Identification of β1,4-galactosyltransferase 1 as a novel receptor for IgA in human mesangial cells

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ABSTRACT

IgA nephropathy is characterised by mesangial deposition of IgA, mesangial cell proliferation and extracellular matrix production. Mesangial cells bind IgA but the identity of the receptors involved remains incomplete. The transferrin receptor (CD71) acts as a mesangial cell IgA receptor and expression is upregulated in many forms of glomerulonephritis, including IgAN. CD71 is not expressed in healthy glomeruli and blocking CD71 does not completely abrogate mesangial cell IgA binding. We have shown that mesangial cells express a receptor that binds the Fc portion of IgA and now report that this receptor is an isoform of β-1,4-galactosyltransferase. A human mesangial cell cDNA library was screened for IgA binding proteins and β-1,4-galactosyltransferase identified. Cell surface expression of the long isoform of β-1,4-galactosyltransferase was shown by flow cytometry and confocal microscopy and confirmed by immunoblotting. IgA binding and IgA-induced mesangial cell phosphorylation of spleen tyrosine kinase and IL-6 synthesis were inhibited by a panel of β-1,4-galactosyltransferase-specific antibodies suggesting IgA binds to the catalytic domain of β-1,4-galactosyltransferase. Glomerular β-1,4-galactosyltransferase expression was increased in IgAN. We propose that β-1,4-galactosyltransferase is a constitutively expressed mesangial cell IgA receptor with an important role in both mesangial IgA clearance and the initial response to IgA deposition.

Keywords: lgA nephropathy, glomerulonephritis, mesangial cells

INTRODUCTION

IgA nephropathy (IgAN) is characterised by mesangial IgA deposition, often accompanied by a mesangial proliferative glomerulonephritis, and is the commonest pattern of primary glomerulonephritis in all countries where renal biopsy is widely practiced.[1] IgA deposition is not necessarily an irreversible phenomenon as it is believed that the mesangium is normally capable of clearing finite amounts of IgA and that processes distinct from IgA deposition are necessary for the development of glomerulonephritis. [2-4]

The principal pathway for mesangial IgA clearance is mesangial cell (MC) receptor-mediated endocytosis and catabolism. [5 6] In most instances IgAN is not associated with a significant glomerular cell infiltrate suggesting that glomerular injury is mediated predominantly through IgA-induced activation of MC and local complement activation. Cross-linking of IgA receptors elicits proliferation and a pro-inflammatory and pro-fibrotic phenotypic transformation in MC. [7-10] Furthermore, exposure to IgA has been shown to result in phosphorylation of a number of key intracellular kinases, including spleen tyrosine kinase (SYK).[11 12] IgA is also capable of altering MC-matrix interactions by modulating integrin expression and this may have an important role in remodelling of the mesangium following glomerular injury. [13]

Human MC (hMC) express at least one IgA receptor, the transferrin receptor (CD71), but not the other IgA receptors thus far characterised.[14-20] CD71 is overexpressed by hMC and in glomeruli of patients with IgAN.[16 21 22] Cross-linking of CD71 by IgA, in concert with the enzyme transglutaminase 2, results in hMC proliferation and secretion of IL-6 and TGF-β.[8 23] CD71 is ubiquitously expressed by dividing cells and it remains unclear whether CD71 is involved in the initiation of glomerular injury or rather amplifies existing hMC activation and glomerular injury in IgAN.[24 25] CD71-

specific antibodies and transferrin only partially inhibit the binding of IgA to hMC supporting the concept that hMC express at least one other type of IgA receptor.[12 16 21] There is preliminary evidence suggesting that this receptor could be an asialoglycoprotein receptor [26], an Fc α/μ receptor [27], an integrin IgA receptor [28] and/or an FcαR.[15]

Here we report that β-1,4-galactosyltransferase 1 (β-1,4-GalT1) can act as an IgA receptor, is expressed by hMC and is found *in vivo* in the glomeruli of healthy and diseased kidneys and that mesangial expression appears increased in IgAN.

