Role of the macrophage galactose lectin in the uptake of desialylated LDL

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Abstract

Desialylated low density lipoprotein (LDL) is rapidly taken up and accumulated by both peripheral blood monocytes and cells isolated from human arterial intima consisting predominantly of smooth muscle cells. It is shown that thioglycollate (TG)-elicited mouse macrophages and mouse peritoneal macrophages stimulated with lipopolysaccharide (LPS) show increased expression of a membrane-bound, galactose-specific lectin that could be responsible for this uptake. In LPS-stimulated macrophages accumulation of desialylated LDL is increased ca. 2.6-fold. Accumulation of acetylated LDL in the same cells is reduced, suggesting that the galactose-specific lectin might be responsible for the uptake of desialylated LDL. Transfection of cells with the mouse macrophage Gal:GalNAc-specific lectin (MMGL) increased their capacity to take up asialofetuin (ASF) and, to a smaller extent, desialylated LDL. The uptake of desialylated LDL was small, most likely due to the high $k_d$ of MMGL for biantennary oligosaccharides as found on LDL, and low concentration of LDL achieved in tissue culture experiments. The data suggest that the expression of galactose-specific lectins can be elevated under inflammatory conditions, and that these receptors could contribute to foam cell formation under conditions of high desialylated LDL concentration, as might be found in arterial intima.

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1. Introduction

Fatty streak formation in the arteries of patients suffering from atherosclerosis has often been attributed to the expression of scavenger receptors in macrophage foam cells leading to the unregulated uptake of modified lipoproteins from the blood [1,2]. Acetylation or oxidation of low density lipoprotein (LDL) in vitro effectively converts LDL into a ligand for the scavenger receptor, but it is not known if oxidation is the primary cause of LDL modification in vivo.

An alternative modification of LDL that might lead to atherosclerosis is desialylation [3]. Apolipoprotein (apo) B-100 isolated from native LDL of normolipidemics was found to be only partially sialylated with approximately half of the N-linked lactosamine oligosaccharides having one exposed Gal residue [4]. Patients with coronary heart disease (CHD) have been shown to have lower levels of sialic acid on LDL apo B [5] which could therefore contain biantennary oligosac-
charides terminating in Gal residues — making them a high affinity ligand for lectin receptors [6]. LDL lipids are also found to be desialylated in CHD patients [5], leading to a lower overall sialic acid content of LDL particles in patient groups in several studies [7,8]. Previous publications have suggested that uptake of desialylated LDL by macrophages could be via scavenger receptors [9], although our data, based on competition with asialofetuin (ASF), argued for uptake by a Gal-specific lectin receptor [10]. The uptake of desialylated LDL has now been examined in macrophages stimulated with different agents. Lipopolysaccharide (LPS)-stimulated macrophages, which have down-regulated scavenger receptors, were found to take up desialylated LDL more avidly than resting macrophages.

Furthermore, we show that LPS-stimulated macrophages express increased amounts of a galactose/galactosamine-specific lectin (Gal/GalNAc-specific lectin). These data argue for a lectin-mediated uptake of desialylated LDL that is independent of the scavenger receptor. In cells transfected with the mouse macrophage Gal/GalNAc specific lectin (MMGL) an increase in the uptake of ASF, and to a smaller extent, desialylated LDL, could be demonstrated. Thus it is possible that the human orthologue of MMGL might be responsible for the uptake of desialylated LDL by macrophages in atheromatous plaque.

2. Experimental procedures

2.1. Materials and methods

Neuraminidase attached to agarose beads, ASF and bovine serum albumin (BSA) were obtained from Sigma, Bicinchoninic acid assay kit and sulfo-NHS-biotin were from Pierce. Antibody against apo B-100 (monoclonal), goat-anti-mouse Ig-rhodamine and re-striction enzymes were purchased from Boehringer Mannheim. Anti-FLAG M2 monoclonal antibody was from KODAK. Secondary antibodies, linked to horseradish peroxidase (HRP), Rainbow molecular weight markers (full range), streptavidin—fluorescein, PD-10 columns (Sephadex G-25 M) and ECL kit and film were from Amersham Pharmacia Biotech. Invitrogen TA cloning kit (pCR 2.1 vector) was obtained from Bresatec, SA. For DNA extractions QIAquick Gel Extraction kit from QIAGEN was used. LipofectAMINE reagent (GibcoBRL) was purchased from Life Technologies, Geneticin (G418) and trichloroacetic acid (TCA) were from Calbiochem. Oligonucleotides for polymerase chain reaction (PCR) and DNA sequencing were obtained from Bresatec. The Glyco fluorescence assisted carbohydrate electrophoresis (FACE) kit was obtained from Gradipore.

