Spleen Tyrosine Kinase Is Important in the Production of Proinflammatory Cytokines and Cell Proliferation in Human Mesangial Cells following Stimulation with IgA1 Isolated from IgA Nephropathy Patients


*J Immunol* 2012; 189:3751-3758; Prepublished online 5 September 2012;
doi: 10.4049/jimmunol.1102603
http://www.jimmunol.org/content/189/7/3751

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Spleen Tyrosine Kinase Is Important in the Production of Proinflammatory Cytokines and Cell Proliferation in Human Mesangial Cells following Stimulation with IgA1 Isolated from IgA Nephropathy Patients


IgA immune complexes are capable of inducing human mesangial cell (HMC) activation, resulting in release of proinflammatory and profibrogenic mediators. The subsequent inflammation, cellular proliferation, and synthesis of extracellular matrix lead to the progression of IgA nephropathy (IgAN). Spleen tyrosine kinase (SYK) is an intracellular protein tyrosine kinase involved in cell signaling downstream of immunoreceptors. In this study, we determined whether SYK is involved in the downstream signaling of IgA1 stimulation in HMC, leading to production of proinflammatory cytokines/chemokines and cell proliferation. Incubation of HMC with IgA1 purified from IgAN patients significantly increased the synthesis of MCP-1 in a dose-dependent manner. There was also significantly increased production of IL-6, IL-8, IFN-γ-inducible protein-10, RANTES, and platelet-derived growth factor-BB. Stimulation of HMC with heat-aggregated IgA1 purified from IgAN patients induced significantly increased HMC proliferation. Both pharmacological inhibition of SYK and knockdown of SYK by small interfering RNA significantly reduced the synthesis of these mediators and inhibited HMC proliferation. Moreover, positive immunostaining for total and phospho-SYK in glomeruli of kidney biopsies from IgAN patients strongly suggests the involvement of SYK in the pathogenesis of IgAN. To our knowledge, we demonstrate, for the first time, the involvement of SYK in the downstream signaling of IgA1 stimulation in HMC and in the pathogenesis of IgAN. Hence, SYK represents a potential therapeutic target for IgAN. The Journal of Immunology, 2012, 189: 3751–3758.
In this study, we investigated whether SYK is expressed in HMC and whether it is involved in downstream signaling of IgA1 stimulation in HMC, leading to synthesis of proinflammatory mediators by HMC and proliferation of HMC.

Materials and Methods

Sample collection
Serum samples were collected from healthy subjects and randomly selected patients with IgAN attending the John Walls Renal Unit, Leicester General Hospital. All patients had biopsy-proven IgAN with microscopic hematuria and/or proteinuria. The study was approved by the local Research Ethics Committee, and all subjects gave their written informed consent for serum collection.

Purification of serum IgA1
Serum was precipitated with an equal volume of 45% ammonium sulfate solution and dissolved in 0.175 mol/l Tris-HCl (pH 7.5). The obtained fraction was incubated with Jacalin–Agarose (Vector Laboratories, Peterborough, U.K.) at room temperature for 90 min. Unbound proteins were removed by extensive washing of the Jacalin–Agarose with Tris-HCl, and IgA1 eluted from the Jacalin with 1 mol/l galactose in Tris-HCl. The IgA1 was then dialyzed against 0.2× PBS, and the total volume reduced to 20% of the original volume and stored at −20°C until use. The purity and concentration of IgA1 fractions were confirmed by SDS-PAGE, Western blot analysis, and ELISA. Axial aggregates of IgA1 purified from patients with IgAN was confirmed by lectin-binding assay using N-acetyl galactosamine-specific lectin Helix aspersa as described previously (4). In all experiments, pooled IgA1 from randomly selected patients or healthy subjects was used. For each batch of IgA1, serum samples from eight subjects were used.

SYK inhibitor
The SYK inhibitor R406 was provided by Rigel Pharmaceuticals (South San Francisco, CA) and AstraZeneca (London, U.K.). The details of this molecule have been reported previously (26).

Primary HMC culture
HMC were purchased at passage 3 (Lonza, Walkersville, MD). HMC were grown in mesangial cell basal medium supplemented with 5% FCS, gentamicin (30 μg/ml), and amphotericin B (15 μg/ml) at 37°C in an atmosphere of 5% CO₂/95% air. Cells grown at passage 5–8 were used in all experiments.

