Expression of mouse alpha-macroglobulins, lipoprotein receptor-related protein, LDL receptor, apolipoprotein E, and lipoprotein lipase in pregnancy

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Abstract The expression of the proteinase inhibitors of the alpha-macroglobulin family and of their clearance receptor was analyzed in the mouse during pregnancy, embryonal development, and adolescence. In total we studied seven partners of a complicated network of interactions in proteolysis and lipid metabolism:alpha-2-macroglobulin, murinoglobulin, the alpha-2-macroglobulin receptor/lipoprotein receptor related protein, the murine equivalent of the receptor associated protein or the 44 kDa heparin binding protein, the *low density lipoprotein receptor*, *apolipoprotein* E, and lipoprotein lipase. The data demonstrate that: *i)* the regulation of expression of mouse tetrameric alpha-2-macroglobulin results in very constant levels, similar to alpha-2-macroglobulin in humans; *ii)* single chain murinoglobulin, not alpha-2-macroglobulin, is subject to regulation of expression during pregnancy, around birth, and in adolescence; *iii)* an important role seems implicated for the alpha-2-macroglobulin receptor in placental lipid metabolism, probably making it the most important lipoprotein receptor to supply the fetus; *iv)* the massive increase in apolipoprotein E synthesis in uterus and placenta accentuate the changed lipid metabolism during pregnancy to an apolipoprotein E-based uptake by the alpha-2-macroglobulin receptor/lipoprotein receptor related protein; *v)* the increased expression of lipoprotein lipase underlines its role in the generation of free fatty acids in uterus and placenta as another mechanism of supply, next to receptor mediated endocytosis of lipoproteins.—**Overbergh, L., K. Lorent, S. Torrekens, F. Van Leuven, and H. Van den Berghe.** Expression of mouse alpha-macroglobulins, lipoprotein receptor related protein, LDL receptor, apolipoprotein E, and lipoprotein lipase in pregnancy. *J. Lipid Res.* 1995. **36:** 1774–1786.

Supplementary key words alpha-2-macroglobulin · murinoglobulin · alpha-2-macroglobulin receptor · alpha-2-macroglobulin receptor associated protein · LDL receptor · apolipoprotein E · lipoprotein lipase · pregnancy · development

The continuously increasing number of mouse strains with molecularly defined, spontaneous mutations, and the even faster growing number of inflicted mutations, proves that mice are the test models of choice for studies of complex interactive systems in vivo. These studies need to delineate the temporal and spatial expression patterns of the components involved by demonstrating the presence of mRNA and/or protein in cells and tissues. The interest in the proteinase inhibitors of the alpha-macroglobulin family and of their receptor, which also plays an important role in lipid metabolism, has shifted the emphasis in this field from structural to biological analysis with the mouse as the prime model at this moment (1–4).

In the mouse two structurally different types of proteinase inhibitors of the alpha-macroglobulin family (AM) have been studied at the protein, the mRNA, and the genomic levels: the tetrameric mouse alpha-2-macroglobulin (MAM) (1, 5) and the single-chain murinoglobulins (MUG) (6–8). A single MAM cDNA and three different MUG isotype cDNAs were molecularly cloned and sequenced (5, 7), while at least four different MUG genes, including a pseudo-gene, were identified (8). This contrasts with the identification of only a single MUG isoform protein in mouse plasma (5–7). In vitro MAM and MUG show only minor differences in inhibitory spectrum (6, 9) despite a completely different primary structure of the bait regions, the proteolytic cleavage site of activation (7, 8). The precise physiological need for

Abbreviations: AM, alpha-macroglobulin; A2M, alpha-2-macroglobulin; MAM, mouse alpha-2-macroglobulin; MUG, murinoglobulin; A2MR, alpha-2-macroglobulin receptor; LRP, low density lipoprotein receptor related protein; LDLR, low density lipoprotein receptor; gp330, glycoprotein 330; LPL, lipoprotein lipase; β-VLDL, β-very low density lipoprotein; apoE, apolipoprotein E; A2MRAP, alpha-2-macroglobulin receptor associated protein; HBP-44, 44 kDa heparin binding protein; pc, post coitum; pp, post partum.

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the presence of the single-chain inhibitors is therefore not explained by the available molecular and structural data. In the prospect of studies with mice in which the MAM and/or MUG genes are experimentally inactivated, the analysis of their expression patterns in vivo will help to answer or focus some of the questions.

The essential cellular partner of the alpha-macroglobulins, the alpha-macroglobulin proteinase-inhibitor complex clearance receptor (A2MR) (10, 11) is identical to the low density lipoprotein receptor related protein (LRP) (12–14). Many more ligands bind to A2MR/LRP in addition to activated A2M (10, 11) and pregnancy zone protein (15): tissue type plasminogen activator and urokinase plasminogen activator complexed to the plasminogen activator inhibitor type 1 (16, 17), lipoprotein lipase (18, 19), β-very low density lipoproteins (β-VLDL) enriched with apolipoprotein E (apoE) (20–23), lactoferrin (24), lipoprotein[a] (25) and *Pseudomonas* exotoxin A (26, 27). This bewildering multifunctional nature of A2MR/LRP raises questions concerning its precise role in the clearance of proteinase-inhibitor complexes and in lipid metabolism. Its essential role in embryogenesis was proven directly by inactivation of the A2MR/LRP gene in mice (2, 3).

Regulation and modulation of A2MR/LRP activity by a 40 kDa protein has been claimed. This protein, first observed as co-purifying with A2MR/LRP, inhibits the binding of most of the ligands summarized above (28–30). By molecular cloning, the human A2M receptor associated protein (A2MRAP) was shown to be the homologue of the 44 kDa mouse heparin binding protein (HBP-44) and of the rat Heymann nephritis antigen (31–35). The genes coding for the human and mouse proteins are structurally almost identical (36). On the other hand, the intracellular occurrence of A2MRAP as well as the different cellular distribution of its mRNA relative to A2MR/LRP mRNA in many mouse tissues (37, 38) and in human placenta (39) adds to the enigma of its function.

Next to A2MR/LRP, the other lipoprotein receptor to be considered is the classical low density lipoprotein receptor (LDLR) that is prominently expressed in liver as well as in most other organs and cells (40, 41). Its key role in cholesterol metabolism, mediating uptake of lipoproteins containing apolipoproteins B and E, is conclusively demonstrated by a large number of diverse mutations causing familial hypercholesterolemia (40). Similarly, in mice with a targeted inactivation of the LDLR gene (42, 43) or with an overexpression of the human LDLR gene (44), profound effects on plasma cholesterol levels and lipoprotein clearance are evident. The relative contribution of LDLR and A2MR/LRP in lipid metabolism is not clear. Their common ligand apolipoprotein E (apoE), synthesized mainly in the liver, but also in a variety of other tissues (45, 46), was included in the present analysis.

