Secretion of pre-beta-migrating apoA-I by cynomolgus monkey hepatocytes in culture

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Abstract Cynomolgus monkey hepatocytes that had been stored frozen were thawed, established in culture, and used to study apoA-I secretion. Protein synthetic activity was low at first, but increased with time, approaching what appeared to be the constitutive levels of the intact liver by day 7. During the first week, cellular RNA levels increased from 5.3 ± 0.3 to 18.6 ± 1.0 $\mu g/10^6$ cells; albumin secretion rates increased from undetectable to 55.4 $\mu g/10^6$ cells per day; apoA-I mRNA levels increased from 174 ± 12 to 564 ± 145 $ng/10^6$ cells; and apoA-I secretion rates increased from undetectable to 2.11 ± 0.27 $\mu g/10^6$ cells per day. Analysis of day 7-conditioned media by agarose electrophoresis, gradient gel electrophoresis-immunoblotting, and column chromatography, showed that the apoA-I produced by the cells was present in three distinct forms. One had an apparent molecular mass greater than 1 million Da, migrated pre-β, and accounted for 11 ± 3 (mean ± SD)% of the total; one had an apparent molecular mass of 104 kDa, had α migration, and accounted for 27 ± 2% of the total; and one had an apparent molecular mass of 50 kDa, migrated pre-β, and accounted for 46 ± 9% of the total. These data support the proposition that the pre-β-migrating, 50 kDa, apoA-I-containing particles identified in the plasma of cynomolgus monkeys are nascent hepatic HDL. —**Castle, C. K., M. E. Pape, K. R. Marotti, and G. W. Melchior.** Secretion of pre-beta-migrating apoA-I by cynomolgus monkey hepatocytes in culture *J. Lipid Res.* 1991. **32:** 439–447.

Supplementary key words apoA-I • pre-β-migrating HDL • nascent HDL

High density lipoproteins have been recognized as one of the major carriers of cholesterol in the plasma for over 40 years, yet their role in cholesterol homeostasis is still not completely understood. They apparently originate as small, disk-shaped, apolipoprotein-phospholipid complexes (1). In primates, the liver is thought to be a principal source of these HDL precursors (2–4), although similar particles may also be formed as a by-product of chylomicron and VLDL catabolism (5, 6). In addition, there is now evidence (7–9) that nascent HDL can be formed in the periphery (in the interstitial fluid of the limbs). It is not clear what the relative contribution of each source is to the plasma HDL pool.

The residence time of nascent HDL in the plasma is not known, nor has a specific plasma HDL-subfraction been identified as nascent HDL. However, we (10) recently identified a pre-β-migrating[3] HDL subfraction in the plasma of cynomolgus monkeys which may be nascent HDL. What appears to be an analogous entity in human plasma had previously been identified and partially characterized (11–18). Under normal conditions the pre-β HDL are present in the plasma at relatively low concentrations, making up less than 5% of the circulating apoA-I pool in primates (10–18). However, with certain types of hyperlipidemia in humans (11, 13–15), or with a diet-induced hypercholesterolemia in monkeys (10), their relative concentration can increase substantially such that in extreme instances they can account for almost 50% of the circulating apoA-I.

In an attempt to gain some insight into the origin of the pre-β HDL in the plasma of primates, we studied apoA-I secretion by cynomolgus monkey hepatocytes that had been cryopreserved (19) and restored to culture. The primary hepatocyte model has a potential advantage over transformed cell lines in that the cells in primary culture might reflect more accurately what is occurring in the liver. We report here the results of studies to characterize the synthesis and secretion of apoA-I in this model; and, show that the apoA-I particles secreted by these cells have certain key characteristics in common with pre-β-migrating apoA-I in the plasma.

Abbreviations: gge, gradient gel electrophoresis; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; FBS, fetal bovine serum; EGF, epidermal growth factor; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; EIA, electroimmunoassay.

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[3]These particles were originally termed "β-migrating" HDL (10), but since they appear to be analogous to the human entity, we will refer to them hereafter as "pre-β-migrating" HDL, so as to conform to the human nomenclature. See also the Discussion section in this report.

METHODS

Hepatocyte isolation, preservation and culture

Cynomolgus monkeys (*Macaca fascicularis*) obtained from the Upjohn colony were used as liver donors. They had consumed only monkey chow and had unremarkable medical histories. The animals were fasted overnight, anesthetized, their livers were removed, and the hepatocytes were isolated and cryopreserved exactly as described previously (19). The hepatocytes used in these studies had been stored for 3 months or less. Just prior to an experiment, the hepatocytes were thawed and established in culture essentially as described previously (19), with the exception that, after the first 24 h in culture (during which they were maintained in the supplemented L-15 medium containing 17% fetal bovine serum (19)), the media formulation was changed such that it no longer contained fetal calf serum. In addition, EGF (20 ng/ml, final concentration; Amgen, Boston) (20) was added. Under those culture conditions the cells remained viable for at least 2 weeks, as measured by morphology and protein synthesis rates. In the absence of EGF and FBS, cell viability was significantly reduced within 2–3 days. When the medium contained FBS (17%) but not EGF, reductions in cell viability were consistently evident by day 8. Under no conditions did we see evidence of increased DNA synthesis or cell proliferation.

Quantification of apoA-I mRNA levels

The total cellular RNA was isolated and the apoA-I mRNA mass was quantified using the internal standard-S1 nuclease assay exactly as described previously (21). This method is capable of detecting changes in apoA-I mRNA levels as small as 10%, and accurately quantifying changes as small as 25%.

