Expression and physical association of Fcα receptor and Fc receptor γ chain in human mesangial cells

Yusuke Suzuki1,2, Chisei Ra2, Kan Saito2, Satoshi Horikoshi1, Shunji Hasegawa2, Toshinao Tsuge1, Ko Okumura2 and Yasuhiko Tomino1

1Division of Nephrology, Department of Medicine and 2Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Abstract

Background. Most intensive investigations on the pathogenesis of IgA nephropathy have focused on the process before IgA deposition and the characteristics of IgA/IgA immune complex (IgA IC), but it still remains uncertain whether mesangial IgA ICs may cause glomerular injuries directly or are only secondary events of another pathological process. To assess the role of IgA ICs in IgA nephropathy, we investigated the characteristics of Fcα receptor (FcαR) and FcR γ chain which is a signalling subunit of FcR in human mesangial cells (MCs).

Methods. Gene expression of FcαR and FcR γ chain of human cultured MCs was examined by RT–PCR and subsequent Southern blot analyses. Sequence analyses after subcloning were also performed for further confirmation. Expression of FcαR and FcR γ chain at the protein level and their physical association in MCs were determined by immunoprecipitation after stimulation of the cells with heat-aggregated IgA.

Results. Two distinct cDNA products were amplified from each cultured MC line. The sequence of the major product of ~900 bp was completely identical to that of FcαR previously described. The smaller product had a 288 bp deletion which corresponded to exon 2 encoding the extracellular domain 2 of FcαR. Gene expression of FcR γ chain was also confirmed. Furthermore, we proved the physical association of FcαR with the FcR γ chain by co-immunoprecipitation under stimulation with a high dose of the heat-aggregated IgA.

Conclusion. These findings suggested that polymeric IgA and/or IgA IC can directly activate MCs via FcαR associated with the γ chain. Our data also indicated that phenotypic variations of FcαR occur on MC, such as splicing forms, the γ chain association and/or the α chain expression itself, which may contribute to the pathogenesis of IgA nephropathy.

Key words: FcαR; FcR γ chain; IgA nephropathy; mesangial cells; physical association

Introduction

IgA nephropathy is found widely and is recognized as the most common form of primary glomerular disease throughout the world. In long-term follow-up studies of patients with IgA nephropathy, 25–40% of them progressed to renal failure within 20 years or more after initial discovery of the disease [1]. An understanding of the sequential events in the pathogenesis of IgA nephropathy, which is a key issue for development of successful therapy, is therefore essential. IgA nephropathy is characterized by predominant mesangial depositions of IgA/IgA immune complex (IgA IC) and elevation of the serum IgA ICs [2–4], and their contribution to pathogenesis has been suggested. Intensive investigations by several groups revealed the characteristics of glomerular or serum IgA in IgA nephropathy. It was confirmed that the glomerular IgA in patients with IgA nephropathy was predominantly anionic polymeric IgA1 [5–7]. Furthermore, it was shown that IgA in patients with IgA nephropathy had structural abnormalities [8].

Most of these studies focused on the process before IgA deposition (such as genetic and immunological background) and the characteristics of IgA ICs. It still remains uncertain whether mesangial IgA ICs may cause glomerular injuries directly, or are only a secondary event of some other pathological process. If IgA ICs had direct effects on glomerular injuries, they would appear to contribute to the pathogenesis by inducing complement activation firstly [9], as in the classical inflammatory cascade of type III (immune complex type) allergy.

On the other hand, mechanisms of allergic responses have been clarified by extensive studies on FcεR (IgE receptor) and FcγR (IgG receptor). In the development of this field, one of the most important findings is that the FcγR γ chain, which initially was discovered as a
subunit of the tetrameric high-affinity receptor complex for the Fc portion of IgE (FcεRI) in mast cells [10,11], is able to associate with other FcRs (FcγRI, FcγRIII, FceRI) [12–16]. Based on this finding, the signal transduction and function of immunoglobulins or ICs via FcRs could be analysed. It was reported that the expected inflammatory response in the reversed passive Arthus reaction was abolished despite an intact complement system in mice with a homologous disruption of the FcR γ chain gene [17]. Although complement activation by IC in type III allergy had been considered as the most important event, this study indicated for the first time that FcRs play critical roles in type III allergy. It has been revealed that FcRs which are expressed on glomerular infiltrating cells and intrinsic cells play critical roles in experimental glomerulonephritis, such as the anti-glomerular basement membrane (GBM) glomerulonephritis model or the New Zealand Black/New Zealand White F1 (NZB/W F1) model [18,19]. Since the same kind of mechanism is suggested for the initiation of immune complex-mediated glomerulonephritis such as IgA nephropathy, we focused on the characteristics of FcεRI which is expressed on MCs.

