Apolipoprotein A-I–cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles

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Abstract The ability of lipid-free human apoA-I expressed by transfected Chinese hamster ovary (CHO) cells to form apoA-I–lipid complexes extracellularly when incubated with CHO cell monolayers was investigated. Lipid-free apoA-I was incubated with nontransfected CHO-C19 cells for 24 h and extracellular assembly products were isolated at d ≤ 1.235 g/ml; approximately 12% of the incubated apoA-I floated at d ≤ 1.235 g/ml when apoA-I was added at 10 μg/ml. The composition of the particles was 51.3% protein, 20.3% phospholipid, and 28.3% cholesterol. Electron microscopy of the apoA-I–lipid complexes revealed that discoidal particles 15.4 ± 4.1 nm diameter predominated but some vesicular particles 34.7 ± 16.8 nm diameter were also in evidence. Nondenaturing gradient gel electrophoresis of the extracellular assembly products formed after 24 h incubation with 10 μg/ml apoA-I showed particle size heterogeneity with major bands at 11.2 and 9.0 nm; additional minor components banded at 7.3, 17.7, and 19.5 nm. This size distribution, as well as composition and electron microscopic structure, is similar to that of complexes isolated from the medium of CHO cells transfected with the human apoA-I gene. The formation of extracellular assembly complexes was concentration-dependent such that at 2 μg apoA-I/ml for 24 h, primarily 7.3 nm complexes were formed; at 4 μg/ml the distribution was more heterogeneous and the major band peaked at 9.2 nm, while at 8 μg/ml the 7.3 nm component was greatly diminished and the 11.2 nm component was the major one. Formation of extracellular assembly products was sensitive to time of incubation when CHO-C19 cells were incubated with 8 μg/ml apoA-I for periods from 1 to 24 h. After 1 h incubation, only the 7.3 nm particle was present; at 4 h, in addition to the 7.3 nm component, a small band was noted at 9.0 nm. After 12 h incubation, the 7.3 and 9.0 nm components were pronounced but a distinct peak at 11.1 nm was also visible; at 24 hr the 11.0 and 9.0 components were dominant and the 7.3 nm particle was a minor component. We propose a precursor–product relationship in the formation of extracellular nascent apoA-I-containing particles where the first complexes are small, 7.3 nm, lipid-poor particles that eventually give rise to larger sized, lipid-enriched complexes.—**Forte, T. M., R. Goth-Goldstein, R. W. Nordhausen, and M. R. McCall.** Apolipoprotein A-I–cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. *J. Lipid Res.* 1993. **34:** 317–324.

Supplementary key words Chinese hamster ovary cells • nondenaturing gradient gel electrophoresis • electron microscopy • phospholipid

Apolipoprotein (apo) A-I is a 243 amino acid protein synthesized primarily in the liver and intestine. It is the major protein associated with high density lipoproteins (HDL) where it constitutes approximately 75% of the HDL protein. Elevated levels of HDL and its associated apoA-I have been correlated with decreased risk for cardiovascular disease. HDL (1–8) and its constitutive proteins (apoA-I and apoA-II) (9–13) have been shown to stimulate cholesterol efflux and net transport from cells thereby initiating the first steps in the putative reverse cholesterol transport pathway. It is by this pathway that HDL is believed to exert its anti-atherogenic effects.

Although it has been clearly demonstrated that apoA-I synthesis occurs almost exclusively in the liver and intestine, the mechanism(s) and site(s) of HDL assembly remain unclear. Suggestive evidence for both the direct secretion of nascent HDL particles (14, 15) and for HDL assembly in the extracellular milieu (5, 9, 13) have been published.

The Chinese hamster ovary (CHO) cell has been successfully transfected with the human apoA-I gene; moreover Mallory et al. (16) showed that the stably transfected CHO 143 clone secretes large quantities of apoA-I. Recently it has been demonstrated that a heterogeneous population of apoA-I–lipid complexes accumulates in the conditioned medium of this clone (17). The lipidated forms of apoA-I consisted of several discrete morphological entities including discs and vesicles. The discoidal particles recovered from the medium were substrates for lecithin:cholesterol acyltransferase, being converted by the enzyme into spherical particles that resembled plasma HDL. In addition to discoidal and vesicular particles, a lipid-poor 7.2 nm particle which could not be resolved by

Abbreviations: CHO, Chinese hamster ovary; HDL, high density lipoprotein.

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electron microscopy, but was identifiable by nondenaturing gradient gel, was observed in the culture medium. The latter component was unique because it possessed phospholipid but no cholesterol.