2.2. Preparation of LDL and modified proteins

Blood was drawn from healthy, fasting normolipidemic individuals having plasma cholesterol levels between 4.2 and 5.09 mmol/l. The blood was added immediately to a concentrated protease inhibitor solution containing, at final dilution, 90 U aprotinin/ml, 20 μg soybean trypsin inhibitor/ml and 3 mM EDTA. The blood cells were removed by centrifugation at 400 × g for 30 min. LDL was then prepared by the rapid procedure of Sattler et al. [11], which involves a single KBr gradient centrifugation in a Beckman TL-100 centrifuge. The LDL was ready in about 3 h after collecting the blood. Small molecules were removed from the LDL prior to use by gel filtration on PD-10 columns and elution with nitrogen-purged phosphate buffered saline (PBS). LDL was desialylated in vitro with neuraminidase attached to agarose beads as described by Camejo et al. [12]. More than 85% of the apo B-associated sialic acid was removed in this process. For binding, uptake and degradation by MMGL-transfected cells, LDL was desialylated for 4 h only to prevent any LDL precipitation. Analysis of apo B-100 oligosaccharides after neuraminidase treatment by FACE was described previously [13].

LDL was acetylated as described previously [10,14] using 6 μl acetic anhydride/mg LDL protein. [125I]Iodination of neuraminidase-treated LDL (NT-LDL) and ASF was carried out with Na125I (2–5 mCi/mg protein) by the iodine monochloride method [15]. Iodinated proteins (specific activity 0.1–1.1 × 106 and 1.6–2.1 × 106 cpm/pmol protein for NT-LDL and ASF, respectively) were separated from unreacted iodine using PD-10 column followed by dialysis for 24 h against PBS.

Biotinylation of proteins was carried out in 50 mM NaHCO3 buffer using sulfo-NHS-biotin in 6-fold molar excess for 1 h at room temperature. The unreacted biotin was removed with PD-10 column followed by dialysis for 24 h against PBS.

2.3. Preparation and use of macrophages

Resident peritoneal macrophages were isolated from Q5 mice as described [16]. For binding studies 0.5–1 × 106 mouse peritoneal macrophages were incubated at 4°C for 4 h with 10 μg/ml [125I]NT-LDL in DMEM containing 1% BSA. The medium was aspirated and the unbound material was removed with five washes in cold PBS. Cells were then scraped off in 200 μl of cold PBS and the radioactivity determined. Protein content was determined by the bicinchoninic acid assay [17].

Degradation of [125I]NT-LDL was determined by incubation of 1 × 106 mouse peritoneal macrophages for 4 h at 37°C with [125I]NT-LDL at a concentration
of 5–10 µg protein/ml. A total of 100 µl of media was removed and 3 M TCA was added to a final concentration of 10%. The sample was vortexed vigorously and incubated for 10 min on ice. After addition of 250 µl of 0.7 M AgNO₃ to remove free iodine [18] the sample was centrifuged at 2000 rpm and the amount of radioactivity in the remaining supernatant was determined.

2.4. Biosensor assay

Approximately 27 ng/mm² of ASF was coupled to the surface of a carboxy-methyl dextran biosensor cuvette using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide according to the manufacturers instructions (Fisons). After quenching and washing in PBS containing 0.05% Tween 20 the binding of solubilised membrane protein was determined in a Fisons IAsys biosensor at 25°C. A total membrane fraction was prepared from the control, elicited or stimulated macrophages by homogenisation of approximately 1 x 10⁸ cells [19] and centrifugation for 1 h at 100 000 x g in a Beckman TL-100 ultracentrifuge. Each membrane pellet was then resuspended in 100 µl of 1% NP-40 containing 20 mM HEPES pH 6.8, 100 mM KCl, 0.1 mM Pefabloc (Boehringer Mannheim), 1 mM benzamidine and 5 µg/ml aprotinin at 4°C (NP-40 buffer). For each assay the cuvette was first equilibrated in 1% NP-40 buffer until a steady baseline was achieved. A total of 50 µl of solubilised membranes were then added (approx. equivalent to 10⁷ cells). After 4 min the membranes were washed out of the cuvette with 1% NP-40 buffer and the new baseline determined.

