Determinants of human apolipoprotein [a] secretion from mouse hepatocyte cultures

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Abstract **Efforts to develop an in vitro model system to analyze apolipoprotein [a] (apo[a]) gene transcription, mRNA translation, and protein secretion have been complicated by the limited tissue and species distribution of apo[a] and the presence of regulatory DNA sequences remote from the apo[a] transcription start site. In the current study we examined primary hepatocytes cultured from apo[a] transgenic mice as a model system for analyzing apo[a] biogenesis. Hepatocytes from mice transgenic for a yeast artificial chromosome (YAC) encoding the entire apo[a] gene in its own genomic context (YAC-apo[a] hepatocytes) were unable to maintain apo[a] expression beyond 48 h of culture. This suggests that the apo[a] promoter was not active in cultured YAC-apo[a] hepatocytes. In contrast, apo[a] expression was maintained for at least 7 days in hepatocytes cultured from mice transgenic for an apo[a] cDNA under control of the mouse transferrin promoter (transferrin-apo[a] hepatocytes). Pulse-chase experiments established that more than 80% of apo[a] synthesized by both transferrin-apo[a] and YAC-apo[a] hepatocytes was degraded prior to secretion, independently of the coexpression of human apoB. Thus, low secretion efficiency appears to be a general characteristic of human apo[a] proteins in mouse liver. Apo[a] secretion was increased somewhat (from 18% to 32%) in the presence of lipoprotein-containing serum. Transformed cell lines derived from transferrin apo[a] hepatocytes retained characteristics of apo[a] secretion similar to those observed in primary cells. Primary and transformed apo[a] transgenic hepatocytes may provide valuable additional models with which to study posttranslational mechanisms regulating apo[a] secretion.**—Wang, J., J. Boedeker, H. H. Hobbs, and A. L. White. **Determinants of human apolipoprotein [a] secretion from mouse hepatocyte cultures.** ***J. Lipid Res.*** **2001.** 42: **60–69.**

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Apolipoprotein [a] (apo[a]) is the unique glycoprotein component of plasma lipoprotein [a] (Lp[a]). Lp[a] is an unusual lipoprotein found only in the plasma of humans, Old World primates (1), and the hedgehog (2). Lp[a] consists of a low density lipoprotein (LDL)-like particle in which apoB-100 is attached to apo[a] by a disulfide bond (3). Lp[a] levels vary more than 1,000-fold among individuals (4). High plasma levels of Lp[a] (>25–30 mg/dl) are associated with an increased risk of cardiovascular disease (5).

Variation in plasma Lp[a] levels is determined primarily by inheritance at the apo[a] gene locus (6). The apo[a] gene is highly polymorphic and encodes a variable number of copies (from ~12 to 51) of a plasminogen kringle 4 (K4)-like domain (7, 8). An inverse correlation between apo[a] size and plasma Lp[a] levels (9, 10) determines approximately 50% of the variation in plasma Lp[a] concentrations (6, 11, 12). Polymorphisms independent of K4 number account for most of the remaining variation in plasma Lp[a] levels (6, 13).

Differences in plasma Lp[a] levels among individuals are determined primarily by differences in Lp[a] production rate (14–16). Lp[a] is produced by the liver (17–19). The majority (20–24), although not all (25, 26), studies support that assembly of apo[a] with apoB to form Lp[a] occurs after secretion of apo[a] by the hepatocyte. The rate of apo[a] secretion is likely determined at multiple levels. Differences in hepatic apo[a] mRNA concentration imply a major role for apo[a] gene transcription rate and/or mRNA stability (27–29). Posttranscriptional mechanisms are also important. Apo[a] is inefficiently secreted by the hepatocyte and a portion of the protein is retained in the endoplasmic reticulum (ER) and degraded by the proteasome (30, 31). Large apo[a] isoforms tend to be degraded to a greater extent than small isoforms (24, 30, 32), accounting at least partially for the inverse correlation between apo[a] size and plasma Lp[a] level. However, not all small apo[a] isoforms are efficiently secreted

Abbreviations: 6AHA, 6-amino hexanoic acid; apo, apolipoprotein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low density lipoprotein; Lp[a], lipoprotein [a]; MTP, microsomal triglyceride transfer protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFM, serum-free medium; SSC, salt-sodium citrate; SV40, simian virus 40; YAC, yeast artificial chromosome.

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(30), presumably because of the presence of destabilizing amino acid sequences in some of these variants (33, 34).

Although plasma Lp[a] levels vary enormously among individuals, levels within an individual remain remarkably constant (4) and, in comparison with other lipoproteins, are remarkably refractory to modulation by dietary and pharmacological intervention. Nonetheless, a variety of factors have been identified that either increase or decrease plasma Lp[a] levels [reviewed in refs. (35–37)]. Both transcriptional and posttranscriptional mechanisms may play roles in regulation of Lp[a] levels by these factors (38–47).

Analysis of factors that influence Lp[a] production rate has been hampered by the limited tissue and species distribution of Lp[a], the low activity associated with apo[a] promoter fragments in vitro (48, 49), and the presence of regulatory sequences >20 kb upstream of the transcription start site (38, 50, 51) and in other regions of the apo[a] gene (39). The development of mice transgenic for a 17 K4 human apo[a] cDNA under the control of the mouse transferrin promoter (23, 52), and subsequently of mice transgenic for both apo[a] and human apoB (Lp[a] transgenic mice) (53, 54), has provided in vivo models to study some aspects of Lp[a] metabolism. The characteristics of human apo[a] synthesis and secretion and Lp[a] assembly in hepatocytes cultured from Lp[a] transgenic mice (55–57) are similar to those documented for endogenous apo[a] in primary baboon hepatocytes (20, 21, 30, 31, 33) and for transfected human apo[a] proteins in transformed liver cell lines (24, 32, 58). One important difference between baboon apo[a] and human apo[a] expressed in mouse hepatocytes, however, is that the extent of human apo[a] presecretory degradation (>80%) (55, 56) is much greater than expected for the relatively small (17 K4) apo[a] protein expressed in mice (30).

