Perlecan Mediates the Antiproliferative Effect of Apolipoprotein E on Smooth Muscle Cells

AN UNDERLYING MECHANISM FOR THE MODULATION OF SMOOTH MUSCLE CELL GROWTH?*

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Apolipoprotein E (apoE) is known to inhibit cell proliferation; however, the mechanism of this inhibition is not clear. We recently showed that apoE stimulates endothelial production of heparan sulfate (HS) enriched in heparin-like sequences. Because heparin and HS are potent inhibitors of smooth muscle cell (SMC) proliferation, in this study we determined apoE effects on SMC HS production and cell growth. In confluent SMCs, apoE (10 μg/ml) increased 35SO4 incorporation into PG in media by 25–30%. The increase in the medium was exclusively due to an increase in HSPGs (2.2-fold), and apoE did not alter chondroitin and dermatan sulfate proteoglycans. In proliferating SMCs, apoE inhibited [3H]thymidine incorporation into DNA by 50%; however, despite decreasing cell number, apoE increased the ratio of 35SO4 to [3H]thymidine from 2 to 3.6, suggesting increased HS per cell. Purified HSPGs from apoE-stimulated cells inhibited cell proliferation in the absence of apoE. ApoE did not inhibit proliferation of endothelial cells, which are resistant to heparin inhibition. Analysis of the conditioned medium from apoE-stimulated cells revealed that the HS PG increase was in perlecan and that apoE also stimulated perlecan mRNA expression by 2-fold. The ability of apoE isoforms to inhibit cell proliferation correlated with their ability to stimulate perlecan expression. An anti-perlecan antibody completely abrogated the antiproliferative effect of apoE. Therefore, these data show that perlecan is a potent inhibitor of SMC proliferation and is required to mediate the antiproliferative effect of apoE. Because other growth modulators also regulate perlecan expression, this may be a key pathway in the regulation of SMC growth.

Apoptosis is a key ligand for several lipoprotein receptors and plays a major role in the hepatic clearance of remnant lipoproteins (1, 2). In recent years, however, several nontraditional functions of apoE have emerged that are related either to its antiatherogenic function or its role in Alzheimer’s disease (3–9). For example, expression of apoE in the vessel decreased atherosclerosis in apoE-null mice without significant changes in plasma lipoproteins (4, 5). Recently, Fazio et al. (5), by transplanting apoE null macrophages into normal C57BL6 mice, increased atherosclerosis without altering lipoprotein profile. How apoE protects the vessel wall from atherogenesis is not clear. Possible antiatherogenic roles of vascular apoE include promotion of reverse cholesterol transport (6), inhibition of lipoprotein oxidation (7), inhibition of lipase-mediated low density lipoprotein retention (8), inhibition of platelet aggregation (9), inhibition of smooth muscle cell (SMC) proliferation (10), and, as we recently showed, increasing endothelial heparan sulfate (HS) (11).

Heparin and HS are biologically active glycosaminoglycans (GAG) composed of alternating residues of uronic acid (glucuronic acid in HS and iduronic acid in heparin) and glucosamine (12). HSPGs have several vasoprotective effects; the best characterized among these is their ability to inhibit SMC proliferation (13–15). Although the antiproliferative effect of apoE has been realized for many years (16–18, 10), how apoE inhibits cell proliferation is not known. Our previous studies showed that apoE increased HSPG production in endothelial cells (11). Thus, it raises the possibility that apoE-mediated inhibition of SMC proliferation may be due to its ability to induce HS production in cells. In the present study, we show that apoE stimulates SMC production of perlecan HSPGs, which mediates the antiproliferative activity of apoE.

