Apolipoprotein E Inhibits Serum-stimulated Cell Proliferation and Enhances Serum-independent Cell Proliferation*

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Independently of its role in lipid homeostasis, apolipoprotein E (apoE) inhibits cell proliferation. We compared the effects of apoE added to media (exogenous apoE) with the effects of stably expressed apoE (endogenous apoE) on cell proliferation. Exogenous and endogenous apoE increased population doubling times by 30–50% over a period of 14 days by prolonging the G1 phase of the cell cycle. Exogenous and endogenous apoE also decreased serum-stimulated DNA synthesis by 30–50%. However, apoE did not cause cell cycle arrest; both apoE-treated and control cells achieved equivalent saturation densities at 14 days. Further analyses demonstrated that exogenous and endogenous apoE prevented activation of MAPK but not induction of c-fos expression in response to serum growth factors. Endogenous (but not exogenous) apoE altered serum concentration-dependent effects on proliferation. Whereas control (non-apoE-expressing) cell numbers increased with increasing serum concentrations (1.6-fold for every 2-fold increase in serum), apoE-expressing cell numbers did not differ as serum levels were raised from 2.5 to 10%. In addition, in low serum (0.1%), apoE-expressing cells had elevated DNA synthesis levels compared with control cells. We conclude that apoE does not simply inhibit cell proliferation; rather, the presence of apoE alters the response to and requirement for serum mitogens.

Apolipoprotein E (apoE) plays an important role in the progression of atherosclerosis (1), and different isoforms are associated with varying risks of Alzheimer’s disease (2). ApoE is a major protein component of several classes of plasma lipoproteins and is capable of binding to a number of other molecules, including cell-surface lipoprotein receptors and heparan sulfate proteoglycans (1, 3). Although apoE can also influence cell proliferation (4–6), research efforts have focused on determining the mechanisms by which apoE affects lipid metabolism, specifically by facilitating lipoprotein transport, promoting cell cholesterol efflux, and enhancing intracellular cholesteryl ester and triglyceride hydrolysis (7–10). ApoE can reach cells from two sources: exogenous apoE (defined as apoE synthesized or provided by a remote source such as plasma apoE or apoE added to experimental media), usually as a component of lipoproteins, or endogenous apoE (defined as apoE produced by cellular apoE gene expression). Since apoE binds to lipids and other molecules (1, 3, 11), exogenous apoE and endogenous apoE can potentially encounter and interact with different groups of molecules in the extracellular environment compared with the intracellular environment. For example, exogenous apoE binds to cell-surface apoE receptors and mediates endocytosis, whereas endogenous apoE is mainly associated with Golgi compartments (1, 12). Thus, it is likely that following endogenous synthesis, apoE has different effects on cell function compared with apoE entering cells from the extracellular environment. In macrophages, exogenous apoE is much less effective than endogenous apoE in promoting cholesterol efflux (7, 13). Although exogenous apoE and endogenous apoE are capable of interacting with cells differently, they both can bind the same cell-surface components such as heparan sulfate proteoglycans (3), thereby exerting similar biological effects.

Exogenous apoE affects the proliferation of various cell types (4–6). Cell proliferation is controlled by protein kinase-based signaling pathways linking cell-surface mitogen receptors with various targets within the cell, including critical transcription regulators. Exogenous apoE has been shown to affect the activities of several protein kinases known to be involved in transmitting mitogenic stimuli, including protein kinases A and C and mitogen-activated protein kinases (MAPKs) (6). On the other hand, endogenous apoE expression is controlled by growth state (14), and/or it is tightly coupled to cell differentiation (15, 16). Endogenous apoE also alters cellular signaling and protein kinase activity (17, 18). The question of whether exogenous apoE and endogenous apoE have similar effects on cell proliferation or differentiation has not been addressed directly in the same cell type.

Previous studies have examined the inhibitory effects of exogenous apoE on short-term cell proliferative responses in the presence of serum stimuli (4–6). In this study, we determined the effects of exogenous apoE and endogenous apoE on cell proliferation under conditions of both serum deprivation and serum stimulation. To compare the effects of exogenous apoE and endogenous apoE on proliferation in the same cell type, we...
stably expressed apoE in a rat fibroblast cell line (F111 cells) that normally does not express apoE. The proliferation properties of parental F111 cells have been previously characterized in detail (19–21). Cells expressing the apoE transgene were defined as E⁺ cells, in contrast to E⁻ cells carrying only the control plasmid (encoding hygromycin resistance). Responses of cells to serum were determined in the presence or absence of exogenous apoE. Our results indicate that although exogenous apoE and endogenous apoE exhibit similar effects on cell proliferation at a fixed serum concentration, cell proliferative responses to apoE differ dramatically under serum-deprived versus serum-stimulated conditions.

