Soluble Low Density Lipoprotein Receptor-related Protein (LRP) Circulates in Human Plasma*

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Our studies have identified a soluble molecule in normal human plasma and serum with the characteristics of the a-chain of the low density lipoprotein receptor-related protein (LRP). LRP is a large multifunctional receptor mediating the clearance of diverse ligands, including selected lipoproteins, various protease inhibitor complexes, and thrombospondin. A soluble molecule (sLRP) has been isolated from plasma using an affinity matrix coupled with methylamine-activated a2-macroglobulin, the ligand uniquely recognized by LRP, and eluted with EDTA. This elute contains a protein that co-migrates on SDS-polyacrylamide gel electrophoresis with authentic human placental LRP a-chain, is recognized by anti-LRP a-chain monoclonal antibodies, and binds the 39-kDa receptor-associated protein (RAP) and tissue plasminogen activator-inhibitor complexes. A similar RAP-binding molecule was detected in medium conditioned for 24 h by primary cultures of rat hepatocytes, suggesting that the liver may be the in vivo source of sLRP. In contrast, immunoprecipitation experiments failed to detect the production of sLRP by cultured HepG2 hepatoma and primary human fibroblast cells. Addition of a soluble form of LRP to cultured HepG2 cells resulted in a significant inhibition of capacity of these cells to degrade tPA, a process that has been demonstrated to be mediated by cell surface LRP. Preliminary data indicate that the concentration of sLRP is altered in the plasma of patients with liver disease. Increased levels of sLRP may antagonize the clearance of ligands by cell-bound LRP, perturbing diverse processes including lipid metabolism, cell migration and extracellular proteinase activity.

The low density lipoprotein receptor-related protein (LRP) has been previously identified as a membrane-bound endocytic receptor (1, 2). Studies have demonstrated that LRP mediates the internalization of multiple, structurally unrelated ligands, including selected lipoproteins, proteinase-inhibitor complexes, plasminogen activators, and thrombospordin (reviewed in Refs. 3 and 4). The binding of all ligands to LRP is inhibited by the receptor-associated protein (RAP), a protein that was co-purified with LRP (2, 5). The range of ligands recognized by LRP suggests that it plays a role in diverse processes including lipid metabolism, cell growth, migration, and tissue invasion. LRP expression is widespread; however, it is most highly expressed in the liver, brain, and placenta. The remarkable degree of cross-species identity conserved in the LRP amino acid sequence (3) and the embryonic lethal phenotype obtained after targeted disruption of the LRP gene in the mouse (6) underscore the biological importance of this molecule.

Here we report the identification of a soluble form of LRP circulating in human plasma. The characterization of this molecule, which maintains the ligand binding characteristics of cell surface LRP, introduces a new dimension to the biology of LRP. Accumulation of soluble LRP in plasma may antagonize the clearance of ligands by cell-bound LRP, perturbing lipid metabolism and cellular processes involving extracellular proteinase activity.