The more recent production of two strains of mice transgenic for yeast artificial chromosomes (YACs) encoding the entire human apo[a] gene in its own genomic context (39, 40) has provided an additional opportunity to investigate factors that regulate apo[a] mRNA levels in vivo (39–42). Hepatocytes cultured from YAC-apo[a] mice may also provide a valuable in vitro model system with which to study factors that regulate human apo[a] gene transcription.

In the current study we further investigated the utility of primary hepatocytes from apo[a] transgenic mice as in vitro model systems to study Lp[a] biogenesis. In particular, we examined whether hepatocytes from YAC-apo[a] mice can be used to study the regulation of apo[a] gene transcription in vitro. We also investigated the factors that influence human apo[a] secretion efficiency in mouse hepatocyte cultures.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA)-linoleate and insulin were from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and delipidated FBS were from Life Technologies (Gaithersburg, MD). [^{35}S]cysteine and Expre^{35}S^{35}S were from DuPont NEN (Boston, MA). Rabbit anti-human Lp[a] antibody was from Cortex Biochemicals (San Leandro, CA). To make anti-mouse apoB polyclonal antibodies, lipoproteins were prepared from mouse plasma by ultracentrifugation and resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. The apoB-100 band was excised and used for injection into rabbits. Sheep anti-human apoB was from Roche Molecular Biochemicals (Indianapolis, IN). Rabbit anti-human albumin was from Dako (Carpinteria, CA), and goat anti-mouse transferrin was from Cappell (Cochranville, PA). The plasmid pSV3neo, encoding the simian virus 40 (SV40) large-T antigen and neomycin resistance (59), was kindly provided by R. Lanford (Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, TX). All other reagents were of analytical grade.

Hepatocyte isolation and culture

Mouse hepatocytes were isolated exactly as described previously (55) and were routinely cultured in a serum-free medium (SFM) formulation (60). Other media used as described for individual experiments, included DMEM-insulin-BSA [DMEM containing 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, insulin (5 μg/ml), and BSA (500 μg/ml) complexed to 0.79% linoleic acid by weight], and DMEM plus 10% FBS. Unless otherwise stated, experiments were performed on cells that had been in culture for 48–72 h.

Mice hemizygous for a 17 K4 human apo[a] cDNA under the control of the mouse transferrin promoter, and homozygous for full-length human apoB (Lp[a] transgenic mice), or hemizygous for a YAC encoding the entire gene for a 13 K4 human apo[a] isoform as well as substantial 5′ and 3′ sequence (YAC-apo[a] mice), were as described (39, 53). Mice hemizygous for the 17 K4 human apo[a] cDNA in the absence of the human apoB transgene (apo[a] mice) were obtained by crossing Lp[a] mice with C57BL6/SJ wild-type mice (Jackson Laboratories, Bar Harbor, ME). The presence of the apo[a] and apoB transgenes was determined by immunoblotting of plasma (see below).

Transformed cell lines

Primary hepatocytes (2×10^5 cells per 35-mm dish) were transfected 24 h after isolation with pSV3neo, as described previously (31). Two days after transfection, the cells were placed in G418 (400 μg/ml). Drug-resistant colonies were picked and expanded by standard techniques. Established cell lines were screened for apo[a] expression by immunoblotting (see below). Cell lines were maintained in SFM or DMEM-10% FBS and passaged every 3–4 days.

Transformation of the hepatocytes resulted in morphological change from the characteristic cuboidal shape of primary hepatocytes to more elongated cells that divided rapidly (data not shown). Cells cultured in SFM maintained their differentiated phenotype, whereas those in DMEM-10% FBS lost expression of many liver-specific proteins (see Fig. 5). Cells grown in both media, however, maintained apo[a] expression for at least 30 passages (highest passage number analyzed).

Radiolabeling, immunoprecipitation, and SDS-PAGE

Hepatocytes were labeled for 16 h with [^{35}S]cysteine and Expre^{35}S^{35}S label, or were labeled for 15 min and chased for various periods in unlabeled medium, as described previously (20). For cells cultured in DMEM-10% FBS, dialyzed serum was used during labeling. Samples were immunoprecipitated and analyzed on 4–10% gradient SDS-polyacrylamide gels after reduction with 2-mercaptoethanol, as described (20), with the exception that a rabbit anti-apo[a] polyclonal antibody was used. Apo[a] bands were quantified by densitometric scanning and

were normalized to total cell protein mass determined by bicinchoninic acid assay (Pierce, Rockford, IL). To determine the extent of apo[a] degradation in pulse-chase experiments, the amount of the apo[a] precursor present at 30 min of chase was taken as a measure of apo[a] synthesis [peak incorporation of counts into the apo[a] precursor is observed at this time point (31, 55)]. The total amount of apo[a] remaining at 6 h of chase (cells plus medium) was then used to estimate the extent of apo[a] degradation, as previously described (31). To determine the extent of apo[a] maturation at 6 h, total mature apo[a] (cells plus medium) was quantified as described above and expressed as a percentage of apo[a] synthesized.

Immunoblotting

Immunoblotting of plasma or medium samples after fractionation on 4–10% gradient gels was performed as described previously (20), with the exception that secondary antibodies were labeled with horseradish peroxidase (Fisher Biotech, Pittsburgh, PA) and detection was by chemiluminescence (Supersignal substrate; Pierce).

