Journal of Cellular Biochemistry 54:299–308 (1994)

Apolipoprotein E: A Potent Inhibitor of Endothelial and Tumor Cell Proliferation

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Abstract Recombinant human apolipoprotein E3 (apoE), purified from *E. coli*, inhibited the proliferation of several cell types, including endothelial cells and tumor cells in a dose- and time-dependent manner. ApoE inhibited both de novo DNA synthesis and proliferation as assessed by an increase in cell number. Maximal inhibition of cell growth by apoE was achieved under conditions where proliferation was dependent on heparin-binding growth factors. Thus, at low serum concentrations (0–2.5%) basic fibroblast growth factor (bFGF) stimulated the proliferation of bovine aortic endothelial (BAE) cells severalfold. The bFGF-dependent proliferation was dramatically inhibited by apoE with an $IC_{50} \approx 50$ nM. Under conditions where cell proliferation was mainly serum-dependent, apoE also suppressed growth but required higher concentrations to be effective ($IC_{50} \approx 500$ nM). ApoE also inhibited growth of bovine corneal endothelial cells, human melanoma cells, and human breast carcinoma cells. The IC_{50} values obtained with these cells were generally 3–5 times higher than with BAE cells. Inhibition of cell proliferation by apoE was reversible and dependent on the time of apoE addition to the culture. In addition, apoE inhibited the chemotactic response of endothelial cells that were induced to migrate by a gradient of soluble bFGF. Inhibition of cell proliferation by apoE may be mediated both by competition for growth factor binding to proteoglycans and by an antiadhesive activity of apoE. The present results demonstrate that apoE is a potent inhibitor of proliferation of several cell types and suggest that apoE may be effective in modulating angiogenesis, tumor cell growth, and metastasis.

Key words: DNA, heparin-binding growth factors, basic fibroblast growth factor, carcinoma cells, angiogenesis

In a normal tissues, cell growth and DNA synthesis are closely controlled by a variety of regulatory factors operating on both positive and negative levels [Weinberg, 1989]. As a normal cell transforms and proliferates to form a solid tumor, it exhibits several characteristics, including induction of new blood vessel formation [Folkman and Shing, 1992; Aznavoorian et al., 1993]. The capacity to induce angiogenesis and neovascularization is typical of many malignant cells and is a prerequisite of solid tumor growth. Moreover, malignant cells produce a variety of factors that stimulate endothelial cell proliferation and migration and cause new capillary beds to form within the tumor nodule [D'Amore, 1988; Folkman and Klagsbrun, 1987; Folkman and Shing, 1992]. In addition to its role in malignant cell growth, abnormal neovascularization is characteristic of other diseases, including neovascular glaucoma, diabetic retinopathy, and rheumatoid arthritis [Folkman and Shing, 1992].

Basic fibroblast growth factor (bFGF) is a strong heparin-binding molecule that is present in virtually all tissues and acts on a number of cell types to stimulate mitogenesis, cell motility, and angiogenesis [Gospodarowicz et al., 1987]. bFGF is a potent inducer of angiogenesis in vivo and in cell culture [Folkman and Shing, 1992; Folkman and Klagsbrun, 1987; Hayek et al., 1987]. bFGF is associated with the heparan sulphate proteoglycans of the extracellular matrix [Vlodavsky et al., 1993], and the matrix appears to be a reservoir for the growth factor [Bashkin et al., 1989]. Cell surface heparan sulfate proteoglycans (HSPG) bind both to bFGF and to the high affinity bFGF receptor [Gallagher and Turnbull, 1992; Kan et al., 1993] and play an essential role in the presentation of

Received September 23, 1993; accepted November 2, 1993.

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bFGF to its receptor [Yayon et al., 1991]. Recent evidence suggests that cell surface HSPG may play a general role in presentation of several growth factors to their receptors, including the receptors for TGFβ [López-Casillas et al., 1993] and MIP-1β [Tanaka et al., 1993]. Some heparin-binding proteins which have antiproliferative activity for endothelial cells, such as platelet factor 4 [Maione et al., 1990], and adhesive proteins such as fibronectin [Homandberg et al., 1986] and thrombospondin [Good et al., 1990; Taraboletti et al., 1990; Vogel et al., 1993a] may play physiological roles in regulating angiogenesis. Both a recombinant heparin-binding domain of thrombospondin and synthetic heparin-binding peptides from thrombospondin mimic the effects of intact thrombospondin on endothelial growth and motility [Vogel et al., 1993a].

Apolipoprotein E (apoE) is a plasma protein that binds with very high affinity to heparin and proteoglycans [Cardin et al., 1988; Mahley, 1988; Mahley et al., 1979; Zhong-Sheng et al., 1993]. ApoE participates in plasma lipoprotein metabolism through its high affinity interaction with cell surface receptors, including the low-density lipoprotein (LDL) receptor, and the LDL receptor-related protein (LRP) [Hertz et al., 1988; Lund et al., 1989; Yamada et al., 1989, 1992].