MATERIALS AND METHODS

Proteins and Reagents—All chemicals were of analytical grade and purchased from BDH (Kilsyth, Australia). Bovine serum albumin (BSA), benzamidine, phenylmethylsulfonyl fluoride, bacitracin, leupeptin, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and HEPES were purchased from Sigma. Electrophoresis reagents were purchased from Bio-Rad. Proteins were iodinated with carrier free Na125I (Australian Radioisotopes, Lucas Heights, Australia) and iodogen reagent (Pierce), according to the manufacturer’s instructions. Protein concentrations were determined using the BCA protein assay (Pierce). Recombinant RAP was synthesized as a glutathione S-transferase fusion protein in Escherichia coli and purified as described previously (5). The RAP-GST/gGex plasmid was a generous gift from Joachim Herz, University of Texas Southwestern Medical Center, Dallas, TX. a2-Macroglobulin (a2M) was purified from human plasma by Zn2+--chelate chromatography and gel filtration, as described previously (7). It was activated by the addition of 0.2 M methylamine, 30 min, room temperature, dialyzed into 20 mM Mes, pH 6.0, and stored at 4 °C. Purified recombinant human tissue plasminogen activator (tPA) was donated by Karl Thomae GmbH (Biberach, Germany). Recombinant plasminogen activator inhibitor-1 (PAI-1) was expressed in E. coli transformed with a plasmid (pMBL11/PAI-1) containing full-length PAI-1 cDNA (Ref. 8, a generous gift of A. Zonneveld, University of Amsterdam) and purified as described previously (9), with additional purification by size exclusion chromatography on Bio-Gel P-60 (Bio-Rad). The anti-LRP a-chain monoclonal antibody (mAb 9G1) and affinity-purified rabbit anti-LRP (R777) were kind gifts of Dr. Dudley Strickland, American Red Cross, Rockville MD (2). A commercial anti-LRP a-chain mAb (number 3402) was also purchased (American Diagnostics, Greenwich, CT). A hybridoma secreting an anti-LRP b-chain COOH-terminal peptide mAb (11H4; ATCC CRL-1936) was obtained...
from the American Type Culture Collection (Rockville, MD). Antibodies were purified from culture supernatant using protein G-Sepharose (Pharmacia Biotech Inc., Uppsala, Sweden), according to the manufacturer's instructions. All cell culture reagents were purchased from ICN (Costa Mesa, CA) and culture ware was from Costar (Cambridge, MA).

**LRP Immunoprecipitation**—Microtiter plates (Maxisorp, Nunc, Denmark) were coated with 1 µg/well rRAP (100 µl) diluted in carbonate buffer, pH 9.6 (2 h, 37 °C). After blocking in assay buffer (HBSC (20 mM HEPES, 0.15 M NaCl, 2 mM Ca²⁺, pH 7.4) containing 0.1% Tween 20, 1% BSA), 30 min, 37 °C, a 100-µl sample (diluted in assay buffer) was added to the well and incubated 2 h at room temperature. After washing, the plates were incubated with 100 µl/well anti-LRP mAb (SG1, 5 µg/ml) in assay buffer, 1 h at room temperature. After washing, 100 µl of 1/1000 dilution rabbit anti-mouse Ig-horseradish peroxidase conjugate (Dako, Carpinteria, CA) was added (30 min, room temperature), and bound antibody was quantitated with 200 µl/well chromogenic substrate (1 mg/ml ABTS, 0.003%, v/v, H₂O₂ diluted in citrate buffer, pH 4.5). Color development was stopped after 20 min by addition of 50 mM EDTA, 20 mM Mes, pH 6.0. Fractions eluting between 21 and 25 ml were pooled and analyzed.

**Affinity Isolation of LRP**—An affinity matrix was prepared by coupling 280 mg of methylamine-activated α₅M to 25 ml of CNBr-Sepharose (Pharmacia Biotech Inc.), according to the manufacturer's instructions. Fresh frozen human plasma (200 ml) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 mM benzotriazol, 1 µM leupeptin) was thawed at 37 °C. The plasma was adjusted to pH 7.4 by addition of 1/10 volume 0.1 M HEPES, pH 7.4, 10 IU/ml heparin, 5 mM Ca²⁺, clarified by centrifugation (4 °C, 20 000g, 30 min), and filtered through a 0.45-µm nitrocellulose filter (Millipore-Waters, Milford, MA). The plasma was mixed with blank Sepharose gel for 1 h at 4 °C. The precleared plasma supernatant was then mixed with methylamine activated α₅M-Sepharose for 6 h at 4 °C. The mixture was washed on a scintillation glass funnel with 250 ml of HBSC, packed into a column and eluted with 25 ml EDTA, 20 mM Mes, pH 6.0. Protease inhibitors (as above) were added to each fraction (1 ml). LRP was also isolated from detergent-solubilized human placental membranes, prepared as described previously (10), using the activated α₅M-Sepharose affinity matrix. The fractions were screened by specific LRP immunopassay and pooled positive fractions that were stored on a 5–15% gradient SDS-polyacrylamide gels (SDS-PAGE) and analyzed by silver staining (11).