2.5. Lipid loading of macrophages

For lipid-loading experiments native (LDL), acetylated (AcLDL), or desialylated-LDL (NT-LDL) was added to the cells at a final concentration of 50 µg/ml in Hanks’ buffered salt solution (HBSS) containing 10% lipoprotein deficient serum (LPDS). The cells were then incubated for 24 h at 37°C. Control cells were prepared under the same conditions without addition of LDL. At the end of this time cholesterol and cholesterol esters were extracted with hexane/methanol as described [20]. HPLC analysis was by a reversed-phase C-18 column (Supelco, 25 x 0.46 cm, 5 µm particle size). Cholesterol and cholesterol esters were detected at 210 nm after elution with acetonitrile/isopropanol (30:70 v/v) at a flow rate of 1 ml/min.

2.6. Western blot

A peptide corresponding in sequence to the carboxyterminal 9 amino acid of MMGL (CEMK-LAKES) [21] was coupled to diphtheria toxin and used to immunise rabbits by multiple intradermal injections. Serum was prepared from blood taken 10 days after the second monthly injection of 300 µg of peptide.

Membranes from various macrophage preparations, or total cell protein was solubilised in SDS gel sample buffer and a portion (approx. 30 and 100 µg, respectively) loaded on a 10 or 12.5% polyacrylamide gel. Transfer onto nitrocellulose and Western blotting was as previously described [22] using 1:1000 antiserum followed by anti-rabbit Ig-HRP (1:10 000) and enhanced chemiluminescence (ECL) detection (Amersham). For the detection of MMGL-FLAG, the KODAK M2 monoclonal anti-FLAG antibody (1:2000), followed by anti-mouse Ig-HRP (1:10 000) and ECL detection were used.

2.7. Cloning MMGL

The cDNA encoding MMGL was obtained from RAW 264.7, a mouse macrophage-like cell line, by PCR using the following oligonucleotides: 5’-GGG TGT CGA CAT GAT ATA CGA AAA CCT CCA GAA C-3’ and 3’-FLAG MMGL: 5’-GGG AAC CTT CTA CTA CTT GTC ATC GTC CTT GTA GTC GCT CTC GTG CAG CCT C-3’. These oligonucleotides amplified the complete open reading frame of MMGL [21] and introduced a sequence encoding the FLAG epitope at the 3’ end and flanking Sal I/Hind III restriction enzyme sites. The PCR product was first cloned into pCR 2.1 vector (Invitrogen) using the overhanging A residues introduced by Taq polymerase. Sequencing revealed a single mutation: 853 T → C that resulted in a change in the amino acid sequence: Val → Ala. This mutation occurs in the carbohydrate recognition domain (CRD), but this amino acid is not preserved in other gal-specific lectin receptors [23]. It is unlikely therefore that this conservative replacement would have any functional significance. The DNA fragment encoding MMGL (945 bp) was cut out from the PCR 2.1 vector with restriction enzymes (Sal I/Hind III), and cloned into pAXneoRX vector.

2.8. Transfecting ECV 304 cells and immunofluorescence analyses

The ECV304 cells were transfected with MMGL cloned in pAXneoRX using the lipofectamine method (GibcoBRL). Transfected cells were selected by growing in media with 600 µg/ml Geneticin (G418); single colonies were trypsinised individually using glass cloning rings and grown in G418-containing media. Levels of expression were assessed by immunolabelling with anti-FLAG antibody followed by goat-anti-mouse Ig-rhodamine. Colonies expressing high levels of MMGL were subcloned to get more homogeneously
expressing cells, then checked by immunolabelling for the cell surface presence of the MMGL-FLAG receptor and active internalisation. The MMGL expression in the transfected cells did not decrease even after ten passages, or freezing and thawing.

For all immunofluorescence analyses, cells were cultured directly on glass coverslips and grown for 2–3 days. For anti-FLAG antibody binding, cells were incubated with the antibody for 1 h at 4°C then fixed with methanol; for internalisation experiments, after incubation at 4°C for 1 h, cells were placed in fresh media and warmed up to 37°C for 1 h prior to fixing in methanol.

To confirm the lectin’s ability to bind and internalise ASF, cells were incubated with biotinylated ASF and anti-FLAG antibody for 1 h at 37°C, in the presence of leupeptin or chloroquine to prevent lysosomal degradation. Incubation with the secondary antibodies, goat-anti-mouse Ig-rhodamine and/or streptavidin–fluorescein, was carried out for 30 min at 37°C.

In some cases cells were fixed in 3% paraformaldehyde ± 0.2% Triton X-100, then incubated with anti-FLAG antibody followed by goat-anti-mouse Ig-rhodamine to look at the steady state distribution of the lectin receptor.