In the present in vitro study, we confirmed the co-existence of FcεRI and FcγR γ chain at the gene and protein levels and documented the characteristics of FcεRI and its physical association with the FcγR γ chain in these cells.

Materials and methods

Cells and cultures

We analysed MCs from four different donors, two primary cultured MCs, one fetal MC (FMC) and one commercially purchased MC (Clonetics, San Diego, CA). Primary cultured MCs from two different nephrectomy specimens were prepared as previously described [20]. FMC was kindly provided by Dr Kopp (NIDR, NIH, Bethesda, MD) [21]. All MCs were characterized morphologically by phase contrast microscopy, positive staining for vimentin, fibronectin, desmin and collagen type IV, and negative staining for factor VIII antigen and cytokeratin [22]. We used MCs at the 4th to 10th passage in RPMI 1640 medium buffered with 25 mM HEPES at pH 7.4 supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 5 μg/ml transferrin and 0.06 U/ml insulin (complete medium). A human monocytic cell line, U937 as control, was obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% FCS.

Reverse transcription (RT), polymerase chain reaction (PCR) and Southern blot analyses

Total cellular RNA from MCs and U937 cells was isolated by the single-step method. Total RNA (2 μg) was reverse transcribed using the SuperScript® Preamplification System (Gibco-BRL) in a reaction mixture containing 1× buffer, 10 mM dithiothreitol (DTT), dNTP (0.5 mM each), 0.5 μg of oligo(dT) primer, Escherichia coli RNase H and Superscript II. FcεRI and FcγR γ chain transcripts were amplified specifically by PCR using primers encompassing the entire FcεRI- and FcγRII chain-coding region (FcεRI sense primer, 5′-ATGGACCCCCAACCAGACCACTCTCTGTGTT-3′; antisense, 5′-TTACTTGCAGACCTTGGTGGTCT- GTGCAA-3′, FcγRI chain sense primer, 5′-ATGATTCC- CAGCATTGCTGTGCTTATGC-3′; antisense 5′-CTATCT GTGGTGGTTTCTCAGGCTAGT-3′) [23,24]. The reaction mixture underwent 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min using a thermal cycler (Perkin Elmer Japan, Applied Biosystems Division). Amplification of β-actin cDNA was also performed as an internal control. The resultant PCR products were analysed on 1.5% agarose gels, and the amplified bands were visualized by staining with ethidium bromide and transferred onto nylon filters for Southern hybridization. We generated each probe for Southern blot analyses by RT-PCR amplification with specific FcεRI and FcγR γ chain primers. These generated probes were also sequenced and confirmed to have the expected FcεRI and FcγR γ chain sequences. They were labelled with digoxigenin (Dig11-dUTP) (Boehringer Mannheim, Germany) using a random primed DNA labelling kit (Boehringer Manheim, Germany) according to the manufacturer’s instructions. The transferred membranes were prehybridized at 65°C for 20 min, and then incubated at 65°C for 15 h with alkaline phosphatase-conjugated anti-digoxigenin antibody and CSPD (DIG Luminescent kit: Boehringer, Anaheim, CA). The reaction was analysed by visual chemiluminescence according to the manufacturer’s instructions [25].

Sequencing of cDNA

Each visualized band was isolated from the gels with a SUPRECM®-01 Kit (Takara Biomedical, Tokyo, Japan). The purified PCR products were cloned into pCRTM II vector (Original TA cloning Kit; Invitrogen Corp., San Diego, CA) and sequenced using the Taq Dye Densit® Termination Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) by an automated DNA sequencer (Applied Biosystems: Model 373A). We analysed the cDNA sequence of 10 clones from different MCs.