EXPERIMENTAL PROCEDURES

Materials—Purified lipid-free apoE was used for experiments. [³H]Thymidine and ECL Western blotting reagents were purchased from Amersham Pharmacia Biotech, and [³²P]dCTP was purchased from PerkinElmer Life Sciences. High-glucose (4.5 mg/ml) Dulbecco's modified Eagle's medium (DMEM) was obtained from Cellgro (carried by Fisher). Calf serum was obtained from Hyclone Laboratories (Logan, UT). Penicillin/streptomycin/glutamine mixture (100 × 10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, 29.2 mg/ml l-glutamine) and 100 U/ml penicillin G (Sigma) were purchased from Life Technologies, Inc. Phosphate-buffered saline (PBS), diethyl pyrocarbonate, and CsCl were purchased from Sigma. Hygromycin B was purchased from Roche Molecular Biochemicals. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from New England Biolabs Inc. (Beverly, MA). Monoclonal anti-apoE antibody E10 was a generous gift from Drs. E. Krul and G. Schonfield (University of Washington). Horseradish peroxidase-labeled secondary antibodies were purchased from Amersham Pharmacia Biotech and New England Biolabs Inc.

Plasmids—An apoE expression vector in which the human apoE cDNA is expressed from the cytomegalovirus immediate-early promoter was a generous gift from Dr. T. Mazzone (University of Chicago) (33). The hygromycin resistance gene cloned into the plasmid pHygGro was regulated by the rat actin promoter (34). Plasmids were transfected into competent Escherichia coli strain DH5 and purified on QIAGEN columns or by CsCl/EtBr banding (35).

Rats—Rat F111 embryonic fibroblasts were maintained in high-glucose DMEM containing 10% calf serum and 100 units/ml penicillin G sodium plus 100 μg/ml streptomycin sulfate. ApoE-expressing cell lines were generated by cotransfecting F111 fibroblasts with the apoE3 expression vector and pHygGro using calcium phosphate-mediated DNA precipitation (36). Control cell lines were generated in parallel by transfecting the parental cells with pHygGro alone. Hygromycin-resistant cells were selected for 1 week in high-glucose DMEM supplemented with 10% calf serum, 100 units/ml penicillin G sodium plus 100 μg/ml streptomycin sulfate, and 200 μg/ml hygromycin and then maintained in the same medium with reduced hygromycin concentrations (100 μg/ml). Three apoE-expressing (E⁺) and three control (E⁻) clones were expanded into cell lines and used for experiments. Similar results were obtained with each clone.

Immunoblot—For detection of apoE synthesis and secretion, cells (60% confluence) were cultured for 24 h in serum-free medium. Both the conditioned medium (concentrated by Amicon Centriprep concentrators, molecular mass cutoff of 10,000 Da) and whole cell extracts (following lysis in 150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 8.0) were resolved by SDS-12% polyacrylamide gel electrophoresis, followed by electrophoretic transfer of proteins to nitrocellulose membranes. ApoE was detected by incubating membranes with the monoclonal anti-human apoE antibody E10, 1:2000 dilution in Tris-buffered saline, 0.1% (v/v) Triton X-100, 1% (v/v) nonfat milk, and 0.1% (v/v) sodium azide. Bound primary antibodies were visualized with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies and ECL reactions (37). To determine MAPK activity, cell extracts were resolved electrophoretically on SDS-10% polyacrylamide gels and transferred to nitrocellulose membranes. 31Δ reporter that was probed with primary antibodies specific to MAPK (1:2000 dilution) or phosphorylated MAPK (1:2000 dilution), followed by incubation with horseradish peroxidase-labeled secondary antibodies and ECL reactions for the detection of total and activated MAPK levels.