2.9. Binding, uptake and degradation of 125I-labelled proteins

ASF and NT-LDL were iodinated using the ICl method [15]. Transfected and untransfected cells were passaged into 6-well plates and grown for 3 days in media containing 10% foetal calf serum (FCS) before the experiments; in some cases cells were pre-incubated with LDL (0.1 mg/ml) 24 h prior to the experiment to downregulate the LDL receptor (LDLR).

For surface binding after equilibrating at 4°C, the cells were incubated with [125I]ASF or [125I]NT-LDL ± competitors (either 100 × excess of ASF or 30–40 × excess of LDL) in 1 ml serum-free media for 2 h at 4°C. Total cell-associated counts were measured in cells incubated with [125I]ASF or [125I]NT-LDL ± competitors for 2 h at 37°C. Medium was then removed by aspiration, and the wells were rinsed 5 × with 2 ml PBS. The cells were dissolved in 1 ml 0.2 M NaOH and the bound radioactivity was determined with a COBRA auto-gamma counter.

The rate of degradation was measured as the increase in TCA-soluble radioactivity accumulating in medium after 18 h incubating with [125I]ASF or [125I]NT-LDL ± competitors at 37°C under a 5% CO2. To 0.9 ml of medium 100 μg of BSA and 0.1 ml of 100% TCA were added and after at least 10 min on ice, the precipitates were centrifuged at 10 000 rpm for 10 min, then the amount of radioactivity was measured in the supernatant fluid.

Protein determination in all experiments was done on cells rinsed 5 × with 2 ml PBS and dissolved in 1 ml 0.2 M NaOH using bicinchoninic acid assay and BSA standard curve. In the case where in each individual experiment the protein concentrations between wells were insignificantly different for given cell line, average of 18–36 (all in triplicates) protein concentrations were used in calculations of cpm/mg cell protein.

3. Results

3.1. Membrane-bound lectins are expressed in TG-elicited and LPS-stimulated macrophages

Expression of membrane bound Gal-specific lectins in mouse macrophages stimulated with different agents was tested by measuring the binding of solubilised macrophage membrane proteins to ASF in a biosensor cuvette. A total membrane fraction was prepared from equal numbers of control, elicited or stimulated macrophages and dissolved in 200 μl of 1% NP-40 buffer. When 50 μl of these preparations were added to the biosensor cuvette, significant binding could only be detected when the membranes were derived from TG-elicited macrophages or from macrophages stimulated with LPS. This was evident from the steeply sloped binding curve, and by the inability to wash off the bound material in PBS–Tween buffer (Fig. 1). In contrast, when solubilised membranes from control or periodate (PI)-elicited macrophages were used, the binding isotherm rapidly plateaued, and the signal returned to baseline on washing the cuvette. Even after addition of 150 μl of control membranes no binding was observed, whereas the signal increased when the amount of membranes from TG-elicited or LPS-stimu-

![Fig. 1. Induction of lectins in macrophage membranes. Mouse peritoneal macrophages (A) untreated, (B) treated with 1 μg/ml lipopolysaccharide (LPS), (C) elicited with thioglycollate (TG) or (D) elicited with periodate (PI) were homogenised and the membrane pellet solubilised in NP-40 buffer. An asialofetuin (ASF)-coated biosensor surface was equilibrated with NP-40 buffer (E) and then the membrane preparation added (M). After 4 min the biosensor cuvette was washed with PBS–Tween (W). The difference in Arc Secs after washing (W-E) represents the amount of membrane protein bound to the cuvette.](image-url)
lated macrophages added to the cuvette was increased (data not shown). Similar results were obtained in two to three different experiments using different biosensor cuvettes.

3.2. Induction of Gal/GalNAc-specific lectin in TG-elicited and LPS-stimulated macrophages

Membrane fractions from control, TG-elicited, PI-elicited and LPS-activated macrophages were immunoblotted with a polyclonal antibody raised against the carboxy terminal nine amino acids of the MMGL[21]. This antibody detected several bands in whole cell lysates from control RAW 264.7 cells which express the protein, but only one band at ca. 39 kDa in purified plasma membrane protein fraction. A band, migrating with the same mobility, was detected in the membranes of TG-elicited macrophages and LPS-stimulated macrophages but not in untreated peritoneal macrophages or PI-elicited macrophages (data not shown), consistent with the lack of Gal-specific binding in these cells (Fig. 1).