Treatment of HMC with different IgA1 preparations, IgG and SYK inhibitor R406
For measurement of cytokines, HMC were cultured in a 24-well culture plate (5 × 10⁵ cells/well) in basal medium to confluence, growth-arrested, and then incubated in RPMI 1640 medium containing 0.5% FCS with total IgA1 isolated from IgAN patients (IgAN–IgA1), IgA1 isolated from healthy control subjects (IgG) or heat-aggregated human IgG (IgG-ag) (Sigma-Aldrich, Gillingham, U.K., aggregated at 63°C for 20 min) or 6 or 24 h. For proliferation experiments, HMC were cultured in a 96-well culture plate (1.5 × 10⁴ cells/well), growth-arrested, and then stimulated with heat-aggregated IgA1 isolated from IgAN patients (algalA1, aggregated at 63°C for 150 min (27)) for 24 h. For the experiments with R406, HMC were preincubated in medium containing either 0.04% DMSO (vehicle) or R406 in 0.04% DMSO for 1 h before further treatment with different IgA1 preparations. For RNA extraction, HMC were harvested after 6 h.

For determination of cytokine concentrations, culture supernatants were collected after 24 h. For proliferation experiments, cell proliferation assays were performed after 24 h. The MTT assay (Sigma-Aldrich) was performed to assess cell viability.

Transfection of HMC with small interfering RNAs
Lipofectamine RNAiMax (Invitrogen, Paisley, U.K.) was used for the transfection with small interfering RNAs (siRNA). siRNA directed against SYK and nonspecific, scrambled, control siRNA were predesigned (Silencer select Predesigned siRNA; Ambion/Applied Biosystems, Paisley, U.K.). HMC were seeded onto 24-well culture plate (3 × 10⁵ cells/well) in antibiotic-free mesangial cell growth media and grown overnight. HMC were then transfected with 50 nM of each siRNA mixed with Lipofectamine reagent in Opti-mem medium (Invitrogen). HMC were maintained for 72 h and then subjected to Western blot analysis. For the cytokine experiments, medium was changed to 0.5% FCS containing RPMI 1640 medium after 48 h of transfection for 24 h before further stimulations. For the proliferation experiments, HMC were transfected in a 100 × 20-mm dish and then seeded onto 96-well culture plate in 0.5% FCS containing RPMI 1640 medium after 48 h of transfection. Further stimulations were performed after 72 h of transfection.

Determination of MCP-1 gene expression by quantitative RT-PCR
Total cellular RNA was isolated using TRIzol reagents (Invitrogen). Two micrograms of RNA was reverse transcribed with the First-Strand cDNA Synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Applied Science, Burgess Hill, U.K.). Real-time PCR was performed on the Mastercycler ep realplex (Eppendorf, Histon, U.K.) using the SYBR green MasterMix (ABgene, Epsom, U.K.). β-Actin served as the internal control. The sequences of PCR primers were as follows: MCP-1, forward primer, 5’-CCACCGCAGAAGTGGGTTCAG-3’, and reverse primer, 5’-CTTGGGTTGTTGAGTGTTG-3’; and β-actin, forward primer, 5’-TCACCACACTGTTGCCCACTTACGA-3’ and reverse primer, 5’-CAGCGGAAGCGGCTCACTTGCCAATGG-3’. The expression of MCP-1 mRNA was analyzed by a relative quantification method, the 2⁻ΔΔCt method.

Determination of cytokine concentrations in culture supernatants
MCP-1 ELISA. MCP-1 was measured by specific ELISA using commercially available paired Abs (R&D Systems, Abingdon, U.K.). The sensitivity of the MCP-1 ELISA was 9.8 pg/ml.

Multiplex cytokine assay
The expression of all other cytokines and chemokines was determined using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, Hemel Hempstead, U.K.). Briefly, the principle of a sandwich immunoassay was combined with Luminex fluorescent bead-based technology (Luminex, Austin, TX), allowing measurement of large numbers of cytokines and chemokines in small sample volumes. The assay was run on a Luminex 200 system using low Photomultiplier Tube settings for broad range standard curve (system calibrated with CAL2 low RPI target value) according to the manufacturer’s instructions.

