Pathogenic role of (S)IgA in IgA nephropathy

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In the middle of every difficulty lies opportunity'

-Albert Einstein

CONTENTS

ABBREVIATIONS

Aa	amino acid
ASGPR	asialoglycoprotein receptor
BSA	bovine serum albumin
CHO	chinese hamster ovary
CTB	cholera toxin B
DC	dendritic cells
Dig	digoxygenin
dIgA	dimeric IgA
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
HMW	high molecular weight
IgA	Immunoglobulin A
IgAN	IgA nephropathy
IL	interleukin
kDa	kilo dalton
KLH	keyhole limpet hemocyanin
mAb	monoclonal antibody
MAC	membrane attack complex
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
MFI	mean fluorescence intensity
MIF	macrophage migration inhibitory factor
mIgA	monomeric IgA
NHMC	normal human mesangial cells
NHS	normal human serum
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SC	secretory component
SDS-PAGE	sodium dodecyl sulphate- poly acrylamide gel eletrophoresis
SIgA	secretory IgA
SNP	single nucleotide polymorphism
SPR	surface plasmon resonance
TGF	transforming growth factor
TNF	tumor necrosis factor

General Introduction

CHAPTER 1

Clinical presentation of IgA nephropathy

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease shows a broad spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA1 in the glomerular mesangium (1-3). In the glomeruli of patients with IgAN mostly high molecular weight IgA1 is detected, sometimes together with IgM and/or C3 (4,5). After renal transplantation recurrent mesangial IgA deposition is observed in 50 % of the patients (6). Case reports have shown that IgA deposits disappear after transplantation of a kidney with IgA deposits into a non-IgAN patient (7). These results strongly suggest that IgAN is not only a disease of the kidney, but also dependent on systemic factors.

Interestingly, patients with IgAN often present macroscopic hematuria following upper respiratory tract infections. Mucosal immune challenge leads to an increased production of IgA in the systemic compartment, probably based on the migration of B cells (the mucosa-bone marrow axis) (8). This mucosa-bone marrow traffic has been confirmed by challenging healthy individuals intranasally with the neoantigen cholera toxin subunit B (CTB) (9). In patients with IgAN a mucosal IgA hyporesponse to mucosal immunization with this neoantigen was observed (9).

Since differences in circulating IgA together with IgA binding to mesangial cells have been proposed to play an important role in the pathogenesis of IgA nephropathy, detailed analysis of circulating IgA and its interactions with cellular receptors is important.

Histopathology of IgA nephropathy

The most common histological lesion seen in renal biopsies from patients with IgAN are focal or diffuse mesangial proliferative glomerulonephritis (10). The initial phase of IgAN is characterized by increase in mesangial matrix but no segmental sclerosis. Focal proliferative lesions comprise the largest subgroup of IgA nephropathy. The histologic changes range from focal and segmental mesangial proliferative glomerulonephritis to focal glomerulonephritis with segmental endocapillary cell proliferation, with or without crescent formation. Associated with these variable expressions of glomerular pathology are variable degrees of tubular atrophy, interstitial fibrosis, and interstitial inflammation comprised of lymphocytes, monocytes/macrophages, and plasma cells. The number of macrophages in the glomeruli correlates with the presence of crescents and proteinuria (11). Furthermore, more monocytes and T-cells were found in biopsies of patients with active disease as compared to those without disease activity (12).

Immunohistochemistry has revealed that IgA deposits mainly consist of the IgA1 subclass (13,14), and commonly occur with co-deposits of C3, IgG, and, less common, IgM (15). The predominance of IgA1 deposits and the specific hinge region of IgA1 with potential O-linked glycosylation sites have initiated a directed search for alterations in glycosylation. Indeed, in the eluate of renal deposits, a specific reduction of O-linked galactosylation has been observed (16,17). Furthermore, with size fractionation of eluted proteins from kidney sections, it was shown that deposited

IgA is mostly high molecular weight of nature (18). Additional evidence for the deposition of high molecular weight IgA was obtained from immunohistochemical analysis of renal tissue (14,19,20).

Composition of IgA

IgA is the most abundant class of immunoglobulin synthesized in humans, with 66 mg of IgA/kg of body weight produced daily compared to 34 mg of IgG and 8 mg of IgM. The half life of IgA in the circulation is 6 days. Almost all circulating IgA (2 mg/ml) is produced in the bone marrow and the liver is involved in the catabolism of the circulating IgA. Only negligible amounts (1 mg/ kg bodyweight/ day) of the total IgA produced in the bone marrow, spleen and lymph nodes (20 mg/ kg bodyweigth/ day) reach the external secretions (21). The other part of the IgA (46 mg/ kg bodyweight/ day) is produced at the mucosal sites and is secreted efficiently as secretory IgA (SIgA).

General composition

Human IgA exists as two isotypes, IgA1 and IgA2, with IgA2 having two allotypic variants: IgA2m(1) and IgA2m(2). The human IgA subclasses differ at 14 amino acid (aa) positions in the α-chain sequence. α-Chains have three constant region domains Cα1 to Cα3 (Figure 1A). IgA2 of the A2m(2) allotype differs from A2m(1) and IgA1 in 6 positions; 2 in Cα1 and 4 in Cα3. The Cα3 domain is the same for IgA1 and IgA2m(1). The Cα2 domain is the same for both A2 allotypes and differs from IgA1.

A major difference between IgA1 and IgA2 occurs in the hinge region. IgA2 molecules lack a 13-aa segment found in the hinge region of IgA1 molecules, which contain 5 potential O-linked carbohydrate sites.

Serum IgA

Human IgA in serum exists with an IgA1: IgA2 ratio of about 9:1 (22,23) and is found in different molecular forms: monomeric IgA (mIgA), composed of two heavy and two light chains; dimeric IgA (dIgA), consists of two IgA molecules linked with a joining (J-) chain. Finally, in serum also additional high molecular weight forms of IgA can be recognized, generally described as polymeric IgA (pIgA). The composition of human serum polymeric IgA is diverse and may include CD89/IgA complexes, IgA immune complexes and IgA-fibronectin complexes (24). In humans, circulating IgA primarily consists of monomeric IgA (mIgA), and only 10-20% of the IgA is found in high molecular weight IgA (dIgA and pIgA) forms. In contrast, in rodents IgA is mostly present in a high molecular weight form (25).

Secretory IgA

Secretory IgA (SIgA) is the major immunoglobulin responsible for protecting the mucosal surfaces. To generate SIgA, dimeric IgA with the attached J-chain is produced in plasma cells close to the epithelium. The epithelial cells express on the basolateral side the polymeric Ig receptor (pIgR) that binds to dIgA; this complex is translocated through the epithelial cell (transcytosis). During transcytosis the extra-

cellular part of the pIgR, the secretory component (SC), is covalently linked to dIgA. At the mucosal surface the secretory component is cleaved from the pIgR and secretory IgA is secreted. Besides the presence of SIgA in the mucosa, low levels (10 µg/ ml) of SIgA can be detected in serum (Figure 1B) (26,27).

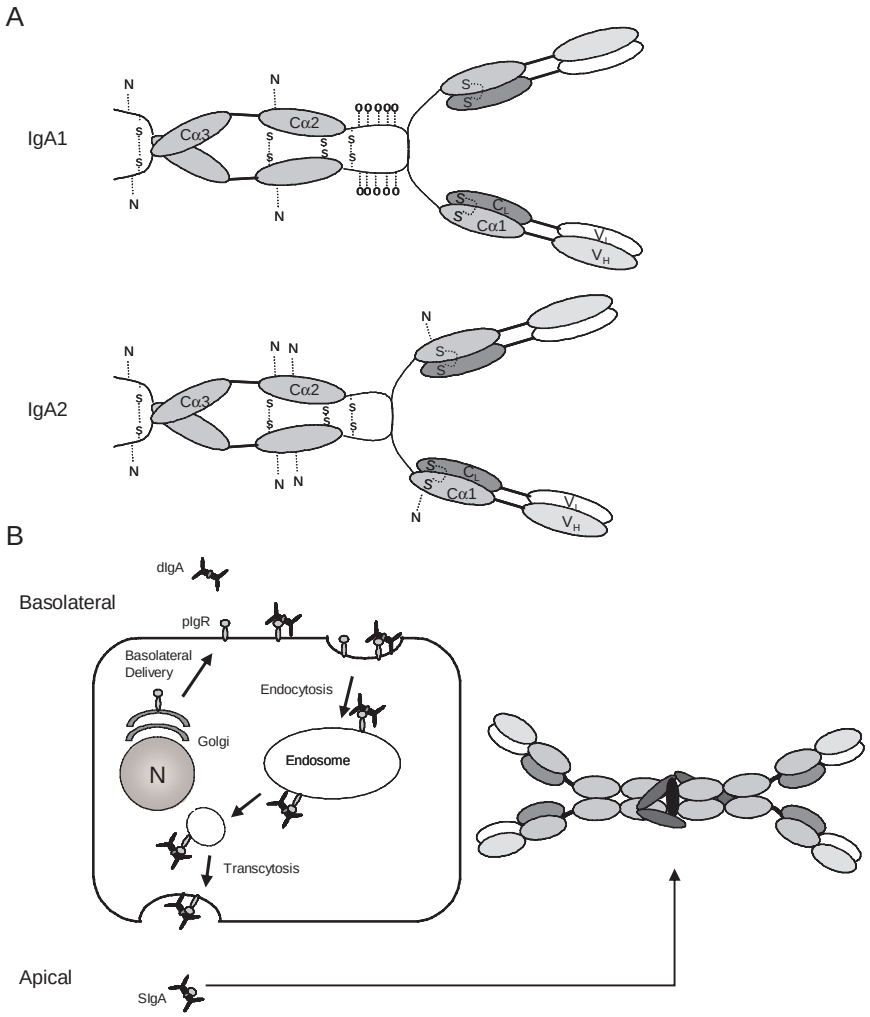

Figure 1: proposed domain structure of human monomeric IgA
A) The α heavy chain contains 3 constant domains Cα1, Cα2 and Cα3 and 1 variable domain VH. The light chain contains 1 constant domain CL and 1 variable domain VL. Positions of disulfide bonds (S), N- (N) and O-(O) linked glycosylation sites are indicated. (adapted from (30)). B) Pathway of pIgR through an epithelial cell (adapted from (104))

Glycosylation of serum IgA

Glycans contribute 6 to 7 % of the total molecular mass of IgA1 and 8 to 10% of the total mass of IgA2 proteins. The higher carbohydrate content in IgA2 proteins is the result of additional N-linked oligosaccharide side chains (28). Human IgA1 contains two conserved N-glycosylation sites in each α-chain (Asn263 and Asn459), while the IgA2 subclass contains an additional two (IgA2m(1)) or three (IgA2m(2)) conserved N-glycans (29). The number, the type and the terminal sugar residues vary between proteins of IgA subclasses but also within one subclass (Figure 2) (30). Serum IgA contains complex type N-linked carbohydrate moieties. Biantennary structures accounted for 86 % of the N-linked glycans on IgA whereas 14 % of the oligosaccharides were multiantennary or extended (31).

IgA1 is one of the few serum proteins and unique among circulating immunoglobulins in having O-glycosylation as well as N-glycosylation sites. These O-glycosylation sites are restricted to the hinge region of IgA1, which contains four to five short chains. The O-glycans are relatively simple sugars in which N-acetylgalactosamine (GalNAc) is O-linked to a serine or threonine residue. The glycan is completed with a terminal galactose (Gal) with or without additional sialic acid residues (NeuNAc) (Figure 2).

Glycosylation of SIgA

The glycosylation of SIgA is different compared to that of serum IgA in several aspects (Figure 2). Modelling of SIgA suggests that the N-glycans on the heavy chain can be masked by the SC (32). This may also result in a different exposure of the O-glycans. Moreover, specific analysis of the glycosylation of the IgA heavy chain present in SIgA, demonstrated different N-glycan structures compared to that of serum IgA. Specifically, terminal GlcNAc residues are present on the majority of the N-glycans of SIgA (32). The O-glycans on the hinge region of the heavy chain of SIgA1 presented a wide range of glycan structures, of which the major part is now characterized (32). Finally, also SC itself is heavily glycosylated.

The J chain (16 kDa) contains a single carbohydrate side chain linked to asparagine. This N-linked glycan is approximately 8 % of the molecular mass of J chain. This chain consists of fucose, mannose, galactose, N-acetylglucosamine and sialic acid. The N-glycan appears to be critical to polymer formation between J chain and IgA monomer subunits (Figure 2) (33).

Free secretory component (SC) was isolated from mucosal secretions as well as associated with SIgA. SC (70 kDa) consists of five immunoglobulin-like domains with approximately 22 % of the total molecular mass of SC contributed by carbohydrates. The 5 to 7 site chains contain N-acetylglucosamine, fucose, mannose, galactose and sialic acid, N-glycosidic linked to the protein backbone (Figure 2) (34).

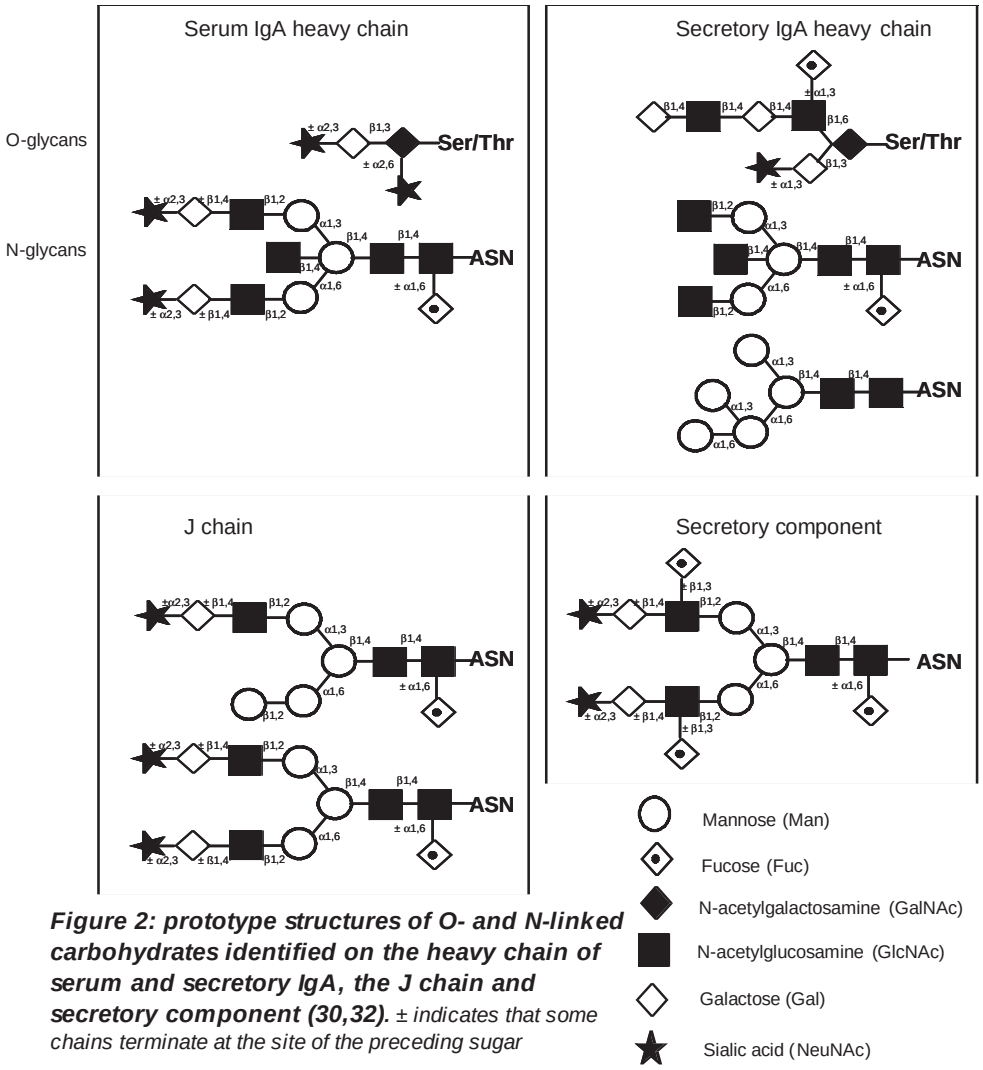

Figure 2: prototype structures of O- and N-linked carbohydrates identified on the heavy chain of serum and secretory IgA, the J chain and secretory component (30,32). *± indicates that some chains terminate at the site of the preceding sugar*

Effector functions of IgA

IgA plays an important role in providing protection at mucosal surfaces. Passive protection by SIgA, secreted by the mucosal immune system, plays a central role in the protection of mucosal surfaces in general. Mechanisms of protection by SIgA at mucosal surfaces are: inhibition of adherence (SIgA appears to surround a microbe and other particulate antigens with a hydrophilic shell that repels attachment to a mucosal surface), agglutination, mucus trapping (SIgA diffuses freely through mucus (35)), neutralization of enzymes and toxins, and interaction with innate antimicrobial factors. On the other site there is increasing evidence that serum IgA is able to trigger effector functions that have the potential to destroy micro-organ-

isms, including: interaction with the complement pathway (although the level of activation differs between isotypes), interaction with Fc receptors on leukocytes, and epithelial cells (Figure 3).

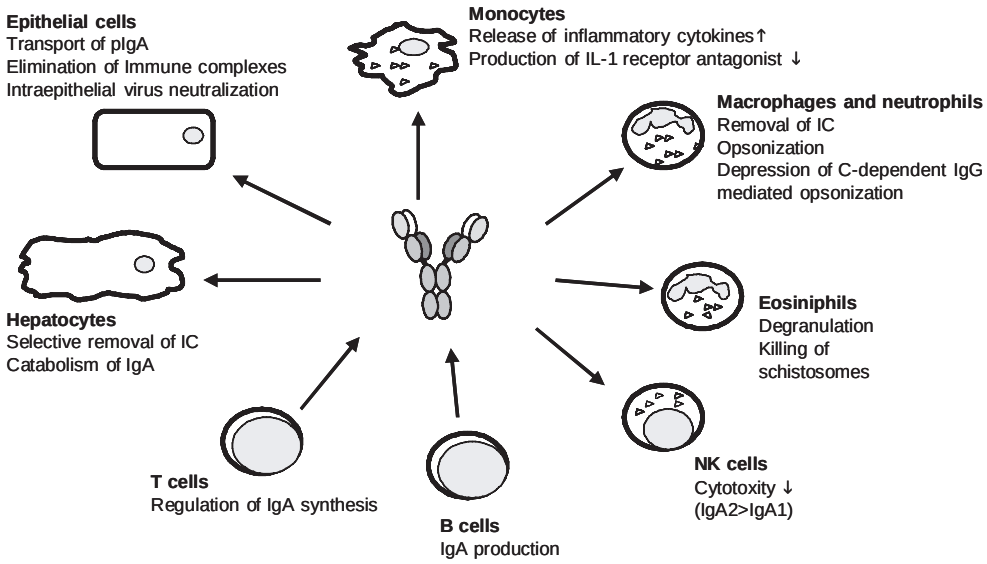

Figure 3: Biological consequences of the interaction of IgA with various cell types (adapted from (30)).

Cells of the myeloid lineage (neutrophils, eosinophils, monocytes, and macrophages) express CD89 through which these cells can be activated by IgA. B cells produce IgA, whereas T cells are important for the regulation of the IgA production. The interaction of IgA with Natural Killer (NK) cells may be mediated by lectin-like receptors for carbohydrate determinants. Epithelial cells transport dIgA to the apical surface where it release SIgA. Hepatocytes are important in the clearance of IgA from the circulation.

Complement activation

The complement system is a key component of our innate immune system and is comprised of a complex of at least 30 proteins and regulators. The liver is the main source of complement synthesis. The complement molecules constitute approximately 5 % of the total serum proteins. Three principle pathways are involved in complement activation, the classical pathway, the alternative pathway and the lectin pathway, each with their own recognition mechanism. These pathways converge at the central component of the complement system, C3. The final common pathway leads to the formation of a protein complex on a complement-activating surface, named the membrane attack complex (MAC) (Figure 4). IgA can activate complement via the alternative pathway and the lectin pathway (36,37). The lectin pathway can be activated via the recognition molecules mannose-binding lectin (MBL), H-ficolin and L-ficolin. IgA can interact with MBL and thereby activate the lectin pathway as demonstrated by activation and deposition of C4 (37), whereas for the ficolins no IgA binding data are available yet.