3.3. Lectins expressed in TG-elicited macrophages are not surface located

Previous studies have shown that desialylated LDL degradation by macrophages is competed by ASF suggesting that the macrophage Gal-specific lectin may be responsible for the uptake of desialylated LDL[10]. Since TG-elicited macrophages exhibited elevated levels of membrane associated lectins in the biosensor assay we tested the ability of these cells to accumulate desialylated LDL by measuring the accumulation of cholesterol esters by reversed phase HPLC (Fig. 2A). Surprisingly it was found that TG-elicited macrophages demonstrated less accumulation than control cells. To determine if this was caused by a decreased rate of uptake of the NT-LDL cells were incubated with 125I-labelled NT-LDL and the release into the medium of TCA soluble radioactivity was measured (Fig. 2B). The TG-elicited macrophages were considerably less able to degrade 125I-labelled NT-LDL, suggesting that the induced lectin receptors might be intracellularly located rather than on the cell surface. This hypothesis was confirmed by measuring the amount of binding of 125I-labelled ASF to the cell surface (Fig. 2C). This experiment indicates that fewer Gal-specific lectins were present on the surface of the TG-elicited macrophages despite the increased lectin associated with total membrane extracts of these cells.

3.4. LPS gives increased uptake of NT-LDL with decreased uptake of AcLDL

Thioglycollate (TG) can contain endotoxin therefore it was possible that the effect of TG-elicitation on lectin expression was due to LPS-activation of the elicited cells. Therefore, the direct effect of LPS on desialylated LDL uptake was also examined. In Fig. 3 it can be seen that LPS-stimulated peritoneal macrophages showed increased levels of desialylated LDL accumulation con-
sistent with the increase in surface expression of lectin receptors detected by the biosensor assay (Fig. 1). In two separate experiments this stimulation of uptake was 2.6- and 6-fold. In contrast, AcLDL accumulation was decreased after LPS-stimulation as previously reported [24,25], presumably due to lowered scavenger receptor expression, and native LDL uptake was not changed by LPS-stimulation (Fig. 3).

3.5. Transfection of ECV304 cells with MMGL

Three lines of evidence suggest that MMGL might be involved in the uptake of desialylated LDL leading to atherosclerosis: (a) macrophages after inflammatory stimulation express a lectin which has been tentatively identified as MMGL; (b) these macrophages accumulate desialylated, but not native LDL; and (c) MMGL is specifically induced in rat cardiac allografts with atherosclerosis [26]. To test this hypothesis, a cDNA fragment encoding the open reading frame of mouse MMGL from a macrophage-like cell line, RAW 264.7 was cloned and used to transfect ECV 304 cells, an immortalised cell line derived from human umbilical vein endothelial cells (HUVEC). The 3’ oligonucleotide used to amplify the MMGL cDNA encoded the FLAG epitope, to provide a convenient tag for immunolocalisation. As a result, the C-terminus of the receptor contained 8 extra amino acid residues. The tag was located in the extracellular domain, MMGL being a type II transmembrane glycoprotein. For transfection experiments ECV 304 cells were used, as these cells have low endogenous MMGL-like activity as measured by binding of [125I]ASF (see below).

To verify that the FLAG-tagged protein expressed in the MMGL transfected cells was normally processed, MMGL-transfected and untransfected ECV 304 cells were solubilised in Laemmlie sample buffer, separated the proteins by SDS-polyacrylamide gel electrophoresis (PAGE) (12.5%) and electrotransferred onto nitrocellulose. The recombinant MMGL-FLAG was identified using the monoclonal anti-FLAG antibody followed by anti-mouse Ig-HRP and compared to the MMGL band detected in RAW cells using an anti-peptide antiserum followed by anti-rabbit Ig-HRP. The size of the bands corresponding to the MMGL-FLAG and MMGL were approximately 39 kDa (data not shown). The control ECV cells did not show any bands with either antibody.

The level of expression of MMGL in the cloned, transfected cell-lines was assessed by indirect immunofluorescence using a monoclonal anti-FLAG antibody. In Fig. 4A a strong surface and intracellular punctate staining was observed in cells fixed in formaldehyde and permeabilised in 0.2% Triton X-100. When formaldehyde fixation in the absence of Triton was used, only the surface staining remained (Fig. 4B) confirming that the punctate staining arose from intracellular vesicles. In order to verify that the surface receptor was capable of being internalised cells were incubated with anti-FLAG monoclonal at 4°C, washed, and then either fixed with methanol (Fig. 4D) or placed in fresh media and incubated at 37°C for 1 h, followed by fixation with methanol (Fig. 4E). Untransfected cells showed no fluorescence at comparable exposures (Fig. 4C). It can be seen that surface MMGL receptor tagged with monoclonal anti-FLAG antibody is capable of being internalised in the transfected cells.