RESULTS

hMC expression library screening

3.22 x 10^5 cfu from a hMC expression library were screened for their ability to increase IgA binding to HEK 293 cells. Background IgA binding to HEK 293 cells was observed which was believed due to CD71 expression. Clone 5S increased IgA-FITC binding to a level equivalent to that seen with the positive control, a clone containing CD89 sequence (Figure 1). Sequencing revealed clone 5S contained 936bp of cDNA sequence coding for the catalytic region of human β-1,4-GalT1 (Figure 2).

β-1,4-GalT1 mRNA and protein expression by hMC

The two mRNA species of β-1,4-GalT1 were amplified from cultured hMC and sequencing confirmed the presence of both the long (cell membrane) and short form (Golgi apparatus) of β-1,4-GalT1 (Figure 3). Immunoblotting of hMC, lysates and cell membranes confirmed the presence of translated long and short forms of β-1,4-GalT1 (Figure 4). A 58 KDa protein corresponding to the short form of β-1,4-GalT1 was seen with total hMC lysate and 9335 (Figure 4A) but not the cell membrane fraction (blot not shown). [29] A 74 KDa protein, consistent with expression of a

glycosylated cell surface variant of β-1,4-GalT1 was best seen with hMC cell membrane fractions and 9286 (Figure 4B).[30]

Mesangial cell surface expression of β1,4 GalT1

Cell surface expression of β-1,4-GalT1 was demonstrated in each of the primary hMC lines studied (n=5) with each of the β-1,4-GalT1-specific antibodies: 5G4 (Figure 5), 1H11 and 1B6 (data not shown). Confocal microscopy of permeabilised hMC stained with 9335 revealed localisation of β-1,4-GalT1 to both the perinuclear region, in association with the Golgi apparatus, and the cell surface of the primary processes (Figure 6A). This pattern of β-1,4-GalT1 expression is identical to that seen in other cells [31]. In non-permeabilised cells β-1,4-GalT1 staining was limited to the cell membrane consistent with expression of the surface expression of the long form of β-1,4-GalT1 (Figure 6B).

Inhibition of IgA binding to hMC by β-1,4-GalT1-specific antibodies and soluble human β-1,4-GalT1

IgA binding to hMC was inhibited both by pre-incubation of IgA-FITC with soluble human β-1,4-GalT1 (Figures 7A & B) and blocking of the catalytic site of cell surface β-1,4-GalT1 (the presumed IgA binding domain of the protein) with 9335 and 1H11 (Figures 7A & C). This demonstrated that soluble β-1,4-GalT1 can compete with cell surface β-1,4-GalT1 to bind IgA and that blocking the catalytic site of β-1,4-GalT1 blocks access of IgA to its ligand binding domain. IgA binding was not completely blocked, supporting previous findings that hMC express more than one IgA receptor.

Inhibition of IgA-induced hMC activation by β-1,4-GalT1 and CD71 specific antibodies

IgA1-induced IL-6 release was significantly reduced with 3191, 3192 and Sc17299 (only data for 3192 shown), which bind to different epitopes in the catalytic region of β-1,4-GalT1, but not with Sc22277 which binds to the stem region of β-1,4-GalT1 (Figure 8A). Inhibition of IL-6 synthesis was not complete, consistent with co-expression of an additional hMC IgA receptor. Expression of CD71 was confirmed as blocking of IgA1-CD71 binding also resulted in partial inhibition of IL-6 synthesis. There was no increase in inhibition of IL-6 release when CD71/β-1,4-GalT1 dual blockade was performed implying that at least one other functionally distinct IgA receptor is likely to exist and that CD71 and β-1,4-GalT1 may share intracellular signalling pathways. To investigate this latter possibility IgA-induced SYK phosphorylation was measured. This was reduced by inhibition of both IgA1-CD71 and IgA1-β-1,4-GalT1 binding (Figure 8B).

