Apolipoprotein E expression in aortic smooth muscle cells: the effect of βVLDL

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Abstract The expression of apolipoprotein E in cultured neonatal rabbit aortic smooth muscle cells was examined. Northern blot analysis determined that there was a single RNA transcript of approximately 1.2 kb. Moreover, a polyclonal antibody against rabbit apolipoprotein E was prepared in a goat and used in immunoprecipitations to demonstrate that the cultured cells secreted apolipoprotein E into the media. A double band typical of apolipoprotein E migrated at apparent molecular masses of 37 and 39 kDa. Analysis of steady-state levels of apolipoprotein E mRNA demonstrated that expression increased as cell seeding density increased. When examined as a function of time in culture, there were two peaks of expression evident 1 day and 8 days after seeding. The addition of βVLDL (β-very low density lipoprotein) to smooth muscle cells increased both [³H]thymidine incorporation into DNA as well as cell number and these increases were accompanied by a decrease in the levels of apolipoprotein E mRNA in cells treated with the lipoprotein for 1 and 7 days. After incubation of the cultures with βVLDL for 1 week, the cells were radiolabeled with [³⁵S]methionine and the media was subjected to immunoprecipitation with anti-apolipoprotein E. The data revealed that the amount of apolipoprotein E secreted into the media decreased in the presence of βVLDL. ▪▪ In summary, these results show that apolipoprotein E expression in aortic smooth muscle cells is regulated by cell density, time in culture, cell proliferative state, and βVLDL addition. These observations may have relevance to the conditions that are known to accompany the development of the atherosclerotic lesion.—**Schreiber, B. M., H. V. Jones, and C. Franzblau.** Apolipoprotein E expression in aortic smooth muscle cells: the effect of βVLDL. *J. Lipid Res.* 1994. **35:** 1177–1186.

Supplementary key words atherosclerosis • apoE mRNA • cell seeding density • ELISA • smooth muscle cell differentiation

Apolipoprotein E is a major constituent of plasma lipoproteins that classically functions in lipid transport and redistribution (reverse cholesterol transport), although other roles have been postulated (1–4). Apolipoprotein E mediates the binding of lipoproteins to the apoB,E (LDL) receptor (5, 6). In its capacity to remove cholesterol from peripheral tissues to the liver, apolipoprotein E probably plays an important role in inhibiting the development and/or progression of atherosclerosis. Synthesis of apolipoprotein E by aortic smooth muscle cells has been shown (7–9) and is likely to contribute to local arterial pools of the apolipoprotein. These cells are known to be intimately involved in atherogenesis as they migrate from the medial layer of the blood vessel to its intima where differentiation results in proliferation and connective tissue synthesis (10). Although Majack et al. (9) have suggested that the proliferative state of the smooth muscle cell influences apolipoprotein E expression, little else is known about the parameters that affect the synthesis of apolipoprotein E by these cells. These studies were undertaken to examine apolipoprotein E expression in cultured aortic smooth muscle cells. Studies from our laboratory have established that cultured neonatal rat cells synthesize and secrete apolipoprotein E (7). Moreover, cells from both rat and rabbit undergo phenotypic changes that result in a multilayered culture with an extensive insoluble extracellular matrix (11–14). Treatment with the atherogenic lipoprotein, βVLDL, causes intracytoplasmic lipid droplet formation reminiscent of the smooth muscle-derived foam cells characteristic of atherosclerosis (15). The experiments reported here demonstrate that the expression of apolipoprotein E mRNA in cultured neonatal rabbit aortic smooth muscle cells is a function of cell density and time in culture. Moreover, apolipoprotein E levels decrease in cell cultures treated with βVLDL.

MATERIALS AND METHODS

Isolation and culture of neonatal rabbit aortic smooth muscle cells

Neonatal rabbit aortic smooth muscle cells were isolated aseptically from the aortae of 3-day-old New Zealand White rabbits (Pine Acres Rabbitry, Brattleboro,

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Abbreviations: βVLDL, β very low density lipoproteins; LDL, low density lipoproteins; LDS, lipoprotein-deficient serum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MF, methionine-free.
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VT). Aortae were removed, stripped to remove the intima, and minced. Digestion with collagenase (Type IA; Sigma Chemical Co., St. Louis, MO) and porcine pancreatic elastase (Type III; Sigma Chemical Co.) was performed as described previously (11, 12, 15, 16), and cells were seeded at a density of 5×10^5 cells/25 cm^2 tissue culture flask in Dulbecco's modified Eagle's medium (J. R. H. Biosciences, Lenexa, KS; cat. no. 56-469-111) supplemented with 3.7 g/l NaHCO$_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM nonessential amino acids (GIBCO Laboratories, Grand Island, NY), and 1 mM MEM sodium pyruvate solution (GIBCO Laboratories; DMEM), and 20% fetal bovine serum (Sigma Chemical Co; FBS). Cells maintained in primary stage for 1 week were passaged after trypsinization with 0.5% trypsin/0.02% EDTA (GIBCO Laboratories). Studies were executed on cells in first or second passages. Cells were plated in DMEM containing 10% FBS at a density of 20×10^3/cm^2 (unless otherwise stated). In experiments in which steady-state levels of apolipoprotein E mRNA were monitored as a function of either time in culture or cell seeding density, the cells were maintained in DMEM containing 10% FBS. In experiments in which the effect of βVLDL was evaluated, 1 day prior to its addition, cells were washed twice with Puck's Saline G, and DMEM containing lipoprotein-deficient serum (LDS) was added. After an additional incubation of 1 day, βVLDL was added. All cultures were fed twice weekly.