IgA receptors

Different IgA receptors are described in literature (Table 1). These receptors belong to two major families of receptors, namely the Ig superfamily and the lectin family. The known receptors for IgA in the Ig superfamily are the FcαRI (CD89), the Fcα/μR and the polymeric Ig receptor. The polymeric Ig receptor is present on epithelial cells and is important for the transcytosis of IgA to the mucosal surfaces (38). The known receptors for IgA in the lectin family are the asialoglycoprotein receptor (ASGPR) and the mannose receptor. The ASGPR is present on hepatocytes and is important for clearance of IgA (39). The ligand specificity for ASGPR is terminal galactose. The other IgA receptor in the lectin family, the mannose receptor, is present on dendritic cells and macrophages, and can bind and internalize SIgA without inducing maturation in dendritic cells (40). This binding of SIgA to the mannose receptor is sugar dependent and can be blocked with mannose, fucose and N-acetylglucosamine.

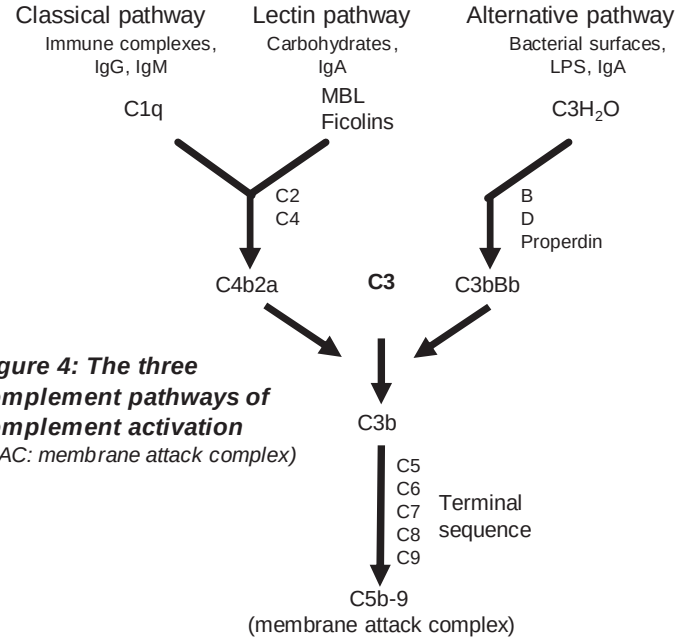

Figure 4: The three complement pathways of complement activation
(MAC: membrane attack complex)

FcαRI (CD89)

FcαRI (CD89) is an IgA receptor, which is constitutively expressed on polymorphonuclear leukocytes (PMN), monocytes, eosinophils, and selected macrophages (41). CD89 is also expressed on Kupffer cells in the liver. It was suggested that CD89 on Kupffer cells provides a second line of defence in mucosal immunity (42). Initially it has been suggested that CD89 might be an IgA receptor at the surface of mesangial cells (43), however it is now widely accepted that CD89 is not expressed by mesangial cells (44-47). CD89 consists of two extracellular Ig-like domains with potential N- and O-linked glycosylation sites, followed by a stretch of hydrophobic

amino acids representing the predicted transmembrane domain, with a positively charged arginine which is essential for association of CD89 with the FcR γ-chain homodimer-signalling subunit (48), and a short cytoplasmic tail devoid of recognition signalling motifs (Figure 5). The protein core of CD89 has a predicted molecular mass of 30 kDa with differential glycosylation at six potential N-linked sites, and the probability of additional O-glycosylation contributing to the variable size observed for the mature receptor, 55-75 kDa on monocytes and neutrophils, 70-100 kDa on eosinophils. The site of interaction between CD89 and IgA was identified in the first extracellular domain of CD89 (49,50) and in the Cα2/Cα3 junction of IgA (51,52).

CD89 participates in different aspects in host defence. CD89 induces phagocytosis of IgA complexed antigens (53), initiates antibody-dependent cellular cytotoxity (54) and CD89 is important for the clearance of IgA from the circulation (48). Upon activation, a soluble form of CD89 is released from the surface of monocytes and monocytic cell lines (55). These soluble CD89 molecules circulate in a complex, covalently linked with IgA, in the high molecular weight fractions of serum IgA (56). Binding studies with different molecular forms of IgA have shown that pIgA binds better to CD89 than mIgA (54,57,58). Furthermore one study suggested that SIgA can only interact with CD89 if MAC-1 (CD11b/ CD18) is present (59).

To study the role of the IgA-CD89 interaction mouse models were used. Although CD89 is described on human myeloid cells, no murine homolog has yet been defined. Therefore transgenic mouse models have been created, including a model in which the CD11b promoter was used (60). In this model human CD89 was highly expressed on macrophages/ monocytes. These transgenic mice develop spontaneously massive mesangial IgA deposits after 12 weeks, suggesting a role for CD89 in IgA nephropathy (60).

Table 1: Receptors with IgA binding capacities

	Receptor	*Ligand*	*Cellular distribution*
Ig family	FcαRI (CD89)	pIgA> mIgA	Myeloid cells
	Fcα/μR	IgA/ IgM	B cells,Macrophages,Mesangial cells
	pIgR	dIgA	Epithelial cells
C-type lectins	ASGPR	IgA	Hepatocytes
	Mannose receptor (CD205)	SIgA	Macrophages,Dendritic cells
	Transferrin receptor (CD71)	IgA1	Mesangial cells

Fcα/μR

The Fcα/μR, located on chromosome 1, is a newly identified receptor for IgA. Transcription of the receptor is demonstrated in several tissues including thymus, spleen (B cells and macrophages, but not on granulocytes, T cells or NK cells), liver, kidney, small and large intestines, testis and placenta (61,62). Furthermore, transcription of the Fcα/μR was described on mesangial cells and was upregulated after stimulation of mesangial cells with IL-1 (63).

The Fcα/μR is a type 1 transmembrane protein with a 32-aa leader sequence, a

423-aa extracellular domain, a 20-aa transmembrane domain and a 60-aa cytoplasmic region. The extracellular domain has four potential sites for NH_2-linked glycosylation (61), leading to a mature protein of 60- 70 kDa. In the extracellular domain of the receptor cysteine residues are identified and it is flanked by the consensus sequence for immunoglobulin-like domains, indicating that this molecule is a member of the immunoglobulin super family. The Fcα/μR shows no significant homology with other proteins. However, in the immunoglobulin like domain there is a motif that is conserved in the first immunoglobulin like domain of the polymeric Ig receptor (Figure 5) (61).

The Fcα/μR mediates endocytosis of immune complex composed of Staphylococcus aureus and IgM anti-S. aureus antibody by primary B lymphocytes (61). The underlying mechanism of this internalization is not yet known. However, experiments with Fcα/μR mutants suggest that the di-leucine motif is important in this process (61). Furthermore, the Fcα/μR acquires the ability to bind IgM and IgA antibodies after stimulation of B cells.

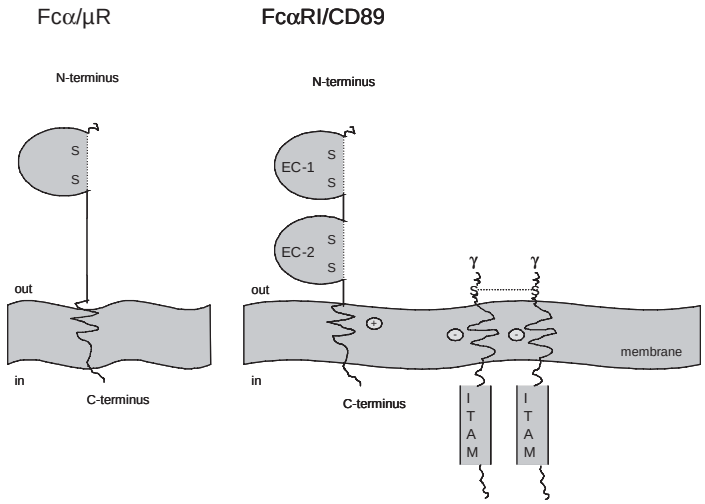

Figure 5: structure of Fcα/μR and of CD89 with the γ-chain.
The extracellular, membrane and cytoplasmic domains of the Fcα/μR and CD89 as well as the γ-chain with its signalling motifs are depicted.

IgA receptors and mesangial cells

The binding of high molecular weight IgA is better to mesangial cells than that of monomeric IgA. However the specific mechanism for the binding and retention of IgA1 remains uncertain. Several findings point to an IgA-specific receptor(s) on mesangial cells (41,64). However none of the known IgA receptors (CD89, ASGPR, pIgR) is expressed on mesangial cells (45,47). Two other receptors have recently emerged as candidate receptors for binding IgA; CD71 (transferrin receptor) and the Fcα/μR (63,65,66). CD71 expression is enhanced in the glomeruli of IgA nephropathy patients and co-localizes with IgA1 deposits. Mesangial cells bind both IgA sub-

classes, whereas CD71 binds only IgA1, suggesting involvement of an additional receptor. Theoretically, it may be the Fcα/μR that can be transcribed by mesangial cells in vitro (63). In contrast, another group found that polymeric IgA1 from IgA nephropathy patients induces macrophage migration inhibitory factor (MIF) and TNF-α in mesangial cells. This induction is probably through an unidentified IgA receptor, as shown by failure to suppress IgA-induced MIF synthesis by blocking IgA receptors with specific antibodies or various ligands to IgA receptors (67).

IgA in IgA nephropathy

Several studies using lectin interactions (Table 2) and fluorophore-assisted carbohydrate electrophoresis (FACE) focused on the analysis of IgA glycosylation, showing aberrant O-glycosylation in circulating IgA from IgAN patients, resulting in increased Tn antigen (GalNAcβ1-Ser/Thr) residues (68-71). This undergalactosylated IgA1 may lead to recognition by IgG antibodies and generation of circulating IgG-IgA1 complexes (72). Furthermore, altered interaction with mesangial cells has been described (73). This aberrantly O-glycosylated IgA is suggested to be dependent on a hampered function of the β1-3 galactosyltransferase (74). Furthermore, it is suggested that downregulation of the β1-3 galactosyltransferase chaperone (Cosmc) is important for the aberrant O-glycosylation in patients with IgAN (75).

Table 2: Reactivity of lectins used for analysis of IgA glycosylation

Name	*Abbreviation*	*Sugar specificity*
Artocarpus integrifolia	Jacalin	Galβ1-3GalNAc
Helix aspersa	HAA	GalNAc
Vicia villosa	VV	GalNAc
Helix pomatia	HPO	GalNAc
Erythrina crystagalli	ECL	Galβ1-4GlcNAc
Ricinus communis	RCA	βGal
Sambucus nigra	SNA	NeuNAc
Peanut agglutinin	PNA	Galβ1-3GalNAc

Levels of plasma IgA1 are elevated in about half of the patients with IgAN (76-78), which appears to be the result of an increased production of this isotype by the bone marrow (79-82) and by a low elimination rate by the liver. Mucosal pIgA plasma cell numbers are normal or even reduced in IgAN (83,84), whereas pIgA antibody levels in mucosal secretions are not elevated and are sometimes lower than controls (9). Furthermore, systemic antigen challenge results in increased titers of circulating pIgA1 antibodies (85,86) with normal levels in mucosal secretions (87).

IgA and mesangial cells

Functional studies with purified IgA from IgAN patients showed that IgA from IgAN patients binds better to mesangial cells than IgA healthy individuals (73),

although this is still controversial (88). The binding of polymeric and aggregated IgA to mesangial cells was stronger as compared to monomeric IgA. Moreover, polymeric IgA with the highest net negative charge is superior in binding to mesangial cells (73). In IgAN circulating aberrantly glycosylated IgA1 has been described. To mimic this IgA, IgA1 was purified with Jacalin and in vitro degalactosylated. The removal of galactose residues from IgA1 isolated with Jacalin increases binding to mesangial cells in vitro (89).

The activation of mesangial cells by IgA1 immune complexes is considered the initiating event in the pathogenesis of IgA nephropathy. Mesangial cell activation was observed in vitro in many instances (90-93). Exposure of mesangial cells to IgA is capable of initiating a proinflammatory cascade involving mesangial cell secretion of IL-1β, TNF-α , IL-6, TGF-β and MIF and the release of the chemokines MCP-1 (CCL2), IL-8, and IP-10 (91,94-97). After stimulation of mesangial cells with degalactosylated IgA the production of these factors is higher as compared to control IgA. In vivo, urinary IL-6 (98), the tubular and interstitial expression of intercellular adhesion molecule type 1 (99), and the intrarenal expression of proinflammatory cytokines and chemokines (100) correlated with renal injury and may have prognostic value.

IgA is also capable of altering mesangial cell-matrix interactions by modulating integrin expression, and this could have an important role in remodeling of the mesangium following glomerular injury (101). There is also evidence that activation of mesangial cells by co-deposited IgG could synergistically contribute to the development of a proinflammatory mesangial cell phenotype and thereby influence the degree of glomerular injury (102). It is not yet clear which specific physicochemical properties of mesangial IgA affect mesangial cell activation; however, there is some in vitro evidence that undergalactosylated IgA glycoforms from patients with IgAN reduce proliferation, increase nitric oxide synthesis and the rate of apoptosis, and enhance integrin synthesis in cultured mesangial cells (101,103). This, together with the overrepresentation of aberrantly glycosylated IgA1 in mesangial IgA, suggests that IgA1 O-glycosylation plays a role in both the deposition of IgA and the subsequent injury.

Scope of this thesis

For a better understanding of the role of IgA and mesangial cells in IgA nephropathy, we focused on different questions in the course of the disease. In chapter 2 and 3 we focused on the possible receptor mechanisms underlying mesangial IgA deposition. Therefore we studied the interaction of IgA with CD89 in different binding assays. We showed a similar association to CD89 for monomeric and polymeric IgA (chapter 2). CD89 is described not to be present on mesangial cells, whereas the recently identified Fcα/μR is suggested to be expressed by mesangial cells. To investigate the role of the Fcα/μR in IgA nephropathy we produced fusion proteins of this receptor and used these fusion proteins for IgA binding studies (chapter 3).

Because it is suggested that IgAN is not only a disease of the kidney, but also dependent on systemic factors we investigated in chapter 4 to chapter 8 which changes in IgA lead to the deposition of IgA in the glomeruli. Therefore, we investi-

gated in chapter 4 the activation of the lectin pathway of complement via IgA in glomeruli of IgAN patients. We show that activation of the lectin pathway of complement in the glomeruli of patients with IgAN is associated with more severe renal disease. In chapter 5 we isolated IgA from patients and controls and separated this IgA in monomeric and polymeric IgA. With these IgA preparations we investigated the differences between monomeric and polymeric IgA between patients and controls including the interaction with lectins and mesangial cells. In this study we observed clear differences between monomeric and polymeric IgA for lectin and mesangial cell interactions, but there were no differences between patients and controls. However, the concentration of SIgA in the polymeric IgA preparations was significantly higher in patients as compared to controls. This suggests that only a minor part of the IgA from patients might be different from controls. Finally, we demonstrated that SIgA is able to bind stronger to mesangial cells than serum IgA, and that SIgA is present in glomerular IgA deposits (chapter 6). To confirm the presence of SIgA in the glomerular IgA deposits we studied the presence of SIgA in biopsies from IgAN patients. We showed in chapter 7 that in 15 % of the cases SIgA is detectable in the glomerular IgA deposits. Finally, chapter 8 summarizes the studies described in this thesis and discusses the relevance of these new findings.

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CHAPTER 2

Monomeric and polymeric IgA show a similar association with the myeloid FcαRI/CD89

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Summary

IgA is found in both mucosal secretions and serum and is the dominant immunoglobulin isotype produced in humans. It exists in different molecular forms, namely monomeric IgA, dimeric IgA, polymeric IgA and secretory IgA, all exhibiting interactions with FcaRI/CD89 to some extent. CD89 is an activating, γ-chain associated, Fc receptor for IgA expressed on myeloid cells. Here, we investigated the interaction of monomeric and polymeric IgA purified from human serum with CD89 using surface plasmon resonance. The results demonstrate a similar association for monomeric and polymeric IgA with CD89. In contrast, monomeric IgA dissociated more rapidly from CD89 than polymeric IgA. Removal of N-glycans from mIgA resulted is an increased association with CD89, whereas the dissociation was more rapid, resulting in binding comparable to that of untreated monomeric IgA. We conclude that the initial interaction of monomeric and polymeric IgA with CD89 is similar, whereas monomeric IgA dissociates more rapidly from CD89. In view of the large excess of monomeric IgA in serum, monomeric IgA will compete for CD89 interaction with polymeric IgA, thereby preventing cell activation initiated by receptor aggregation contributing to the anti-inflammatory role of IgA.

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INTRODUCTION

Immunoglobulin A (IgA) is the predominant immunoglobulin isotype, and plays a critical role in protecting the host against environmental pathogens and antigens encountered at mucosal surfaces (1). In secretions, secretory IgA (SIgA) is generated during transcytosis of dimeric IgA (dIgA) by epithelial cells, ultimately leading to its association with the extracellular part of the polymeric Ig receptor (secretory component) (2). In humans, IgA in the circulation primarily consists of monomeric IgA (mIgA), but about 10-20% of the IgA is found in dimeric or polymeric IgA (pIgA) forms, while in rodents IgA is mostly present in a polymeric form (3). Furthermore, IgA consists of two subclasses namely IgA1 and IgA2. IgA1 contains ten potential O-glycosylation sites and two N-glycosylation sites and IgA2 does not contain O-glycosylation sites but contains two or three additional N-glycosylation sites. In vitro deglycosylation of IgA leads to self-aggregation, suggesting that underglycosylation of IgA may contribute to generation of high molecular weight IgA (4).

The transmembrane molecule FcαRI (CD89) has been identified as receptor for the Fc portion of human IgA (5). This receptor is constitutively expressed on polymorphonuclear leukocytes (PMN), monocytes, eosinophils, dendritic cells and a subset of macrophages (5-7), as well as on Kupffer cells in the liver, where it has been suggested to provide a second line of defence (8). CD89 consists of two extracellular Ig-like domains followed by a stretch of hydrophobic amino acids representing the predicted transmembrane domain, with a positively charged arginine which is essential for association of CD89 with the FcR γ-chain homodimeric signalling subunit (9), and a short cytoplasmic tail devoid of recognition signalling motifs. The protein core of CD89 has a predicted molecular mass of 30 kDa with differential glycosylation at six potential N-linked sites, and the probability of additional O-glycosylation contributing to the variable size observed for the mature receptor (55-110 kDa) (10). CD89 has been reported to bind both IgA1 and IgA2 with similar affinity (Ka ~10^6 M-1). The site of interaction between CD89 and IgA was identified in the first extracellular domain of CD89 (11,12) and in the Cα2/Cα3 junction of IgA (13,14).

CD89 participates in different aspects in host defence. CD89 induce phagocytosis of IgA complexed antigens (15), initiates antibody dependent cellular cytotoxity (16) and CD89 is important for the clearance of IgA from the circulation (9). Binding studies with different molecular forms of IgA have shown that pIgA binds stronger to CD89 than mIgA (16-18).

Furthermore, one study suggested that secretory IgA (SIgA) can only interact with CD89 if MAC-1 is present (19). For most of these binding studies total IgA was purified from serum as a source for mIgA followed by production of pIgA by an artificial aggregation process (17). Alternatively, mIgA was generated from SIgA via a chemical treatment (16).

The purpose of present study was to study the interaction of mIgA and pIgA in their physiological conformation with CD89 in a quantitative manner. Therefore, these two biological forms of IgA were directly isolated from serum and their interaction with CD89 was studied by ELISA and surface plasmon resonance. Furthermore, we studied a potential role for IgA glycosylation in the differential interaction of the molecular forms of IgA with CD89. The results indicate that the initial

interaction of mIgA and pIgA with CD89 is comparable, but since mIgA dissociates more rapidly from CD89, the final outcome is that pIgA remains associated with CD89 for an extended time, thereby potentially increasing its signalling potency.