MATERIALS AND METHODS

Heparinase I and heparitinase (heparinase III) and chondroitin ABC lyase were purchased from Seikagaku America Inc. (Bethesda, MD). Aqueous solutions of [35S]sulfate were from Amersham Pharmacia Biotech. [3H]Leucine and [3H]glucosamine were from NEN Life Science Products. ApoE3 was either purchased from Calbiochem (La Jolla, CA) or purified from conditioned medium of Chinese hamster ovary cells transfected with apoE cDNA (19) by heparin-agarose chromatography. ApoE2 and apoE4 isoforms were from Calbiochem. Perlecan antibody was from Zymed Laboratories Inc. (South San Francisco, CA).

Cells—Rat and human aortic SMCs were kindly provided by Dr. L. Rabbani (Department of Medicine, Columbia University) (20). Data with rat SMCs are presented below. Initial experiments were also performed with human SMCs, and similar results were obtained. SMCs were grown in basal medium supplemented with growth factors, basic fibroblast growth factor and epidermal growth factor (Clonetics, San smooth muscle cell; GAG, glycosaminoglycans; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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Diego, CA. Bovine aortic endothelial cells were isolated and cultured as described (21). The cells (5–15 passages) were grown in minimal essential medium containing 10% fetal bovine serum (Life Technologies, Inc.).

**Metabolic Labeling**—PGs were radiolabeled with either [35S]sulfate or [3H]leucine for the indicated time periods. Medium PGs were collected and purified by DEAE-cellulose chromatography (see below). Cell-associated PGs were assessed by extracting cells with 50 mM Tris buffer, pH 7.4, containing 4 mM urea, 1% Triton X-100, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. To study the effects of apoE, confluent SMCs were incubated in culture medium containing [35S]SO₄ and the indicated concentrations of apoE for 24 h. Cell and medium PG levels were assessed.  

**DEAE Cellulose Chromatography of PGs**—To determine changes in PGs, DEAE-cellulose chromatography was performed as described previously (21, 22). A DEAE-cellulose column was equilibrated with 50 mM Tris buffer, pH 7.4, containing 4 mM urea, 0.1 mM NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% CHAPS. The column was washed with the same buffer and with buffer containing 0.5 M NaCl, and PGs were eluted with the same buffer containing 0.5 M NaCl. Fractions containing radioactivity ([35S]SO₄) were pooled and dialyzed against minimal essential medium overnight and counted. To determine the relative proportion of HSPGs and chondroitin and dermatan sulfate PGs, an aliquot of the pooled fraction was incubated in 50 mM sodium acetate buffer, pH 5.2, with 1 unit/ml each of heparinase and heparitinase or with 0.5 units of chondroitin ABC lyase for 16 h at 37 °C. The reaction mixture was precipitated either with 0.5 volumes of 1% cetylpyridinium chloride or with 3 volumes of ethanol to precipitate undigested GAG. Radioactivity in the supernatant and pellet was determined.

**SMC Proliferation**—To determine the effects of apoE or HS on SMC proliferation, cells were plated at low density (8 × 10⁴/well) and cultured for 24–48 h in the presence or absence of apoE or HSPGs. Cell number was counted with hemacytometer, and net growth was determined (15). Alternatively, SMCs were cultured in the above conditions; cells were then labeled with [3H]thymidine for 6 h, and radioactivity incorporated into the DNA was determined by trichloroacetic acid precipitation of the cell lysate.

**Determination of Perlecan Protein and mRNA**—To determine changes in perlecan protein, control and apoE-treated (10 μg/ml) SMCs were labeled with [3H]leucine for 24 h (steady state). PGs were isolated from SMC medium and purified by DEAE-cellulose chromatography. Purified PGs were immunoprecipitated with an anti-perlecan antibody (100-fold diluted), and immunoprecipitates were analyzed by 5% SDS-PAGE. Perlecan (molecular mass, ~550 kDa) was identified by autoradiography.

For Northern blotting, a 497-base pair polymerase chain reaction product representing domain I of perlecan (forward and reverse primers with sequences 5’-GGCTGAGGGCTACGATGG-3’ and 5’-TGGCCAGGCCTGGAACT-3’, respectively) was generated by reverse transcription-polymerase chain reaction of endothelial cell RNA. Northern blotting of total RNA from control and apoE-treated (5 μg/ml for 24 h) SMCs was performed using 35P-labeled perlecan probe. RNA load was normalized by determining 18 S RNA.