Cell numbers were determined with a Coulter Counter (Coulter Electronics Inc.; Hialeah, FL).

Isolation and characterization of βVLDL

βVLDL was isolated and characterized as previously described by density gradient ultracentrifugation (d < 1.006 g/ml) from serum of male New Zealand White Rabbits fed a diet rich in cholesterol and peanut oil (16). The protein concentration was determined as described previously (15).

Preparation of lipoprotein-deficient serum (LDS)

LDS was prepared from FBS by ultracentrifugation at a density of 1.21 g/ml as described previously (15). The final protein concentration of media containing LDS was equivalent to that of 10% FBS.

Analysis of [³H]thymidine incorporation into DNA

Cells were plated into 96-well flat-bottom tissue culture plates in DMEM containing 10% FBS. Media were removed from subconfluent cultures and cells were washed twice with Puck's Saline G. DMEM containing LDS was then added to the wells, which were incubated overnight, at which time βVLDL was added. After the cells were allowed to incubate with the lipoprotein for 20 h, the media were removed, and the wells were washed with serum-free DMEM. Cells were radiolabeled for 4 h with 100 μl

[³H]thymidine (20 μCi/ml; 5 Ci/mmol; DuPont NEN Research Products, Boston, MA) in serum-free DMEM. Cells were harvested and [³H]thymidine incorporation into DNA was assessed as described previously (16).

Analysis of steady-state levels of apolipoprotein E mRNA

Cells were plated into 75 cm^2 cell culture flasks in DMEM containing 10% FBS and the total cellular RNA was extracted as described below. In experiments in which βVLDL was added, 1 day prior to its addition, media were removed and the cells were washed twice with Puck's Saline G. Medium containing 10% LDS was added, and on the following day, βVLDL was added (0, 10, 100 μg/ml). At the appropriate times, total cellular RNA was harvested from pooled flasks as described below. Each experiment was repeated at least two times using a fresh βVLDL preparation and a different set of primary cells.

Total cellular RNA was extracted essentially as described by Chomczynski and Sacchi (17). Cells were washed with phosphate-buffered saline (PBS), pH 7.4, and then scraped into 1.0 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. One-tenth ml 2 M sodium acetate (pH 4.0), 1.0 ml water-saturated phenol, and 0.2 ml chloroform–isoamyl alcohol 49:1 were added sequentially. The mixture was cooled for 15 min on ice, and then centrifuged at 10,000 g for 20 min at 4°C. One ml isopropanol was added to the supernatant, which was then placed at −20°C overnight. After centrifugation, the pellet was washed with 75% ethanol and then was dried under a stream of N$_2$, dissolved in water, and stored at −20°C.

Steady-state levels of apolipoprotein E mRNA were determined by Northern blot analysis of total cellular RNA. The RNA samples (15 μg/lane) were denatured and electrophoresed through a 1% agarose, 6% formaldehyde gel (18). Electrophoresis was performed at a constant voltage of approximately 35 volts for 500–600 volt-h. The gel was then washed in water and stained with ethidium bromide (1 ng/ml) to evaluate loading.

The gel was transferred to a hybridization membrane (BioTrace HP; Gelman Sciences, Ann Arbor, MI) electrophoretically in 10 mM Tris, 0.59 mM EDTA, 5 mM Na acetate (pH 7.8), and baked at 80°C for at least 30 min. The blots were hybridized according to the manufacturer's instructions, except that a final concentration of 50% deionized formamide at 42°C was used. Denatured radiolabeled DNA probe was added (5×10^6 dpm) and hybridization was carried out for at least 16 h. Washes at room temperature were performed using first 1 × SSC, 0.1% SDS and then 0.4 × SSC, 0.1% SDS. The blots were exposed to film (Kodak XAR-5) with intensifying screens at −80°C.

DNA probes were labeled with [³²P]dCTP by random

priming using the instructions supplied by the commercial manufacturer of the kit. A near full-length cDNA probe encoding rabbit apolipoprotein E was kindly supplied by Dr. Vassilis Zannis of Boston University School of Medicine. For comparison, blots were also probed for actin with a 700-base pair cDNA [pAC-16; (19)] and for histone H3 with a genomic fragment encoding amino acids 57–125 [pRAH3.2; (20)].

