Synthesis of TNF-α by mesangial cells cultured with polymeric anionic IgA—role of MAPK and NF-κB


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Abstract
Background. Deposition of polymeric IgA1 (pIgA) in kidney mesangium is the hallmark of IgA nephropathy (IgAN). Current consensus is that a fraction of IgA1 molecules in the circulation of IgAN patients exhibit aberrant structures or properties that may lead to their deposition. Our previous findings suggest that the anionic property of IgA1 may play a role in mesangial IgA1 deposition in patients with IgAN. In the present study, the functional consequences of the binding of anionic polymeric IgA1 to human mesangial cells (HMCs) were investigated.

Methods. Anionic polymeric IgA1 from IgAN patients and healthy subjects was isolated by sequential jacalin affinity chromatography, size exclusion chromatography using size exclusion and MonoQ ion exchange chromatography. HMCs were cultured with purified anionic polymeric IgA1 and the release of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was examined by enzyme-linked immunosorbent assay. The signalling pathways involved in anionic pIgA-mediated HMC activation were examined by immunoblotting. Standard electrophoretic mobility shift assay (EMSA) was used to further examine whether the transcriptional factor NF-κB is associated in the signalling process. To define the mechanism of TNF-α and IL-6 production, HMCs were cultured with anionic pIgA in the presence or absence of p42/p44 mitogen-activated protein kinase (MAPK) inhibitor (PD98059), NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) or NF-κB blocking permeable peptides SN50 (P < 0.01). The increased synthesis of IL-6 by anionic pIgA in HMC was reduced by inhibitor to NF-κB or p42/p44 MAPK and was abolished by the simultaneous presence of inhibitors to p42/p44 MAPK and NF-κB. The up-regulation of TNF-α was partially suppressed by inhibitor to NF-κB but not PD98059.

Conclusion. Our results suggest that polymeric anionic IgA1 could activate HMC and increase the synthesis of TNF-α and IL-6. While both the p42/p44 MAPK and NF-κB pathways are essential in regulating the anionic pIgA-induced synthesis of IL-6, TNF-α synthesis mediated by anionic pIgA is partly dependent on NF-κB.

Keywords: anionic IgA; human mesangial cell; IgA nephropathy; polymeric IgA

Introduction
IgA nephropathy (IgAN) is a common cause of end-stage renal failure worldwide. Deposition of polymeric IgA1 in kidney mesangium is the hallmark of IgA nephropathy (IgAN). A putative nephritogenic role of the deposited IgA has been ascribed to the structure of the IgA1 and the circulating IgA1-containing immune complexes. Human IgA1 molecules contain a highly unusual structure, the hinge region, which is located between the first and second constant region domains. The hinge region consists of serine, threonine and proline. In human IgA1, the serine and the threonine are O-linked to N-acetylgalactosamine (GalNAc). The GalNAc in turn carries galactose (Gal) through a β1,3 linkage. Either the Gal or GalNAc can be attached with or without a sialic acid (NeuNAc, N-acetylmuramic acid). IgA1 eluted from the biopsy in IgAN are polymeric and anionic in nature [1]. Animal experiments revealed a preferential mesangial
deposition of immune complexes of anionic charge suggesting that the electrostatic charge of immune complexes is important in the mesangial binding. The deposition of pIgA1 and IgA1-containing immune complex (IgA1-IC) is associated with glomerular inflammation. We and other investigators have shown that pIgA1 and IgA1-IC activated mesangial cells to produce pro-inflammatory cytokines including IL-6, transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), monocyte chemoattractant-protein 1 (MCP-1), interleukin-8 (IL-8) and macrophage migration inhibitory factor (MIF) [2–4].

IgA1 molecules with altered carbohydrate structure may modulate mesangial cell reactivity. Deglycosylated IgA1 enhances zvβ3 integrin expression by mesangial cells [5]. Deglycosylated IgA1 also decreases mRNA expression and protein synthesis of vascular endothelial growth factors (VEGFs) in mesangial cells through up-regulation of iNOS activity [6]. Gal-deficient IgA1 is prone to self-aggregation and forms large complexes that cannot be effectively removed by the catabolic pathway [7]. In vitro study revealed that binding of aggregated or pIgA to mesangial cells in IgAN led to increased expression of NF-κB [4]. The IgA1 molecules from patients with IgAN have aberrant glycosylation. The overall charge of the aberrant IgA1 molecules is inevitably affected by increased negatively charged sialic acid in the carbohydrate side chains of the IgA1 hinge region. Recent studies have focused on the roles of aberrant glycosylated IgA1 in the pathogenesis of IgAN. The charge of IgA1 molecules may be important in the pathogenesis of the disease although receiving less attention. In the present study, we examined the effect of anionic pIgA on the production of IL-6 and TNF-α in cultured human mesangial cells. The signalling mechanisms involved were also studied.