Glomerular expression of β-1,4-GalT1 in human kidney

Low level mesangial expression of β-1,4-GalT1 (*) was seen by immunofluorescence (IF) and immunohistochemistry (IHC) in kidneys without evidence of glomerular disease (Figure 9A & B); thin membrane nephropathy (Figure 9C & D) and membranous nephropathy (Figure 9E & F) consistent with the constitutive expression of β-1,4-GalT1 by mesangial cells *in vivo*. Mesangial β-1,4-GalT1 staining was increased in IgAN (Figure 9G, H & I). In parallel, glomeruli were isolated by laser capture microdissection (LCMD) and the presence of mRNA for the long form of β-1,4-GalT1 was confirmed by RT-PCR and sequencing (data not shown).

DISCUSSION

Studies examining IgA binding to hMC and IgA-induced hMC activation acknowledge that IgA receptors apart from the transferrin receptor (CD71) must exist.[16 21 32] Importantly, glomerular CD71 expression has not been found in healthy

subjects, expression *in vivo* is limited to glomeruli showing active inflammation, and is not restricted to IgAN.[22] This is consistent with CD71 being ubiquitously expressed by proliferating cells.[24 25] It is unlikely therefore that CD71 plays a role in either constitutive mesangial IgA clearance or the initiation of glomerular injury in IgAN. Rather CD71 is likely to have a role in amplifying glomerular injury once MC activation has occurred. We and others have reported the presence of novel hMC IgA receptors but the nature of these receptors has until now remained unknown. [5 17 18 33] Through successive rounds of hMC cDNA library screening we have identified the *N*-glycosylating enzyme β-1,4-GalT1 as a novel IgA receptor and demonstrated its expression in normal and diseased glomeruli. There have been reports of β-1,4-GalT1 acting as a cell surface receptor for IgA on a number of different cell types [34-36]. It is therefore not unreasonable to propose that β-1,4-GalT1 similarly functions as a hMC IgA receptor.

β-1,4-GalT1 is one of at least seven polypeptides that catalyse the transfer of galactose from the sugar nucleotide donor uridine 5′-diphospho-galactose (UDP-Gal) in a β1,4 linkage to glycoside residues with a terminal *N*-acetylglucosamine (GlcNAc) moiety.[37] The β-1,4-GalT1 gene is unusual in that it encodes two protein isoforms that differ in their cytoplasmic domains. These two isoforms are generated by differential translation initiation from two in-frame translation initiation sites.[38 39] The long β-1,4-GalT1 isoform consists of 399 amino acids, whereas the short isoform, a product of a downstream AUG translation initiation site, lacks 13 amino acids at the *N* terminus.[38 40] The two isoforms have overlapping but distinct biological properties. Both isoforms are able to function during glycoprotein processing in the Golgi complex but only the long isoform, with its additional 13 amino acid cytoplasmic sequence, is able to function as a signalling type II cell membrane receptor [31 41-44] where it plays important

roles in cell-to-cell and cell-to-extracellular matrix interactions as well as cell activation and proliferation.[45 46 47 48]β-1,4-GalT1 is not unique in having multiple subcellular distributions, there is evidence for cell surface expression of other glycosyltransferases including an *N*-acetylgalactosaminyl transferase,[49] a sialyltransferase,[50] and two fucosyltransferases,[51 52] all of which participate in cellular interactions by binding to ligands on other cells or in the extracellular matrix. Cell surface expression of β-1,4-GalT1 is characteristically increased on the leading edge of a number of cell types during cell adhesion where it facilitates cell spreading and migration through an interaction with extracellular matrix components and an association with the cytoskeleton.[53 54] Consistent with these studies hMC surface expression of β-1,4-GalT1 was concentrated in lamellipodia.[41 44 53 54]

Cell surface β-1,4-GalT1 functions as a lectin-like receptor owing, in part, to the absence of UDP-galactose, which enables low affinity, stable binding between β-1,4-GalT1 and appropriate glycoside ligands on glycoproteins in the extracellular environment.[46] Our data suggests that β-1,4-GalT1 binds IgA at its catalytic site through recognition of IgA GlcNAc residues. This is supported by the observations that the original clone identified during library screening contained the entire coding sequence for the catalytic region of β-1,4-GalT1 and that only antibodies specific to this region of β-1,4-GalT1 are capable of blocking IgA binding to hMC and IgA1-induced IL-6 synthesis. IgA1 contains two conserved *N*-glycosylation sites in each α-chain Fc region (Asn^{263} and Asn^{459}) and IgA1 Fab *N*-glycosylation has also been reported.[55] Precise definition of the Fc *N*-linked glycans of IgA1 is lacking but available data suggests they are predominantly of the biantennary complex type with a core fucose, bisecting GlcNAc, or both.[55 56] β-1,4-GalT1 mediated hMC binding of

