Human Placenta Secretes Apolipoprotein B-100-containing Lipoproteins*

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Supply of lipids from the mother is essential for fetal growth and development. In mice, disruption of yolk sac cell secretion of apolipoprotein (apo) B-containing lipoproteins results in embryonic lethality. In humans, the yolk sac is vestigial. Nutritional functions are instead established very early during pregnancy in the placenta. To examine whether the human placenta produces lipoproteins, we examined apoB and microsomal triglyceride transfer protein (MTP) mRNA expression in placental biopsies. apoB and MTP are mandatory for assembly and secretion of apoB-containing lipoproteins. Both genes were expressed in placenta and microsomal extracts from human placenta contained triglyceride transfer activity, indicating expression of bioactive MTP. To detect lipoprotein secretion, biopsies from term placentas were placed in medium with [35S]methionine and [35S]cysteine for 3–24 h. Upon sucrose gradient ultracentrifugation of the labeled medium, fractions were analyzed by apoB-immunoprecipitation. 35S-labeled apoB-100 was recovered in d ~ 1.02–1.04 g/ml particles (i.e. similar to the density of plasma low density lipoproteins). Electron microscopy of negatively stained lipoproteins secreted from placental tissue showed spherical particles with a diameter of 47 ± 10 nm. These results demonstrate that human placenta expresses both apoB and MTP and consequently synthesizes and secretes apoB-100-containing lipoproteins. Placental lipoprotein formation constitutes a novel pathway of lipid transfer from the mother to the developing fetus.

Lipids from the maternal circulation are vital to sustain fetal growth and brain development. In addition to essential fatty acids, fetal development is dependent on cholesterol, glycolipids, and lipid-soluble vitamins. Although fatty acids can passively diffuse across the placental barrier (1), specific mechanisms for delivery of other lipids, including cholesterol, remain to be determined (1). Elucidation of the molecular machinery involved in lipid transport to the fetus will potentially help understand states of fetal overgrowth (e.g. in maternal diabetes) or intrauterine growth restriction.

The most efficient system for delivery of lipids from a tissue to the blood is the formation and secretion of apolipoprotein B (apoB)-containing lipoproteins. The apoB-containing lipoproteins can contain large amounts of cholesterol and triglycerides and also serve as carrier of essential lipids such as lipophilic vitamins and glycolipids (2–4). The importance of apoB-containing lipoprotein secretion is evident in the liver and intestine, where both apoB and microsomal triglyceride transfer protein (MTP) are needed to export large amounts of lipids for usage in peripheral tissues (2, 5, 6).

In rodents, the yolk sac plays an essential role in lipid transport to the fetus during the major part of pregnancy. The yolk sac of mice and rats express the apoB gene (7, 8) and synthesize apoB-containing lipoproteins (9, 10). The importance of lipoprotein secretion from the yolk sac in mice is emphasized by the observation that both apoB and MTP knockout mice die in utero (11, 12). In humans, apoB is also expressed in the yolk sac (13). However, the human yolk sac is a rudimentary organ and looses its nutritional functions early in embryogenesis (14). Instead, the substrate transfer from the maternal to the fetal circulation occurs in placenta. We speculated that even though rodent placentas express only very low levels of apoB and MTP (7, 8), the transfer of lipids from mother to fetus in humans could be mediated, at least in part, by apoB-containing lipoprotein secretion from the placenta. This hypothesis was addressed by characterizing human term placental biopsies with respect to mRNA and protein expression of MTP and apoB and secretion of newly formed apoB-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Tissues and Cells—Biopsies from human term placenta (~0.3 g) were obtained after elective cesarean section in healthy women with non-pathological pregnancies. Biopsies were placed in medium on ice (for metabolic labeling studies and electron microscopy) or frozen in liquid N2 (for mRNA and MTP activity studies) within 7–15 min after childbirth. All mothers gave informed, written consent, and the local ethics committee approved the study protocol (KF 01–048/01). Mouse hearts were taken from three 6-month old male C57Bl/6 mice (M&B, Ry, Denmark) that had been housed at the Panum Institute, University of Copenhagen and fed standard laboratory chow (Altromin no. 1314; Altromin, Rugsarden, Denmark).

