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Proinflammatory cytokines regulate FcαR expression by human mesangial cells *in vitro*

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SUMMARY

IgA nephropathy (IgAN) is defined by the predominant deposition of IgA immune complexes (IC) in the glomerular mesangium. Interaction between IgA immune complexes and mesangial cells (MC) could be a linchpin for the genesis of IgAN. We studied the modulation of MC expression of IgA receptors (FcαR) by selected cytokines. Binding of ^{125}I-IgA to quiescent human MC showed $2{\cdot}55 \times 10^5$ sites/cell with an affinity (Ka) of $3{\cdot}2 \times 10^7$ M^{-1}. Addition of selected recombinant cytokines had no significant influence on Ka, but increased the number of sites/cell relative to unstimulated cells. Northern hybridization using the pHuFcαR cDNA probe showed time-dependent increases in mRNA expression in stimulated *versus* control cells. IL-6 and tumour necrosis factor-alpha (TNF-α) had a biphasic effect on the FcαR mRNA level; at 48 h, IL-6 increased steady state mRNA levels about six-fold relative to control, TNF-α increased mRNA four-fold, and interferon-gamma (IFN-γ) induced FcαR mRNA two-fold. By reverse transcriptase-polymerase chain reaction (RT-PCR), the FcαR expressed on human MC appears highly homologous to that expressed by U937 cells. Altered FcαR expression in response to cytokines may influence the pathogenesis of IgAN by affecting deposition and/or clearance of IgA-IC in the mesangium.

Keywords IgA nephropathy mesangial cells Fcα receptor cytokines

INTRODUCTION

IgA nephropathy (IgAN) is a prevalent form of glomerulonephritis, defined by the abundance of IgA relative to other immunoglobulin classes within glomerular immune deposits [1] that accumulate exclusively or predominantly in the mesangium [1,2]. Consequently, the interaction of IgA with the mesangial cells (MC) that populate this portion of glomeruli is a focal point for current investigation.

Mesangial cells, specialized pericytes which occupy a central position in the glomeruli, synthesize several autocrine and/or paracrine growth factors and cytokines [3–5]. MC also take up macromolecules, including immune complexes (IC) [6,7]. Previous studies have demonstrated the expression by cultured MC of receptors (R) for the constant region of IgG, structurally and functionally similar to the FcγRII and FcγRIII expressed by granulocytes [8–11].

Human neutrophils [12,13], monocytes and macrophages [12,14,15], eosinophils [16,17] and lymphocytes [18–20] all bind IgA. The granulocytes also engulf IgA-coated particles, release inflammatory cytokines (e.g. IL-6 and tumour necrosis factor-alpha (TNF-α)) and reactive oxygen metabolites in response to IgA-IC and mediate IgA antibody-dependent cell-mediated cytotoxicity [13,21–25]. Recently, the cDNA sequence for one IgA receptor expressed by human myeloid cells, FcαR, was published [26]. Stimulation of monocytes and/or neutrophils by phorbol myristyl acetate (PMA), TNF-α, IL-1, or endotoxin (lipopolysaccharide (LPS)) increases FcαR expression [27–30], whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) increase the affinity of FcαR on neutrophils [31]. Different effects of interferon-gamma (IFN-γ) upon FcαR expression by monocytes are reported. Shen *et al.* [28] reported that IFN-γ down-regulates FcαR expression by monocytes, but Pfefferkorn & Yeaman [32] demostrated that IFN-γ up-regulates FcαR expression by U937 cells.

Recently, binding of IgA to rat MC was described by two different laboratories [33,34]. The binding characteristics differ markedly, suggesting the possibility that MC bear two distinct receptors for IgA. The function of these receptors on MC is not known fully as yet, but uptake and degradation of IgA aggregates by MC has been demonstrated [35]. Conceivably, therefore, these receptors serve to prevent accumulation of IgA-IC or aggregates in the glomeruli.

Since IL-6, TNF-α and IFN-γ are increased in IgAN and some models of glomerulonephritis, and TNF-α and IFN-γ are also known modulators of the expression of FcαR by human monocytes, we

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speculated that these cytokines could influence the accumulation of IgA in the mesangium and/or alter glomerular function via effects on FcαR expression by MC. Accordingly, we examined the influence of these selected cytokines upon IgA binding to and FcαR mRNA expression by MC in culture.