3.6. Binding, association and degradation of [125I]ASF

To determine if the MMGL receptor was functional in the transfected cells, the surface binding, uptake into endosomal compartments, and release of low molecular weight degraded [125I]ASF were measured. For surface binding, the cells were incubated with [125I]ASF for 2 h at 4°C (Fig. 5A). The transfected cells (solid bars, Fig. 5A) showed between 2.5-fold ($P = 0.04$) (Fig. 5A, bars 1 and 3) and 3-fold ($P = 0.01$) increase in surface binding in two separate experiments compared to untransfected cells. Adding 100-fold excess unlabelled ASF decreased binding of [125I]ASF by 60% ($P = 0.06$) (Fig. 5A, bars 1 and 2) and 30% ($P = 0.0002$) in these experiments, respectively, reducing binding to levels similar to those seen in untransfected cells (Fig. 5A, bars 3 and 4).

The total cell associated counts were also measured after incubating cells with [125I]ASF for 2 h at 37°C, as an indication of the ability of cells to internalise ligand into endocytic compartments (Fig. 5B). After 2 h incubation with ligand, endosomal pathways are saturated but little lysosomal degradation has occurred. In transfected cells (solid bars, Fig. 5B), total cell associated radioactivity increased nearly 10-fold ($P < 0.0001$) com-
Degradation of $^{125}$IASF in transfected and untransfected cells was estimated by measuring the release of TCA soluble radioactivity into the medium after incubating cells with $^{125}$IASF for 18 h at 37°C (Fig. 5C). In medium incubated with transfected cells, the level of degradation products was increased 3-fold ($P < 0.0001$) (Fig. 5C, bars 1 and 3) and the increase was abolished by competition with 100-fold excess of non-radioactive ASF (Fig. 5C, bar 2). Similar results were obtained in four separate experiments.

### 3.7. Binding and uptake of ASF by indirect immunofluorescence

In Fig. 5 it can also be seen that competition with an excess of non-radioactive ASF caused a significant decrease in the binding, uptake and degradation of $^{125}$IASF by untransfected ECV304 cells, suggesting that also some endogenous receptors for ASF are present on ECV cells. ECV304 cells were chosen for the transfection experiments because they have low levels of endogenous Gal lectin activity compared to other cells (e.g. CHO cells, data not shown). To confirm that the increased binding and internalisation of ASF in the transfected cells compared to the untransfected was due to the expression of functional MMGL, cells grown on coverslips were incubated with biotinylated ASF and anti-FLAG antibody for 1 h at 37°C in the presence of chloroquine to inhibit lysosomal degradation. The cells were then fixed with methanol, and labelled with goat-anti-mouse Ig-rhodamine and streptavidin–fluorescein, so the ligand and the antibody could be monitored in the same cells using different excitation and emission wavelengths. In Fig. 6 it can be seen that the cells taking up large quantities of biotinylated ASF (Fig. 6A), are the same cells that show high levels of staining for the lectin receptor (Fig. 6B).

Fig. 4. Immunofluorescence of positive transfected cells showing surface and/or internal staining. Cells were fixed in (A) 3% paraformaldehyde ± 0.2% Triton X-100 and (B) in 3% paraformaldehyde without Triton X-100, then incubated with anti-FLAG antibody followed by goat-anti-mouse Ig-rhodamine showing the intracellular and surface steady state distribution of the lectin, respectively; (C) labelling of control (untransfected) ECV 304 cells with goat-anti-FLAG antibody bound at 4°C followed by methanol fixation; (D) labelling of positive transfected cells with goat-anti-mouse Ig-rhodamine after anti-FLAG antibody binding at 4°C for 1 h followed by methanol fixation; and (E) anti-FLAG antibody uptake at 4°C for 1 h followed by incubation at 37°C for 1 h in fresh medium, methanol fixation and labelling with goat-anti-mouse Ig-rhodamine as described in Section 2.
3.8. Binding, association and degradation of $[^{125}\text{I}]$NT-LDL

All LDL particles isolated from plasma are recovered in a partially sialylated state [13] however very few particles contain apo B-100 with biantennary oligosaccharides in their di-desialylated form [4]. The Gal-specific lectins have 1000-fold lower $k_d$ for di-desialylated biantennary oligosaccharides (i.e. with two exposed Gal residues) than for mono-desialylated oligosaccharides (with only one exposed Gal) [6]. Therefore, to convert native LDL into a higher affinity ligand for MMGL, freshly isolated LDL was treated with neuraminidase bound to agarose beads for 4 h. This time was sufficient to remove most of the apo B-bound sialic acid and to generate di-desialylated oligosaccharides. Fig. 7 shows FACE analysis of neutral oligosaccharides isolated from LDL protein after treatment with neuraminidase for various times. The di-desialylated oligosaccharide was identified by its mobility compared to a glucose ladder and a standard A0 (Fig. 7A, last lane), generated by mild acid hydrolysis of mono- and di-sialylated oligosaccharides from apo B. The degree of desialylation of LDL at each time point was estimated from the ratio of the intensities of the di-desialylated oligosaccharide band to one of the high mannose chains, Man$_8$GlcNAc$_2$ (Fig. 7B). It has been found that longer treatment with neuraminidase does not remove any additional sialic acid and is detrimental, as it causes LDL to aggregate and precipitate.