Preparation of goat anti-rabbit apolipoprotein E antiserum

Apolipoprotein E for use as an immunogen was prepared from βVLDL. The lipoprotein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described below (except that a 4.5% stacking gel was used). The apolipoprotein E band was cut from the gel which was then extracted in 0.125 M Tris (pH 6.8) containing 0.001 M EDTA, 10% glycerol, and 0.1% SDS. Extracts were then dialyzed against water, lyophilized, and stored frozen to time of use. Atlantic Antibodies (Stillwater, MN) was supplied with the purified apolipoprotein E which was used to immunize a goat. Multiple boosts (100 μg) were administered during a period of approximately 1 year until the titer by enzyme-linked immunosorbent assay (ELISA) was acceptable (see Results section). The goat was then bled and the immunoglobulin fraction was prepared by immunoprecipitation with ammonium sulfate (21). Preimmune serum or normal goat serum (Sigma Chemical Co.; Goat Serum Donor Herd cat. no. G6767) were treated similarly and used as controls in both ELISA and immunoprecipitation protocols.

ELISA

In order to determine the titer of the goat anti-apolipoprotein E antiserum, a competitive ELISA was developed. All incubations were performed in a humidified chamber. Apolipoprotein E (0.1 μg/well in 0.1 ml PBS) was added to ethanol-washed microtiter trays and incubated overnight at 4°C. Wells were then washed 3 times with 0.2 ml PBS (pH 7.4) containing 0.001% Tween 80 (PBS-Tween), and blocking was achieved by the incubation of 0.2 ml fatty acid-free bovine serum albumin (Sigma Chemical Co.; cat. no. A7511; 1 mg/ml in PBS-Tween) for 1 h at 37°C, at which time the wells were washed 3 times with 0.2 ml PBS-Tween. After removal of the last wash, apolipoprotein E (10 μg/well in 25 μl PBS-Tween) or PBS-Tween (blank wells) was added. Goat anti-rabbit apolipoprotein E antiserum or normal goat serum was added (25 μl), and the trays were incubated at room temperature for 2 h. Wells were then washed 3 times with 0.2 ml PBS-Tween. Alkaline phosphatase-conjugated rabbit anti-goat IgG [100 μl 1:1,000 dilution (Sigma Chemical Co.; cat. no. A-7650)] was added and incubated for 1 h,

and the wells were again washed 3 times with PBS-Tween. Wells were then incubated with 100 μl substrate buffer (28.2 mg phosphatase substrate; Sigma Chemical Co.; cat. no. 104-O) in glycine buffer] for 30 min at room temperature at which time 5 μl 3 N NaOH was added and color development was evaluated with an ELISA plate reader.
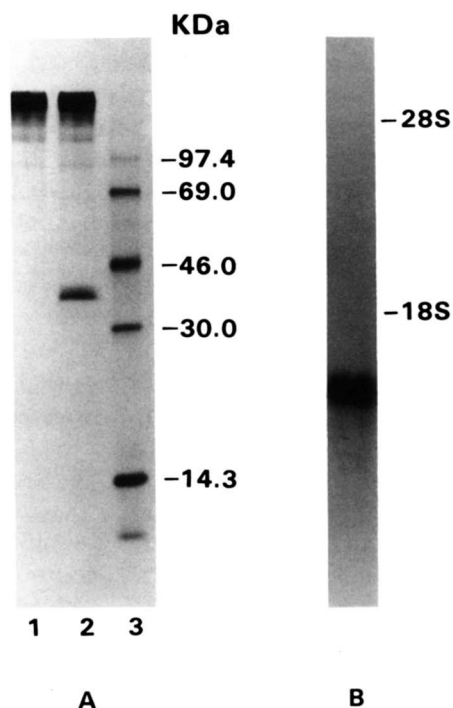
Immunoprecipitation for apolipoprotein E

Labeling of media proteins with [^{35}S]methionine. Prior to the addition of [^{35}S]methionine, media were removed and the cells were washed twice with Puck's Saline G. Methionine-free DMEM (Gibco Laboratories cat. no. 320-1970AJ) supplemented with 2 mM L-glutamine (Gibco Laboratories cat. no. 320-5039), 3.7 g/l NaHCO$_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM nonessential amino acids, and 1 mM MEM sodium pyruvate solution (MF-DMEM) was then added and the cells were incubated for 30 min, at which time the media were removed and the cells were refed with MF-DMEM containing [^{35}S]methionine (40 μCi/ml; New England Nuclear, Boston, MA; [^{35}S]Protein Labeling Mix; cat. no. NEG-072). The cells were radiolabeled for 4 h and then placed on ice. Proteolytic inhibitors were added to the media to achieve final concentrations of 5 mM EDTA, 10 mM ϵ-amino caproic acid, 10 mM N-ethylmaleimide, 50 μM phenylmethylsulfonyl fluoride, and 300 μM p-hydroxymercuribenzoate. Samples were then dialyzed against water and lyophilized. This experiment was repeated 2 times using fresh βVLDL preparations and different sets of primary cells. Duplicate media samples were analyzed for apolipoprotein E by immunoprecipitation followed by SDS-PAGE.