Preparation of antibodies

We generated a mouse anti-human FcεRI monoclonal antibody (AAR1) [16] and rabbit anti-human FcγRII chain polyclonal antibody (anti-FcγRII chain antibody) by immunizing rabbits with a synthetic peptide of the FcγRII intracellular region (anti-FcγR monoclonal antibody) was kindly provided by Dr Shirasawa (Department of Molecular Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan).

Metabolic labelling

Before metabolic labelling, U937 cells (3–4×10⁶) as controls were pre incubated with phorbol 12-myristate 13-acetate (PMA; 10⁻⁷ mol/l) for 4 h and interferon-γ (IFN-γ) (400 U/ml) for 28 h to enhance FcεRI and FcγR γ chain expression [16,26]. MCs (2×10⁷) and U937 cells were starved in cysteine-free RPMI 1640 medium supplemented with 5% dialysed FCS and incubated for 3 h in a humidified CO₂ incubator (5% CO₂) at 37°C to purge intracellular pools.
of cysteine. After washing with phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 supplemented with 5% dialysed FCS, 35S-labelled cysteine (0.1 mCi/ml, American Radiolabeled Chemicals Inc., St. Louis, MO) and each stimulant (U937 cells: PMA/IFN-γ, MC: heat-aggregated IgA), and then incubated at 37°C for 20 h. Heat-aggregated myeloma IgA (Organon Teknika Co., Durham, NC) was prepared by heat treatment at 63°C for 150 min and added to stimulate MCs at the concentration of 10 μg/ml (low dose) and 500 μg/ml (high dose) for 20 h. U937 cells were stimulated with PMA (10−7 mol/l) and IFN-γ (400 U/ml).

**Immunoprecipitation**

The labelled cells were washed and lysed in the lysis buffer (1% digitonin/25 mmol/l Tris–HCl/150 mmol/l NaCl and protease inhibitors) on ice for 45 min. After centrifugation at 15 000 g at 4°C for 15 min, the supernatants of the cell lysates (MC; 2×107, U937 cells; 3–4×107) were pre-cleared with 30 μl of protein G–Sepharose 4B beads (Pharmacia P-L Biochemicals Inc., Milwaukee, WI) at 4°C for 3 h under constant rotation. The pre-cleared lysates were then subjected to immunoprecipitation with AAR1 (10 μg/100 μl sample), anti-FcR monochlonal antibody (50 μg/100 μl sample) or anti-Fcγ chain antibody (50 μg/100 μl sample). The immunoprecipitates were collected on protein G–Sepharose 4B beads (30 μg), washed with the digitonin buffer, and extracted from the beads by boiling in Laemmli sample buffer containing 2% SDS with or without 2-mercaptoethanol. They were analysed by SDS–PAGE (10–20% gradient gel; ACI Japan Co., Kanagawa, Japan). Molecular mass markers were purchased from Bio-Rad Laboratories (Hercules, CA).

**Results**

**Gene expression of FcR and FcR γ chain in MCs**

Two distinct cDNA products were amplified from U937 cells and each cultured MC with the FcR primers (Figure 1). RT–PCR products from MCs and U937 cells gave rise to a major band of ~900 bp, corresponding to the expected full-length FcR. The smaller cDNA products had a length of ~600 bp. Both of these PCR products from MCs were hybridized with a cDNA probe encoding the full-length FcR, indicating that these cDNA products are homologous to FcR on U937 cells. Several batches of MCs, even the same cloned MC but at different passages, preferentially expressed smaller products (Figure 1, lanes 4, 5, 6 and 8), in contrast to U937 cells (Figure 1, lane 1). However, we could not find significant correlation between these splicing patterns and the cell passages, even with further studies of other cloned MCs.