MATERIALS AND METHODS

IgA purification and iodination

Human monoclonal IgA1 protein, precipitated from the serum of a patient with multiple myeloma by 40% saturated ammonium sulphate, was re-dissolved in PBS, extracted in Seroclear Reagent (Calbiochem, La Jolla, CA), and dialysed extensively against Dulbecco's PBS (DPBS). Dimeric (d)IgA was obtained by iterative gel filtration on tandem Superose 6 ($30 \times 1{\cdot}5$ cm) columns (Pharmacia, Uppsala, Sweden), and purity and size were verified by SDS–PAGE. A portion of the dIgA was radiolabelled with ^{125}I by the chloramine T method [36] to a specific activity of $2{\cdot}5–5 \times 10^6$ ct/min per μg.

Control cells

The R1.1 (mouse lymphoma) cell line, negative for FcαR [37], was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. The human monocytic cell line U937, a kind gift from Dr M. L. Tykocinski (Case Western Reserve University, Cleveland, OH), was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). To induce FcαR expression, some cultures of these cells were stimulated with 10^{-7} M PMA (Sigma, St Louis, MO) for 12 h [26,27].

Mesangial cell culture

Different explant cultures of human MC, kind gifts from Drs J. R. Sedor and M. Simonson (Case Western Reserve University) and Dr L. Gesualdo (The University of Bari, Bari, Italy), were cultured in T-75 flasks and maintained as already described [9]. At confluency, MC were subcultured and used from passages 3 to 10 for different experiments. The cells were characterized as intrinsic MC using previously reported criteria [38]. Primary explant cultures of MC from glomeruli isolated from Sprague-Dawley rats and BALB/c mice were established as described [9,39].

Binding studies

Mesangial cells, cultured in Multiscreen-HA 96-well filtration plates (Millipore Corp., Bedford, MA), were made quiescent by serum starvation (0·5% FBS) for 24 h. Cells were then incubated in media containing human (h) recombinant (r) IL-6 (2 ng/ml; Genzyme Diagnostics, Cambridge, MA), TNF-α (2 ng/ml; Collaborative Biochemical Products, Bedford, MA) or IFN-γ (5 ng/ml; Collaborative Biochemical), or in medium alone, for up to 48 h. After incubation, the cells were washed with binding buffer (0·5% bovine serum albumin (BSA), 0·05% NaN_3/DPBS) and then incubated in this solution at 4°C for 30 min. Cells were incubated (in triplicate wells) with increasing concentrations of ^{125}I-dIgA in ice cold binding buffer at 4°C for 8 h, followed by rapid washing of the wells using a vacuum manifold (Millipore). Individual membranes were punched in the Multiscreen multiple puncher assembly (Millipore) and counted in a gamma spectrometer. In 13% of the wells, randomly selected, cells were fixed with 1% glutaraldehyde, permeabilized using 0·5% Triton X-100 (Boehringer Mannheim, Indianapolis, IN), and stained with haematoxylin (Zymed, San Francisco, CA) for direct counting. The data from these experiments were analysed by Inplot-4 software (GraphPad Software, San Diego, CA) using non-linear regression, and subjected to Scatchard analysis using Cricket Graph (Cricket Software, Inc., Philadelphia, PA).

Northern hybridization technique

Confluent serum starved (0·5% FBS for 24 h) MC in T-75 flasks were incubated in medium with or without cytokines, as above, for 3–48 h. Total RNA, isolated from cells using an RNA STAT-60 kit (TEL-TEST 'B', Inc., Friendswood, TX), was subjected to electrophoresis on a 1·2% agarose/formaldehyde gel, then blotted (by capillary action) onto a positively charged nylon membrane (Boehringer Mannheim). Membranes were air dried for at least 30 min, baked at 120°C for 30 min, and prehybridized and hybridized according to Ausubel *et al.* [40] to ^{32}P-labelled cDNA probe (pHuFcαR), a kind gift from Dr C. R. Maliszewski [26]. After stringent washing (1×5 min at room temperature in $2 \times$ SSC/0·5% SDS; 2×20 min at room temperature in $2 \times$ SSC/0·1% SDS; 2×20 min at 68°C in $1 \times$ SSC/0·1% SDS; 2×20 min at 68°C in $0{\cdot}1 \times$ SSC/0·1% SDS), the membranes were exposed to X-OMAT AR x-ray films (Eastman Kodak, Rochester, NY) at –80°C for 1–3 days.