MATERIAL AND METHODS

IgA purification

Serum from 6 individuals was used for IgA purification, according to methods described before (20). In brief, serum was applied to an anti-IgA (HisA 43, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) affinity column. The column was washed with 0.5 x PBS and 0.3 M NaCl, and subsequently IgA was eluted with 0.1 M glycine/ 0.3 M NaCl (pH 2.8). Directly after elution the fractions were neutralized with 1 M Tris pH 8.0. The eluted protein fractions that contained IgA, as assessed by ELISA (21), were pooled and dialysed against PBS containing 2 mM EDTA.

Isolated IgA was size-separated with a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands) into polymeric IgA and monomeric IgA. These pools were analysed for total IgA content using ELISA (21).

Secretory IgA that was used in some experiments was obtained from Sigma.

ELISA

Binding of IgA to Fc-$(CD89)_2$ was analysed by coating ninety-six well Nunc Maxisorb microtitre plates (Gibco/Invitrogen, Carlsbad, CA) with Fc-$(CD89)_2$ (2 µg/ml) in carbonate buffer (pH 9.6) (100 µl/well) overnight at room temperature. After coating, the plates were washed with PBS/ 0.05 % Tween. Plates were incubated with different concentrations of IgA in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgA was detected using mouse anti-human IgA (4E8), followed by HRP-conjugated goat anti-mouse IgG (Dako, Heverlee, Belgium), both diluted in PBS/ 1 % BSA/ 0.05 % Tween and incubated for 1 hour at 37 ºC. Enzyme activity of HRP was developed using ABTS (2, 2'-azino-bis (3-ethyl benzathioline-6-sulphonic acid)) (Sigma, St. Louis, MO). The O.D. at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, USA).

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis was carried out using a BIAcore instrument (Biacore AB, Uppsala, Sweden). A CM5 sensor chip (BIAcore AB) was coupled with recombinant CD89 (10000 response units (RU)), chimeric Fc-$(CD89)_2$ (10000 RU), and BSA (10000 RU), following manufacturers' instructions. Binding assays were performed at flow rates of 5 µl/min using HBS EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20). A 10 µl aliquot of analytes was injected and subsequently allowed to dissociate for 10 minutes. Subsequently, the surfaces were regenerated with 0.1 M glycine/ 0.3 M NaCl, pH 2.8. Early binding was defined as the response units at 450 seconds, and late binding was defined as the response units at 1100 seconds.

Another CM5 sensor chip was coupled with BSA (200 RU) and chimeric Fc-$(CD89)_2$ (250 RU). Kinetic assays were performed at flow rate of 30 µl/min and 180 µl IgA was injected. Data collected for each experiment were analyzed in a bivalent model using biaevaluation software (mIgA 180 kDa and pIgA 360 kDa).

Glycosidase treatment of IgA

Monomeric IgA (800 µg) was first digested with N-glycosidase F (PNGase F), cloned from *flavobacterium meningosepticum* and expressed in *E. coli* (6 U/ 200 µg IgA, Roche) in 0.02 M sodium phosphate buffer pH 7.2 with 2 mM EDTA. The first sample, to be used as control, was IgA with incubation buffer (mock IgA); the second sample was IgA with enzymes (treated IgA). After 72 hours of incubation at 37ºC this IgA was further digested with Neuraminidase from *Arthrobacter ureafaciens* (6 mU/200 µg IgA, Roche, Mannheim, Germany),β (1-3,4,6) galactosidase isolated from a cloned gene expressed in *E. coli* (7 mU/ 200 µg IgA, Prozyme), or combinations of the enzymes. This deglycosylation step was performed in 50 mM sodium acetate pH 5.6 for 72 hours at 37ºC. After treatment, samples (10 µg) were analysed by loading on a 10 % reduced SDS-PAGE gel and Coomassie stained.

HAA ELISA

To confirm deglycosylation, the deglycosylated IgA was assessed for binding to biotinylated *Helix Aspersa* (HAA, Sigma) lectin, known to recognize terminal GalNAc, was performed. Ninety-six well NUNC Maxisorp microtitre plates were coated with 2 µg/ml IgA in carbonate buffer (pH 9.6) (100 µl/well), overnight at room temperature. After washing with PBS/ 0.05 % Tween and blocking for one hour 37 ºC with PBS/ 1 % BSA, the plate was incubated with 5 µg/ml biotinylated HAA in PBS/ 1 % BSA/ 0.05 % Tween. Binding of HAA to IgA was detected with HRP-conjugated streptavidin (Zymed). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. was measured at 415 nm.

Western blot analysis

IgA preparations (3 µg) were subjected to 10 % SDS-PAGE under reducing conditions, followed by semi-dry blotting to PVDF (immobilin-P, Millipore, Bedford, MA). Blots were blocked for 2 hours in PBS/ 0.1 % Tween/ 5 % BSA. Blots were subsequently incubated with 2 µg/ml biotinylated HAA in PBS/ 0.1 % Tween/ 2.5 % BSA overnight at 4ºC. After washing with PBS/ 0.1 % Tween, blots were incubated with HRP-conjugated streptavidin (Zymed) for 2 hours at room temperature. After extensive washing bands were visualized with Supersignal (Pierce Chemical Co., Rockford, IL) and exposure to HyperfilmTM films (Amersham Pharmacia).

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed rank test. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Dose dependent binding of IgA to Fc-$(CD89)_2$ in ELISA

To investigate the interaction of different molecular forms of IgA with CD89, IgA was purified from serum using a monoclonal anti-IgA affinity column and the eluate was size fractionated by gel filtration (Figure 1A). Pools containing mIgA or pIgA were obtained and assessed for the binding to Fc-$(CD89)_2$ in ELISA (Figure 1B). The binding of IgA was dose-dependent over a wide range of concentrations. Binding of pIgA to CD89 was much stronger than that of mIgA, the latter requiring a

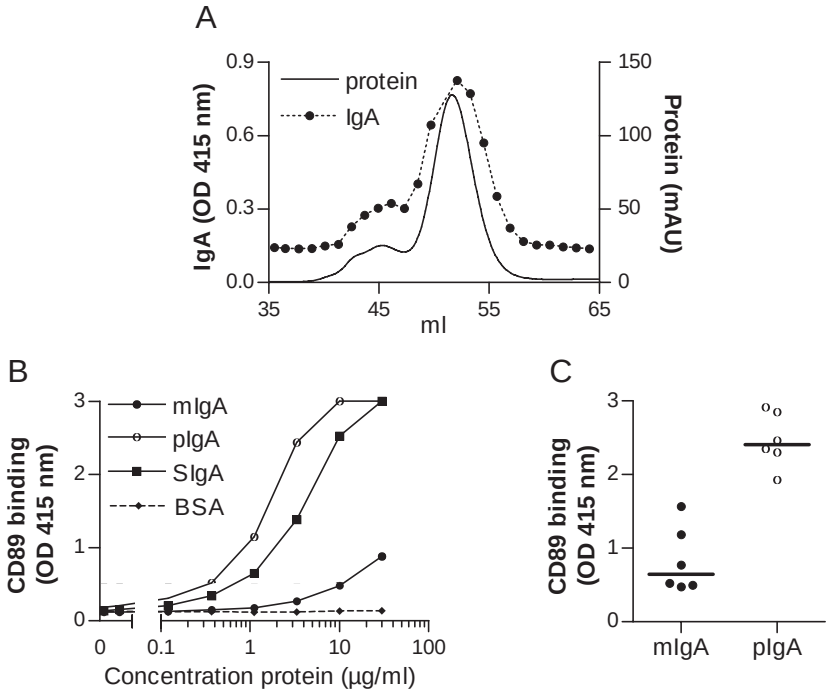

Figure 1: Binding of pIgA and SIgA to CD89 in ELISA is stronger than that of mIgA.

A) IgA was affinity purified and size-fractionated on a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA by ELISA. B) Isolated IgA was pooled in pIgA fraction (39-47.5ml) and mIgA fraction (47.5-57ml). Binding of mIgA, pIgA and SIgA to immobilized Fc-(CD89)$_2$ was measured in ELISA. C) From 6 individuals, IgA was purified and size fractionated as above. The binding of IgA (10 µg/ml) to Fc-(CD89)2 was measured in ELISA.

50-fold higher concentration to reach the same level of binding. We have reported earlier that in serum low concentrations of SIgA are present (22). Therefore we also tested the binding of SIgA to CD89. The binding of SIgA to CD89 was similar to that of pIgA, whereas it was much stronger than the binding of mIgA.

Using the same procedure, mIgA and pIgA were isolated from serum of 6 different individuals and tested for binding to Fc-(CD89)$_2$ in ELISA at a fixed concentration (Figure 1C). In all cases, pIgA bound stronger to Fc-(CD89)$_2$ compared to mIgA.

Difference in IgA binding is determined by dissociation from immobilized CD89 and not by association

The interaction of mIgA or pIgA with CD89 was evaluated using a biosensor. Two channels of a CM-5 sensor chip were coupled with recombinant CD89 and chimeric Fc-(CD89)$_2$. A third control channel was coupled with BSA. Monomeric IgA and polymeric IgA exhibited strong binding to both CD89 (not shown) and Fc-(CD89)$_2$

(Figure 2). The association of pIgA and mIgA to Fc-$(CD89)_2$, was similar while the dissociation was more rapid for mIgA compared to pIgA which led to a higher late binding level for pIgA (Figure 2A). SIgA binding was assessed as well and it was found that the association of SIgA to CD89 was lower compared with mIgA and pIgA. The binding of SIgA remained stable, comparable to that of pIgA (Figure 2A).

Similar results were obtained from IgA preparations from 6 different individuals (Figure 2B). The profiles of the binding of IgA isolated from 6 different individuals to Fc-$(CD89)_2$ were also analysed for the early (450 s) and late binding (1100 s). Monomeric and polymeric IgA, applied at identical concentrations, showed similar early binding to Fc-(CD89)2 (Figure 2B, C). The late binding assessed by SPR, in agreement with the measurements in ELISA, showed a stronger binding of pIgA to CD89 compared to mIgA (Figure 2B, C).

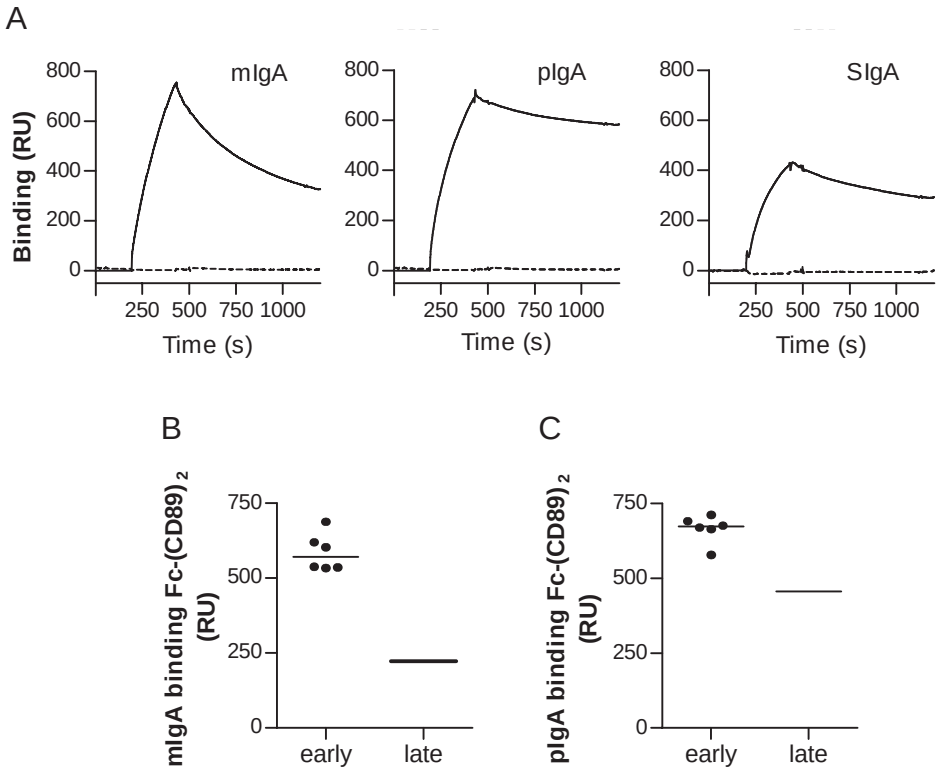

Figure 2: Real time binding of IgA to CD89.

A) Binding profile of mIgA (200 µg/ml), pIgA (200 µg/ml), isolated from serum, and SIgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. From 6 individuals, IgA was purified and size fractionated as above. The binding of mIgA B); (200 µg/ml) and pIgA C); (200 µg/ml) to Fc-$(CD89)_2$ was measured with SPR at two different time points (early (450 s) and late (1100 s) binding). Results represent response units of binding to Fc-$(CD89)_2$ after subtraction of response units obtained with BSA.

To determine the binding constants of the interaction of IgA with CD89, another CM-5 sensor chip was coupled with a low concentration of Fc-$(CD89)_2$ and BSA, and the binding of different concentrations of mIgA and pIgA was measured. To investigate the dissociation constants (kd) for mIgA and pIgA we made use of a bivalent model with a molecular mass of 180 kDa for mIgA and 360 kDa for pIgA. As presented above the dissociation of mIgA was more rapid compared with pIgA. Kinetic analysis showed a lower affinity for mIgA (kd = $3.9x10^{-4}$) compared to pIgA (kd = $2.6x10^{-4}$). These dissociation constants explain why pIgA ultimately remains associated with CD89 for a longer period, and also provide a rationale for the higher binding of pIgA as compared to mIgA to CD89 in ELISA.

Binding of deglycosylated IgA to CD89 is not changed while association is increased

Results presented above demonstrate that the interaction between pIgA and CD89 is more stable than the interaction between mIgA and CD89. Since previously published results suggest that underglycosylation promotes the production of polymeric IgA (4), and since the glycosylation of IgA has been implicated in its interaction with CD89 (23), we postulated that the glycosylation differences between monomeric and polymeric IgA could account for their differential interaction with CD89. Furthermore, underglycosylated IgA has been shown in serum of IgA nephropathy patients (24,25). This patient-derived IgA shows enhanced exposure of terminal GalNAc on O-linked glycans, which can be recognized by a GalNAc-specific lectin, *Helix Aspersa* (HAA). Previously, it has been reported that HAA might specifically bind to high molecular weight proteins in human serum (26), but interaction with pIgA has not been studied. The binding of HAA lectin to immobilized highly purified monomeric and polymeric IgA was studied by ELISA. We observed a dose-dependent binding of both mIgA and pIgA with HAA, with mIgA exhibiting significantly less binding to HAA as compared to pIgA (Figure 3A). These results suggest that pIgA, compared with mIgA, has smaller O-linked glycans with increased exposure of GalNAc.

To investigate whether exposure of terminal O-linked GalNAc might lead to an increased interaction of IgA with CD89, we treated mIgA with neuraminidase and galactosidase. Furthermore, to evaluate a possible role of N-glycans in the binding of IgA to CD89, mIgA was treated with PNGase F to remove N-linked sugars. These enzymatic treatments resulted in a clearly reduced MW of the IgA heavy chain as shown by SDS-PAGE, without affecting the light chain which is known to be non-glycosylated (Figure 3B). Increased exposure of terminal GalNAc after treatment of mIgA with neuraminidase and galactosidase was confirmed by a lectin-blot using biotinylated HAA (Figure 3B, C) and by using an ELISA system (Figure 3D). As expected, removal of N-glycans with PNGase F did not result in increased exposure of GalNAc (Figure 3B-D). Interestingly we observed that treatment of mIgA with neuraminidase alone already increased HAA reactivity, suggesting the presence of undergalactosylated O-linked glycans on mIgA.

To investigate whether exposure of terminal GalNAc or removal of N-glycans might enhance the interaction between IgA and CD89, we tested these well charac-

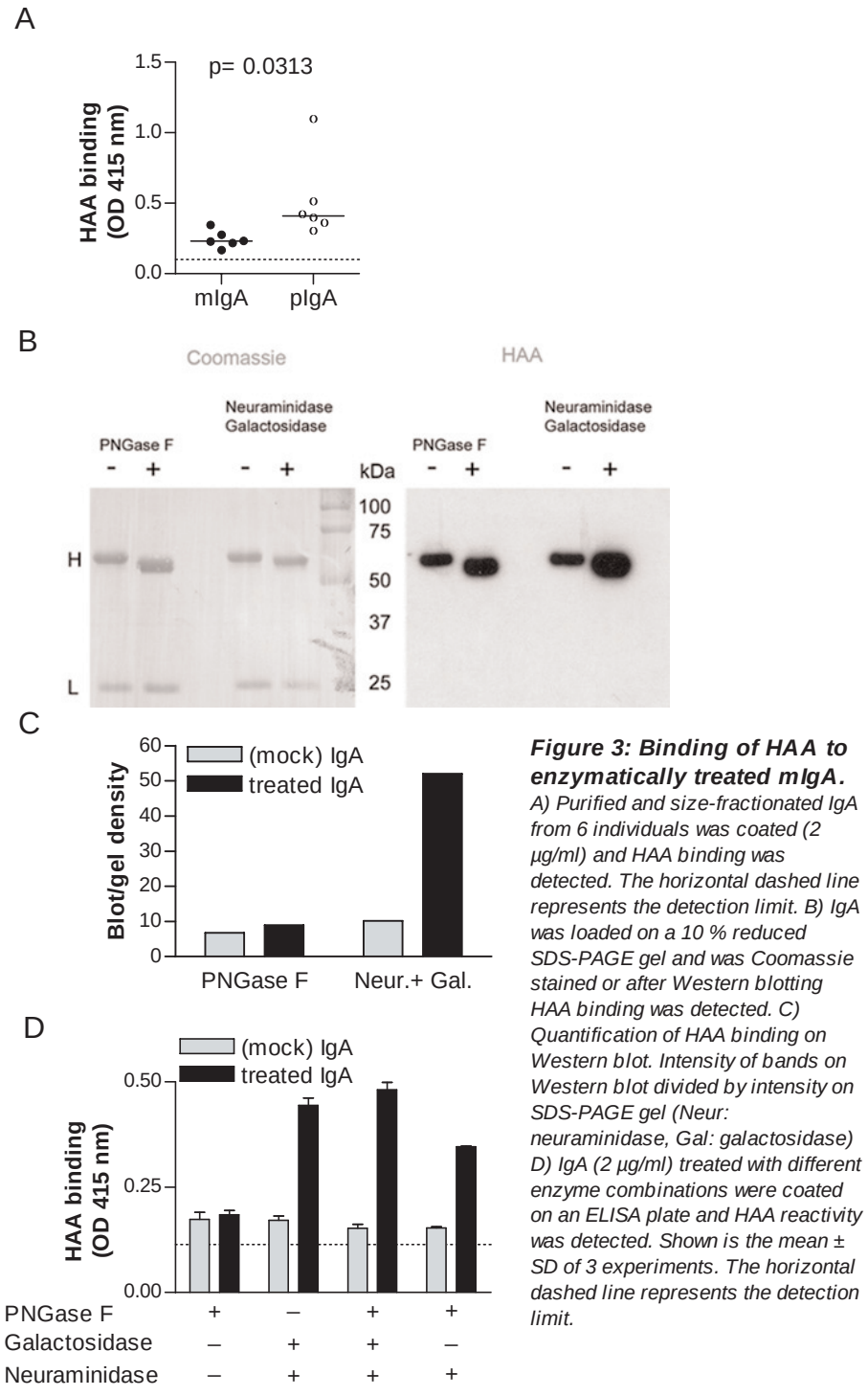

Figure 3: Binding of HAA to enzymatically treated mIgA.
A) Purified and size-fractionated IgA from 6 individuals was coated (2 µg/ml) and HAA binding was detected. The horizontal dashed line represents the detection limit. B) IgA was loaded on a 10 % reduced SDS-PAGE gel and was Coomassie stained or after Western blotting HAA binding was detected. C) Quantification of HAA binding on Western blot. Intensity of bands on Western blot divided by intensity on SDS-PAGE gel (Neur: neuraminidase, Gal: galactosidase) D) IgA (2 µg/ml) treated with different enzyme combinations were coated on an ELISA plate and HAA reactivity was detected. Shown is the mean ± SD of 3 experiments. The horizontal dashed line represents the detection limit.

terized deglycosylated IgA preparations for binding to Fc-$(CD89)_2$ using ELISA and SPR. Treatment of mIgA with combinations of neuraminidase, galactosidase and/or PNGase F did not change the binding of IgA to CD89 in ELISA (Figure 4A) and this was confirmed by showing a similar level of binding to CD89 in the late phase of SPR (Figure 4B, C). However, the early binding of mIgA after removing the N-glycans was increased compared to mock treated IgA or IgA treated with other enzymes (Figure 4B, D). These results suggest that N-glycans negatively affects the interaction of IgA with CD89.