Northern blotting

Total RNA was prepared from hepatocytes by using TriZol (GIBCO, Grand Island, NY) according to the manufacturer instructions. Aliquots (10 μg) of RNA were separated on 1.2% agarose/1.1% formaldehyde gels and transferred to nylon membranes (Nytran Supercharge; Schleicher & Schuell, Keene, NH) by capillary blotting. Blots were probed with a plasmid containing the 17 K4 human apo[a] cDNA (pRKha17) (58) labeled with ^{32}P by random priming, using the Rediprime labeling kit (Amersham, Arlington Heights, IL). Prehybridization was performed for 1 h at 65°C in Rapid-hyb buffer (Amersham). Hybridization was in the same buffer overnight at 65°C with a probe concentration of 5×10^5 cpm/ml. Blots were washed twice for 20 min at room temperature in 5× salt-sodium citate (SSC)-0.1% SDS, followed by two washes for 15 min in 1× SSC-0.1% SDS, once for 15 min in 0.1× SSC-0.1% SDS, and once for 15 min in 1× SSC-1% SDS. Blots were then exposed to X-ray film at −80°C with two intensifying screens. Blots were stripped for 10 min at 100°C in 1% SDS and reprobed with a fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (61) (2.5×10^5 cpm/ml), as described above.

RESULTS

Hepatocytes from three different strains of mice were used in this study: *1*) mice transgenic for a cDNA encoding a 17 K4 human apo[a] isoform, under the control of the mouse transferrin promoter (apo[a] mice); *2*) mice transgenic for both the 17 K4 apo[a] isoform and the entire human apoB gene (Lp[a] mice); and *3*) mice transgenic for a YAC encoding the entire gene for a 13 K4 human apo[a] isoform, including 40 kb of 5′ and 200 kb of 3′ sequence (YAC-apo[a] mice) (for details, see Materials and Methods).

Influence of human apoB coexpression on apo[a] secretion

Previous studies demonstrated that the 17 K4 human apo[a] isoform is inefficiently secreted from hepatocytes cultured from Lp[a] transgenic mice; >80% of the protein was subjected to proteasome-mediated, ER-associated degradation (ERAD) (56, 57). Lp[a] transgenic hepatocytes synthesize and secrete human apoB-100 and apoB-48, in addition to the endogenous mouse apoB protein and human apo[a]. It is possible that the coexpression of human apoB in these cells contributed to the low secretion efficiency of apo[a], perhaps because of competition for ER chaperone proteins or because of nonproductive associations between apo[a] and apoB in the ER lumen (25). To determine the effect of human apoB coexpression on apo[a] secretion, we analyzed secretion of the 17 K4 apo[a] isoform in hepatocytes that do not express human apoB (apo[a]-only transgenic hepatocytes).

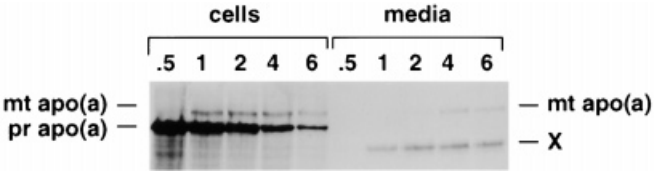

Fig. 1. Influence of human apoB coexpression on apo[a] secretion. Hepatocytes isolated from apo[a]-only mice were labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased for between 0.5 and 6 h in unlabeled medium, as described in Materials and Methods. Apo[a] in the cells and medium at each time point was analyzed by immunoprecipitation and 4–10% SDS-PAGE. The positions of the precursor (pr) and mature (mt) forms of apo[a], and of a protein nonspecifically immunoprecipitated from the culture medium (X), are indicated.

Hepatocytes isolated from apo[a]-only mice were labeled for 15 min and chased for various periods up to 6 h in unlabeled medium. Apo[a] was immunoprecipitated from the cell lysates and culture medium and analyzed by SDS-PAGE (**Fig. 1**). Apo[a] is synthesized as a lower molecular weight, ER-associated precursor that undergoes processing to a higher molecular weight form before secretion. The increase in apo[a] molecular weight on maturation is due to Golgi specific modification and addition of *N*- and *O*-linked glycans (20). At the early chase time points, the ER-associated apo[a] precursor was readily detected in the cell lysates (Fig. 1). The amount of precursor remained relatively stable until the 2-h chase time, after which time point the amount declined. By 6 h of chase the majority of the apo[a] precursor had disappeared from the cells (Fig. 1). At 60 min of chase, the higher molecular weight, mature form of apo[a] appeared in the cell lysates and by 4 h could also be detected in the culture medium (Fig. 1). Only a small portion of the precursor underwent maturation, however, and by 6 h of chase <5% of apo[a] synthesized during the 15-min pulse had been secreted into the medium (Fig. 1). The vast majority of apo[a] (81 ± 7%; mean ± SD, n = 3) was degraded. The control proteins albumin and transferrin were quantitatively secreted from the cells (data not shown). The kinetics of apo[a] maturation and secretion and the extent of apo[a] degradation were similar to those observed for apo[a] in Lp[a] transgenic hepatocytes [see below and ref. (56)]. Coexpression of human apoB is therefore not responsible for the low secretion efficiency of apo[a] from Lp[a] hepatocytes.

Expression of apo[a] in YAC-apo[a] hepatocyte cultures

The extent of presecretory degradation varies enormously among apo[a] allelic variants (24, 30, 32). It was therefore possible that the low secretion efficiency of apo[a] observed in our experiments was specific to the 17 K4 apo[a] isoform expressed in the transferrin-apo[a] mice. Alternatively, low secretion efficiency could be a general characteristic of human apo[a] isoforms expressed in mouse hepatocytes. To address this issue, we examined the secretion of a second apo[a] isoform in hepatocytes from YAC-apo[a] transgenic mice that express a 13 K4 apo[a] gene in its own genomic context.

To determine the optimum time point at which to analyze apo[a] secretion from the YAC-apo[a] hepatocytes, we examined secretion of the 13 K4 apo[a] isoform into the culture medium at various time points after hepatocyte isolation. Medium from duplicate plates of cells was collected every 24 h and analyzed by immunoblotting with an anti-human apo[a] antibody (**Fig. 2A**). For comparison, apo[a] secretion from Lp[a] hepatocytes was also examined.