Purified NT-LDL was iodinated and incubated with the transfected and untransfected cells to determine the role of MMGL in the binding, uptake and degradation of desialylated LDL. Prior to the experiments, cells were grown for 24 h in medium containing 10% FCS supplemented with 0.1 mg/ml human LDL to ensure that the LDLR was fully downregulated.

Binding of $[^{125}\text{I}]$NT-LDL at 4°C to MMGL-transfected cells (solid bars, Fig. 8) was increased 21% (Fig. 8A, bars 1 and 3) and 65% compared to untransfected cells in two separate experiments. Addition of 100-fold excess unlabelled ASF caused the binding of $[^{125}\text{I}]$NT-LDL in the transfected cells to decrease to levels similar to untransfected cells (Fig. 8A, bars 1 and 2), suggesting that the lectin receptor is responsible for some binding of $[^{125}\text{I}]$NT-LDL. However, the majority of the binding was due to residual LDLRs as excess LDL reduced binding in all cells by 50–80%. The reason for the unexpectedly high level of LDLR binding might be its higher affinity at 4°C compared to 37°C [27].

Internalisation of NT-LDL into the endocytic pathway was measured as before by the association of ligand with cells after 2 h incubation at 37°C. In MMGL-transfected ECV cells a small increase in cell associated $[^{125}\text{I}]$NT-LDL was observed compared to untransfected cells (Fig. 8B, bars 1 and 3). Adding a
100-fold excess of unlabelled ASF, reduced cell associated counts in transfected cells by 36% (Fig. 8B, bars 1 and 2) and 80% in two separate experiments indicating a role for MMGL rather than the LDLR in this internalisation. Addition of excess of unlabelled LDL substantially reduced the cell associated radioactivity (Fig. 8B, bars 1 and 5) indicating that over half of the cell associated radioactivity was due to uptake by LDLRs.

Degradation of [¹²⁵I]NT-LDL in MMGL transfected cells, measured by TCA soluble release of radioactivity into the medium over 18 h, was increased by 30% (Fig. 8C, bars 1 and 3) and 15% in two separate experiments compared to the untransfected cells (Fig. 8C, bars 1 and 3). This increase was abolished by competition with 100-fold excess of non-radioactive ASF, reducing it to levels similar to those observed using untransfected cells (Fig. 8C, bars 1 and 2). Addition of unlabelled LDL still caused a reduction in detected counts (30–50% in Fig. 8C, bars 5–8). The smaller effect of LDL in these longer-term experiments is presumably due to consumption of the LDL during the experiment.

Although a trend was observed in Fig. 8 suggesting a role for Gal-specific lectins in the internalisation of desialylated LDL, the P-values for increased binding, uptake and degradation of [¹²⁵I]NT-LDL in transfected compared to untransfected cells were not significant (P = 0.07 to P = 0.29).

4. Discussion

It has recently been shown that uptake of desialylated LDL by mouse peritoneal macrophages could be competed by ASF, suggesting the involvement of a galactose-specific receptor [9]. Several lectins have been described in activated macrophages where they are thought to take part in the recruitment of macrophages during inflammatory reactions [28]. The Gal/GalNAc-
specific lectin is a type II membrane spanning protein with homology to the hepatic asialoglycoprotein receptor (ASGPR) [21,29]. It is induced in arterial allografts and by TG-elicitation of macrophages, but is not expressed significantly in other tissues or in bone-marrow derived macrophages stimulated with a variety of cytokines [26].

It has been shown that functional Gal-binding receptors are induced in TG-elicited macrophages and by LPS-activation of mouse peritoneal macrophages. An anti-peptide antibody raised in rabbits was able to detect a 39 kDa protein after both TG-elicitation and LPS-activation, suggesting that the mouse macrophage Gal/GalNAc-specific lectin was indeed present, although the action of other lectins as well can not be ruled out. PI elicitation, which did not result in elevated lectin expression by the biosensor assay, did not give a cross-reacting band of this mobility.