HepG2 cells were grown in a humidified atmosphere of 90% air and 10% CO2 at 37 °C in Dulbeco’s modified Eagles’s medium with GlutaMAX-I, 4500 mg/l glucose, and pyridoxine and without sodium pyruvate (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The cells were split 1:4 or 1:5 twice a week. Labeling experiments were carried out with subconfluent monolayered HepG2 cells in 6-well Multi-dishes (9.6 cm² well) (Nunc AS, Roskilde, Denmark).

mRNA Expression—Total RNA was isolated from human placental biopsies and HepG2 cells with TRIzol (Invitrogen) and used for cDNA

The abbreviations used are: apoB, apolipoprotein B; HDL, high density lipoprotein; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein.

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synthesis and quantitative real-time PCR analysis of apoB and MTP mRNA expression with a Lightcycler (Roche A/S, Hvidovre, Denmark) (15, 16). The primers used for MTP and apoB mRNA amplification have already been described (17). For amplification of β-actin the primers were: h-β-actin-5′-GAAGATTTGCGATGACGGTAT-3′ and h-β-actin-3′-TCTGTGGCATCAGAAGACT-3′.

**Placental Microsomal Triglyceride Transfer Activity—**Extracts of microsomal proteins were prepared from biopsies of two human placentas, pig heart, and pig liver by homogenization of ~100 mg of each tissue separately in 1 ml of buffer (50 mM Tris-Cl, 50 mM EDTA, 5 mM EDTA, and 0.02% protease inhibitor; Roche A/S) with a Potter-Elvehjem homogenizer (Buch & Holm A/S, Herlev, Denmark). The total protein concentration in each homogenate was determined with the bicinchoninic acid protein assay (Pierce, Copenhagen, Denmark) using bovine serum albumin as standard. The protein concentration was adjusted to 1.75 mg/ml, and placental homogenates were centrifuged for 60 min at 100,000 g in a Beckman Optima LE-80K ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). The supernatant (i.e. the microsomal fraction) was added to 1/10 volume of 0.54% sodium deoxycholate, pH 7.5, and incubated on ice for 30 min, followed by overnight dialysis at 4 °C against 15 mM Tris, 40 mM NaCl, 10 mM EDTA, and 0.02% sodium pyruvate and protease inhibitors (Complete Mini; Roche A/S) with 7% fetal bovine serum, 2.0 mM GlutaMAX-I (Invitrogen), [methionine- and cysteine-free Dulbecco’s modified Eagle’s Medium (Sigma)] and 0.23 mCi [35S]Promix in each well. Cell debris was removed by brief centrifugation.

**Lipid Metabolism Studies—**Each human placental biopsy (~0.3 g) was placed in a 2.0-ml Eppendorf tube, minced into ~1-mm3 pieces with scissors, and washed three times with 1.0 ml of incubation medium [methionine- and cysteine-free Dulbecco’s modified Eagle’s Medium (Sigma)] with 7% fetal bovine serum, 2.0 mM GlutaMAX-I (Invitrogen), 2.0 mM sodium pyruvate and protease inhibitors (Complete Mini; Roche A/S) before adding 1.4 ml of incubation medium with 0.59–0.88 mM of [35S]Promix (Amersham Biosciences). In some experiments, the incubation media also contained 0.81 mM oleic acid complexed with bovine serum albumin (molar ratio 2:1) (Sigma) or 0.41 mM fatty acid-free bovine serum albumin (Sigma). The tube was placed at 37 °C on a shaking table at 250 rpm. The time from child delivery to incubation of the placentas was 45–65 min. After 3–24 h, the tubes were pelleted by centrifugation (10,000 g for 1 min at 4 °C), and the medium was collected. HepG2 cells were pre-incubated for 1 h with incubation medium without Complete Mini followed by incubation for 22–24 h with 1.5 ml of incubation medium, with or without 0.81 mM oleic acid, and with 0.23 mM [35S]Promix in each well. Cell debris was removed by brief centrifugation.

