cell Proliferation and Migration. Primary mouse smooth muscle cells were isolated from the aorta of C57BL/6 mice using a modification of the procedure of Mimura et al. (*19*), as described previously (*12*). The primary aortic smooth muscle cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Cells between passages 1 and 5 were used for experiments.

Cell quiescence was induced by incubation in DMEM containing 0.4% fetal bovine serum for 48 h at 37 °C prior to experiments. ApoE inhibition of PDGF-stimulated smooth muscle cell proliferation was evaluated on the basis of incorporation of BrdU into cellular DNA after quiescent smooth muscle cells were incubated with 10 ng/mL PDGF−BB and various concentrations of apoE as previously described (*8*, *12*). The effect of apoE on smooth muscle cell migration was determined by preincubating the cells with apoE for 30 min at 37 °C prior to their addition to the top chamber of a tissue-cultured Transwell polycarbonate membrane with 8 μm pores in 24-well plates. Cell migration was assessed by the number of cells that migrated to the bottom surface of the filter toward the lower Transwell chamber containing 10 ng/mL PDGF−BB. All experiments were performed in triplicate and were repeated at least five times with different apoE and smooth muscle cell preparations.

RESULTS

The impact of various apoE isoforms on smooth muscle cell proliferation was assessed by incubating quiescent primary mouse smooth muscle cells with PDGF in the presence or absence of increasing concentrations of recombinant human apoE2, apoE3, or apoE4. Cell proliferation was determined after 24 h on the basis of the incorporation of BrdU into cellular DNA. Results showed that apoE3 was the most effective isoform in inhibition of PDGF-stimulated smooth muscle cell proliferation. Significant inhibition of PDGF-stimulated smooth muscle cell proliferation was observed at an apoE3 concentration of 7.5 μg/mL, increasing

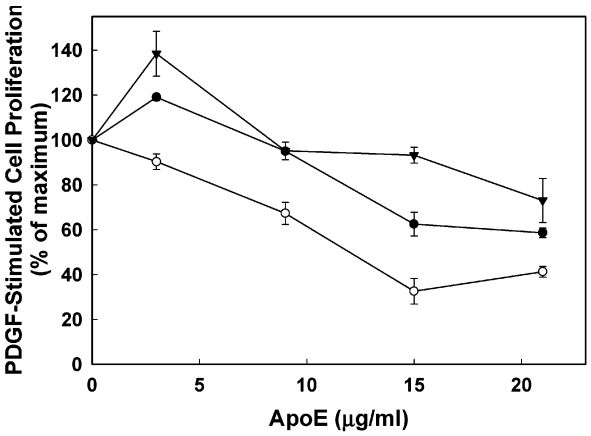

FIGURE 1: ApoE inhibition of PDGF-stimulated smooth muscle cell proliferation. Serum-starved mouse smooth muscle cells were incubated for 24 h in 96-well plates (2.5×10^3 cells/well) in the presence of 10 ng/mL PDGF−BB and recombinant human apoE2 (filled circles), apoE3 (open circles), or apoE4 (filled triangles). Ten microliters of a 10 mM BrdU solution was added 5 h prior to the end of the experiments. Cell proliferation was determined on the basis of the incorporation of BrdU into cellular DNA as determined by the BrdU cell proliferation ELISA kit from Roche diagnostics. Maximum stimulation, determined by PDGF-induced BrdU incorporation into cellular DNA in the absence of apoE, ranged from 2.5- to 4-fold above background level in five different experiments. The data are the mean ± SD from triplicate assays in five different experiments.

to >50% inhibition observed at concentrations higher than 15 μg/mL (Figure 1). The human apoE2 isoform was less effective, with no significant inhibition of cell proliferation observed at 7.5 μg/mL. However, higher concentrations of apoE2 were found to partially inhibit PDGF-stimulated smooth muscle cell proliferation, with a maximum level of approximately 40% inibition achieved at concentrations >15 μg/mL (Figure 1). The apoE4 variant was the least effective of the apoE isoforms in inhibiting PDGF-stimulated cell proliferation. No significant inhibition of smooth muscle cell proliferation was observed with apoE4 at concentrations up to 15 μg/mL, with only a marginal 20% inhibition observed at 22 μg/mL (Figure 1). At higher concentrations (~50 μg/mL), all three apoE isoforms effectively inhibited PDGF-stimulated smooth muscle cell proliferation by >85% (data not shown).