**Western and Ligand Blots**—Prior to Western and ligand blot analysis samples were electrophoresed on 6% SDS-PAGE minigel for 2 h at 150 V. The gels were electrophoretically transferred to polyvinylidene difluoride membrane (NEN Life Science Products) and incubated, according to the manufacturer's instructions, with 5 µg/ml mAb and 1/2000 dilution rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dako). Bound antibody was visualized with chemiluminescence reagent (Renaissance, NEN Life Science Products) and exposed to film (Hyperfilm-MP, Amersham Corp.). For ligand blotting, blots were blocked in 5% milk, HBSC (0.1% Tween 20 and incubated 4 h at room temperature). The blots were washed on a scintillation glass funnel with 250 ml of HBSC, packed into a column and eluted with 25 ml EDTA, 20 mM Mes, pH 6.0. Protease inhibitors (as above) were added to each fraction (1 ml). LRP was also isolated from detergent-solubilized human placental membranes, prepared as described previously (10), using the activated α₅M-Sepharose affinity matrix. The fractions were screened by specific LRP immunopassay and pooled positive fractions that were stored on a 5–15% gradient SDS-polyacrylamide gels (SDS-PAGE) and analyzed by silver staining (11).

**Immunoprecipitation**—HepG2 cells and HFF were plated in 60-mm Petri dishes in 10 ml of culture medium. When the cells were 80% confluent, the monolayers were washed twice with phosphate-buffered saline and incubated in 4 ml of cysteine/methionine-free EMEM (10% FBS) for 20 min at 37 °C. The cultures were then pulsed by incubation, 1 h, in the presence of 66 µCi/ml [³⁵S]methionine/cysteine (Trans³⁵S-label, ICN Biomedical). Long term labeling was conducted overnight in the presence of 20% cysteine/methionine complete EMEM. The chase period (0, 30, 60, 180, or 240 min) was initiated by the addition of 4 ml of EMEM, 10% FBS containing 0.2 µCi unlabeled cysteine/methionine. Medium was collected and centrifuged 1200 × g for 5 min. Cell monolayers were washed twice with phosphate-buffered saline and lysed in the dish by the addition of 4 ml of 1% Triton, HBSC + protease inhibitors (as above) and clarified by centrifugation 4000 × g for 10 min at 4 °C. Samples were mixed with 25 µl of protein G-Sepharose (1:1 slurry, 30 min, 4 °C) and then incubated (4 °C, 2 h) in the presence or absence of 5 µg of anti-LRP α-chain mAb. After incubation with 25 µl of protein G-Sepharose (1:1 slurry, 30 min, 4 °C), the Sepharose was washed five times in 20 ml MES, pH 6, 0.5 M NaCl, 0.1% Triton X-100, and suspended in 50 µl of SDS-PAGE sample buffer and electrophoresed on a 5–15% gradient SDS-PAGE gel. Gels were fixed in 10% acetic acid.
acid, 50% methanol, soaked in Amplify (Amersham Life Science, Inc., Little Chalfont, United Kingdom), and dried before exposing to film for 3 days.

Degradation of 125I-tPA by HepG2 Cells—HepG2 cells plated in 24-well culture dishes were washed three times in EMEM, 0.2% FBS and incubated in 2 ml of medium containing 3 nM 125I-tPA in the presence or absence of the following ligands: 1) 1 μM unlabeled tPA, 2) 2 μg/ml (3.3 nM) purified placental LRP, and 3) 10 μg/ml (16.7 nM) purified placental LRP. After incubation for appropriate times, binding medium was removed and mixed with an equal volume of 20% trichloroacetic acid containing 4% phosphotungstic acid, incubated on ice for 10 min, centrifuged 10,000 × g for 5 min, and a 1-ml aliquot of supernatant was counted in a γ-counter to determine counts/min of 125I-tPA degraded from triplicate cultures at each time point.