The peak of apo[a] secretion from Lp[a] transgenic hepatocytes occurred 48–72 h after isolation and apo[a] secretion was maintained for at least 7 days (Fig. 2A and data not shown). Secretion of both human and endogenous mouse apoB-48 and apoB-100 was also maintained throughout this period (Fig. 2A and data not shown). Hepatocytes isolated from YAC-apo[a] transgenic mice also maintained endogenous apoB secretion for at least 4 days (the longest time point examined; Fig. 2A and data not shown). In contrast, the YAC hepatocytes were unable to maintain high levels of apo[a] secretion beyond 24 h of culture. By 72 h, apo[a] was almost undetectable in the medium of these cells (Fig. 2A). A number of different culture media containing various combinations of serum, growth factors, and other additives were unable to maintain apo[a] secretion from YAC hepatocytes beyond 72 h (data not shown). Addition of human growth hormone to the medium, which increases hepatic apo[a] mRNA levels in the YAC-apo[a] mice (42), also was unable to maintain apo[a] secretion (data not shown).

Immunoprecipitation of apo[a] from hepatocytes labeled with a short pulse of [^{35}S]methionine established that the decline in apo[a] secretion from the YAC-apo[a] hepatocytes correlated with a drastic decrease in the amount of apo[a] synthesized by 48 h of culture (Fig. 2B). Northern blotting established that the 7.5-kb mRNA produced by the 13 K4 apo[a] gene was readily detected in YAC hepatocytes that had been in culture for 24 h, but by 3 days was essentially undetectable (Fig. 2C). Levels of GAPDH mRNA in these cells did not decline over the same time period (Fig. 2C). In the Lp[a] transgenic hepatocytes, levels of the 9.5-kb 17 K4 apo[a] mRNA transcript remained relatively constant from 1 to 4 days in culture (Fig. 2C). These results are consistent with the loss of apo[a] expression in YAC-apo[a] hepatocytes after 24 h in culture being due to loss of activity of the apo[a] promoter in vitro.

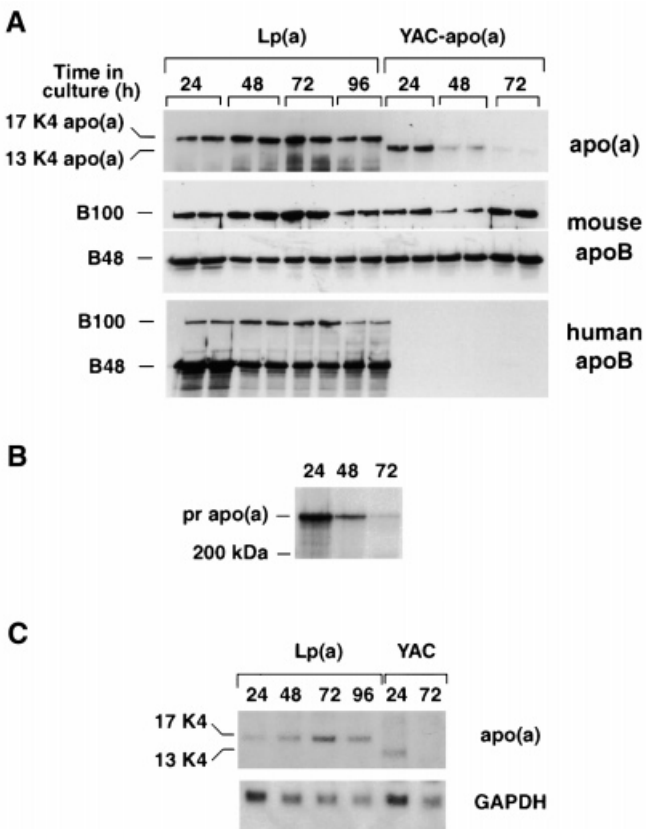

Fig. 2. Expression of apo[a] in Lp[a] and YAC-apo[a] transgenic hepatocytes. A: Culture medium from duplicate plates of Lp[a] and YAC-apo[a] hepatocytes was collected every 24 h after plating, up to 96 h. Aliquots were resolved on a 4–10% SDS-polyacrylamide gel and immunoblotted with antibodies against human apo[a] (top), mouse apoB (middle), or human apoB (bottom). For mouse apoB, a longer autoradiographic exposure is presented for apoB-100 versus apoB-48. The positions of the 17 K4 and 13 K4 apo[a] proteins, and of apoB-100 and apoB-48, are indicated. B: YAC-apo[a] hepatocytes that had been in culture for 24, 48, or 72 h were labeled for 15 min and apo[a] was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE, as described in Materials and Methods. The positions of the apo[a] precursor (pr apo[a]) and of the 200-kDa molecular mass marker, are indicated. C: RNA isolated from Lp[a] and YAC-apo[a] hepatocytes that had been in culture for between 24 and 96 h was analyzed by Northern blotting with an apo[a] cDNA probe (top) or rat GAPDH cDNA probe, as described in Materials and Methods. The positions of the mRNAs for the 17 K4 and 13 K4 forms of apo[a] are indicated.

Low secretion efficiency is a general characteristic of human apo[a] isoforms in mouse hepatocytes

To examine the efficiency of secretion of the 13 K4 apo[a] isoform that was synthesized by the YAC-apo[a] hepatocytes, pulse-chase experiments were performed with hepatocytes that had been in culture for 20 h, a time point at which apo[a] synthesis and secretion were still readily detectable (Fig. 2).