RT–PCR in combination with Southern blot analysis using a cDNA probe encoding the full-length FcR γ chain confirmed the gene expression of the FcR γ chain in most MCs in the same way as in U937 cells (Figure 2). The intensity of the hybridization signal with the FcR γ chain probe was different at different passages even in the same cloned MCs, as shown in Figure 2. There was no correlation between passage number and the signal intensity of FcR γ chain in all cloned MCs. Furthermore, no significant correlation was found between the intensity of hybridization signals for FcR and FcR γ chain.

**Sequence analyses**

We further subcloned the PCR products and sequenced them to confirm the gene expression of FcR in MCs. cDNA fragments from several clones (>3 of each MC) were purified from the agarose gel and subcloned into pCRTM II vector. The sequence of the major product of 873 bp was completely identical to the sequence of FcR cDNA previously described [23]. The products of the smaller band (585 bp) included a 288 bp deletion which corresponded to exon 2 encoding the extracellular domain 2 of FcR [27–29], suggesting that alternative splicing forms occurred in MCs.

The sequence of the RT–PCR products by FcR γ chain primers was also confirmed to be completely identical to that of γ chain cDNA previously reported [24].

**Characterization of the anti-human FcR antibody (AAR1)**

As shown in Figure 3, the binding of fluorescein isothiocyanate (FITC)-labelled AAR1 to FcR on CHO/FcR cells was inhibited by pre-treatment with IgA, in a dose-dependent manner. Pre-treatment with AAR1 was also able to inhibit IgA binding. Furthermore, this monoclonal antibody worked well in Western blot analyses and immunostaining. The
Fig. 3. Competition between AAR1 and human IgA for the binding to FcαR on CHO/FcαR cells. FACS analysis revealed that the binding of FITC-labelled AAR1 to FcαR was inhibited by pre-treatment with IgA in a dose-dependent manner.

characterization of this antibody will be described in detail elsewhere (K. Saito et al., in preparation).

Expression and physical association of FcαR and FcRγ chain in MCs

MCs and U937 cells were metabolically labelled with [35S]cysteine and lysed with a non-ionic detergent, digitonin, which is capable of maintaining some non-covalent protein interactions. The lysates were subjected to immunoprecipitation with AAR1 and anti-FcRγ chain antibody, and the immunoprecipitates were resolved by SDS–PAGE (10–20% gradient gels). From the lysates of MCs treated with a low dose of heat-aggregated IgA (10 μg/ml) as well as from those of untreated MCs, these antibodies did not immunoprecipitate protein species at the predicted size of FcαR or FcRγ chain (data not shown). In contrast, from the lysates of MCs treated with a high dose of heat-aggregated IgA (500 μg/ml), anti-FcRγ chain antibody co-immunoprecipitated the 50–70 kDa FcαR glycoprotein and 20 kDa FcRγ chain under unreduced conditions (Figure 4A, lane 8). A 10 kDa protein (FcRγ chain monomer) was detected, with disappearance of the 20 kDa protein under reduced conditions (Figure 4C, lane 5), which is a characteristic of FcRγ chain homodimers. However, AAR1 did not immunoprecipitate the FcRγ chain nor FcαR from the same lysates (Figure 4A, lane 6). We confirmed that AAR1, which recognizes the IgA-binding site of FcαR, could not bind to FcαR when a high dose of heat-aggregated IgA occupied FcαR, probably because the affinity of AAR1 is lower than that of aggregated IgA. Therefore, we further tried immunoprecipitation with anti-FcαR polyclonal antibody, which recognizes the intracellular domain of FcαR, and succeeded in co-immunoprecipitating FcαR and FcRγ chain under the same conditions at the predicted size (Figure 4B, lane 4). The 10 kDa protein under reduced conditions was also detected, with disappearance of the 20 kDa protein recognized as FcRγ chain homodimers (Figure 4C, lane 6).