Determination of sLRP Concentration in Human Plasma—The LRP immunoassay was used to determine the concentration of sLRP in human plasma. A standard curve was prepared by diluting affinity-purified placental LRP in the concentration range 0.125–2.5 μg/ml. sLRP concentrations were calculated from the standard curve using a four parameter curve fit. A preliminary study was conducted to determine the effects of blood additives and storage conditions on the estimation of LRP concentration by the LRP immunoassay. To determine the range of LRP in normal human plasma, citrated plasma was obtained from 50 healthy blood donors giving informed consent. In addition, citrated plasma samples (n = 45) were collected in 2 M-Sepharose by SDS-PAGE (reducing) revealed the presence of a single chain molecule which co-migrates with the α-chain of LRP (Mr 500,000) isolated from human placental membranes (Fig. 2A). The high molecular weight protein appears to be structurally and functionally related to human LRP α-chain, as it is recognized by an anti-LRP mAb (8G1) on Western blot and binds 125I-RAP in a ligand blot (Fig. 2A). In contrast, a mAb specific for the COOH terminus of LRP β-chain (Mr 85,000) failed to specifically recognize any species in the enriched plasma fraction either by specific immunoassay (data not shown) or Western blot (Fig. 2B). A second, distinct anti-LRP mAb detected sLRP in the enriched plasma fraction, providing further evidence that the affinity eluate of human plasma contains a molecule closely related to LRP (Fig. 2C). A molecule of identical electrophoretic mobility was also recognized by an affinity-purified rabbit anti-human LRP polyclonal antibody (R777, a generous gift of Dr. Dudley Strickland, American Red Cross; data not shown).

sLRP Is Secreted by Primary Cultures of Rat Hepatocytes—Ligand blot analysis of culture medium conditioned by primary cultures of rat hepatocytes revealed the presence of a 125I-RAP-binding protein that co-migrated on SDS-PAGE with human placental LRP and a cell-associated molecule in cellular lysates of cell rat hepatocytes (Fig. 3A). Prior to analysis the conditioned medium was concentrated 16-fold by ion exchange chromatography on DEAE-Sephacel. Immunoprecipitation of metabolically labeled human HepG2 and HFF cells with an anti-LRP α-chain antibody (8G1) failed to detect the accumulation of a soluble form of LRP in the supernatant medium after a 4-h chase (Fig. 3B). Cell-associated LRP was readily detected at each chase time point, i.e., from the end of the 1-h pulse onwards. Long term labeling experiments (up to 24 h) also failed to detect detectable amounts of LRP in medium conditioned by these cells (data not shown).

A Soluble Form of LRP Is Able to Perturb the Degradation of tPA by HepG2 Cells—The uptake and degradation of tPA has been demonstrated to be mediated by LRP and can be inhibited by the LRP antagonist RAP (14). Ligand blot analysis demonstrates that both the purified placental LRP and the plasma-
A Soluble Form of LRP

FIG. 3. sLRP is released by cultured primary rat hepatocytes. A, rat hepatocytes were cultured for 7 days in serum-free medium on matrigel to maintain a differentiated phenotype characteristic of adult liver. Samples, prepared as described under “Materials and Methods,” were electrophoresed on 6% SDS-PAGE gels for ligand blot analysis. Blots were incubated with 125I-RAP to detect LRP in human placental lysate eluted from DEAE-Sephacel (lane 2), hepatocyte cell lysate (lane 2), hepatocyte cell lysate eluted from DEAE-Sephacel (lane 3), and rat hepatocyte conditioned medium concentrated 16-fold on DEAE-Sephacel (lane 4). B, monolayer cultures of human HepG2 and foreskin fibroblast cells were biosynthetically labeled by incubation at 37 °C in 35S-cysteine/methionine containing medium. At selected chase time points, medium and cells were collected and immunoprecipitated with an anti-LRP mAb, as described under “Materials and Methods.” Immunoprecipitates were electrophoresed on 5–15% gradient SDS-PAGE gels and processed for autoradiography. Autoradiographs from the 4-h chase time point are shown above. + indicates immunoprecipitate obtained with anti-LRP mAb, and − indicates negative control in which no antibody was present. The position of specifically immunoprecipitated LRP α-chain is marked by the arrow.

derived sLRP bind 125I-tPA-PAI-1 complexes (Fig. 4A). Fig. 4B demonstrates the inhibition of 125I-tPA degradation by HepG2 cells when assays were conducted in the presence of 10 μg/ml (16.7 nM) purified placental LRP.