ApoE is synthesized in many tissues, including the liver, intestine, adrenal glands, kidney, lung, spleen, testes, ovary, and brain [Mahley, 1988]. Many studies have demonstrated important roles for apoE in lipid transport in various tissues including liver, brain, peripheral nerves, arterial walls, and plasma. However, it is synthesized by a number of cell types that do not *necessarily* participate in cholesterol homeostasis [Boyles et al., 1989; Hui et al., 1980]. ApoE also exhibits biological activities that are not obviously related to lipid transport [Mahley, 1988]. For example, ApoE, synthetic peptides from apoE, and apoE-containing lipoproteins are potent suppressors of lymphocyte activation by mitogens and antigens [Cardin et al., 1988; Hui et al., 1980; Dyer et al., 1991]. Its activity to suppress formation of atherosclerotic lesions in Watanabe hyperlipidemic rabbits was not associated with a lowering of plasma cholesterol levels. Recently, expression of the type 4 allele of apoE was associated with late onset familial Alzheimer disease [Strittmatter et al., 1993]. These observations suggest that other activities of apoE, including its heparin binding activity, may be biologically relevant.

We report here that recombinant apoE is a potent inhibitor of endothelial cell proliferation and motility. Furthermore, apoE suppresses proliferation in vitro of two types of tumor cells that depend on neovascularization for malignant progression in vivo. ApoE competes with bFGF for binding to HSPG on endothelial cells. In addition to its ability to inhibit growth by competing with heparin-dependent growth factors, some of the antiproliferative effects of apoE may result from an antiadhesive activity.

MATERIALS AND METHODS

Materials

Recombinant human apoE3 isoform was expressed in *E. coli* and purified as previously described [Vogel et al., 1985]. bFGF was obtained from Collaborative Research (Bedford, MA) and Bachem (Richmond, CA). A recombinant fragment of human fibronectin (FN33), containing the cell-binding domain of human fibronectin, amino acids 1329–1722 was prepared as previously described [Werber et al., 1990; Vogel et al., 1993b]. A 28 kD recombinant fragment from the heparin-binding domain of human thrombospondin-1 spanning amino acids 1–242 and synthetic peptides from the type I repeats of thrombospondin were prepared as described [Guo et al., 1992; Vogel et al., 1993a]. A 30 amino acid peptide containing a tandem repeat of apoE residues 141–155(LRKLRKRLLRDADDL) [Dyer et al., 1991; Dyer and Curtiss, 1991] was prepared with a Biosearch 9600 peptide synthesizer using standard tBoc chemistry.

Cell Culture

Bovine aortic endothelial cells (BAE cells) were provided by Dr. E. Gallin (AFRRI, Bethesda, MD) and were used at passages 5–10. BAE cell cultures were maintained in low glucose DMEM containing 10% FCS, 4 mM glutamine, 50 μg/ml ascorbic acid, and 500 U/ml each of penicillin G and streptomycin sulfate. Media components were obtained from Biofluids Inc. (Rockville, MD). BAE cells were grown at 37°C in 5% CO_2. The media were changed every 2–3 days. Bovine corneal endothelial cells (BCE cells) were used at passages 2–8 [Munjal et al., 1990]. BCE cell cultures were maintained at 34°C in 5% CO_2 in the same medium but without ascorbic acid and including 2.5 μg/ml amphotericin B. Human A2058 melanoma cells [Todaro et al., 1980] and human MDA MB435 breast carcinoma cells

(American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium, containing 10% FCS. Mouse N18TG2 neuroblastoma cells were provided by Dr. Z. Vogel (The Weisman Institute of Science, Rehovot). CHO-K1 (ATCC CCL61) cells were obtained from the American Type Culture Collection (Rockville, MD).

Thymidine Incorporation into DNA

Confluent monolayers of cells were washed once in PBS and starved in 0.5% FCS-containing medium for 48 h. Cells were harvested using trypsin, washed in medium containing 10% FCS, resuspended in medium containing 0.1% BSA, and seeded in 24-well plates at 20,000 cells/well in the presence of the indicated concentrations of FCS, growth effectors, and 2.5 μCi/well of thymidine[methyl-^{3}H] (86.1 Ci/mmol; Dupont NEN, Boston, MA). The assays were terminated after 30–40 h by washing the cells two times with 1 ml of PBS, fixing with 0.3 ml of methanol/acetic acid (3:1), washing two times with 0.5 ml of 80% ethanol, and air drying. Cells were extracted from the wells by incubation with 300 μl of trypsin/EDTA for 1 h at 37°C and 30 min at room temperature followed by the addition of 100 μl of 1% SDS. The radioactivity of the extracted material was quantified in a scintillation counter.