Quantification of apoA-I and albumin secretion rates

The apoA-I concentration of conditioned media was quantified by immunoassay using a monospecific, polyclonal antibody raised in rabbits against cynomolgus monkey apoA-I. Western immunoblotting analysis (22) against monkey serum proteins separated by SDS-PAGE showed a single band that co-migrated in the gel with authentic cynomolgus monkey apoA-I.

Usually, 1–2 million hepatocytes were sustained per 60-mm Petri dish. The volume of medium in each dish was 3 ml and the media were changed daily. To quantify the apoA-I present in media that had been exposed to the cells for 24 h, a 20–100-μl aliquot was taken, brought to a final volume of 300 μl with Tris-buffered saline (TBS), and bound to nitrocellulose paper using a slot blot apparatus (Schleicher & Schuell, Keene, NH). The free binding sites on the nitrocellulose paper were then blocked and the primary antibody was allowed to react with the apoA-I present on the paper followed by a radiolabeled secondary antibody exactly as described in some detail previously (10). The secondary antibody (affinity-purified goat-anti-rabbit IgG) was purchased from Boehringer Mannheim Biochemicals (Indianapolis) and radiolabeled with ^{125}I as described below. After incubation with the secondary antibody, the nitrocellulose paper was washed extensively in blocking buffer (10), the area corresponding to each slot was cut out, and the radioactivity contained in that portion was measured in a gamma counter. The apoA-I in a given portion was determined from a standard curve relating apoA-I mass to radioactivity bound to the nitrocellulose paper. Blank slots were run with each set of determinations for background correction.

Various dilutions of a serum standard pool were run in duplicate with each set of unknowns and the apoA-I concentrations of the unknowns were expressed as a percentage of the serum standard. The parameters of the standard curves varied slightly from assay to assay, but for a typical curve they were $y = -1.55x^2 + 234x + 1389$, where y is the cpm bound and x the mass of apoA-I applied to the slot. The correlation coefficient exceeded 0.99 in every instance. The absolute mass of apoA-I in the serum standard was determined exactly as described previously (23). The effective range of the assay was from 3 to 75 ng of apoA-I per slot. The assays were usually run in duplicate and the curve describing the relationship between duplicates was $y = 0.99x + 37$, $r = 0.998$.

The secondary antibody was radiolabeled with ^{125}I using Pierce Iodo-Beads (Pierce Chemical Co., Rockford, IL). Usually, one Iodo-Bead and 1 mCi of Na^{125}I (Amersham Corporation, Arlington Heights, IL) were added to 100 μl of PBS in a 1-ml conical Eppendorf vial. The contents of the vials were mixed by vortexing, and 20 μl of affinity-purified IgG (protein concentration, 1 mg/ml) was added. The vials were vortexed again and allowed to sit at room temperature for 15–20 min. The Iodo-Bead was then removed from the vial and the contents were passed through a PD-10 desalting column (Pharmacia LKB Biotechnology, Piscataway, NJ). The column eluant was collected in 500-μl aliquots and the radiolabeled IgG was usually eluted in fractions 6 and 7 (2.5–3.5 ml). The radiolabeled IgG was then dialyzed against PBS to remove residual non-protein-bound iodine.

The albumin secretion rate was measured by electroimmunoassay (EIA) essentially as described previously (24–26). The antibody (anti-human albumin; Sigma Chemical Company, St. Louis, MO) concentration in the gel was 0.25%. The conditioned media (9.5 μl, undiluted) was electrophoresed for 16–18 h at 2.5 volts/cm. The gels were washed, dried, and stained exactly as described previously (24–26). Various dilutions of serum (for which the albumin concentration had been determined by the dye-binding method of Rodkey (27) as modified by

Doumas, Watson, and Biggs (28)) were run in duplicate, in the same gel with the unknowns, and a standard curve was constructed relating rocket height with concentration.

Electrophoresis and immunoblotting

Agarose electrophoresis followed by immunoblotting was used to determine the electrophoretic mobility of the various apoA-I-containing fractions in the conditioned media, and was performed exactly as described previously for plasma (10).

Gradient gel electrophoresis followed by immunoblotting was performed to determine the size distribution of the secreted apoA-I-containing fractions. Pharmacia 4–30% gradient gels were used for the electrophoresis, which was performed as described previously (24), except that the electrophoresis was only allowed to proceed for 14 h and the gels were not fixed. After electrophoresis, the gels were removed from the cassette and washed with transfer buffer (20 mM Tris, 150 mM glycine) for 1 h. The buffer was changed at 20-min intervals. The proteins were then transferred to nitrocellulose paper (0.45 μM, Schleicher & Schull) by electroblotting (30 volts for 24 h; buffer temperature, 4°C) using a Transphor Electrophoresis Unit (Hoefer Scientific, San Francisco).

Previous studies had shown that by 14 h, the various apoA-I-containing bands had essentially reached their maximum migration distance in the gel, yet the efficiency with which they transferred to the nitrocellulose paper was still adequate, i.e., longer electrophoresis times had no detectable effect on the estimates of Stoke's diameter, but appeared to significantly reduce the efficiency of transfer.

The apoA-I bands on the nitrocellulose paper were identified immunochemically exactly as described previously (10). The size distribution of the various apoA-I bands was determined by comparison of their migration distance in the gel to that of proteins of known Stokes' diameter exactly as described by Nichols, Krauss, and Musliner (29) except that the apoA-I-containing bands were identified by autoradiography rather than protein staining.

Column chromatography

The distribution of apoA-I among the various sized particles was quantified by column chromatography using A-0.5M and A-15M agarose (Bio-Rad Laboratories, Richmond, CA) essentially as described previously (10). Nine ml of culture medium that had been with the cells for 24 h (usually between days 6 and 7) was applied to the column in each instance. The column flow rate was 18 ml/h and 7-ml fractions were collected. The apoA-I content of each column fraction was quantified by the slot-blot immunoassay described above.