Plasma sLRP Levels Are Increased in Patients with Liver Disease—sLRP levels were measured in healthy subjects to define the normal concentration range. The plasma sLRP concentration estimated using the LRP immunoassay did not vary significantly when blood from healthy donors was collected into a dry tube (serum, 6.9 μg/ml), EDTA (6.5 μg/ml), or citrate (6.8 μg/ml), when samples were assayed at 10–20-fold dilution in Ca2+–containing assay buffer. Addition of 10 IU/ml heparin to the assay buffer to prevent postdilution clotting also did not interfere with the estimation of sLRP concentration. The range of sLRP concentration detected in the plasma of healthy subjects (n = 50; mean 6.1 ± 1.2 μg/ml; range 3.7–10.8 μg/ml), patients with abnormal liver function (n = 45; mean 7.1 ± 3.44 μg/ml, range 2.0–22.4 μg/ml), and patients with NIDDM (n = 49; mean 6.5 ± 0.9 μg/ml, range 5.0–9.0 μg/ml) is shown in Fig. 5. The plasma concentration of sLRP was significantly altered (varied by >2 S.D.) from the normal concentra-

FIG. 4. A soluble form of LRP inhibits the degradation of 125I-tPA by HepG2 cells. A, ligand blot analysis confirms that placental LRP is able to bind 125I-labeled tPA-PAI-1 complexes in a ligand blot (lane 1). Similarly, the circulating molecule enriched from human plasma by α2M-Sepharose affinity chromatography binds 125I-tPA-PAI-1 complexes in a ligand blot (lane 2). The specificity of binding is indicated by the absence of binding in the presence of excess unlabeled tPA (lane 3). The faster migrating, second band binding 125I-tPA-PAI-1 in lane 2 was also recognized by the anti-LRP mAb 8G1 (lane 4) and frequently appears after storage of the affinity eluate. B, HepG2 monolayers were incubated at 37 °C with 4 nM 125I-tPA diluted in Dulbecco’s modified Eagle’s medium, 1% BSA in the presence or absence of competitors as indicated. Endocytosis and degradation of the ligand were indicated by the appearance of trichloroacetic acid-soluble counts/min in the culture supernatant. Each symbol represents the average of triplicate determinations ± S.D.

FIG. 5. The plasma concentration of sLRP is altered in patients with liver disease. The normal concentration range of sLRP in the plasma of healthy subjects (n = 50), estimated using the LRP immunoassay, is shown alongside that detected in patients (n = 45) displaying abnormal liver function and patients with NIDDM (n = 49). The normal concentration range of plasma sLRP concentration is indicated by the hatched area on the graph; this area corresponds to the mean concentration in the healthy subjects (6.1 μg/ml ± 2 S.D. (±2.4)).
abnormal liver function test did not correlate with disease etiology nor with the level of any plasma proteins assayed, including bilirubin, alkaline phosphatase, transaminases, γ-glutamyltransferase or 5'-nucleotidase.

**DISCUSSION**

LRP belongs to the low density lipoprotein receptor family, the members of which share many structural and functional characteristics (3). The α-chain of the heterodimeric LRP contains multiple, Ca2+-dependent ligand binding domains and is noncovalently bound on the cell surface to the membrane-spanning β-chain (15). The characteristics of the soluble molecule circulating in human plasma suggest it is a molecule closely related to the α-chain of LRP: it displays Ca2+-dependent binding to two established LRP ligands RAP and activated α2M, a ligand uniquely recognized by LRP; it is a single chain molecule which co-migrates on SDS-PAGE with authentic human placental LRP α-chain; and it is recognized by two distinct anti-LRP α-chain monoclonal antibodies. We were unable to detect the intracellular COOH terminus of the LRP β-chain in the affinity-isolated sLRP. However, it is possible that a truncated β-chain may be associated with the soluble α-chain. Precise determination of the exact structure of plasma sLRP and its relative affinity for the various LRP ligands will only be possible with a highly purified preparation of sLRP. These studies are currently under way. The presence of a circulating LRP-like molecule is not confined to human plasma, and a similar molecule has been detected in plasma and serum from a variety of mammals and the chicken.2

