Lyso-Gb3 activates Notch1 in human podocytes

Maria D. Sanchez-Niño1,2,3, Daniel Carpio4, Ana Belen Sanz1,2,3, Marta Ruiz-Ortega1,2,3, Sergio Mezzano4 and Alberto Ortiz1,2,3,*

1IIS-Fundacion Jimenez Diaz, School of Medicine, UAM, Madrid, Spain, 2IRSIN, Madrid, Spain, 3REDINREN, Madrid, Spain and 4Unidad de Nefrología, Instituto de Medicina, Universidad Austral de Chile, Valdivia, Chile

*To whom correspondence should be addressed at: Unidad de Dialisis, IIS-Fundacion Jimenez Diaz, Av Reyes Católicos 2, 28040 Madrid, Spain. Tel/Fax: +34 915504800; Email: aortiz@fjd.es

Abstract
Podocyte injury is an early feature of Fabry nephropathy, but the molecular mechanisms of podocyte injury are poorly understood. Lyso-Gb3 accumulates in serum in Fabry disease and increases extracellular matrix synthesis in podocytes. We explored the contribution of Notch1 signaling, a mediator of podocyte injury, to lyso-Gb3-elicited responses in cultured human podocytes. At clinically relevant concentrations, lyso-Gb3 activates podocyte Notch1 signaling, resulting in increased active Notch1 and HES1, a canonical Notch transcriptional target. A γ-secretase inhibitor or specific Notch1 small interfering RNA (siRNA) inhibited HES1 upregulation in response to lyso-Gb3. Notch1 siRNA or γ-secretase inhibition also prevented the lyso-Gb3-induced upregulation of Notch1, Notch ligand Jagged1 and chemokine (MCP1, RANTES) expression. Notch siRNA prevented the activation of nuclear factor kappa B (NFκB), and NFκB activation contributed to Notch1-mediated inflammatory responses as the NFκB inhibitor, parthenolide, prevented lyso-Gb3-induced chemokine upregulation. Notch1 also mediates fibrogenic responses in podocytes as Notch siRNA prevented lyso-Gb3 upregulation of fibronectin mRNA. Supporting the clinical relevance of cell culture findings, active Notch1, Jagged1 and HES1 were observed in Fabry kidney biopsies. Lyso-Gb3 elicited similar responses in mouse kidney. In conclusion, lyso-Gb3 promotes Notch1-mediated inflammatory and fibrogenic responses in podocytes that may contribute to Fabry nephropathy.