Cell Proliferation Assay

Endothelial cell proliferation was measured using the CellTiter 96™ colorimetric assay (Promega, Madison, WI). Five thousand cells per well were plated into 96-well tissue culture plates (Costar) in medium containing 0.5 or 5% FCS and the indicated concentrations of growth effectors. Cells were maintained at 34°C for BCE cells or at 37°C for BAE cells in 5% CO_2. After 72 h, 15 μl of dye solution was added to each well, and the plates were incubated for an additional 4 h. Solubilization solution (100 μl) was added to each well, and the absorbance at 570 nm was determined after 24 h as described by the manufacturer.

Chemotaxis

Chemotaxis of endothelial cells was determined in modified Boyden chambers as previously described [Taraboletti et al., 1990]. Trypsinized BCE cells were resuspended in complete medium and allowed to recover in suspension for 2.5–3 h. Cells were collected by centrifugation, suspended in DMEM, 0.1% BSA, and added at 1.5–2 × 10^6 cells/ml to the upper wells of the chemotaxis chambers. Trypsinized BAE cells were resuspended briefly in complete medium, centrifuged, resuspended at 1 × 10^6 cells/ml in DMEM containing 0.1% BSA, and used immediately. Chemotaxis of both cell strains was measured after incubation at 37°C in 5% CO_2 for 4.5–5 h.

Adhesion and Binding Assays

After trypsinization, cells were resuspended in complete medium and allowed to recover for 1 h at 25°C with gentle rocking. Adhesion to immobilized proteins and peptides were determined as previously described [Roberts et al., 1987; Guo et al., 1992]. Binding of ^{125}I-bFGF [Neufeld and Gospodarowicz, 1985] or ^{125}I-apoE to the endothelial cells was determined as previously described [Vogel et al., 1993a; Guo et al., 1992]. ^{125}I-proteins were added and incubated with the cells on a rotating table for 1 h at 20°C. Bound radioactivity was determined after centrifugation of the cells through oil. Binding of ^{125}I-apoE to heparin was determined using an immobilized heparin-bovine serum albumin conjugate as previously described [Guo et al., 1992].

RESULTS

Inhibition of DNA Synthesis in Endothelial Cells

Addition of bFGF to sparse freshly plated BAE cell cultures that had been serum starved for 48 h stimulated the incorporation of [^{3}H]-thymidine severalfold relative to control (Fig. 1). DNA synthesis was strongly inhibited by addition of apoE to the culture. Inhibition was dose-dependent, and the degree of inhibition was inversely related to the serum concentration. At 1% FCS, 50 nM apoE inhibited bFGF-induced DNA synthesis by 95%, whereas at 2.5% FCS comparable inhibition (83%) was attained only at a tenfold higher concentration of apoE. Inhibition was also dependent on the time of addition of apoE to the culture. Using BAE cells growing in 1% FCS, 0.5 μM apoE inhibited bFGF-stimulated thymidine incorporation by 65% when added at the time of cell stimulation, 34% when added after 15 h, and 10% when added after 22 h (results not shown). Inhibition was reversible in that cell proliferation was restored (50%) when the apoE was removed after 22 h of cell stimulation. Heat denaturation of

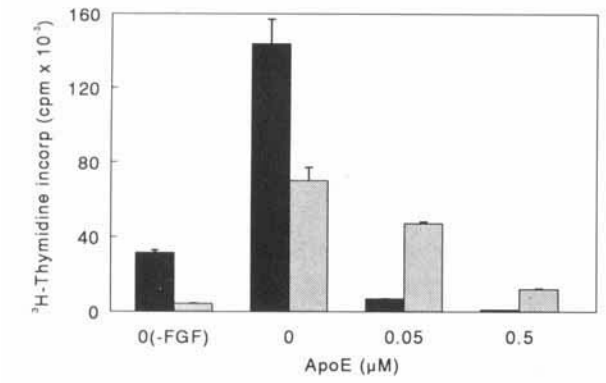

Fig. 1. Inhibition of bFGF-induced DNA synthesis in bovine aortic endothelial cells by apolipoprotein E. Serum starved BAE cells were plated at 2 × 10^4 cells/well in the presence of [^{3}H]-thymidine and 1% FCS (solid bars) or 2.5% FCS (gray bars) in the absence of bFGF (−FGF) or in the presence of 10 ng/ml bFGF and the indicated concentrations of apoE. Thymidine incorporation was quantified as described in Materials and Methods. Results are presented as mean ± SD.