Primers for reverse transcriptase-polymerase chain reaction

Synthetic oligonucleotides (Biosynthesis Inc., Lewisville, TX) served as nested 5′ and 3′ primers. The 3′ outer primer (3′-CTTGTGGTTCACAGACGTTC-5′) includes an antisense sequence (nucleotides 880–900), and the 5′ outer primer (5′-CCATGCCTTTCATATCTGCC-3′) includes a sense sequence (nucleotides 118–138) of the published FcαR plasmid sequence. The expected polymerase chain reaction (PCR) product of 782 bp has PstI and HindIII restriction sites, which were used for further identification. The 3′ middle primer (5′-CATCAA-CACCAGACCCCGATCTGCAGAGAG-3′) is an antisense sequence (nucleotides 420–450) and the 5′ middle primer (5′-GATCGGGGTCTGGTGTTGATG-3′) is a sense sequence (nucleotides 429–450) of the FcαR cDNA. As an internal control for reverse transcriptase (RT)-PCR, we used a set of primers for human β-actin (Stratagene, La Jolla, CA).

Analysis of FcαR mRNA using PCR

Total RNA (2 μg; prepared as described above) from each cell type was incubated in an RT reaction using the Gene Amp RNA PCR kit (Perkin-Elmer, Norwalk, CT) according to the manufacturer's instructions. The downstream Fcα receptor primer was used at 1 μM for reverse transcription. Cycles of PCR were performed after completion of RT by addition of the second primer at 1 μM. PCR products were resolved on a 1·2% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet light box (Fotodyne Inc., Hartland, WI).

RESULTS

Effect of cytokines on IgA binding to human MC

At 4°C, IgA binding to human MC is a monotonic function of IgA concentration (Fig. 1). Scatchard analysis of the binding data from five independent experiments using four different cell cutures indicated a single population of receptors, with an average Ka of $3{\cdot}2 \pm 0{\cdot}30 \times 10^7$ M^{-1} (Table 1). Quiescent cells, without cytokine stimulation, bear $2{\cdot}55 \pm 0{\cdot}36 \times 10^5$ sites/cell (Table 1).

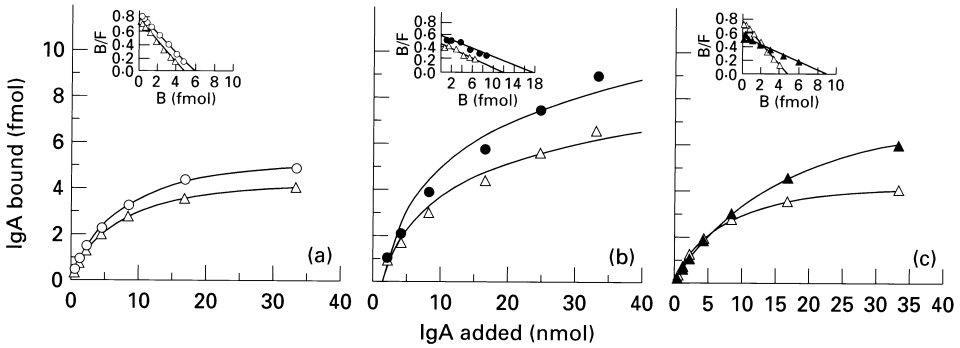

Fig. 1. Binding of ^{125}I-IgA to human mesangial cells (MC). In each panel, the binding to unstimulated cells (Δ) is repeated, and compared with cells stimulated with IL-6 (○, a), tumour necrosis factor-alpha (TNF-α) (●, b) or IFN-γ (▲, c). The Scatchard plots of each binding curve (inset to each panel) are linear (all $t > 12{\cdot}3$, all $P < 0{\cdot}001$ by linear least squares regression), indicating a single population of receptors in all cases. Although the apparent binding affinity varies somewhat between experiments (e.g. the cells stimulated with TNF-α and IFN-γ depicted in this single experiment differ from unstimulated), there was no systematic change in affinity over all experiments (see Table 1).

