We reported previously that a 116-kDa lipoprotein lipase (LPL)-binding protein from endothelial cells has sequence homology to the amino-terminal region of apolipoprotein (apo) B. We now tested whether endothelial cells synthesize apoB mRNA and protein. Primers were designed to the human apoB cDNA sequence and reverse transcription polymerase chain reaction was performed using total RNA isolated from bovine and human endothelial cells. With primers to the 5' region of the apoB mRNA (amino-terminal region of apoB protein) expected size PCR products were generated from both bovine and human endothelial cells as well as from mouse liver RNA, which was used as a control. Primers designed to the 3' region of apoB mRNA generated PCR products from human endothelial cells and HepG2 cells but not from bovine or mouse cells. These data suggest that endothelial cells contain full-length apoB mRNA and that the 5' or the amino-terminal region of apoB is highly conserved from mouse to human. This was confirmed by direct sequencing of the mouse and bovine PCR products. To test whether apoB protein was produced, bovine endothelial cell proteins were metabolically labeled with [35S]methionine/cysteine or [3H]leucine and immunoprecipitated with anti-human apoB antibodies. Using extracts from cells labeled for 1 h, monoclonal antibody 47, directed to the low density lipoprotein receptor binding region of apoB, precipitated a protein of approximate molecular mass 550,000, the size of full-length apoB. Immunoprecipitation of the 550-kDa protein was abolished in the presence of added unlabeled low density lipoprotein. From cells labeled for 16 h, a 116-kDa protein was immunoprecipitated by polyclonal anti-apoB antibodies. This protein was partly released from cells by heparin treatment. Pulse-chase analysis showed that the 116-kDa fragment appeared at the same time as the full-length apoB began disappearing. The immunoprecipitated 116-kDa fragment also bound labeled LPL on ligand blot, further suggesting that it is an amino-terminal fragment of apoB. Incubation of endothelial cells with oleic acid (0.25 and 0.5 mM) did not significantly alter the production of either the full-length apoB or the 116-kDa fragment. These data show that endothelial cells synthesize apoB. The full-length apoB appears to be cleaved to form a 116-kDa fragment that can function as a LPL-binding protein.

Apolipoprotein (apo) B is the major apoprotein present in circulating plasma lipoproteins, including chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) (1, 2). ApoB exists in two isoforms, apoB-100 and apoB-48. ApoB-100, a large glycoprotein with an approximate molecular mass of 550 kDa is synthesized in liver; apoB-48, which arises by mRNA editing, is primarily synthesized in intestine. ApoB-100 has several functional and structural domains. It contains hydrophobic domains throughout its length that are believed to be involved in lipid binding (3). The carboxyl-terminal region of the protein has domains that bind to LDL receptors (4, 5) and to heparin (6).

The amino-terminal region of apoB is the relatively hydrophilic part of apoB. It contains six cystine disulfide bridges making it highly globular (1, 2). Models of apoB suggest that this region of apoB extends away from the lipid core of LDL (7, 8). Although the amino-terminal, but not the COOH-terminal, region of apoB is preserved in the two circulating forms of apoB, apoB-100 in VLDL and LDL, and apoB-48 in chylomicrons, no function has been attributed to this region. Previous studies from our laboratory showed that lipoprotein lipase (LPL) binds to the amino-terminal region of apoB (9, 10). A 116-kDa LPL-binding protein isolated from bovine aortic endothelial cells had sequence homology with regions near the amino terminus of apoB (9). Monoclonal antibodies (mAb) that recognize the amino-terminal, but not the carboxyl-terminal, region of apoB inhibited LPL binding to endothelial cells (9). Thus, we postulated that LPL binding to endothelial cells may involve two molecular interactions, the well known LPL association with heparan sulfate proteoglycans (11–13) and a second protein-protein interaction between LPL and apoB (9).