The site of apoA-I-lipid complex formation in CHO cell culture medium, as well as in other cell culture models, has not been resolved. Model systems used to investigate HDL synthesis, including rat primary liver cells (18), perfused monkey liver (19), human hepatoma-derived liver cell lines (20, 21), and the CHO 143 clone (17), have a common feature, namely, the majority of apoA-I isolated from the medium is not associated with lipid. Because lipid-free apoA-I is the major form of secreted apoA-I, it has led investigators to suggest that HDL may be assembled extracellularly by interaction of apoA-I with other lipoproteins and/or with cell membranes. The latter interaction is difficult to assess in liver cell cultures and liver perfusions because apoA-I, as well as other apolipoproteins and lipoproteins, are simultaneously secreted into the medium. CHO cell cultures provide a model wherein one can test the interaction of CHO 143 clone-derived lipid-free apoA-I with nontransfected CHO cells. In the present study lipid-free apoA-I isolated from the conditioned medium of the CHO 143 clone was used to examine assembly and particle size heterogeneity of nascent, extracellularly formed HDL.

MATERIALS AND METHODS

Culture of CHO cells transfected with human apoA-I gene and isolation of lipid-free apoA-I fraction

The stable transformed CHO clone, CHO:pMTAIR143 (CHO 143 clone) which expresses human apoA-I, was kindly provided by Dr. Joanne Mallory, California Biotechnology Inc., Mountain View, CA. The transfected cells were cultured in Dulbecco's modified Eagle's medium/Coon's F12 (1:1) with 10% fetal bovine serum (FBS) as previously described (16). When cells were confluent, the monolayers were rinsed with Hanks' balanced salt solution and grown in serum free medium in the presence of 15 mM HEPES, 80 μM $ZnSO_4$, and 30 μM $FeSO_4$. Conditioned medium was harvested each day for 5–7 consecutive days and was passed through a 0.2-μm filter to remove debris. Medium was pooled and EDTA (1 mg/ml) and gentamicin sulfate (0.1 mg/ml) were added. Pooled conditioned medium was concentrated approximately 100-fold by ultrafiltration, adjusted to d 1.25 g/ml with sodium bromide, and ultracentrifuged in a Beckman 50.3 rotor (40,000 rpm, 48 h, 4°C). The top 1 ml containing the floating, lipid-associated apoA-I ($d \leq 1.235$ g/ml) was removed by aspiration; the 5 ml protein-rich infranatant containing the lipid-free apoA-I was collected and dialyzed extensively against saline–Tris–EDTA (150 mM, 10 mM, and 0.27 mM, respectively) and sterile-filtered. The amount of apoA-I in the $d > 1.235$ g/ml fraction was determined by ELISA (21).

Incubations with lipid-free apoA-I fraction

To test whether the lipid-free apoA-I from transfected CHO cells is lipidated extracellularly by interaction with cells, known quantities of apoA-I were incubated with nontransfected CHO-C19 cells. Although conditioned medium from transfected CHO cells is complex, lipid-free apoA-I was not isolated from the $d > 1.235$ g/ml fraction prior to incubation in order to approximate conditions occurring in the transfected CHO culture medium and to minimize handling. Aliquots of this protein-rich fraction with known apoA-I concentration were used in the various incubations. CHO-C19 cells were grown in 850 cm^2 roller bottles with DMEM/F12 and 10% FBS. Prior to the onset of the incubation experiment, the monolayer was rinsed with Hanks' balanced salt solution and serum-free medium (DMEM/F12) was added to the bottles. This medium was removed after 24 h incubation and discarded to assure the removal of residual contaminating FBS lipoproteins. At the onset of the incubation experiments, 70 ml of fresh serum-free medium containing a known amount of apoA-I was introduced into each flask and the monolayers were incubated as described in figure legends. At the conclusion of the experiment, conditioned medium was collected and immediately placed on ice. Gentamicin sulfate (0.1 mg/ml), EDTA (1 mg/ml), and PMSF (0.5 mM) were added and cell debris was removed by filtration. Conditioned medium was concentrated approximately 35-fold by ultrafiltration. The concentrated medium was adjusted to d 1.25 g/ml and subjected to ultracentrifugation as indicated above; the $d \leq 1.235$ g/ml fraction containing lipid-associated apoA-I complexes was removed by aspiration. Both the $d \leq 1.235$ and $d > 1.235$ g/ml fractions were extensively dialyzed against saline–Tris–EDTA prior to carrying out further analyses. Recovery of apoA-I protein in incubations with 8 μg/ml apoA-I ranged from 71 to 106% after 24 h incubation.