Calculations

Table 2 contains, for comparison, the apoA-I secretion rates of the cultured hepatocytes (measured directly) and estimates of the rate at which that protein is secreted by the cynomolgus monkey liver (in vivo). The latter estimates were determined by assuming that: *1*) the total body apoA-I synthesis rate in the male cynomolgus monkey was 75 mg/day (obtained from reference 23); *2*) the liver contributed 63% (47 mg/day) to the total apoA-I entering the plasma each day (based on data in reference 2 that show that the liver contains 63% of the apoA-I mRNA in the monkey); and *3*) the liver of a cynomolgus monkey accounts for 1.8 ± 0.2% of its body weight (a value we obtained using necropsy data from 10 male cynomolgus monkeys that were part of another study, and whose mean weight was 4.6 kg). We further assumed, using data from Williams et al. (30) for the cynomolgus monkey, that the liver RNA content was 6.2×10^{-3} g/g of liver; the liver DNA content was 2.0×10^{-3} g/g of liver; and that there was 5.45×10^{-12} g of DNA (diploid)/hepatocyte. Therefore, an 82.8 gm liver (4600×0.018) would contain a total of 0.513 g of RNA. If one assumes an RNA:DNA ratio of 3:1, it follows that there are 1.69×10^{-11} g RNA/cell, 3.0×10^{10} cells per liver, and that the apoA-I secretion rate is 1.6×10^{-9} mg/cell per day.

RESULTS

Effect of cryopreservation on apoA-I and albumin secretion

Cynomolgus monkey hepatocytes, obtained by collagenase perfusion of the liver, were stored frozen as described previously (19). To study apoA-I production, the cells were thawed, established in culture, and the apoA-I mRNA levels and the secretion rate of the protein were measured at several times.

As shown previously (19), cryopreservation for up to 6 months did not abolish the hepatocytes' capacity to synthesize and secrete either apoA-I or albumin. **Table 1** shows the albumin secretion rates as well as the total cellular RNA levels measured from the time the cryopreserved cells were established in culture through day 13. **Fig. 1** shows the apoA-I mRNA levels and the apoA-I secretion rates over the same interval. It is clear from Table 1 and Fig. 1 that the hepatocytes' ability to synthesize and secrete apoA-I and albumin was initially comprised. However, by the 3rd day in culture the amount of both the total RNA and the apoA-I mRNA had reached what appeared to be their constitutive levels; and by the 5th day in culture the albumin and apoA-I secretion rates had also plateaued. It is noteworthy that the apoA-I secre-

TABLE 1. Changes in albumin secretion and cellular RNA levels with time after cryopreserved hepatocytes were restored to culture

	Days in Culture						
	1	3	5	7	9	11	13
				μg/10⁶ cells per day			
Albumin secretion[a]	nd	32.8 ± 9.5	58.6 ± 25.1	55.4 ± 28.3	49.8 ± 22.4	46.2 ± 18.1	36.4 ± 16.0
				μg/10⁶ cells			
RNA[b]	5.3 ± 0.3	15.4 ± 1.4	15.7 ± 1.7	18.6 ± 1.0	20.4 ± 3.0	17.9 ± 3.1	16.1 ± 4.5

[a]Mean ± range/2; nd, not determined. To convert to μg/mg cell protein per day, multiply by 1.16.
[b]Mean ± range/2.

tion rate did not reach its maximum until 3 or more days after the apoA-I mRNA levels reached their maximum; an indication that, at least initially, mRNA transcription was not the rate-limiting step in the apoA-I secretion process.

These data indicated that cryopreserved cells, restored to culture, were capable of secreting substantial quantities of apoA-I. To determine how apoA-I secretion by the cells in culture related to that by the intact liver, we compared the apoA-I mRNA levels, the total RNA levels, and the apoA-I secretion rates in the cells with that in the cynomolgus monkey liver. Those data are shown in **Table 2**. Note that, in each instance, what appeared to be the constitutive level in the cell closely approximated that of the intact liver, suggesting that the cryopreserved hepatocytes might be an acceptable model with which to study apoA-I metabolism in vitro.

Electrophoretic mobility and size distribution of the apoA-I containing particles in the conditioned media

In addition to determining the apoA-I secretion rate, we also characterized the apoA-I-containing particles in the conditioned media of the cryopreserved hepatocytes. **Fig. 2** shows an agarose electrophoresis immunoblot of conditioned media from one of the hepatocyte preparations used for Fig. 1. Note that the antibody detected increasing quantities of apoA-I through day 7, and that in every instance the majority of that apoA-I migrated pre-β (in exactly the same position that cynomolgus monkey LDL migrates (10)). Slight amounts of α-migrating material were detected by the antibody, and the α-migrating apoA-I appeared to resolve into a distinct band by day 9. Nonetheless, during the first 2 weeks in culture, the pre-β-migrating particles were the predominant apoA-I containing lipoproteins secreted by these hepatocytes.

Fig. 3 shows the size of the apoA-I-containing particles present in the conditioned media as measured by gradient gel electrophoresis (gge)-immunoblotting. Three major bands could be detected on the autoradiogram when day 6 and day 8 conditioned media were analyzed. The principal band migrated near the bottom of the gel and had an estimated molecular mass of 50 kDa (Stokes' diameter 6.9 nm). Two-dimensional (agarose-gge) electrophoresis-immunoblotting (not shown) demonstrated that those 50-kDa particles were, in fact, the pre-β-migrating particles evident in Fig. 2.