Western blotting
Whole cell lysates were prepared using radioimmunoprecipitation lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with a protease inhibitor mixture and phosphatase inhibitor. The lysates were incubated at 4°C for 20 min and then centrifuged at 14,000 rpm at 4°C for 20 min. The protein concentration of the supernatants was measured by the Bradford protein assay (Pierce, Thermo Fischer Scientific, Loughborough, U.K.). Equal amounts of protein extracts were separated by 10% SDS-PAGE (NaDodSO₄–PAGE) and transferred to Amersham Hypermill ECL (GE Healthcare, Amersham, U.K.). After blocking with 6% dry milk in TBS, membranes were incubated under agitation with total SYK Ab (4D-10; Santa Cruz Biotechnology), phospho-SYK (Tyr525/526) Ab (2711; Cell Signaling Technology, Danvers, MA), or anti-actin Ab (Sigma-Aldrich) diluted in 6% BSA containing TBS overnight at 4°C. Membranes were then washed with TBST (TBS with 0.05% Tween 20) and incubated with peroxidase-conjugated secondary Abs (Dakocytomation, Ely, U.K.) diluted in blocking buffer (1:3000) for 1 h at room temperature. Membranes were washed again in TBST, and then, Western blots were developed using the Amersham ECL plus reagents (GE Healthcare).

Cell proliferation ELISA using BrdU
After stimulation of HMC with algA1 for 24 h, cell proliferation ELISA using BrdU was performed using the cell proliferation ELISA kit (Roche Applied Science, Rotkreuz, Switzerland), according to the manufacturer’s instructions.

Immunohistochemistry of kidney biopsies from patients with IgAN
Renal biopsies were performed according to the clinical need of the patients in Hammersmith Hospital. The patients gave written consent for use of biopsied tissues surplus to diagnostic analysis for research. The protocols were approved by local research ethics committee. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues. Sections were subject to heat-induced Ag retrieval in 0.1 M citrate buffer (pH 6) and then sequentially blocked in 0.3% hydrogen peroxide and 20% goat serum (Sigma-Aldrich). Sections were then incubated with primary Abs for total SYK (N-19; Santa
Results

Effect of SYK inhibitor R406 on the synthesis of MCP-1 by HMC following stimulation with IgAN–IgA1

HMC were incubated with IgAN–IgA1, cIgA1, or aIgG at different concentrations for 24 h, and the concentration of MCP-1 was measured in culture supernatants by ELISA. The synthesis of MCP-1 following stimulation with IgAN–IgA1 was significantly upregulated in a dose-dependent manner. At the same concentration of each stimulus, the concentration of MCP-1 was significantly higher following stimulation with IgAN–IgA1 than cIgA1 or aIgG (Fig. 1A). There were no significant differences in cell viability, assessed by MTT assay, at different concentrations of IgAN–IgA1, cIgA1, or aIgG (data not shown). In the next experiment, HMC were preincubated with either vehicle or R406 at three different concentrations for 1 h, and then, IgAN–IgA1 at 50 μg/ml was added to the media. Quantitative RT-PCR was performed using total RNA extracted from HMC after incubation with IgAN–IgA1 for 6 h. The expression of MCP-1 mRNA was significantly upregulated in cells treated with either IgAN–IgA1 alone or vehicle and IgAN–IgA1. In the cells treated with R406, the upregulation of MCP-1 mRNA expression following stimulation with IgAN–IgA1 was significantly inhibited in a dose-dependent manner (Fig. 1B).

Concentrations of MCP-1 in the culture supernatants from HMC following stimulation with IgAN–IgA1, under treatment with vehicle or R406, were consistent with the MCP-1 mRNA expression in each condition (Fig. 1C). Treatment with R406 reduced the IgAN–IgA1-induced production of MCP-1 significantly in a dose-dependent manner. There were no significant differences in cell viability, assessed by MTT assay, in different treatment conditions (data not shown).