Compositional analyses

ApoA-I was determined by ELISA essentially as previously described (21). Protein was determined by Markwell's modified method of Lowry (22); phospholipid was determined by the method of Bartlett (23), and cholesterol by gas–liquid chromatography according to the procedure of Hindriks, Wolthers, and Greon (24).

Electrophoretic analyses and electron microscopy

The size distribution of particles was determined by nondenaturing gradient gel electrophoresis on 4–30% gels (Pharmacia) according to the method of Blanche et al. (25). Standards used to calibrate the gels consisted of the globular proteins thyroglobulin, apoferritin, lactate

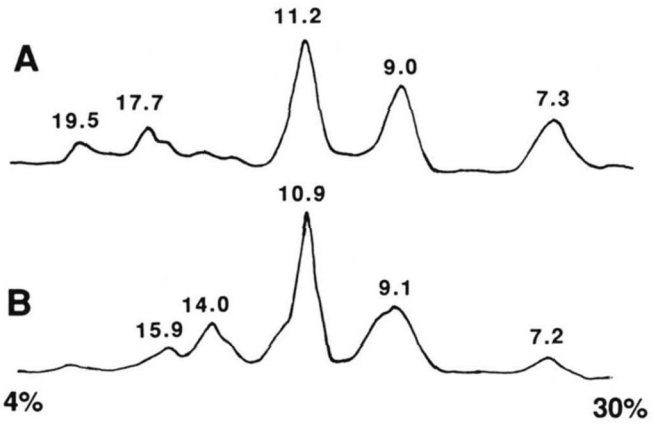

Fig. 1. Nondenaturing gradient gel (4–30% polyacrylamide) scans of d<1.235 g/ml fractions isolated from culture medium. (A) ApoA-I–lipid complexes isolated from medium of nontransfected CHO-Cl9 cells incubated with 10 μg/ml apoA-I for 24 h. These components are referred to as extracellular assembly products. (B) The distribution of apoA-I–lipid complexes isolated from transfected CHO cell medium after 24 h incubation with serum-free medium is shown for comparison. Numbers over peaks indicate banding position of complexes in nm; this is a relative indicator of particle diameter as the major components are discoidal in shape and the standard curve was constructed using globular proteins.

dehydrogenase, and albumin; peak positions reported in nanometers (nm) are based on the diameter of the globular standards. Proteins associated with the complexes were analyzed by SDS-polyacrylamide gel electrophoresis on 4–20% gradient gels. Western blot analyses of SDS-PAGE and agarose gels were carried out as previously described (20).

Samples were dialyzed against ammonium acetate buffer, pH 7.4, and stained with 2% sodium phosphotungstate for evaluation by electron microscopy according to previously described procedures (26).

RESULTS

Structure and size distribution of extracellular assembly products

Lipid-free apoA-I incubated with CHO-C19 cells for 24 h formed apoA-I–lipid complexes that floated at d ≤1.235 g/ml; we have termed such complexes "extracellular assembly products." The extracellular assembly products consisted of several discrete components when examined by nondenaturing gradient gel electrophoresis as seen in **Fig. 1A.** Major peaks banded at 11.2 nm and 9.0 nm while additional components banded at 7.3, 17.7, and 19.5 nm. This profile is similar to that previously reported by us for complexes isolated at d≤ 1.235 g/ml from medium of transfected CHO cells after 24 h incubation; for comparison, such a profile is seen in Fig. 1B.

Consistent with the gradient gel profile (Fig. 1A), the electron microscopic morphology of the extracellular assembly products was heterogeneous (**Fig. 2A**). The predominant morphologies were discoidal particles (15.4 ± 4.1 nm diameter; 4.6 ± 0.03 nm thickness, mean of three experiments) which formed rouleaux; some larger vesicular structures (34.7 ± 16.8 nm) were also in evidence. This morphological heterogeneity is similar to that which we reported for apoA-I products isolated from transfected CHO cell medium (17).