Preincubation of human MC with human recombinant cytokines (IL-6, TNF-α and IFN-γ) increased the binding of IgA to these cells (Fig. 1a,b,c). Although individual experiments differed to some degree, none of the cytokines significantly influenced Ka overall. However, MC stimulated with IL-6 and TNF-α for 48 h consistently showed at least a 60% increase in the number of sites per cell, whereas IFN-γ increased the number of sites per cell by 40% relative to control (Table 1). In a limited number of experiments, MC in suspension demonstrated binding comparable to that observed with adherent cells (data not shown).

Effect of cytokines on expression of FcαR mRNA in human MC

In order to assess the role of transcription of FcαR in increasing IgA binding to MC, we employed Northern hybridization. Stimulation of human MC with selected human recombinant cytokines (IL-6, TNF-α, or IFN-γ) increased steady state FcαR mRNA after a 48 h incubation (Fig. 2). Several time course experiments (Fig. 3) demonstrated that IL-6 most profoundly affected MC FcαR mRNA; a transient increase of three-fold peaking after 6 h decreased to nearly the control level at 16 h, followed by a second rapid rise in FcαR mRNA levels to almost six-fold at 48 h. TNF-α stimulation was similar to IL-6, but the first phase peaked earlier, at 3 h, and the second phase was both more gradual and less intense, reaching a level of four-fold that of control at 48 h. The MC were somewhat less responsive to IFN-γ; both the early (6 h) and the later (48 h) responses were only two-fold that of basal.

Table 1. Effect of 48 h culture with IL-6, tumour necrosis factor-alpha (TNF-α), IFN-γ on human mesangial cell (MC) FcαR expression*

Stimulus	Sites/cell† (10^5 molecules)	Affinity (Ka)† ($\times 10^7$) (M^{-1})
None ($n = 4$)	$2{\cdot}55 \pm 0{\cdot}36$	$3{\cdot}2 \pm 0{\cdot}3$
IL-6, 2 ng/ml ($n = 2$)	$4{\cdot}03 \pm 1{\cdot}38$‡	$4{\cdot}5 \pm 3{\cdot}1$
TNF-α, 2 ng/ml ($n = 3$)	$4{\cdot}13 \pm 0{\cdot}87$‡	$3{\cdot}3 \pm 0{\cdot}1$
IFN-γ, 5 ng/ml ($n = 2$)	$3{\cdot}60 \pm 0{\cdot}39$	$1{\cdot}4 \pm 0{\cdot}2$

*Human MC were grown in 96-well plates to confluence, and then serum-starved for 24 h. Cells were incubated in the presence or absence of cytokines for 48 h, and then binding studies using ^{125}|-IgA were performed as mentioned in Materials and Methods.

† Data are shown as mean ± s.e.m.

‡ Denotes a statistically significant difference ($P < 0{\cdot}05$) from control (no stimulus) by Fisher's least significant difference and by Dunnett's *t*-test under a one-way analysis of variance ($F = 3{\cdot}971$, with the stimulus as the independent variable). The affinity values were not significantly different ($F = 0{\cdot}413$) among the different stimulus conditions, although a relatively large variance was observed for IL-6 in the two experiments.