Immunoprecipitation. Lyophilized media samples were solubilized in PBS containing 0.5% SDS, 0.5% Triton X-100, and 0.25% deoxycholate (pH 7.4), and then incubated with the immunoglobulin fraction (100–400 μg) prepared from either goat anti-rabbit apolipoprotein E or normal goat serum for 2 h at 37°C. IgGsorb (1:6 dilution; the Enzyme Center, Malden, MA) was then added, and the reaction was incubated for 30 min at room temperature. The pellet was then washed once with PBS containing 1% SDS, 1% Triton X-100, and 0.5% deoxycholate (pH 7.4) plus 1 mg/ml bovine serum albumin, followed by 3 washes in the same buffer without albumin. After removal of the supernatant from the last wash, samples were subjected to SDS-PAGE as described below.
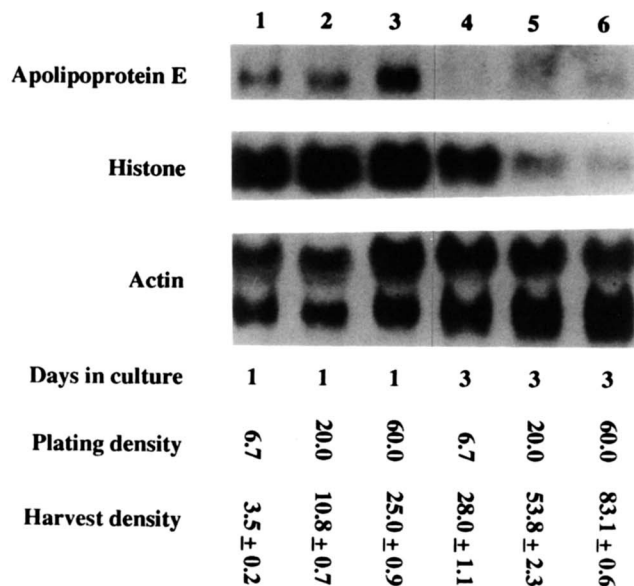
SDS-PAGE. SDS-PAGE was performed essentially as described by Laemmli (22). A 3% stacking gel and 12% running gel were used. Fluorography was performed essentially as described by Bonner and Laskey (23). The fluorograms were scanned on a Molecular Dynamics Computing Densitometer equipped with ImageQuant Software v2.0.

RESULTS

In order to utilize a homologous system incorporating βVLDL isolated from cholesterol-fed rabbits and smooth muscle cells derived from neonatal rabbits, it was first necessary to determine whether the rabbit cells synthesize apolipoprotein E [since earlier studies were performed in cultures derived from rat aortae (7)]. Therefore, a polyclonal antiserum directed against rabbit apolipoprotein E was prepared in a goat and screened using the ELISA protocol. The titer of the final trial bleed was greater than $1/20 \times 10^{-3}$. To determine whether the rabbit smooth muscle cells synthesize and secrete apolipoprotein E into the culture media, cells were incubated in 1% FBS for 3 days, and then radiolabeled with [^{35}S]methionine. The medium was harvested and subjected to immunoprecipitation using the immunoglobulin fraction of the antiserum. **Figure 1A** shows the results of such an assay in which SDS-PAGE was performed on the immunoprecipitated material. A double band typical of apolipoprotein E migrated at apparent molecular masses of 37 and 39 kDa (24). At times, a single diffuse band was visualized instead
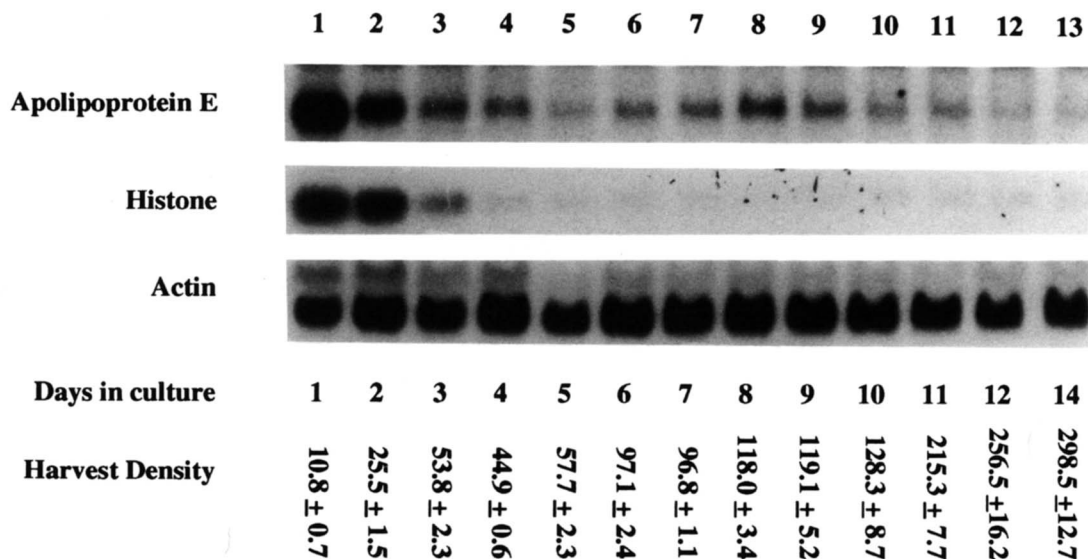