IgA through Fc located *N*-glycans is consistent with our and others previous findings of Fcα dependant binding and activation of hMC.[15 17 33 57]

Ligand-induced aggregation of β-1,4-GalT1 results in a range of cell-type specific intracellular signal cascades. Similar to integrins β-1,4-GalT1 associates with the cytoskeleton and evidence suggests that the scaffolding function of Src Suppressed C Kinase Substrate (SSeCKS) serves to orchestrate some of the signaling and cytoskeletal functions associated with β-1,4-GalT1.[58] Cross-linking of surface β-1,4-GalT1 in fibroblasts results in tyrosine phosphorylation of focal adhesion kinase (FAK) and disorganisation of actin stress fibres[44]. There is evidence supporting SSeCKS as a downstream mediator of protein kinase C-controlled actin-based cytoskeletal architecture in mesangial cells.[59] Furthermore, expression of tensin, a focal adhesion protein known to associate with FAK and vinculin, is increased in areas of mesangial expansion in IgAN suggesting that the expression of tensin is associated with extracellular matrix production.[60] We have previously shown that IgA induces phosphorylation of SYK in hMC.[11] In this paper we show that inhibiting IgA-CD71 and IgA-β-1,4-GalT1 binding inhibits IgA-induced SYC phosphorylation, suggesting that both receptors may share intracellular signalling pathways. This intracellular crosstalk may explain why we were unable to show an additive effect of dual receptor blockade in our *in vitro* system. The SYK inhibitor fostamatanib is currently in Phase II trials in IgAN (ClinicalTrials.gov Identifier: NCT02112838) and it is plausible that one potential therapeutic target of this drug could be IgA-β-1,4-GalT1 (and IgA-CD71) induced hMC activation.

β-1,4-GalT1$^{-/-}$ mice show semilethality before weaning, however, those mice that survive develop an IgAN-like disease associated with elevated levels of circulating IgA immune complexes and mesangial IgA deposition, mesangial matrix expansion,

and electron-dense deposits in the paramesangial regions. [61-64] One possible explanation for this phenotype is that loss of cell surface β-1,4-GalT1 severely impairs IgA clearance pathways both in and out of the kidney resulting in persistence of IgA immune complexes in the circulation and impaired mesangial IgA clearance. This currently, however, remains untested.

There is clearly now a need to better understand the functional role of cell surface β-1,4-GalT1 in hMC, its importance in maintaining glomerular homeostasis and, in particular, determining how hMC interact with the mesangial matrix and proteins, such as IgA, in health and disease.

METHODS

Antibodies

β-1,4-GalT1-specific antibodies were kind gifts from Prof Barry Shur (Emory School of Medicine, Atlanta, USA; 9335 and 9286) and Dr Peter Delves (UCL, London; 5G4, 1B6, 1H11). In addition, rabbit polyclonal antisera was raised against the catalytic domain of β-1,4-GalT1 (3191 and 3192) and two monoclonal antibodies obtained from Santa Cruz Biotechnology (Sc17299 and Sc22277). The binding domain for each antibody is shown in Figure 2. FITC labelled human serum IgA and goat anti mouse-FITC $F(ab)_2$ were from Perbio Science UK Ltd. Goat anti mouse IgG-Cy3 and FITC-streptavidin were from Zymed. Biotinylated anti rabbit IgG was from Vector Laboratories.

Cell lines

Human embryonic kidney cells (HEK 293) were obtained from Invitrogen Life Technologies and primary cultures of human mesangial cells (hMC) were isolated as described previously. [15] Clonetics™ mesangial cells were obtained from Lonza, UK. All experiments were performed on hMC between passages 5 and 9. All cells were

maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and detached using an enzyme-free cell dissociation buffer (Gibco®).