Effect of IgAN–IgA1 and R406 on other mediators produced by HMC

The effect of stimulation with IgAN–IgA1 at 50 μg/ml and treatment with R406 at two different concentrations on the synthesis of a panel of cytokines and chemokines by HMC was analyzed using a multiplex cytokine assay. Among the 27 cytokines and chemokines tested, the synthesis of IL-6, IL-8, IP-10, RANTES, and platelet-derived growth factor (PDGF)-BB was significantly increased following stimulation with IgAN–IgA1 for 24 h. Treatment with R406 significantly downregulated the synthesis of these cytokines and chemokines (Fig. 2).

Effect of knockdown of SYK using siRNA on the synthesis of MCP-1 and other mediators by HMC

To verify the inhibitory effect of R406 on SYK, HMC were transfected with either siRNA directed against SYK (SYK siRNA) or nonspecific, scrambled control siRNA. Cells were incubated for 72 h in complete medium before Western blot analysis. Western blot analysis showed clearly reduced expression of SYK in HMC transfected with SYK siRNA compared with HMC transfected with control siRNA or untreated HMC (Fig. 3A). For the stimulation experiments, cells were grown arrested after 48 h of transfection and stimulated with IgAN–IgA1 at 50 μg/ml for 24 h. Knockdown of SYK in HMC by transfection with SYK siRNA significantly reduced the IgAN–IgA1-induced production of MCP-1, whereas transfection with control siRNA did not affect the synthesis of MCP-1 (Fig. 3B). We then looked at the effect of SYK siRNA on the synthesis of other mediators by HMC using a multiplex cytokine assay. Among the 27 cytokines and chemokines tested, the synthesis of IL-6, IL-8, IP-10, RANTES, and PDGF-BB was significantly reduced by the transfection with SYK siRNA but not by the transfection with control siRNA (Fig. 4).
vehicle or R406 at three different concentrations for 1 h before
stimulation with aIgA1 at 200 µg/ml for 24 h. The proliferation of
HMC was significantly inhibited by treatment with R406 in a dose-
dependent manner (Fig. 5A). To verify the effect of R406, HMC
were transfected with either SYK siRNA or control siRNA and then
incubated with aIgA1 200 µg/ml for 24 h. The proliferation of
HMC was significantly inhibited by treatment with R406 in a dose-
dependent manner (Fig. 5A). To verify the effect of R406, HMC
were transfected with either SYK siRNA or control siRNA and then
incubated with aIgA1 200 µg/ml for 24 h. The proliferation of

**FIGURE 2.** Effect of stimulation with IgAN–IgA1 and treatment with R406 on the synthesis of cytokines and chemokines by HMC was analyzed by multiplex cytokine assay. The synthesis of IL-6, IL-8, MCP-1, IP-10, RANTES, and PDGF-BB was increased significantly following stimulation with IgAN–IgA1 at 50 µg/ml. Treatment with R406 at 0.2 and 2 µM significantly reduced the synthesis of these cytokines following stimulation with IgAN–IgA1. The results are representative of three experiments and reported as mean ± SD. *p < 0.01, †p < 0.05.

**FIGURE 3.** Concentrations of MCP-1 in the culture supernatants from HMC incubated with IgAN–IgA1 after transfection with siRNA against SYK or nonspecific scrambled control siRNA are shown. (A) Western blot analysis performed after 72 h of transfection shows clearly reduced expression of SYK in HMC transfected with SYK siRNA compared with HMC transfected with control siRNA or nontransfected HMC. Data are shown as the ratio of arbitrary intensity units for SYK to actin. (B) Concentrations of MCP-1 in the culture supernatant from nontransfected HMC increased significantly following stimulation with IgAN–IgA1 at 50 µg/ml. Transfection with SYK siRNA significantly reduced the synthesis of MCP-1 (p = 0.0006). Transfection with control siRNA did not affect the synthesis of MCP-1. The results are representative of three experiments and reported as mean ± SD.
HMC was significantly inhibited by knockdown of SYK by transfection with SYK siRNA but not by control siRNA (Fig. 5B). Activation of SYK in HMC following stimulation with IgAN–IgA1

To investigate whether SYK is activated following stimulation with IgAN–IgA1, HMC were incubated with IgAN–IgA1 at 200 μg/ml for various time periods, and Western blot analysis for phosphorylation of SYK was performed with total cell lysates. The phosphorylation of SYK was increased after stimulation with IgAN–IgA1 for up to 5 min and then decreased afterward when normalized with the β-actin expression level (Fig. 6)