The next set of experiments compared the effectiveness of the various apoE isoforms in inhibition of smooth muscle cell migration toward PDGF. In view of previous studies showing that low concentrations of apoE were sufficient to inhibit smooth muscle cell migration in comparison to its inhibition of cell proliferation (*9*), a concentration range of 0−3 μg/mL was used for the current experiments. Results showed no difference among the various apoE isoforms, at all concentrations tested, in their ability to inhibit smooth muscle cell migration toward PDGF (Figure 2). Approximately 50% inhibition of PDGF-stimulated smooth muscle cell migration was achieved at <1 μg/mL apoE2, apoE3, and apoE4. Complete inhibition was achieved with a 3 μg/mL quantity of any one of the three common apoE isoforms (Figure 2).

DISCUSSION

Receptors on the smooth muscle cell surface capable of binding apoE include the LDL receptor, LRP, and heparan

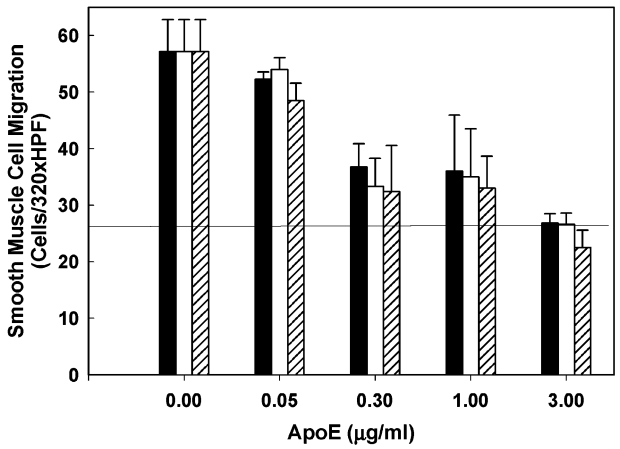

FIGURE 2: ApoE inhibition of PDGF-directed smooth muscle cell migration. Quiescent smooth muscle cells were incubated with recombinant human apoE2 (solid bars), apoE3 (open bars), or apoE4 (hatched bars) for 30 min at 37 °C prior to their addition to the top chamber of Transwell membranes in 24-well 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 10 ng/mL PDGF−BB, were determined after a 4 h incubation period. The horizontal line at 26 cells/320 × HPF represents the basal level of smooth muscle cells that migrated to the bottom chamber in the absence of PDGF. The data represent the mean ± SD of triplicate samples from five different experiments.

sulfate proteoglycans. Previous studies have shown that binding to the LDL receptor requires apoE reconstitution with lipids into apoE−lipid complexes (*20*). However, the requirement for lipid reconstitution for apoE interaction with LRP has been controversial. Although most studies exploring apoE interaction with LRP have utilized apoE-enriched β-VLDL, lipid-free apoE was found to behave similarly as lipid-reconstituted apoE in inducing LRP-mediated neuronal apoptosis (*21*). Lipid-free apoE was also found to interact with LRP in solid-phase binding assays and in ligand blots (*22*, *23*). In addition, LRP-mediated cellular uptake and degradation of lipid-free apoE have also been reported (*24*). In the current study, we showed that lipid-free apoE2, apoE3, and apoE4 were all effective in inhibiting PDGF-stimulated smooth muscle cell migration. In previous studies using antibodies directed against LRP as well as smooth muscle cells lacking in LRP expression, we demonstrated that this apoE inhibitory activity is mediated through its interaction with LRP (*10*). Taken together, our data support the hypothesis that lipid-free apoE is capable of binding LRP and that this interaction does not show apoE isoform preferences. The lack of isoform preference in apoE interaction with LRP is also consistent with previous reports showing similar ability of apoE2, apoE3, and apoE4 in enhancing β-VLDL binding to LRP (*25*, *26*).