Methods

Materials

Rosewell Park Memorial Institute Medium (RPMI 1640 medium) and fetal bovine serum were obtained from Life Technologies (Rockville, MD, USA). Jacalin agarose was obtained from Pierce (Rockford, IL, USA). Superose fast protein liquid chromatography (FPLC) column was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Consumables for electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Monoclonal mouse anti-actin was obtained from Lab Vision (Fremont, CA, USA). Antibodies to total and phosphorylated p42/p44 mitogen-activated protein kinase (MAPK), pan protein kinase C (PKC), p38-MAPK and c-Jun N-terminal kinase (JNK) were obtained from Cell Signaling Technology (Beverly, MA, USA) and secondary antibodies for immunoblotting were obtained from Dako (Carpinteria, CA, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

Patients and controls

Thirty Chinese patients (17 males and 13 females) with a clinical and renal immuno-pathological diagnosis of primary IgAN were studied. The histological diagnosis was made at least 18 months prior to the study and their serum creatinine remained stable over the previous 12 months. Their proteinuria ranging from 0.4 to 2.6 g/day, and were between 20 and 50 years of age (mean ± SD, 29.5 ± 8.5 years). IgA nephropathy was diagnosed by the presence of predominant granular IgA deposits, mainly in the glomerular mesangium and occasionally along the peripheral capillary basement membrane by immunofluorescence studies, as well as mesangial electron dense deposits in ultrastructural examination. Systemic lupus erythematosus, Henoch–Schonlein purpura and hepatic disease were excluded by detailed clinical history, examination and negative laboratory testing for hypocomplementaemia, anti-DNA antibody or hepatitis B virus surface antigen. Their endogenous creatinine clearance was >65 ml/min/1.73 m². Venous blood was collected from each patient at clinical quiescence (a period of no macroscopic haematuria or mucosal infection and urinary erythrocyte count <10 000/ml in un-centrifuged urine). The serum was isolated and frozen at −20°C until for isolation of IgA by a jacalin-agarose affinity column. Serum IgA1 concentration was determined by nephelometry. Thirty healthy subjects (18 males and 12 females), comparable in age and race with no macroscopic haematuria or proteinuria, were used as controls. Serum was similarly collected from these individuals.

Culture of human mesangial cell

Isolation and characterization of human mesangial cells were performed as previously described [8]. Glomeruli were prepared from the cortex of human cadaveric kidney judged to be unsuitable or transplantation or from the intact pole of kidneys removed for circumscribed tumour. Histological examination of these kidney samples revealed no renal pathology. Glomerular cells were grown in RPMI 1640 medium supplemented with glutamine (2mmol/l), N-[2-hydroxyethyl]-piperezine-N’-[2-ethanesulfonic acid] (HEPES) (10 mmol/l), penicillin (50 U/ml) streptomycin (50 μg/ml) and 12% fetal calf serum in an atmosphere of 5% CO2–95% air. Mesangial cells have astellate appearance and grow in clumps. They show a network of intracellular fibrils of myosin and they contract in the presence of 1 nmol/l of angiotensin II. Mesangial cells from a single nephrectomy sample at fourth to seventh passage were used in our experiments.

Purification of polymeric and monomeric IgA

Jacalin-binding protein (JBP) was purified using a jacalin–agarose affinity column and monomeric IgA and polymeric IgA were fractionated at room temperature by the FPLC [Pharmacia, Uppsala, Sweden] as described previously [8]. The identity of IgA after FPLC was confirmed by anti-IgA affinity chromatography and an IgA sandwich enzyme-linked immunosorbent assay (ELISA). Two pooled fractions from fractions #20 to #33 (polymeric IgA fractions) and from fractions #34 to #50 (monomeric IgA fractions) were prepared for further analysis. Polymeric IgA was...
high-molecular mass IgA with molecular weights between 250 and 1000 kDa containing secretory component and C3 as shown by solid-phase assays. Monomeric IgA was low-molecular mass IgA with molecular weights between 100 and 250 kDa containing neither secretory component nor C3. The content of IgG in the fraction was measured by an anti-IgG ELISA. The pooled fractions were dialysed and concentrated to 2 ml with Centricon [Amicon, Beverly, MA, USA] and stored at −70 °C until use. The purity of IgA fractions was confirmed by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and ELISA [3].