Lipoprotein lipase (LPL) is a glycoprotein that plays a central role in lipoprotein metabolism by hydrolyzing the triglycerides from chylomicrons and β-VLDL lipoproteins, generating free fatty acids, which are used directly for energy or for storage (47, 48). LPL also functions as a ligand for A2MR/LRP, thereby enhancing the uptake of β-VLDL (18, 19). Recently, LPL was shown to interact with A2M directly (49), although the functional implications of this interaction are not clear.

We report the analysis of the differential expression of MAM and MUG mRNA and proteins during pregnancy and adolescence in the mouse. Furthermore, at different stages of pregnancy and thereafter, we have analyzed mRNA isolated from liver, placenta, and uterus for expression of apoE and the apoE binding receptors A2MR/LRP and LDLR. Our results demonstrate that A2MR/LRP is likely to exert an important role in lipid metabolism, rather than in homeostasis of proteinase activity. Its expression in placenta and uterus is totally differently regulated relative to LDLR during the physiologically perturbing period of pregnancy. Moreover, the expression patterns of A2MR/LRP, LDLR, apoE, and LPL demonstrate a redistribution of metabolism of circulating triglycerides during pregnancy that become primarily hydrolyzed in and transferred to the uterus and placenta. Furthermore, in this study we could only identify arguments for a role of the murinoglobulins, the single-chain proteinase inhibitors and not for the tetrameric alpha-2-macroglobulin in pregnancy and during infantile life, which substantiates the normal phenotype of mice in which the MAM gene has been inactivated (1, and unpublished results).

MATERIALS AND METHODS

cDNA probes

The cDNA clone mLDLRc90 for mouse LDLR was kindly provided by M. Hofker (Leiden, The Netherlands). The 700 bp EcoRI insert was used as a probe (50). The mouse apoE cDNA clone, pmEUC18 was obtained from S. Tajima (Osaka, Japan) (51). To detect A2MR/LRP mRNA, a 1.4 kb EcoRI cDNA fragment was used as a probe corresponding to positions 776–2197 of the mouse cDNA (52). The cDNA probe used to detect LPL mRNA was a 1 kb PstI restriction fragment isolated from the coding region of rat LPL (provided by J. Auwerx). cDNA probes for MUG, MAM, and HBP-44 were generated by PCR-amplification: MUG, position 1777–2262 (MUG1 cDNA) (7); MAM, position 1188–1758 (mouse cDNA) (5); HBP-44, position 8–1078 (mouse cDNA) (33). The PCR fragments were isolated

in low melting agarose or purified on silica. A 2.0 kb human β-actin cDNA probe (Clontech Lab, Palo Alto, CA) was used as a control in northern blotting to allow for normalization of mRNA loading. Labeling of the probes was performed by hexanucleotide-mediated incorporation of [^{32}P]dCTP, as described before (7, 37).

Plasma and tissue samples

All plasma and tissue samples were taken from the C57/Black mouse strain. The following organs were dissected from male, female, and pregnant female (at day 14 pc) mice for total RNA extractions: heart, liver, lung, spleen, kidney, cerebrum, cerebellum, thymus, salivary glands, muscle, testicles, ovaria, placenta, and uterus. Blood was taken by heart puncture in heparinized glass capillaries or syringes from anesthetized animals of the indicated age. Plasma was collected after centrifugation at 1400 *g* for 5 min at room temperature.

Plasma samples for rocket immuno-electrophoresis and livers for total RNA extractions were isolated from C57/Black mice, at the different timepoints defined as neonatal (days 1 and 7), infantile (days 14 and 21), juvenile (day 28), and in puberty (day 35). At day 1, the day at which the pups were born, plasma and livers from 12 pups from two litters were pooled. At the other timepoints, plasma and liver from three male and three female mice were isolated and analyzed individually.

The different stages analyzed during pregnancy were days 4, 7, 10, 13, 16, and 19 pc with day 1 pc being the morning on which the copulation plugs were observed. Pregnancy at day 4 pc was ascertained by flushing the uterus for the presence of blastocysts. For the post-implantation stages, pregnancy was checked by isolation of the embryos, which were used for other experiments.

RNA extraction

Total RNA was extracted from different tissues of C57/Black mice. Isolated tissues were immediately frozen in liquid nitrogen and stored at −70°C until used. RNA extractions were performed by the guanidinium isothiocyanate method, as described before (37, 38). Tissue was mechanically homogenized in a motor-driven homogenizer (Virtis, Gardiner, NY), in denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% (w/v) sarcosyl, 0.1 M 2-mercaptoethanol). Addition of 0.1 volume of 2 M sodium acetate, pH 4.0, 1.0 volume of water-saturated phenol, and 0.2 volumes of chloroform (containing 4% isoamyl alcohol), was followed by thorough mixing and subsequent cooling on ice for 15 min. Samples were centrifuged at 39000 *g* for 20 min at 4°C. The aqueous phase, containing RNA, was mixed with an equal volume of isopropanol, and placed at −20°C for 1 h to precipitate the RNA. The RNA was pelleted at 3300 *g* at 4°C for 30 min, resuspended again in denaturation solution, and precipitated with isopropanol under the same conditions. After centrifugation, and washing in 75% ethanol (3 times) the purified RNA was resuspended in 100% formamide or in DEPC-treated water, and stored at −70°C. The concentration of RNA in the samples was measured spectrophotometrically at 260 nm.

PolyA mRNA was isolated by treating 75 μg of total RNA, by affinity-purification on oligo dT-coupled magnetic beads, according to the manufacturer (Dynal A.S., Oslo, Norway).