In a separate set of control experiments, conditioned medium was harvested from CHO-C19 cells and was incubated with lipid-free apoA-I in order to determine whether cell debris or material secreted by the nontransfected CHO-C19 cells may provide a lipid source for the extracellular assembly process. No apoA-I-containing particles of d≤1.235 g/ml were formed, although in a highly concentrated sample some electron microscopic

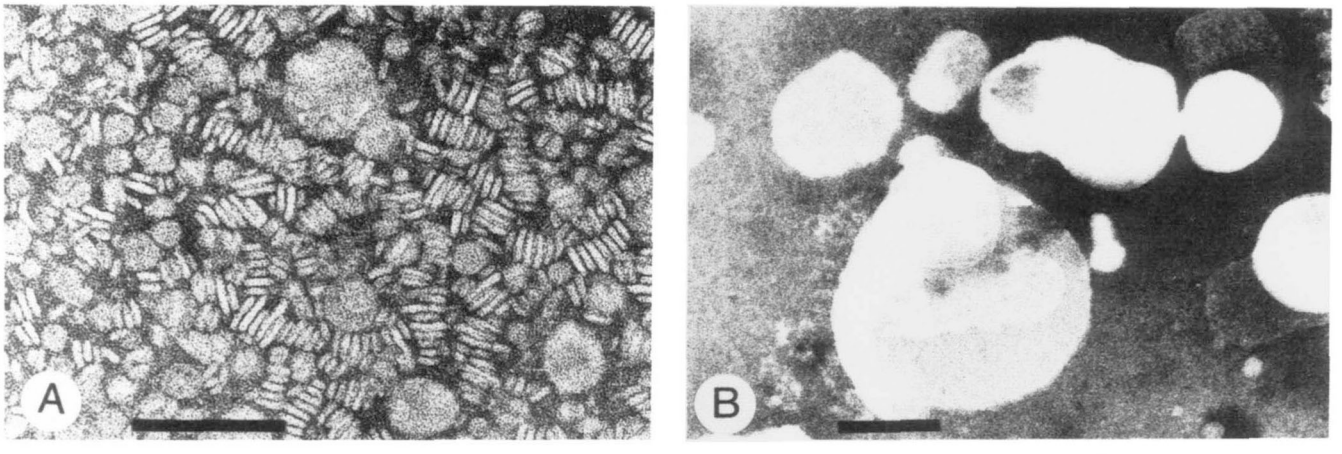

Fig. 2. Electron micrographs of negatively stained fractions. (A) Extracellular assembly products harvested from medium after incubation of nontransfected CHO-Cl9 cells with 10 μg/ml apoA-I for 24 h. Discoidal particles are the predominant components. Some vesicular particles are also in evidence. (B) The d<1.235 g/ml fraction after incubation of serum-free conditioned medium from nontransfected CHO-Cl9 cells (24-h harvest) with apoA-I (i.e., incubation carried out in the absence of cells). A 10-μl aliquot of d<1.235 g/ml fraction was allowed to dry completely on the grid in order to identify structures which were extremely sparse. Bar markers represent 100 nm.

TABLE 1. Composition of apoA-I-lipid complex

Source	Protein	Phospholipid	Cholesterol
CHO-Cl9[a]	51.3 ± 3.8	20.3 ± 1.2	28.3 ± 5.0
Transfected CHO[b]	51.3	18.8	29.9

[a] n = 3.
[b] From ref. 17.

fields showed the presence of large, irregular structures that probably represent cell debris (Fig. 2B). These incubations suggest that intact cells were required for formation of extracellular assembly products.

Composition of extracellular assembly products

The composition of the extracellular assembly products generated after 24 h incubation is shown in **Table 1.** The protein to lipid ratio (1:1) of the extracellular assembly products is similar to that of plasma HDL. The lipid moiety of the d ≤ 1.235 g/ml apoA-I complexes consists of phospholipid and unesterified cholesterol; this composition is compatible with the predominance of discoidal particles. The composition of the extracellular assembly products, moreover, is comparable to that reported for the apoA-I-lipid complex that accumulates in the medium of transfected CHO cells (17).

Evaluation of the protein moiety of the extracellular assembly product by SDS-PAGE (**Fig. 3**) indicates that the protein is predominantly apoA-I although a faint band close to 70 kD is also present (Fig. 3B). The immunoblot with anti-apoA-I (Fig. 3D) indicates that both bands are positive for apoA-I, suggesting that the larger molecular weight band may represent an aggregated form of apoA-I. Thin-layer chromatography was used to evaluate phospholipid species; the major phospholipid was phosphatidylcholine, while small quantities of phosphatidylethanolamine and phosphatidylserine were also present (data not shown). This phospholipid composition was similar to that of complexes isolated from transfected CHO cell medium.