Separation of polymeric and monomeric IgA fractions using Mono-Q ion exchange chromatography

Polymeric IgA1 or monomeric IgA1 pooled fraction was first dialysed with binding buffer (20 mM Tris–HCl, pH 8.0) before applying onto a Mono Q HR 5/5 column [8]. The column was first equilibrated with 20 ml of binding buffer and the bound IgA was eluted with 12.5 ml of elution buffer (a linear salt gradient from 0 to 1 M NaCl in 20 mM Tris–HCl, pH 8.0). Sub-fractions of 250 μl were collected throughout the elution. Fifty microliters of the eluted sub-fractions were stored for cell lysate binding ELISA. The rest of sub-fractions eluted with 0.2–0.4 M NaCl in 20 mM Tris–HCl were pooled as polymeric or monomeric IgA1 bearing less anionic charges (pIgA P1 or mIgA P1). The sub-fractions eluted with 0.4–0.55 M NaCl in 20 mM Tris–HCl were pooled as polymeric or monomeric IgA bearing more anionic charges (pIgA P2 or mIgA P2). The pooled sub-fractions were dialysed and concentrated to 500 μl. IgA concentration in the pooled sub-fractions was assayed by a sandwich ELISA as described previously [19]. The endotoxin content in the IgA preparations was determined using Limulus amoebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD, USA). The endotoxin level for all IgA preparations was <1 pg/mg protein. To ensure lipopolysaccharides (LPS) is not involved in the IgA-induced cytokine release, five randomly selected pIgA P2 fractions from patients were further treated with Detoxi-Gel endotoxin removing column (Pierce). The effect of pIgA P2 preparations with or without Detoxi-Gel treatment on TNF-α or IL-6 release was compared. The pooled sub-fractions were then stored at −70 °C until used.

Quantification of metabolic activity, cell proliferation, viability and apoptosis of HMC cultured with IgA preparations

The metabolic activity of HMC cultured with IgA preparations were measured by a commercial colorimetric kit (Chemicon International, Temecula, CA, USA) based on cleavage of the tetrazolium salt WST-1 [2-(4-iiodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono sodium salt]. Briefly, growth-arrested HMCs were seeded into 96-well plates (0.25 × 10^5 cells per well) before exposure to IgA preparation (50 μg/ml) for 48 h. WST-1 reagent was then added and incubated at 37 °C for further 2 h. The absorbance was measured using 450 nm as the primary wavelength and 600 nm as the reference wavelength. Results were expressed as percentage changes in absorbance compared with that of the medium control (defined as HMC incubated with plain culture medium). The cytotoxic effect of IgA preparations on HMC was measured by LDH assay kit (Roche Diagnostic, Indianapolis, IN, USA) according to manufacturer protocol. Results were expressed as percentage changes in relative LDH release (absorbance ratio between LDH release and the total intracellular LDH) compared with that of the medium control. HMC proliferation was measured using BrdU incorporation assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer’s protocol. Results were expressed as percentage changes in absorbance compared with that of the medium control. To examine whether IgA induces apoptosis in HMC, activation of caspase 3 in HMC cultured with different fractions of IgA was determined using the caspase 3 activity fluorometric immunosorbant enzyme assay kit (Roche Diagnostics) according to manufacturer’s protocol and the results were expressed as fluorescence unit.

Treatment of human mesangial cells with different IgA preparations

HMC were grown to confluence, the culture medium was removed and RPMI medium containing 0.1% vol/vol FBS were added to the cells for 48 h prior to further culture experiments. The cells were exposed to IgA preparations at increasing concentrations (0, 25, 50, 100 or 200 μg/ml) for 48 h at 37 °C. In order to study whether TNF-α or IL-6 synthesis by mesangial cells was associated with p42/p44 MAPK or/and NF-κB, similar experiments were performed in cells pre-incubated with inhibitors to p42/p44 MAPK (PD98059, 25 mM) or/and NF-κB before stimulating with different IgA preparations (50 μg/ml). For all experiments, the culture supernatant was collected and stored at −70 °C for ELISA determination of TNF-α and IL-6.

ELISA of TNF-α and IL-6 in culture supernatants

A sandwich ELISA was performed according to the manufacturer’s protocol (Bender MedSystems, Vienna, Austria) to quantify the level of immuno-reactive TNF-α and IL-6 in cell culture supernatants. The detection sensitivity for TNF-α and IL-6 was 5 pg/ml and 1.6 pg/ml and the intra-batch coefficient of variation was ±7.8 and 6.3%, respectively.