Fig. 2. The effect of cell seeding density on steady-state levels of apolipoprotein E mRNA. Total cellular RNA was extracted 1 day (lanes 1, 2, and 3) and 3 days (lanes 4, 5, and 6) after seeding. Plating densities are expressed as cell number $\times 10^{-3}/cm^2$ and include 6.7×10^3 cells/cm² (lanes 1 and 4), 20.0×10^3 cells/cm² (lanes 2 and 5), or 60.0×10^3 cells/cm² (lanes 3 and 6). Fifteen µg RNA from each sample was subjected to Northern blot analysis using probes to detect either apolipoprotein E, histone H3, or actin. At harvest times, cells from equivalent 4 cm² wells were trypsinized and counted with the aid of a Coulter Counter. Cell counts are expressed as mean cell number $\times 10^{-3}/cm^2 \pm$ SEM (n = 4).

Fig. 1. Apolipoprotein E is expressed in cultured neonatal rabbit aortic smooth muscle cells. A) Serum-deprived cells were radiolabeled with [^{35}S]methionine. The media was harvested, subjected first to immunoprecipitation (2.5×10^6 cpm) using the immunoglobulin fraction of either the pre-immune serum (lane 1) or the anti-apolipoprotein E antiserum (lane 2), and then to SDS-PAGE as described in Materials and Methods. The migration of markers (lane 3) is shown and their molecular masses are designated. B) Total cellular RNA was extracted from quiescent cells and 15 µg RNA was subjected to Northern blot analysis using a rabbit apolipoprotein E cDNA probe (the migration of 28S and 18S rRNA is designated).

of the two bands. Using Northern blot analysis it was also determined that apolipoprotein E expression could be detected at the level of mRNA (Fig. 1B); the cDNA hybridized with a single RNA species of approximately 1.2 kb.

Once it had been established that apolipoprotein E was synthesized by the cultured neonatal rabbit aortic smooth muscle cells, studies were performed to monitor the expression of steady-state levels of apolipoprotein E, histone H3, and actin mRNA (**Fig. 2**) as a function of cell seeding density. The expression of apolipoprotein E and total cell number from cultures harvested 1 day and 3 days after plating at densities of 6.7, 20.0, and 60.0×10^3 cells/cm² was examined. The data in Fig. 2 (lanes 1, 2, and 3) show that one day after plating, steady-state levels of apolipoprotein E increased as cell plating density increased. Three days after plating, the cell number increased in cultures seeded at all densities, however, apolipoprotein E mRNA expression decreased in all cultures (lanes 4, 5, and 6) when compared to the day 1 levels. The density-dependent effect was still apparent, in that expression of apolipoprotein E was evident in cultures derived from cells plated at the highest density (lane 6) but not at the lowest (lane 4). Steady-state levels of histone H3 mRNA decreased with time in culture for the

Fig. 3. The effect of time in culture on steady-state levels of apolipoprotein E mRNA. Total cellular RNA was extracted 1–14 days after seeding with 20.0×10^3 cells/cm², and 15 μg RNA from each sample was subjected to Northern blot analysis using probes to detect either apolipoprotein E, histone H3, or actin (note that lanes 1 and 3 are represented on Fig. 2). At harvest times, cells from equivalent 4 cm² wells were trypsinized and counted with the aid of a Coulter Counter. Cell counts are expressed as mean cell number $\times 10^{-3}$/cm² $\pm$ SEM (n = 4 except on d. 11, n = 3).

cells seeded at the higher densities, demonstrating that cell proliferation began to decrease in these cultures.

As steady state mRNA levels of apolipoprotein E decreased from day 1 to day 3, it appeared that time in culture also influenced apolipoprotein E expression. Moreover, the cell number in cultures harvested 1 day after plating with the highest seeding density (Fig. 2; lane 3) was virtually identical to cultures harvested 3 days after plating with the lowest seeding density (Fig. 2; lane 4), i.e., approximately 25×10^3 cells/cm²; however, steady state mRNA levels were greater in the high density, day-1 cells than the low density, day-3 cells suggesting that cell density does not solely dictate apolipoprotein E expression. Therefore, the expression of apolipoprotein E as a function of time was evaluated. Cell cultures were seeded with the middle dose of cells, i.e., 20.0×10^3 cells/cm². Total cellular RNA was harvested at various times during the lifetime of the culture and analyzed for steady-state levels of apolipoprotein E, histone H3, and actin mRNA (**Fig. 3**). Cell proliferation was also monitored and these data are presented as well. Steady-state mRNA levels of the cell cycle-related gene, histone H3, were high very early in the lifetime of the culture, indicating that the cells were most actively proliferating at this time. A complicated pattern of expression of apolipoprotein E mRNA that is not totally dependent on cell proliferative state (or total cell number) was apparent, however. The apolipoprotein E transcript was expressed initially at very high levels 1 day after seeding. There was a gradual decrease followed by another peak of expression after the cells had reached confluence 8 days after seeding.