Labeled medium from placenta or HepG2 cells was subjected to discontinuous sucrose gradient ultracentrifugation. The sucrose gradient (20, 21) was formed by layering from the bottom of 13.2 ml of 15% sucrose, 5 ml of 25% sucrose, 5 ml of 50% sucrose, 5 ml of 25% sucrose, 5 ml of 12.5% sucrose containing 1 ml of the sample and 3 ml of phosphate-buffered saline. Complete Mini (1 tablet/7 ml) was added to all solutions in experiments with placental medium. After ultracentrifugation (35,000 rpm at 70–71 h at 12 °C with a Beckman SW41 Ti rotor in a Beckman Optima LE-80K ultracentrifuge), the gradients were unloaded from the top of the tube into six fractions: 1 ml (top fraction), 4 × 1.5 ml, and 5 ml (bottom fraction), and the density of each fraction was determined by weight.

Apob was recovered by immunoprecipitation from 500 µl of each fraction and the unfraccionated labeling medium. At first, each sample was precipitated with 20 µl of Protein A/G PLUS-Agarose (0.5 ml agarose/2.0 ml; Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min followed by pelleting of the beads by centrifugation (2 min at 13,000 × g). A polyclonal human apoB-100 antibody (DakoCytomation, Glostrup, Denmark) was added to the supernatant and the mixture was incubated for 12–16 h before Protein A/G PLUS-Agarose (20 µl) was added. All incubations were carried out at 4 °C on a rocking-type mixer. After 2 h further, the tubes were centrifuged (2 min at 13,000 × g), and the pellet was washed three times with Tris-buffered saline before being subjected to SDS-PAGE with Novex 4–20% tris-glycine gels (Invitrogen). After drying of the gels, 35S-labeled proteins were visualized with a FUJIX BAS2000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan).

**Electron Microscopy—**Medium containing placental lipoproteins was prepared by incubating placental tissue with oleic acid for 24 h as described for the metabolic labeling studies; however, no fetal bovine serum was added, and instead of Promix, non-radioactive methionine (100 µg/ml; Sigma) and cysteine (500 µg/ml; Sigma) were used. The medium was adjusted to a density of 1.100 g/ml with NaBr. 5 ml of density-adjusted medium was overlayered with a 1.063 g/ml NaBr solution in a 13.2-ml Ultra-Clear tube and ultracentrifuged in the Beckman SW41 Ti rotor at 40,000 rpm and 20 °C for 20 h. Thereafter, formvar-coated copper grids (400 mesh, Ref. 25; Ax-Lab, Copenhagen, Denmark) were placed on the liquid surface of each tube for 3 min.

Lipoproteins were visualized using negative staining with 2% phosphotungstic acid, pH 7.0 (22–24), and a Zeiss EM 900 electron microscope with a Mega View camera system. To compare placental and plasma lipoproteins, we isolated VLDL (d < 1.019 g/ml), LDL (1.019 < d < 1.063 g/ml), and HDL (1.063 < d < 1.21 g/ml) from human plasma by sequential ultracentrifugation and visualized them as described above.

**RESULTS**

**MTP and apoB Expression in the Human Placenta—**Real-time reverse transcription-PCR analyses were used to establish whether the genes necessary for lipoprotein secretion, apoB and MTP, are expressed in human placenta. Both gene products are present in biopsies from human placentas (Fig. 1A). Placental biopsies taken adjacent to the umbilical cord, at the periphery of the placenta, or between the two showed no sys-
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**Fig. 2. Secretion of apoB-100-containing LDL from human placenta.** Human placental biopsies (A and B) or cultured HepG2 cells (C and D) were incubated with [35S]methionine and [35S]cysteine. The media were subsequently subjected to sucrose density gradient ultracentrifugation. ApoB was isolated from each of six density fractions by immunoprecipitation and analyzed by SDS-PAGE and filmless autoradiographic analysis. The relative intensity of the [35S]apoB band is shown below each fraction. In some experiments, oleic acid was added to the incubation medium (A and C). The polyclonal anti-apoB antibody also precipitated several proteins of smaller size than apoB in both placental tissue and HepG2 cells. The nature of these proteins has not been investigated. The results are representative of at least three independent experiments.