Northern blotting

RNA was separated by denaturing gel electrophoresis, using 10 μg of total RNA or approximately 1.5 μg of polyA mRNA per lane. Prior to loading the RNA was denatured at 70°C for 10 min in 50% deionized formamide, 1 × MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 8) and 6% formaldehyde. The RNA samples, containing 0.1 μg/μl ethidium bromide, were separated electrophoretically in a 1% agarose gel containing 6% formaldehyde, run for 5 h at 60 V in 1 × MOPS buffer. Capillary transfer to Hybond N nylon membrane (Amersham, UK) was performed overnight in 10 × SSPE buffer (1.5 M sodium chloride, 100 mM sodium phosphate, 10 mM EDTA, pH 7.4). RNA was fixed to the membrane by baking for 20 min at 80°C, followed by UV-crosslinking for 45 sec (Stratalinker 1800, Stratagene, Heidelberg, Germany). Filters were prehybridized at 42°C, for 6 h or overnight in 5 × SSPE, 5 × Denhardt's solution (100 μg/ml polyvinylpyrrolidone, 100 μg/ml bovine serum albumin, 100 μg/ml ficoll-400), 0.5% SDS, 50% deionized formamide, 100 μg/ml denatured sperm DNA, 50 μg/ml heparin and hybridized in the same solution supplemented with 10% dextran sulphate at 42°C overnight with addition of 1–2 million cpm/ml of the indicated radiolabeled probe. The blots were washed in 0.3 × SSPE/0.5% SDS at 60°C for 1 h. Stripping of the filters in 0.5% SDS, 40 mM Tris, pH 7.8, for 15 min at 80°C was performed between hybridization for MUG and MAM mRNA, as both show characteristic transcripts of the same size. Autoradiography was done by exposure to Hyperfilm MP (Amersham, UK) using intensifying screens at −70°C for 2 h up to 7 days. Appropriately exposed autoradiographs were scanned densitometrically and the values were normalized to the signal obtained with the β-actin cDNA probe. The values were expressed as mean ± standard error (SE). The significance of differences between mean values in pregnant and non-pregnant controls were evaluated by the one-sided *t*-test.

Rocket immuno-electrophoresis

Heparinized mouse plasma from C57/Black female, male, pregnant, neonatal, infantile, juvenile, and puber mice was collected from anesthetized animals by cardiac puncture. Plasma from non-pregnant, adult female C57/Black mice was included as a control. Rocket immuno-electrophoresis was carried out on glass plates, in 0.8% agarose gels in 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4 (TBE) containing 2.5% (v/v) of specific rabbit antiserum against MUG or MAM. After electrophoresis (2.5 V/cm) overnight, the gels were soaked in H_2O, dried, stained with Coomassie Brilliant Blue, and destained by diffusion. Original antisera against MUG and MAM were prepared by immunization of rabbits with the isolated proteins (7, 53). The absolute concentrations for MAM and MUG were obtained from comparison with known amounts of the proteins, purified as described previously (5, 7). Concentrations as low as 0.05 mg/ml can be detected by this method, with an intra-assay variation of less than 5%, while the inter-assay variation is minimized by using the purified proteins as standards in each determination.

RESULTS

Tissue distribution of MUG and MAM mRNA in adult mice

The following organs from male, female, or pregnant (day 14 pc) C57/Black mice were extracted for total RNA and northern blotting: heart, liver, lung, spleen, kidney, cerebrum, cerebellum, thymus, salivary glands, muscle, testis, ovaria, placenta, and uterus. Hybridization was performed with cDNA probes for MUG and MAM. The MUG cDNA probe used is specific for the different MUG mRNAs and does not cross-react with MAM mRNA under the conditions used (7, 8). The only organ yielding the characteristic 5 kb MUG mRNA signal was the liver, in both male and female mice as well as in pregnant mice (results not shown). Similarly, the liver was the only organ expressing the 5 kb MAM mRNA (results not shown). These results conclusively established the liver as the only organ expressing MUG and MAM mRNA in adult mice.

Expression of MUG and MAM during development

MUG and MAM displayed a fundamentally different temporal expression pattern during development: MAM mRNA was expressed in fetal liver from day 13 pc on, while MUG mRNA was not detected during embryogenesis (37). Rocket immuno-electrophoresis now demonstrated the presence of MAM protein in embryo blood plasma while MUG protein was confirmed to be absent from the embryonal circulation at days 17 and 19 pc (results not shown).

The evolution of expression of MUG and MAM in neonatal, infantile, juvenile, and puber mice was further studied by northern blotting of total liver RNA while their blood plasma level was quantitated by immuno-electrophoresis. The level of circulating MAM in neonatal mice (day 1) was about 1 mg per ml plasma (**Fig. 1**). This corresponds to less than a third of adult levels (between 3 and 4 mg/ml) and these are not yet reached at puberty (day 35) (Fig. 1). The MAM protein levels in neonatal and adolescent mice are not a direct reflection of the MAM mRNA levels in liver which remain nearly constant during the 5 weeks after birth (Fig. 1, **Fig. 2**). This observation indicates either a progressively more efficient translation of MAM mRNA or, more likely, less consumption of MAM protein as the mice develop.

MUG protein and mRNA were barely detectable during neonatal and infantile life (Fig. 1, Fig. 2). In the third week at the time of weaning, MUG was present in plasma, and adult levels of nearly 1 mg/ml were, similar to MAM, also not yet reached at puberty (day 35) (Fig. 1). At all time-points during adolescence, higher levels of hepatic MUG mRNA were observed in male relative to female mice, which was reflected in a similar difference in the MUG protein plasma levels (Fig. 1, Fig. 2).

Expression of MUG and MAM in pregnant female mice

Plasma levels of the alpha-macroglobulins were measured in pregnant and lactating C57/Black mice at days

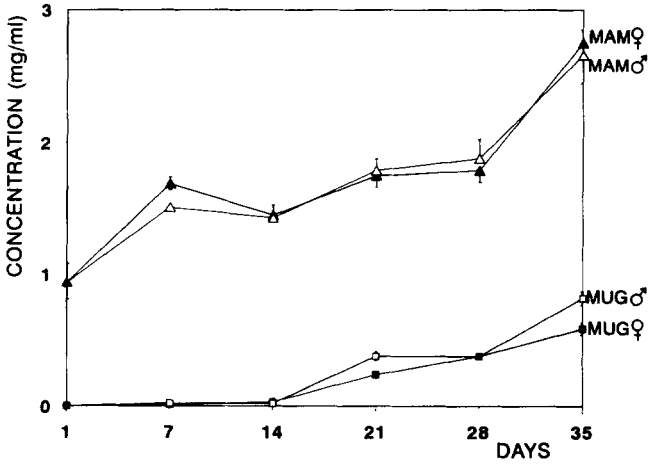

Fig. 1. Plasma levels of MAM and MUG in newborn and adolescent mice. Plasma was isolated from newborn and adolescent C57/Black mice at different days after birth. The plasma level of MAM and MUG was determined by rocket immuno-electrophoresis, using specific antisera. Each time point represents the mean (± SE) of three individual measurements, separately performed for male and female mice, as indicated. Standard errors that fall within the symbols are not shown. Absolute concentrations (in mg/ml) are represented in ordinate (see text for details).