Similar to the apo[a] and Lp[a] hepatocytes, the majority of apo[a] synthesized by the YAC-apo[a] hepatocytes was degraded prior to secretion (**Fig. 3**). Immediately after a 15-min pulse, radiolabeled apo[a] precursor was readily detected in the cell lysates. By 6 h of chase, how-

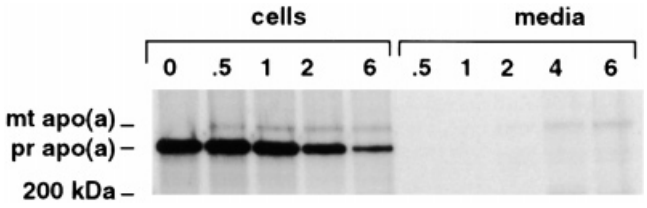

Fig. 3. Apo[a] is inefficiently secreted from YAC-apo[a] hepatocytes. Hepatocytes isolated from YAC-apo[a] transgenic mice were labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased for the indicated times as described for Fig. 1. Apo[a] in the cells and medium at each time point was analyzed by immunoprecipitation and 4–10% SDS-PAGE. The positions of the precursor (pr) and mature (mt) forms of apo[a], and of the 200-kDa marker are indicated.

ever, the majority of the precursor had disappeared from the cells and <5% of the apo[a] had been secreted (Fig. 3). The vast majority of the newly synthesized 13 K4 apo[a] isoform appeared to be retained and degraded inside the cells (70% and 97% degraded at 6 h and 8 h of chase, respectively, in two independent experiments). In contrast, the control proteins albumin and transferrin were quantitatively secreted from the cells (data not shown). Because the 13 K4 apo[a] isoform was isolated from a human individual with high circulating Lp[a] levels (39) it seems unlikely that the extensive presecretory degradation is a characteristic peculiar to the isoform under study and is more likely a general characteristic of human apo[a] isoforms in mouse hepatocytes.

Serum increases apo[a] secretion from primary hepatocytes

The SFM used to culture the hepatocytes used in experiments described above contains little lipid (28 μM linoleic acid). Studies of transformed rat and human liver cell lines suggest that apo[a] secretion can be increased by adding lipid to the culture medium (43). To determine whether the efficiency of apo[a] secretion from primary mouse hepatocytes could be increased by addition of lipid, we compared the synthesis and secretion of apo[a] in Lp[a] hepatocytes that had been cultured in SFM or in DMEM-10% FBS for 48 h (**Fig. 4**).

Steady-state labeling of hepatocytes cultured in SFM or in DMEM-10% FBS revealed substantially more apo[a] in the medium of cells grown in DMEM-10% FBS than in the medium of cells grown in SFM (Fig. 4A). Secretion of both human and endogenous mouse apoB-100 was also significantly increased in DMEM-10% FBS compared with SFM (Fig. 4A). Cells grown in SFM or in DMEM-10% FBS each maintained a highly differentiated phenotype, with albumin, transferrin, and α_1-antitrypsin (α_1-AT) readily observed in the media of both sets of cultures (Fig. 4A).

To examine the reason for the increase in apo[a] secretion in the presence of serum, pulse-chase analysis was performed (Fig. 4B and C). Similar amounts of apo[a] precursor were labeled during a 15-min pulse under the different culture conditions (ratio of precursor at 30 min of chase in DMEM-10% FBS vs. SFM, 1.3:1), demonstrating a similar rate of apo[a] synthesis (Fig. 4B). By 6 h of chase, however, 3-fold more mature apo[a] was recovered in the medium of cells grown in DMEM-10% serum than in the medium of cells grown in SFM (Fig. 4B). The percentage of apo[a] that underwent maturation by 6 h of chase in cells cultured in DMEM-10% FBS was significantly greater than that in cells grown in SFM: 34 ± 14% versus 18 ± 6% (mean ± SD, n = 5), respectively (Fig. 4C). However, under both culture conditions, the majority of apo[a] was still subject to presecretory degradation (61 ± 15% vs. 68 ± 10% degradation by 6 h in DMEM-10% FBS and SFM, respectively).

To determine whether the increase in apo[a] secretion in cultures grown in DMEM-10% FBS was due to the presence of serum or to the absence of factors in the SFM, we analyzed the efficiency of apo[a] maturation and secretion under conditions in which cells were cultured and labeled in SFM and then chased in SFM plus 10% FBS. An increase in apo[a] maturation was also observed under these conditions compared with cells chased in SFM alone (percent maturation at 6 h = 32 ± 9%, mean ± SD, n = 4; Fig. 4C), although this did not reach significance. Because serum was added only to the chase in this experiment, the results suggest that the effect of serum on apo[a] secretion was posttranslational.

Dialysis or heat treatment of FBS did not affect its ability to increase apo[a] secretion from primary hepatocytes. However, delipidated serum was unable to increase apo[a] secretion (Fig. 4D). Thus, the influence of serum on apo[a] secretion appeared to be dependent on the presence of fatty acids or other lipid components.

Apo[a] secretion from transformed mouse hepatocytes

Use of primary hepatocytes as a model to study Lp[a] biogenesis requires that hepatocyte isolations be performed on a continual basis, a process that is costly in terms of both time and animals used. To facilitate our analyses of apo[a] synthesis and secretion we derived transformed cell lines from Lp[a] hepatocytes by transfection with a plasmid (pSV3neo) encoding the SV40 large-T antigen (see Materials and Methods). The cell lines were maintained in the same SFM used to culture the primary hepatocytes or were adapted to culture in DMEM-10% FBS.

For transformed cells grown in SFM, the characteristics of apo[a] synthesis and secretion were similar to those documented in primary hepatocytes, with the vast majority of apo[a] subject to presecretory degradation (83 ± 8%, mean ± SD, n = 6; **Fig. 5B**). As in primary hepatocytes (56, 57), apo[a] degradation in the transformed cells was inhibited by the proteasome inhibitor lactacystin, and apo[a] secretion efficiency was markedly increased in the presence of the lysine analog 6-amino hexanoic acid (6AHA; data not shown).