The origin of the 116-kDa apoB fragment found in cultured endothelial cells was uncertain. It might have been synthesized by the endothelial cells, or it could have originated in the culture medium. ApoB is extremely susceptible to proteolysis and lipid-free amino-terminal fragments of apoB have been found in serum (14, 15). Much of the apoB-100 that is synthesized by cells undergoes intracellular degradation (16). However, a 85-kDa amino-terminal apoB fragment escapes intracellular degradation and is secreted by the human liver cell line HepG2 (17, 18). Although full-length apoB is degraded by apoB-transfected Chinese hamster ovary cells, these cells will secrete amino-terminal apoB fragments (19). Conceivably, the liver or other organs could synthesize fragments of apoB that circulate in the plasma and then attach to the luminal endothelial surface. Alternatively, apoB fragments could be synthesized by endothelial cells.

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The experiments described here provide evidence that cultured endothelial cells produce both a full-length and an amnio-terminal fragment of apoB.

MATERIALS AND METHODS

Endothelial Cells—Bovine aortic endothelial cells (BAEC) were isolated and cultured as described (20). The cells (5–15 passages) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc.). Human aortic endothelial cells (HEC, passage 3) were purchased from Clonetics Corp. (San Diego, CA) and were cultured in endothelial cell growth medium (catalog number CC-3024, Clonetics) containing 2% fetal bovine serum, 10 ng/ml human epidermal growth factor, and 12 μg/ml of bovine brain extract. These cells were positive for factor VIII (von Willebrand's) antigen and uptake of di-acetylated LDL, endothelial cell markers.

Oligonucleotides—The following primers were designed (Designer-PCR software, Research Genetics, Huntsville, AL) based on the human apoB sequence (21). Downstream (DS) primers were used in the reverse transcription reaction and DS together with upstream (US) primers were used in the PCR reaction: US1 (nucleotides nt 249–268), 5′-GCC GAT TCA AGC ACC TCC G 3′; DS1 (complementary to nucleotides 441–422), 5′-CAG GGT TGA AGC CAT A 3′; DS2 (complementary to nucleotides 883–864), 5′-GCT TCC TCT TAG CTT GCA G 3′; US2 (nucleotides 12,355–12,372), 5′-GGG TCT CTT ATG ATT ATG T 3′; DS3 (complementary to nucleotides 12,735–12,716), 5′-CGG AAA CTG CAA TCT GGG G 3′.

RNA Isolation and RT-PCR—RNA from confluent monolayers of endothelial cells was isolated using total RNA isolation reagent (TRI-ZOL, Life Technologies, Inc.). RT-PCR was performed using GeneAmp RNA PCR kit from Perkin-Elmer (Rosche Laboratories, Branchburg, NJ). Amplification was carried out for 30 cycles, and PCR products were analyzed by 2% agarose gels (23). For PCR at the amino-terminal region of apoB, primers US1 and DS1 were used. The expected product size is 193 bp. For PCR near the carboxyl terminus (3′-terminus), primers US2 and DS3 were used that would give a PCR product of 381 bp. Double-stranded sequencing of PCR products was performed using Applied Biosystems automated sequencing apparatus (Sequetech Corp., Mountain View, CA).

Screening of Bovine Intestine cDNA Library—A 1-kilobase fragment of apoB (representing approximately 7% of the amino terminus of apoB) was generated from a pCMV5- apoB plasmid (gift of Dr. Z. Yao) (22) by Stul/NotI digestion. The fragment was 32P-labeled by the random primer labeling procedure (Boehringer Mannheim) according to manufacturer's instructions. 32P-Labeled apoB cDNA was used to screen a 100,000-fold dilution of a bovine intestine cDNA library. Positive clones were isolated and DNA from viral supernatants was prepared by standard protocols (23). PCR of bovine apoB was performed using primers US1 and DS2. The expected product size is 635 bp. DS2 was designed such that sequence for a larger part of apoB at the amino-terminal region could be obtained.

Antibodies—Rabbit anti-human apoB polyclonal antibody was kindly provided by Drs. X. Wu and H. Ginsberg of this department. Monoclonal anti-human apoB antibody, mAb47, was provided by Dr. L. Curtiss (Scripps Research Institute, CA). mAb47 has epitopes at the receptor binding domain of human apoB (24) and also binds to bovine LDL (9).