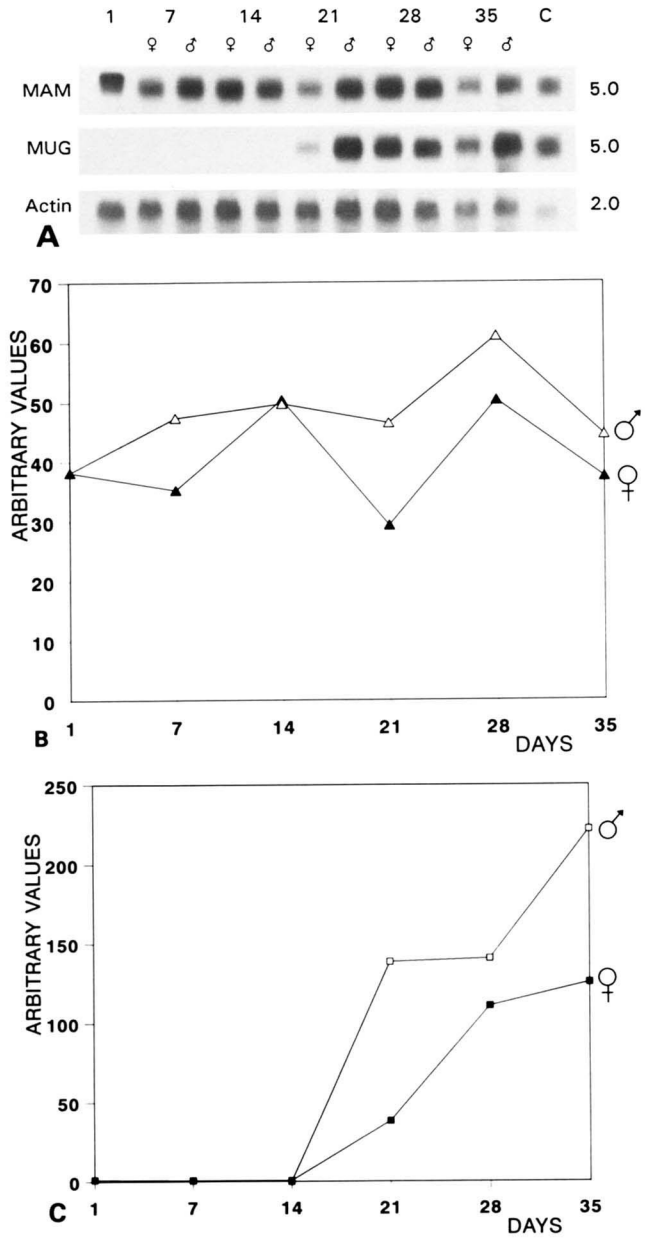

Fig. 2. Northern blotting of liver mRNA from neonatal and adolescent mice, probed for MAM and MUG. Panel A: Northern blotting of total liver RNA consecutively hybridized with cDNA probes specific for MAM, MUG, and β-actin. Total liver RNA was extracted at the indicated time points (days after birth). C, control liver RNA, extracted from an adult, male C57/Black mouse. The size of the mRNA is indicated on the right in kilobases. Panels B and C: Graphical representation of quantitation by densitometric scanning of the northern blots probed for MAM mRNA (panel B) and MUG mRNA (panel C). Arbitrary units are used that were obtained by normalization to the signal obtained for β-actin in each lane. Each time point is the calculated mean of three independent determinations of RNA extracts from individual mice on different blots, performed for male and female mice as indicated.

4, 7, 10, 13, 16, and 19 pc and at day 1 and 7 pp. Blood plasma protein and liver RNA was isolated and analyzed from the same animals.

Maternal hepatic MUG mRNA levels decreased significantly over the course of pregnancy: a 3-fold down-regulation was observed, with the lowest level of only about 30% of non-pregnant controls, reached between day 10 and 16 pc ($P < 0.010$) (**Fig. 3**). Parturition was characterized by a remarkable and pronounced transient increase of the hepatic mRNA levels, attaining normal levels on the day of birth. Afterwards, MUG mRNA levels dropped again to about 60% of control levels, 7 days pp (Fig. 3). This pattern of MUG mRNA expression was reflected in the circulating MUG protein, with the lowest concentration of only 0.2 mg/ml at day 10 to 16 pc, compared to 0.6 to 0.8 mg/ml in non-pregnant female mice ($P < 0.001$) (**Fig. 4**). An increase in circulating MUG levels was observed towards parturition, although the rise in protein level was less pronounced than that seen for the liver MUG mRNA levels (Fig. 4). These observations extend previous findings (54) and demonstrate that this remarkable maternal

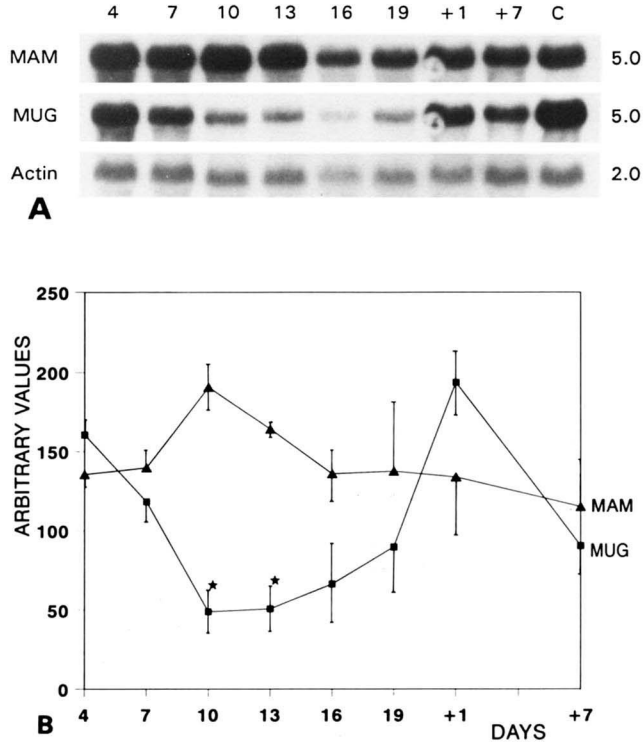

Fig. 3. Northern blotting of liver mRNA probed for MAM and MUG during pregnancy. Panel A: Northern blotting of total liver RNA consecutively hybridized with cDNA probes specific for MAM, MUG, and β-actin. Total liver RNA was extracted at the different time points during pregnancy and post partum as indicated. The size of the mRNA is indicated on the right in kilobases. Panel B: Graphical representation of quantitation by densitometric scanning of the northern blots, normalized to the β-actin signal. Each time point is the calculated mean (± SE) of three determinations of individual mice on different blots, performed for female mice as indicated. Statistically significant difference ($P < 0.010$) relative to non-pregnant female controls are denoted by an asterisk.