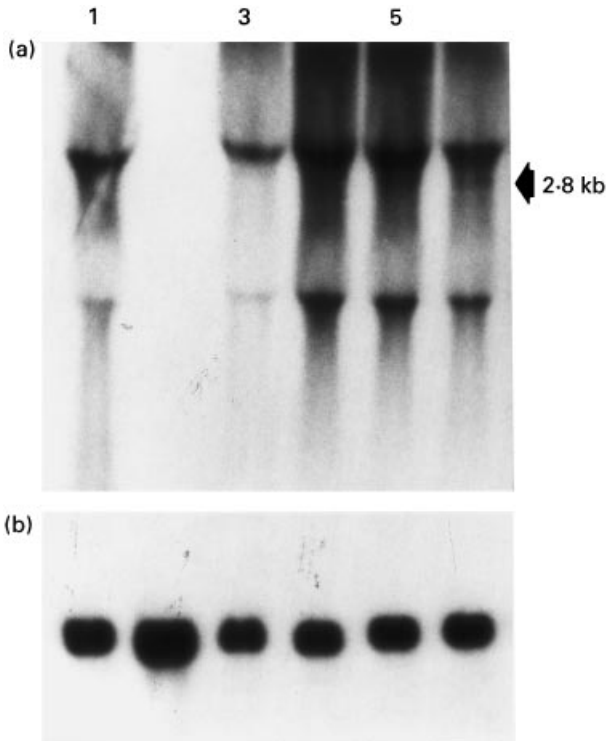

Fig. 2. Northern blot analysis. (a) The pHuFcαR probe hybridized to 30 μg of total RNA from phorbol myristyl acetate (PMA)-stimulated U937 cells (lane 1), but not to that from R1.1 cells (lane 2). Relative to the hybridization seen with RNA from unstimulated human mesangial cells (MC) (lane 3), there is increased signal in cells stimulated for 48 h with IL-6 (2 ng/ml, lane 4), tumour necrosis factor-alpha (TNF-α) (2 ng/ml, lane 5) or IFN-γ (5 ng/ml, lane 6). In both U937 cells and human MC, the transcript is recognized as a 2·8 kb band with a weaker signal at 2·6 kb. (b) The same blot was stripped and probed for GAPDH to control for equal loading of RNA in each lane.

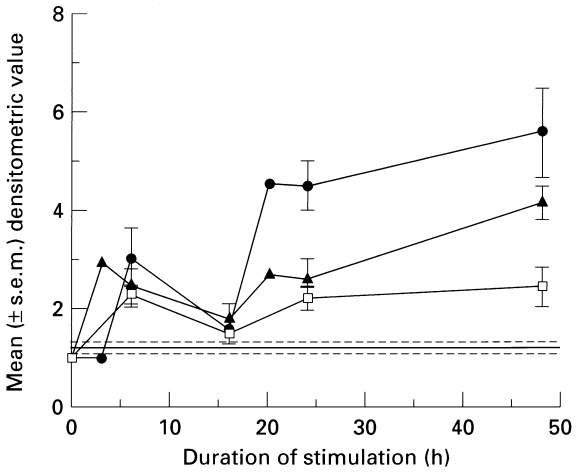

Fig. 3. Time course of FcαR mRNA expression in cultured human mesangial cells (MC) stimulated with selected cytokines. Densitometry values on the ordinate are normalized to the mean control value (unstimulated cells harvested at the beginning of each experiment indicated by the solid horizontal rule). The control level at all time points was within 12% of the value at zero time; the confidence interval for multiple determinations for unstimulated cells is indicated by the dashed horizontal lines. Data are expressed as mean ± s.e.m. of multiple independent experiments. The 3 h and 20 h observations were made in only one experiment, and hence bear no error bars. Multiple inferences were drawn from one-way analysis of variance ($F = 13{\cdot}2$) at 6, 24 and 48 h, and all stimuli elicited a significantly higher ($P < 0.01$) hybridization to the probe than the control value (referenced to stimulated cells harvested at 0 time) by Dunnett's *t*-test and Fisher's protected *t*-test. ●, IL-6; ▲, TNF-α; □, IFN-γ.