Amplification of the cDNA library and isolation of clone DNA

The hMC cDNA expression library was synthesised using mRNA isolated from hMC and the expression vector pCMVSPORT 6, the clones had been electroporated into E.coli EMDH10B resuspended in 80% SOC/20% glycerol and stored at -80°C. 1µl of the library stock was diluted in SOC to a final concentration of 3.22 x 10^5 cfu/ml. 50µl of this suspension was spread over each of twenty 150mm Luria agar plates containing ampicillin (50µg/ml). Plates were incubated at 37°C until the appearance of small colonies. Colony samples were lifted from the plates using nylon filters (Amersham Pharmacia). Plates were incubated at 37°C for 4 hours to allow the colonies to regrow before being stored at 4°C. Clones from each filter were scraped into 6ml Luria broth containing ampicillin (50µg/ml) and incubated at 37°C, with shaking, for 4 hours. Plasmid DNA from each culture was isolated using S.N.A.P. Miniprep Kit (Invitrogen Life Technologies).

Transient transfection and expression of clones from the cDNA library in HEK 293 cells

HEK 293 cells were chosen as the transfection recipients for library screening as they had been shown capable of similar post-translational modification of synthesised proteins as adult mesangial cells. This was considered important as the glycosylation of any putative receptor may play an important role in binding to human IgA1. Cells were seeded in 150cm^2 flasks at 70% confluency and transfected with library DNA using Lipofectamine (Invitrogen Life Technologies) as per the manufacturer's instructions. Transfected cells were incubated at 37°C, 5% CO_2 for 48 hours to allow expression of the cDNA insert.

Screening of transfected HEK 293 cells for IgA binding by flow cytometry

Transfected cells were detached and suspended in 5ml of media and incubated at 37ºC, 5% CO_2 for 30 minutes. Cells were washed with ice-cold PBA (phosphate buffered saline, 0.1% bovine serum albumin, 0.01% sodium azide), divided between 2 tubes, centrifuged at 1,000 rpm for 5 minutes, at 4ºC and incubated on ice in the dark with either 50µl PBA or IgA-FITC (50µg/ml) for 45minutes. Cells were then washed in ice-cold PBA and suspended in PBA with propidium iodide (PI, Molecular Probes, 5µg/ml). For each round of transfectant screening a positive (HEK 293 cells transfected with cDNA for CD89) and negative (HEK 293 cells transfected with empty plasmid) control were included.

Cell-associated fluorescence intensity was evaluated using a FACScan flow cytometer coupled to CellQuest software (Becton Dickinson). Dead cells were live gated out by PI staining and a minimum of 10^4 cells were analysed. Cell-associated fluorescence is expressed either as arbitrary units of median channel fluorescence (MCF), where the primary antibody was unconjugated, or molecules of equivalent soluble fluorescein (MESF) when IgA-FITC was used. For MESF a linear regression analysis of mean fluorescence intensity versus fluorescein molecules per particle was performed using quantitative fluorescein microbead standards (Becton Dickinson), and the resulting formula was used to determine MESF [65].

Repetitive screening of the hMC cDNA library for IgA binding clones

Reference plates containing clones exhibiting the greatest IgA binding when expressed in HEK 293 cells were progressively subdivided and plasmids from each section transfected into HEK 293 cells and screened by flow cytometry. This was repeated until it was possible to isolate single colonies from the reference plates. At

this point plasmid DNA from single clones was transfected into separate flasks of HEK 293 cells and IgA binding confirmed by flow cytometry.

Sequencing

Sequencing of IgA-binding clones and β-1,4-GalT 1 transcripts was performed by The Protein and Nucleic Acid Laboratory (University of Leicester) using an Applied Biosystems 3730 automated sequencer. Sequences were compared with the NCBI database using the Basic Local Alignment Search Tool (BLAST).