In LPS-activated macrophages, but not in TG-elicited macrophages, we have demonstrated increased desialylated LDL uptake, presumably because the lectins induced in TG-elicited macrophages are not accessible at the cell surface. This would not be surprising in view of the large amount of phagocytosis occurring in TG-elicited cells and the similar reports of latent receptors by other groups [30,31]. The receptor responsible for internalisation of desialylated LDL was not the scavenger receptor, since LPS-stimulation of macrophages caused an increase in cholesterol ester accumulation from desialylated LDL whilst inhibiting the uptake of acetylated LDL in the same cells. It has been previously shown that the scavenger receptor is down regulated under these conditions [24,25]. In addition, previous reports that desialylated LDL might be taken up by scavenger receptors [9] are unlikely to be true, as scavenger receptors normally take up ligands with increased negative charge [32] whereas desialylated LDL has lost acidic sialic acid residues.

In a previous study [33] it was shown that high concentrations of LPS (> 10 μg/ml) could cause an increased uptake of LDL, suggesting an increase in the expression of the LDLR. At a concentration of 1 μg/ml, however, no increase of LDL uptake was observed in agreement with our data (Fig. 2). Funk et al. [34] have reported an increase in LDL uptake at LPS concentrations as low as 1 μg/ml, but the LDL was not characterised for degree of desialylation, and direct measurements of receptor concentration were not reported. In this study it was possible to measure an upregulation of the Gal/GalNAc-specific lectin after treatment with 1 μg/ml LPS concomitant with an increase in cholesterol-ester accumulation after incubation of mouse peritoneal macrophages with NT-LDL. The lack of increase in LDL uptake makes it most probable that the increased uptake of desialylated LDL was via the lectin receptor.

Transfection of ECV cells with DNA encoding for MMGL resulted in a cell line expressing high levels of surface Gal/GalNAc-specific lectin. This lectin was...
shown to act as a receptor capable of binding cell surface ligand and delivering it to lysosomes by immuno-fluorescence detection of biotinylated ASF uptake, and by the increased binding, uptake and degradation of [125I]ASF in MMGL transfected cells compared to untransfected control. By using desialylated LDL as ligand a small, but non-significant metabolism of desialylated LDL by MMGL receptors that is independent of the LDLR has also been demonstrated.

Although the LDLR was downregulated by growing cells in high-serum media, it was estimated from the amount of LDL bound at 4°C that approximately 4–7000 LDLRs remain on the cell surface. A similar calculation using the 4°C binding of ASF suggests that approximately 100,000 MMGL are present per cell. Although these figures are both underestimates (since one has not extrapolated to infinite ligand concentration using a Scatchard analysis) they give a measure of the relative numbers of these two receptors on the transfected cells. Despite the excess of MMGL receptors the LDLR is still able to compete for a substantial portion of desialylated LDL binding due to its low dissociation constant (2 × 10⁻⁹ M [27]). Binding of glycoproteins to the Gal-specific lectins depends strongly on the structure of the carbohydrate chains: mono-, bi-, tri- and tetraantennary Gal-terminal oligosaccharides bind with increasing affinities, with dissociation constants of 10⁻³, 10⁻⁶, 5 × 10⁻⁹ and 10⁻⁹ M, respectively [6]. The kₐ of MMGL for the bi-antennary di-desialylated oligosaccharides present in NT-LDL, therefore, would be ca. 10⁻⁶ M, over 500 times less than that of LDL for the LDLR.

In the experiments only 0.03 μM NT-LDL was added, of which approximately 85% (0.025 μM) contained di-desialylated biantennary oligosaccharides, thus the concentration of ligand for the MMGL would be much less than its kₐ. LDL in serum is present at a 64-fold higher concentration (ca. 1.6 μM [35]) and in atherosclerotic lesions, desialylated LDL may be specifically retained through interactions with proteoglycans and other matrix components [12]. Under these conditions the uptake by the Gal-specific lectin will be increased since it has a high kₐ, while that of the LDLR will remain constant. It has been shown that MMGL is drastically and specifically upregulated in rat cardiac allografts with atherosclerosis, suggesting that this receptor might be a marker for chronic rejection [24]. The hypothesis was that this receptor might also account for lipid accumulation if it could bind to circulating desialylated LDL. The data demonstrate that relatively little desialylated LDL is taken up by a Gal-specific lectin at concentrations achievable in tissue culture experiments due to the low affinity of these receptors. It remains a possibility, however, that a human orthologue of MMGL might contribute to atherosclerosis in humans in the microenvironment of the arterial intima.

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