We were also interested in how the atherogenic lipoprotein, βVLDL, would affect apolipoprotein E ex-

pression in smooth muscle cells. In a previous report, we demonstrated that the cell number in cultures of neonatal rabbit aortic smooth muscle cells treated with βVLDL was greater than their nontreated counterparts (15). In order to pursue this finding, the effect of βVLDL on [³H]thymidine incorporation into DNA of actively proliferating cells was examined. **Figure 4** demonstrates that βVLDL induced a dose responsive increase in
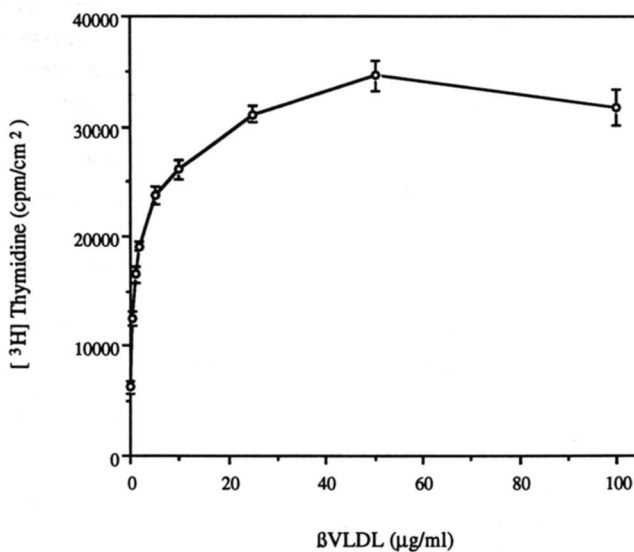
Fig. 4. The effect of βVLDL on [³H]thymidine incorporation into DNA of actively proliferating cultures. One day prior to the addition of βVLDL, sub-confluent cells were treated with LDS overnight, and then βVLDL was added for 20 h, at which time [³H]thymidine incorporation into DNA was determined as described in Materials and Methods. Data are expressed as mean cpm/cm² $\pm$ SEM (n = 10, control; n = 5, all others).
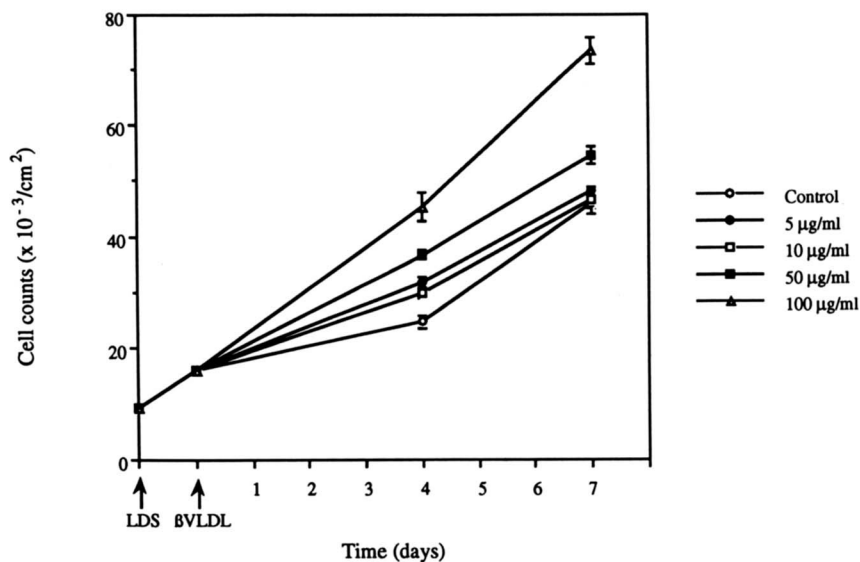
Fig. 5. The effect of βVLDL on cell proliferation. One day prior to the addition of βVLDL, sub-confluent cells were treated with LDS overnight, and then 0 (control; open circles), 5 (closed circles), 10 (open squares), 50 (closed squares), or 100 μg βVLDL/ml (open triangles) was added for 4 days and 7 days, at which time cells were trypsinized and counted with the aid of a Coulter Counter. Data are expressed as mean cell count $\times$ 10^{-3}/cm^2 $\pm$ SEM (n = 4).

[3H]thymidine incorporation into DNA that reached a plateau at approximately 25–50 μg βVLDL/ml. Moreover, **Fig. 5** shows that lipoprotein treatment caused an increase in cell number; in comparison to control cultures, the data show that 4 days after the addition of 5, 10, 50, and 100 μg βVLDL/ml, cultures contained 28.8%, 21.4%, 49.1%, and 83.8% more cells, respectively.