Microscopy—Cell morphology was examined under an Olympus CK2 inverted microscope, and images were recorded by an Olympus C-35 AD-4 camera. The differential interference contrast images were taken under magnification ×100. For experiments aimed to identify the Golgi regions, cells maintained in coverslip bottom dishes were fixed and incubated with 10 μM NBD-C6 dissolved in DMEM containing 0.68 mg/ml fatty acid-free bovine serum albumin and 1% NAD at 37 °C. Cells were then washed with DMEM containing 1% NAD at 37 °C and submitted for imaging analysis. For identifying the intracellular neutral lipid, cells in coverslip bottom dishes were incubated with 100 ng/ml Nile red and observed immediately. For apoE immunostaining, cells in coverslip bottom dishes were fixed for 30 min with 2% formaldehyde and then washed four times with PBS. Cells were permeabilized with saponin (500 μg/ml), 50 μg/ml purified lipid-free apoE, and 20 μM Nile red in PBS for 20 min. Prior to incubation, the anti-apoE antibody was pre-absorbed with E⁻ cell protein extracts to reduce nonspecific binding. ApoE immunodetection was performed as follows: 1) monoclonal anti-apoE primary antibody E10 (1:2000 dilution) incubation (1 h); 2) three washes with 5% calf serum and 500 μg/ml saponin in PBS (5 min/wash); 3) rhodamine-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution in 5% calf serum and 500 μg/ml saponin in PBS) incubation (1 h); and 4) two washes with 5% calf serum and 500 μg/ml saponin in PBS (5 min/wash) and one wash with PBS (10 min). For actin staining, formaldehyde-fixed cells were extracted by acetone (20 °C, 3–5 min), followed by two PBS washes and a 25-min incubation in PBS and 1% bovine serum albumin blocking solution. Cells were then stained with goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at 1:40 dilution in PBS and 1% bovine serum albumin for 20 min. Fluorescent images were obtained using a Zeiss LSM410 confocal laser scanning system attached to a Zeiss Axiovert 100TV inverted microscope. For observation of fluorescence signals based on the excitation/ emission wavelengths of the dyes (rhodamine, 570/590 nm; Nile red, 552/563 nm; and phallacidin, 505/512 nm), appropriate combinations of excitation laser and emission filter settings were used. Images were recorded with a Pentium PC computer with Zeiss LSM software for image enhancement and analysis.

Cell Proliferation Rates—Cells (500/well) were plated in 12-well tissue culture plates (Corning Inc., Corning, NY), and cell number was determined from days 3 to 14. Media (with or without apoE) were changed every 3 days throughout the course of the experiments. For experiments aimed to identify the Golgi and submit for sequencing, cells incubated with exogenous apoE, 10 μg/ml purified lipid-free apoE were added at every media change (every 3 days). At the indicated time points, cells were trypsinized and detached from the plates, and cell number was counted using a hemocytometer. Results represent three parallel experiments.

Fluorescence-activated Cell Sorter Analyses—10⁴ cells suspended in 50°C and then washed with 1 ml of 80% ethanol and incubated at 4 °C for 30 min. Cells were then pelleted and resuspended in 500 μl of 0.1 mg/ml propidium iodide and 0.6% Nonidet P-40, followed by incubation with 500 μl of 2 mg/ml RNase A at room temperature for 30 min in the dark.

DNA Synthesis—For experiments measuring DNA synthesis, 1 μCi/ml [³H]thymidine was added to the experimental medium and incubated for 8 h. Following the newly synthesized DNA at the end of the incubation, cells were placed on ice and washed twice with ice-cold PBS, and cell macromolecules including DNA were precipitated for 1 h with ice-cold 10% trichloroacetic acid. Cell precipitates were washed twice with ice-cold 10% trichloroacetic acid to remove free [³H]thymidine and then dissolved in 1 N NaOH (37 °C, 2 h). Trichloroacetic acid-insoluble radioactivity was quantified by scintillation counting. Experiments were repeated three times, and similar results were obtained.