Introduction
Fabry disease is a rare X-linked hereditary disease caused by mutations in the alpha-galactosidase gene (GLA) gene encoding the lysosomal enzyme alpha-galactosidase (1). Disease manifestations are a consequence of the intracellular and extracellular accumulation of glycosphingolipids (2). Initial symptoms develop in childhood. However, diagnosis is often delayed until potentially life-threatening organ involvement develops in the kidney, heart or central nervous system. Fabry nephropathy is a progressive proteinuric kidney disease of metabolic origin (3,4). Thus, conceptually, it is reminiscent of diabetic nephropathy, also a proteinuric kidney disease consequence of a different metabolic disorder characterized by hyperglycemia and accumulation of glucose degradation products (3). However, the precise cellular and molecular mechanisms linking glycolipid accumulation to tissue injury and disease manifestations in Fabry disease are not fully understood (5,6). The lack of an adequate animal model has hindered progress in understanding the pathogenesis and development of optimal therapy (5). Enzyme replacement therapy (ERT) is the current cornerstone of Fabry disease management (1,4,7). However, because of delayed diagnosis, ERT is frequently started once tissue injury is well advanced. In a recent series, mean time from symptoms onset to diagnosis was 20–30 years for patients with different rates of kidney disease progression, and mean age at start of ERT was 35–42 years (8). To put these figures into perspective, mean age at end-stage kidney disease and initiation of renal replacement therapy was 40 years in a larger series (9). At the start of ERT, mean proteinuria levels were clearly pathological for all groups (0.7–1.7 g/g creatinine) (8). In this regard, ERT is less efficacious in improving patient outcomes when started after tissue injury and specifically, pathological albuminuria or tissue fibrosis has developed. Proteinuria is a marker of podocyte injury and focal segmental glomerulosclerosis, a form of tissue fibrosis (10). The lower efficacy of metabolic defect
correction once target organ injury has already developed is not surprising. The therapy aimed at correcting the metabolic defect in established diabetic nephropathy does not prevent kidney disease progression, and add-on nephroprotective therapies are needed (11). Thus, ERT should be complemented by add-on therapies aimed at modifying the underlying pathogenesis of tissue injury. Targeting the renin–angiotensin system (RAS) reduces proteinuria in Fabry nephropathy and may slow the loss of renal function, whereas vitamin D receptor activators protect cultured podocytes from glycolipids and decrease albuminuria in Fabry patients (7,12–15). However, symptoms of Fabry disease such as hypertension and bradycardia may limit the tolerability of RAS inhibitors. Novel therapeutic approaches based on a better understanding of pathogenic events are needed to optimize patient outcomes. Podocyte injury is an early feature of both Fabry and diabetic nephropathy (16,17). In young Fabry patients, podocyte, but not endothelial glycolipid, deposits correlated with albuminuria and age, foot process effacement as a sign of podocyte injury is observed and higher cumulative ERT doses resulted in both decreased podocyte deposits and reduced albuminuria (16,17). Focal segmental glomerulosclerosis, a consequence of podocyte injury, is observed in Fabry disease even before albuminuria becomes pathological (18). However, the molecular mechanisms of podocyte injury are poorly understood. We hypothesized that similar to the situation in diabetic nephropathy, metabolites accumulated in Fabry disease may elicit responses in kidney cells, leading to activation of secondary mediator of tissue injury (5). Lyso-Gb3 is a circulating bioactive glycolipid accumulated in Fabry disease (19). Even after initiation of ERT, podocytes may be exposed to excess glycolipid levels: after 5 years of ERT, only some biopsies showed evidence of podocyte clearance (17). In this regard, understanding the effects of lyso-Gb3 on podocytes may provide novel therapeutic approaches to protect podocytes even in ERT-treated patients. Similar to high glucose concentrations, lyso-Gb3 increased the expression of type IV collagen and fibronectin in a transforming growth factor (TGF)-β1-dependent manner in non-Fabry cultured human podocytes (14). High glucose concentrations also directly elicit proinflammatory and lethal responses in cultured renal cells, including glomerular podocytes (20–22), and we hypothesized that lyso-Gb3 may induce a similar range of responses.

The Notch signaling pathway consists of several receptors, Notch1 through Notch4, their membrane-bound ligands of the DSL (Delta, Serrate, LAG-2) family and other non-canonical ligands (23). Upon ligand binding, Notch undergoes a series of proteolytic cleavages, and γ-secretase generates a free cytoplasmic domain protein, the Notch intracellular domain (active Notch, NICD). The NICD translocates to the nucleus and promotes transcription of target genes such as the basic helix-loop-helix protein HES. Notch1 activation causes podocyte injury and kidney fibrosis (24–26). Evidence of Notch1 activation was observed in podocytes in human diabetic nephropathy and focal segmental glomerulosclerosis (25,27), active Notch1 overexpression in podocytes caused albuminuria and progressive glomerulosclerosis in mice (25,28) and γ-secretase targeting ameliorated proteinuria in rats (25). Among the factors known to activate Notch signaling, TGF-β1 increased Notch1 activation and expression of the Notch ligand Jagged1 in cultured podocytes (25).

As both activators of Notch signaling in podocytes, such as TGF-β1, and the consequences of Notch activation (albuminuria, podocyte injury, glomerulosclerosis and kidney fibrosis) are relevant for Fabry nephropathy, we have now explored the potential of lyso-Gb3 to activate Notch1 signaling in cultured human podocytes as well as the functional consequences of Notch1 activation. Specifically, we have focused on inflammation as a poorly studied but recognized feature of Fabry disease (29–33).

**Results**

Lyso-Gb3 activates Notch1 signaling and upregulates Notch1 expression in podocytes

As cleaved Notch1 and Notch2 are considered stronger activators of canonical target gene transcription than cleaved Notch3 or Notch4 (34) and Notch1 promotes podocyte injury in vivo (25), we focused on the role of Notch1 in the podocyte response to lyso-Gb3. Notch1 was activated in cultured human podocytes exposed to 100 nM lyso-Gb3, as demonstrated by western blot for the active, cleaved form of Notch1 (Fig. 1A). This concentration of lyso-Gb3 was chosen on the basis of previously published dose-response studies when the cell culture model was set up and clinical relevance (14,19,35). Besides inducing activation of constitutively expressed Notch1, lyso-Gb3 may further contribute to Notch1 activation by increasing Notch1 expression (Fig. 1B and C). The time-course suggests that activation of constitutively expressed Notch1 precedes the upregulation in Notch1 gene expression. Moreover, Notch1 activation was followed by upregulation of the mRNA and protein expression of HES1, a canonical transcriptional target of Notch (Fig. 1D and E).