The electrophoretic mobility of the extracellular assembly product formed after 24 h incubation was examined by agarose electrophoresis (**Fig. 4**). The complexes had alpha mobility which is similar to that of isolated plasma HDL. The same mobility was noted for conditioned medium which contained both lipid-associated and unas-

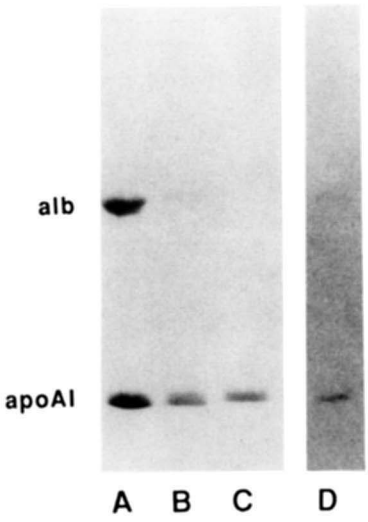

Fig. 3. SDS-polyacrylamide (4–20%) gel electrophoresis of apoA-I-lipid complexes. (A) Standards: albumin (alb) and apoA-I; (B) extracellular assembly complex isolated after incubation of nontransfected CHO-Cl9 cells with 10 μg/ml apoA-I. The major band co-migrates with apoA-I; however, a minor component is also present just above the region corresponding to albumin. The Western blot for this same fraction shown in (D) indicates that the larger sized band also reacts with apoA-I antibody. (C) ApoA-I-lipid complex harvested from transfected CHO cell medium after 24 h incubation is shown for comparison.

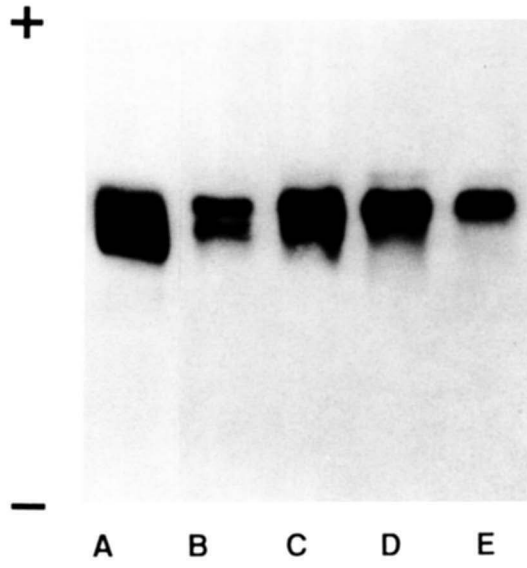

Fig. 4. Electrophoretic mobility of extracellular assembly products (24 h incubation with 10 μg/ml apoA-I) compared with mature plasma HDL. Fractions were electrophoresed on Beckman Paragon gels and then transferred to nitrocellulose and probed with apoA-I antibody. (A) Plasma HDL; (B and C) extracellular assembly products isolated at d < 1.235 g/ml from two separate incubations with CHO-Cl9 cells; although the majority of particles are discoidal, they have the same migration pattern as the spherical plasma HDL; (D and E) concentrated, unfractionated, conditioned medium containing both lipid-associated and lipid-free apoA-I; the majority of apoA-I in this fraction is lipid-free apoA-I but the migration pattern is essentially the same as that for the complexed apoA-I.

sociated apoA-I where the latter was the major form of apoA-I.

Effect of apoA-I concentration and time of incubation on the heterogeneity of apoA-I-lipid complexes

Increasing the amount of apoA-I incubated with CHO-C19 cells for 24 h increased the amount of apoA-I recovered in the d≤1.235 g/ml fraction (**Fig. 5**). Maximum complex formation occurred between 8–10 μg apoA-I per ml of medium. At 8 μg/ml apoA-I, after 24 h incubation, 12.4 ± 1.5% of total apoA-I floated at d≤1.235 g/ml where previous studies with transfected CHO cells revealed that 16 ± 1.9% of the apoA-I accumulating in the medium floated at this density (17). To assess whether the concentration of apoA-I influenced the distribution of the extracellular assembly complexes, the d≤1.235 g/ml fractions from cells incubated with 2, 4, and 8 μg/ml apoA-I were analyzed by nondenaturing gradient gel electrophoresis; resulting scans are seen in **Fig. 6.** During 24 h incubation with low levels of apoA-I (2 μg/ml) the major extracellular assembly product was the 7.3 nm component; however, small quantities of particles that banded at 11.6 and 9.4 nm were also present. With increasing amounts of apoA-I there was a progressive increase in intensity of the larger-sized components that banded at 11.2 and 9.0 nm and a concomitant decrease in intensity of the smallest, 7.3 nm particle. The changes in particle size distribution as a function of apoA-I concentration suggest a precursor–product relationship between the small 7.3 nm particle and the larger 11 and 9 nm particles.