Discussion

FcRs are composed of multiple subunits in which ligand-binding and signal-transducing functions are localized in distinct subunits [30]. The FcRγ chain is a transmembrane protein in the form of a disulfide-linked homodimer, shown to have an ITAM (immunoreceptor tyrosine-based activation motif) containing two tyrosine residues within the cytoplasmic region, which are phosphorylated by tyrosine kinases on receptor engagement and are critical for FcR functioning [30,31]. Furthermore, the FcRγ chain is essential for surface expression of FcεRI and the low affinity IgG receptor, FcγRIII-A [31]. In contrast to these receptors, transfection experiments indicated that the IgA receptor, FcαR, does not require FcRγ chain for membrane expression [23]. However, it was reported that FcαR on U937 cells associated with the FcRγ
Fig. 4. Expression and physical association of FcεR and FcR \( \gamma \) chain in human MCs. U937 cells and MCs were metabolically labelled with \(^{35}\)S-cysteine in the presence of IFN-\( \gamma \)/PMA or a high dose of heat-aggregated IgA and solubilized with lysis buffer containing 1% digitonin (see Materials and methods). The cell lysates were immunoprecipitated with AAR1, anti-FcR \( \gamma \) chain antibody, anti-FcεR polyclonal antibody or control antibodies. (A) The immunoprecipitates with AAR1 and anti-FcR \( \gamma \) chain antibody were subjected to SDS–PAGE (10–20% gradient gels) under unreduced conditions. Lanes 1 and 5, mouse IgG1 (G1); lanes 2 and 6, AAR1 (\( \alpha \)); lanes 3 and 7, normal rabbit serum (N); lanes 4 and 8, anti-FcR \( \gamma \) chain antibody (\( \gamma \)). (B) The lysates were precipitated with anti-FcεR polyclonal antibody (\( p\alpha \)) in lanes 2 and 4, and normal rabbit serum (N) as controls in lanes 1 and 3, under unreduced conditions. (C) FcR \( \gamma \) chain under reduced conditions. The same lysates as in (A) or (B) were subjected to SDS–PAGE under reduced conditions. Lanes 1 and 4, AAR1 (\( \alpha \)); lanes 2 and 5, anti-FcR \( \gamma \) chain antibody (\( \gamma \)); lanes 3 and 6, anti-FcεR polyclonal antibody (\( p\alpha \)).

It was reported previously that MCs possessed a receptor that specifically bound IgA through the Fc region [32]. In this study, we confirmed the presence of FcεR in MCs at the gene and protein levels and showed that the cDNA sequences of FcεR from MCs were completely identical to the published sequences [23]. Furthermore, we detected two distinct FcεR transcripts by RT–PCR amplification of RNA from cultured MCs and following Southern blot analyses. The nucleotide sequence of the larger PCR product of 873 bp was matched completely by that of the cDNA which was described previously in myeloid cells, corresponding to the expected full length. Sequence analyses of the smaller PCR products revealed deletions of 288 nucleotides, which were produced by alternative splicing of exon 2 encoding extracellular domain 2 [27]. Indeed, this splicing site is observed at the last nucleotide of exon 1 and at the first nucleotide preceding the transmembrane/cyttoplasmic domain, resulting in the complete lack of exon 2 transcript in the smaller PCR products. The existence of several FcεR isoforms has been described in neutrophils, monocytes and alveolar macrophages [27,28]. In this study, Southern blot and sequence analyses of the PCR-amplified products from MCs provided evidence of the existence of at least two FcεR isoforms also in MCs. It was interesting that in Southern blot analyses, the smaller products caused
by the deletion of extracellular domain 2 of FcαR exhibited stronger signals than the longer products from the same cloned MCs. Because extracellular domain 2 is the binding domain for IgA, it is suggested that transcriptional regulation may work in MCs and contribute to the biological and/or pathological response to IgA or IgA IC binding. Further experiments are required to determine this transcriptional regulation in MCs.