Signalling molecules involved in HMC activated with anionic pIgA

HMC were grown to confluence in 6-well culture plate (1 × 10^5 cells per well). The cells were growth arrested with RPMI containing 0.1% vol/vol FBS for 48 h. After changing new medium, the cells were exposed to 50 μg/ml of IgA preparations for 30 min. At the end of incubation, cells were harvested for preparation of total cell or nuclear lysate as described subsequently.

Western blot analysis

HMC were lysed with lysis buffer containing protease inhibitor cocktails (Sigma). The cell extracts were pelleted at 150,000 g for 60 min to remove cell debris. The protein concentrations were measured by a modified Lowry method using bovine serum albumin as standard (DC protein assay.
Anionic IgA1 increase TNF-\(\alpha\) and IL-6 release by HMC

kit, BioRad). Ten micrograms of total protein from the extract or immune-precipitated pellet from 10\(^6\) cells were electrophoresed through a 15% SDS-PAGE gel before transferring to a PVDF membrane. After blocking for 1 h at room temperature in blocking buffer (1% gelatin in PBS with 0.05% Tween-20), the membrane was incubated for 16 h with monoclonal anti-actin antibody (1:1000); rabbit anti-phospho p42/44 MAPK (1:5000), rabbit anti-phospho pan PKC antibody (1:4000) or rabbit anti-INK antibody (1:1000) in PBS-Tween. The membrane was washed and incubated for 2 h at room temperature with a peroxidase-labelled goat anti-rabbit or anti-mouse immunoglobulin (Dako). After further washing, the membrane was detected with ECL chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). For semi-quantitative determination of protein expression, western blotting images for some experiments were scanned on a flatbed scanner and the density of the bands was quantitated using ImageQuant software (Molecular Dynamic, Sunnyvale, CA, USA). Densitometry results were reported as percentages of medium control after normalization with the average arbitrary integrated values of the actin signal.

Electrophoretic mobility shift assay (EMSA)

Standard EMSA was used to further examine the role of the transcription factors activator protein-1 (AP-1) and NF-\(\kappa\)B in anionic plgA-induced HMC activation. For EMSA of NF-\(\kappa\)B, HMCs were cultured in T75 tissue culture flask and, upon confluence, treated with different IgA preparations (50\(\mu\)g/ml) for 1 h. In parallel experiment, HMCs were incubated with 25 \(\mu\)M PDTC (Sigma) or 100 \(\mu\)g/ml cell membrane-permeable peptides (SN50M or SN50) for 30 min before cultured with plgA P2 from IgAN Patients. For EMSA of AP-1, HMCs were treated with different IgA preparations (50\(\mu\)g/ml) for 1 h. In parallel experiment, HMCs were incubated with inhibitor to p42/p44 MAPK (PD98059, 25 mM) for 30 min before cultured with plgA P2 from IgAN Patients. At the end of the incubation, nuclear extract was prepared using NE-PER nuclear extraction reagent (Pierce) and stored at \(-70^\circ\)C until assay. Gel shift oligonucleotide for NF-\(\kappa\)B (AGTTGAGGGGACCTTCCTCC AGGC) or AP-1 (CGCTTGATGACTCAGCCGGAA) was biotinylated using Biotin 3’ end labelling kit (Pierce) and the EMSA was carried out with the LightShift chemiluminescent EMSA kit (Pierce) according to manufacturer’s instruction.

Statistics. All data (cell culture experiments) were expressed as means ± SD. Inter-group differences for continuous variables were assessed by the unpaired \(t\)-test. The IL-6/ TNF-\(\alpha\) protein synthesis in HMC following exposure to different concentrations of IgA preparations was analysed with multivariate ANOVA and Bonferroni’s method was applied to control for multiple testing. All \(P\) values quoted are two-tailed and significance is defined as \(P < 0.05\).

Results

Separation of IgA1 into differently charged fractions

Separation of plgA1 or mIgA1 into two different pooled fractions (plgA P1 and plgA P2, mIgA P1 and mIgA P2) was carried out by ion exchange chromatography using MonoQ column and the bound IgA1 was eluted using gradient increases in ionic strength with NaCl. Figure 1 depicts the results of SDS-PAGE of the isolated pooled fractions under reducing (Figure 1A) and non-reducing condition (Figure 1B). There was no polymeric IgA co-purified with the plgA P1 and P2 fractions and no monomeric IgA was co-purified with the plgA P1 and plgA P2 fractions. Under reducing SDS-PAGE, the IgA molecules in all pooled fractions were resolved to heavy and light chains of predicted molecular weight (55 and 23 kDa). The concentration of IgA1 of differently charged fractions was shown in Table 1. The IgA1 concentration in sub-fractions plgA P1, plgA P2 and mIgA P1 of IgAN patients was significantly higher than the comparable fractions from the control subjects (\(P < 0.05\)). There was no significant difference in the amount of mIgA P2 between the IgAN patients and controls. The amount of more anionic plgA P2 fraction represented <5% of total purified IgA1 in IgAN patients and controls.