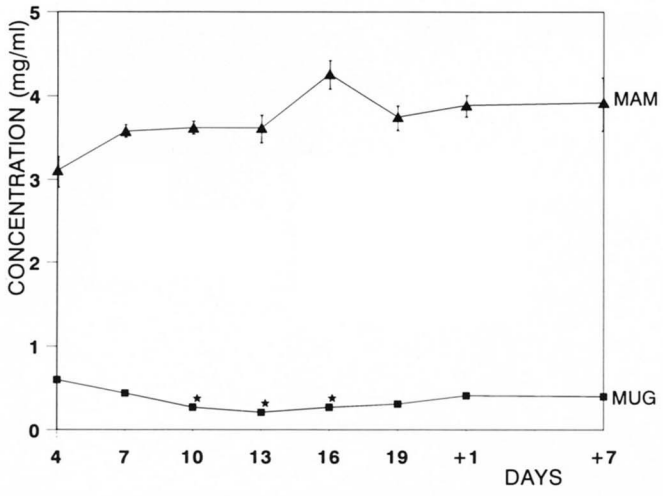

Fig. 4. Maternal plasma levels of MAM and MUG during pregnancy. Plasma samples were isolated at the indicated time points during pregnancy, indicated on the abscissa. The level of MAM and MUG was determined by rocket immuno-electrophoresis, using specific antisera. Each time point represents the calculated mean (± SE) of three individual measurements. Values for the standard errors that are within the size of the symbols are not shown. Statistically significant differences ($P < 0.001$) relative to non-pregnant female controls are denoted by an asterisk. Absolute concentrations are represented in ordinate (see text for details).

rebound of MUG expression at the time of birth was controlled transcriptionally.

MAM mRNA as well as circulating MAM protein levels were maintained nearly unchanged during the entire course of pregnancy, although a minor increase of not more than 1.5-fold was observed (Fig. 3, Fig. 4).

Expression of A2MR/LRP, HBP-44, LDLR, apoE, and LPL during pregnancy

The expression of mRNA coding for A2MR/LRP, HBP-44, LDLR, apoE, and LPL was determined during pregnancy and lactation in different mouse tissues. Total RNA from livers of pregnant and lactating C57/Black mice was extracted at days 4, 7, 10, 13, 16, and 19 pc and at day 1 and 7 pp. PolyA mRNA was extracted from placenta at day 12 and 19 pc and from uteri at days 12 and 19 pc and at the day of birth. When separating the non-term placentas from the uteri, placental remnants left in the uterine tissue were judged to contribute less than 10% of the uterine tissue.

The characteristic 15 kb A2MR/LRP mRNA (37, 52) was detected in liver, placenta, and uterus (**Fig. 5, Fig. 6, Fig. 7**). Densitometric scanning and normalization relative to β-actin mRNA levels demonstrated that maternal liver A2MR/LRP mRNA levels were somewhat decreased during the first week of pregnancy, after which non-pregnant values were restored. After day 13 pc, however, a more pronounced abrupt 3-fold reduction in A2MR/LRP mRNA was observed in the three individual mice analyzed at each time point. This lower level was maintained until and beyond parturition. Return to normal levels was not rapid because at day 7 pp the levels of liver A2MR/LRP mRNA were only about 50% of control, non-pregnant levels (Fig. 5). In uterus the A2MR/LRP mRNA levels did not vary significantly (Fig. 7). In placenta on the other hand, a dramatic 7-fold increase in the A2MR/LRP mRNA concentration was observed at day 19 pc relative to day 12 pc (Fig. 6).

The two transcripts coding for HBP-44 were 1.8 and 3.6 kb in size (33, 37, 38). In liver and uterus no signifi-

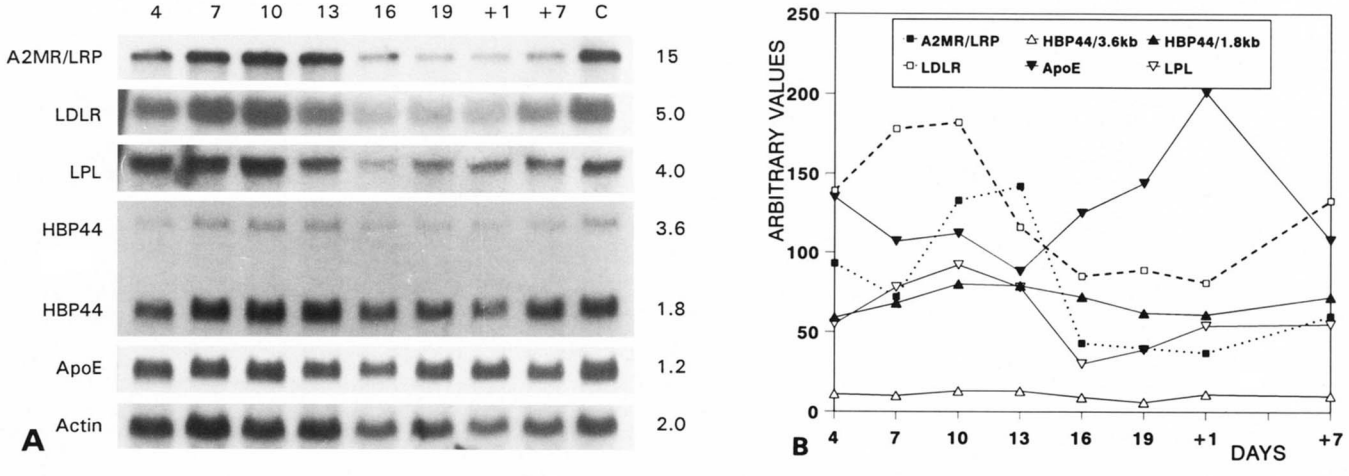

Fig. 5. Northern blotting of total liver RNA from pregnant and lactating female mice, probed for A2MR/LRP, LDLR, LPL, HBP-44, and apoE. Panel A: Northern blotting of total liver RNA consecutively hybridized with cDNA probes specific for A2MR/LRP, LDLR, LPL, HBP-44, and apoE. Total liver RNA was extracted at the time points indicated from pregnant female C57/Black mice. The size of the respective mRNA is indicated on the right in kilobases. Panel B: Graphical representation of quantification by densitometric scanning of the northern blots, normalized to the β-actin signal. Each time point is the calculated mean of three determinations of individual mice on different blots.