Placenta expresses lipase activity (26). We therefore considered the possibility that lipoproteins from placenta might in fact be secreted as VLDL and subsequently converted to LDL as a result of hydrolysis of the triglyceride moieties. To examine the extent of lipolytic conversion of VLDL to LDL, medium containing [35S]-labeled VLDL was harvested from HepG2 cells and incubated with human placenta or mouse heart tissue (which expresses high levels of lipoprotein lipase (27)) for 24 h. The incubation with mouse heart tissue lead to disappearance of the [35S]apoB-100 VLDL particles; only a faint band corresponding to [35S]apoB-100 LDL was seen (Fig. 3C). In contrast, incubation with placental tissue only produced partial redistribution of [35S]apoB-100 from VLDL to LDL (Fig. 3B). This supports the conclusion that placenta, both with and without oleic acid supplementation, mainly secretes apoB-100 in particles with LDL density. However, it also indicates that some newly formed VLDL may have been converted to LDL upon secretion and therefore escaped detection as [35S]VLDL in our analysis of the medium.

To see placental lipoproteins, placental biopsies were incubated with serum-free medium for 24 h before isolation of d < 1.063 g/ml lipoproteins. Electron microscopy of negatively stained placental lipoproteins showed spherical lipoproteins of 47 ± 10 nm (mean ± S.D., n = 65) (Fig. 4A). This result was seen in two independent experiments. The placental lipoproteins were unexpectedly larger than plasma LDL (mean diameter, 23 ± 7 nm) on similarly prepared grids (Fig. 4B). The diameter of the plasma d < 1.019 g/ml lipoproteins varied considerably and was on average 78 ± 64 nm.

**DISCUSSION**

This study shows that human term placenta produces and secretes apoB-100-containing lipoproteins. The maternal li-
population profile changes with an increase of triglyceride and cholesterol levels in all lipoprotein fractions during pregnancy (1, 28). This makes the mother the obvious donor of lipid moieties to placenta. The quadruplication of the fetal weight from the 26th gestational week to delivery is caused mainly by lipid accumulation (29). We therefore suspect that the fetus is on the receiving end of placental lipoprotein production. In support of this idea, Parker et al. (30) found that the LDL-cholesterol concentration was 10% higher in the umbilical artery than in the umbilical vein.

The present results add placenta to a growing list of organs that produce apoB-containing lipoproteins. In addition to liver and intestine, recent data suggest that cardiac lipoprotein formation plays an integrated role in cardiac lipid metabolism (16, 17, 19, 31) and the kidney is a major lipoprotein producing organ in the chicken (32). We have also detected apoB mRNA in the mouse kidney and would not be surprised if the kidney was found to secrete apoB-containing lipoproteins in mammals. However, it is unlikely that lipoprotein synthesis is important in all cells (e.g. as a generally expressed pathway for removal of excess triglycerides) simply because the apoB expression levels are extremely low or undetectable in tissues other than those discussed above, including skeletal muscle, adipose tissue, lung, and spleen (7, 33).

The present data suggest that placenta may produce apoB-containing lipoproteins with an efficacy per gram tissue that is 2% of that in the adult liver (a rough estimate based on the relative MTP activity and MTP mRNA contents in placenta versus pig liver and cultured human liver cells). Because the placenta weighs 4 times more than the fetal liver, this result implies that ~8% of the apoB-containing lipoproteins in fetal plasma might be derived from the placenta. This estimate is, of course, rather speculative. Nevertheless, it illustrates that lipoproteins synthesized in placenta may contribute significantly to the plasma pool of apoB-containing lipoproteins in the fetus.