**Data Analysis**—Results are expressed as mean ± S.D. Experiments were done in triplicate and repeated at least once. Statistical analyses were performed by Student’s t test to determine the significance of change. A significance difference was considered for p values equal to or less than 0.05.

**RESULTS**

**ApoE Increases Sulfate Incorporation into SMC HSPGs**—Previous studies showed that addition of apoE increased HSPG production in endothelial cells but not in macrophages, which predominantly synthesize chondroitin and dermatan sulfate PGs (15). Similarly, HSPGs represent only ~25% of total PGs synthesized by SMCs. To determine whether apoE increased HS PGs in SMCs, confluent monolayers of SMCs were incubated with apoE (10 μg/ml) for 16 h, and PGs in cellular and secreted pools were determined following purification. In different experiments, the total PG level in cells was increased by 15–22% and in medium by 25–30% in apoE-treated cells (Fig. 1A). Because HSPGs in the media can act as inhibitors of cell proliferation, we determined HSPGs in control and apoE media. The heparinase-sensitive radioactivity, representing HSPGs, was increased by 108% in apoE-treated cells (Fig. 1B). The amounts of chondroitin and dermatan sulfate PGs, which constitute ~75% of total medium PGs, were not altered by apoE-treatment. These data show that apoE treatment of SMCs results in an increase specifically in HSPGs. As in endothelial cells (15), this increase was found to be primarily due to an increase in synthesis (not shown).

**Apoptosis**—To determine changes in perlecan protein, control and apoE-treated (10 μg/ml) SMCs were labeled with [3H]leucine for 24 h (steady state). PGs were isolated from SMC medium and purified by DEAE-cellulose chromatography. Purified PGs were immunoprecipitated with an anti-perlecan antibody (100-fold diluted), and immunoprecipitates were analyzed by 5% SDS-PAGE. Perlecan (molecular mass, ~550 kDa) was identified by autoradiography.

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We also determined the effect of apoE on $[^3H]$glucosamine incorporation into PGs. ApoE increased $[^3H]$glucosamine incorporation into HSPGs by about 3.5-fold (Fig. 1C). Thus, the ratio of $[^3H]$glucosamine to $[^35]SO_4$ in HSPGs was increased approximately by 1.75, suggesting that although HS GAG were increased by apoE, these HS are relatively under-sulfated.

**ApoE Inhibits SMC Proliferation** —The above experiments were done on confluent SMCs. We next determined apoE effects on proliferating SMCs. Previous studies showed that apoE inhibits SMC proliferation stimulated by serum or platelet-derived growth factor (10). The growth medium in the current experiments contained serum, basic fibroblast growth factor, and epidermal growth factor. In different experiments, the addition of apoE to the medium inhibited cell proliferation by 45–55% (both cell number and $[^3H]$thymidine incorporation; Fig. 2A) in 24 h. This inhibition was greater than that previously observed with 25 µg of apoE (10).

We examined whether apoE altered HSPGs in proliferating cells. Because the cell number is decreased by apoE, by comparing the ratios of $[^35]SO_4$ to $[^3H]$thymidine, we determined the amount of PGs per cell in control and apoE-treated cells (Fig. 2B). Despite decreasing the cell number, apoE increased the ratio of $[^35]SO_4$ to $[^3H]$thymidine (from 2 to 3.46), suggesting increased HSPGs per cell.

**HSPGs from ApoE-treated Cells Are Potent Antiproliferatives** —We first examined whether apoE-treated cells contained antiproliferative substances in the medium. Conditioned medium was collected from control and apoE-treated SMCs, and apoE was removed by immunoprecipitation (Fig. 3, inset). These media were then added to subconfluent SMCs, and cell growth was determined after 24 h (Fig. 3A). Control conditioned medium (CCM) inhibited SMC growth by 18% compared with control medium. Conditioned medium from apoE-treated cells (ECM) inhibited cell proliferation by 51%, suggesting that apoE treatment stimulated the production of antiproliferative substances.