To investigate this putative precursor–product relationship further, the time course of complex formation was studied in incubations using 8 μg/ml apoA-I. **Fig. 7** summarizes these studies and indicates that at 1 h the smallest component, 7.3 nm, was essentially the only assembly product. With increasing time of incubation, additional components appeared in a sequential fashion where the

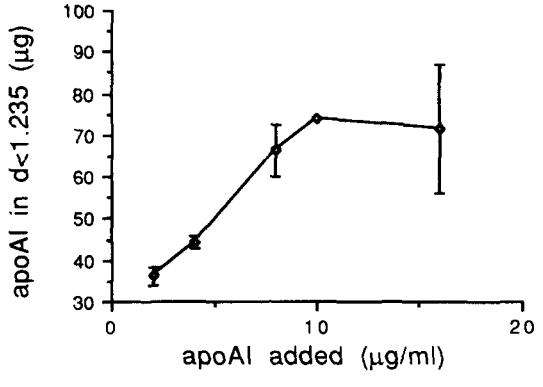

Fig. 5. ApoA-I concentration dependence on formation of complexes that float at d 1.235 g/ml (n = 3). Known amounts of apoA-I were incubated with nontransfected CHO-C19 cells for 24 h before harvesting the medium.

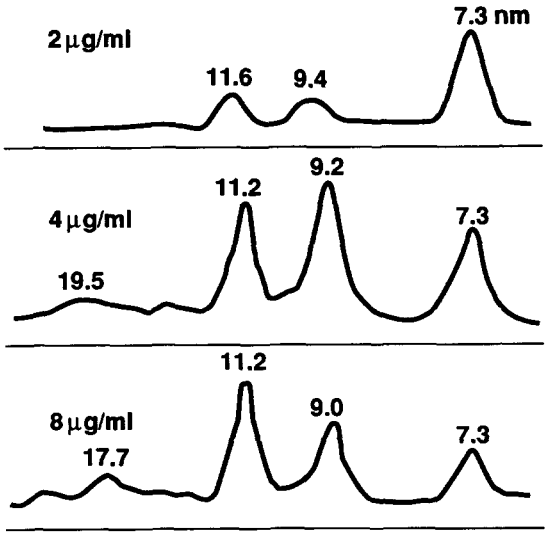

Fig. 6. Effect of apoA-I concentration on particle size distribution of apoA-I-lipid complexes. Nontransfected CHO-C19 cells were incubated for 24 h. The isolated d<1.235 g/ml fraction was analyzed on 4–30% nondenaturing gradient gels. Scans of gels indicate that at low concentrations the major apoA-I complex bands at 7.3 nm. With increasing amounts of apoA-I in the incubation medium there is a progressive shift to larger particles.

9.0 nm particle appeared first followed by the 11.0 nm particle. At 24 h the typical pattern consisting primarily of the larger components (11.0 and 9.0 nm) predominated. The time-dependent appearance of small 7.3 nm particles followed by that of larger, discoidal particles again sup-

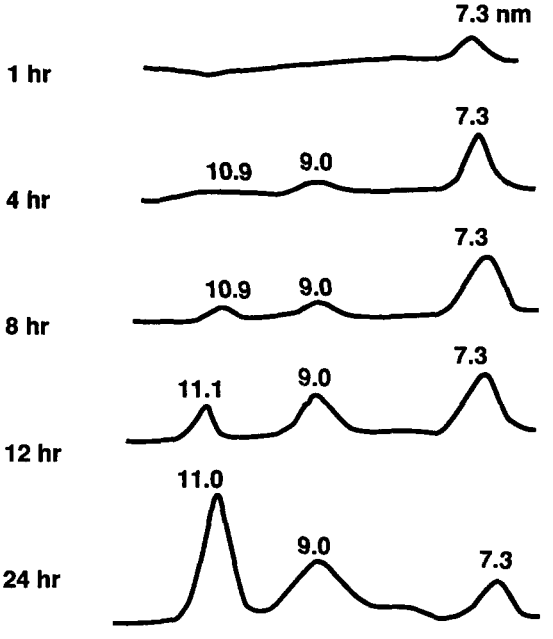

Fig. 7. Effect of time of incubation with 8 μg apoA-I/ml on extracellular complex formation. At 1 h the complex is mainly the 7.3 nm component. With increasing time additional components are evident; the relative peak height of the 7.3 nm component decreases as the peak height of the 9.0 and 11.0 nm particles increases.

ports a precursor-product relationship between the small 7.3 nm particle and the discoidal ones.