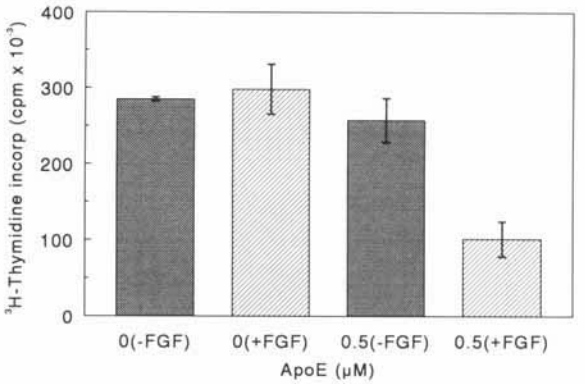

Fig. 2. Effect of apolipoprotein E on DNA synthesis of preattached BAE cells. BAE cells at 1 × 10^5/well were allowed to attach for 18 h in medium containing 10% FCS and then serum starved for 24 h. [^{3}H]-thymidine was added in medium containing 0.5% FCS, and incorporation was determined in the absence (−FGF, gray bars) or presence of 10 ng/ml bFGF (+FGF, hatched bars) and the indicated concentrations of apoE. Results are presented as mean ± SD.

apoE abolished its inhibitory activity (results not shown), indicating that the antiproliferative activity was specific for the native protein. ApoE purified from human plasma had identical activity to the recombinant protein (results not shown). ApoE had no apparent effect on protein synthesis prior to the inhibition of DNA synthesis, based on measurements of [^{35}S]-methionine incorporation after labelling for 6 h in the presence of 0.5 μM apoE (results not shown).

When cells were plated at high density (1 × 10^5 cells/well) and allowed to preattach to the substratum, addition of bFGF only weakly stimulated thymidine incorporation (Fig. 2). The higher basal incorporation may indicate that the cells were not entirely quiescent after 24 h starvation or that endogenous bFGF was stimulating DNA synthesis. Although bFGF had little direct effect on DNA synthesis under these conditions, it increased the sensitivity of the cells to apoE. Thus, 0.5 μM apoE only inhibited thymidine incorporation into cells grown in the absence of bFGF by 10%, whereas in the presence of 10 ng/ml bFGF, thymidine incorporation was inhibited by 66%.

ApoE also inhibited DNA synthesis in BCE cells stimulated by bFGF (Fig. 3). Stimulation of thymidine incorporation by bFGF in BCE cells was observed in the absence of serum, and inhibition by apoE was strongest under these conditions. As was observed in BAE cells, increasing the serum concentration reduced the effectiveness of inhibition by apoE. In contrast to the

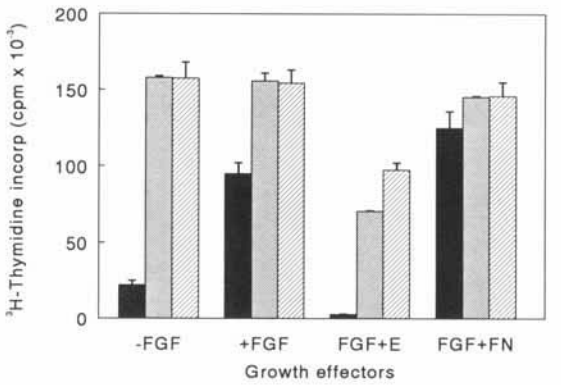

Fig. 3. Effect of apolipoprotein E on DNA synthesis of bovine corneal endothelial cells. Serum starved BCE cells were plated at 2 × 10^4 cells/well in the presence of [^{3}H]-thymidine and 0% (solid bars), 1% (gray bars), or 2% FCS (striped bars) alone (−FGF) or with 10 ng/ml bFGF (+FGF), 10 ng/ml bFGF and 0.5 μM apoE (FGF + E), or 10 ng/ml bFGF and 0.5 μM recombinant cell-binding domain of fibronectin (FGF + FN). Incorporation is presented as mean ± SD.

effect of apoE, addition of the 33 kD recombinant fragment of fibronectin did not inhibit DNA synthesis in BCE cells.

Inhibition of Tumor Cell Growth by ApoE

Inhibition of DNA synthesis by apoE was not restricted to endothelial cells. Two types of tumor cells (Fig. 4) and smooth muscle cells (results not shown) were also sensitive to growth inhibition by apoE, but N18TG2 neuroblastoma or CHO-K1 cells were not. The addition of 0.5 μM apoE suppressed growth by 35% and 65% in

A2058 melanoma and MDA MB435 breast carcinoma cells, respectively. Inhibition of DNA synthesis in both types of cells, however, required higher concentrations of apoE than were required to inhibit endothelial cell growth.

Reversal of apoE Inhibition by the Cell-Binding Domain of Fibronectin

We observed that all cell types that were sensitive to growth inhibition by apoE exhibited changes in morphology when treated with apoE. The cells generally spread less and acquired a more rounded shape. Similar phenomena were observed previously with other inhibitors of endothelial cell proliferation, including a heparin-binding fragment of thrombospondin-1 [Vogel et al., 1993a], and the glycoprotein SPARC [Hasselaar and Sage, 1992]. ApoE had no effect on growth of cells in suspension, such as human megakaryocytes (T. Vogel, unpublished data). These data suggested that apoE may regulate proliferation, in part, by disrupting cell-matrix interactions, as proposed by Ingber and Folkman [1989].