We next studied the functional relevance of Notch1 for lyso-Gb3 signaling by silencing Notch1 with specific small interfering RNAs (siRNAs) (Fig. 2A). Notch1 silencing significantly prevented the upregulation of HES1 in response to lyso-Gb3 at the protein (Fig. 2B) and mRNA (Fig. 2C) levels. Furthermore, lyso-Gb3 upregulation of the Notch ligand Jagged1 was also prevented by Notch1 silencing (Fig. 2D).

These data indicate that lyso-Gb3, a glycolipid accumulated in Fabry disease, activates Notch1 signaling in normal human podocytes, raising the possibility that Notch1 signaling may be involved in the pathogenesis of Fabry disease. However, siRNA-based therapies have not yet been implemented in the clinic. In contrast, γ-secretase inhibitors are undergoing clinical trials (36). Thus we tested the modulation of the podocyte response to lyso-Gb3 by the γ-secretase inhibitor GSI IX, a pharmacological inhibitor of Notch activation. GSI IX prevented lyso-Gb3-induced expression of the canonical Notch1 target gene HES1 at the mRNA (Fig. 3A) and protein levels (Fig. 3B). Thus, Notch1 signaling activation in podocytes in response to lyso-Gb3 may be prevented by either Notch1 siRNA or γ-secretase inhibitors.

Notch1 activation in response to lyso-Gb3 promotes a proinflammatory response in podocytes

As lyso-Gb3 was previously shown to promote a fibrogenic response in podocytes (14), we now explored the impact of lyso-Gb3 on another key process in kidney injury, inflammation. In cultured podocytes, lyso-Gb3 induced the expression of MCP-1 and RANTES mRNA, two chemokines involved in proteinuric kidney disease (Fig. 4A and B) (37,38). In order to study the role of Notch1 signaling in lyso-Gb3-induced inflammatory responses, we explored the response of inflammatory gene expression to Notch1 or γ-secretase targeting. Specific Notch1 siRNA prevented the upregulation of MCP-1 mRNA (Fig. 4C) and protein (Fig. 4D) and RANTES mRNA (Fig. 4E) in response to lyso-Gb3. GSI IX also prevented the increase in MCP-1 and RANTES mRNA (Fig. 4F and G), thus confirming that chemokine expression induced by lyso-Gb3 in podocytes is mediated by a γ-secretase-dependent process. Taken together, these data suggest that Notch1 signaling mediates the proinflammatory response to lyso-Gb3 in human podocytes.
Figure 1. Lyso-Gb3 activates Notch1 in cultured human podocytes. Podocytes were stimulated with 100 nM lyso-Gb3 for different time periods and mRNA and cellular protein isolated and analyzed. (A) Lyso-Gb3 induces Notch1 cleavage to yield an active fragment (cleaved Notch1). Note that the Notch1 cleavage precedes changes in mRNA expression shown in (C) and (D). Quantification and representative western blot. (B) Lyso-Gb3 upregulated uncleaved Notch1 protein. Representative western blot. (C) Lyso-Gb3 upregulated Notch1 mRNA expression, qRT-PCR. (D) Lyso-Gb3 upregulated HES1 mRNA expression, qRT-PCR. (E) Lyso-Gb3 upregulated HES1 protein expression. Quantification and representative western blot. Data are mean ± SD of three or four independent experiments. *P < 0.005 versus control and **P < 0.05 versus control.
Lyso-Gb3 activates NFκB in podocytes in a Notch1-dependent manner

As we observed a role for Notch1 in proinflammatory responses elicited by lyso-Gb3, we explored whether lyso-Gb3 and Notch1 recruited the transcription nuclear factor kappa B (NFκB), a key regulator of inflammatory responses (39). Both MCP-1 and RANTES are bona fide targets of NFκB (39). Parthenolide inhibits the nuclear translocation of the NFκB protein RelA and thus inhibits canonical NFκB activation (40). Parthenolide prevented lyso-Gb3 induction of MCP-1 and RANTES mRNA expression (Fig. 5A and B), indicating that this is a canonical NFκB activation-dependent response. Lyso-Gb3 increased NFκB DNA-binding activity, peaking at 24 h
as assessed by electrophoretic mobility shift assay (EMSA) (Fig. 5C). This increase in NFκB DNA-binding activity was prevented by Notch1 siRNA (Fig. 5C). These results suggest that lyso-Gb3 activation of Notch1 results in increased NFκB DNA binding that leads to inflammatory responses in podocytes.