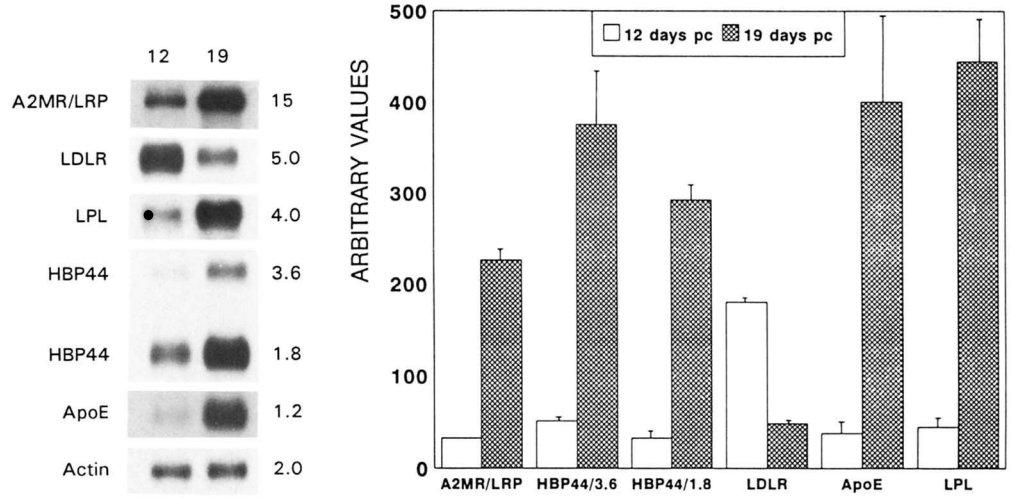

Fig. 6. Northern blotting of placental mRNA probed for A2MR/LRP, LDLR, LPL, HBP-44, and apoE during pregnancy. Panel A: Northern blotting of placental mRNA consecutively hybridized with cDNA probes specific for A2MR/LRP, LDLR, LPL, HBP-44, and apoE. Placental mRNA was extracted at days 12 and 19 pc. The size of the respective mRNA is indicated on the right in kilobases. Panel B: Graphical representation of quantification by densitometric scanning of the northern blots, normalized to the β-actin signal. Each time point is the calculated mean (± SE) of three determinations of individual mice on different blots.

cant alterations were noted during the course of pregnancy (Fig. 5, Fig. 7). Remarkably, in placenta the levels of both transcripts were about 8-fold increased at day 19 pc relative to day 12 pc (Fig. 6), an increase that is very reminiscent of that observed for A2MR/LRP mRNA. Apparently no difference in regulation was evident for the two mRNA transcripts which in the mouse are derived from one gene by using differential polyadenylation signals (33, 36).

The hepatic 5 kb LDLR mRNA transcript (41) is expressed according to a pattern that is very similar to A2MR/LRP mRNA described above: a minor decrease in mRNA concentration during the first week of pregnancy, which was restored at mid-gestation. Thereafter, a pronounced decrease of about 2- to 3-fold was noted from day 13 pc on. This lower level was further maintained until parturition and only restored partially at day 7 pp (Fig. 5). In placenta and uterus the regulation of

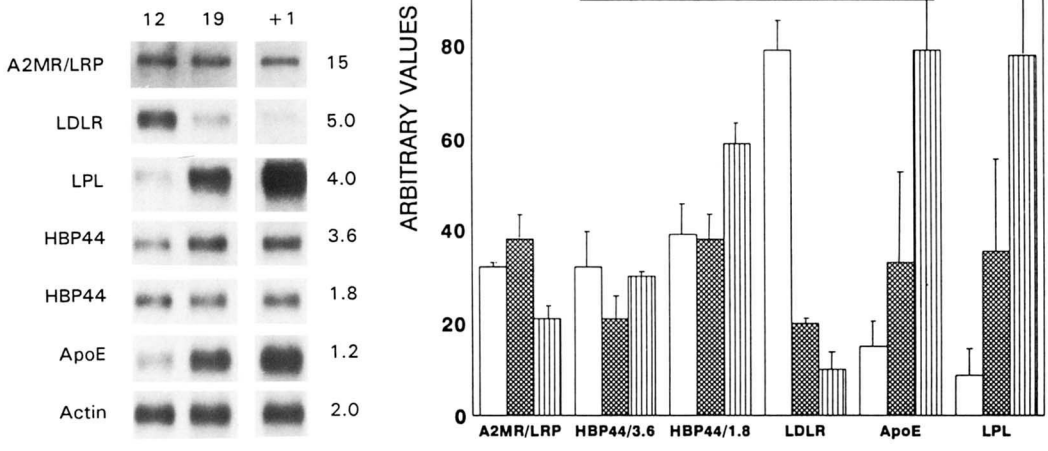

Fig. 7. Northern blotting of uterus mRNA probed for A2MR/LRP, LDLR, LPL, HBP-44, and apoE. Panel A: Northern blotting of uterus mRNA consecutively hybridized with cDNA probes specific for A2MR/LRP, LDLR, LPL, HBP-44, and apoE. Uterus mRNA was extracted at days 12 and 19 pc, and at the day of birth. The size of the respective mRNA is indicated on the right in kilobases. The signal of the 3.6 kb transcript of HBP-44 mRNA was much weaker than in other tissues, hence a longer exposure was used to compare its relative intensity with that of the more abundant 1.8 kb HBP-44 mRNA transcript. Panel B: Graphical representation of quantification by densitometric scanning of the northern blots, normalized to the β-actin signal. Each time point is the calculated mean (± SE) of three determinations of individual mice on different blots.

expression of the two receptors was much different: a clearcut down-regulation of LDLR mRNA levels by a factor of 4 and 8, respectively, during the later stage of pregnancy (Fig. 6, Fig. 7). Thus, in placenta and in uterus, but not in liver, the levels of LDLR mRNA were regulated quite differently from those of A2MR/LRP.