To further examine the role of cell-matrix interactions in the activity of apoE, inhibition of breast carcinoma cell proliferation by apoE was studied using cells plated on wells in the presence of the cell-binding domain of fibronectin (Fig. 4B). DNA synthesis was stimulated approximately twofold relative to cells plated in the absence of fibronectin. No further stimulation of DNA synthesis was obtained by addition of bFGF to this culture. In the absence of fibronectin, bFGF stimulated thymidine incorporation by 25% (Fig. 4B, solid bars). Corresponding to their insensitivity to bFGF, the inhibition of DNA synthesis by apoE was much weaker in cells grown on a fibronectin matrix (20% inhibition) than in cells grown without fibronectin (65% inhibition). A similar effect of fibronectin on apoE-inhibited proliferation was also observed with BAE and BCE cells (results not shown).

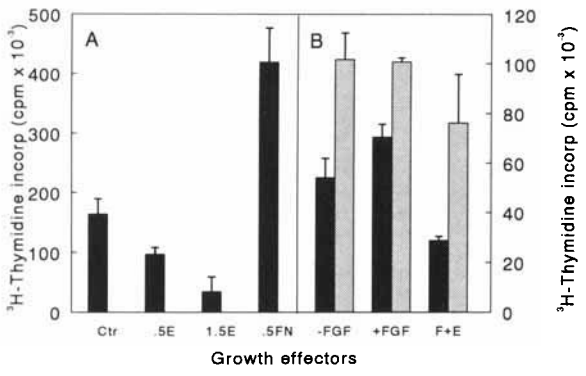

Fig. 4. Effect of apolipoprotein E and fibronection on DNA synthesis in tumor cells. A2058 melanoma cells (**A**) or MDA 435 breast carcinoma cells (**B**) were tested for DNA synthesis as indicated in Materials and Methods. Following serum starvation, the cells were replated in wells in the presence of fresh media containing 0.5% FCS, ^{3}H-thymidine, and growth effectors as indicated, added either at time zero or following 22 h of incubation to allow cell attachment (A and B, respectively). A: 20 ng/ml bFGF alone (Ctr) or together with 0.5 or 1.5 μM apoE (.5E and 1.5E, respectively), or 0.5 μM 33 kD fragment of fibronectin (.5FN). B: With (+FGF, 10 ng/ml) or without bFGF (−FGF), or with bFGF and 0.5 μM apoE (F + E). At time zero, cells were plated either without (solid bars) or with 15 μg/well of the 33 kD fragment of fibronectin (hatched bars). Radioactivity incorporated was measured following 40 h of incorporation as indicated in Materials and Methods.

Inhibition of Proliferation by ApoE and ApoE Peptide

The inhibitory effects of apoE on cell growth were confirmed using a colorimetric cell proliferation assay (Fig. 5). As was observed by thymidine incorporation, the inhibition of BAE cell proliferation by apoE was strongest in low serum, with an $IC_{50} \approx 0.1$ μM. Increasing the serum concentration to 5% increased the IC_{50} approximately tenfold. A synthetic peptide from apoE, which is a dimer of residues 141–155, was previously shown to inhibit proliferation of lymphocytes [Dyer et al., 1991]. The peptide also inhibited BAE cell proliferation (Fig. 5), with an $IC_{50} \approx 3$ μM at 0.5% serum. Increasing the serum concentration also increased the IC_{50} for this peptide approximately tenfold.

Effect of ApoE on Cell Motility

In addition to its effects on cell growth, bFGF stimulates motility of endothelial cells [Folkman and Shing, 1992; Vogel et al., 1993a]. bFGF-stimulated chemotaxis of BAE cells was inhibited in a dose-dependent manner by apoE (Fig. 6). Basal motility of the cells in the absence of added growth factor was also inhibited by apoE, but inhibition of net stimulated motility was greater than the inhibition of basal motility.

ApoE Inhibits bFGF Binding to Heparin and Endothelial Cells

Heparin-binding proteins may recognize distinct sequences on heparin or heparan sulfate chains [Gallagher and Turnbull, 1992]. To determine whether bFGF and apoE recognized simi-

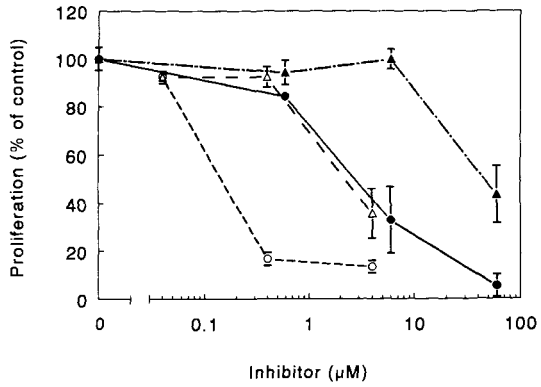

Fig. 5. Effect of apolipoprotein E or apolipoprotein E dimeric peptide on proliferation of BAE cells. BAE cells were plated at 5×10^3 cells/well and grown in the presence of 0.5% FCS (circles) or 5% FCS (triangles) and 20 ng/ml bFGF with the indicated concentrations of apoE synthetic peptide (closed symbols) or recombinant apoE (open symbols). Cell proliferation was determined after 72 h using the CellTiter 96 assay. Cell numbers are presented as a percent of control determined at the respective serum concentrations in the absence of apoE or peptide, mean ± SD, n = 3.