The next two most prominent bands on the autoradiogram had estimated molecular masses of greater than 1 million Da (Stokes' diameter unknown) and 104 kDa (Stokes' diameter 7.7 nm). The greater than 1 million Da band, evident at the top of the gel, migrated pre-β (as determined by the agarose-gge two-dimensional electrophoresis) and may represent apoA-I that had adsorbed to LDL (10). The 104 kDa band was shown by 2-D electrophoresis to correspond with the α-migrating lipoproteins evident in Fig. 2.

Because the efficiency with which different lipoproteins transfer from gradient gels to nitrocellulose paper can vary substantially with lipoprotein size and composition,

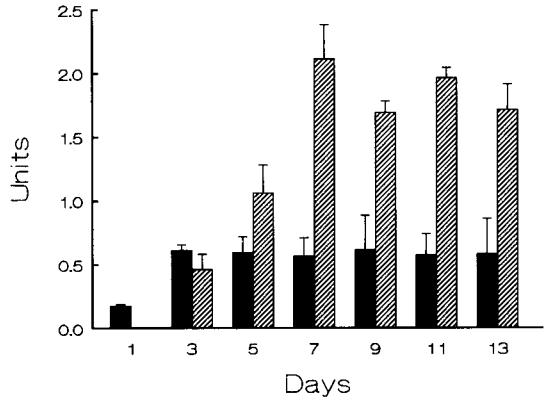

Fig. 1. The apoA-I mRNA content and secretion rate of the protein at various times after cryopreserved hepatocytes were established in culture. Cryopreserved hepatocytes from two cynomolgus monkeys were established in culture and the apoA-I mRNA content of the cells (ng/10^6 cells; solid bars) and the rate at which the protein was secreted (μg/10^6 cells/day; hatched bars) were followed daily for 2 weeks. The mean and range (bars) at various times during that period are shown. The patterns are typical of that seen when hepatocytes that had never been frozen were used.

TABLE 2. Comparison of apoA-I mRNA levels, total RNA levels, and apoA-I synthesis rates in cryopreserved hepatocytes in culture with that in the intact liver

	mRNA	Total RNA	Synthesis Rate
	pg/μg total RNA	*μg/10⁶ cells*	*μg/10⁶ cells per day*
Liver	40 ± 5[a]	16.9[b]	1.6[c]
Hepatocytes[d]	37 ± 4	17.4 ± 3	1.8 ± 0.2

[a]Mean ± SD; n = 5 monkeys.
[b]From Williams et al. (30).
[c]Calculated as described in Methods (calculations).
[d]The values used for apoA-I mRNA and total RNA are the mean (± SD) for days 3–13 (see Fig. 1 and Table 1); the value for synthesis rate is the mean (± SD) for days 7–9 (see Fig. 1).

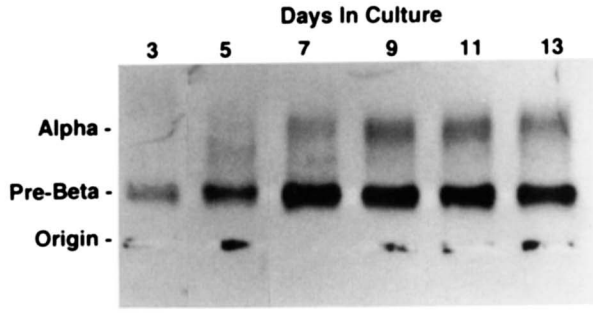

Fig. 2. Agarose electrophoresis-immunoblots of conditioned media at various times after cryopreserved hepatocytes were established in culture. The apoA-I in the conditioned media at each of the times shown represents that secreted during the previous 24 h.

we estimated the relative amounts of the various apoA-I-containing fractions in the conditioned media by column chromatography, rather than by the intensity of the bands on the autoradiogram. **Fig. 4** shows the apoA-I distribution obtained when conditioned medium was fractionated on a 4% agarose column. Two major apoA-I peaks are evident: one eluted with the apoB-containing lipoproteins; the other eluted with albumin. The former probably corresponds to the greater than 1 million Da entity evident at the top of the gge-immunoblots (Fig. 3), and appears from these distributions to account for approximately 17% of the total apoA-I secreted by these cells. The remaining apoA-I eluted as a single peak on the 4% agarose column, but probably represents the 104 kDa and 50 kDa entities that are not separated by this method.

Fig. 5 shows the elution pattern when day 7-conditioned media were passed through a column of 10% agarose. Note that the apoB-containing lipoproteins, and the apoA-I-containing lipoproteins of >1 million Da, eluted in the void volume. The remaining apoA-I-containing lipoproteins eluted as three distinct peaks on the 10% column: the first (elution volume = 275 ml), had an estimated molecular mass of 290 kDa and accounted for 16 ± 6 (mean ± SD) % of the total apoA-I; the second (elution volume = 305 ml) had an estimated molecular mass of 120 kDa and accounted for 27 ± 2 % of the total apoA-I; the third (elution volume = 345 ml) had an estimated molecular mass of 50 kDa and accounted for 46 ± 9% of the total apoA-I.

Based on these data, we would propose that the peak eluting at 345 ml corresponds to the 50 kDa band on the autoradiogram; that eluting at 305 ml to the 104 kDa band on the autoradiogram; and that in the void volume to the band at the top of the gel. There was no band evident on the autoradiogram that corresponded to the peak eluting at 275 ml. Since plasma lipoproteins in this size range (290 kDa) transfer relatively efficiently, we interpret these data as indication that no such particles were present in the gradient gel. The fact that those particles were evident in the column fractions may indicate that they are some type of aggregate that is disrupted by the forces with which the particles were driven through the gradient gels, but not the relatively gentle sieving process taking place in the column. Alternatively, they may represent some type of column-induced artifact.