Proliferation of HMC cultured with anionic plgA

Figure 2A and B depicts the metabolic activity of HMC and cell viability following incubation with different IgA preparations for 48 h determined by the

![Fig. 1. SDS-PAGE analysis of purified pooled IgA fractions.](https://academic.oup.com/ndt/article-abstract/23/1/72/1925901/fig1)
WST kit and LDH assay. Polymeric IgA from patients with IgAN significantly increased the metabolic activity in HMC \((P < 0.05)\) (Figure 2A). HMC remained viable when exposed to various IgA fractions at concentrations of 50 \(\mu\)g/ml (Figure 2B). Polymeric IgA from patients with IgAN significantly increased the cell proliferation in HMC \((P < 0.05)\) as determined by BrdU incorporation assay (Figure 2C). Results of caspase 3 assay indicate that there was no apoptosis when HMCs were exposed to all IgA preparation tested (Figure 2D).

### Table 1. IgA1 concentration (mg/ml) of MonoQ separated fractions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IgAN</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>plgA P1</td>
<td>0.201</td>
<td>0.032</td>
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<tr>
<td>plgA P2</td>
<td>0.104</td>
<td>0.024</td>
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<tr>
<td>mlgA P1</td>
<td>1.156</td>
<td>0.258</td>
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<tr>
<td>mlgA P2</td>
<td>0.454</td>
<td>0.097</td>
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\(*P < 0.05\) vs comparable fraction from control subjects.

**Effect of anionic plgA on the synthesis of TNF-\(\alpha\) and IL-6 by HMC**

Growth arrested HMCs were cultured with IgA preparations at 50 \(\mu\)g/ml for 48 h. The release of TNF-\(\alpha\) and IL-6 in HMC was determined by ELISA. Anionic plgA (plgA P2) from patients with IgAN significantly up-regulated TNF-\(\alpha\) and IL-6 release in HMC (Figure 3). Less anionic plgA fraction (plgA P1) from IgAN patients only slightly increased the TNF-\(\alpha\) and IL-6 synthesis in HMC and the differences were not statistically significant. Compared with other IgA preparations, the release of TNF-\(\alpha\) by HMC cultured with anionic plgA from patients with IgAN was significantly higher \((P < 0.05)\). The release of IL-6 by HMC cultured with anionic plgA from patients with IgAN was also significantly higher than other IgA preparations from IgAN patients and controls. Anionic polymeric IgA1 preparations up-regulated TNF-\(\alpha\) and IL-6 release in a dose- and time-dependent manner (Figures 4 and 5). The up-regulatory effect of anionic plgA preparation (plgA P2) from patients with IgAN was significantly higher as compared with less anionic plgA (plgA P1) and mlgA.
Anionic IgA increase TNF-α and IL-6 release by HMC

Preparations \((P < 0.05)\). Although HMC from a single donor was used in the present study, our preliminary data had shown that pIgA P2 from patients significantly enhanced TNF and IL-6 release by HMC obtained from three other donors when compared with pIgA P2 from control subjects (data not shown).

To ensure that lipopolysaccharides (LPS) is not involved in the IgA-induced TNF-α or IL-6 release, selected pIgA P2 fractions from patients were further treated with Detoxi-Gel endotoxin removing column. Detoxi-Gel treatment did not alter the levels of TNF-α or IL-6 release induced by pIgA P2 preparations (data not shown).

**Fig. 3.** Release of TNF-α and IL-6 by HMC cultured with different IgA1 preparations. (A) The release of TNF-α was significantly increased in HMC cultured with 50 μg/ml anionic plgA from patients with IgAN for 48 h \((P < 0.05 \text{ vs HMC cultured with similar IgA1 fraction from control})\). The release of TNF-α by HMC cultured with anionic plgA from patients with IgAN was significantly higher compared with HMC cultured with all other IgA preparations \((P < 0.05)\). The basal level (HMC cultured in plain medium alone) of TNF-α was 58 ± 14 pg/2 x 10⁵ cells. (B) The release of IL-6 was significantly increased in HMC cultured with 50 μg/ml anionic plgA from patients with IgAN for 48 h \((P < 0.05 \text{ vs HMC cultured with similar IgA1 fraction from control})\). The release of IL-6 by HMC cultured with anionic plgA from patients with IgAN was significantly higher compared with HMC cultured with all other IgA preparations \((P < 0.05)\). The basal level of IL-6 was 8 ± 1.6 pg/2 x 10⁵ cells. The results for IgA-induced TNF-α or IL-6 release represent mean ± SD of 30 patients and controls.