Expression of SYK in kidney biopsies from patients with IgAN

We then investigated whether SYK is activated in the kidney biopsies from patients with IgAN by immunohistochemistry for total

FIGURE 4. Effect of knockdown of SYK using siRNA on the synthesis of other mediators by HMC was analyzed by multiplex cytokine assay. The synthesis of IL-6, IL-8, MCP-1, IP-10, RANTES, and PDGF-BB was increased significantly following stimulation with IgAN–IgA1 at 50 μg/ml. The synthesis of these mediators by HMC transfected with SYK siRNA was significantly downregulated compared with HMC transfected with control siRNA. The results are representative of three experiments and reported as mean ± SD. *p < 0.05.

FIGURE 5. Effect of SYK inhibition by R406 and knockdown of SYK by SYK siRNA on the proliferation of HMC following stimulation with algA1 was shown. (A) The proliferation of HMC was significantly increased following stimulation with algA1 at 200 μg/ml. Treatment with R406 significantly reduced HMC proliferation in a dose-dependent manner. Treatment with vehicle did not significantly affect the HMC proliferation. *p < 0.0001, *p = 0.0016. (B) Transfection with SYK siRNA significantly inhibited the proliferation of HMC following stimulation with algA1 at 200 μg/ml, whereas transfection with control siRNA did not affect the proliferation of HMC following stimulation with algA1. The results are representative of three experiments and reported as mean ± SD. *p < 0.0001.
SYK and phospho-SYK. The levels of both total and phospho-SYK were increased in glomeruli of kidney biopsies from patients with IgAN. In the crescentic IgAN, total and phospho-SYK were detected in both the glomerular tuft and the crescent. In the negative controls (kidney biopsy from patients with minimal change disease), two images of the same glomerulus showed very slight staining for total Syk but not for phospho-SYK (Fig. 7).

Discussion

In this study, we demonstrate that IgA1 from patients with IgAN activates HMC and induces the synthesis and release of a number of proinflammatory mediators and the proliferation of HMC. This is consistent with previous data showing stimulatory effects of IgA1 on HMC (9–12, 14, 27). Although we used total serum IgA1, which is a mixture of polymeric and monomeric IgA1, for these experiments, we were able to see a clear difference in stimulatory effects on HMC between IgAN–IgA1 and cIgA1. It is unlikely that potential contaminants during the IgA1 purification process induced the observed stimulatory effects, because patient and healthy subject IgA1 were prepared in exactly the same manner. Exposure to alG did not result in any stimulatory effects in HMC, suggesting that the stimulatory effect of IgAN–IgA1 was not induced by binding of the IgG component of IgA IC to HMC. Using total serum IgA1 purified from patients with IgAN, we were not able to induce proliferation of HMC. Previous study showed circulating IC containing aberrantly glycosylated IgA1 isolated from patients’ sera stimulated mesangial cell proliferation (14). Because of technical issues, we were not able to isolate circulating IC from patients’ sera. However, using heat-aggregated patient IgA1 according to the previous study (27), we were able to induce the proliferation of HMC.

Binding of IgA1 IC to mesangial cells with subsequent cell proliferation and release of proinflammatory and profibrotic mediators are widely accepted as key events in the pathogenesis of IgAN (15, 28). Previous studies suggest that these mediators play a significant role in the pathogenesis and progression of IgAN. Mesangial cell-derived mediators induce proliferation of proximal tubular epithelial cells and synthesis of inflammatory mediators (29). Moreover, mesangial cell-derived TNF-α and platelet-activating factor downregulate podocyte markers, resulting in altered glomerular permeability and enhanced proteinuria in IgAN (30, 31). These data indicate that such mediators play an important role in the cross-talk between the different cell types present in glomeruli. In clinical studies of IgAN, patients with moderate to severe disease showed much higher renal MCP-1 mRNA and protein expression, and higher levels of urinary MCP-1, compared with patients with mild and nonprogressive disease. Urinary levels of IL-6 and IL-8 also correlate with histological lesions and levels of proteinuria (32–34). Furthermore, levels of renal gene expression of various cytokines and chemokines, including TNF-α, IL-1β, IL-6, IL-8, and IFN-γ, correlate with the pattern and extent of histological lesions and clinical findings (35). Another study showed that serum PDGF-DD levels were elevated in patients with IgAN, although the levels of renal PDGF-DD mRNA expression did not differ between patients and control subjects (36). In this study, stimulation of HMC with IgA1 purified from patients with IgAN significantly increased synthesis of IL-6, IL-8, MCP-1,