Identification of β-1,4-GalT1 mRNA in hMC

RNA from hMC was isolated using RNA 4-pcr kit (Ambion) and cDNA synthesised using the Reverse Transcriptase System (Promega). cDNA coding for human β-1,4-GalT 1 was amplified using the following primers (3'ATGAGGCTTCGGGAGCCGC 5' (PRIMER A) to amplify the long transcript; 3'ATGCCAGGCGCGTCCCTACAG 5' (PRIMER B) to amplify the short transcript and 3'GACCTGAATTAGCCAGGGA 5' (PRIMER C), reverse primer for both amplicons-see Figure 2). Reaction mixtures were heated at 95ºC for 10 minutes then 40 cycles of 95ºC 30 seconds, 70ºC for 1 minute and 72ºC for 30 seconds and resolved by 1% agarose gel electrophoresis.

Identification of β-1,4-GalT1 protein expression by hMC by immunoblotting

hMC cell lysates were prepared from 75cm^2 flasks of 80% confluent hMCs as previously [66] For hMC membrane preparations hMC cell lysates were subjected to further centrifugation at 24,000rpm for 30 minutes at 4ºC. The supernatant was discarded and membrane pellet resuspended in SDS-PAGE reducing buffer.

Cell lysates and membrane fractions were resolved by SDS-PAGE under reducing conditions followed by transfer onto nitrocellulose membranes, and immunoblotted according to Towbin, Staehelin and Gordon.[67] Membranes were stained with with β-1,4-GalT1 specific antibodies (9335 and 9286) and specific antibody binding detected

using horseradish peroxidase-conjugated goat anti rabbit Igs. Immunoblots were developed using enhanced chemiluminescnce (ECL detection kit, Amersham Pharmacia).

Identification of cell surface β-1,4-GalT1 expression by hMC by flow cytometry

Confluent hMCs were detached, washed in ice-cold PBA and incubated in duplicate on ice with 10μl of β-1,4-GalT-1 specific monoclonal antibody (5G4, 1H11 or 1B6) or PBA (negative control) for 45 minutes. After two washes in ice-cold PBA hMC were incubated on ice with 10μl of goat anti mouse-FITC $F(ab)_2$ for 45 minutes. hMC were resuspended in PBA with PI and hMC surface IgA staining measured by flow cytometry. Dead cells were live gated out by PI staining and a minimum of 10^4 hMC were analysed.

Confocal microscopy for β-1,4-GalT1 expression by hMC

hMCs were cultured to 50% confluence in 8 chambered slides. After washing with PBS cells were fixed with 3.8% paraformaldehyde at RT for 15 minutes. Following three washes in PBS hMC were incubated with PBS, 3% BSA, with or without 0.2% Triton X-100 (to permeabilise the hMC cell membrane) for 1 hour at RT and then incubated overnight at 4ºC with the β-1,4-GalT1-specific 9335 diluted in blocking solution with 5% goat serum. After three washes in PBS with or without 0.2% Triton X-100 hMC were incubated with goat anti rabbit-Cy3 at RT in the dark for 1hr. hMC were then washed three times in PBS and mounted with DakoCytomation fluorescent mounting medium (Dako). The fluorescence was visualised using a Leica TCS4d laser scanning confocal microscope and Scanware.

Inhibition of IgA binding to hMC by soluble β-1,4-GalT1

hMCs were detached, washed with ice-cold PBA and incubated with 50μl PBA, IgA-FITC, IgA-FITC with 0.02-0.1 U/ml of human β-1,4-GalT 1 or IgA-FITC with 315

U/ml of lysozyme, and incubated on ice in the dark for 30 minutes. In each case IgA-FITC was at a final concentration of 50μg/ml and there had been prior mixing of the IgA-FITC with lysozyme / β-1,4-GalT1 for 30 minutes at RT before being added to hMC. After incubation hMC were washed with ice-cold PBA, resuspended in PBA with PI and hMC IgA surface staining measured. Dead cells were live gated out by PI staining and a minimum of 10^4 hMC were analysed. Experiments were performed in duplicate and on three separate occasions.