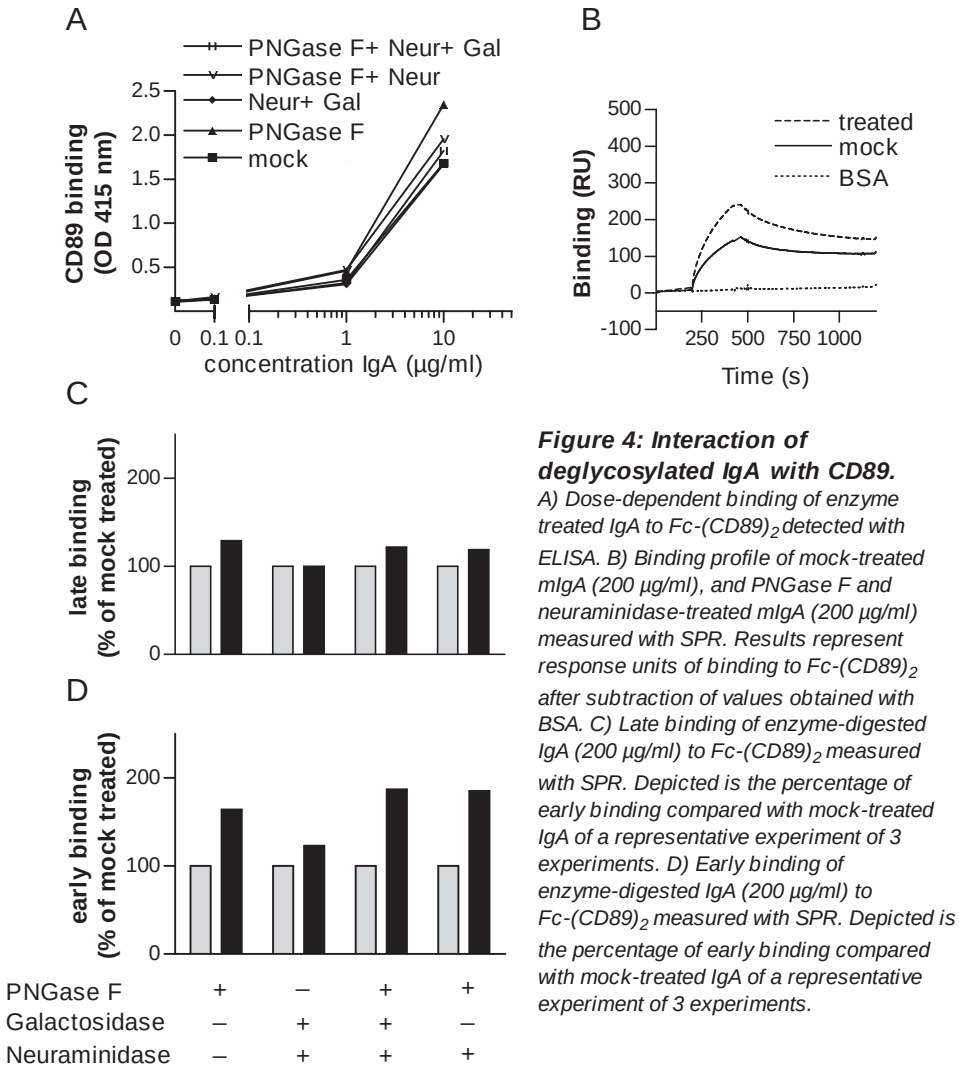

Figure 4: Interaction of deglycosylated IgA with CD89.
A) Dose-dependent binding of enzyme treated IgA to Fc-$(CD89)_2$ detected with ELISA. B) Binding profile of mock-treated mIgA (200 µg/ml), and PNGase F and neuraminidase-treated mIgA (200 µg/ml) measured with SPR. Results represent response units of binding to Fc-$(CD89)_2$ after subtraction of values obtained with BSA. C) Late binding of enzyme-digested IgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. Depicted is the percentage of early binding compared with mock-treated IgA of a representative experiment of 3 experiments. D) Early binding of enzyme-digested IgA (200 µg/ml) to Fc-$(CD89)_2$ measured with SPR. Depicted is the percentage of early binding compared with mock-treated IgA of a representative experiment of 3 experiments.

DISCUSSION

The present study provides evidence that the initial binding of mIgA and pIgA to CD89 by SPR were similar. However the dissociation of mIgA is more rapid than that of pIgA leading to a more sustained binding of the latter to CD89. Furthermore we show that SIgA is able to bind to CD89 without the presence of accessory molecules. It seems that N-glycans are important for the initial interaction of mIgA to CD89 because removal of the N-glycans by enzymatic treatment of mIgA resulted in enhanced association of IgA with CD89, whereas the final binding level remained the same as that for mock treated IgA. Taken together, these data provide more insight in the interaction of different molecular forms of IgA with CD89.

Binding studies with IgA and CD89 using mutagenesis and chemical modification showed that R82 and H85 in the FG loop of the first domain of CD89 are essential for the binding activity of CD89 (11). In a model (27), based on the KIR structure, it was suggested that the ligand binding site of CD89 is located in the first domain and not in the second domain, as described for other Fc receptors. The crystal structure of the IgA/CD89 recently confirmed these findings (28). The binding site for CD89 on IgA seems to overlap with that of bacterial IgA-binding proteins, suggesting involvement of the Cα2/Cα3 interdomain regions (29). Furthermore, using recombinant IgA molecules it has been shown that the presence of certain regions in both the Cα2 and Cα3 domains of IgA are required for efficient interaction with CD89 and for triggering of this receptor (14). Such a model (27) is consistent with a study that revealed a 2:1 stoichiometry for the CD89-IgAFc interaction (30,31). Furthermore, the crystal structure of the IgA1Fc-CD89 complex also showed that IgA could be in complex with two CD89 molecules (30). Our data indicate that mIgA molecules are able to interact similar with CD89 as pIgA, in contradiction with earlier suggestions (16,17).

In the circulation a large excess of mIgA is present and therefore CD89 might be permanently occupied with mIgA, thereby competing for CD89 binding by pIgA and prevents cell activation. The interaction of mIgA with CD89 might also be sufficient for internalization of mIgA via CD89, thereby creating intracellular CD89-IgA complexes which then can be secreted in the circulation (20).

In IgA nephropathy (IgAN), the most common primary glomerulonephritis with IgA deposits in the glomeruli, an altered O-glycosylation of the IgA1 molecule is described (24,32-34). Patient IgA1 contains smaller O-linked glycans with more terminal GalNAc and this could play a role in the deposition of IgA1 in the mesangial area of these patients (24,25,35). IgA treated with neuraminidase and galactosidase resembles the underglycosylated IgA as described in IgA nephropathy (24). In our studies we did not find a difference in binding of IgA treated with neuraminidase and galactosidase to CD89 as compared to control IgA. However after treatment of IgA with PNGase F, to remove the N-glycans, the initial interaction of mIgA with CD89 was enhanced, suggesting an important role for the N-glycans on IgA in the initial interaction with CD89. Several different N-glycans are described to be present on IgA, among which high mannose type N-linked glycan chains (36). These high mannose N-glycans may be likely candidates for the interaction of IgA with MBL (37). Recent studies have suggested the presence of MBL in association with IgA in the

mesangial area of patients with IgAN (38,39).

In literature it is still controversial whether N-glycans present on IgA do influence the interaction with CD89. One study showed that IgA with a mutation in ASN-263 in IgA, which lacks the Cα2 N-glycan, led to loss of binding to CD89 (23). However, another study showed a normal binding of the IgA1 mutant N263A to CD89 (32). In the first study mutant IgA was expressed in insect cells. This could lead to differences in the glycan structure (23). In the present study we show that deglycosylation of IgA could lead to an increase in early binding and therefore more interaction in the circulation, however this will not lead to an increase in late binding, which is in agreement with the data observed with mutant N263A. In the crystal structure of the IgA-CD89 complex (28), the observed N-glycans on IgA approach within 8 Å, but do not directly contact, the receptor (28). In CD89, a mutation in an amino acid that is not directly in contact with IgA resulted in an 11-fold loss of affinity (28). Because of the presence of the N-glycans on IgA close to these binding sites it could be that the N-glycans hamper the IgA/ CD89 interaction, but more research is needed to confirm this.

In summary we have shown that mIgA is able to interact with CD89 in a similar way as pIgA but that the interaction is more stable for pIgA than for mIgA. Furthermore we show that the N-glycans of IgA are important for the initial interaction with CD89. This binding of mIgA to CD89 could explain the observed presence of IgA-CD89 complexes in serum.

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CHAPTER 3

Comparable binding of monomeric and polymeric IgA to the novel IgA Fc receptor, Fcα/μR.

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Summary

Aberrant interaction between IgA and IgA-receptors has been implicated as an important pathogenic factor in IgA nephropathy (IgAN). Several IgA receptors have been characterized but most of these receptors are not present on mesangial cells. Recently a novel Fc receptor for IgA (Fcα/μR) has been identified in B cells and macrophages. It has been shown that Fcα/μR is abundantly expressed in the kidney. In this study we produced recombinant human Fcα/μR proteins and investigated their interaction with human IgA and IgM.

Using RT-PCR we showed that human renal mesangial cells express Fcα/μR and that the expression is upregulated following IL-1 stimulation of the mesangial cells. Human Fcα/μR chimeric proteins (Fcα/μRIg), consisting of the extracellular domain of Fcα/μR and the human IgG1 Fc-tail, were generated and produced in CHO cells. The interaction of Fcα/μRIg with IgA was tested with two different assays. First the binding of different forms of IgA and IgM to Fcα/μRIg was measured directly by ELISA. In a second assay the competition with binding of labelled mIgA to Fcα/μRIg by different forms of IgA and IgM were investigated. All forms of IgA and IgM showed a dose dependent binding to Fcα/μRIg. The binding of monomeric and polymeric IgA to Fcα/μRIg were comparable. Enzymatically deglycosylated IgA obtained by PNGase F and neuraminidase treatment showed a reduced interaction with the Fcα/μRIg. In conclusion, using recombinant chimeric proteins, we showed that IgA binds to Fcα/μR and that the interaction of IgA with Fcα/μR is partially dependent on glycosylation of IgA. This study provides additional insight in the interaction of IgA with Fcα/μR.

INTRODUCTION

Receptors for the Fc portion of immunoglobulins are present on different cell types of the immune system and play important roles in a wide array of immune responses, like antibody dependent cellular cytotoxity and phagocytosis. IgA is the predominant isotype produced and it plays a critical role in the protection of the host against environmental pathogens at mucosal surfaces (1).

IgA nephropathy (IgAN) is the most common type of primary glomerulonephritis worldwide. The disease presents a spectrum of clinical symptoms, leading to progressive renal failure in a substantial proportion of patients. The hallmark of the disease is deposition of IgA in the glomerular mesangium (2-4). In glomeruli of patients with IgAN mostly high molecular weight IgA1 is detected, sometimes together with IgM and/or C3 (5-7). The deposition of IgA in the mesangium is associated with renal inflammation, however extensive studies still have not revealed a specific mechanism of deposition.

Binding of IgA to mesangial cells has been studied before (8,9) and has revealed that the binding of polymeric IgA to mesangial cells is stronger than that of monomeric IgA. Moreover, recently our group showed that secretory IgA, present in low concentrations in the circulation, has the best binding to mesangial cells in vitro as compared to serum IgA (10). Several studies have shown that aberrant O-glycosylation of circulating IgA from IgAN patients, may result in increased Tn antigen (GalNAcβ1-Ser/Thr) expression (11-13) and that binding of IgA from patients with IgAN to mesangial cells is better as compared to that of IgA from healthy individuals (9). However, this finding is still controversial (14).

Several IgA receptors have been described in literature. The best-known receptors, the polymeric Ig receptor (pIgR), the asialoglycoprotein receptor and CD89 have already been described to be absent on mesangial cells (15-17). Recently a novel IgA receptor, Fcα/μR, located on chromosome 1, has been identified. Expression of transcripts of this receptor has been demonstrated in several tissues including kidney, small intestine, lymph node and appendix (18,19). Furthermore, transcription of the Fcα/μR was demonstrated in mesangial cells, and shown to be upregulated by IL-1, a cytokine implicated in the pathogenesis of IgAN (20). Studies in mice have shown surface expression of Fcα/μR in mature but not in immature B lymphocytes. Furthermore, stimulated B lymphocytes were able to bind IgA and IgM (19). Of interest is the finding that rat IgA and both human and rat IgM binds to mouse Fcα/μR (18). However, the interaction of human Fcα/μR with human IgA and IgM has not been reported until now.

Therefore, the aim of the present study was to analyse the interaction of human IgA and IgM to recombinant human Fcα/μR. We show that two different recombinant Fcα/μRs react with IgM and with different molecular forms of IgA in ELISA and that monomeric IgA has similar binding as polymeric IgA to Fcα/μR. Furthermore, the binding of monomeric IgA to Fcα/μR is affected by its glycosylation.

MATERIAL AND METHODS

Production of Fcα/μRIg

Two forms of Fcα/μRIg fusion protein were produced following cloning of the complete extracellular part of the receptor into the pME-Neo Fc vector (long Fcα/μRIg) (21) or of the N-terminal Ig domain without the long stalk (short Fcα/μRIg). The XHO-1 restriction site in the linker between the fusion product and the human IgG1 Fc tail and Eco-RI at the beginning of the extracellular part of the receptor were used. PCR was used to amplify the extracellular part of the Fcα/μR using a pcDNA3.1/ His/ TOPO vector containing the whole receptor (20) (Table 1) and PWO polymerase (5U/μl, Roche). The purified product obtained after agarose gel electrophoresis was subcloned in the pME-Neo Fc vector previously used for the production of Fc-$(CD89)_2$ (21) and the identity and orientation were confirmed by sequencing and restriction digestion. The vector was subsequently introduced into Chinese hamster ovary (CHO) cells using electroporation and selected for neomycin resistance with G418 (400 μg/ml, Gibco). Single clones with stable expression were obtained, and clones with the highest production as determined by ELISA were selected (long Fcα/μRIg: 4 μg/ml, short Fcα/μRIg 1.7 μg/ml). Supernatants of CHO cells producing long or short Fcα/μRIg were applied to a prot G or HiTrap Mabselect SuRe columns and after washing the column with PBS, bound proteins were eluted with 0.1M citric acid pH 3.0. Fractions were neutralized immediately with 1M Tris-HCl pH 10, and tested for human IgG content by ELISA.

Table 1: Primer sequences

		Sequence (5'-3')
Long Fcα/μRIg	Forward	CTT CGA ATT CAT GCC CCT CTT CCT CAT ACT GTG CC
	Reverse	TCA ACT CGA GCC GAG AGC TGC TTT CAT CTT CTG G
Short Fcα/μRIg	Forward	CTT CGA ATT CAT GCC CCT CTT CCT CAT ACT GTG CC
	Reverse	ATT ACC TCG AGC CCA GCA GCT GGA GTG GCT GT
Fcα/μR	Forward	GAC AAC TAC CAA GGC TGA TAG G
	Reverse	TCT GTC CCT CAG GGT CCT GGA T

Cytospin preparations

Cells were harvested by trypsin digestion and subsequently incubated with 1 % paraformaldehyde for 30 min at 4 ºC. After washing with PBS, the cells were centrifuged onto glass slides and air dried. After blocking with PBS/ 1 % BSA for 1 hour RT, the cytospin preparations were incubated with 10 μg/ml monoclonal anti human IgG (HB43, ATCC) for 1 hour RT. Binding of HB43 was visualized with Oregon Green conjugated goat anti mouse IgG (Molecular probes, Leiden, The Netherlands).

IgG ELISA

In order to determine the chimeric protein concentrations we used an IgG ELISA as described before (22). Plates were coated with rabbit anti human IgG (Jackson, West Grove, PA, USA) in carbonate buffer (pH 9.6) (100 μl/ well) overnight at RT. Subsequently, the plates were washed with PBS/ 0.05 % Tween. Plates were incubated with chimeric protein or IgG in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgG was detected with goat anti human IgG biotin, followed by streptavidin conjugated to HRP (Zymed).

Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured.

RNA extraction and RT-PCR

Total RNA was extracted from mesangial cells using RNeasy mini kit (Qiagen, Valencia, CA). OD260/280 ratio was measured to determine the quantity and purity of RNA preparations. Fixed amounts of total cellular RNA (1 µg) were reverse transcribed into cDNA by oligo(dT) priming, using M-MLV reverse transcriptase (Gibco/Life Technologies, Breda, The Netherlands). PCR to detect the human Fcα/µR was performed with specific primers (Table 1) using AmpliTaq DNA polymerase (Applied Biosystems, Roche, Mannheim, Germany). The PCR reaction was performed as described before (20). The cDNA samples were also subjected to PCR for GAPDH as an internal control (23). PCR products were resolved on 1 % agarose gels and bands were visualized by ethidium bromide staining.

Purification of IgA, IgG and IgM

Serum was used as a starting source for IgA purification, using methods described before (24). In brief, serum was applied to an anti-IgA (clone HisA 43, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) affinity column. The column was washed sequential with 0.5 x PBS and 1 M NaCl, and subsequently bound IgA was eluted with 0.1 M glycine/ 0.3 M NaCl (pH 2.8). Directly after elution the fractions were neutralized with 1 M Tris pH 8.0. The eluted protein fractions that contained IgA, as assessed by ELISA (25), were pooled and dialysed against PBS containing 2 mM EDTA.

Purified IgA was size-separated into monomeric IgA (mIgA) and polymeric IgA (pIgA) using a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands). Pools of monomeric and polymeric IgA were made and analysed for total IgA content using ELISA (25).

Secretory IgA (SIgA) was obtained from Sigma. Human IgG and IgM, free of IgA, were isolated as described before (26).

Assessment of immunoglobulin binding to Fcα/µRIg

Binding of IgA and IgM to Fcα/µRIg constructs was analysed by ELISA. Ninety-six well Nunc Maxisorb microtitre plates (Gibco/Invitrogen, Carlsbad, CA) were coated overnight with long Fcα/µRIg (5 µg/ml) in carbonate buffer (pH 9.6) (100 µl/well) at room temperature. Subsequently, the plates were washed with PBS/ 0.05 % Tween and incubated with different concentrations of IgA or IgM in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgA was detected with biotin labelled monoclonal anti human IgA (4E8), bound IgM with biotin labelled monoclonal anti human IgM (HB57). After incubation of one hour the plates were washed and bound conjugates were detected with streptavidin conjugated to HRP. Enzyme activity of HRP was developed using ABTS (2, 2'-azino-bis (3-ethyl benzathioline-6-sulphonic acid)) (Sigma, St. Louis, MO). The O.D. at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, USA).

In order to compare the interaction of different immunoglobulins to the Fcα/µRIg constructs we set up a competition ELISA. For this purpose, the dose dependent binding of dig labelled mIgA was tested to immobilized long and short Fcα/µRIg. Subsequently, plates were coated with long Fcα/µRIg (5 µg/ml) in carbonate buffer (pH 9.6) (100 µl/well) overnight at room temperature and after extensive washing with PBS/ 0.05 % Tween, incubated with IgA,

IgM or IgG in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After one hour, dig-conjugated mIgA (final concentration 1 μg/ml) was added to the plate. The amount of mIgA-dig bound was detected subsequently using $F(ab)_2$ anti-Dig Abs conjugated to HRP.