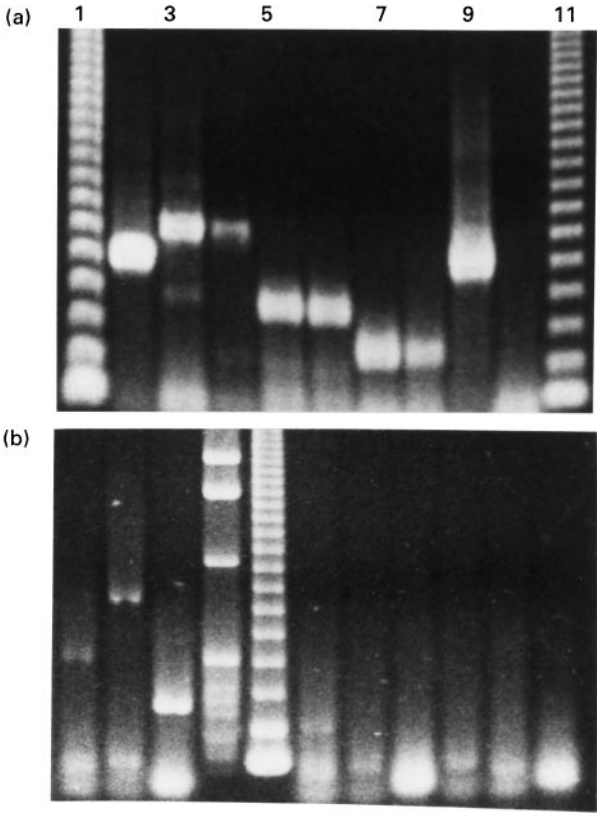

Fig. 4 Gel electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) products from RNA derived from U937 cells, human mesangial cells (MC), rat MC or mouse MC using three sets of primers for FcαR (see Materials and Methods). (a) Lanes 1 and 11 contain a 123 bp DNA ladder, and lanes 2 and 9 contain RT-PCR products of U937 and human MC, respectively, developed with primers for β-actin as an internal control for the reaction. Lanes 3, 5 and 7 contain RT-PCR fragments produced by RNA from U937 cells, lanes 4, 6 and 8 contain PCR fragments produced by RNA from human MC, and lane 10 contains RT-PCR products from R1.1 cell RNA as a negative control. Lanes 3, 4 and 10 were derived from amplification with the 3′–5′ outer primer pair; lanes 3 and 4 reveal the expected 782 bp product. Lanes 5 and 6 were derived after RT-PCR with the 3′ outer and 5′ middle primer pair, whereas lanes 7 and 8 were developed from RT-PCR with the 3′ middle and 5′ outer primer pair; the expected 470 bp and 332 bp products are seen with both U937 cells and human MC. (b) Lanes 1, 2 and 3 contain RT-PCR products from U937 cell RNA, lanes 4 and 5 contain size markers (1 kb and 123 bp ladders), lanes 6–8 are RT-PCR products from rat MC and lanes 9–11 are RT-PCR products from mouse MC using the same three sets of primers employed in (a). The expected amplificands observed with U937 cells are not seen with MC derived from rats or mice.

Partial analysis of FcαR mRNA sequence

Reverse transcription and PCR amplification of several preparations of RNA derived from human MC with the outer primer pair consistently yielded the expected 782 bp fragment, as seen with PMA-stimulated U937 cells (Fig. 4a, lanes 3 and 4). Interestingly, no amplificand was derived from total RNA from rat or mouse MC (Fig. 4b, lanes 6 and 9) or from R1.1 cells (Fig. 4a, lane 10) with this first set of primers (3′ – 5′ outer pair). With two additional sets of primers, spanning the open reading frame of FcαR cDNA from U937 cells, human MC produced the same appropriately sized fragments as U937 cells (Fig. 4a, lanes 5–8). RT-PCR of RNA from rat and mouse MC did not give rise to any of the expected fragments (Fig. 4b, lanes 7, 8, 10 and 11). Digestion of the 781 and 332 bp amplificands derived from human MC by PstI and/or HindIII produced the same pattern of cleavage bands as the corresponding amplificands derived from U937 cells (data not shown). These results strongly suggest that FcαR expressed in human MC is homologous to FcαR expressed in U937 cells. On the other hand, it seems that a different sequence exists for FcαR expressed on murine and rat MC.

DISCUSSION

The affinity, number of sites per cell, transcript size and restriction site content of the FcαR exhibited by human MC closely resemble those described for human monocytes and the U937 monocyte line. Our Northern hybridizations show a major band at 4 kb, with two minor bands at 2·8 and 2·6 kb, in constant proportions. Both U937 cells and human MC always showed the same banding pattern, which did not change with RNA loading, ranging from 10 to 30 μg per lane. Maliszewski *et al.* [26] previously reported a duplex of 2·6 and 2·8 kb after hybridization of mRNA from monocytes and granulocytes with FcαR cDNA. We speculate that the 4 kb bands in our blots are specific bands, as reported by Maliszewski *et al.* [26] for neutrophils. In addition, we observed a lower molecular weight band, probably a catabolic product of FcαR mRNA, as suggested by the existence of two AUUUA motifs in the 3′ untranslated region of the putative mRNA sequence for FcαR [41,42]. In RT-PCR with three different sets of primer pairs, human MC mRNA and U937 cells produced the same amplificands, with the predicted sizes and PstI and HindIII restriction sites, which suggests that the mRNA sequence of FcαR expressed on human MC is the same as that in U937 cells.