In order to determine the effect of βVLDL on apolipoprotein E expression, steady-state mRNA levels were determined in cultures that had been maintained in LDS in the presence or absence of the lipoprotein for up to 1 week. **Figure 6** shows that, upon addition of the lipoprotein, there was a decrease in steady-state levels of apolipoprotein E mRNA which was evident after 24 h and persisted for 1 week in cells continuously maintained in the presence of the βVLDL. Additional experiments showed that down-regulation of apolipoprotein E expression was evident 2 h after the addition of the lipoprotein (data not shown). For comparison, the effect of βVLDL on histone H3 and actin expression is also presented. The proliferative response (demonstrated in Figs. 4 and 5) was accompanied by an increase in expression of histone H3 mRNA at the 24 h time point, whereas at the later time point, levels of histone H3 mRNA were equivalent in control and βVLDL-treated cultures. Therefore, despite the persistence of the decrease in apolipoprotein E expression, βVLDL may only activate a single round of DNA replication.

Once it was determined that steady-state levels of apolipoprotein E mRNA decreased in response to βVLDL, it was of interest to establish whether synthesis

and secretion of the protein were also affected. Therefore, cells were incubated in LDS in the absence or presence of either 10 or 100 μg βVLDL/ml for 1 week, at which time the cultures were radiolabeled with [35S]methionine, and immunoprecipitated with goat anti-apolipoprotein E immunoglobulins. **Figure 7** shows that the amount of radiolabeled apolipoprotein E found in the media decreased in the presence of βVLDL. Densitometric scans of duplicate samples treated with 0 (control), 10 μg βVLDL/ml,
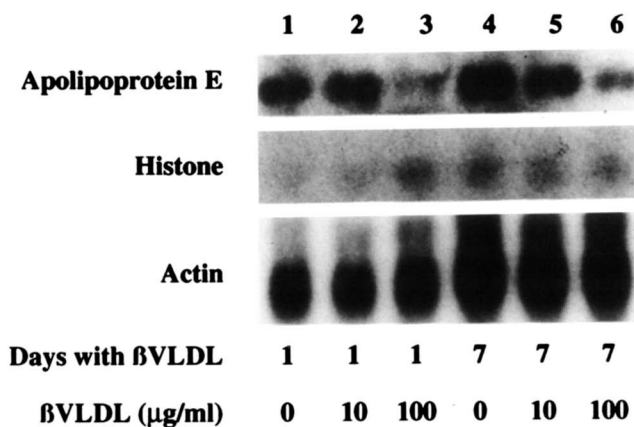
Fig. 6. The effect of βVLDL on steady-state levels of apolipoprotein E mRNA. One day prior to the addition of βVLDL, sub-confluent cells were treated with LDS overnight. Total cellular RNA was extracted 1 day (lanes 1, 2, and 3) or 7 days (lanes 4, 5, and 6) after the addition of 0 (control; lanes 1 and 4), 10 μg βVLDL/ml (lanes 2 and 5), or 100 μg βVLDL/ml (lanes 3 and 6), and 15 μg RNA from each sample was subjected to Northern blot analysis using probes to detect either apolipoprotein E, histone H3, or actin.
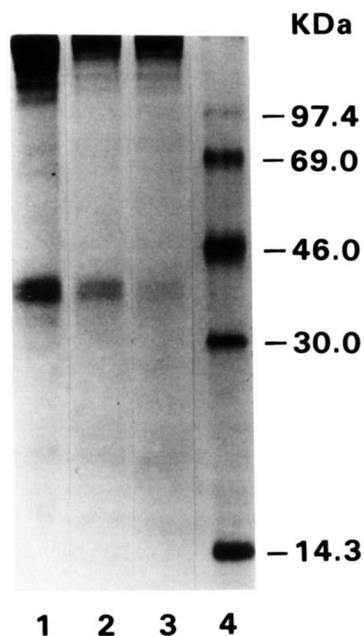
Fig. 7. The effect of βVLDL on apolipoprotein E in the media. One day prior to the addition of βVLDL, sub-confluent cells were treated with LDS overnight. Cells were radiolabeled with [^{35}S]methionine 7 days after the addition of 0 (control; lane 1), 10 µg βVLDL/ml (lane 2), or 100 µg βVLDL/ml (lane 3). Media was harvested, subjected first to immunoprecipitation (0.25×10^6 cpm) using the immunoglobulin fraction of the anti-apolipoprotein E antiserum, and then to SDS-PAGE as described in Materials and Methods. The migration of markers (lane 4) is shown and their molecular masses are designated.

or 100 µg βVLDL/ml show that relative densities of the apolipoprotein E bands were 23.3, 9.5, and 7.8, respectively.

DISCUSSION

Recent work has established that neonatal rat and rabbit aortic smooth muscle cells in culture represent an excellent model for studying the functionality of this cell type in vivo (11–13, 15, 16, 25, 26). The cells from both species grow and become multilayered cultures, forming the "hills and valleys" growth pattern typical of smooth muscle cells in culture. Extensive extracellular matrices consisting of large quantities of insoluble elastin, collagen, as well as proteoglycans are produced. We have recently reported that these cells metabolize βVLDL and accumulate cholesteryl esters (15).