Glycosidase treatment of IgA

In order to investigate whether the IgA glycosylation is important for the interaction with Fcα/μR, mIgA was enzymatically deglycosylated. Therefore, monomeric IgA (800 μg) was first digested with N-glycosidase F (PNGase F), cloned from *flavobacterium meningosepticum* and expressed in *E. coli* (6 U/ 200 μg IgA, Roche) in 0.02 M sodium phosphate buffer pH 7.2 with 2 mM EDTA. The first sample, to be used as control, was IgA with incubation buffer (mock IgA (800 μg)); the second sample was IgA with enzymes (treated IgA (800 μg)). After 72 hours of incubation at 37 ºC this IgA was further digested with neuraminidase from *Arthrobacter ureafaciens* (6 mU/200 μg IgA, Roche, Mannheim, Germany), β (1-3,4,6) galactosidase isolated from a cloned gene expressed in *E. coli* (7 mU/ 200 μg IgA, Prozyme), or combinations of the enzymes. This deglycosylation step was performed in 50 mM sodium acetate pH 5.6 for 72 hours at 37 ºC. After treatment, samples (10 μg) were analysed by loading on a 10 % reduced SDS-PAGE gel and Coomassie stained.

Flow cytometry

NHMC (normal human mesangial cells, Cambrex, USA) were expanded according to the protocol provided by the manufacturer. Experiments with NHMC were performed in RPMI with 10 % FCS, 1 % non-essential amino acids, 0.5 % transferrin/insulin/selenium, 1 % sodium pyruvate, 1 % L-glutamine (all purchased at Gibco/ life Technologies, Paisley, Scotland). Cells were harvested by trypsinization (0.02 % (w/v) EDTA/ 0.05 % (w/v) trypsin in PBS from Sigma (St. Louis, MO)), and washed with FACS buffer (0.5 x PBS containing 1 % BSA/ 2.8 % glucose/ 0.01% NaN3) and incubated with monomeric and polymeric serum IgA, and SIgA (Sigma). Following incubation for 1 hour at 4 ºC, cells were washed and incubated for one hour at 4 ºC with monoclonal anti-IgA Ab 4E8 (IgG1) (25). IgA binding was visualized using PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and assessed for fluorescence intensity by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

Binding of IgA to mesangial cells was also determined following activation of mesangial cells for 48 hours with IL-1 (10 ng/ml).

RESULTS

Construction of chimeric forms of Fcα/μR

In order to investigate the interaction of different molecular forms of IgA with Fcα/μR, two different Fcα/μRIg fusion proteins were generated. Long Fcα/μRIg was generated by fusion of the complete extracellular part of the receptor with the human IgG1 Fc tail, whereas short Fcα/μRIg was generated by fusion of the extracellular part containing the Immunoglobulin-like domain with the human IgG1 Fc tail (Figure 1A). Recombinant soluble long and short Fcα/μRIg were produced in CHO cells. Because no specific reagents were available for detection of human Fcα/μR, we tested the cells (Figure 1B) and supernatants for the presence of the IgG Fc-tail. Untransfected cells were negative for the human IgG1 Fc tail, whereas both fusion

proteins were produced by a number of cell lines.

Supernatants of the two transfected cell lines were applied to protein G columns and after acid elution one peak each was collected and tested in the human IgG ELISA (Figure 1C). The human IgG ELISA was positive for both human IgG and the chimeric proteins.

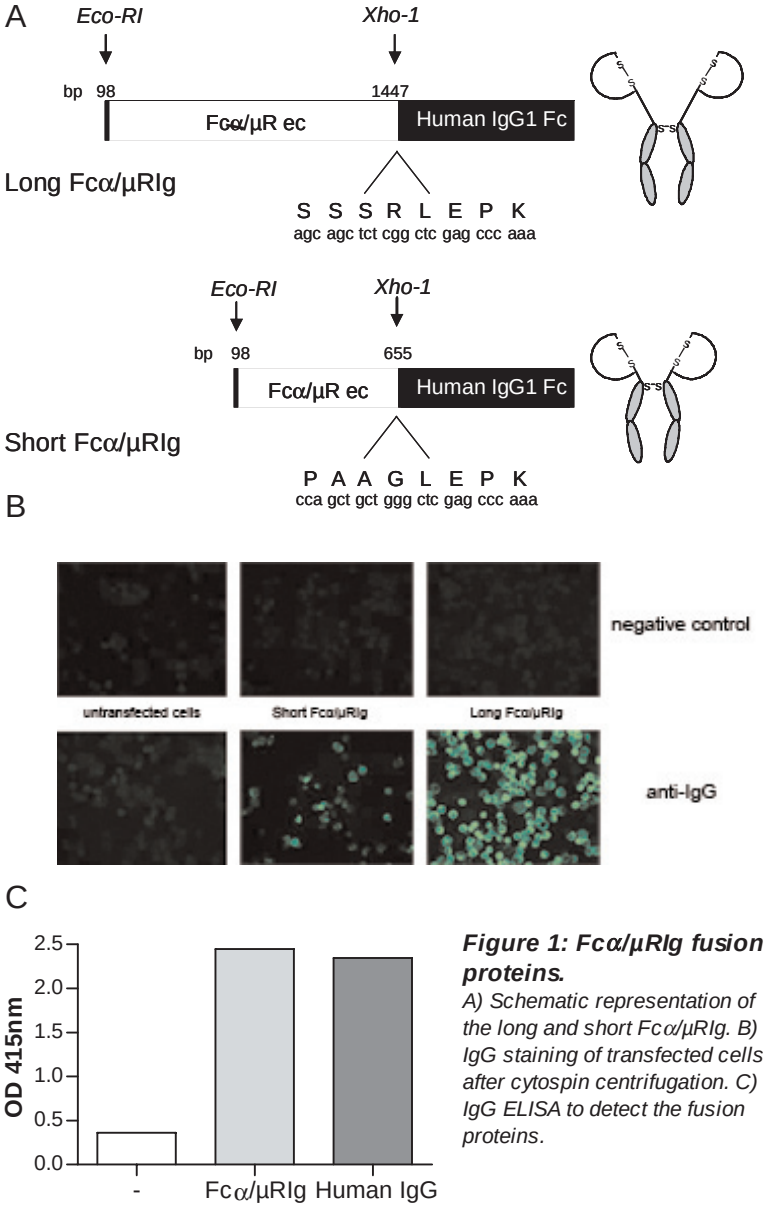

Figure 1: Fcα/μRIg fusion proteins.
A) Schematic representation of the long and short Fcα/μRIg. B) IgG staining of transfected cells after cytospin centrifugation. C) IgG ELISA to detect the fusion proteins.

Dose dependent binding of IgA and IgM to Fcα/μR in ELISA

To investigate the interaction of different molecular forms of IgA with Fcα/μR, long Fcα/μRIg was coated in ELISA plates and binding of IgA and IgM was assessed. The binding of IgA was dose-dependent over a wide range of concentrations (Figure 2A). Binding of monomeric and polymeric IgA to Fcα/μR were similar. We have reported earlier that in serum low concentrations of SIgA are present (10). Therefore we also tested the binding of SIgA to Fcα/μR. Binding of purified serum IgA to Fcα/μR was stronger than that of SIgA. Also IgM exhibited a dose dependent binding to Fcα/μR (Figure 2B).

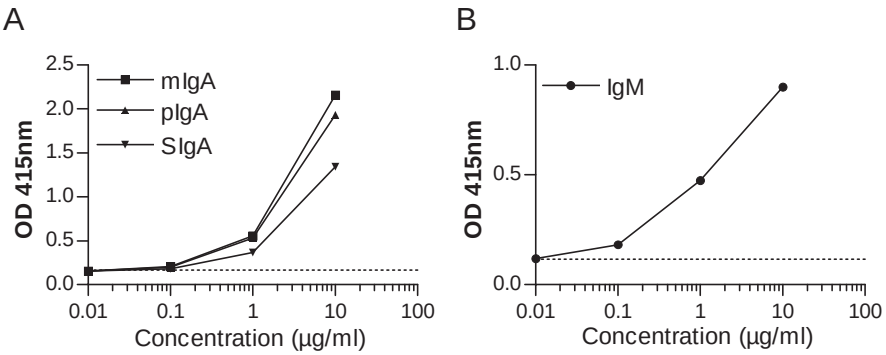

Figure 2: Binding of IgA and IgM to Fcα/μR in ELISA.
A) Dose dependent binding of IgA to immobilized long Fcα/μRIg (5 μg/ml). IgA binding was detected with a monoclonal anti-human IgA antibody B) Dose dependent binding of IgM to immobilized long Fcα/μRIg (5 μg/ml). IgM binding was detected with a monoclonal anti-human IgM antibody. Dotted line represents the negative control.

Inhibition of binding of mIgA-Dig to Fcα/μR by monomeric and polymeric IgA

Results presented above demonstrated that several forms of IgA and IgM bind to Fcα/μR. However, the detection antibody for binding of IgA and IgM were different and therefore it was not possible to compare the relative amounts of binding of IgA and IgM to Fcα/μR. To obtain more quantitative comparison of the binding of IgA and IgM to Fcα/μR, a competition ELISA was set up. First, the dose dependent binding of mIgA-Dig to Fcα/μR was tested (Figure 3A). Binding of mIgA-Dig to Fcα/μR was dose dependent and the binding of mIgA-Dig to Fcα/μR was similar for long Fcα/μRIg and short Fcα/μRIg. Short Fcα/μRIg contains the immunoglobulin domain of the Fcα/μR without the long extracellular stalk, whereas long Fcα/μRIg consists of the whole extracellular part of the Fcα/μR. The similar binding of mIgA-dig to long Fcα/μRIg and short Fcα/μRIg, suggests the involvement of the immunoglobulin like domain of Fcα/μR for binding to mIgA.

Based on the results, we then selected a dose of 1 μg/ml mIgA-Dig for subsequent competition experiments with different forms of IgA and IgM (Figure 3B). The binding of mIgA-Dig to Fcα/μR was inhibited with mIgA and pIgA in a dose dependent manner, while IgG was unable to influence the binding of mIgA-Dig to Fcα/μR. Furthermore, SIgA showed a reduced capacity to inhibit mIgA-Dig binding to

Fcα/μR. Using IgM as a competitor; we did find some inhibition even at relatively low concentrations. However, IgM inhibited the mIgA-Dig binding with a maximum of 30 %, indicating that SIgA and IgM have a much lower affinity for Fcα/μR than mIgA or pIgA.

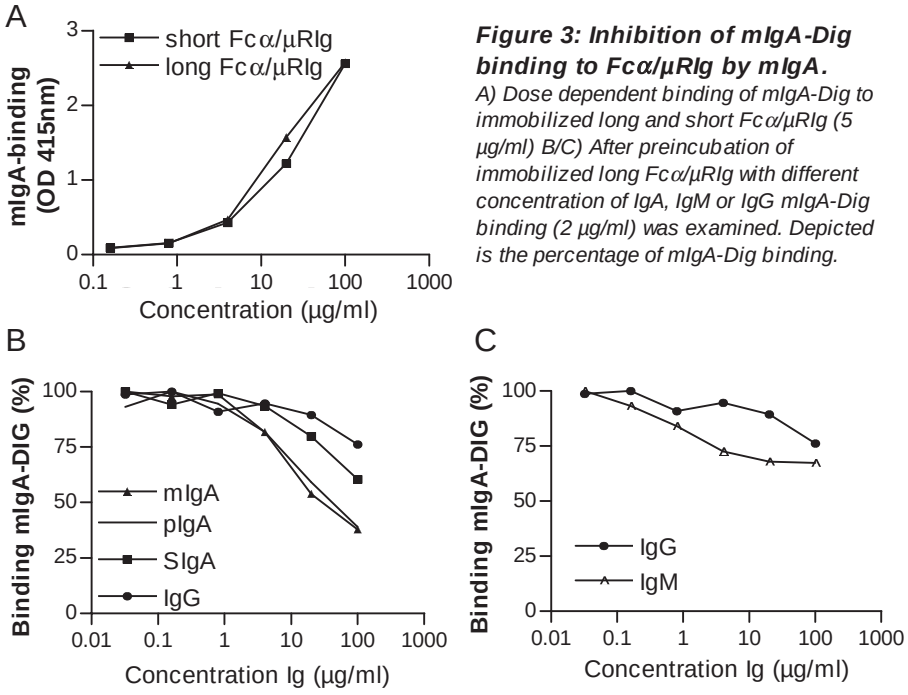

Figure 3: Inhibition of mIgA-Dig binding to Fcα/μRIg by mIgA.
A) Dose dependent binding of mIgA-Dig to immobilized long and short Fcα/μRIg (5 μg/ml) B/C) After preincubation of immobilized long Fcα/μRIg with different concentration of IgA, IgM or IgG mIgA-Dig binding (2 μg/ml) was examined. Depicted is the percentage of mIgA-Dig binding.

Deglycosylation of mIgA is associated with reduced binding to Fcα/μR

Since previously published results suggest that glycosylation of IgA can be important for the interaction with its receptors (27,28), we investigated whether glycosylation differences of IgA affect its interaction with Fcα/μR. Therefore, IgA was treated with neuraminidase, galactosidase, PNGase F or with combinations of these enzymes (28), and tested this treated IgA for binding to Fcα/μR (Figure 4). Treatment of mIgA with a combination of galactosidase and neuraminidase did not alter the binding of IgA to Fcα/μR (Figure 4). Removal of the N-glycans of IgA by treatment of IgA with PNGase F resulted in reduced inhibition of the binding of mIgA-Dig to Fcα/μR. This reduction in binding of IgA to Fcα/μR was even more pronounced after treatment of IgA with a combination of PNGase F and neuraminidase. These results suggest that N-glycans and sialic acids positively affect the interaction of IgA with Fcα/μR.

No difference in interaction of IgA with stimulated mesangial cells

We hypothesized that after IL-1 stimulation the IgA binding to mesangial cells was increased. Therefore, we first analyzed the IgA binding to mesangial cells with-

out stimulation of the mesangial cells (Figure 5A). After incubation of different molecular forms of IgA with mesangial cells the binding was examined by FACS. Binding of mIgA to mesangial cells was very low. In contrast, pIgA and SIgA showed a clear binding to mesangial cells.

The results presented above indicate IgA binding to mesangial cells. It has been suggested that Fcα/μR is present on mesangial cells, and thereby could play a role in IgAN (20). Therefore, we investigated whether Fcα/μR was detectable in mesangial cells (Figure 5B). The Fcα/μR transcript was present in mesangial cells and was up-regulated after stimulation of mesangial cells with IL-1, as described before (20).

The binding of IgA to mesangial cells was examined after mesangial cell stimulation with IL-1. Although the transcript of Fcα/μR is up-regulated in mesangial cells after IL-1 stimulation, there was no increase in IgA binding after stimulation of mesangial cells (Figure 5C). This was observed with all different molecular forms of IgA.

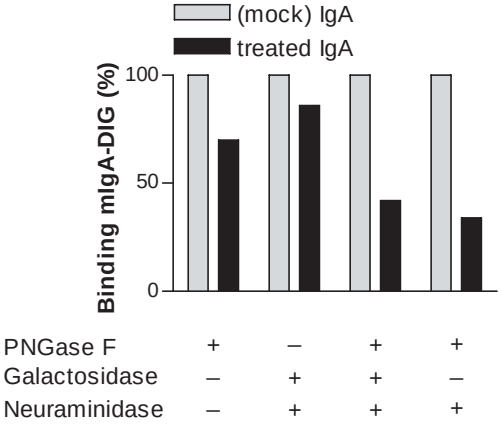

Figure 4: Interaction of deglycosylated IgA with Fcα/μR.
A) Inhibition of mIgA-Dig binding (2 μg/ml) with mock treated IgA (10 μg/ml) or deglycosylated IgA (10 μg/ml) to immobilized long Fcα/μRIg, depicted is the percentage of mIgA-Dig inhibition B) mIgA-Dig binding after preincubation of immobilized long Fcα/μRIg with different concentration mock treated or PNGase F and neuraminidase treated IgA. Depicted is the percentage of mIgA-Dig binding.

DISCUSSION

To our knowledge this is the first time that the interaction of human IgA and human IgM to human Fcα/μR was studied directly. In literature it has been suggested that the binding of mouse IgM to mouse Fcα/μR was stronger as compared to the binding of mouse IgA (18). In the present study we cannot confirm the mouse study in the human situation, in that the interaction of human IgA and human IgM with human Fcα/μR was strongest for IgA. Furthermore, this interaction of IgA with Fcα/μR was affected by the glycosylation of IgA. The binding of IgA and IgM was similar for both fusion proteins investigated, suggesting an important role for the immunoglobulin-like domain of the receptor in the interaction with IgA and IgM.

The mouse Fcα/μR is homologous to the human Fcα/μR (18). Strong binding of rodent IgM and IgA to mouse Fcα/μR was observed, but in contrast to rodents in humans 90 % of circulating IgA is monomeric (29). In this study we found that the interaction of human Fcα/μR was similar for monomeric and polymeric IgA. This

interaction of IgA with Fcα/μR is different as compared to the interaction of IgA with another IgA receptor of the Ig superfamily, the FcαRI/CD89 (28,30-32).

Fcα/μR has been described to be present on mesangial cells and was suggested to play a role in IgAN. With size fractionation of eluted proteins from kidney sections, it was shown that deposited IgA was mostly of high molecular weight nature (5). Furthermore, elution of deposits from glomeruli from a patient with IgA nephropathy, revealed a high accumulation of SIgA in the IgA deposits (10). Moreover, in vitro binding studies showed that high molecular weight IgA bound better to mesangial cells than monomeric IgA. In the present study we show that the Fcα/μR interacts similar with monomeric and polymeric IgA. Furthermore we showed an up-regulation of Fcα/μR transcript in mesangial cells after IL-1 stimulation. However, high molecular weight IgA binds better to mesangial cells as compared to monomeric IgA and we could not detect differences in IgA binding to IL-1 stimulated mesangial cells. Altogether this suggests an additional receptor on mesangial cells.

In IgAN an aberrantly O-glycosylated IgA1 molecule has been described. With enzymatic treatment of IgA we investigated whether the interaction of IgA with

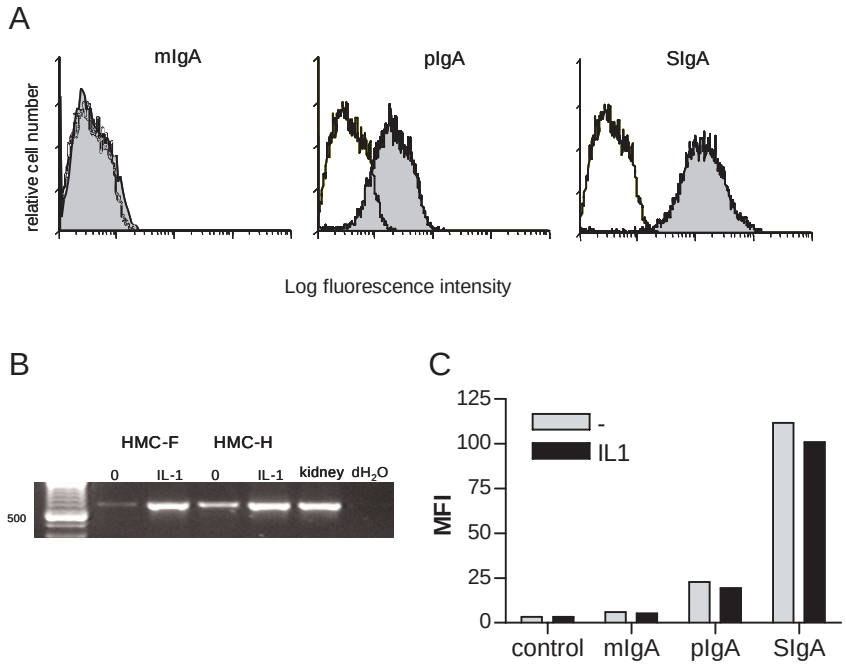

Figure 5: Interaction of IgA with stimulated mesangial cells.
A) mesangial cells were incubated with different molecular forms of IgA (200μg/ml) and assessed for IgA binding by flow cytometry. Filled histograms represent the binding of IgA, the open histograms represent the control staining with secondary antibodies B) mRNA expression of Fcα/μR in mesangial cells with and without IL-1 stimulation. Kidney was used as positive control and H_2O as negative control. C) FACS analysis with IL-1 stimulated mesangial cells and different molecular forms of IgA. Depicted is the mean fluorescence intensity (MFI).