Transformed cells cultured in DMEM-10% FBS secreted substantially more apo[a] than those cultured in SFM (Fig. 5A). In general, however, SFM maintained the cells with a more highly differentiated phenotype; transformed cells grown in SFM secreted human apoB-48 and apoB-100, as well as endogenous mouse apoB-48, apoB-100, transferrin, and albumin (Fig. 5A). In contrast, cells

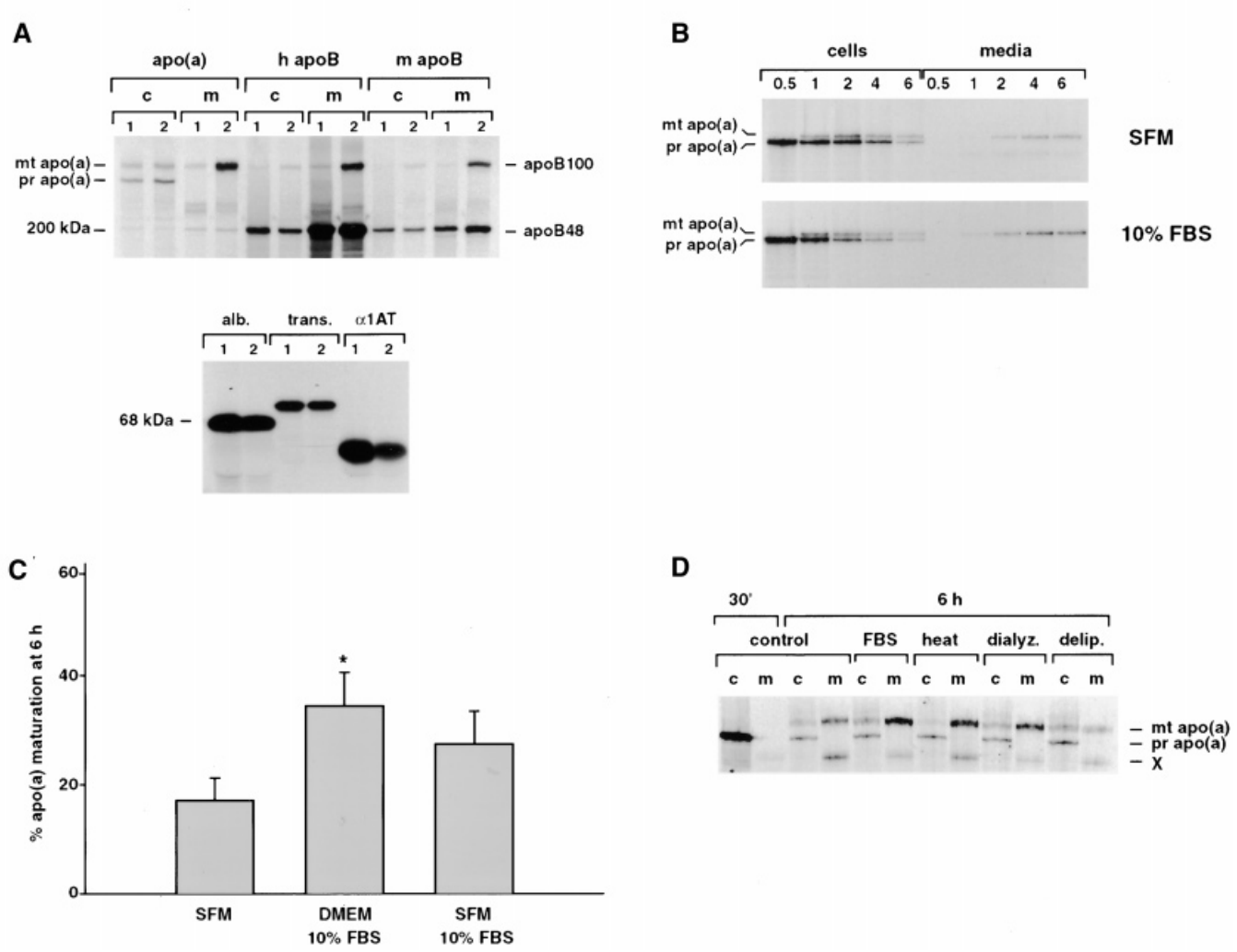

Fig. 4. Influence of serum on apo[a] secretion from Lp[a] transgenic hepatocytes. A: Top: Hepatocytes cultured for 24 h in SFM (lanes 1) or in DMEM-10% FBS (lanes 2) were labeled overnight as described in Materials and Methods. Apo[a], human (h) apoB, and mouse (m) apoB were then imunoprecipitated from cells (c) and medium (m) and analyzed by 4–10% SDS-PAGE. Bottom: Aliquots of labeled medium were immunoprecipitated with antibodies to albumin (alb.), transferrin (trans.), or α_1-antitrypsin (α1AT) and analyzed by 8% SDS-PAGE. The position of the 68-kDa molecular mass standard is indicated. B: Apo[a] secretion from hepatocytes cultured for 48 h in SFM or DMEM-10% FBS was analyzed by pulse-chase exactly as described in the legend to Fig. 1. C: Percentage of apo[a] that had undergone maturation by 6 h of chase in Lp[a] hepatocytes cultured in SFM, DMEM-10% FBS, or cultured in SFM and chased in SFM plus 10% FBS. Experiments were performed and the percent maturation was calculated as described in Materials and Methods. Error bars represent the SE of at least four independent determinations. * $P < 0.05$ versus SFM. D: Lp[a] transgenic hepatocytes were labeled for 15 min, and then chased for 30 min or 6 h in medium containing 10% FBS, heat-treated FBS (80°C, 10 min), dialyzed FBS, or delipidated FBS. Apo[a] in the cells (c) and medium (m) was analyzed by immunoprecipitation and SDS-PAGE. X denotes a protein nonspecifically recovered in immunoprecipitates of culture medium.

grown in DMEM-10% FBS secreted no detectable apoB, and the levels of albumin and transferrin in the medium were drastically reduced (Fig. 5A).

Pulse-chase analysis revealed a substantially higher level of apo[a] synthesis in the cells cultured in DMEM-10% FBS compared with those grown in SFM (the exposure times of the autoradiographs in Fig. 5B were 8 and 1 day for cells in SFM and DMEM-FBS, respectively). Similar to primary hepatocytes, the portion of apo[a] that underwent maturation and secretion was also greater in cells cultured in DMEM-10% FBS than in cells grown in SFM (percent maturation at 6 h was 6% and 56% for cells cultured in SFM and DMEM-FBS, respectively; Fig. 5B). A correspondingly greater portion of apo[a] was degraded in cells cultured in SFM versus DMEM-10% FBS (82% vs. 39% degraded by 6 h, respectively). Thus, the higher level of apo[a] secretion from transformed cells grown in DMEM-FBS appeared to be due to a combination of an increase in apo[a] synthesis rate and an increase in its efficiency of secretion.