RNA Extraction and Gel Electrophoresis—Cells were plated in 100-mm tissue culture dishes in the growth medium overnight. After experimental treatments (2.5% calf serum ± 10 μg/ml apoE), cells were washed twice at room temperature with 0.1% diethyl pyrocarbonate-treated PBS and then harvested in Trizol reagent (3 ml/dish; Life Technologies, Inc.). RNA was extracted following the manufacturer’s instructions. RNA concentration was determined by spectrophotometry at A₂₆₀ (RNA 10 μg/ml) was dissolved in water and ethidium bromide-containing sample buffer (5 Prime → 3 Prime, Inc., Boulder, CO), followed by boiling in a water bath for 5 min. Samples were loaded and resolved on 1.2% agarose gels (containing 40 μg MOPS, pH 7.0, 10 μg/ml ethidium bromide, and 1.1% formaldehyde). At 1.2% agarose, the gels were examined under UV light to ensure equivalent RNA loading and integrity. Gels were then gently washed at room temperature with diethyl pyrocarbonate-treated water for 30 min to remove ethidium bromide.

Northern Blot Analyses—RNA samples on gels were transferred to nylon membranes (Strategene, La Jolla, CA) in 10× SSC (1.5 × NaCl)
RESULTS

Isolation of ApoE-expressing Cell Lines—To compare the effects of exogenous apoE added to cells via the culture medium with the effects of endogenous apoE (synthesized in and secreted from cells), we established stably transfected derivatives of the rat F111 embryonic fibroblast cell line. Multiple clones were isolated that expressed either apoE and hygromycin resistance (referred to as E- cells) or hygromycin resistance only (referred to as E' cells). We confirmed that E’ cells synthesized and secreted apoE by immunoblot analyses of whole cell extracts and concentrated conditioned medium from these clones (Fig. 1A) and by indirect immunofluorescence (Fig. 1B). E’ cells synthesized and secreted an ~34-kDa protein that comigrated with purified apoE. Based on the signal intensities of apoE standards obtained in these immunoblots, we estimate that E’ cells secrete ~40 ng of apoE/10^6 cells/day and contain ~25 ng of intracellular apoE/mg of total cell protein. No apoE, either intracellular or secreted, was detected in E’ cells. Although E’ cells clearly secrete apoE, cell-associated apoE was not localized with the Golgi apparatus (Fig. 1, B and C). It is also noteworthy that little apoE was detected at the cell surface (Fig. 1B).

Interesting morphological changes were observed in E+ cells. Compared with E- cells (Fig. 2A), E+ cells (Fig. 2C) had shorter cell bodies, resulting in a more oval shape, as well as less distinct nuclear boundaries when viewed by phase-contrast microscopy and a prominent perinuclear accumulation of phase-bright vesicles. Despite the above changes, similar to E- cells (Fig. 2B), E+ cells maintained a prominent actin filament network (Fig. 2D). In E+ cells (Fig. 2G), the perinuclear accumulation and increase in size of phase-bright vesicles relative to similar vesicles in E- cells (Fig. 2E) were more apparent when viewed by differential interference contrast microscopy. In E- (Fig. 2, E and F) and E+ (G and H) cells, these vesicles were not co-localized with the Golgi apparatus. However, these vesicles did stain positive with Nile red (data not shown), indicating the presence of neutral lipid. Despite the apparent formation of larger lipid-containing vesicles or droplets in E+ cells, there was no measurable difference in whole cell lipid content (triglyceride and cholesterol ester) between E- and E+ cells (data not shown).

The same morphological changes were seen in each of three independent E’ clonal isolates, but were never seen in E- cells exposed to 10 μg/ml exogenous apoE, even after prolonged periods (up to 14 days). Thus, the presence of apoE within these fibroblasts had substantial effects on cellular architecture that could not be mimicked by exposure to exogenous apoE.
ApoE Inhibits F111 Cell Proliferation—To determine whether apoE (both exogenous and endogenous) has a sustained effect on cell proliferation, we compared the proliferation rate of E− cells, in the presence or absence of exogenous apoE, with that of E+ cells over a period of 14 days (Fig. 3). Cells that expressed apoE had significantly slower proliferation rates during mid-to-late log growth (days 5–11) (Fig. 3A). Very similar results were seen when exogenous apoE was added to the growth medium of E− cells (data not shown). In both cases, apoE increased population doubling times by 30–50%, resulting in a 4–7-fold reduction in cell number on days 5 and 7 (Fig. 3B). Despite the reduction in proliferation rates by day 14, the cell densities of apoE-exposed cultures were equal to the saturation density of cultures not exposed to apoE. Therefore, apoE expression or addition to the culture medium did not arrest cell proliferation.