The 1.2 kb apoE mRNA (38, 51) was detected in liver, placenta, and uterus (Fig. 5, Fig. 6, Fig. 7). In all three organs an increased apoE mRNA concentration was observed as pregnancy progressed, reaching maximal levels at parturition, which in the liver were no more than 2-fold higher than control (Fig. 5). Much greater amounts were synthesized in placenta and uterus, however, as apoE mRNA levels were increased as much as 5- and 10-fold, respectively, in uterus and placenta (Fig. 6, Fig. 7).

The LPL mRNA, with a size of 4.0 kb (38, 55, 56) was expressed at a low level in adult liver. During pregnancy, this level further diminished, showing a very similar, although less pronounced regulation pattern compared to A2MR/LRP and LDLR: a minor increase of about 1.7-fold was observed in the first week of pregnancy, after which a 3-fold down-regulation was noted, with lowest levels around day 16 pc (Fig. 5). In placenta and uterus, much more pronounced changes were observed, with a marked increase in LPL mRNA of about 10-fold in both tissues, towards the end of pregnancy (Fig. 6, Fig. 7).

DISCUSSION

Expression of MAM and MUG

The intrinsic reason for the presence of either type, let alone for two types of macroglobulin proteinase inhibitors in rodents, i.e., tetrameric and monomeric forms, is unknown. In the mouse, MAM and MUG are closely related proteins with extensive sequence identity, except in the bait region that determines the inhibitory spectrum of these proteinase inhibitors (5, 7, 8). Nevertheless, their proteinase inhibitory capacity, when tested with a limited set of proteinases, proved to be comparable (6, 9). To circumvent structural studies that are inherently limited and to gain insight in the A2M system in vivo, we have analyzed the expression of these components in vivo during different stages of the lifespan of the mouse. The present report is part of an extensive study, including targeted inactivation of the MAM and MUG genes to generate mice deficient in either or both inhibitors (1).

The present data demonstrate that in the mouse both MAM and MUG mRNA are expressed exclusively in the liver but starting at very different time points: in the second week of embryonal development and during infantile life just before weaning, respectively. Postnatally, the MAM mRNA levels in liver are constant while circulating MAM protein levels increase progressively to adult levels of about 3 mg/ml (Fig. 1, Fig. 2). This indicates that consumption of MAM decreases as the animals grow, which can be due, but only in part, to the increased expression of MUG. This situation is different from humans, in which A2M plasma levels at birth and in the first years of life are higher than in adults (57, 58). The abrupt increase in liver MUG mRNA, paralleled closely by the plasma levels of MUG just before weaning, suggests a hormonal regulation of MUG gene transcription.

In the mouse, maternal A2M is stably expressed over the entire period of pregnancy, which is very similar to its human counterpart (58 and references therein). The expression of the murinoglobulins, on the other hand, is characterized by an important decline in the second week of pregnancy and a transient recovery around parturition. This pattern confirms previous findings (54) and again suggests hormonal regulation of transcription of the MUG gene(s). Physiologically, the meaning of the decreased MUG concentration in the second week of pregnancy is not clear. It might be aimed at reducing inactivation of proteases to allow further invasion of the placenta into maternal tissues. The transient recovery around parturition which is observed in MUG mRNA, is barely reflected in MUG blood plasma levels, indicating a high consumption of maternal MUG around birth. This could be of importance in protecting the female against proteolytic enzymes released from the uterus and detaching placenta during birth. Indeed, a variety of proteinases have been identified in term placenta in humans (59, 60) and mice (61, 62) that could play a role in the separation of the placenta from the uterine tissue, i.e., collagenases (59). Cysteine proteinases (cathepsin B, H, and L) and their specific inhibitors are also found in term placenta in humans (63) and mice (62), but it should be stressed at this point that this is a rather unexplored and unknown aspect of the physiology of pregnancy.

In all the conditions tested, the murinoglobulin isoform 1 (7) appeared to be the one involved as transcripts of the MUG2 gene were only detected at very low levels by northern blotting or PCR. Transcripts from other MUG genes were never detected. Analysis of MUG protein isolated from plasma demonstrated, by N-terminal sequencing, that only MUG1 protein is circulating (7). Although this type of analysis is indirect, the consistent finding of MUG1 mRNA and protein do indicate that MUG1 is the major, if not the only, murinoglobulin that is physiologically important in the conditions studied.

Expression of A2MR/LRP, LDLR, and HBP-44

Changes in lipid metabolism occurring during pregnancy and lactation have been observed consistently in humans (64–66) and experimental animals (67–70) but the physiological implication is not completely understood. In rats, hyperlipidemia is associated with late pregnancy and is sustained in lactating rats, but normal levels are established within 2 days after weaning (69, 70). By analyzing the expression pattern of lipoprotein receptors we aimed at a better understanding of the complex interactions in pregnancy and lactation. To this end we have studied mRNA levels of the proteins as an indicator of their expression, fully aware of the caveat that differences in mRNA levels are not per se reflected in similar changes in protein production levels.

The hepatic expression of A2MR/LRP and LDLR, as well as of LPL, showed a very similar regulation during mouse pregnancy: a marked decrease in mRNA expression in the last week of pregnancy. These data demonstrate that the liver, which in non-pregnant conditions is the major tissue for lipoprotein transformation and metabolism, has a marked decrease in uptake capacity of lipoproteins by both LDLR and A2MR/LRP. Further, the noted decrease in LPL mRNA expression in the liver should also contribute by diminished lipolysis and uptake of fatty acids. As a consequence, the levels of circulating lipoproteins are expected to increase, especially the VLDL-triglycerides, which has indeed been documented in humans (64–66) and in experimental animals (69–71). The contribution of increased production of lipoproteins is to be taken into account but to our knowledge has not been quantified.

The comparable decrease in hepatic expression of A2MR/LRP, LDLR, and LPL mRNA contrasts with their differential regulation in placenta and uterus: from day 12 to 19 pc the 4-fold decrease in placental LDLR mRNA seems to be more than balanced by the 7-fold increase in placental A2MR/LRP mRNA. In uterus, A2MR/LRP mRNA levels showed only minor alterations, while LDLR mRNA again decreased dramatically about 8-fold. LPL mRNA levels in placenta as well as in uterus, on the other hand, were very strikingly increased in late pregnancy.