The similar characteristics of apo[a] synthesis, secretion, and degradation in the transformed cells and the ability to regulate apo[a] secretion from these cells with serum and lysine analogs (data not shown) suggest they may provide a useful additional in vitro system for the analysis of apo[a] biogenesis.

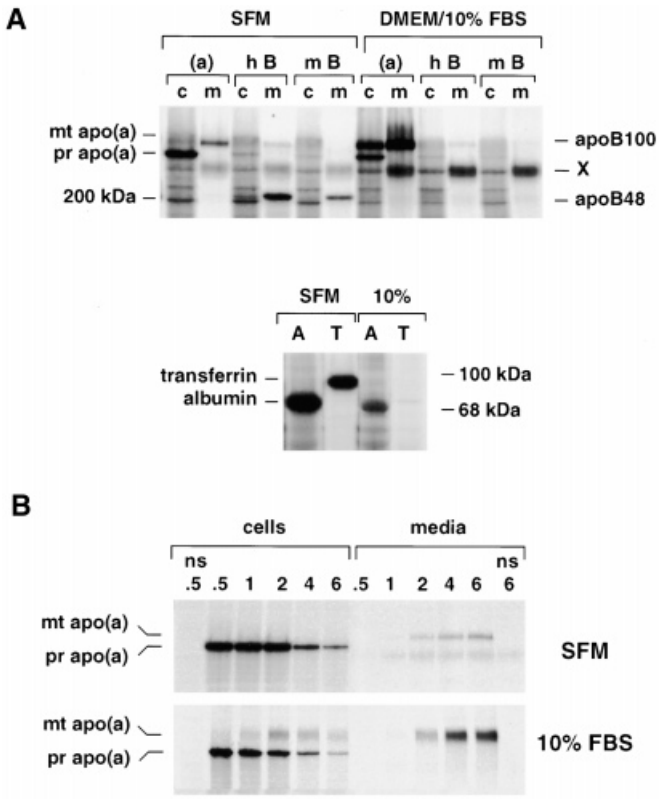

Fig. 5. Apo[a] expression in transformed mouse hepatocyte cell lines. Transformed cells were derived from Lp[a] transgenic hepatocytes as described in Materials and Methods. Cells from a single clone were grown in SFM or in DMEM-10% FBS. A: Cells were labeled overnight with [^{35}S]methionine and [^{35}S]cysteine as described in Materials and Methods. Top: The cell lysates (c) and medium (m) were immunoprecipitated with antibodies to apo[a] [(a)], human apoB (h B), or mouse apoB (m B) and analyzed by 4–10% SDS-PAGE. The positions of the precursor (pr) and mature (mt) forms of apo[a], apoB-100, apoB-48, the 200-kDa molecular mass marker and a protein nonspecifically precipitated from the culture medium (X) are indicated. Bottom: Aliquots of labeled medium were immunoprecipitated with antibodies against albumin (A) or transferrin (T) and analyzed by 8% SDS-PAGE. Positions of molecular mass markers are indicated to the right. B: Apo[a] synthesis and secretion were analyzed by pulse-chase as described in the legend to Fig. 1. Cells and medium were immunoprecipitated with anti-apo[a] antibodies or with preimmune serum (ns) and analyzed by SDS-PAGE as described in Materials and Methods. An 8-fold longer autoradiographic exposure is presented for cells in SFM versus DMEM-10% FBS.

DISCUSSION

Attempts to determine the molecular mechanisms regulating human Lp[a] production rate have been hampered by the lack of suitable in vitro model systems. In the current study we examined the expression of human apo[a] in primary cultures of hepatocytes derived from three strains of apo[a] transgenic mice, to assess their suitability as model systems to study the regulation of apo[a] biogenesis. Our results suggest that the apo[a] promoter in YAC-apo[a] transgenic mice does not retain activity in vitro, at least under the conditions used in this study. However, primary and transformed hepatocytes derived from Lp[a] and apo[a]-only transgenic mice, in which apo[a] mRNA synthesis is directed by the mouse transferrin promoter, may provide useful models with which to analyze post-translational regulation of apo[a] secretion.

Currently, no ideal in vitro model system exists for the analysis of apo[a] gene transcription. As far as we are aware, no transformed human liver cell line retains sufficient expression of the endogenous apo[a] gene. Human apo[a] promoter constructs show low activity in vitro (48, 49). In addition, not all the gene regulatory regions have been identified (39) and some exist far upstream from the transcription start site (38, 50, 51). Primary human hepatocytes, which can now be purchased commercially, provide a potential model system and have been used successfully to study apo[a] secretion (26). However, human donors expressing high levels of apo[a] cannot be preselected. In addition, hepatocytes used over a course of experiments will necessarily be derived from different donors who express a variety of different apo[a] alleles at different levels. Primary hepatocytes from other primate species provide a good alternative system; baboon and cynomolgus monkey hepatocytes have been used successfully to study apo[a] expression and secretion in vitro (20, 46). Again, however, access to liver tissue is limited. For these reasons, we investigated the use of hepatocytes isolated from YAC-apo[a] transgenic mice as a model system to study human apo[a] gene expression. However, we were unable to retain apo[a] expression in YAC-apo[a] hepatocytes beyond 48 h of culture. Northern blotting established that the levels of apo[a] mRNA were drastically decreased in the YAC hepatocytes over time, suggesting loss of activity from the apo[a] promoter. Addition of human growth hormone, which increases apo[a] mRNA levels in the YAC-apo[a] transgenic mice (42), was unable to retain expression (data not shown). Baboon hepatocytes grown in the same SFM as used in the current study maintain expression of the endogenous apo[a] gene for at least 3 weeks in culture (A. L. White and R. E. Lanford, unpublished observation). This may suggest that the expression of human and baboon apo[a] is regulated differently. Alternatively, it is possible that not all of the elements controlling human apo[a] expression are contained in the YAC clone, or that the mouse does not maintain appropriate expression of the human apo[a] gene. Apo[a] expression in vivo in YAC-apo[a] mice is regulated in a more dramatic fashion than in humans in response to dietary and hormonal factors (39–42), which may support one of the latter two possibilities. Loss of apo[a] gene expression in vitro, however, does suggest that the apo[a] gene can be regulated. Future studies will attempt to define conditions that will maintain apo[a] expression in mouse hepatocytes in vitro. However, currently this system does not provide a suitable model for the analysis of apo[a] gene regulation.