The increased population doubling time of apoE-exposed cells was associated with a prolongation of the G1 phase of the cell cycle. E− and E+ cells were fixed, and their DNAs were stained with propidium iodide 48 h after replating. Note that plating densities for this experiment were considerably higher than in the extended proliferation assays. As a result, by 48 h, cell populations were in early-to-mid log growth. Relative DNA content was quantified by flow cytometry (Fig. 3C). At 48 h post-plating, 70% of E− cells had a 2N DNA content (and thus were in the G1/G0 phase of the cell cycle), 18% had a 4N DNA content (G2/M phase), and 12% had DNA levels intermediate between 2N and 4N (cells in S phase). In contrast, 85% of the E+ cells were in G0/G1, with compensatory decreases in the number of cells in S phase (from 12 to 10%) and G2/M (from 18 to 5%; p < 0.05). There were few cells in either population with less than 2N DNA levels, indicating that apoE expression did not increase apoptotic DNA fragmentation.

ApoE Attenuates Growth Factor Signaling—Prolongation of the G1 phase of the cell cycle is frequently associated with decreased responsiveness to mitogens. Indeed, several investigators have demonstrated that exogenous apoE inhibits short-term proliferative responses to mitogens of endothelial cells, Kaposi’s sarcoma cells, and smooth muscle cells (4–6). To see whether exogenous apoE and endogenous apoE decreased F111 cell responsiveness to serum mitogens, we measured [3H]thymidine incorporation into E− cells, into E+ cells, and into E− cells treated with exogenous apoE. Cells were arrested in G0 by serum starvation (0.1% calf serum for 48 h) and grown in 0.1 or 10% calf serum and 1 μCi/ml [3H]thymidine with or without exogenous apoE (10 μg/ml) for 40 h, and incorporation of [3H]thymidine was quantified. The results of these experiments uncovered two effects of apoE. First, total incorporation of [3H]thymidine into DNA and the -fold induction of [3H]thymidine incorporation in response to serum were significantly reduced by apoE (Fig. 4). Serum stimulated [3H]thymidine incorporation into E− cells by 25-fold. Exogenous apoE reduced the serum response of E− cells so that [3H]thymidine incorporation increased by only 7-fold. Serum treatment had modest effects on E+ cells, increasing [3H]thymidine incorporation by 4-fold. Second, in the presence of apoE (and in particular, endogenous apoE), serum-starved cells continued to incorporate [3H]thymidine into DNA. As a result, despite the pronounced decreases in -fold stimulation in response to serum treatment, apoE decreased overall thymidine incorporation only by 30% (exogenous apoE) or by 50% (endogenous apoE).
ApoE Inhibits MAPK Activation, but Not c-fos Expression—Among the major mitogens present in serum are peptide growth factors that are ligands for receptor tyrosine kinases, in particular, the potent fibroblast mitogen platelet-derived growth factor (PDGF). Numerous studies have established that growth factors drive G1 progression by activating cytosolic and nuclear protein kinases that subsequently activate specific transcription factors. Key targets include the p42 and p44 MAPKs and the c-fos proto-oncogene (39, 40). We determined whether apoE altered the overall levels of serum- or PDGF-induced tyrosine phosphorylation, MAPK activity, and c-fos expression. Cells (both E- and E+ cells) were serum-starved for 48 h and then treated with either 10 ng/ml PDGF or 10% calf serum (with or without exogenous apoE) for 10 min. Both total tyrosine phosphorylation (A) and MAPK activation (B; amount of phospho-MAPK (MAPK-PO4) relative to the amount of total MAPK (MAPK-tol)) were assessed by immunoblotting. In C, serum-starved E- and E+ cells were treated with 0.1 or 3% calf serum ± 10 μg/ml exogenous apoE for 30 min, after which total RNA was extracted, and c-fos and L30 mRNA levels were determined by Northern blot analysis. ApoE (both exogenous and endogenous) prevented MAPK activation without detectably affecting tyrosine phosphorylation or c-fos mRNA expression.

FIG. 5. Effects of apoE on growth factor signaling. E- and E+ cells were maintained in 0.1% calf serum (CS) for 48 h. Cells were then stimulated with 10 ng/ml PDGF or 10% calf serum in the presence or absence of 10 μg/ml exogenous (exog.) apoE for 10 min. Tyrosine phosphorylation (A) and MAPK activation (B; amount of phospho-MAPK (MAPK-PO4) relative to the amount of total MAPK (MAPK-tol)) were assessed by immunoblotting. In C, serum-starved E- and E+ cells were treated with 0.1 or 3% calf serum ± 10 μg/ml exogenous apoE for 30 min, after which total RNA was extracted, and c-fos and L30 mRNA levels were determined by Northern blot analysis. ApoE (both exogenous and endogenous) prevented MAPK activation without detectably affecting tyrosine phosphorylation or c-fos mRNA expression.