Inhibition of IgA binding to hMC by anti-β-1,4-GalT1 antibodies

hMCs were detached, washed with ice-cold PBA and incubated on ice with 10μl of the β-1,4-GalT1-specific 9335 or 1H11, rabbit serum alone or rabbit anti-cytokeratin antibody (negative control) for 30 minutes. hMC were washed with ice-cold PBA and incubated on ice with 10μl of IgA-FITC (2mg/ml) in the dark for 30 minutes. After washing with ice-cold PBA, hMC were resuspended in PBA with PI and hMC IgA surface staining measured. Experiments were performed in duplicate and on three separate occasions.

Inhibition of IgA-induced IL-6 synthesis and spleen tyrosine kinase phosphorylation in hMC by anti-CD71 and anti-β-1,4-GalT1-specific antibodies

Confluent hMCs were serum starved for 24h before being exposed for 5 minutes (SYK phosphorylation) or 48h (IL-6 synthesis) to IgA (50μg/ml) either alone or following a 15 min pre-incubation with CD71 or β-1,4-GalT1-specific antibodies. IgA was purified, as previously described, from pooled serum samples. [15] At the end of the experiment cell lysates and tissue culture supernatants were collected and analysed for phospho-SYK by western blotting and IL-6 concentration by ELISA respectively, and the protein content of each well measured using the Bradford protein assay (Bio-Rad).

Data was expressed as relative expression of phosphor-SYK/β-actin and fold difference of IL-6 concentration per mg/cell protein over media only control.

Laser capture microdissection and glomerular expression of β-1,4-GalT1 in human kidney

Multiple sections (5μm for IF/IHC; 10 μm for LCMD) were cut from fresh frozen and paraffin embedded kidney blocks from healthy subjects (normal poles of three kidneys removed for renal cell carcinoma) and cases of thin membrane nephropathy, membranous nephropathy and IgAN. The presence of the β-1,4-GalT1 was determined by staining with 9335 by both IF and IHC as previously described. [68] All sections were treated in exactly the same way and randomly mixed in the different staining runs with the technician blind to the diagnosis. For LCMD glomeruli were located and captured using the Arcturus PixCell II LCMD system. Glomeruli from a minimum of six sequential 10 μm sections from each kidney were collected. RNA was isolated, cDNA synthesised and cDNA coding for human β-1,4-GalT 1 was amplified as described for hMC.

Statistical Analyses

Statistical analyses were performed using a two- tailed t-test. Results are expressed as the mean + SEM. A *P*-value of <0.05 was considered significant. All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

DISCLOSURES

None.

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Legends to Figures

Figure 1

IgA-FITC binding to HEK 293 cells increases after transfection with clone 5S (thick line) to a similar extent to that seen with a clone containing CD89 sequence (dotted line) and the two histograms overlap. IgA-FITC binding to HEK 293 cells transfected with an empty vector is also shown (thin line). Background IgA binding to HEK 293 cells was observed which we believe was due to expression of CD71.

Figure 2

Diagrammatic representation of the structure of both the long and short forms of β-1,4-GalT1 showing the region of the protein encoded by clone 5S, the protein domain recognised by each of the antibodies and the sites of complementarity of primers used in the studies.

Figure 3

Agarose gel electrophoresis of RT-PCR products derived from human mesangial cells. cDNA were amplified using PCR primers for both the long and short transcripts of β-1,4-GalT1. The predicted PCR products were obtained for both the long (cell membrane) and short (Golgi apparatus) forms of β-1,4-GalT1 mRNA and identities confirmed by sequencing.

Figure 4

Human mesangial cell lysates were immunoblotted with the β-1,4-GalT1-specific 9286, which recognises the 13 amino acid tail of the long protein, and 9335 which recognises the catalytic region of β-1,4-GalT1. A 58 kDa band consistent with the short form of β-1,4-GalT1 was observed with 9335. As the short form of β-1,4-GalT1 represents over 90% of total cellular β-1,4-GalT1 the long form of β-1,4-GalT1 is barely seen at 74 KDa at this exposure. Human mesangial cell membranes from 2

different donors were immunoblotted with 9335 and 9286 and 74 KDa bands consistent with the long form of β-1,4-GalT1 were observed (immunoblot with 9286, an antibody specific for the long form of β-1,4-GalT1 is shown) .