Fcα/μR was glycosylation dependent. After treatment of IgA with neuraminidase and galactosidase, to generate a glycan structure as described to be present on IgA from IgAN patients, no difference in the interaction of IgA with Fcα/μR was observed. However, after removal of the sialic acid and N-glycan residues a clear reduction in binding of IgA to Fcα/μR was observed, suggesting that the charge of the sialic acid residues together with the N-glycan structure is important for the IgA- Fcα/μR interaction. However, it is not clear whether the carbohydrates itself or the possible change in confirmation as a result of the treatment play a role in this process.

Mouse Fcα/μR can mediate endocytosis of immune-complexes composed of IgM and S. aureus (18). The underlying mechanism of this internalization is not known yet, but with receptor mutants it is suggested that the di-leucine motif is important in this process (18). For human Fcα/μR it is not known whether the receptor is able to internalize immune-complexes with IgA or IgM and if so then which mechanism could be of importance for this process. Therefore it would be very interesting to investigate, whether human Fcα/μR is also able to mediate endocytosis of IgA immune-complexes.

The Fcα/μR immunoglobulin like domain has the same motif as the first extracellular immunoglobulin like domain of the polymeric Ig receptor (pIgR) (18). IgA and IgM are also able to bind to pIgR, and this binding is described to be on the first extracellular domain of the pIgR (33). The binding of IgA and IgM to pIgR is restricted to IgA (dimeric IgA (dIgA)) and IgM containing the J chain (34). The binding of IgA and IgM was similar for the long Fcα/μRIg and the short Fcα/μRIg, suggesting an important role for the immunoglobulin like domain of the Fcα/μR for the interaction with IgA and IgM. As mentioned before the binding of IgA to the pIgR is restricted to dIgA (34), whereas monomeric IgA has the best interaction with Fcα/μR, suggesting another role for the Fcα/μR as compared to the role of pIgR.

In conclusion, we showed that IgA and IgM are able to interact with Fcα/μR. A similar binding of monomeric and polymeric IgA to Fcα/μR was observed. Furthermore, we showed that this interaction is affected by the glycosylation of the IgA molecule. The mechanism of interaction of IgA with Fcα/μR is still not clear however our data suggest an important role for the immunoglobulin like domain of the extracellular part of the Fcα/μR. This study provides more insights in the interaction of IgA with Fcα/μR.

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CHAPTER 4

Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease

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Summary

IgA nephropathy (IgAN) is characterized by glomerular co-deposition of IgA and complement components. Earlier studies have shown that IgA activates the alternative pathway of complement, whereas more recent data also indicate activation of the lectin pathway. The lectin pathway can be activated by binding of mannose-binding lectin (MBL) and ficolins to carbohydrate ligands, followed by activation of MBL-associated serine proteases (MASPs) and C4. We studied the potential role of the lectin pathway in IgAN.

Renal biopsies of IgAN patients (N = 60) showed mesangial deposition of IgA1 but not IgA2. Glomerular deposition of MBL was observed in 15 out of 60 cases with IgAN (25 %) and showed a mesangial pattern. All MBL-positive cases, but none of the MBL-negative cases, showed glomerular co-deposition of L-ficolin, MASPs and C4d. Glomerular deposition of MBL and L-ficolin was associated with more pronounced histological damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis and interstitial infiltration, as well as with significantly more proteinuria. IgAN patients with or without glomerular MBL deposition did not show significant differences in serum levels of MBL, L-ficolin or IgA, nor in the size distribution of circulating IgA. Furthermore, in vitro experiments showed clear binding of MBL to polymeric but not monomeric patient IgA, without a significant difference between both groups.

Together, these findings strongly point to a role for the lectin pathway of complement in glomerular complement activation in IgAN, and suggest a contribution for both MBL and L-ficolin in the progression of the disease.

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INTRODUCTION

IgA nephropathy (IgAN) is a common renal disease primarily characterized by mesangial deposition of IgA (reviewed in (1,2)). Mesangial IgA deposition induces an inflammatory process, involving mesangial proliferation, interstitial damage and proteinuria, which slowly progresses to sclerosis and end-stage renal failure in about one third of cases. Several effector mechanisms are likely to be involved in the induction of renal inflammation and damage, including direct interactions between IgA and mesangial cells (3-5) and IgA-mediated complement activation (1,6,7).

IgA deposition in IgAN is commonly associated with deposition of complement factors, most often C3, the terminal complex C5b-9, and properdin (8,9). Furthermore, increased levels of soluble split products of activated C3 have been observed in the circulation of IgAN patients (10-12). These findings suggest involvement of the alternative pathway. Indeed, in vitro studies with IgA from humans and rodents, as well as in vivo studies in rats, have indicated that IgA can directly activate the alternative pathway of complement (6,13-15).

The complement system can be activated via three pathways, the classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the cleavage of C3, followed by activation of the common terminal pathway and formation of C5b-9. The classical pathway involves binding of C1q to e.g. immune complexes, leading to generation of the C3 convertase C4b2a. The more recently discovered lectin pathway of complement is activated following an interaction of the plasma lectins mannose-binding lectin (MBL), L-ficolin or H-ficolin with their carbohydrate ligands (16-19). This leads to activation of MASPs (MBL-associated serine proteases) present in a pro-enzymatic complex with these lectins. Activated MASP-2 generates C4b2a, followed by C3 cleavage. In contrast to the classical pathway and the lectin pathway, the alternative pathway leads to activation of C3 in a C4-independent way, involving factors B and D and properdin.

Until now, the mechanism of complement activation in IgAN is still incompletely defined. Next to the presence of factors indicative for alternative pathway activation, several studies have shown glomerular deposition of C4, as well as circulating C4 activation products in a subpopulation of IgAN patients (10,20). Since in vitro studies indicated that IgA can not activate the classical complement pathway, and evidence for C4 activation in IgAN was observed in the absence of C1q deposition, activation of the lectin pathway of complement was hypothesized. Deposition of MBL in association with IgA, as a marker for lectin pathway activation, was reported in a subpopulation of IgAN patients by several authors (21-23), but these findings were questioned by others (24). Furthermore, the relation of glomerular MBL positivity with parameters of renal damage and complement activation via the lectin pathway was inconsistent between the different studies.

Recently, we reported that human polymeric serum IgA can bind to human MBL (25). This interaction involves the lectin domain of MBL, and results into activation of C4 and C3. Therefore, we hypothesize that C4 activation in IgAN may result from binding of serum MBL-MASP-2 complexes to IgA deposited in the renal mesangium. In the present study we have analyzed the possible contribution of the lectin path-

way of complement to activation of C4 and induction of renal damage in a well-defined set of renal biopsies of IgAN patients. Next to MBL, also deposition of the functionally related molecule L-ficolin was studied. The results indicate activation of the lectin pathway, involving both MBL and L-ficolin, in a subpopulation of IgAN patients, which is strongly associated with markers of disease progression.

MATERIAL AND METHODS

Patients and biopsies

Renal biopsies were selected from patients with IgA nephropathy of whom a renal biopsy was taken between January 2001 and December 2003. Patients were selected if adequate tissue was obtained for diagnostics (at least 8 glomeruli in light microscopy sections; complete immunohistology and electron microscopy examination), and if sufficient frozen material was available for additional staining after immunodiagnosis (at least 6 glomeruli in at least 15 (5 m thick) tissue sections). Cases with Henoch-Schönlein purpura, systemic lupus erythematosus, liver cirrhosis or other systemic diseases were excluded. In total, 60 biopsies were selected for evaluation.

Among selected patients, 69 % were males and 31 % females. Serum creatinine levels ranged from 44 to 972 µmol/l at the time of renal biopsy. Creatinine clearance was calculated according to the Cockroft formula (range 10-160 ml/min). Presentation at time of renal biopsy was as following: 58 % urinary abnormalities, 32 % chronic renal failure, and 10 % acute renal failure. Chronic renal failure was defined as serum creatinine value >124 µmol/l or creatinine clearance <80 ml/min in at least three determinations prior to renal biopsy and further confirmed during hospitalization. Acute renal failure was defined as an abrupt decline in glomerular filtration rate detected at patient admission, without previous altered examinations and without ultrasonographic signs of chronic renal injury.

Urine samples (24 hour collection) and serum samples were obtained at time of renal biopsy.

For additional immune fluorescence studies, a more recent series of renal biopsies was collected from IgAN patients (N = 25) using the same selection criteria as described above. From these patients, detailed clinical and histological information has not been collected.

Serological analysis

Serum IgA concentration was measured by standard nephelometric immunoassay. MBL complex activity was assessed by ELISA using coated mannan as a ligand and detecting the activation of purified C4, as described before (26) with some modifications (27). MBL complex activity was expressed in arbitrary units per ml based on the activity of a normal human pool serum. MBL concentrations were assessed by sandwich ELISA as described previously (25). Concentrations of L-ficolin were assessed using a similar protocol. In brief, plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with mAb GN5 (5 µg/ml; mouse mAb against human L-ficolin) or mAb 3E7 (5 µg/ml; mouse IgG1 anti-human MBL) in coating buffer (100 mM Na2CO3/ NaHCO3, pH 9.6). After each step, plates were washed with PBS containing 0.05 % Tween 20. Residual binding sites were blocked by incubation with PBS containing 1 % BSA. Serum samples and subsequent detection antibodies were diluted in PBS containing 1 % BSA and 0.05 % Tween 20, and incubated for one hour at 37 °C. Primary antibodies were mAb 3E7 and mAb GN5, respectively, conjugated to digoxygenin (dig) (from

Roche Diagnostics, Almere, The Netherlands). Binding of mAb was detected using HRP-conjugated sheep anti-dig antibodies (Fab fragments, from Roche). Concentrations of MBL and L-ficolin were calculated using serial dilutions of a standard serum with a known concentration of MBL (kindly provided by Dr. P. Garred, Kopenhagen, Denmark) or L-ficolin (kindly provided by Dr. D.C. Kilpatrick, Edinburgh, UK), respectively.

Purification of serum IgA and assessment of MBL binding to IgA

IgA was purified starting from 350 µl of patient serum (N = 12). The serum was directly applied to an IgA affinity column (Sepharose beads conjugated to mAb HisA43 anti-human IgA, kindly provided by Dr. J. van den Born, Amsterdam, the Netherlands), using Veronal-buffered saline containing 2 mM EDTA as a running buffer. IgA was eluted using 0.1 M Glycin / 0.3 M NaCl (pH 2.8) and fractions were immediately neutralized by 1 M Tris pH 8.0. Fractions were assessed for IgA content and the IgA peak was pooled and concentrated until approximately 350 µl. These samples were further purified on a Sephadex 200 gel filtration column (25 ml, from GE Healthcare, Munich, Germany), using the same running buffer. Fractions of 0.3 ml were collected.

On basis of the protein profile, fractions were selected that contain polymeric, dimeric and monomeric IgA, respectively. IgA concentration in these fractions was quantified by IgA sandwich ELISA. Increasing concentrations of IgA were coated on ELISA plates and binding of purified human MBL was assessed as described previously (25) using dig-conjugated mAb 3E7 for detection. In parallel, the amount of immobilized IgA was detected using biotinylated goat anti-human IgA (Dako, Glostrup, Denmark), followed by HRP-conjugated streptavidin (Zymed, Invitrogen, Breda, The Netherlands).

The relative amounts of polymeric, dimeric, and monomeric serum IgA were quantified on basis of the protein profile obtained following gel filtration of purified IgA, using calculation of the area under the curve.

Immunohistology and immunofluorescence

For immunofluorescence and immunoperoxidase stainings, the unfixed renal tissue was embedded in OCT compound (Sakura Tissue-tek, Bayer, Leverkusen, Germany), snap-frozen in a mixture of isopentane and dry-ice and stored at -80 °C. Subsequently, 5 µm sections were placed on slides and stored at -20 °C until immunostaining.

We used mouse monoclonal antibodies directed against the following molecules: MBL (mAb 3E7 (28), mAb 1C10 and mAb 2A9 (29)), L-ficolin (mAb GN4, mAb GN5 (30)), MASP-1/3 heavy chain (mAb 1E2 and mAb 4C2 (31,32)), C4 binding protein (mAb from Quidel, San Diego, CA, USA), C5b-9 (aE11, from Abcam, Cambridge, UK), IgA1 (mAb NI69-11 from Nordic Tilburg, The Netherlands (33)) and IgA2 (mAb 14-3-26 from Becton Dickinson, Erembodegem, Belgium and mAb NI512 from Nordic (33)). Rabbit polyclonal antibodies were applied for detection of C3 (FITC-labeled anti-human C3c, from Dako), C4d (from Biomedica, Vienna, Austria (34)) and MASP-2 (a MASP-2-specific antibody generated in the laboratory of Nephrology using the recombinant protease domain of MASP-2 ((35,36)) kindly provided by Dr. P. Gal, Institute of Enzymology, Budapest, Hungary; to be described elsewhere).

For indirect immunofluorescence, after fixation in cold acetone, tissues were incubated sequentially with the primary antibody and the proper fluorescently labeled secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse Ig or goat anti-rabbit Ig, Molecular

Probes). Slides were finally mounted with an anti-fading aqueous mounting medium (Fluorsave, Calbiochem).

Immunoperoxidase staining of MBL was performed as previously described (37). Briefly, after incubation with 0.5 % avidin (Sigma Chimica, Milan, Italy) and 0.01 % biotin (Sigma), to suppress endogenous avidin-binding activity, tissue sections were fixed in a methanol-H2O2 solution to block endogenous peroxidase. After washing, sections were incubated with primary antibody, followed by biotinylated anti-mouse antibody (Zymed), and peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (DAB, from Sigma), sections were counterstained with Harry's hematoxylin, dehydrated, and mounted in Permount.

In both immunofluorescence and immunoperoxidase methods, specificity of antibody labeling was demonstrated concurrently using proper control immunoglobulins (Zymed).

Evaluation of renal tissue

Evaluation of renal tissue was performed blindly by two independent observers. For immunostaining, tissues were scored as negative (0) or positive (1), according to the detection of staining in the majority of glomeruli, in at least 3 tubular cross-sections per field, and in vessel endothelium.

For histology, sections were stained using standard periodic-acid Schiff (PAS), periodic-acid-silver methenamine (PASM), and/or Trichrome techniques. Mesangial proliferation was scored as 1+ when mild-moderate (i.e. between 4 and 6 cells per mesangial area) and 2+ when intense (more than 6 cells per mesangial area). Extracapillary proliferation, global sclerosis, and segmental sclerosis were calculated as percentage of the total number of glomeruli. Interstitial infiltration and fibrosis were scored 0 when absent, 1+ when mild (involving < 30 % of the interstitium), 2+ when moderate (30-60 % of the interstitium involved), or 3+ when intense (when present in > 60 % of the renal interstitium). Hyalinosis of the vessel wall was indicated when absent (0) or present (1).

Statistical analysis

Data were compared between IgAN patients showing positive and negative glomerular staining for MBL, respectively. Frequency analysis was performed using Chi-square test. Other comparisons were evaluated using the Mann Whitney U test. The Spearman Rank correlation coefficient was used to analyze correlation. Differences were considered statistically significant when P was below 0.05.

RESULTS

Evidence for glomerular lectin pathway activation in IgA nephropathy

The presence of MBL was examined in 60 renal biopsies from IgAN patients. Glomerular staining for MBL was observed in a predominantly mesangial pattern in 15 biopsies (25 %, Figure 1A, C) whereas glomeruli in 45 IgAN biopsies stained negative for MBL (Figure 1D). A similar positive staining was observed using three different mAb directed against MBL, whereas staining with an isotype control mAb was completely negative (Figure 1B).

Next we examined the presence of other molecules involved in the lectin pathway of complement. All renal biopsies with positive glomerular staining for MBL also

showed glomerular staining for L-ficolin, as shown by two different mAb, whereas glomerular L-ficolin staining was negative in all MBL-negative cases (Table 1, Figure 2). Similarly, all cases positive for MBL and L-ficolin, but none of the negative cases, showed positive mesangial staining for the common heavy chain of MASP-1 and MASP-3, using two mAb, and for C4d and C4 binding protein (Table 1, Figure 2). The majority of biopsies in both MBL-negative and MBL-positive groups showed deposition of C3, whereas C1q was negative (Table 1).

All IgAN biopsies showed a typical mesangial deposition of IgA1 (Table 1, Figure 2). In contrast, we were unable to show a positive staining for IgA2, using two different IgA2-specific mAb (Table 1, Figure 2), although these antibodies showed clear positive staining on renal biopsies from lupus nephritis patients (data not shown).

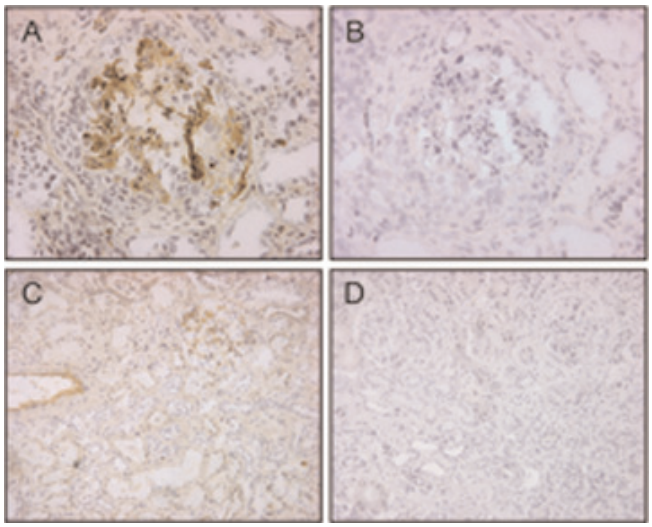

Figure 1. Glomerular MBL deposition in IgAN patients. *Biopsies from patients with IgAN were stained with mAb 1C10 (A), mAb 3E7 (C, D) or an isotype control mAb (B). Figures A, C, and D are derived from different patients who showed positive (A, C) or negative (D) staining for MBL. Figures A and B are from the same patient. Please note tubular and vascular staining for MBL in C, in addition to glomerular staining.*

In order to further extend the analysis of complement activation in IgAN, deposition of MASP-2, being the C4-cleaving enzyme of the lectin pathway, and C5b-9, as the final product of the terminal pathway of complement, was evaluated. Due to a shortage of renal tissue, an additional series of 25 renal biopsies was used for these studies. From these biopsies, 6 showed positive glomerular staining for MBL (24 %), confirming the results presented above in an independent study. All MBL-positive biopsies showed positive glomerular staining for MASP-2 (Figure 2), whereas MASP-2 was negative in all other biopsies. Staining for C5b-9 was clearly positive in a mesangial pattern in all IgAN biopsies. However, in MBL-positive biopsies, staining was observed with a high intensity (Figure 2), whereas intensity showed a marked variation in MBL-negative biopsies (not shown).

Glomerular lectin pathway activation in IgA nephropathy is associated with markers of disease progression

Data indicated above show that IgAN patients can be divided in two groups, with (25 %) and without (75 %) evidence for glomerular activation of the lectin pathway of complement. These two patient groups were further characterized.