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When we looked specifically at activation of p42/p44 MAPKs by serum and PDGF, a different picture emerged (Fig. 5B). Stimulation of E- cells resulted in activation of MAPK in a manner that paralleled overall changes in tyrosine phosphorylation. Either in E+ cells or when added to E- cells, apoE dramatically reduced the activation of MAPK. This was true in response to both PDGF and serum stimulation.

In many situations, following activation in the cytosol, MAPKs translocate to the nucleus and contribute to transcriptional regulation of target genes. Following mitogenic stimulation of resting cells, critical nuclear targets are the immediate-early growth response genes, including the c-fos proto-oncogene. Increased c-fos mRNA levels were seen within 15 min of stimulating serum-starved E- cells as well as E+ cells. Peak c-fos mRNA levels were reached 30 min after stimulation. Despite the effects of apoE on [3H]thymidine incorporation, cell cycle progression, and MAPK documented above, no inhibition of c-fos induction was observed in the presence apoE (Fig. 5C).

Vogel et al. (4) previously proposed that apoE inhibits proliferation by competing with fibroblast growth factors for heparin binding. This model leads to the prediction that at a fixed concentration of apoE, increased levels of growth factor should
increase proliferation. We tested this prediction by measuring the proliferation of E\(^+\) cells, of E\(^+\) cells treated with apoE (10 \(\mu\)g/ml), and of E\(^+\) cells in different serum concentrations (Fig. 6). The proliferation rate of E\(^+\) cells, either in the absence or presence of exogenous apoE, increased incrementally in response to increases in serum from 2.5 to 10%. At days 7 and 9, each 2-fold increase in serum concentration resulted in an \(\approx 1.6\)-fold increase in cell number (Fig. 6, A and B). In contrast, the proliferation rate of E\(^+\) cells was much less dependent on serum concentration. As shown in Fig. 6C, the increase in serum concentration from 2.5 to 10% did not significantly increase cell number on day 7 or 9. Note that the number of E\(^+\) cells is three to seven times more than the number of E\(^+\) cells under each condition at days 7 and 9. Therefore, although it is possible that competition with fibroblast growth factor or other serum factors for heparin-binding sites might explain part of the anti-proliferative effect of apoE, it is unlikely to account for all of the actions of apoE, in particular, when apoE is expressed endogenously.

ApoE Stimulates DNA Synthesis in the Absence of Serum—In the presence of apoE, F111 cells had elevated levels of DNA synthesis in the absence of serum (Fig. 4, compare open bars). This latter result was examined in more detail (Fig. 7). First, we confirmed that apoE elevated basal \(\text{[3H]}\)thymidine incorporation in the absence of calf serum using three independent isolates (one pool of many clones, E\(^-\)1 and E\(^+\)-1; and two isolated clones, E\(^-\)2, E\(^-\)3, E\(^-\)2, and E\(^-\)3) of transfected cells expressing hygromycin resistance or expressing both hygromycin resistance and apoE. For the control cell lines, basal \(\text{[3H]}\)thymidine incorporation ranged from 4500 to 8000 dpm incorporated per dish. Addition of exogenous apoE variably increased \(\text{[3H]}\)thymidine incorporation, with responses ranging from 110% in clone E\(^-\)1 to a 300% increase (from 5200 to 16,100 dpm) in clone E\(^-\)3. In clonal isolates of apoE-expressing cells, basal \(\text{[3H]}\)thymidine incorporation was dramatically elevated. In these cells, incorporation ranged from 11,200 dpm/dish for the uncloned pool to \(\approx 30,000\) dpm/dish for the clonal isolates. ApoE expression and secretion were quantified in the two monoclonal E\(^+\) cell lines. The two clones had comparable levels of cell-associated apoE (27.7 and 29.2 ng of apoE/mg of cell protein, respectively); however, the level of apoE in the conditioned medium from clone E\(^-\)3 (1700 ng of apoE/mg of cell protein) was about five times higher than in the medium from clone E\(^-\)2 (320 ng of apoE/mg of cell protein). Despite differences in apoE secretion, similar levels of DNA synthesis were observed in the two apoE-expressing clones. Therefore, apoE has two apparently disparate effects on proliferation. On the one hand, apoE allowed serum-deprived cells to remain in the cell cycle. On the other hand, apoE extended the time of G\(_1\) progression, resulting in longer population doubling times. This effect was associated with significantly attenuated responses to serum growth factors and/or PDGF.