![FIGURE 6.](image1)

![FIGURE 7.](image2)
IP-10, RANTES, and PDGF-BB by HMC. These are all mediators involved in inflammation and cell proliferation in renal disease (37). Blocking the synthesis of these mediators and the proliferation of HMC in response to binding of IgA1 to HMC may, therefore, have great relevance to the treatment of IgAN.

Pharmacological inhibition of SYK by R406 effectively reduced the synthesis of the above-mentioned mediators and also the proliferation of HMC following stimulation with IgA1 purified from patients with IgAN. Although the effect of R406 is likely to be mediated through selective inhibition of SYK, it is possible that the compound also affects other kinases, such as Lck, c-Kit, and Fct3 (18). We therefore verified the results by knockdown of the SYK gene using SYK siRNA. Both pharmacological inhibition of SYK and knockdown of the SYK gene showed consistent inhibitory effects on the synthesis of various mediators and the proliferation of HMC.

Furthermore, we were able to demonstrate the activation of SYK in the glomeruli of kidney biopsies from patients with IgAN using immunohistochemistry for phospho-SYK, which strongly suggests the involvement of SYK in the pathogenesis of IgAN. However, it is unclear which cells expressed SYK in the glomeruli, because we did not perform double staining to identify cell types expressing SYK. Both migrating and intrinsic cells in glomeruli may have expressed SYK. Our results from the Western blot analysis for total and phospho-SYK clearly demonstrate the expression of SYK in mesangial cells.

To date, it is still controversial which receptors and mechanisms are involved in the binding of IgA1 to mesangial cells. The latest study suggest that complex interaction of IgA1, soluble CD89 receptor, transferrin receptor 1, and transglutaminase 2 is required for the binding of IgA1 leading to the activation of mesangial cells (38). Whether SYK is involved in the downstream signaling of this complex interaction remains to be further investigated.

The SYK inhibitor R788 (prodrug of R406) has been studied in patients with rheumatoid arthritis (RA) in phase II clinical trials with overall favorable outcomes. The adverse events included diarrhea, increase in systolic blood pressure, and neutropenia (24, 39). RA is a disease with complex pathogenic mechanisms involving several inflammatory cascades and recruitment of inflammatory cells. Inhibition of SYK by R788 may result in the inhibition of a wide range of downstream functional responses following immunoreceptor activation, including cell proliferation and differentiation, and effector functions such as cytokine release, leading to favorable outcomes in RA (40).

To our knowledge, this is the first report showing the expression of SYK in HMC and its involvement in the cellular responses to IgA1 stimulation. We demonstrate in our study that the synthesis of proinflammatory and proliferative mediators by HMC and the proliferation of HMC can be effectively reduced by the inhibition of SYK. Use of a SYK inhibitor may, therefore, be a novel therapeutic strategy in IgA nephropathy, which is also well known for its complex pathogenic mechanisms including inflammation, proliferation, and fibrosis.

Acknowledgments
We thank Dr. Simona Deplano, Dr. Maria Fragiadaki, and Kapil Kampe for technical advice and support, Prof. Terry Cook and Prof. John Feehally for valuable advice on this project, Tricia Higgins for technical assistance, and Dr. Andreas Jehle and Prof. Jürg Steiger for generous support.

Disclosures
M.J.K. received research funding from Agen. F.W.K.T. has received research project grants from Roche Palo Alto, AstraZeneca, and Baxter Biosciences. E.S.M. is employed by Rigel Pharmaceuticals and owns stock and/or stock options for Rigel Pharmaceuticals. C.D.P. has received research funding from Cycelac, UCB Celltech, Proxicagen, and GlaxoSmithKline. The other authors have no financial conflicts of interest.

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