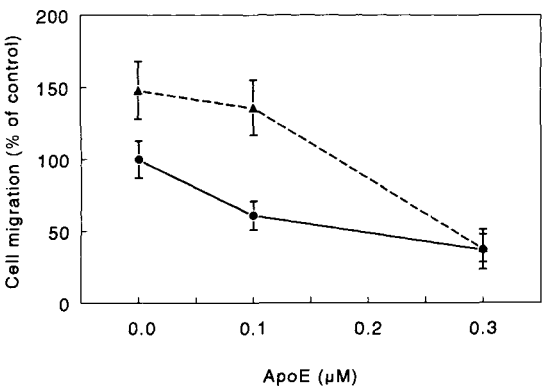

Fig. 6. Inhibition of chemotaxis of BAE cells by apolipoprotein E. Chemotaxis was determined in modified Boyden chambers as described in Materials and Methods. The number of cells migrated per field is presented as mean ± SD, n = 3, in the presence of the indicated concentrations of apoE added to the upper chamber with the cells (●) or with the indicated concentrations of apoE in the upper chamber and 30 ng/ml bFGF added to the lower chamber (▲).

lar heparin sequences, the ability of bFGF to inhibit binding of [^{125}I]apoE to immobilized heparin was determined (Fig. 7). Binding of radiolabelled apoE was strongly and completely inhibited by bFGF. Note that the concentrations of bFGF required to displace radiolabelled apoE were significantly lower than the concentration of unlabelled apoE required to displace radiolabelled apoE. Thrombospondin and a recombinant 18 kD fragment derived from the amino-

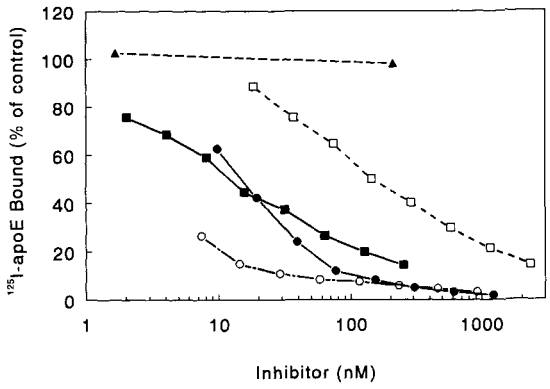

Fig. 7. Inhibition of ^{125}I-apolipoprotein E binding to heparin by bFGF and other heparin-binding proteins. Binding of ^{125}I-apoE to immobilized heparin-BSA was determined as described in Materials and Methods. Inhibition by the indicated concentrations of apoE (●), bFGF (○), thrombospondin (■), 18 kD recombinant heparin-binding domain of thrombospondin (□), or laminin (▲) is presented as percent of control binding to heparin-BSA without inhibitors and is the mean of duplicate determinations.

terminus of thrombospondin, which were shown previously to compete with bFGF for binding to heparin [Vogel et al., 1993a], also competed with apoE for binding to heparin. In contrast, laminin binds to heparin but did not inhibit apoE binding to heparin. The dimeric apoE peptide 141–155 also inhibited binding of apoE to heparin-BSA (results not shown). Binding of ^{125}I-bFGF to heparin, however, was less sensitive to inhibition by apoE than by other heparin-binding proteins [Vogel et al., 1993] (results not shown), suggesting that the sequence in heparin recognized by apoE is also recognized by bFGF but additional sequences that do not bind apoE are also recognized by bFGF.

Adhesion of BCE cells to a heparin-binding peptide from thrombospondin [Guo et al., 1992] was also inhibited by apoE (Fig. 8). A 50% inhibition of adhesion was observed at approximately 0.1 μM apoE. ApoE was approximately fivefold more potent on a molar basis than the recombinant 18 kD heparin-binding domain from thrombospondin.

To examine the role of HSPGs in binding of apoE to the cell surface, binding of [^{125}I]-apoE to the cells was measured in the presence of heparin and several heparin-binding molecules (Fig. 9). Binding of apoE to all four cell types was significantly inhibited by heparin. Inhibition of apoE binding by bFGF paralleled that by heparin. Binding of apoE to BCE, melanoma, and breast carcinoma cells was inhibited 80–90% by

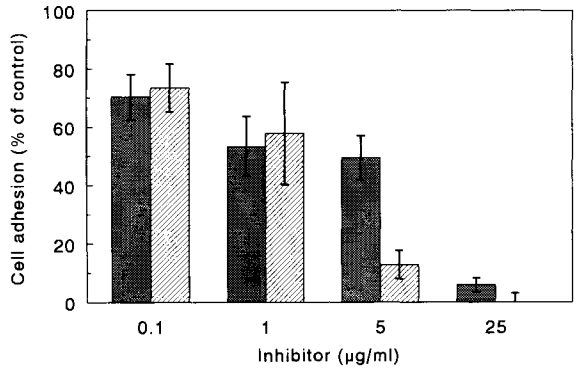

Fig. 8. Inhibition of BCE cell adhesion to the heparin-binding peptide SHWSPWSSCSVT by apoE or recombinant heparin-binding domain of thrombospondin. BCE cell adhesion was determined using 2×10^5 cells/well to acrylic discs coated with 200 μg/ml of synthetic peptide in the presence of the indicated concentrations of 28 kD recombinant heparin-binding domain of thrombospondin (gray bars) or apoE (striped bars). Results are presented as mean ± SD, n = 3, and are normalized to control adhesion determined in the absence of inhibitors.