Notch1 also mediates fibrogenic responses in podocytes

The pro-fibrogenic activity of Notch1 has been extensively studied in renal cells (26). As we previously observed that lyso-Gb3 upregulated the gene expression of extracellular matrix (ECM) proteins such as fibronectin (14), we now explored whether Notch1 activation contributed to fibrogenic responses. Indeed, upregulation of fibronectin gene expression induced by lyso-Gb3 was also prevented by Notch1 siRNA (Fig. 6).

Expression of Notch1, Jagged1 and HES1 in kidney biopsies from Fabry patients

As in cultured podocytes lyso-Gb3 activated the Notch1 pathway, we next explored the expression of Notch1 signaling pathway proteins in human Fabry disease kidneys. No expression of Notch1, active Notch1, Jagged1 or HES1 was observed in control kidneys. However, staining for Notch1 (Fig. 7A), active Notch1 (Fig. 7B), Jagged1 (Fig. 7C) and the Notch1 target gene HES1 (Fig. 7D) was observed in kidney biopsies from Fabry patients. The staining pattern was suggestive of glomerular podocyte and tubular cell expression.

Figure 3. Inhibition of γ-secretase prevents the expression of the canonical Notch target gene HES1 in response to lyso-Gb3 in cultured human podocytes. (A) The γ-secretase inhibitor GSI IX (10 μM) prevents HES1 mRNA upregulation induced by 100 nM lyso-Gb3 for 24 h. qRT-PCR. *P < 0.005 versus control and **P < 0.01 versus lyso-Gb3 alone. (B) The γ-secretase inhibitor GSI IX prevents HES1 protein upregulation induced by exposure to 100 nM lyso-Gb3 for 24 h. Quantification and representative western blot. *P < 0.01 versus control and **P < 0.02 versus lyso-Gb3 alone. Data are mean ± SD of three or four independent experiments.

Systemic lyso-Gb3 administration effects on murine kidneys in vivo

Systemic administration of lyso-Gb3 to mice resulted in whole kidney upregulation of MCP-1, RANTES, HES1 and Jagged mRNA expression at 24 h, a time point consistent with cell culture data (Supplementary Material, Fig. S1A–D). This was associated with increased numbers of F4/80 macrophages in glomeruli compared with controls (Supplementary Material, Fig. S1E).

Discussion

The main findings are that lyso-Gb3 activates Notch1 signaling in podocytes and this leads to NFκB activation and transcription of proinflammatory and ECM genes. Given accumulating evidence for a role of Notch1 in podocyte injury and kidney fibrosis, these data suggest that Notch1 should be explored as an add-on therapeutic target in Fabry disease, on top of ERT.

Proteinuria is a key prognostic indicator of renal outcomes both in ERT-naïve and in ERT-treated Fabry patients (8,41). Proteinuria is usually a manifestation of podocyte injury, and podocytes are cleared of glycolipids by ERT slowly if at all (10,17,42). In young Fabry patients, podocyte deposits volume density and foot process width increased with age and correlated directly with proteinuria, whereas endothelial deposits did not (16). Foot process effacement is a manifestation of podocyte injury observed in Fabry children with minimal albuminuria (18). Hence, podocyte injury has been proposed to play a pivotal role in the development and progression
of Fabry nephropathy (16). Support for this concept comes from the observation that podocytes from some young patients can be cleared by several years of ERT, and this was associated with regression of ‘moderately increased albuminuria’ (17). Podocyte deposits are less responsive to ERT in adults and may need more than 5 years of continued ERT to show any clearance. The clinical correlate is lack of improvement of proteinuria by ERT in adults. In contrast, kidney endothelial cells and fibroblast deposits are cleared within 6–12 months of ERT (42). Thus, it is relevant to study the adverse effects of glycolipids on podocytes, even in the ERT era.
Plasma lyso-Gb3 is dramatically increased in classically affected male Fabry patients and is also increased in females, whereas it is undetectable in healthy controls (19,35,43). These characteristics may contribute to a cross-talk between cells with glycolipid deposits and those that have been cleared or are deposit-free and contribute, together with the key role of the distribution of X-inactivation involving the wild-type allele of the GLA in kidney cells, to the occurrence of end-stage renal disease in some females (9,44,45). Lyso-Gb3 elicited responses and recruited secondary mediators of injury in normal (non-Fabry) podocytes. Thus, in normal human podocytes, lyso-Gb3 dose- and time-dependently increased the expression of the fibrogenic cytokine TGF-β1 and increased ECM (fibronectin and type IV collagen) synthesis in a TGF-β1-dependent manner (14). This