Despite this clear evidence for the presence of FcαR on MCs, expression of FcεR on MCs seemed to be down-regulated with no stimulation. Without any stimulation, the amount of FcεR transcripts of MCs was small in comparison with that of U937 cells, and it was difficult to detect expression of FcεR by Northern blot and immunoprecipitation in MCs at rest. FcγRIII also seems to be expressed on MCs in the same manner [33]. The stimuli which enhance FcεR expression in MCs are still unclear, except for a few reports suggesting IgA itself [32] or cytokines [34]. In our experiments, IFN-γ, which is known to promote FcεR expression in U937 cells [16], and each of the other cytokines tested failed to enhance FcεR expression in MCs. Although we could detect surface expression of FcεR on MCs by fluorescence activated cell sorting (FACS) analyses upon stimulation with several cytokine combinations, this expression was very unstable. In addition, we have preliminary evidence that a mouse anti-human FcεR monoclonal antibody (AAR1), which may recognize the IgA Fc-binding site of FcεR, was capable of enhancing the gene expression of FcεR in MCs. However, these effects on FcεR expression were dependent on clones of MCs. Although further study will be needed to determine the mechanisms of FcεR up-regulation, a Spanish group recently demonstrated Ca2+ mobilization in MCs by aggregated IgA via FcεR, which was not affected by pre-incubation with galactose and N-acetyl-galactosamine, and hypothesized that sugars might not play an important role in some functional events triggered by aggregated IgA [35]. These data suggested that IgA binding to FcεR and subsequent cross-linking of the receptor may increase the expression of FcεR on MCs independently of the molecular structure of IgA (e.g. glycosylation state).

The present study also demonstrated the gene expression of the FcγR γ chain in MCs. The nucleotide sequence of the FcγR γ chain of MCs was completely identical to that of myeloid cells. We further confirmed the physical association of the FcγR γ chain homodimer with FcγR on MCs for the first time. FcγRIII expression on cytokine-stimulated MCs was demonstrated [33] and its association with the FcγR γ chain was also suggested because FcγRIII requires the FcγR γ chain for its membrane expression. Although the FcγR γ chain is not essential for the cell surface expression of FcγR [23], a transfection experiment with FcεR and FcγR γ chain on B cells demonstrated that signal transduction through the FcγR also required the functional association with the FcγR γ chain [36]. If IgA IC is able to trigger some biological functions of MCs directly via FcεR, physical association with a signalling molecule will be required for FcεR on MCs. Our immunoprecipitation results using a high dose of the heat-aggregated IgA indicated that polymeric IgA and/or IgA IC could activate MCs directly via FcεR associated with the γ chain. Indeed, this is in good agreement with previous reports on FcεR-mediated triggering of both proximal (tyrosine phosphorylation and intracellular Ca2+ mobilization [35]) and distal (cytokine production [37,38]) signal transduction responses in MCs, which were induced strongly by aggregated IgA [37]. We could detect the functional association of the FcεR–FcγR only upon treatment with a high dose of aggregated IgA. It was reported recently that the binding affinity of aggregated IgA for FcεR depends on the extent of its polymerization (aggregation) [39]. A high dose of aggregated IgA may contain relatively more optimal aggregated IgA which efficiently up-regulates the FcεR expression on MCs after its binding. It is likely that we could detect the FcγR γ chain association with FcεR only when FcεR was expressed on MCs in a sufficient amount. FcεR on MCs may work functionally in the limited situation.

We hypothesized that the occurrence of polymeric IgA in the glomerular mesangial areas, regardless of the mechanisms of deposition, circulating IC or its in situ formation with multivalent antigens, may induce cross-linking of FcεR on MCs and activate the signalling cascade mediated by the FcγR γ chain, which triggers a variety of cellular effector functions (e.g. cytokine production, cell proliferation). It has been shown that the glomerular IgA deposits in patients with IgA nephropathy are mostly polymeric in nature, suggesting a contribution to the pathogenesis of this disease.

In this study, we characterized the expression of FcεR on MCs and suggested the mechanisms of mesangial response to glomerular IgA ICs via FcεR on MCs. Furthermore, it is also important that phenotypic variations of IgA receptors occur on MCs, because it is possible that the phenotypic variations (differences in quantities of several splicing forms, or of the γ chain association and/or the α chain expression itself) may contribute to the pathogenesis of IgA nephropathy. For an understanding of the molecular mechanisms of IgA nephropathy, further studies will be required to ascertain the physiological or pathological factors, including genetic background and organ specificity, which enhance the expression of FcεR on MCs selectively. Characterization of FeRs which are expressed on other intrinsic glomerular cells or infiltrating cells are also of importance.

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