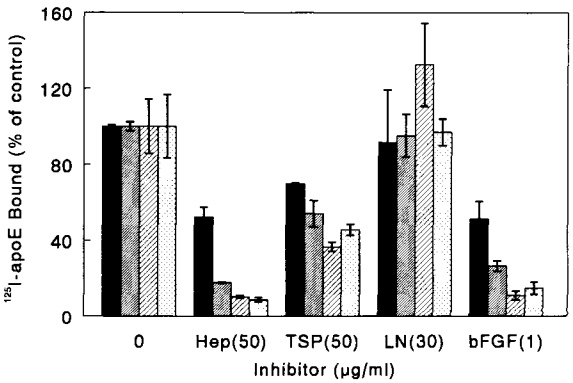

Fig. 9. Inhibition of ^{125}I-apolipoprotein E binding to cells by heparin-binding proteins. Binding of ^{125}I-apoE to BAE cells (2×10^5, solid bars), BCE cells (2×10^5, gray bars), MDA MB435 breast carcinoma cells (2×10^5, striped bars), or A2058 melanoma cells (2×10^5, dotted bars) was determined as described in Materials and Methods. Binding was determined in the presence of the indicated concentrations of heparin (Hep), thrombospondin (TSP), laminin (LN), or bFGF and is presented as a percent of control binding in the absence of inhibitors, mean ± SD, n = 3.

1 μg/ml bFGF, whereas binding to BAE cells was inhibited only 50%. The heparin-binding matrix protein, thrombospondin, partially inhibited apoE binding to the four cell types. Laminin did not significantly inhibit binding of apoE to any of the cell types examined, consistent with its lack of effect on binding of apoE to heparin. Although bFGF quantitatively inhibited [^{125}I]-apoE binding, unlabelled apoE was a relatively weak inhibitor of [^{125}I]-bFGF binding to endothelial cells. In the presence of 1.5 μM apoE, binding of [^{125}I]-bFGF to BCE and BAE cells was inhibited 11 ± 2% and 9 ± 5%, respectively. Since 85% and 96% of [^{125}I]-bFGF binding to the respective cells was inhibited by heparin in the same experiment, bFGF probably binds to some sites on endothelial HSPGs that are not recognized by apoE, consistent with the differences observed for inhibition of heparin binding.

DISCUSSION

In the present study we have demonstrated that recombinant human apoE, a plasma protein generally involved in cholesterol and lipoprotein metabolism [Mahley, 1988], is also a selective and highly effective inhibitor of the proliferation of several types of cells, including endothelial and tumor cells. At least part of this activity is due to competition with growth factors for interaction with cell surface HSPGs. Its inhibitory effects on endothelial cell proliferation, motility, and adhesion suggest a role for apoE as an antiangiogenic factor and further suggest that apoE may be useful in inhibiting pathological neovascularization.

ApoE inhibits cell proliferation stimulated by bFGF or serum. Much greater inhibition is obtained, however, under conditions where proliferation is ultimately dependent on bFGF. ApoE also inhibits growth of tumor cells, including human melanoma and breast carcinoma cells, but not growth of an anchorage-independent cell line. Inhibition is reversible and requires the native protein, but it can be mimicked by a synthetic peptide dimer derived from apoE that contains a tandem repeat of residues 141–155. Because proliferation of some cell types is not inhibited by apoE and growth of apoE-sensitive cells is restored by the addition of fresh medium, apoE suppression of growth is not due to cytotoxicity. Moreover, inhibition by apoE is highly dependent on the time at which apoE is added to the culture and is most effective before progression into the S phase, in a manner similar to the inhibitory activities of the heparin-binding domain of TSP [Vogel et al., 1993a] and the glycoprotein SPARC [Hasselaar and Sage, 1992]. The activity of recombinant apoE is also specific in that the cell-binding domain of human fibronectin, a recombinant protein of similar size that is highly effective in inhibiting fibronectin/fibrinogen receptors on platelets [Vogel et al., 1993b], did not inhibit growth in this system.