Metabolic Labeling and Immunoprecipitation—Confluent monolayers of endothelial cells were incubated either with [35S]methionine/cysteine mixture (Translabel ™) or with [3H]leucine (Amersham Corp.). Following labeling, media were collected and cells were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 50 μg/ml each of leupeptin and pepstatin and 0.5 mM PMSF (lysis buffer) for 6 h at 4 °C. Cell lysates were centrifuged for 20 min at 14,000 rpm in a microcentrifuge, and the supernatants were used for immunoprecipitation. In some experiments following labeling, cells were washed with PBS containing 10 units/ml of heparin prior to lysis of cells. Proteins in the medium, cell lysates, and heparin-released fractions were immunoprecipitated with anti-apoB antibodies (25) and analyzed by 3–12% SDS-polyacrylamide gel electrophoresis (9). Gels were immersed in Autoradiot (National Diagnostics, Atlanta, GA) for 1 h, dried, and autoradiographed.

RESULTS

Endothelial Cells Synthesize ApoB mRNA—We tested whether endothelial cells express apoB message by performing RT-PCR. Primers to the 5′ region of apoB that are complementary to nucleotides 249–268 in exon 3 and nucleotides 422–441 in exon 4 were used. Because these primers span an intron, a different size product will be generated from any DNA contamination. RT-PCR of total RNA from BAEC and HEC resulted in the generation of the expected 193-bp PCR product (Fig. 1A). Human liver cell line HepG2 RNA and mouse liver RNA were used as positive controls. In addition to the 193-bp product, some lower molecular mass products were also observed in PCR reactions from BAEC and HEC. Southern blotting with labeled HepG2 PCR product showed binding with only the 193-bp product from BAEC (not shown). These results show
that endothelial cells express apoB mRNA. Yeast RNA did not produce the PCR product and no products were generated from endothelial cell RNA when reverse transcriptase was omitted in the reaction (not shown).

We then tested whether a full-length apoB mRNA is expressed by these cells. Primers to nucleotides (12,355–12,372 and 12,735–12,716) in the 5′ region of human apoB were used. Using these primers expected size PCR products (381 bp) were generated only from HEC and HepG2 cells (Fig. 1A). No PCR products were generated from BAEC or mouse liver RNA. No PCR products were generated from HEC RNA when reverse transcriptase was omitted from the reaction (HEC without RT). These results suggest that HEC express a full-length apoB mRNA. The primers designed to human apoB mRNA did not react with bovine and mouse mRNA sequences. Thus, the 5′-terminal region of apoB mRNA (amino-terminal region of apoB) appears to be more conserved from mouse to human than the 3′ or carboxyl-terminal region. Alternative possibilities, although less likely, are that BAEC synthesize only the 5′-terminal part of the apoB or that another protein with homology to the 5′-terminal region of apoB is synthesized by bovine endothelial cells.

The Amino Terminus of ApoB Is Highly Conserved—To further confirm that the amino-terminal region of apoB is highly conserved, PCR products from bovine endothelial cells and mouse liver were sequenced and compared with human apoB sequence. Bovine apoB showed a 92–94% nucleotide and amino acid homology in this amino-terminal region, and mouse apoB was 80–85% homologous, suggesting that this region of apoB is very highly conserved. PCR products from another bovine source, bovine intestine library, were also sequenced. The sequence showed a 88–90% homology for the region of nucleotide 460 to nucleotide 860, strongly suggesting that it is a highly conserved region.

Immunoprecipitation of ApoB—In our previous studies we used BAEC to characterize LPL binding to cells and to identify the 116-kDa apoB like LPL-binding protein. We therefore used BAEC in the following experiments to examine whether full-length apoB and a 116-kDa apoB fragment are synthesized by these cells.

Because apoB is rapidly degraded in several cell lines, we initially used short labeling times that would allow us to precipitate an apoB size protein (Fig. 2A). Cells were labeled for 1 h, and cell extracts were immunoprecipitated by either polyclonal apoB antibody or mAb47. A protein of the size of full-length apoB was immunoprecipitated by mAb47. The immunoprecipitation of this large apoB size protein was competed when unlabeled LDL was used in the reaction (mAb47 + LDL). No smaller size products were precipitated by mAb47. Polyclonal antibody did not precipitate either a full-length or a 116-kDa fragment of apoB (not shown). These data suggest that endothelial cells produce full-length apoB.