The observed decline in LDLR mRNA expression in mouse placenta is consistent with a similar, although less pronounced decrease in placental LDLR mRNA expression with progressing pregnancy in humans (72). The 7-fold increase in placental A2MR/LRP mRNA expression in late pregnancy demonstrates that the placental expression of A2MR/LRP mRNA is regulated differently, indeed opposite to that of LDLR mRNA. Unlike LDLR, expression of A2MR/LRP is not regulated by sterols (73), nor was any evidence obtained for the presence of sterol responsive elements in the A2MR/LRP promoter (74). The high placental A2MR/LRP mRNA expression substantiates in vitro studies on trophoblasts, showing the appearance of A2MR/LRP as cytotrophoblasts differentiate into syncytiotrophoblasts and an inverse response of A2MR/LRP and LDLR expression to cAMP (75). In human trophoblasts in situ, intense immunofluorescent staining was observed for A2MR/LRP on the apical role of syncytiotrophoblasts throughout pregnancy, but no quantitative data are available (39). Our quantitative results, demonstrating a 7-fold increase of mouse placental A2MR/LRP mRNA expression in late pregnancy, are highly suggestive for a major role of A2MR/LRP in lipid metabolism during pregnancy. A comparable up-regulation, although situated in the second week of pregnancy, was recently observed for mouse placental VLDLR mRNA, another member of the LDLR family that binds apoE-containing VLDL (76–78).

In addition, placental A2MR/LRP will also contribute to the uptake and clearance of A2M- and especially MUG-proteinase complexes formed around parturition, as indicated by the increased consumption of MUG during this period, as discussed above.

As far as HBP-44 is concerned, this enigmatic component of the A2MR/LRP system displayed an expression pattern that was very similar to that of A2MR/LRP during pregnancy. In placenta both transcripts were significantly up-regulated while in the uterus they were maintained at fairly constant levels. On the other hand, the constant hepatic HBP-44 mRNA levels further contrasted with the strongly down-regulated A2MR/LRP mRNA levels. This could suggest that HBP-44 might exert an extra inhibition of ligand binding to A2MR/LRP, under the assumption that HBP-44 is indeed capable of reaching the ligand binding sites on the receptor in vivo. Consistent with this notion is the dissociated expression pattern of A2MR/LRP and A2MRAP in human trophoblasts, where a role for A2MR/LRP is suggested in trophoblast invasion, its activity being regulated by the expression of A2MRAP (39). Analysis of the expression pattern of gp330, another member of this receptor family which binds HBP-44 and postulated to be regulated by it (26), might yield further insight of this system, although it could make matters even more complex. This is favored by and could partially explain the differential cellular expression pattern of A2MR/LRP and HBP-44 in different mouse tissues (37). Mice in which the different receptors or the HBP-44 gene are inactivated are expected to yield more definite answers in this regard.

Expression of LPL and apoE

The striking increase of LPL mRNA in mouse uterus and placenta, demonstrates that its regulation is compa-

rable to the situation in rats (71). The marked changes in tissue specific expression of LPL during pregnancy demonstrate a major redistribution in metabolism of lipoproteins and in the fate of circulating triglycerides: they are diverted from uptake by the liver to uptake by the uterus and placenta and possibly by other tissues. Free fatty acids, generated through extracellular hydrolysis of VLDL by the high amounts of LPL at the maternal side of the placenta (79), are transferred directly across the placenta, driven by the generated transplacental free fatty acid gradient, as also suggested in humans (66), rats (80), and guinea pigs (81). These fatty acids must indeed provide the necessary energy and building blocks not only for the developing fetus, but for placenta and uterus as well.

The regulation of expression of apoE is another aspect of lipid metabolism to understand how its levels of expression relate to expression of receptors for apoE-lipoproteins. The increase in liver apoE mRNA with progressing pregnancy is minor, relative to the dramatic 10- and 5-fold increase in apoE mRNA levels in placenta and uterus, respectively. This must result in higher plasma levels of apoE-containing lipoproteins in late pregnancy and must enhance the increased uptake of VLDL, as observed in mice overexpressing rat apoE (73, 82). Also, in humans, term placental membranes show a high binding capacity for VLDL, compared to LDL or HDL, although the identity of the receptor(s) was not established (83). Furthermore, mice in which the apoE gene is silenced by targeting showed profound effects on their lipoprotein clearance, as demonstrated by the very high plasma cholesterol levels, mainly in the VLDL and β-VLDL fraction, as well as by the marked increase in apolipoprotein B-48 (84–86). Not unimportantly, it is our experience that apoE-deficient mice are problematic in reproduction when kept in an open animal house as opposed to normal reproduction under specific pathogen-free conditions. It will be of considerable interest to learn about the expression pattern of both receptors in these transgenic mice during pregnancy, similar to studies here in normal mice.

Conclusions

Regulation of expression of the two members of the alpha-2-macroglobulin family in the mouse is exclusively restricted to the liver, a situation that is different from human and rat. The temporal regulation of MAM and MUG is, however, completely different. MAM is expressed pre-natally in embryonic liver and is very constant during pregnancy, while MUG is subject to regulation during pregnancy, around birth, and in infantile life. This regulation suggests a role for MUG especially around birth, in the inhibition and clearance of proteolytic enzymes released from uterus and detaching placenta.

The documented changes in the expression patterns of A2MR/LRP, LDLR, LPL, and apoE demonstrate that the uptake of cholesterol and lipids in the placenta is provided by increased uptake of apoE-VLDL via A2MR/LRP as well as by increased fatty acid uptake generated by LPL overexpression. Thus, chylomicrons and VLDL, highly enriched in apoE, are subject to hydrolysis by LPL at the maternal side of the placenta and the generated fatty acids freely cross the placenta. Furthermore, the resulting β-VLDL particles are taken up by A2MR/LRP and other receptors such as VLDLR and eventually gp330 to be degraded in the lysosomes, and the lipids and acids are released into the cells. This process is again enhanced by high levels of LPL at the maternal side of the placenta.

In addition, the increased expression of A2MR/LRP in placenta will be important in the uptake and clearance of other known, or as yet unknown, ligands. That A2MR/LRP is essential for normal mouse embryonic development was shown by the fact that mice homozygous for an inactivated A2MR/LRP gene are not viable (2, 3). Therefore, this effect could be in part or even totally due to malfunctioning of the placental uptake of ligands by A2MR/LRP. The possibility that a defective clearance of A2M- or MUG-proteinase complexes contributes to this system will be investigated in mice with a null mutation in the MAM and MUG genes (1).

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