The ability of lipid-free apoE in binding LRP and inhibiting smooth muscle cell migration is clearly different from the requirement of lipid reconstitution for apoE binding to the LDL receptor. Interestingly, the apoE monoclonal antibody 1D7, with epitope localized to residues 140−150 of apoE (*27*), was capable of suppressing lipid-free apoE inhibition of smooth muscle cell migration (*12*) as well as apoE−lipid complex binding to the LDL receptor (*27*). This latter observation suggested that both processes are mediated by the same receptor binding domain of apoE. Thus, the different ability between LDL receptor and LRP to interact with lipid-free apoE may be due to a structural difference in the ligand binding domains of these two receptors. The high-affinity binding of apoE-containing lipoproteins to the LDL receptor requires multiple interactions between each LDL receptor with multiple apoE in each lipoprotein particle (*28*, *29*). Accordingly, lipid-free apoE is not capable of this high-affinity interaction, whereas apoE's reconstituted into lipid complexes containing multiple apoE's per particle are amenable to this high-affinity interaction (*29*). The defective LDL receptor binding activity of apoE2 is due to the arginine-to-cysteine substitution at residue 158, causing a different salt bridge arrangement that results in conformational constraint on the receptor binding domain when the apolipoprotein is embedded in a lipid−protein complex (*30*, *31*). Lipid-free apoE2 does not suffer from this conformational constraint, and thus the receptor binding domain may be accessible for interaction with LRP. The similar efficiency between lipid-free apoE2, apoE3, and apoE4 in interacting with the receptor binding domain-specific apoE monoclonal antibody (*27*) and in inhibiting smooth muscle cell migration is supportive of this hypothesis.

The current study also revealed that apoE2 and apoE3 were more effective than apoE4 in inhibiting PDGF-stimulated smooth muscle cell proliferation. We have shown previously that apoE inhibition of smooth muscle cell proliferation is mediated through its binding to heparan sulfate proteoglycans (*12*). Other work revealed that heparan sulfate proteoglycans do not discriminate between apoE2, apoE3, and apoE4 in binding characteristics (*32*). However, heparan sulfate proteoglycan-mediated intracellular accumulation of apoE was much lower for apoE4 than either apoE2 or apoE3 (*33*). Whether the differential apoE isoform effects on inhibition of smooth muscle cell proliferation are related to the intracellular accumulation of this apolipoprotein remains to be determined.

The lower efficiency of apoE4, in comparison with apoE2 and apoE3, to suppress growth factor-induced smooth muscle cell proliferation adds to a growing list of mechanisms by which inheritance of the apolipoprotein ϵ4 allele contributes to higher risk of a number of diseases (*34*). Previous studies have shown that the ϵ4 allele is associated with Alzheimer's disease (*35*). One mechanism by which apoE4 contributes to Alzheimer's disease is its inhibition of neurite outgrowth and depolymerization of microtubules (*36*). As stated previously, the apolipoprotein ϵ4 allele is also associated with significantly greater risk of cardiovascular disease (*2*, *16*, *17*). One mechanism by which apoE4 contributes to cardiovascular risk can be explained by its assocation with elevated plasma cholesterol level (*17*). However, the association of the apolipoprotein ϵ4 allele with cardiovascular risk remains highly significant even after adjustment for cholesterol level was made (*2*). The apolipoprotein ϵ4 allele is also associated with intima-media thickening of the carotid arteries (*37*), ischemic stroke of the large vessel (*38*), and restenosis after coronary angioplasty (*39*, *40*). The association of the ϵ4 allele with these disorders is also evident even after adjustments for other risk factors including plasma and LDL cholsterol levels were made. Since intimal smooth muscle cell hyperplasia is a hallmark of these vascular diseases, the inefficiency of apoE4, in comparison with apoE2 and apoE3, in inhibiting growth factor-induced smooth muscle cell proliferation may partially account for its increased risk of these

diseases. Finally, apoE has been reported to inhibit proliferation of a number of different cell types in addition to the smooth muscle cells (*41*). The association of the ϵ4 genotype with breast cancer risk reported recently (*42*) may be attributed to the reduced efficiency of apoE4 in inhibiting cell growth.

The current study utilized lipid-free apoE at concentrations of 0−3 μg/mL and 0−20 μg/mL to explore the potential difference among the apoE isoforms in inhibiting smooth muscle cell migration and proliferation, respectively. These apoE concentrations are within the normal physiological concentration of 20−50 μg/mL apoE in human plasma (*16*). Although most, if not all, of the apoE in circulation is associated with lipoproteins, apoE is also synthesized and secreted locally in the vessel wall by smooth muscle cells (*43*−*45*). The secreted apoE may be retained by the extracellular matrix at the cell surface similar to that observed in the liver (*46*, *47*). Whether apoE protection against smooth muscle migration and proliferation in vivo (*7*, *48*) is due to inhibition by the locally derived lipid-free apoE or by circulating apoE associated with lipoproteins remains to be determined.

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