The present data support two mechanisms for apoE interactions with the cell. The first is di-

rect competition of apoE with growth factors, such as bFGF, for binding to HSPG receptors on the cell. We have demonstrated that apoE and bFGF compete for binding to heparin and to heparin-inhibitable binding sites on the cell surface. bFGF requires interaction with HSPG for high affinity binding to the signal transducing bFGF receptor [Yayon et al., 1991; Gallagher and Turnbull, 1992]. Furthermore, the bFGF receptor may also interact directly with HSPG [Kan et al., 1993], and apoE could potentially compete for this interaction as well. However, the data presented herein provide direct support only for competition with bFGF for binding to HSPG. The incomplete inhibition by apoE of bFGF binding to endothelial cells contrasts with the strong inhibition observed previously using heparin-binding recombinant fragments and peptides derived from thrombospondin [Vogel et al., 1993a] and suggests that apoE may inhibit proliferation by an additional mechanism.

A second possible mechanism for inhibition of responses to bFGF is through disruption of cell-matrix interactions by apoE, as suggested by the effects of apoE on cell spreading in the present studies and on neurite outgrowth when added to cultures of dorsal root ganglion neurons [Handelmann et al., 1992]. Inhibition of adhesion may result from binding competition between apoE and other adhesive proteins for cell surface or matrix HSPGs [Zhong-Sheng et al., 1993; Guo et al., 1992; Lilly-Stauderman et al., 1993]. Alternatively, apoE may indirectly inhibit cell-matrix interactions. Two types of protein receptors for apoE have been described [Hertz et al., 1988; Lund et al., 1989; Yamada et al., 1989, 1992]. Occupancy of these receptors by apoE could transduce signals that could directly inhibit proliferation or indirectly inhibit by "inside out" signaling through integrin receptors that mediate adhesion to the extracellular matrix [Hynes, 1992]. The activity of the apoE peptide does not differentiate between these mechanisms, since the peptide binds both to the LDL receptor [Dyer and Curtiss, 1991] and to heparin.

The effect of apoE in the presence of low serum concentrations is greater than when added in the presence of high serum. This observation may indicate that some apoE determinants are more available or optimally positioned when apoE is not bound to lipoproteins. Binding of apoE to lipoproteins in the serum could also increase the sequestration of the bound apoE via the LDL and LRP receptors, with subsequent degradation of the apoE in lysosomes. Low serum concentrations or an excess of apoE may also allow apoE to successfully compete with LDL for binding to HSPGs on the endothelial cell surface. The binding of LDL to HSPG may function to present these particles to the high affinity LDL and LRP receptors for internalization [Zhong-Sheng et al., 1993]. Thus, apoE may also be antiproliferative at low serum concentrations by inhibiting LDL uptake by the cells and limiting the availability of lipids essential for growth.

Several recent studies have shown an important role of apoE in the pathogenesis of atherosclerosis [Plump et al., 1993; Zhang et al., 1992]. We and others have shown that intravenous administration of apoE in hyperlipidemic rabbits resulted in reduced plasma cholesterol levels [Yamada et al., 1989; Mahley et al., 1989]. More recently, following a sustained intravenous administration of apoE into Watanabe heritable hyperlipidemic rabbits, progression of atherosclerosis and both the number and size of related lesions were significantly reduced [Yamada et al., 1992]. In these studies, however, plasma lipid levels did not change, suggesting a direct effect of apoE at the lesion site. Similarly, Badimon et al. [1990] have shown that intravenous injection of apoE-containing HDL caused regression of preestablished atherosclerosis in cholesterol fed rabbits.

One proposed mechanism for direct action of apoE in lesions is through its interaction with lipoprotein lipase. Lipoprotein lipase is a key enzyme in lipoprotein triglyceride metabolism produced by macrophages and adipocytes. Addition of the enzyme to subendothelial matrix markedly increases LDL binding. ApoE displaces LDL bound to the lipase but does not displace lipase bound to subendothelial matrix [Saxena et al., 1993]. ApoE complexed in HDL has similar activity, which may clarify its role in removing cholesterol from peripheral tissues in "reverse cholesterol hemostasis" [Mahley, 1988]. Based on the present data, apoE may also inhibit cell proliferation in developing atherosclerotic lesions. These data may provide a basis for direct modulation by apoE of intimal smooth muscle proliferation [Mahley, 1988]. Further studies are needed, however, to define the contribution of this mechanism to the in vivo activities of apoE.

Inhibition of growth factor binding to HSPGs and inhibition of heparin-dependent cell-matrix

interactions may explain the antiproliferative activity of apoE for endothelial and tumor cells in vitro. Activities of apoE in regulating cholesterol homeostasis are probably mediated by its binding to lipoprotein receptors as well as to HSPGs and lipoprotein lipase. ApoE can therefore interact with multiple cellular and matrix components and modulate several cellular functions. The antiproliferative and antimigratory activities of apoE provide a new approach to investigate the effects of apoE on endothelial cells and may contribute to understanding the complex physiological functions of this protein.

ACKNOWLEDGMENTS

We thank Dr. Elaine Gallin for providing aortic endothelial cells. This work was supported in part by National Institutes of Health grant R01 EY09092 to D.A.B.

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