**DISCUSSION**

In this study, we examined both long- and short-term effects of apoE on cell proliferation. In addition, we have systematically compared the effects of apoE added to growth medium with the effects of stably expressing apoE in target cells. Specifically, this study demonstrates that both endogenous apoE and exogenous apoE (a) decrease proliferation rates (but not to 0) over an extended period, (b) inhibit activation of MAPKs and stimulation of DNA synthesis by serum, and (c) increase DNA synthesis in serum-deprived cells. Our results also confirm the data of others (4–6) demonstrating that apoE, independently of its role in lipid transport and metabolism, exerts an anti-proliferative effect on cells and attenuates serum growth factor signaling. However, our results extend these previous studies in three important ways. First, we demonstrated that the effects of exogenous apoE and endogenous apoE differ in both quantitative and qualitative ways. These differences are likely to reflect underlying differences in the mechanism of apoE action when presented to cells in these distinct ways. Second, we demonstrated that the effects of apoE are prolonged. A significant finding in this respect is that apoE exposure does not result in growth arrest. Although apoE prolongs progression through the G\(_1\) phase of the cell cycle, it does not cause cells to exit the cycle and become quiescent or to stop at either the G\(_1\)/S or G\(_2\)/M checkpoint. Third, apoE (in particular, endogenous apoE) dissociates serum growth factor signaling from cell proliferation. This is evident when one considers both the inhibitory effect of apoE on serum-stimulated progression from G\(_0\) through G\(_1\) and into S phase and the observation that E\(^+\) cells continue to synthesize sig-
significant amounts of DNA in the near absence of serum growth factors.

**Do Exogenous ApoE and Endogenous ApoE Act via Distinct Mechanisms?**—In a previous study comparing the effects of exogenous apoE and endogenous apoE on lipoprotein uptake and metabolism by mouse macrophages, we demonstrated clear differences in the response to apoE from these different sources (28, 29). We attributed the difference in action to the restriction of exogenous apoE and endogenous apoE to distinct subcellular compartments; exogenous apoE was detected in endocytic vesicles, whereas endogenous apoE was found in the secretory pathway. In this study, exogenous apoE and endogenous apoE shared the ability to slow proliferation rates, to elevate (or sustain) basal DNA synthesis in low serum, and to inhibit acute growth factor signaling. Endogenous apoE was more effective than exogenous apoE in eliciting the first two responses. It is clear that endogenous apoE can affect additional cellular functions that are not sensitive to the amount of exogenous apoE used in this study, including changes in cellular morphology and the reduction in the requirement for serum mitogens. The broader action of endogenous apoE might result from its ability to target an additional signaling pathway (other than leading to MAPK activation). Recent studies have shown that apoE may inhibit cell proliferation through receptor-independent mechanisms (41), but modulate cell migration through apoE-receptor-dependent pathways, although which of the family of apoE receptors is involved is not known (41, 42). Whether similar mechanisms are responsible for the effects of apoE on cell proliferation and the morphological changes observed in this study requires further investigation.