Figure 5. Lyso-Gb3 induces NFκB activation and NFκB-mediated chemokine mRNA expression. NFκB activation is prevented by Notch1 siRNA in cultured human podocytes. (A and B) Pre-incubation with 10 μM parthenolide prevented the upregulation of chemokine mRNA induced by stimulation with 100 nM lyso-Gb3 for 24 h as assessed by qRT-PCR. (A) MCP-1 mRNA and (B) RANTES mRNA. *P < 0.02 versus control and **P < 0.04 versus lyso-Gb3 alone. (C) NFκB activation in human podocytes treated with 100 nM lyso-Gb3 for 24 h. EMSA representative of three independent experiments. Other data are mean ± SD of three or four independent experiments.
fibrogenic response of podocytes to lyso-Gb3 reminds of podocyte responses to high glucose concentrations in the extracellular milieu (46). In this regard, high glucose concentrations also promote a proinflammatory response in podocytes and activate Notch receptors (23, 24). Similarly, we have now observed that lyso-Gb3 activates Notch1 and that Notch1 mediated the proinflammatory and fibrogenic response of podocytes to lyso-Gb3. In this regard, TGF-β1 and Notch1 are known to interact to promote kidney fibrosis (23, 25, 26) and Notch signaling mediates some pro-fibrotic effects of TGF-β1 in tubular cells (47).

Notch1 has been suggested to be a key contributor to kidney injury (23). Elevated levels of Notch ligands and receptors are detected in proteinuric kidney diseases such as diabetic nephropathy, membranous nephropathy, lupus nephritis and crescentic glomerulonephritis (25, 27, 48). Notch promotes experimental kidney tubulointerstitial fibrosis (26). Mice with elevated expression of Notch1 in podocytes develop albuminuria and glomerulosclerosis and die early (28). Taken together, these data indicate that the Notch pathway plays a key role in podocyte injury and kidney fibrosis (23), and our data add Fabry disease to the list of nephropathies in which Notch may play a role.

Although inflammation is present in Fabry human kidney biopsies (49) and there is also evidence of systemic inflammation, the triggers of inflammation in Fabry disease are not well characterized (30–32). Proinflammatory cytokine production by peripheral blood mononuclear cells is increased in Fabry patients (30). Invariant natural killer T cells are phenotypically and functionally altered in Fabry patients (31). Systemic inflammation may persist despite ERT (32, 33), and thus, accumulated metabolites such as lyso-Gb3 that are not normalized by ERT may be potential triggers of persistent inflammation. Oxidative stress in blood cells and circulating IL-6 and TNF-α were still higher in ERT-treated Fabry patients than in controls (32). Finally, systemic inflammation has been associated with recurrent stroke in Fabry patients on ERT (33). We now provide evidence linking lyso-Gb3 to an inflammatory phenotype in Fabry disease and identify Notch1 as a secondary activator of the inflammatory response.

Lyso-Gb3 upregulated the canonical Notch1 target HES1 in podocytes. The function of HES1 in podocytes has not been characterized. However, podocytes are terminally differentiated cells that share with neurons morphological features and the expression of certain proteins (50). HES1 downregulates the differentiation of neurons and could play a similar dedifferentiating role in podocytes (51). The identification of Notch1 as a mediator of inflammatory and potentially dedifferentiating responses may have in the future therapeutic consequences. Although in vivo siRNA delivery to specifically target Notch1 is still many years away from clinical use, several γ-secretase inhibitors are undergoing clinical trials in malignancy and Alzheimer (36). In this regard, the γ-secretase inhibitor GSI IX prevented lyso-Gb3-induced expression of the canonical Notch1 target gene HES1 and of chemokines.