DISCUSSION

The site and mechanism of apolipoprotein lipidation is an area of great interest because lipidation steps are functionally important for regulating transport and metabolism of lipids. Lipidation events also influence the physical-chemical properties of nascent lipoproteins. Studies on assembly of apoB-containing lipoproteins have shown that this protein is lipidated intracellularly and that secretion of triglyceride requires the participation of apoB (27-29). It is not well established, however, that the smaller apolipoproteins, particularly apoA-I and apoA-II, are assembled as nascent lipoproteins within the cell. Hussain et al. (18) demonstrated that in rat primary liver cells in monolayer culture, a large proportion of the secreted apoA-I is in the lipid-free form but that the protein could associate with mature plasma HDL upon incubation with rat serum. These studies demonstrated that rat lipid-free apoA-I had the capacity to bind normally to HDL and thus is lipidated by association with pre-formed HDL. There was no evidence, however, that the lipid-free apoA-I formed nascent, i.e., discoidal particles. Later studies by Hussain et al. (30) with mouse C127 cells transfected with the apoA-I gene indicated that approximately 11% of the secreted protein floated in the absence of plasma, suggesting that in this model system some apoA-I is lipidated but it was difficult to assess whether the particles were generated intracellularly or extracellularly. The same considerations are true for 3T3 cells transfected with the human apoA-I gene where up to 25% of the secreted protein floated at a density < 1.21 g/ml (31) but the site of the lipidation was unknown.

Early studies by Stein and Stein (9) indicated that apoHDL was able to stimulate cholesterol efflux from ascites cells; additionally, efflux was greater when apoHDL was complexed with phospholipid. Human apoA-I is known to interact with isolated microsomal membranes and extract phospholipid from such membranes along with a small amount of membrane cholesterol (32). The apoA-I-lipid complexes formed in the latter incubations consisted of particles with a protein:lipid ratio similar to that shown for our CHO-C19 extracellular assembly product. However, apoA-I-lipid complexes formed by incubations with microsomal membranes contained only 4% cholesterol compared with 28% found in the CHO-C19 extracellular assembly product. This difference may be attributed to variability in the amount of cholesterol associated with different membrane compartments where microsomal membranes possess less cholesterol than plasma membranes.

It was suggested by Nunez and Swaney (32) that plasma membranes were poor donors of lipids as incubation of apoA-I with red cell ghosts did not result in apoA-I-lipid complexes. Recent reports, however, with aortic endothelial and smooth muscle cells (12) have shown that apoA-I can remove phospholipid and cholesterol from such cells and that the mechanism is not dependent on internalization of apoA-I by the cells, suggesting extracellular assembly of apoA-I-lipid complexes. Studies by Hara and Yokoyama (13) with cholesterol-laden macrophages indicated that purified apoA-I stimulated efflux and net transport of cholesterol from these cells. The products generated contained phospholipid and cholesterol and were termed "nascent HDL." Although the morphology of the apoA-I-lipid product was not described, the composition and density of the particle was consistent with a discoidal particle. From the various studies with apoA-I incubated with cells one can infer that apoA-I stimulates net efflux of cholesterol and phospholipid from cells and that the resultant product represents a form of nascent HDL. It is unclear, however, whether such products represent a homogeneous population of particles and whether the primordial particle is the nascent discoidal one suggested, but not demonstrated, in the above studies. The present study strongly suggests that nascent particles formed in this process are heterogeneous in morphology and that all nascent particles are not necessarily discs. It appears that an important precursor in the process of extracellular HDL assembly is a small 7.3 nm component.

We have previously reported (17) that the conditioned medium from transfected CHO cells grown in serum-free medium contained several distinct populations of particles. One consisted of large vesicular structures associated with seven to eight apoA-I molecules, another consisted of discoidal complexes that banded on nondenaturing gradient gels at 9.0 and 11.0 nm and contained three and four apoA-I molecules per particle, respectively. The third population, which represented a minor fraction floating at $d \leq 1.235$ g/ml, was a small particle banding at 7.2 nm; this component was composed of 94% apoA-I protein and 6% phospholipid and was considered a lipid-poor complex. It was not clear from experiments with transfected CHO cells whether the apoA-I-lipid complexes were formed intracellularly and whether there was a structural relationship between the various populations. Although we cannot completely rule out direct secretion of nascent apoA-I-containing particles by transfected CHO cells, the present study suggests that the nascent apoA-I particles described in our earlier studies were assembled extracellularly as the products from incubations with CHO-C19 cells were identical with transfected CHO cell products with regard to composition, banding position on nondenaturing gradient gels, and electron microscopic structure.