The doubling of the number of sites/cell in response to TNF-α that we observed in human MC also corresponds closely to the response to TNF-α by human monocytes and U937 cells [28,30]. Although IL-6 did not increase FcαR expression by monocytes or U937 cells at the protein or transcriptional level [28], IL-6 did stimulate MC expression of FcαR mRNA, and increased the protein detectable on these cells to a degree comparable to the effect of TNF-α on peripheral blood monocytes [30]. IFN-γ increased FcαR mRNA expression by MC (Figs 2 and 3), but its effect upon FcαR expression by monocytes was controversial [28,32]. Collectively, these observations suggest that the intracellular signals transduced in response to IL-6 and IFN-γ, and/or the regulatory response elements in the FcαR gene, differ in MC *versus* monocytes. Further examination of these issues will prove enlightening.

In this study, both IL-6 and TNF-α exerted a biphasic effect upon MC expression of FcαR mRNA. In fact, biphasic stimulation of several other genes, such as junB and ACTH, has been observed in response to IL-6 or TNF-α [43–45].

Previously, two different laboratories demonstrated FcαR expression by rat MC, but with differing IgA binding characteristics [33,34]. The discrepancies between these two groups are probably not based simply on technical differences. Possibly, distinct molecules, each capable of binding IgA with different specificity, are demonstrated by the two groups. Our observed binding of homologous IgA to human MC supports the view that the receptor described by us and Gomez-Guerrero *et al.* [33] is similar to the monocyte/macrophage FcαR. Although our binding parameters lie midway between those reported by the other two groups [33,34], we did not detect a change in the affinity of FcαR in MC after stimulation, as was seen with neutrophils responding to GM-CSF and G-CSF [31].

We did not observe the Northern hybridization of the pHuFcαR probe to RNA derived from rat MC, as reported by Gomez-Guerrero *et al.* [33], and none of the three primer pairs produced RT-PCR amplification products from rat or mouse RNA. Accordingly, it seems that genetic differences exist among MC FcαR. Presumably, homology between rat and human FcαR is sufficient to allow adequate Northern hybridization under the stringency conditions used by Gomez-Guerrero *et al.* [33], but not under our conditions, or with oligomeric primers in RT-PCR.

Although IL-6 was the most potent stimulus of steady-state RNA expression, followed by TNF-α and IFN-γ, IL-6 was no more effective than TNF-α upon the number of IgA binding sites; again, IFN-γ was relatively weak. These differences in response hierarchy cannot be ascribed to structural changes; a single class of receptor, with unchanged affinity, was observed regardless of the stimulus. The various stimuli may differentially affect apparent translational efficiency (via enhancers, RNA 'editing,' post-translational modification or offsetting differences in transcriptional rate and RNA stability), partitioning of protein within intracellular compartments, or the rate of protein catabolism [29,46]. For example, the protein that binds the AUUUA sequence present at two sites in the 3′ untranslated region of the FcαR may be differentially regulated by the different cytokines [41,42].

The function of FcαR on human MC is still unknown, but may contribute to the pathogenesis of IgAN. Aggregated IgA elicits MC release of inflammatory factors such as superoxide anion, platelet-activating factor (PAF), IL-6 and TNF [33–35,47,48], reminiscent of the monocyte/macrophage response to IgA-IC [13,21–25,49]. Thus, deposited IgA-IC may be phlogistic in the mesangial environment. Given the increased IL-6 and perhaps TNF-α production by MC in response to aggregated IgA [35], the capacity of these cytokines to stimulate FcαR expression by MC suggests a positive feedback cycle favouring disease progression. Alternatively, the FcαR on MC might promote the clearance of deposited IgA-IC from the glomeruli, since IgA aggregates are taken up and catabolized by MC over time [35]. In this context, the up-regulation of FcαR on MC might have a salutary effect in IgAN, whereas failure of mesangial clearance could favour accumulation of IgA-IC within glomeruli, and thereby promote disease progression.

Collectively, prior reports and the data herein suggest that alteration in the level of FcαR on MC is likely to ensue during the course of IgAN. In turn, changes in the extent of IgA binding to MC may be a pivotal element in the onset and/or course of this disease. Therefore, the regulation of FcαR expression by MC in response to cytokines, and the mechanisms whereby these receptors evoke mediator release from MC, need further elucidation.

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