Lyso-Gb3 elicited pathologic responses when used at concentrations found in the circulation of Fabry patients in target cells without pre-existing glycolipid deposits. It can then be hypothesized that lyso-Gb3 originating in hard-to-reach-by-ERT cells in the body may have adverse effects on cells that have been cleared of deposits. Indeed, the dose–response range active in cell cultures corresponded to the circulating lyso-Gb3 concentration range found both in treatment-naive and in ERT-treated Fabry patients. This suggests that our observations are relevant for patients on ERT, in whom lyso-Gb3 levels are known not to normalize (35).

Diabetes is common in the general population and, despite current therapeutic approaches, remains the most frequent cause of end-stage renal disease (11, 52); it is likely that some individuals with hypomorphic GLA gene variants have concurrent diabetes (53). The potential relevance of our current findings for these patients merits further studies. In this regard, our findings would be consistent with possible aggravation of the kidney disease phenotype of patients with cardiac variants of Fabry disease who also develop diabetes mellitus. The cardiac variants of Fabry disease are associated with moderately increased levels of plasma lysoGb3 and with subclinical, mild or late-onset evidence of kidney involvement, but this expectation might be radically changed by the coexistence of diabetes mellitus.

In conclusion, lyso-Gb3 activates Notch1 signaling in non-Fabry podocytes, suggesting that circulating lyso-Gb3 at concentrations that can be found in ERT-naive and ERT-treated Fabry patients may have a pathogenic role in Fabry nephropathy. Among lyso-Gb3-elicted, Notch1-mediated effects, we find increased expression of canonical Notch targets such as HES1, ECM-encoding genes such as fibronectin which may contribute to fibrosis and activation of the canonical NFκB pathway resulting in chemokine secretion which may contribute to inflammation (Fig. 8). These responses may contribute to progressive kidney injury in Fabry nephropathy and may be subject to therapeutic intervention in combination with ERT.

**Materials and Methods**

**Cell culture and reagents**

Human podocytes are an immortalized cell line transfected with a temperature-sensitive SV40 gene construct and a gene encoding the catalytic domain of human telomerase (14, 54). At a permissive temperature of 33°C, cells remain in an undifferentiated proliferative state and divide. Raising the temperature to 37°C results in growth arrest and differentiation to the parental podocyte phenotype. Undifferentiated podocyte cultures were maintained at 33°C
in RPMI 1640 medium with penicillin, streptomycin, ITS (insulin, transferrin, selenite) and 10% fetal calf serum. Once cells reached 70–80% confluence, they were fully differentiated by culture at 37°C for at least 14 days (14,54). Cells were cultured in serum-free medium 24 h prior to the addition of stimuli and throughout the experiment. γ-Secretase inhibitor IX (1–10 µM, GSI IX, Calbiochem) or parthenolide (10 µM) was added 1 h before lyso-Gb3 (Sigma, St Louis, MO, USA) and was not toxic (cell viability assay MTS-PMS, Promega, and morphological assessment of apoptosis). Unless otherwise specified, in the cell culture model, we have developed of podocyte injury in Fabry disease, lyso-Gb3 was used at a concentration of 100 nM. This concentration is clinically relevant, as circulating lyso-Gb3 has been reported to be in the 0–50 nM range for heterozygous females, 100–400 nM for homozygous males and 50–150 nM for ERT-treated patients (19,35).

**Animal model**

Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57/BL6 female mice (12–14-week-old) from the IIS-Fundacion Jimenez Diaz animal facilities received a single i.p. injection of lyso-Gb3 in dimethyl sulfoxide or vehicle and were sacrificed 24 h later (n = 4 per group). The dose was adjusted for an estimated extracellular volume of 10 ml and a hypothetical concentration of lyso-Gb3 in the extracellular volume of 100 mM, simulating the cell culture conditions. Kidneys were perfused in situ with cold saline before removal. Half kidney from each mouse was fixed in buffered formalin, embedded in paraffin and used for immunohistochemistry, and the other half was snap-frozen in liquid nitrogen for RNA and protein studies.