Density of the apoA-I-containing particles in the conditioned media

To determine which, if any, of the apoA-I particles in the media were of a density in the lipoprotein density range, day 6-conditioned media were adjusted to density 1.225 g/ml and ultracentrifuged at 200,000 *g* for 40 h. The only apoA-I-containing particles that floated under those conditions were the greater than 1 million Da fraction. Thus, the other apoA-I-containing particles secreted by the hepatocytes were either disrupted by the centrifugation process or were of density greater than 1.225 g/ml.

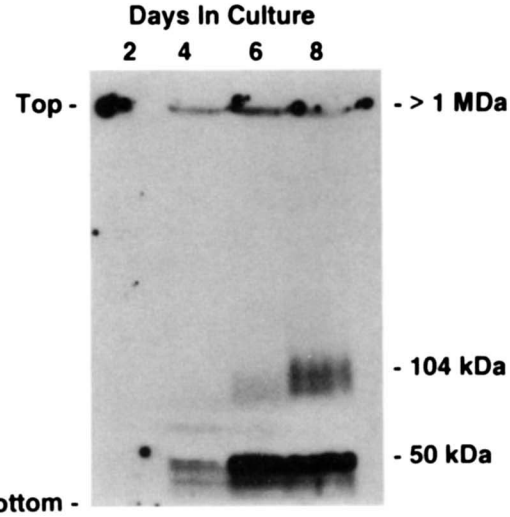

Fig. 3. Polyacrylamide gradient gel electrophoresis of conditioned media at various times after cryopreserved hepatocytes were established in culture. The apoA-I in the conditioned media at each of the times shown represents that secreted during the previous 24 h. Note that the appearance of the 104 kDa entity coincides with the appearance of the α-migrating band in Fig. 2.

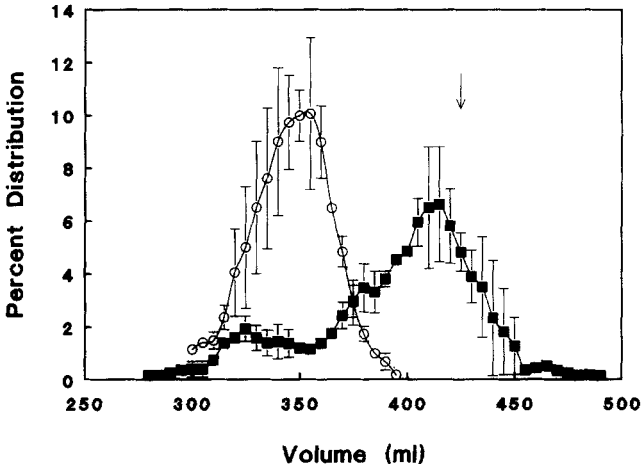

Fig. 4. ApoA-I and apoB distribution in day 7-conditioned media fractionated by agarose (4%) column chromatography. The culture media were changed on day 6, collected 24 h later, and immediately passed through a column of Biogel A-15 M. The distributions of apoB (○) and apoA-I (■) were determined by slot-blot immunoassay. The hepatocytes were from the same two cynomolgus monkeys used for Fig. 1. The mean and range (bars) are shown. The void volume of the column (taken at the elution peak) was 175 ml; the total volume was 501 ml. The arrow shows where the albumin peak eluted. No apoA-I eluted in or near the void volume. That indicates that the pre-β-migrating apoA-I evident in Fig. 2 was not associated with VLDL.

DISCUSSION

ApoA-I synthesis and secretion

The data reported here indicate that cryopreserved hepatocytes might be an appropriate model with which to study the anabolism and secretion of hepatic HDL. Hepatocytes from cynomolgus monkeys that had been stored frozen for several weeks were found, upon restoration to the culture conditions described here, to secrete significant quantities of apoA-I. Although the apoA-I secretion rates and cellular mRNA levels were relatively low initially, both increased after a few days in culture to levels that compared favorably with the levels in the liver. Albumin production by these cells was also substantial, and it is noteworthy that the steady-state albumin:apoA-I ratio in the conditioned media (27 ± 14) compared well with that in the plasma of the donor monkeys (30 ± 9). Thus, at least in this regard, the cryopreserved cells appear to reflect what occurs in vivo.

Our experience using fresh (never frozen) hepatocytes from several cynomolgus monkeys indicated that the low RNA level evident in the cells during the first 48 h was more a result of the cell isolation process (the removal of the liver, the collagenase perfusion, and the subsequent preparation of the cells for culture) than of the cryopreservation, i.e., although the culture conditions were somewhat different for the fresh cells than reported here for the cryopreserved cells, the apoA-I secretion patterns from days 1–7 were virtually identical. Thus, we feel that the perturbations in cellular function induced by cryopreservation were relatively minor compared to those produced by the isolation process. Nonetheless, it is noteworthy that in both cases it required 6–7 days in culture before the cells had attained a steady-state with regard to both apoA-I and albumin secretion. It is not clear whether this is a characteristic unique to primate hepatocytes, or our isolation procedures; however, we would urge caution in interpreting apolipoprotein secretion data from hepatocytes maintained in culture for less than 6–7 days, regardless of the animal species from which they were obtained.

A particularly interesting finding from these studies was that the apoA-I mRNA levels reached a steady-state in the cultured hepatocytes that was identical to that in the intact liver. This conclusion was based on a method for measuring mRNA levels that is relatively precise and accurate (21). If this observation is correct, it suggests that the constitutive levels of apoA-I mRNA in cynomolgus monkey hepatocytes is approximately 40 pg/μg of RNA. Our experience to this point has been that those levels do not vary substantially unless cell homeostasis is severely disturbed. Thus, hepatic apoA-I mRNA levels may remain relatively constant, and any variations in the hepatic secretion rate of the protein may be regulated post-transcriptionally. However, additional studies using liver biopsies will be necessary to confirm that perception.