LRP is most highly expressed in the liver, and analysis of culture medium conditioned by primary cultures of rat hepatocytes revealed the presence of an sLRP-like 125I-RAP-binding protein. Our experiments could not detect the release of a soluble form of LRP from the human hepatoma cell line HepG2, nor from cultured normal human fibroblasts, suggesting that the release of sLRP is not a constitutive property of all cultured cells. The absence of sLRP in medium conditioned by the hepatoma cell line suggests that the production of sLRP may be associated with the more differentiated phenotype of the primary hepatocytes cultured on matrigel (16). Further studies are required to investigate the factors regulating the production of sLRP.

The detection of sLRP in hepatocyte-conditioned medium provides a model system for the further characterization of sLRP and the elucidation of the mechanism generating the soluble form. A wide variety of receptors and other plasma membrane proteins have been identified as having soluble counterparts in serum (17). There are examples in the literature of soluble receptors liberated by proteolytic cleavage of receptor exodomains (18, 19) and those that derive from differential splicing of a common mRNA transcript or transcription of closely related, but distinct, genes (20). In the case of LRP, another potential mechanism for the generation of sLRP could be the disruption of the noncovalent bond that anchors the α-chain to the membrane-spanning β-chain (15). A soluble form of gp330, another member of the low density lipoprotein receptor family, has been detected in the supernatant of a yolk sac carcinoma cell line (21). Interestingly, in this cell line gp330 was present on the cell surface and in supernatant as a complex with RAP. No further characterization of the cellular and molecular events regulating gp330/RAP release has been reported.

Soluble receptors, generally, have reduced ligand affinity constants compared with their membrane-bound counterparts, and the circulating receptor concentration may be insufficient to effectively compete ligand binding to the cell-bound molecule (22). Our preliminary experimental indicates that the addition of soluble purified placental LRP at a physiologically relevant concentration (10 μg/ml) was able to inhibit the degradation of tPA by cultured HepG2 cells, a process that has been demonstrated previously to be mediated by cell surface LRP (14). This indicates that the sLRP may act as a competitive inhibitor of ligand uptake by cell surface-bound LRP. The inhibition of tPA uptake by soluble LRP may have negligible consequences to the level of extracellular proteolysis, because the enzyme is rapidly inactivated before endocytosis by complex formation with plasminogen activator inhibitor-1 (PAI-1), present in large excess in the extracellular matrix of HepG2 cells (23, 24). However, LRP mediates the uptake of many biologically active molecules, including the potent angiogenesis inhibitor, thrombospondin (25), apoE-enriched lipoproteins (26), lipoprotein-lipase (27), and is able to bind directly active tPA (28) and urokinase PA (29). Increased levels of soluble LRP may extend the half-life of these active ligands and influence diverse biological processes, including lipid metabolism, cell growth, and migration and extracellular proteinase activity.

The identification of a soluble molecule with the characteristics of LRP introduces a new dimension into the biology of this unique molecule. Further studies are required to understand the biochemical mechanisms involved in the generation of sLRP and establish its physiological role. The concentration of plasma sLRP appears to alter in some patients with impaired liver function. A more extensive study is required to substantiate this preliminary data and elucidate the functional implications of the moderate changes. As LRP has been implicated to be overexpressed in a range of other pathological processes, including atherosclerosis (30) and Alzheimer’s disease (31), the functional or prognostic implications of plasma sLRP concentration requires investigation.

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