MBL-positive and negative cases had a similar male/female distribution and a similar age at the time of the renal biopsy (Table 2). However, patients with glomerular MBL-positivity presented twice as often with renal failure at the time of renal biopsy, mostly chronic renal failure ($P < 0.0001$, Table 2). Furthermore, MBL-positive cases showed significantly more proteinuria than MBL-negative IgAN patients, whereas, in contrast, less MBL-positive IgAN patients suffered from episodes of macroscopic hematuria (Table 2, Figure 3A, B). In our IgAN patient population, macroscopic hematuria seems to be associated with a more benign disease course,

Table 1. Molecular composition of glomerular deposition in IgA nephropathy*

MBL staining	L-ficolin	MASP-1/3	C4d	C4 binding protein	C1q	C3	IgA1	IgA2
Neg (N = 45)	0%	0%	0%	0%	0%	82%	100%	0%
Pos (N = 15)	100%	100%	100%	100%	0%	60%	100%	0%

*The data indicates the percentage of biopsies with positive staining for a certain marker. Positive staining means clear positivity in the majority of glomeruli. Neg: negative, Pos: positive

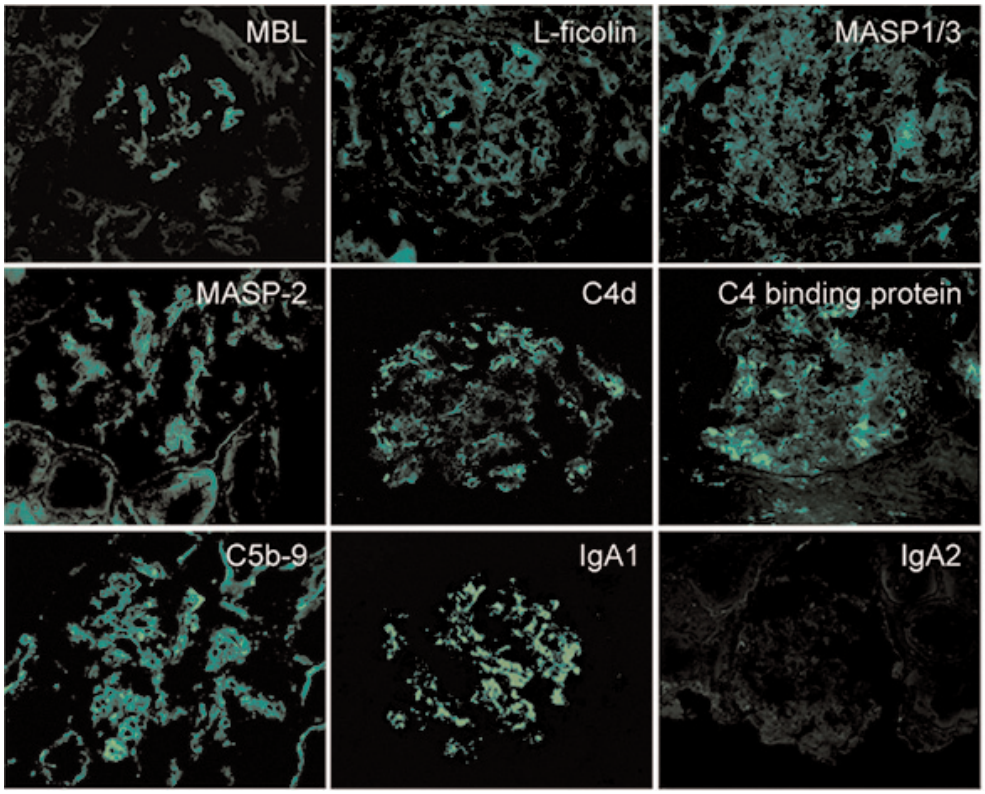

Figure 2. Lectin pathway activation in IgAN.
Renal tissue from IgAN patients was stained for the presence of MBL (mAb 3E7), L-ficolin (mAb GN4), MASP-1/3 (mAb 1E2), MASP-2, C4d, C4 binding protein, C5b-9, IgA1 and IgA2 (mAb NI512), as indicated. Representative images are shown.

since patients with macroscopic hematuria showed significantly less proteinuria than patients without macroscopic hematuria, also when MBL-negative cases were analysed only (P = 0.04, not shown). MBL-positive cases also showed a higher serum creatinine level than MBL-negative IgAN patients, although the creatinine clearance was not significantly different (Table 2). However, the latter parameter could be analyzed for only part of the patients in our study.

Data presented above suggest that glomerular MBL-positivity in IgAN is associated with a more severe disease. This is strongly supported by semi-quantitative histological evaluation of renal damage, demonstrating that biopsies from IgAN patients with positive glomerular staining for MBL had more intense mesangial proliferation as well as significantly more extracapillary proliferation, global sclerosis and interstitial infiltration than MBL-negative cases (Figure 3C-F, Table 3). Increased glomerular and tubulointerstitial damage in MBL-positive cases, as compared to MBL-negative cases, is illustrated in Figure 4.

Table 2. Clinical and laboratory data from IgA nephropathy patients

Parameter	MBL-negative	N	MBL-positive	N	P
Age at renal biopsy (yr;median)	35	36	27	12	0.28
Female gender (%)	36	36	17	12	0.21
Renal failure (% chronic)	33 (73 % chronic)	45	67 (80 % chronic)	15	**<0.0001**
Proteinuria (gram/24 hr;median)	0.7	45	2.5	15	**0.004**
Macroscopic hematuria present (%)	46	39	13	15	**0.025**
Serum creatinine (µmol/l;median)	97	45	133	15	**0.042**
Creatinine clearance (ml/min;median)	92	25	61	10	0.43
Serum MBL (ng/ml;median)	1299	28	873	12	0.8
MBL complex activity (U/ml;median)	738	29	865	12	0.27
Serum L-ficolin (µg/ml;median)	2.6	28	1.82	12	0.15
Serum IgA (mg/ml;median)	2.54	36	2.28	15	0.37
Monomeric serum IgA (%;median)	73	6	73	6	0.7
Dimeric serum IgA (%;median)	23	6	20	6	0.39
Polymeric serum IgA (%;median)	4	6	5.4	6	0.09

MBL-negative cases and MBL-positive cases are defined on basis of glomerular staining. The size distribution of purified serum IgA is expressed as % of total IgA. All data were obtained at the time of the renal biopsy.

No difference between the two groups was found for the presence of vascular lesions. Glomerular staining for MBL was significantly associated with staining for MBL in vessels and tubuli (Table 3 and Figure 1C). However, tubular and vascular staining for MBL was only weakly associated with mesangial proliferation (P = 0.03), and no association was found with proteinuria or other parameters of disease activity. Furthermore, tubulointerstitial staining for MBL was not clearly associated with markers of complement activation (not shown).

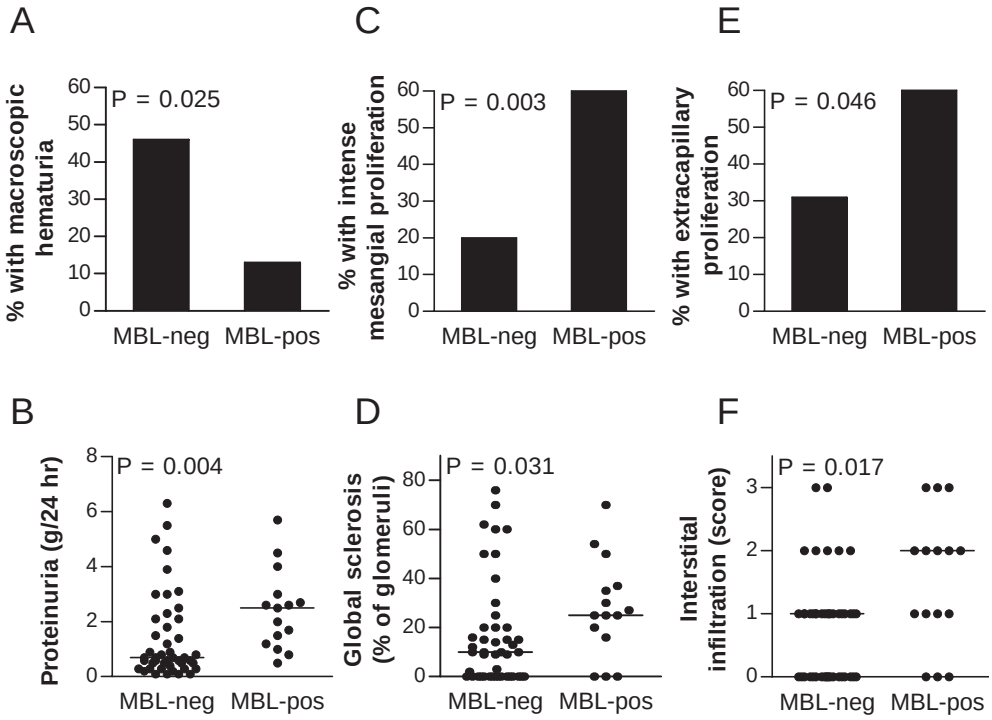

Figure 3. MBL positivity is associated with markers of renal damage.
IgAN patients were divided in two groups on basis of glomerular positivity for MBL. Clinical (A, B) and histological data (C-F) presented are scored as explained in Materials and Methods. Horizontal lines in B, D, and F represent the median. Statistical analysis was performed as described in Tables 2 and 3.

Table 3. Histological data from IgA nephropathy patients

Parameter	MBL-neg (N = 45)	MBL-pos (N = 15)	P
Intense mesangial proliferation (% of cases)	20	60	**0.003**
Extracapillary proliferation present (% of cases)	31	60	**0.046**
Global sclerosis (% of glomeruli;median)	10	25	**0.031**
Segmental sclerosis (% of glomeruli;median)	0	8	0.069
Interstitial infiltration (0-3 scale scoring;median)	1	2	**0.017**
Interstitial fibrosis (0-3 scale scoring;median)	1	2	0.12
Vessel lesions present (% of cases)	33	40	0.64
Vascular MBL staining positive (% of cases)	36	73	**0.01**
Tubular MBL staining positive (% of cases)	22	80	**0.0001**

MBL-negative cases and MBL-positive cases are defined on basis of glomerular staining

Characterization of circulating MBL, L-ficolin and IgA

We further examined whether the observed dichotomy in IgAN patients with respect to glomerular lectin pathway activation could be associated with certain properties of the lectin pathway of complement and /or the IgA in the circulation.

Serum levels of MBL are genetically determined and highly variable in the human population (27). A similar variability was observed for IgAN patients in our study, without a significant difference between cases showing positive and negative staining for MBL (Figure 5A and Table 2). Serum levels of L-ficolin showed less variability, and also did not distinguish between both groups (Figure 5B and Table 2). As a functional parameter, we assessed MBL complex activity, being a measure of the ability of the MBL-MASP-2 complex to activate C4. MBL complex activity showed a similar distribution in both groups (Figure 5C and Table 2), and was strongly correlated to MBL serum concentration (Figure 5D). As expected, three patients with clear MBL deficiency (Figure 5D) showed negative glomerular staining for MBL.

Since these data exclude the simple explanation that lectin pathway deficiency explains the lack of glomerular lectin pathway activation in most IgAN patients, we further studied whether properties of serum IgA may explain the observed dichotomy. The concentration of serum IgA was not significantly different between both groups (Figure 5E and Table 2). To address the question more in depth, serum IgA was purified from serum from IgAN patients. From patients with and without glomerular MBL deposition, we selected 6 patients in each group. Patients with a (possible) MBL deficiency were excluded. IgA was purified from serum and subjected to gel filtration. The protein profile clearly reveals the presence of polymeric,

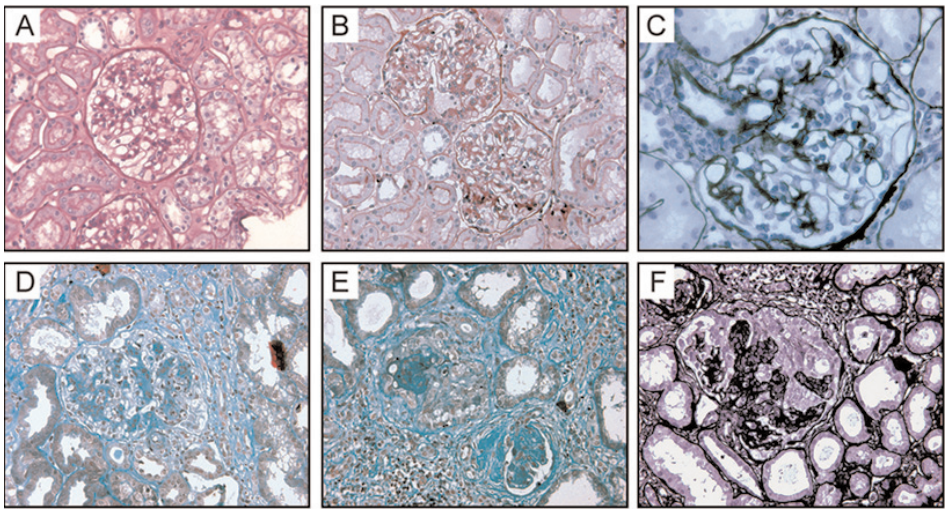

Figure 4. Renal histological damage in IgAN patients.
Renal histology from three patients without glomerular MBL deposition (A-C) shows glomeruli affected by mild mesangial proliferation and mild mesangial matrix expansion. No major tubulo-interstitial lesions are visible. Images D-F represent patients with glomerular MBL deposition (N = 3). Glomerular injury is characterized by intense mesangial proliferation and mesangial matrix expansion (D), segmental and global sclerosis (E), and extracapillary proliferation (F), whereas tubular dilations, interstitial infiltration and fibrosis are evident in the interstitium (D-F). Sections were stained with PAS (A, B), PASM (C, F) or trichrome (D, E) techniques.

dimeric and monomeric IgA (Figure 6A). The size distribution of serum IgA in IgAN patients with and without glomerular MBL deposition showed a low variability without any significant differences (Figure 6B and Table 2).

Subsequently, we studied the binding of purified MBL to patient IgA of different sizes. Therefore, increasing concentrations of monomeric, dimeric, and polymeric IgA were coated on ELISA plates, and binding of MBL was assessed. The results showed a high variability in the MBL binding properties of IgA from different donors, although similar amounts of IgA were immobilized on the ELISA plates (Figure 7A). Monomeric IgA did not show MBL binding, whereas strong but donor-dependent MBL binding could be observed to polymeric IgA (Figure 7B). MBL binding to dimeric IgA was significantly less than that to polymeric IgA ($P < 0.01$). Quantification of the binding of MBL per unit immobilized IgA did not reveal a significant difference between patients with and without glomerular MBL positivity, respectively (Figure 7B). However, two patients whose IgA showed strong MBL binding both showed positive deposition of MBL in the glomerulus.

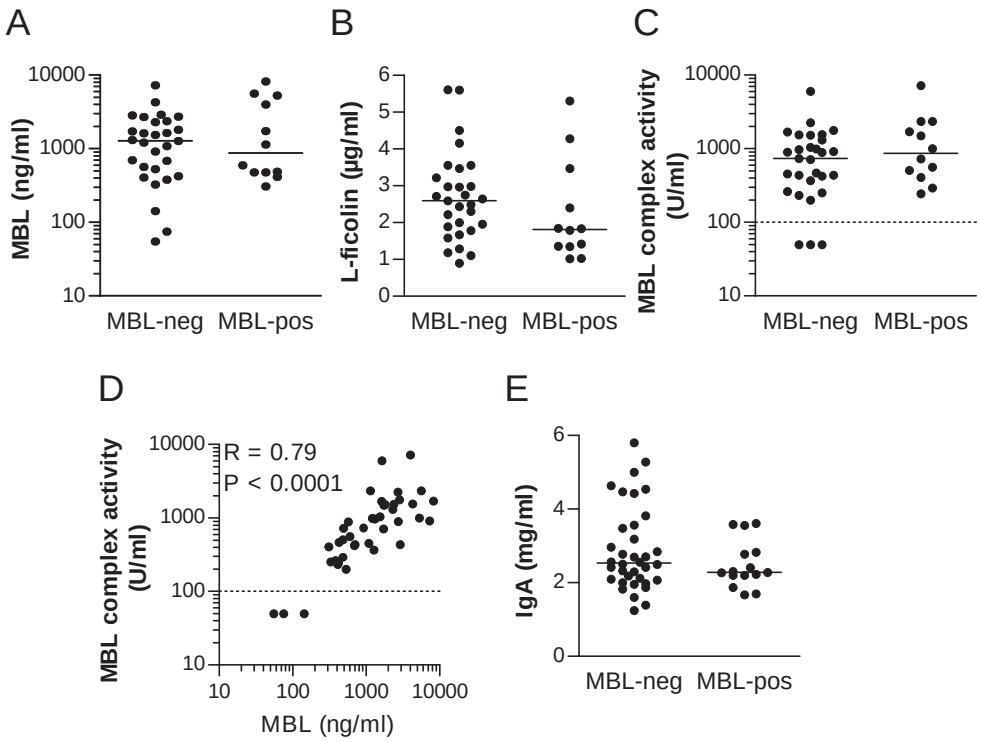

Figure 5. Circulating MBL, L-ficolin, and IgA in IgAN patients. *Concentrations of MBL (A), L-ficolin (B) and IgA (E), as well as MBL complex activity (C) were quantified in serum of IgAN patients as described in Materials and Methods. IgAN patients were divided in two groups on basis of glomerular positivity for MBL. Horizontal solid lines represent the median, and dashed lines indicate the detection limit. Correlation between MBL concentration and MBL complex activity is shown in D.*

DISCUSSION

Data from the present study indicate that IgAN patients can be divided in two groups on the basis of the pattern of complement activation. About 75 % of IgAN patients show negative glomerular staining for MBL, L-ficolin, MASP, C4d and C4-binding protein, indicating that C3 and C5b-9 activation in these patients most likely occurs via the alternative pathway. In contrast, 25 % of IgAN patients show glomerular deposition of MBL, L-ficolin, MASP, C4d but not C1q, which is strongly indicative of activation of complement via the lectin pathway of complement. Importantly, the clinical and histological data clearly indicate that activation of the lectin pathway of complement is associated with more severe renal damage.

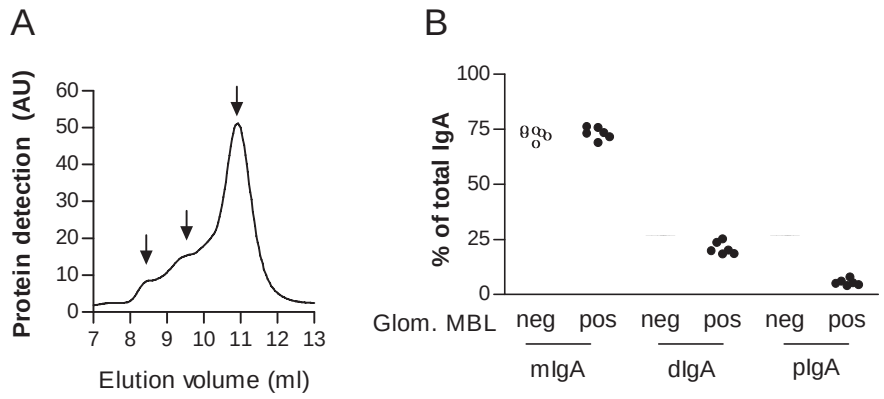

Figure 6. Size distribution of circulating IgA. *A. Typical example of a protein profile obtained after gel filtration of purified IgA. Position of polymeric, dimeric and monomeric IgA is indicated. B. Quantification of IgA size distribution of IgA from 12 IgAN patients showing positive or negative glomerular staining for MBL.*

Using a well-defined polyclonal antibody to C4d, which is now frequently used in the diagnostic evaluation of renal transplant rejection (34), we observed deposition of glomerular C4d in IgAN exclusively in association with molecules of the lectin pathway. An identical pattern was observed for deposition of C4-binding protein, a regulatory protein that was also previously reported as a sensitive marker for the presence of C4 (20). Activated C4 is most likely generated by MASP-2, the key complement-activating enzyme of the lectin pathway, which was shown to be present in the mesangial area in association with MBL.

Next to strongly suggesting activation of C4 via the lectin pathway in a subpopulation of IgAN patients, our data indicate glomerular activation of C3 and C5b-9 via the alternative pathway in the majority of IgAN patients, independently of C4. The latter aspect is in agreement with a number of in vitro studies showing alternative pathway activation by IgA from human and rodent species (6,13,15).

We assume that mesangial MBL and L-ficolin are derived from the circulation as preformed complexes with MASPs, although local production of molecules of the lectin pathway can not be excluded (38). Our previous in vitro studies showed that MBL is able to interact via its lectin domain with purified polymeric serum IgA (25).

Further support for an interaction between MBL and IgA is provided by studies in Henoch Schönlein Purpura nephritis (39,40). In the present study, we show that MBL binds to polymeric IgA from IgAN patients. It is possible that MBL binds to glycans present on the IgA heavy chains. IgA consists of two subclasses, IgA1 and IgA2, of which the former dominates in the circulation (90 % IgA1). However, mucosal IgA may contain up to 65 % IgA2 (7). Interestingly, circulating IgA1 from IgAN patients was reported to have aberrant glycosylation of O-linked glycans, potentially involved in recognition by lectins (41).