Characterization of the secreted particles

We recently reported (10) that significant quantities of the apoA-I in the plasma of cynomolgus monkeys migrated with the β-lipoproteins during agarose electrophoresis, rather than with typical HDL (which

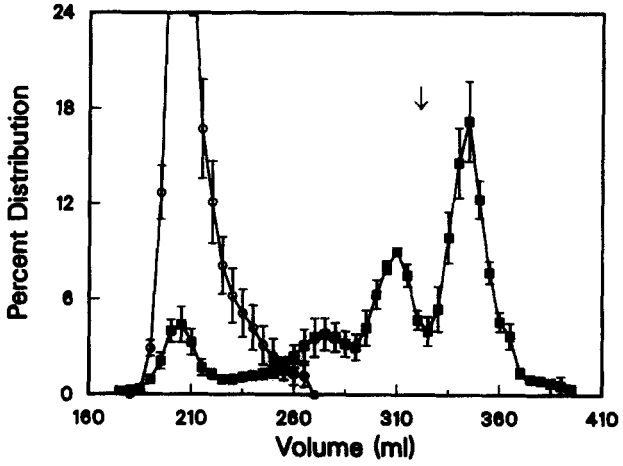

Fig. 5. ApoA-I and apoB distribution in day 7 conditioned media fractionated by agarose (10%) column chromatography. Cryopreserved hepatocytes from three cynomolgus monkeys were used. The apoB (○) and apoA-I (■) were determined by slot-blot immunoassay. The mean and SEM (bars) are shown. The void volume of the column was 200 ml; the total volume was 480 ml. The arrow shows where the albumin peak eluted.

migrates α in this system); and, that the amount of β-migrating apoA-I in the plasma increased markedly when the monkeys were made hypercholesterolemic. The β-migrating apoA-I was separated into two fractions. One, termed apoA-I(LDL), represented a pool of apoA-I that appeared to circulate associated with cynomolgus monkey LDL; the other, termed apoA-I(50kD), appeared to circulate in a complex with an apparent molecular mass of 50 kDa. We have been unable to establish conclusively whether the apoA-I(50kD) is free apoA-I, circulating as dimers, or very small, very high density lipoproteins. However, we suggested that the LDL-associated apoA-I might represent apoA-I(50kD) particles that had adsorbed to the LDL. Indeed, we were able to show that when free apoA-I was added to hypercholesterolemic plasma, some of the added apoA-I did adsorb to the LDL in that plasma.

What appears to be an analogous particle was identified in the plasma of humans several years ago (11). The human entity has been fairly well characterized (11–18) and appears to be very small lipoprotein, with a molecular mass of approximately 70 kDa (15). It migrates slightly faster than human LDL during agarose electrophoresis, and has thus been termed pre-β-migrating apoA-I. However, when we ran human and monkey plasma side-by-side in our system (data not shown), the human pre-β apoA-I migrated to almost exactly the same position as cynomolgus monkey β-migrating apoA-I. Furthermore, both co-migrated exactly with the monkey LDL, but slightly faster than human LDL. Therefore, even though their names imply otherwise, the 70 kDa particle from the human (15–17) and the 50 kDa particle from the monkey (10) are identical with respect to their behavior during agarose electrophoresis. As a result, we will hereafter refer to the monkey entity as pre-β-migrating apoA-I so as to conform to the nomenclature applied to the human entity.

An important question regarding the pre-β-migrating apoA-I concerns their origin. Are they nascent lipoproteins secreted by the liver that will subsequently be transformed into the large, α-migrating HDL? Are they surface remnants, produced during the catabolism of triglyceride-rich lipoproteins? Or, are they nascent HDL, produced in the periphery as part of the reverse cholesterol transport process (31)? The fact that the majority of the apoA-I secreted by the hepatocytes used for this study behaved exactly like the plasma apoA-I(50kD) (i.e., had a molecular mass of 50 kDa, and migrated pre-β during agarose electrophoresis) suggests that the liver might be one source of those small HDL.

Interestingly, a pre-β-migrating apoA-I particle has also been identified in the peripheral lymph from humans and dogs (7–9). However, those studies showed that the concentration of those particles in the lymph was higher than in the plasma. Since it does not seem reasonable that nascent HDL from the liver would be in higher concentration in the interstitial fluid than in the plasma, those investigators concluded that the lymph pre-β-migrating apoA-I particles were probably created in the interstitium. Thus, as suggested above, there may be more than one source of the pre-β particles in the plasma.

In addition to the 50–70 kDa HDL, two larger pre-β-migrating apoA-I species were identified in human plasma by Castro and Fielding (16). The first, a 325 kDa lipoprotein, may represent the 70 kDa entity that has expanded in size as a result of fusion and additional lipid accumulation (16). The second, an even larger particle, represents an apoA-I-CETP-LCAT-apoD complex that is probably involved in the esterification of free cholesterol and its subsequent transfer to the mature HDL (17). When conditioned media from the hepatocyte cultures were analyzed, no apoA-I-containing particles analogous to either of the latter two lipoproteins were evident on the gge-immunoblot autoradiograms. We ran a substantial number of gge-immunoblots, using a variety of conditions (which included transfer times of from a few hours up to 24 h) and were not able to identify any particles in conditioned media in the size range of the 325 kDa particle described by Castro and Fielding (16), even though particles of that size range from plasma transferred quite efficiently. Therefore, we do not believe that lipoproteins analogous to the 325 kDa entity or the apoA-I-CETP-LCAT-apoD complex exist in the conditioned media from these cells. Furthermore, if Castro and Fielding (16) are correct in their proposition that the 325 kDa lipoproteins are fusion products of the 70 kDa particles, and if that process is catalyzed by some transforming factor (32, 33), then one might not expect to see them in the absence of that factor. We would propose that this is the case with the conditioned media, i.e., that the levels of transforming factor, LCAT, and CETP and mature HDL are so low, that the HDL maturation process is essentially blocked, and the apoA-I(50kD) particles accumulate.