**Fig. 4.** Time- and dose-course study of the effect of different IgA1 preparations from patients with IgAN on the release of TNF-α by HMC. Anionic polymeric IgA1 preparations up-regulated TNF-α release in a (A) dose- and (B) time-dependent manner. For dose-course study, the results were expressed as fold increase of TNF-α release by HMC cultured with different concentration of IgA preparation for 48 h in relation to the TNF-α release by HMC cultured with plain medium for 48 h. For time-course study, HMCs were cultured with 50 μg/ml of different IgA preparations. The results were expressed as fold increase of TNF-α release by HMC in relation to the TNF-α level in HMC cultured with plain medium (medium control) at time zero. The results represent the mean ± SD of five separate experiments. \(*P < 0.05 \text{ vs medium control.}\)

Anionic plgA preparations activated p42/p44 MAPK but not PKC, JNK or p38 MAPK pathways in HMC. (Figure 6). The nuclear translocation of NF-κB in HMC after exposure to plgA P2 was also examined by EMSA (Figure 7A). Both PDTC and SN50, but not the mutant peptide, SN50M, blocked the nuclear translocation of NF-κB. Findings from mesangial cells cultured in medium alone used as controls were shown in Figure 7B. Other additional controls including cells treated with PDTC or peptides (SN50 or SN 50M) alone or unlabelled κB oligonucleotide also revealed no shifting of the NF-κB complex by EMSA (electrophoretic gel not shown). Activation of AP-1 was observed in HMC after exposure to plgA P2 (Figure 7C) and the activation was blocked by treatment with p42/p44 MAPK inhibitor PD98059 (Figure 7D).

**Inhibition of p42/p44 MAPK or NF-κB**

To examine whether anionic plgA-induced TNF-α and IL-6 synthesis in HMC was mediated through p42/p44 MAPK or/and NF-κB-associated pathways, HMCs were treated with p42/p44 MAPK inhibitor PD98059 (25 mM), PDTC or the cell membrane-permeable peptide, SN50 (that inhibits the translocation of the active NF-κB complex into the nucleus), for 1 h before

**Activation of MAPK, AP-1 and NF-κB by anionic polymeric IgA**

We investigated whether anionic plgA activated the p42/p44 MAPK, PKC, JNK and p38-MAPK signalling pathways in HMC by immunoblotting studies.
and during incubation with anionic pIgA. As depicted in Figure 8, the increased synthesis of IL-6 and TNF-α and by anionic pIgA in HMC was significantly diminished in the presence of PDTC ($P < 0.01$) or NF-κB blocking permeable peptides, SN50 ($P < 0.01$). The increased synthesis of IL-6 by anionic pIgA in HMC was reduced by inhibitor to NF-κB or p42/p44 MAPK and was abolished by the simultaneous presence of inhibitors to p42/p44 MAPK and NF-κB. The up-regulation of anionic TNF-α was only partially suppressed by inhibitor to NF-κB but not by PD98059.

**Discussion**

This is the first study examining the functional consequence following the binding of anionic polymeric IgA1 from patients with IgAN to human mesangial cells. The absence of messenger RNA encoding for CD89, asialoglycoprotein receptor (ASGPR) and polymeric-immunoglobulin receptor (pIgR) in cultured HMC suggests that the predominant binding of polymeric IgA1 to HMC is mediated by other novel receptors or unidentified mechanisms [9]. We had previously demonstrated that pIgA with the highest negative charge binds more to HMC and the presence of polyanion decrease the binding [8]. Our findings suggest that the anionic property of IgA may play a role in mesangial activation in patients with IgAN. Keeping with this, we had demonstrated in the present study that anionic pIgA from patients with IgAN induced significant release of IL-6 and TNF-α by cultured mesangial cells. The release of IL-6 in HMC induced by anionic pIgA was dependent on the activation of p42/p44 MAPK and NF-κB while similar release TNF-α by HMC was partly associated with NF-κB but independent of p42/p44 MAPK.

Experimental animal model of IgAN with infusion of preformed IgA immune complexes had provided evidence that circulating proinflammatory cytokines may play an aggravating role in the lesions [10]. Amongst these cytokines, TNF-α is able to induce or aggravate renal damage in glomerulonephritis. Enhancement of glomerular damage by TNF-α has been observed in various experimental models of non-IgA nephritis [11,12]. TNF-α is a primary mediator.