Our previous results showed the presence of a labeled 116-kDa LPL-binding protein in cells incubated overnight with [35S]methionine (20). To determine whether a 116-kDa apoB fragment was synthesized by cells, BAEC were labeled for 16 h with [35S]methionine, and proteins were immunoprecipitated with polyclonal apoB antibodies. ApoB antibodies precipitated a 116-kDa protein in both the heparin-released fraction (HR) and cell extracts (Fig. 2B). This band was not found when immunoprecipitation was performed in the presence of excess unlabeled LDL (HR + LDL). This suggested that the 116-kDa protein was a fragment of apoB. As has been described by others (25), we occasionally observed a fragment of molecular mass ~250 kDa that was precipitated by nonspecific antibodies and was not competed by LDL (not shown).

We showed previously that LPL binds to amino-terminal, but not COOH-terminal, fragments of apoB (9, 10). To confirm that
the 116-kDa fragment was an amino-terminal fragment of apoB, we performed ligand blotting with biotinylated LPL (9). Immunoprecipitates obtained from polyclonal apoB antibodies were analyzed by SDS-PAGE and transferred to nitrocellulose (Fig. 2C). In this figure the nonspecific 250-kDa protein is also seen. Biotinylated LPL bound to the 116-kDa fragment precipitated by polyclonal apoB antibodies, suggesting that the 116-kDa apoB is an amino-terminal fragment.

Pulse-Chase Analysis of ApoB—To determine if the 116-kDa fragment of apoB arises by proteolytic cleavage of full-length apoB, we carried out pulse-chase experiments. Cells were labeled with [3H]leucine for 1 h and chased for up to 2 h in the absence of label. Immunoprecipitations were performed with both mAb47 to identify full-length apoB (B-100) and with polyclonal apoB antibodies to identify the 116-kDa fragment (Fig. 3). A 550-kDa band was immunoprecipitated by mAb47 in the 1-h labeled cells. In up to 1 h of chase, no significant loss in the intensity of this band was observed. In 2 h, however, the intensity decreased significantly. In proteins precipitated with the polyclonal antibody, a 116-kDa protein was evident at 2 h, but not earlier. Therefore, as the apoB-100 size band decreased, the 116-kDa protein became evident. These data suggest that the 116-kDa protein arises from proteolysis of apoB-100.

Effect of Oleic Acid on Endothelial ApoB Production—Studies in HepG2 cells showed that incubation with oleic acid resulted in decreased degradation and increased secretion of apoB in lipoprotein particles (25, 27, 28). In endothelial cells no detectable apoB was found either in the total medium or in a lipoprotein (d < 1.21) fraction (not shown). We next tested whether oleic acid will affect apoB production by endothelial cells. Cells were incubated with 0.25 and 0.5 mM oleic acid for 30 min, and cells were then labeled with [3H]leucine for 1 or 4 h. Media and cell extracts were immunoprecipitated with polyclonal apoB antibodies or mAb47. The intensity of the 116-kDa apoB was not significantly affected by treatment with oleic acid in 4-h labeled cells (Fig. 4, polyclonal, lanes 6 and 8). No apoB, full-length or 116 kDa, was observed in the medium of control and oleic acid-treated cells (lanes 4, 5, 7, and 9). Similarly in 1-h labeled cells full-length apoB was not significantly different in control and oleic acid-treated cells (lanes 1–3). These results suggest that endothelial apoB production is not regulated by oleic acid.

DISCUSSION

Since the major function for apoB is assembly of lipoproteins, it is generally assumed that cells that do not make lipoproteins do not synthesize apoB. However, other investigators have found apoB mRNA and protein in tissues other than liver and intestine (29, 30). Our interest in apoB stems from the observation that cultured BAEC contained a 116-kDa amino-terminal fragment of apoB (9). Initial experiments to detect apoB mRNA by Northern blotting were not successful (not shown). We then used the more sensitive PCR, using primers designed to the 5′ region of human apoB mRNA. Much to our surprise we obtained PCR products from total RNA of endothelial cells. With 2μg of total RNA, PCR products from endothelial cells could be generated in 20 cycles compared with 15 cycles for HepG2 RNA. Although BAEC often produced an easily visible PCR product, occasional batches of cells were negative. We suspected that this was because some BAEC lost the ability to synthesize apoB due to repetitive passage or, perhaps, other factors related to their culture conditions.