In the present study, apolipoprotein E expression in the cultured neonatal rabbit aortic smooth muscle cells was examined in the presence and absence of βVLDL. The increase in apolipoprotein E mRNA with increasing cell seeding density establishes this parameter as a regulator of apolipoprotein E expression. The pattern of time-dependent expression during 2 weeks in culture suggests that apolipoprotein E is regulated by smooth muscle cell

differentiation, a process which is still not completely understood. It has been shown that vascular smooth muscle cells undergo phenotypic changes in culture that are characterized by a loss of contractility followed by cell proliferation and biosynthesis of extracellular matrix components (27–30). It has been suggested that these modifications occur during the process of formation of the atherosclerotic plaque as the smooth muscle cell migrates from the intima to the medial layer of the blood vessel (31–33). Cultured smooth muscle cells demonstrate changes in α-smooth muscle actin and myosin heavy chain as a function of cell growth state and density (34, 35); however, it has been suggested that the relationship between cell proliferation and differentiation is complex, and loss of contractile proteins is not absolutely essential for DNA synthesis to proceed (34–37). Components of the extracellular matrix have also been shown to influence smooth muscle cell differentiation (30, 38–42) and the accumulation of components such as the insoluble elastin and glycosaminoglycan-rich extracellular matrix in our culture system is likely to contribute to phenotypic alterations. The data demonstrate that apolipoprotein E expression is influenced in a complex fashion by smooth muscle cell differentiation. At the time of seeding, apolipoprotein E expression is high and begins immediately to decline. At approximately 1 week in culture, the neonatal aortic smooth muscle cells show another peak in apolipoprotein E expression, which may be associated with the multilayering process. It is interesting to speculate on the possibility that such changes in apolipoprotein E may modulate smooth muscle cell differentiation. Apolipoprotein E mRNA levels were highest during the first few days in culture which is the most active period of cell proliferation. This is in contrast to findings by Majack et al. (9) who showed that heparin's growth-inhibiting properties cause an increase in apolipoprotein E mRNA. Moreover, these workers (9) showed that apolipoprotein E secretion is stimulated in cells rendered quiescent by serum deprivation. Our data (not shown) also show that FBS induced a decrease in apolipoprotein E mRNA expression in serum-deprived cells; however, as cell proliferation decreases with time in culture, cells maintained in 10% FBS express very little apolipoprotein E mRNA (Fig. 2; days 10–14) in comparison to actively proliferating cells (Fig. 2; days 1–3). Therefore, the relationship between apolipoprotein E expression and cell proliferation is complex and it appears that the point in the cell cycle in which cell proliferation is blocked may critically affect apolipoprotein E expression.

We have also examined the effect of βVLDL-induced cell proliferation on apolipoprotein E expression. When injury to the artery occurs, cells can be exposed to lipoproteins which can potentially lead to alterations in smooth muscle cell phenotype (43). The major lipoprotein that accumulates in the plasma of cholesterol-fed animals

is βVLDL (44). βVLDL is also known to accumulate in the plasma of patients with Type III hyperlipoproteinemia, a disease associated with premature atherosclerosis (45). In recent years, the potential contribution of this lipoprotein to changes associated with atherosclerosis has been suggested by studies in smooth muscle cells (15, 46, 47) as well as mononuclear phagocytes (16, 48). βVLDL receptors on foam cells (49) isolated from the aorta have been identified. Manderson et al. (50) injected βVLDL into rabbits in concentrations that led to small transient increases in cholesterol and demonstrated lipid accumulation in smooth muscle cells and mononuclear leukocytes, as well as infiltration by the latter cell type into injured arteries. We have previously shown that βVLDL induces mononuclear phagocytes to secrete a growth-potentiating activity for cultured neonatal rat aortic smooth muscle cells (16). Moreover, in a recent report (15) and in this study, βVLDL was shown to directly affect proliferation of rabbit smooth muscle cells. Although apolipoprotein E synthesis increases in mouse peritoneal macrophages and human monocytes treated with LDL, acetylated LDL, or βVLDL (51–54), this report demonstrates that lipoprotein can decrease the expression of apolipoprotein E by cultured neonatal aortic smooth muscle cells, conceivably via its ability to increase cell proliferation. The lipoprotein-induced decrease in apolipoprotein E expression was evident at the level of steady-state mRNA expression as well as newly synthesized and secreted protein. It is known that apolipoprotein E functions as a regulator of cholesterol distribution (1). The βVLDL-induced decrease in apolipoprotein E levels reported here suggests that this role may be jeopardized by atherogenic lipoproteins as smooth muscle cells proliferate during atherogenesis. One can hypothesize that lipoprotein infiltration may contribute not only to smooth muscle cell proliferation characteristic of lesion development, but also to decreases in apolipoprotein E synthesis that could ultimately lead to further deposition of lipid due to a compromised capacity for export from the vessel wall. Knowledge of how aortic smooth muscle cell apolipoprotein E synthesis is regulated is likely to enhance our understanding of lipid metabolism in the normal and diseased artery.

In conclusion, the expression of steady-state levels of apolipoprotein E mRNA in cultured neonatal rabbit aortic smooth muscle cells is regulated by cell density, time in culture, and βVLDL addition. Undoubtedly, the observed changes in apolipoprotein E are the result of a complex interplay between positive and negative regulators of differentiation and cell proliferation. ∎

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