![Fig. 5.](https://example.com/fig5.png) **Fig. 5.** Time-and dose-course study of the effect of different IgA1 preparations from patients with IgAN on the release of IL-6 by HMC. Anionic polymeric IgA1 preparations up-regulated IL-6 release in a (A) dose- and (B) time-dependent manner. For dose-course study, the results were expressed as fold increase of IL-6 release by HMC cultured with different concentration of IgA preparation for 48h in relation to the IL-6 release by HMC cultured with plain medium for 48h. For time-course study, HMCs were cultured with 50 μg/ml of different IgA preparations. The results were expressed as fold increase of IL-6 release by HMC in relation to the IL-6 level in HMC cultured with plain medium (medium control) at time zero. The results represent the mean ± SD of five separate experiments. $^* P < 0.05$ vs medium control.

![Fig. 6.](https://example.com/fig6.png) **Fig. 6.** Immunoblotting study of lysate prepared from HMC exposed to differently charged IgA1 fractions from patients with IgAN and controls. (A) Anionic pIgA preparations activated MAPK but not PKC, JNK and p38-MAPK pathways in HMC. There was no activation (phosphorylated proteins) of all signalling molecules examined when HMC was cultured with plain medium alone (data not shown). Results are representative of five independent experiments. (B) Compared with all other IgA preparations, the degree of activation of p42/p44 MAPK in HMC by anionic pIgA from patients with IgAN was significantly higher ($^* P < 0.05$). Activation of p42/p44 MAPK in HMC by polymeric IgA preparations from patients with IgAN (black bar) was significantly stronger ($^* P < 0.05$) than by polymeric IgA preparations from control. Results are expressed as the ratio of arbitrary unit (phospho-p42/p44 MAPK over total MAPK) obtained from densitometry scanning. Data are expressed as means ± SD of five separate experiments.
in the inflammatory process and has strong immuno-modulating properties. Reduction of proteinuria, glomerular damage, infiltration of leukocytes and expression of vascular adhesion molecules were found in TNF-α knockout mice exposed to nephrotoxic agent [11]. We had previously demonstrated that pIgA is capable of inducing MIF and TNF-α production in human mesangial cell, which may play a major pathogenic role in IgAN [2]. Induction of MIF can be partially blocked by neutralizing antibody to TNF-α, suggesting the possibility that up-regulation of MIF synthesis in human mesangial cells is mediated via an amplifying proinflammatory loop involving TNF-α.

In the present study, we further documented that pIgA-mediated TNF-α production by human mesangial cell was mainly restricted to an anionic subfraction of pIgA. The increased production of TNF-α in HMC induced by anionic pIgA could play an important role in the pathogenesis of IgAN. Using in vitro culture model, we had demonstrated that TNF-α and angiotensin II (AngII) are released from glomerular mesangial cells following initial deposition of ‘pathogenetic’ pIgA [13]. This is followed by proliferation of the mesangial cell and infiltration of

Fig. 7. EMSA detection of AP-1 and NF-κB activation. (A) The translocation of NF-κB into the nucleus of HMC after exposure to pIgA P2 was detected by EMSA. The signal of NF-κB was much more intense for pIgA P2 preparations from patients when compared with control. (B) Both PDTC and SN50, but not the mutant peptide SN50M, blocked the nuclear translocation of NF-κB activated by pIgA P2 from patients. Results are representative of five independent experiments. (C) The translocation of AP-1 into the nucleus of HMC after exposure to pIgA P2 was detected by EMSA. The signal of AP-1 was much more intense for pIgA P2 preparations from patients when compared with control. (B) The p42/p44 MAPK inhibitor (PD98059) blocked the nuclear translocation of AP-1 activated by pIgA P2 from patients. Negative control includes EMSA with the addition of 100-fold molar excess of unlabelled oligonucleotides into the reaction mixture. Results are representative of five independent experiments.
immuno-competent cells. A cross-talk network initiated by TNF-α, AngII and other soluble factors will orchestrate interactions between infiltrating immuno-competent cells and the resident renal cells, forming a major driving force of tubulointerstitial injury.