It is possible to explain the enhanced effectiveness of endogenous versus exogenous apoE on common targets by proposing that higher and more sustained concentrations of apoE at the cell surface were achieved following local synthesis and secretion than were achieved following addition of exogenous apoE to the culture medium. Human plasma contains ~36 µg/ml apoE, largely bound to lipoproteins (43). The minimal exogenous apoE concentration that consistently inhibited cell proliferation in our studies was 5 µg/ml, whereas E+ cells secreted on average only ~100 ng of apoE/ml/day. ApoE is a heparin-binding protein and therefore would be expected to stay bound to cell-surface heparin-containing proteoglycans. Such tight binding would result in an underestimation of the levels of apoE secreted, and the effective concentration acting on the cell surface would be significantly higher. However, immunological localization of apoE in E+ cells does not support this; the majority of apoE appeared to be cytoplasmic and not cell surface-associated (Fig. 1B). In macrophages, endogenous apoE and exogenous apoE follow separate intracellular trafficking routes. Endogenous apoE is associated with Golgi structures, whereas exogenous apoE is found in endocytic vesicles (28). In the E+ cells studied here, there was only minimal overlap between immunoreactive apoE and staining with Golgi markers (Figs. 1, B and C). Although these assays do not afford the resolution necessary to determine whether the bulk of endogenous apoE was cytosolic or vesicular (endocytic or pre-Golgi secretory), they raise the possibility that a significant fraction of endogenous apoE might be in a non-secretory compartment. We suggest it is likely that this intracellular apoE interacts with cytoskeletal components that are not accessible to exogenous apoE and that these interactions cause reorganization of cytoskeletal structures and alter signal transduction.

**How Does ApoE Affect Proliferation?**—Two possible mechanisms have been proposed to account for the anti-proliferative effects of apoE. In one, apoE acts directly to inhibit growth factor signaling. Browning et al. (5) have proposed that apoE, by virtue of its high affinity for heparin sulfate, interferes with growth factor signaling by competing for cell-surface binding sites. This model is unlikely to explain our results for two reasons. First, this mechanism should be limited to inhibiting signaling by mitogens that depend on heparin sulfate binding for efficient signaling, e.g. the fibroblast growth factors. However, in our assays, apoE was broadly effective, blocking the action of the mixture of mitogens present in calf serum and the action of purified PDGF. Second, apoE (exogenous and endogenous) inhibited MAPK activation, but failed to prevent either serum or purified PDGF stimulation of tyrosine phosphorylation or induction of c-fos expression. We showed previously that transcriptional activation of c-fos in F111 cells depends predominantly on signaling to the nucleus via phosphatidylinositol 3-kinase and c-Jun N-terminal kinase (21). Therefore, the different effect of apoE on MAPK activation and c-fos induction indicates that only a specific subset of signaling pathways activated by receptor tyrosine kinases are affected by apoE.

Paka et al. (44) have proposed an alternative mechanism in which apoE exerts a positive effect on synthesis of the heparin sulfate proteoglycan perlecan, which exerts the measured anti-proliferative effects on apoE. Although we cannot rule out a
role for perlecan in mediating the sustained effects of apoE (especially in \(E^+\) cells), the ability of exogenous apoE to rapidly inhibit growth factor signaling is not compatible with the delay (24–48 h) required for increased expression and synthesis of perlecan (44).

At present, we do not know how apoE disrupts growth factor signaling. An important clue might come from our demonstration that although apoE expression slows proliferation and inhibits mitogenic signaling, it does not result in growth arrest. Indeed, apoE expression enhances aspects of proliferation, notably DNA synthesis in the absence of external mitogens. Two precedents for such apparently dichotomous activities are worth noting. The first precedent comes from results that closely parallel ours, in which Chen and Gardner (45) demonstrated that retinoic acid has the ability both to stimulate mitogenesis of aortic smooth muscle cells and to inhibit the mitogenic action of endothelin on these same cells. Thus, retinoic acid inhibits signaling required for early G1 progression (mitogen activation of MAPK), but increases expression of cyclin D, which is required for the progression from late G1 into S phase. The second precedent comes from studies on oncogenic transformation. Acute expression of many oncocenes such as \(v\)-src and \(v\)-ras activates mitogenic signaling cascades and active transcription of target genes. However, in cells that have been transformed by stable expression of these proteins, signaling in response to exogenous growth factors is inhibited (46–48). It is possible that endogenous apoE acts in an analogous, albeit much more constrained manner, resulting in uncoupling of cell cycle progression from dependence on positive extracellular signals.

Abnormalities in the control of cell proliferation and differentiation are associated with a number of diseases, including atherosclerosis. Hyperproliferation of smooth muscle cells contributes to the progressive formation of atherosclerotic plaques (31). Accumulation of apoE in atherosclerotic lesions (32) is believed to be anti-atherogenic by promoting cellular cholesterol efflux and increasing lipid metabolism (7, 10). The previous observation that apoE inhibits smooth muscle cell proliferation (6) and our current finding that the anti-proliferative effect of apoE is sustained over long periods provide an additional mechanism for the anti-atherogenic action of apoE.

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