We next determined whether HSPGs in apoE-conditioned medium mediated inhibition of cell proliferation. PGs from control and apoE-conditioned media were purified by DEAE-cellulose chromatography. This procedure also removed any remains of apoE from the medium (not shown). Equal amounts ($[^35]SO_4$ cpm) of purified PGs were then added to subconfluent SMCs in growth medium, and cell growth was determined. PGs from apoE-treated cells (Fig. 3B, E-PG) inhibited SMC proliferation to an extent similar to that of apoE. PGs from control cells (Fig. 3B, C-PG), although at a similar level, inhibited SMC proliferation by only 18%. These data suggest that HSPGs from apoE-treated SMCs are antiproliferative.

**ApoE Inhibits SMC Proliferation Stimulated by Lysolecithin** —We previously showed that lysolecithin and oxidized low density lipoprotein treatment decreases extracellular HSPGs (23, 24), and others have shown that these agents stimulate SMC proliferation (10, 25). We therefore determined whether apoE ability to increase HSPGs would block lysolecithin effects on SMC proliferation. Incubation of SMCs with lysolecithin decreased $[^35]SO_4$ incorporation into PGs by 36% (not shown). Concomitant with this decrease, lysolecithin increased $[^3H]$thymidine incorporation into DNA (Fig. 4). Lysolecithin effects on SMC proliferation were completely abolished in the presence of apoE. These data suggest that agents that modulate HSPGs influence cell proliferation.

**ApoE Increases Perlecan Production in SMCs** —We next characterized the antiproliferative HSPGs in apoE-stimulated cells. Perlecan is the major HSPG secreted by vascular cells (26). To determine whether apoE increased perlecan secretion,
DEAE-cellulose-purified, [3H]leucine-labeled (core protein-labeled) HSPGs from control and apoE-treated cells were immunoprecipitated by anti-perlecan antibody and analyzed by SDS-PAGE and autoradiography (Fig. 5). The radioactivity associated with a protein of Mr 550,000 (perlecan has a core protein of ~400,000 containing three HS chains of Mr ~500,000–70,000) was increased by apoE. Concomitant with protein increase, apoE also increased perlecan mRNA by greater than 2-fold. These data suggest that the antiproliferative HSPG in SMC medium is perlecan.

Effects of ApoE Isoforms—We next studied the effects of apoE isoforms to determine whether antiproliferative activity correlated with increase in perlecan HSPGs (Fig. 6). ApoE3, the most common isoform of apoE, showed maximum stimulation on perlecan production and inhibition on cell proliferation (45%). ApoE2 and apoE4 did not significantly increase perlecan HSPGs or inhibit cell proliferation. These data further show that the antiproliferative effect of apoE correlates with its ability to stimulate perlecan HSPGs.

The Antiproliferative Effect of ApoE Requires Perlecan—We next determined whether the antiproliferative effect of apoE is mediated by perlecan. Subconfluent SMCs were incubated with control medium or apoE medium containing nonspecific antibody or anti-perlecan antibody (Fig. 7A). Perlecan antibody did not affect cell growth under control conditions. ApoE inhibited [3H]thymidine incorporation into DNA approximately by 48%. In the presence of perlecan antibody, this inhibition was reduced to about 9%. We performed the same experiment with human vascular SMCs (Fig. 7B). ApoE inhibited SMC proliferation by 71%. Perlecan antibody, however, under control conditions stimulated SMC proliferation by 30–35%. The effect of apoE was completely reversed by perlecan antibody. These data suggest that perlecan mediates the antiproliferative effect of apoE both in rat and human SMCs.