**Figure 7.** Expression of Notch pathway proteins in human Fabry disease. Immunohistochemistry of human Fabry biopsies shows increased expression of (A) total Notch1, (B) active Notch1, (C) Jagged1 and (D) HES1. No staining was found in human control biopsies. Original magnification 20× and 40×. Podocyte staining was less conspicuous than in the other cell types due to the extensive glycolipid deposits that resulted in a honeycomb pattern of empty vacuoles.
Protein studies

For western blot, cells were homogenized in lysis buffer and separated by 10 or 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing conditions, as described previously (55). Primary antibodies were rabbit polyclonal anti-Notch1 (1:500, Abcam), anti-cleaved Notch1 (ICN1) (Val1744 from Abcam) and rabbit polyclonal anti-HES1 (1:1000, Cell Signaling). Secondary antibodies were appropriate horseradish peroxidase (HRP)-conjugated antibodies (1:2000, Amersham, Aylesbury, UK). Blots were then probed with mouse monoclonal anti-α-tubulin (1:2000, Sigma), and levels of expression were corrected for minor differences in loading.

MCP-1 levels in the culture supernatants were quantified with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems), according to the manufacturer’s instructions.
Real-time reverse transcription–polymerase chain reaction (RT–PCR)

RNA was isolated using Trizol reagent (Invitrogen, Paisley, UK) (56). About 1 μg RNA was reverse-transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCRs were performed on the ABI Prism 7500 sequence detection PCR system (Applied Biosystems), according to the manufacturer’s protocol using the DeltaDelta Ct method (56). Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays were from Applied Biosystems.

Transfection with siRNA

Cells were grown in six-well plates (Costar, Cambridge, MA, USA) and transfected with a mixture of 20 nmol/ml Notch1 siRNA (Santa Cruz, CA, USA), Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 (Invitrogen) (57). After 18 h, cells were washed and cultured for 6 h in complete medium, and they were next serum-depleted for 24 h before addition of stimulus. This time point was selected from a time-course of decreasing Notch1 protein expression in response to siRNA. A negative control scrambled siRNA provided by the manufacturer did not reduce Notch1 protein.

EMSA

EMSA was carried out as described previously (56). Cells were resuspended in buffer A and homogenized. Nuclei and cytosolic fractions were separated by centrifugation at 1000g for 10 min. Nuclei (pellet) were washed twice in buffer A and resuspended in the same buffer, with a final concentration of 0.39 mol/l KCl. Nuclei were extracted for 1 h at 4°C and centrifuged at 100 000g for 30 min. Supernatants were dialyzed in buffer C, cleared by centrifugation and stored at −80°C. The protein concentration was determined by the bichinchoninic acid method, and transcription factor DNA-binding activity was assayed.

Immunohistochemistry

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation at the Division of Nephrology (Austral University, Valdivia, Chile). Control human kidney specimens (n = 5) were taken from normal portions of renal tissue from patients who underwent surgery because of localized renal tumors. Two renal biopsies from male Fabry patients were studied, yielding similar results: a 27-year-old with serum creatinine 4.7 mg/dl and proteinuria 4.9 g/24 h and a 69-year-old with serum creatinine 4.2 mg/dl and proteinuria 0.69 g/24 h. Immunohistochemistry was carried out in paraffin-embedded tissue sections 5 μm thick. The primary antibody was goat polyclonal anti-Notch1 (1:100, Santa Cruz), rabbit anti-cleaved Notch1 (1:200, Abcam), goat polyclonal anti-Jagged1 (1:50, Santa Cruz) and mouse monoclonal anti-HES1 antibody (1:200, Abcam). Sections were counterstained with Carazzi antibody. The number of glomerular F4/80-positive macrophages was quantified in 20 randomly chosen fields (<40) using Image-Pro Plus software. Samples were examined in a blinded manner.

Statistical analysis

Data are mean ± standard deviation. Mann–Whitney, two-sided t-test or one-way analysis of variance was applied to detect differences between groups. A P < 0.05 was considered statistically significant.

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

Funding

The research was supported by the grants FIS PI13/00047, CP14/00133, ISCIII-RETIC RE6iNREN RD12/0021, Comunidad de Madrid S2010/BMD-2378, CYTED IBERERC, Programa Intensificación Actividad Investigadora (ISCIII) to A.O. and Miguel Servet to M.D.S.-N. and A.B.S., FONDECYT Chile 1120480 to S.M. We thank Maria Eugenia Burgos for technical help.

References