PCR using primers to the 5′ region was positive across species, from human to cow to mouse. When the 5′ region of apoB was sequenced from the PCR products and from bovine intestinal library, the degree of interspecies homology was quite striking. We were surprised at the extent of conservation in the amino-terminal region, 90–95% between human and bovine and 80–85% between mouse and human both at nucleotide and amino acid levels. Previous studies by Matsumoto et al. (31) showed that rat apoB was 83% homologous to human apoB in the region comprising amino acids 138–522. Such conservation during evolution suggests that the region near the amino terminus has an important function.

High interspecies homology has been reported for two other regions of apoB. The LDL receptor binding domain (nucleotides 9623–10,442) is highly conserved. This region has a 75% ho-
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mology at the nucleotide level between mouse and human and 79.9% between human and pig (32). The mRNA editing region of apoB (nucleotides 6640–6720) is also highly conserved. Homology in this region varies from 88 to 93% among different species and humans (33). In our studies, using the primers to the 3′ terminal region of human apoB mRNA, PCR was only positive with HepG2 and HEC. Thus, the primers chosen for the 3′ terminal reaction were species specific. However, these data suggested that endothelial cells contain full length apoB mRNA.

Not only do endothelial cells contain apoB mRNA, but they also synthesize apoB protein. Full length apoB was precipitated from labeled BAEC using mAb47. This immunoprecipitated band was found with short-term labeling of the cells. Longer chase times decreased the amount of full-length apoB, suggesting that this protein was degraded or converted to proteolytic fragments. One of these, a fragment of 116 kDa, was found with longer labeling. Our pulse-chase data suggest that the 116 kDa protein comes from apoB-100. The 116 kDa band was most evident as the larger full-length apoB band decreased in intensity. Although we attempted to block proteolysis with N-acetyl-leucyl-leucyl-norleucinal, the cysteine protease inhibitor used in HepG2 cells (16, 34), these experiments were unsuccessful. Lower concentrations of ALLN had no effect on the cells, while higher concentrations (e.g. 40 μg/ml used in other studies) were toxic to the endothelial cells. Therefore, the site and the events leading to the apoB protelysis are not clear.

Although a full-length apoB was produced in the cells, no detectable amount was secreted into the medium. This may be due to lack of machinery in endothelial cells for lipoprotein assembly and secretion. Immunoblot analysis of total microsomes isolated from and human and bovine endothelial cells showed no detectable microsomal triglyceride transfer protein, a necessary component in the secretion of apoB lipoproteins by liver and intestine (35). Thus lipoprotein production does not appear to be the purpose of apoB production by endothelial cells. This may be the reason why oleic acid had no effect on the production of endothelial apoB.

HepG2 cells secrete a smaller, 85-kDa apoB proteolytic fragment (19). Other proteolytic fragments, including one that is approximately 116 kDa, are also found in these cells. These proteins, however, are less abundant than the 85-kDa protein. Differences either in the apoB (bovine versus human) or in cells (HepG2 versus endothelial) might be responsible for the generation of different size apoB fragments. In this regard, a recent study reported that human endothelial cells contain both 116- and 85-kDa LPL-binding proteins (36). Thus, it is possible that human and bovine endothelial cells process apoB differently.

In our experiments mAb47, but not polyclonal antibodies, precipitated full-length apoB. The same polyclonal antibodies, however, precipitated full-length apoB from HepG2 cells (not shown). This may be a reason why no full-length apoB was precipitated in our 16-h labeling experiments (Fig. 2B). The inability of the polyclonal antibody to precipitate bovine full-length apoB could be because the polyclonal antibodies contain subtypes of antibodies that recognize several epitopes in the human apoB that may not have been conserved in the bovine apoB. In addition, the 116-kDa protein could have competed for those epitopes in the amino-terminal region of apoB that are conserved, making the polyclonal antibodies less likely to work well in immunoprecipitating bovine full-length apoB.

The 116-kDa protein was released from the cells by treatment with heparin. This suggests that the protein is on the cell surface and, therefore, able to interact with LPL. Moreover, it suggests that this apoB fragment is either associated with proteoglycans or is attached to the cells via an ionic interaction.

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