DISCUSSION

HSPGs are thought to be important for blood vessel homeostasis, blood clotting, atherosclerosis, and atherosclerosis. Atherosclerotic vessels have reduced HSPGs, and previous studies have shown that apoE–HDL increased endothelial HS, which in turn could decrease the occurrence of events related to atherosclerosis (11). ApoE was able to increase secretion of HSPGs in both endothelial cells (11) and SMCs (present study). Because subendothelial matrix HSPGs produced by apoE-treated endothelial cells showed strong inhibition of SMC growth, we postulated that actions of vascular apoE would regulate SMC proliferation in the subendothelial space (11). Our current data show direct effects of apoE on SMC HSPGs and thus identify a mechanism for the known antiatherogenic effect of apoE.

The present data strongly suggest that the antiproliferative effect of apoE is due to induction of perlecan HSPGs in SMCs.

1) HSPGs isolated from apoE-stimulated cells inhibited proliferation better than those from control SMCs. 2) ApoE countered the effects of lysolecithin, which is known to decrease extracellular HSPGs. 3) The antiproliferative effect of apoE isoforms correlated with their ability to stimulate perlecan HSPGs. 4) An anti-perlecan antibody blocked the inhibitory effect of apoE on SMC growth, showing that perlecan is required for the antiproliferative effect of apoE. Moreover, apoE did not inhibit proliferation of endothelial cells, which are not sensitive to HSPG inhibition (27) ([3H]thymidine: control, 4780 ± 270 cpm; apoE, 5540 ± 330 cpm). Our data are also consistent with the observation that both apoE (28) and HSPGs (29) inhibit mitogen-activated protein kinase, a key signaling pathway in cell growth.

ApoE treatment increased both perlecan mRNA and protein. Perlecan, the major HSPG of endothelial cells and SMCs (26), consists of a core protein of Mr ~450,000 to which three HS...
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Subconfluent rat SMCs (A) or human SMCs (B) were incubated with medium alone (control), medium containing 10 μg/ml perlecan antibody (Pab), medium containing 10 μg/ml apoE (E), or apoE and perlecan antibody (E+Pab) for 24 h. [3H]Thymidine incorporation was determined.

![Graph A: Rat SMC](image)

![Graph B: Human SMC](image)

**Fig. 7.** The antiproliferative effect of apoE requires perlecan. Subconfluent rat SMCs (A) or human SMCs (B) were incubated with medium alone (control), medium containing 10 μg/ml perlecan antibody (Pab), medium containing 10 μg/ml apoE (E), or apoE and perlecan antibody (E+Pab) for 24 h. [3H]Thymidine incorporation was determined.

Perlecan was shown to negatively correlate with SMC proliferation (30), and it was shown to inhibit Oct-1, a growth-related transcription factor (31). In certain cell types, however, blocking perlecan production via antisense DNA inhibited cell growth (32–34). It is conceivable that perlecan under normal conditions is required for matrix assembly and cell growth; however, excess perlecan in medium that is not deposited into the matrix may block growth factor binding and activity. Support for this comes from the observations that perlecan core protein can bind cell surface integrins and support cell growth (35) and that serum induces perlecan production at early time periods but decreases at later time periods (36).

It is not clear why excess perlecan remained in the medium. It is conceivable that the amount of perlecan in the matrix is saturating, leading to accumulation in the medium. Alternatively, perlecan produced by apoE-stimulated cells is different. The data shown in Fig. 1C suggest that HS chains in perlecan are under-sulfated. Perlecan interaction with surrounding matrix proteins, such as laminin and collagen, requires both core protein and HS chains (37). It is conceivable that reduced sulfation affects perlecan HS interactions with laminin or other perlecan molecules, thereby reducing its ability to incorporate into matrix.