The results from both the concentration-dependence and time-dependence studies are very provocative because both suggest that extracellular assembly products

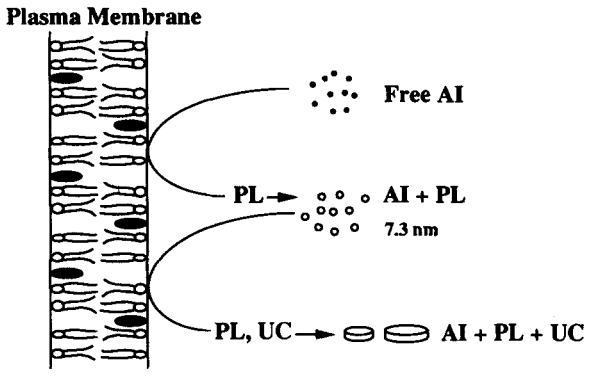

Fig. 8. Schematic showing proposed extracellular events accounting for the heterogeneity of nascent HDL formed during extracellular assembly. The lipid-free apoA-I (A-I) interacts with the cell membrane and associates with phospholipid (PL) to form a lipid-poor apoA-I–phospholipid complex. This complex recruits unesterified cholesterol (UC) and/or additional phospholipid from the cell; the product is a disc that bands at 9.0 nm. Further lipidation and/or fusion of particles results in the formation of larger discs that band at 11.0 nm.

are assembled in a stepwise manner where the 7.3 nm particle is the precursor for larger, lipid-rich particles. Although insufficient material was available for compositional studies, it is likely that the 7.3 nm particle isolated from incubations with nontransfected CHO-C19 cells was identical to the lipid-poor particle isolated from transfected CHO cells for, in both cases, the small particle was not resolved by electron microscopy. At short incubations only the 7.3 nm particle is in evidence in the incubation medium; with increasing time of incubation (Fig. 7) larger complexes appeared such that the 9.0 nm component (likely to contain three apoA-I molecules based on our original transfected CHO incubations) appeared first followed by the larger 11.0 nm complex (with four apoA-I molecules). The kinetics of extracellular assembly product formation suggest a precursor–product relationship between lipid-poor, apoA-I–phospholipid complexes and the lipid-rich, apoA-I–phospholipid–cholesterol complexes. Although speculative, a possible mechanism to explain extracellular assembly and heterogeneity of nascent HDL is shown in the schematic in **Fig. 8.** According to this schematic, apoA-I interacts with cell membranes and removes phospholipid from the plasma membrane thus forming lipid-poor, 7.3 nm apoA-I–phospholipid complexes. The accumulation of a critical mass of lipid-poor, 7.3 nm particles is probably necessary before cholesterol efflux from cell membranes is stimulated, accounting for the appearance of the discoidal complexes containing phospholipid plus cholesterol. The discoidal particles banding at 9.0 nm may be precursors of the particles banding at 11.0 nm; conversion of nascent particles containing three apoA-Is to those containing four apoA-Is probably involves a fusion step as suggested by the progressive changes seen with increasing amounts of apoA-I in the medium. Saturation of the extracellular assembly process at 8–10 μg apoA-I/ml suggests that under our serum-free incubation conditions, availability of membrane phospholipid and cholesterol is limited. Restricted availability of membrane lipids may also account for the low lipid association efficiency where only 12% of the apoA-I is lipidated after 24 h incubation. The sequence, however, of nascent HDL extracellular assembly suggests that the primary step is formation of a small apoA-I–phospholipid complex; cholesterol removal occurs only after the initial phospholipidation of apoA-I. Formation of apoA-I–phospholipid complexes prior to cholesterol removal is consistent with the early observations of Stein and Stein (9) that addition of phospholipid to apoHDL enhanced cholesterol removal from ascites cells.

Because of their small size, lipid-free apoA-I and the lipid-poor, 7.3 nm apoA-I particles are expected to readily filter into the interstitial space. Indeed, Reichl et al. (33) have demonstrated that a lipid-poor apoA-I is present in interstitial fluid. Lipid-free apoA-I has been detected in plasma (34) at a concentration of approximately 75 μg/ml which is considerably higher than the effective concentration of apoA-I used in the present study. Although speculative, extracellular assembly of apoA-I with membrane phospholipid followed by the subsequent recruitment of membrane cholesterol suggests that lipid-free apoA-I may play a role in reverse cholesterol transport and the prevention of atherosclerosis.

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