Figure 5

Human mesangial cells were stained with a panel of β-1,4-GalT1-specific antibodies and cell surface staining measured by flow cytometry. Cell surface expression of β-1,4-GalT1 was observed with all β-1,4-GalT1-specific antibodies and shown is the cell surface staining with 5G4 (thick line). The negative control (secondary antibody only) is shown as the thin line.

Figure 6

Confocal microscopy demonstrated perinuclear localisation of β1,4 GalT1 (solid arrows) in permeabilised hMC consistent with Golgi localisation of the short form of β1,4 GalT1 (6A). Cell surface expression of β1,4 GalT1 along the primary processes of hMC was seen in both permeabilised and non-permeabilised hMC (dashed arrows, 6A and 6B). Scale bars 10μm.

Figure 7

Human mesangial cells were exposed to increasing concentrations of IgA-FITC and IgA binding was inhibited either by pre-incubation of IgA-FITC with soluble human β1,4 GalT1 or an irrelevant protein (lysozyme) or pre-incubation of human mesangial cells with β1,4 GalT1-specific antibodies or control antibodies (rabbit serum or rabbit anti-cytokeratin) (7A). Cell-associated fluorescence, as molecules of equivalent soluble fluorescein (MESF), was calculated and IgA-FITC binding calculated as a percentage of the corresponding uninhibited IgA-FITC binding. Data are expressed as means $\pm$ SEM of three different experiments performed in duplicate (* $p < 0.05$).

Representative histograms are included: 7B IgA-FITC binding to hMC (thick line), IgA-FITC binding after preincubation with human recombinant β1,4 GalT1 (thin line) and lysozyme (dotted line). 7C IgA-FITC binding to hMC (thick line), IgA-FITC binding after preincubation with 9335 (thin line) and rabbit serum (dotted line).

Figure 8

8A. Human mesangial cells were exposed to IgA1 (50 μg/ml) for 48h in the presence of a range of potential inhibitors of IgA1-β1,4 GalT1 and IgA1-CD71 binding and IL-6 synthesis measured. Inhibition of IgA1-induced hMC IL-6 synthesis was observed with antagonism of IgA1-CD71 binding and IgA1-β1,4 GalT1 (catalytic region) binding but not IgA1-β1,4 GalT1 (stem region) binding. There was no increase in inhibition of IL-6 release when IgA1-CD71 binding was combined with IgA1-β1,4 GalT1 (catalytic region) inhibition suggesting both receptors may share common intracellular signalling pathways. Data are expressed as means $\pm$ SEM of four different experiments performed in duplicate (* $p < 0.001$).

8B. Human mesangial cells were incubated with IgA1 (50 μg/ml) for 5 minutes in the presence of inhibitors of IgA1-β1,4 GalT1 and IgA1-CD71 binding and activation of spleen tyrosine kinase (SYK) measured by Western blot analysis for phospho-SYK in total mesangial cell lysates. Inhibition of IgA1-induced SYK phosphorylation, normalised to β-actin expression level, was observed with antagonism of IgA1-CD71 binding and IgA1-β1,4 GalT1 (catalytic region) binding.

Figure 9

Normal human renal cortex was stained with the β-1,4-GalT1-specific antibody 9335 and in all cases a mesangial pattern of glomerular expression of β-1,4-GalT1 was seen by immunofluorescence, confirming *in vivo* constitutive expression of β-1,4-GalT1 by hMC (A and B). Sections from 2 patients with thin membrane nephropathy (C & D)

and 2 patients with membranous nephropathy (E & F) were also stained for β-1,4-GalT1 expression and low level expression was similarly observed (1 glomerulus from each kidney biopsy shown). There was a clear increase in β-1,4-GalT1 glomerular staining in kidneys from 3 patients with IgAN (G, H, I): 1 glomerulus from each kidney biopsy shown. Some staining of proximal tubule cells, particularly of the luminal brush border, was also seen in all sections suggesting that β-1,4-GalT1 may also play a role in tubular protein reabsorption. Scale bars 100μm.