The antiproliferative effect of perlecan is likely due to the HS chains. Although it is not entirely clear how HS inhibits cell proliferation, several mechanisms have been proposed (29, 38–40). We are surprised, however, by the observation that perlecan antibody, which reacts with domain III of perlecan, completely blocked perlecan effect. Domain III is thought to mediate cell adhesion (26), and attachment to the matrix and spreading is a key part of cell growth. Perlecan antibody, as shown in Fig. 7 in control conditions, either did not affect or stimulated SMC growth. However, when added during the seeding of SMCs, perlecan antibody inhibited SMC growth by >40% (not shown). We propose that perlecan in matrix is required for cell growth and that excess unincorporated perlecan may engage the SMC surface molecules involved in cell attachment and spreading. Studies have shown both adhesive and antiadhesive functions for perlecan (26, 41, 42), and the current studies may offer an explanation why this occurs.

Vascular cells produce a variety of growth promoters and inhibitors (43). Physiologically relevant agents that stimulate SMC proliferation include platelet-derived growth factor, thrombin, oxidized low density lipoprotein, and lysolecithin. Vascular cell derived growth inhibitors include transforming growth factor-β, nitric oxide/cGMP, and apoE. Going through published literature on perlecan regulation, we identified an interesting possibility that perlecan may be the key for modulation of SMC growth. Platelet-derived growth factor (44), thrombin (45), serum (36), oxidized low density lipoprotein, and lysolecithin (23, 24), which stimulate SMC growth, decrease perlecan. In contrast, the antiproliferative agents transforming growth factor-β (46), apoE (present study), and even heparin (47) stimulate perlecan expression. Thus, we postulate that modulation of perlecan is key to regulation of cell growth.

The observation that apoE up-regulates perlecan may have implications in other physiological and pathological processes. Perlecan is known to modulate angiogenesis (48). It remains to be determined whether apoE could be an angiogenic factor in vivo. ApoE induction of perlecan may also have implications in the pathogenesis of Alzheimer’s disease. Brains of patients with Alzheimer’s disease accumulate deposits of β-amyloid protein. The β-amyloid protein-containing deposits in the vessel wall are primarily associated with SMCs, endothelial cells, and the surrounding matrix, and studies showed that perlecan is associated with the β-amyloid protein deposits (49). It remains to be determined whether apoE can induce perlecan production in neuronal cells, and if this occurs, it is conceivable that production of soluble perlecan may compete for β-amyloid protein binding to matrix perlecan. Soluble HS-like molecules can inhibit amyloid progression in mice (50).

How apoE stimulates perlecan and what cell surface molecule(s) mediates apoE actions remain to be determined. Based on previous studies, both HSPGs and receptor-associated protein-sensitive pathways may mediate apoE effects on perlecan (11). ApoE-β-very low density lipoprotein, which binds poorly to HSPGs (51), does not inhibit DNA synthesis (52), and RAP at high concentrations could affect apoE binding to HSPGs (53); thus, it is conceivable that cell surface HSPGs directly mediate apoE effect (54). It should be noted that demonstration of requirement for cell surface HSPGs in mediating the antiproliferative effect of apoE is difficult as agents that interfere with cell surface HSPGs, such as heparinase, heparin, and chlorate,
independently inhibit cell proliferation (55, 56). Cell surface syndecan is beginning to be recognized as a signaling receptor (57). Alterations in the phosphorylation state of syndecan may affect cell growth. It remains to be determined whether apoE alters syndecan phosphorylation and whether this is required for its antiproliferative effect. ApoE is also known to stimulate nitric oxide and cGMP production (9), which are antiproliferative (58). Although the role of this pathway in SMC proliferation was not determined (28), preliminary results showed that perlecan can increase HSPG production in SMCs (not shown). Perlecan promoter has cAMP responsive elements (46). Thus, it is conceivable that increased cGMP or cAMP will induce transcription of perlecan mRNA through activation of specific transcription factors.

In summary, our data show that the antiproliferative effects of apoE are mediated by perlecan. We postulate that modulation of perlecan is a key step in regulating SMC growth. Factors that increase perlecan inhibit cell growth, whereas those that decrease perlecan stimulate cell growth.

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