As expected, oleic acid increased the buoyancy of newly secreted apoB-containing lipoproteins from HepG2 cells (25). However, oleic acid failed to increase the buoyancy of the apoB-containing lipoproteins from placental biopsies. It is interesting that a similar lack of effect of oleic acid has been observed when studying the secretion of apoB-containing lipoproteins from oleic acid-perfused mouse hearts (31). Moreover, the present data suggest that the density of placental lipoproteins was only mildly affected by postsecretional lipolysis (or preferential uptake of newly secreted VLDL), because the buoyancy of HepG2-cell derived VLDL changed only partially after incubation with placental biopsies. Ultrastructural analyses have shown that the size of nascent VLDL particles in mouse yolk sac cells diminishes in the late stages of pregnancy (embryonic day 13.5) (7). Because the present experiments were carried out with term placentas, we cannot exclude the possibility that the placenta produces larger and more buoyant lipoproteins at earlier time points during gestation. It is puzzling that the size of placental lipoproteins was similar to that of plasma VLDL remnants, whereas the density resembled that of plasma LDL. Because we saw no 35S-labeled proteins other than apoB-100 after immunoprecipitation of the apoB-containing lipoproteins, we suspect that the high density might reflect differences in the lipid composition of placental and plasma lipoproteins.

*Fig. 3. Hydrolysis of VLDL by human placenta.* To investigate in vitro hydrolysis of VLDL by placenta, [35S]apoB-100 VLDL was harvested from HepG2 cells (A) and incubated with either 0.3 g of minced human placental (B) or mouse heart tissue (C) at 37 °C for 24 h. The media were subsequently subjected to sucrose density gradient ultracentrifugation. ApoB was isolated from each of six density fractions by immunoprecipitation and analyzed by SDS-PAGE and filmless autoradiographic analysis. The relative intensity of the [35S]apoB band is shown below each fraction.

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2 Emil D. Bartels and Lars B. Nielsen, unpublished data.
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Although similarly sized placental lipoproteins were seen in two independent experiments, the apparent discrepancy could also reflect an artifact of the preparation of placental lipoproteins for electron microscopy (34). What is the importance of placental lipoprotein in the fetus? Perhaps the function as vehicle is not so much for triglycerides as it is for other lipid soluble molecules, e.g. cholesterol, glycolipids, and lipid soluble vitamins. Comprehensive studies of knock-out mouse models for the two genes involved in apoB-containing lipoprotein synthesis and secretion (MTP and apoB) have shown that homozygous embryos in both cases die shortly after birth (35, 41, 42). The principal role of α-tocopherol transfer protein is to mediate the incorporation of α-tocopherol into newly formed apoB-containing lipoproteins (4). It is interesting that, in humans, the placenta shows the second highest tissue expression of the α-tocopherol transfer protein gene (surpassed only by the liver) (40). Despite this, persons with mutations in the α-tocopherol transfer protein gene (which causes severe vitamin E deficiency after birth) or with apoB deficiency (i.e. abetalipoproteinaemia or familial hypobetalipoproteinaemia) have been reported to develop symptoms only postnatally (35, 41, 42). This implies that pathways other than LDL formation also can convey lipid and vitamin E transport into the developing human fetus. Another mechanism could involve the APT-binding cassette transporter 1 (ABC-AI), which is highly expressed in the placenta (43) and mediates efflux of both cholesterol and vitamin E to HDL (44). Indeed, vitamin E in the fetal circulation is found both in HDL and LDL lipoproteins (45).

Although the present findings cast light on a new aspect of transplacental lipid transport, it also undeniably highlights unanswered questions such as: what is the lipid composition of placental lipoproteins and is the secretion regulated? Some of these questions could be conveniently addressed in cell culture studies. Real time-PCR studies of apoB and MTP expression in isolated placental trophoblast cells suggested that the trophoblasts are capable of making lipoproteins (data not shown). However, when we cultured two trophoblast-derived cancer cell lines (BEWO and JAR), we found only very low expression levels of apoB and MTP mRNA in those cells. Thus, future studies of human placental lipoprotein production most likely will have to employ primary cultures of trophoblast cells (46) or ex vivo dual perfusion of isolated cotyledons (47).

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