It is noteworthy that we did observe apoA-I particles of apparent molecular mass 290 kDa when conditioned media were passed through 10% agarose columns. However, as pointed out in Results, the total absence of such particles on the gge-immunoblot autoradiograms suggests that they might represent some type of aggregate that is disrupted during passage through the gradient gel, or a column-induced artifact. Therefore, it is not clear at this point whether a 290 kDa, apoA-I-containing lipoprotein is actually secreted by the cells.

Finally, we did identify a very high molecular mass, apoA-I-containing particle by both gge-immunoblotting and column chromatography. Those particles were trapped in the top of the 4–30% gradient gels, and eluted in the void volume when passed through a 10% agarose column. They co-eluted with the apoB and apoE (in the LDL molecular weight range) when passed through the 4% agarose column. These high molecular weight, apoA-I-containing

particles present in the conditioned media may be comparable to the apoA-I(LDL) present in plasma. We could not determine from these studies whether the apoA-I was secreted in association with the LDL, or whether it adsorbed to the LDL particle after its secretion.

If, as we are suggesting here, the apoA-I-containing particles secreted by these cells are nascent HDL, typical of those secreted by the intact liver, the question arises as to the potential effect of the culture conditions (especially as regards the lipid content of the media) on the apoA-I size distribution. These data, for example, were from cells grown in serum-free media, devoid of added lipid. However, under these conditions, the cells can be seen by light microscopy to accumulate significant numbers of lipid droplets by the fifth day in culture (possibly due to the large amount of carbohydrate in the culture media). Thus, it would not appear that the intracellular lipid concentration limited the secretion rate or size distribution of the nascent lipoproteins. Nonetheless, in a separate series of experiments, we evaluated the size distribution of the apoA-I secreted by heaptocytes that were grown in medium containing fetal bovine serum (17% by volume) for 6 days and then switched to serum-free media for the next 24 h. (The serum-free conditions were necessary because our antibody to cynomolgus monkey apoA-I cross-reacted with the apoA-I in the FBS.) The size distribution of those apoA-I-containing particles was identical to that reported here. These observations suggest that the size distribution of nascent HDL may be largely independent of the lipid content of the hepatocytes, a deduction in general agreement with the conclusions of Hamilton et al. (34) who studied nascent HDL from liver perfusates of orotic acid-fed rats.

In conclusion, these data show that, upon restoration to culture, cryopreserved hepatocytes from the cynomolgus monkey synthesize and secrete substantial quantities of apoA-I, and that the apoA-I has some key characteristics in common with the pre-β-migrating apoA-I shown to be present in human and monkey plasma. The apoA-I containing particles in the conditioned media also have some characteristics in common with the apoA-I-containing particles obtained by perfusion of monkey livers. Jones et al. (35), for example, detected significant quantities of apoA-I, with an apparent molecular mass that was less than albumin (and in the general size range of the 50 kDa particles described here) in the uncentrifuged perfusate. Thus, it seems plausible that the primate liver secretes particles of roughly the same size as those we identified in the conditioned media from primate hepatocytes. That supports the proposition that some, if not all, of the pre-β-migrating apoA-I in the plasma are nascent hepatic HDL.

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REFERENCES

1. Gotto, A. M., Jr., H. J. Pownall, and R. J. Havel. 1986. Introduction to plasma lipoproteins. *Methods Enzymol.* **128:** 3–41.
2. Sorci-Thomas, M., M. M. Prack, N. Dashti, F. Johnson, L. L. Rudel, and D. L. Williams. 1988. Apolipoprotein (apo) A-I production and mRNA abundance explain plasma apoA-I and high density lipoprotein differences between two nonhuman primate species with high and low susceptibilities to diet-induced hypercholesterolemia. *J. Biol. Chem.* **263:** 5183–5189.
3. Johnson, F. L., J. Babiak, and L. L. Rudel. 1986. High density lipoprotein accumulation in perfusates of isolated livers of African green monkeys. Effects of saturated versus polyunsaturated dietary fat. *J. Lipid Res.* **27:** 537–548.
4. Babiak, J., H. Tamachi, F. L. Johnson, J. S. Parks, and L. L. Rudel. 1986. Lecithin:cholesterol acyltransferase-induced modifications of liver perfusate discoidal high density lipoproteins from African green monkeys. *J. Lipid Res.* **27:** 1304–1317.
5. Tall, A. R., and D. M. Small. 1978. Plasma high density lipoproteins. *N. Engl. J. Med.* **299:** 1232–1236.
6. Tall, A. R., P. H. R. Green, R. M. Glickman, and J. W. Riley. 1979. Metabolic fate of chylomicron phospholipids and apoproteins in the rat. *J. Clin. Invest.* **64:** 977–989.
7. Reichl, D., T. M. Forte, J. L. Hong, D. N. Rudra, and J. Pflug. 1985. Human lymphedema fluid lipoproteins: particle size, cholesterol and apolipoprotein distributions, and electron microscopic structure. *J. Lipid Res.* **26:** 1399–1411.
8. Sloop, C. H., L. Dory, and P. Roheim. 1987. Interstitial fluid lipoproteins. *J. Lipid Res.* **28:** 225–237.
9. Lefevre, M., C. H. Sloop, and P. S. Roheim. 1988. Characterization of dog prenodal peripheral lymph lipoproteins. Evidence for the peripheral formation of lipoprotein-unassociated apoA-I with slow pre-B electrophoretic mobility. *J. Lipid Res.* 29: 1139–1148.
10. Melchior, G. W., and C. K. Castle. 1989. Apolipoprotein A-I metabolism in cynomolgus monkey. Identification and characterization of B-migrating pools. *Arteriosclerosis.* **9:** 470–478.
11. Schonfeld, G., A. Baily, and R. Steelman. 1978. Plasma, apolipoprotein A-I and A-II levels in hyperlipidemia. *Lipids.* **13:** 951–959.
12. Kunitake, S. T., K. J. LaSala, and J. P. Kane. 1985. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. *J. Lipid Res.* **26:** 549–555.
13. Daerr, W. H., U. Minzalaff, and H. Greten. 1986. Quantitative determination of apolipoprotein A-I in high density lipoproteins and 'free' apolipoprotein A-I by two-dimensional agarose gel lipoprotein-'rocket' immunoelectrophoresis of human serum. *Biochim. Biophys. Acta.* **879:** 134–139.
14. Neary, R. H., and E. Gowland. 1987. Stability of free apolipoprotein A-I concentration in serum, and its measurement in normal and hyperlipidemic subjects. *Clin. Chem.* **33:** 1163–1169.
15. Ishida, B. Y., J. Frolich, and C. J. Fielding. 1987. Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer. *J. Lipid Res.* **28:** 778–786.
16. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-B-migrating high density