The cytokine IL-6 is also implicated in the pathogenesis of IgAN through its role in stimulating mesangial cell proliferation and synthesis of extracellular-matrix macromolecules [14,15]. IL-6 was the most probable risk factor for the aggravation of renal damage in IgAN and urinary IL-6 activity correlate well to the diagnosis, pathology and prognosis of the IgAN [16]. Imbalance in serum proinflammatory cytokines, including over-production of IL-6, is associated with disease progression in IgAN [17]. Herein, we showed that anionic pIgA stimulate mesangial cell proliferation and activated HMC to release more IL-6 suggesting anionic pIgA could play a role in the pathogenesis in IgAN. Although TNF-α and AngII are central regulators of the inflammatory process in IgAN, the IL-6 system can further promote inflammatory events through the activation and proliferation of lymphocytes, leukocyte recruitment and the induction of the acute-phase protein response not only in mesangial area where deposition of pathogenic pIgA takes place, but also at distinct sites such as tubulointerstitial area.

We next examined the signal transduction pathways involved in anionic pIgA-induced TNF-α and IL-6 synthesis by HMC. Previous study demonstrated that aggregated IgA from patients with IgAN induced release of intracellular calcium, phosphorylation of p42/p44 MAPK and mesangial cell proliferation [18]. Our present data confirm this observation and extend them by showing that anionic pIgA activated p42/p44 MAPK but not PKC, JNK or p38-MAPK pathways in HMC. Recognition sequence for the transcription factor NF-κB is present in many genes that are important in immune and inflammatory reaction. NF-κB is also the most prominent transcription factors modulating target genes in response to TNF-α. Since anionic pIgA up-regulated the production of TNF-α, we speculated that NF-κB could be involved in the signal transduction pathway of mesangial cell activation by anionic pIgA. As expected, our data showed that anionic pIgA-induced nuclear translocation of NF-κB. Using inhibitors to p42/p44 MAPK and NF-κB, we further demonstrated that the increased synthesis of IL-6 by anionic pIgA in HMC was NF-κB- and p42/p44 MAPK-dependent since the synthesis of IL-6 could only be abolished by the simultaneous presence of both inhibitors. Different signal transduction pathways are involved in regulation of IL-6 gene and various transcription factors can interact with the corresponding binding sites on IL-6 promoter to initiate IL-6 mRNA transcription. The finding that inhibitors to NF-κB only partially suppressed anionic pIgA induced-IL-6 production by HMC suggested that other signalling mechanisms are operational in controlling IL-6 production. Our data also showed that activation of p42/p44 MAPK is essential in AP-1 activation and modulating anionic pIgA induced-IL-6 production by HMC and this could be related to AP-1. Binding site for AP-1 is present in the promoter region of IL-6. AP-1 can be induced by p42/p44 MAPK through activation of the transcription factors ternary-complex factors (TCFs) that induce the transcription of FOS and JUN genes, thereby increasing the number of AP-1 complexes and activating AP-1 target genes including IL-6 [19]. This is the first report showing that

![Fig. 8. Effect of PD98059 and NF-κB inhibitors on anionic pIgA induced release of TNF-α and IL-6 by HMC. The increased release of (A) TNF-α and (B) IL-6 and by HMC cultured with anionic pIgA in patients (black bar) and control (grey bar) was significantly diminished in the presence of PDTC and NF-κB blocking permeable peptides SN50 (*P < 0.01 vs HMC incubated with patients' or controls' pIgA P2 without the presence any inhibitors). The increased release of IL-6, but not TNF-α, by HMC cultured with anionic pIgA was reduced in the presence of PD98059 and was abolished with the simultaneous presence of inhibitors to p42/p44 MAPK and NF-κB. The results represent mean ± SD of 30 patients and controls.](https://academic.oup.com/ndt/article-abstract/23/1/72/1925901)
Anionic IgA1 increase TNF-α and IL-6 release by HMC

the transcriptional regulation of IL-6 production in HMC by anionic pIgA is under independent control of AP-1 and NF-κB.

The up-regulation of TNF-α by anionic pIgA in HMC was partially suppressed by inhibitor to NF-κB but not by PD98059, suggesting that an alternative pathway distinct from NF-κB and p42/p44 MAPK is implicated. Similar finding had been reported in human alveolar macrophage (HAM) [20]. Incubation of HAM with pIgA resulted in enhanced TNF-α release. The activation of NF-κB in HAM by pIgA contributed partly to the up-regulation of TNF-α by pIgA. Further investigation is warranted to find out the alternative pathway involved in anionic pIgA-mediated up-regulation of TNF-α in HMC.

In conclusion, anionic pIgA from patients with IgAN can activate the p42/p44 MAPK, AP-1 and NF-κB signal transduction pathways and trigger the up-regulation of IL-6 release. Our results support that anionic pIgA-mediated-TNF-α and IL-6 release by HMC may contribute to the pathology and disease progression of IgAN.

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References


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