We have previously characterized primary hepatocytes isolated from Lp[a] transgenic mice as a model to study posttranslational regulation of human Lp[a] biogenesis (55, 56). This system provides the opportunity to analyze human apo[a] secretion and Lp[a] formation in a primary hepatocyte background, using the same apo[a] iso-

form over many experiments and without the need for human tissue. It also allows observations made in vivo in the transgenic mice to be interpreted at the molecular level in vitro. For example, we previously demonstrated that apo[a] secretion from mouse hepatocytes is increased in the presence of the lysine analog 6AHA. 6AHA appears to act as a chemical chaperone for apo[a], increasing apo[a] secretion and decreasing its intracellular degradation (56). Frank et al. (62) demonstrated an increase in plasma apo[a] levels in apo[a] transgenic mice after administration of lysine analogs, suggesting that these compounds may also increase apo[a] secretion in vivo. Thus, results obtained in our in vitro system appear to reflect the in vivo situation, at least in the mouse.

In comparison with studies in baboon hepatocytes (30), however, we found that the 17 K4 human apo[a] isoform expressed in the Lp[a] hepatocytes is subject to more extensive presecretory degradation than would be expected from its relatively small size. It was not clear whether this represented an intrinsic characteristic of human apo[a] or was a function of its expression in mouse cells. No detailed analysis of the extent of apo[a] degradation in human cells has been published, although studies by Nassir and colleagues (43) suggest that a portion of a 17 K4 human apo[a] isoform is degraded in HepG2 cells. We considered the possibility that coexpression of human apoB in the Lp[a] hepatocytes may contribute to the low secretion efficiency of apo[a], perhaps due to competition for ER chaperone proteins or nonproductive associations between apo[a] and apoB in the ER lumen (25). However, apo[a] was secreted at similarly low efficiency from hepatocytes derived from apo[a]-only mice that do not express the human apoB transgene. Analysis of a second, 13 K4, human apo[a] isoform in hepatocytes from YAC-apo[a] mice also demonstrated a low secretion efficiency of apo[a]. This isoform was cloned from an individual with a high plasma Lp[a] level (72 mg/dl) (39). In contrast, plasma levels of apo[a] achieved in the YAC-apo[a] mice are only ~8 mg/dl (39), suggesting that apo[a] may also escape the hepatocyte less readily in vivo in the mouse. Thus, although we cannot rule out that an amino acid sequence variation was introduced into the transgene during generation of the YAC mice that decreased apo[a] secretion efficiency, it appears that low secretion efficiency is a general characteristic of human apo[a] isoforms in mouse hepatocytes. It is possible that mice, that do not normally synthesize the apo[a] protein, may not have the machinery in place for high level apo[a] secretion.

We also considered the possibility that the low secretion efficiency of apo[a] in mouse hepatocytes was related to the culture conditions. Nassir et al. (43) reported that apo[a] secretion efficiency is increased by oleic acid in transformed human and rat hepatocytes in culture. Conversely, inhibitors of the microsomal triglyceride transfer protein (MTP) decrease apo[a] secretion, suggesting that apo[a] secretion is coupled to triglyceride synthesis and lipoprotein assembly (43). Addition of serum to our primary hepatocyte cultures resulted in a moderate increase in apo[a] secretion efficiency, although the majority of apo[a] was still directed toward ERAD. Consistent with the study by Nassir et al., delipidated serum did not increase apo[a] secretion from primary cultures, suggesting that lipoproteins or fatty acids were responsible for the effect. The mechanism of the effect is unclear. Nassir et al. suggested that, similar to apoB, translocation of apo[a] across the ER membrane may be coupled to lipoprotein assembly. However, we have been unable to demonstrate the presence of untranslocated apo[a] in baboon (31) or mouse hepatocytes (data not shown), even in the presence of proteasome inhibitors to prevent apo[a] degradation. In addition, apo[a] is secreted from cells that do not synthesize MTP or lipoproteins (58). Apo[a] synthesis and secretion was also clearly uncoupled from that of apoB in our transformed cell lines; cells grown in serum maintained an elevated level of apo[a] secretion despite the drastically reduced level of apoB expression. Furthermore, the effect of serum in our experiments was posttranslational, and, based on the electrophoretic mobility of the apo[a] precursor (20), all apo[a] is *N*-glycosylated and thus completely translocated into the ER lumen before the start of the chase in pulse-chase experiments. Further study will be required to delineate the mechanism of action of serum/fatty acids on apo[a] secretion.

Transformed cell lines derived from our primary hepatocytes showed similar characteristics of apo[a] secretion and degradation as observed in primary cells, including the regulation of apo[a] secretion by serum (Fig. 5) and 6AHA (data not shown). These cell lines may thus also provide a valuable tool to study factors regulating apo[a] secretion in the mouse.

In conclusion, our studies demonstrate that apo[a] expression is not maintained in cultures of hepatocytes from YAC-apo[a] mice, at least under the conditions examined in this study. However, both primary and transformed mouse hepatocytes may provide valuable model systems for the study of posttranslational mechanisms regulating apo[a] secretion and Lp[a] biogenesis. This will be particularly important in allowing observations made in vivo in the mouse to be analyzed at the molecular level in vitro.

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