lipoprotein. *Biochemistry.* **27:** 25–29.
17. Francone, O. L., A. Gurakar, and C. Fielding. 1989. Distribution and functions of lecithin:cholesterol acyltransferase and cholesterol ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell derived cholesterol. *J. Biol. Chem.* **264:** 7066–7072.
18. Kunitake, S. T., G. C. Chen, S-F. Kung, J. W. Schilling, D. A. Hardman, and J. P. Kane. 1990. Pre-beta high density lipoprotein. Unique disposition of apolipoprotein A-I increases susceptibility of proteolysis. *Arteriosclerosis.* **10:** 25–30.
19. Sun, E. L., D. G. Aspar, R. G. Ulrich, and G. W. Melchior. 1990. Cryopreservation of cynomolgus monkey (*Macaca fascicularis*) hepatocytes for subsequent culture and protein synthesis studies. *In vitro.* **25:** 147–150.
20. Richman, R. A., T. H. Claus, S. J. Pilkis, and D. L. Friedman. 1976. Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes. *Proc. Natl. Acad. Sci. USA.* **73:** 3589–3593.
21. Pape, M. E., K. R. Marotti, and G. W. Melchior. 1990. An improved method for precise quantitation of cellular and tissue apolipoprotein A-I mRNA levels by use of an internal standard. *J. Lipid. Res.* **31:** 727–733.
22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
23. Melchior, G. W., C. K. Castle, T. J. Vidmar, H. Greg Polites, and K. R. Marotti. 1990. ApoA-I metabolism in cynomolgus monkeys: male–female differences. *Biochim. Biophys. Acta.* **1043:** 97–105.
24. Melchior, G. W., H. N. Baker, C. R. Abee, and P. S. Roheim. 1984. Density gradient characterization of the high density lipoproteins in cholesterol-fed hyper- and hyporesponding patas monkeys (*Erythrocebus patas*). *J. Lipid Res.* **25:** 979–990.
25. Baker, H. N., D. A. Eggen, G. W. Melchior, P. S. Roheim, G. T. Malcom, and J. P. Strong. 1983. Lipoprotein profiles in rhesus monkeys with divergent responses to dietary cholesterol. *Arteriosclerosis.* **3:** 223–232.
26. Melchior, G. W., S. C. Willard, and M. T. Brandon. 1985. ApoE levels and distribution in rhesus monkeys consuming an atherogenic diet. *Atherosclerosis.* **58:** 65–80.
27. Rodkey, F. L. 1965. Direct spectrophotometric determination of albumin in human serum. *Clin. Chem.* **11:** 478–487.
28. Doumas, B. T., W. A. Watson, and H. G. Biggs. 1971. Albumin standards and the measurement of serum albumin with bromcresol green. *Clin. Chim. Acta.* **31:** 87–96.
29. Nichols, A. V., R. M. Krauss, and T. A. Musliner. 1986. Nondenaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol.* **128:** 417–431.
30. Williams, D. L., T. C. Newman, G. S. Shelness, and D. A. Gordon. 1986. Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. *Methods Enzymol.* **128:** 671–689.
31. Reichl, D., and N. E. Miller. 1989. Pathophysiology of reverse cholesterol transport. *Arteriosclerosis.* **9:** 785–797.
32. Rye, K-A, and P. J. Barter. 1986. Changes in the size and density of human high density lipoproteins promoted by a plasma-conversion factor. *Biochim. Biophys. Acta.* **875:** 429–438.
33. Rye, K-A. 1989. Interaction of the high density lipoprotein conversion factor with recombinant discoidal complexes of egg phosphatidylcholine, free cholesterol, and apolipoprotein A-I. *J. Lipid Res.* **30:** 335–346.
34. Hamilton, R. L., L. S. S. Guo, T. E. Felker, Y-S Chao, and R. J. Havel. 1986. Nascent high density lipoproteins from liver perfusates of orotic acid-fed rats. *J. Lipid Res.* **27:** 967–978.
35. Jones, L. A., T. Teramoto, D. J. Juhn, R. B. Goldberg, A. H. Rubenstein and G. S. Getz. 1984. Characterization of lipoprotein produced by the perfused rhesus monkey liver. *J Lipid Res.* **25:** 319–335.