Although it is generally believed that IgA deposits in IgAN consist almost exclusively of IgA1 (1,2,7,33,42,43), biopsies from IgAN patients may also contain IgA2 (44). A recent study in this field by Hisano et al. (23) showed that about 50 % of a

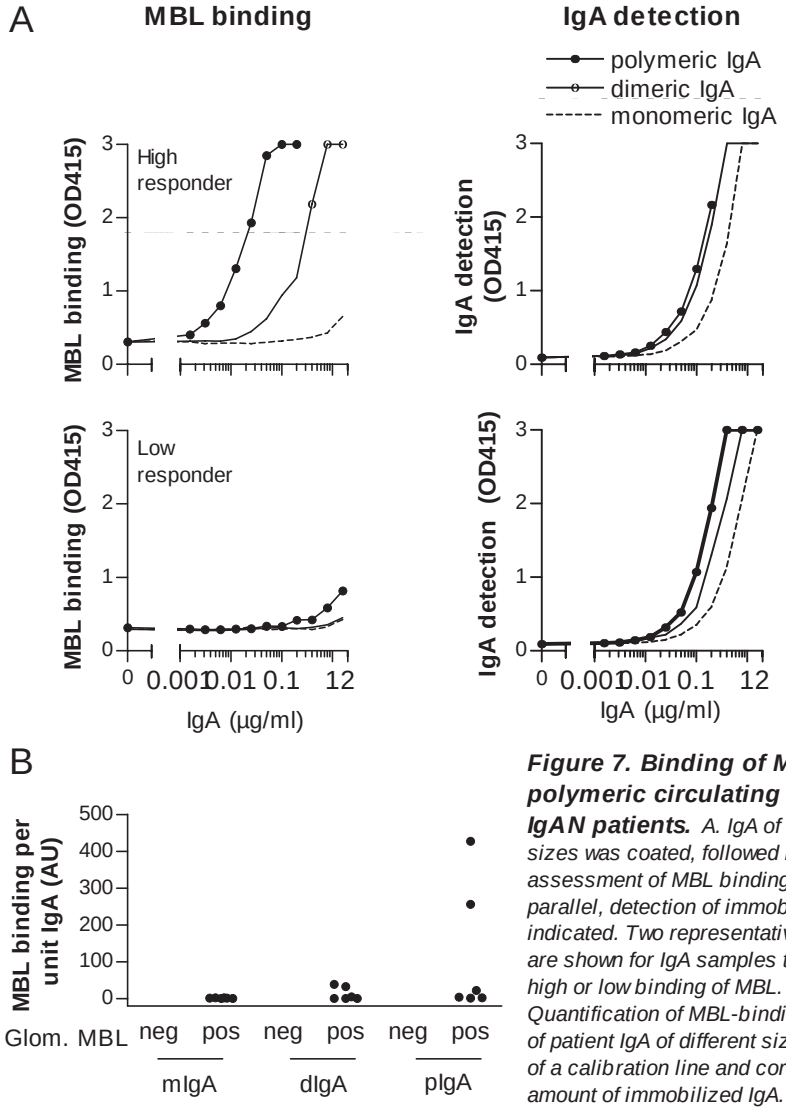

Figure 7. Binding of MBL to polymeric circulating IgA from IgAN patients. *A. IgA of different sizes was coated, followed by assessment of MBL binding and, in parallel, detection of immobilized IgA, as indicated. Two representative examples are shown for IgA samples that show a high or low binding of MBL. B. Quantification of MBL-binding properties of patient IgA of different sizes, on basis of a calibration line and corrected for the amount of immobilized IgA.*

Japanese population of IgAN patients showed deposition of IgA2 next to IgA1, whereas glomerular MBL deposition was exclusively observed in these IgA2-positive patients. Similar results were recently reported in Henoch-Schönlein purpura nephritis (40). Stimulated by these striking observations, we investigated the glomerular presence of IgA2 in our patient population, using two different IgA2-specific mAb, including mAb 14-3-26 that was also used by Hisano et al. (23). However, we were not able to detect any glomerular positivity for IgA2 in IgAN biopsies. This difference might be due to ethnical differences between patient populations. Furthermore, although we tried several amplification methods to acquire a signal for IgA2, it could still be that IgA2 is present in tiny amounts and/or masked by other molecules, thereby hampering detection.

IgA deposited in the renal mesangium of IgAN patients is classically accepted as being polymeric IgA (45). Polymeric IgA contains several other molecular components, which are currently only partially identified (45,46). It can not be excluded that MBL binds to a glycosylated molecule associated with polymeric IgA and present in the mesangial deposits of IgAN patients. An alternative explanation would be that MBL binds to injured tissue in the glomerulus, which is in line with the binding of MBL to apoptotic and necrotic cells (47).

To our best knowledge, data from the present study are the first indication for a role of L-ficolin in disease. In vitro data indicate that ligand specificities of MBL and L-ficolin do only partially overlap (16,17,48). However, recent experiments in our group indicate that L-ficolin also binds to polymeric IgA (Roos et al. manuscript in preparation). MBL and ficolins have an important role in innate immune defense. However, evidence is increasing that MBL and the lectin pathway of complement can also be harmful for the host as mediators of inflammation. In this respect, MBL has been proposed to be involved in ischemia/reperfusion injury (49), diabetic nephropathy (50) and ulcerative colitis (51). In the present study, we observed that IgAN patients with glomerular MBL deposition and lectin pathway activation showed significantly more renal damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis, interstitial infiltration, and proteinuria. An association of glomerular MBL positivity with decreased renal function and increased mesangial proliferation and proteinuria was also reported by Matsuda et al. (21) but could not be confirmed by studies from Endo et al. (22) and Hisano et al. (23). In Henoch-Schönlein purpura nephritis, MBL deposition was associated with increased progression of renal disease (40). Increased disease progression in association with glomerular lectin pathway activation may possibly be ascribed to pro-inflammatory activation products of the complement system, including C5a and C5b-9, and potentially also to a direct effect of MBL.

Similar to several other studies (21-23), we observed lectin pathway activation only in a subpopulation of IgAN patients. The majority of IgAN patients without mesangial MBL deposition did not show any evidence for deficiency of MBL or L-ficolin. Interestingly, serum concentrations of MBL in this population of IgAN patients (N= 41, median = 1213 ng/ml) were significantly higher than those in a population of Caucasian healthy controls (N = 190; median = 677 ng/ml, P = 0.002, data not shown). This difference could be explained by genetic differences and/or by conditions associated with renal disease.

We hypothesize that the difference between IgAN patients with and without mesangial MBL deposition is based on differential availability of the ligand, potentially related to differences in IgA glycosylation. In order to further examine this hypothesis, we investigated the binding of purified MBL to polymeric, dimeric and monomeric IgA from IgAN patients being either positive or negative for glomerular MBL deposition. This analysis revealed binding of MBL to polymeric serum IgA with a large inter-individual variation, but the degree of MBL binding could not be directly related to glomerular lectin pathway activation. However, mesangial IgA is not necessarily similar to serum IgA, and furthermore, it could be that glomerular IgA is differentially accessible in IgAN patients. We assume that the observed dichotomy in IgAN patients is due to several factors acting in combination.

In conclusion, the current study shows that MBL and the lectin pathway of complement are involved in complement activation in a subpopulation of patients with IgAN, which is highly likely to play an adverse role in the disease. In vitro data point to a role for polymeric IgA in lectin pathway activation. Precise identification of the ligand for MBL and L-ficolin in the mesangium, which is presumably present and/or accessible in only part of the patients with IgAN, will provide novel insight in the pathogenesis of IgAN and may provide novel therapeutic options to treat disease progression.

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CHAPTER 5

Differential glycosylation of polymeric and monomeric IgA: a possible role in glomerular inflammation in IgA nephropathy

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Summary

IgA nephropathy (IgAN) is characterized by mesangial deposition of polymeric IgA1 and complement. Complement activation via MBL and the lectin pathway is associated with disease progression. Furthermore, recent studies have indicated a possible role for secretory IgA. IgAN is associated with abnormalities in circulating IgA, including aberrant O-linked glycosylation. The present study characterized and compared functional properties and N-linked glycosylation of highly purified monomeric and polymeric IgA from IgAN patients and controls. Total serum IgA was affinity-purified from patients (n= 11) and controls (n= 11) followed by size-separation. Polymeric but not monomeric IgA contained secretory IgA, and its concentration was significantly higher in IgAN patients than in controls. Both in IgAN patients and in controls, IgA binding to the GalNAc-specific lectin HAA and to MBL was much stronger for polymeric than for monomeric IgA. Furthermore, binding of IgA to mesangial cells was largely restricted to polymeric IgA. Binding of polymeric IgA to mesangial cells resulted in increased production of IL-8, predominantly with IgA from IgAN patients. Quantitative analysis of N-linked glycosylation of IgA heavy chains showed significant differences in glycan composition between monomeric and polymeric IgA, including the presence of oligomannose exclusively on polymeric IgA. In conclusion, binding and activation of mesangial cells, as well as lectin pathway activation is a predominant characteristic of polymeric IgA as opposed to monomeric IgA. Furthermore, polymeric IgA has different N-glycans, which may recruit lectins of the inflammatory pathway. These results underscore the role of polymeric IgA in glomerular inflammation in IgAN.

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INTRODUCTION

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease leads to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA in the glomerular mesangium, together with markers of complement activation (1,2). It is generally thought that this mesangial IgA mainly consists of IgA1 and is mostly polymeric (3). The composition of polymeric forms of IgA in serum is diverse and may include dimeric IgA, secretory IgA (SIgA), CD89 (FcαRI) /IgA complexes, IgA immune complexes and IgA-fibronectin complexes (4-6). Serum dimeric IgA consists of two IgA molecules linked with J chain, whereas SIgA in addition contains secretory component, derived from the mucosal epithelium.

Deposition of circulating IgA in the mesangium leads to renal inflammation, potentially involving direct interactions of IgA with resident and infiltrating cells in the glomerulus, as well as complement activation. The inflammatory process results in renal injury. Although the mechanism of IgA deposition in the renal mesangium of IgAN patients has been a subject of intensive research during the last decades, the pathogenesis of IgAN is still incompletely characterized. A number of studies provided evidence for a mesangial IgA receptor, which is involved in mesangial cell activation by IgA in vitro (7-9).

Glomerular IgA deposition is associated with activation of the complement system (5), involving the alternative pathway and the lectin pathway of complement (10). Recent studies indicate deposition of MBL, a major recognition molecule of the lectin pathway of complement, in a subpopulation of patients in association with a more severe renal injury (10,11), whereas in vitro studies demonstrated binding of MBL to polymeric serum IgA (12). Glomerular complement activation can enhance renal injury via the pro-inflammatory effects of the complement activation cascade.

Studies in IgAN patients who received a renal transplant showed recurrence of mesangial IgA deposition in a high number of cases (13). Vice versa, the accidental transplantation of a kidney with mesangial IgA deposition into a recipient without IgAN resulted in spontaneous disappearance of IgA deposits following transplantation (14). These studies strongly suggest that IgAN is a systemic disease rather than a disease of the kidney.

On basis of these data, abnormalities in IgA are hypothesized to be involved in the pathogenesis of IgAN. Therefore, circulating IgA from IgAN patients has been extensively studied. Serum from IgAN patients contains higher concentrations of IgA (15,16). Recently, our group showed low concentrations of circulating secretory IgA in IgAN patients and controls, whereas IgAN patients with a high serum concentration of SIgA showed more hematuria (17). Furthermore, SIgA accumulated in glomerular IgA deposits, suggesting a pathogenic role for SIgA in IgAN (17). Several studies also focused on IgA glycosylation, showing aberrant O-glycosylation on circulating IgA from IgAN patients, resulting in increased Tn antigen (GalNAcα1-Ser/Thr) residues (18,19). This undergalactosylated IgA1 may lead generation of circulating IgG-IgA1 complexes (20). O-linked glycans are present on IgA1 but not IgA2, whereas IgA1 and IgA2 heavy chains both contain several N-glycosylation sites (21). The galactosylation of the N-glycans is not different between IgAN

patients and controls (22). However, the complete structure of N-linked glycans on IgA has not been studied in IgAN.

Functional studies with purified IgA from IgAN patients suggested an increased interaction of IgA from IgAN patients with mesangial cells as compared to IgA from healthy individuals (23), although this is still controversial (24). Furthermore, after stimulation of mesangial cells with IgA from IgAN patients, the production of proinflammatory cytokines and chemokines was shown to be increased (25), possibly involving (26,27) undergalactosylation of IgAN IgA (28).

The aim of the present study was to characterize and compare the molecular composition and functional properties of monomeric and polymeric serum IgA from IgAN patients and controls. Therefore, we analysed highly purified total serum IgA from patients and controls in a number of aspects that are potentially important in the pathogenesis of IgA nephropathy, including interaction with lectins and mesangial cells. The results show clear functional differences between naturally occurring polymeric and monomeric serum IgA both for patients and controls. The most obvious difference that was noted between IgA isolated from IgAN patients and from controls was an increased fraction of SIgA in polymeric IgA from IgAN patients. Furthermore, we demonstrate that polymeric IgA differs from monomeric IgA in its composition of N-linked glycans.

MATERIAL AND METHODS

Human subjects

In the present study, we obtained serum from 11 healthy volunteers and 11 patients with primary IgAN (Table 1). All patients had biopsy-proven IgA nephropathy. None of these patients had clinical or laboratory evidence of Henoch Schönlein purpura, systemic lupus erythematosus or liver disease, or received immunosuppressive therapy. A healthy control group was selected and matched for gender. The mean age of the controls was somewhat lower; however we had no indications that this affects the biochemical properties of IgA. Renal function was non stable in 5 of the 11 patients with serum creatinine ranging from 203 to 366 µmol/L. The study was approved by the ethical committee of the Leiden University Medical Center. All individuals gave informed consent.

Table 1 Clinical characteristics of the patients with IgA nephropathy and control persons

	IgAN patients	controls
Number	11	11
Male/ female	8/3	9/2
Mean age (*years)*	48	33
Mean serum creatinine (*µmol/L;* range)	190 (82-366)	ND
Proteinuria (0 to 3+; median)	1+	ND
Erythrocyturia (0 to 5+; median)	1+	ND

IgA purification

IgA was precipitated from serum using $(NH4)_2SO_4$ at 50 % saturation, followed by affinity chromatography using HisA43 (mAb against human IgA, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) coupled to CNBR-activated Sepharose 4FF (Amersham). A column of 15 ml was loaded with gamma globulin precipitate corresponding to 10 ml serum, using 0.5 x PBS as a running buffer, followed by washing with 90 ml 0.5 x PBS. Fractions of 3 ml were collected. To remove non-specifically bound proteins, the column was washed with 70 ml 1 M NaCl. Finally, bound IgA was eluted with 100 ml 0.1 M glycine/ 0.3 M NaCl (pH 2.8). Fractions were neutralized with 1 M Tris (pH 8.0). Fractions that contained IgA, as assessed by ELISA (29), were pooled, dialysed against PBS containing 2 mM EDTA, and applied to a mixture of protein G/ anti-human IgM (HB57)-Biogel A5 to remove residual contaminating IgG and IgM, followed by concentration and size-separation with a HiLoadTM 16/60 HR200 Superdex prep grade gel filtration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands), run in 50 mM NH_4HCO_3. Fractions were assessed for the presence of IgA and total protein. On basis of the protein profile, IgA-containing fractions were pooled into polymeric IgA (eluted at 44-50 ml) and monomeric IgA (eluted at 50-60 ml). These pools were analysed for total IgA, IgA1 and IgA2 content using ELISA (29). The percentage of polymeric IgA was quantified using calculation of the area under the curve on basis of the gel filtration profile.

MBL binding ELISA

MBL was purified from pooled plasma obtained from healthy human donors, as described before (12), resulting in a preparation of MBL in complex with its associated serine proteases (MASPs). MBL binding was studied by ELISA, in which 5 µg/ml IgA, or human serum albumin (HSA) as a control, was coated , followed by blocking with PBS/BSA, incubation with MBL (2 µg/ml) and detection of MBL binding as described (12). For inhibition experiments, MBL was pre-incubated with MgEGTA (10 mM), D-mannose or L-mannose (100 mM, from Sigma).

Activation of C4 via the lectin pathway

Activation of C4 by MBL-MASP complexes was measured as described before (12). In brief, incubation of MBL-MASP-complexes on coated IgA was followed by incubation with purified C4 and detection of C4 binding.

HAA binding

IgA was assessed for binding to biotinylated *Helix Aspersa* (HAA, Sigma) lectin, known to recognize terminal GalNAc. NUNC Maxisorp plates were coated with 5 µg/ml IgA or HSA as a control, in carbonate buffer (pH 9.6), overnight at room temperature. After washing with PBS/ Tween and blocking for one hour with PBS/ 1 % BSA, wells were incubated with 5 µg/ml biotinylated HAA in PBS/ 1 % BSA/ 0.05 % Tween. Binding of HAA was detected with HRP-conjugated streptavidin (Zymed). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured.

SIgA ELISA

In order to quantify SIgA levels in isolated IgA, a sandwich ELISA specific for SIgA was used as described before (17). Briefly, plates were coated with a mAb against secretory com-

ponent (NI194-4), followed by incubation with IgA and detection of IgA binding.

Glycosidase treatment of IgA

Detection of undergalactosylated IgA with lectins could be hampered by the presence of sialic acids. To get a clear and full picture of the galactosylation, we treated IgA with neuraminidase and checked for binding to HAA and MBL. IgA (5 µg/ml) and HSA were coated, followed by blocking, and subsequent incubation with 100 mM sodium acetate pH 5.0, with or without 10 mU/ml Neuraminidase from Arthrobacter ureafaciens (Roche, Mannheim, Germany), for 3 hours at 37 ºC. Subsequently HAA and MBL binding were assessed as described above.

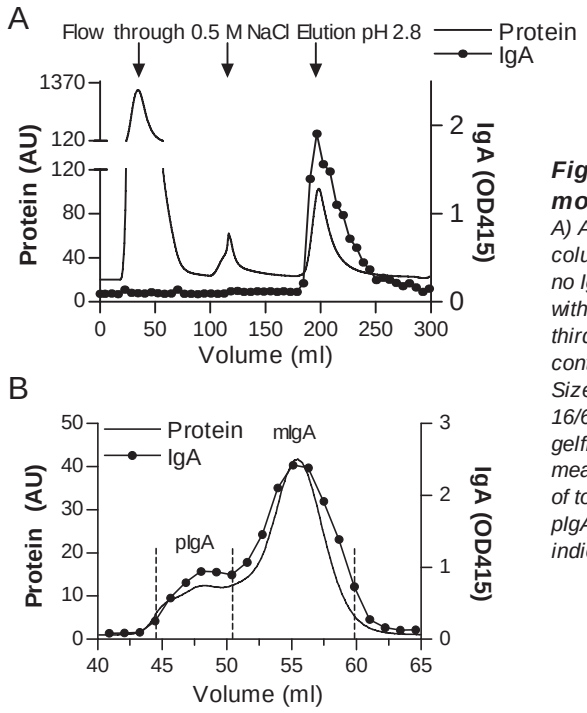

Figure 1. Purification of monomeric and polymeric IgA.
A) Affinity purification of IgA with an anti-IgA column. First peak is flow through (protein, no IgA), the second peak is after washing with 0.5 M NaCl (protein, no IgA), and the third peak is after acid elution. This peak contains IgA as detected with ELISA. B) Size-fractionation of IgA on a HiLoadTM 16/60 HR 200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA by ELISA. IgA was pooled in pIgA (44-50 ml) and mIgA (50-60 ml) as indicated.

Cell culture

Normal human mesangial cells (NHMC, Cambrex, USA) were expanded according to the protocol provided by the manufacturer in mesangial cell basal medium with supplements (Cambrex, USA). Experiments with NHMC were performed in RPMI with 10 % FCS, 1 % non-essential amino acids, 0.5 % transferrin/insulin/selenium, 1 % sodium pyruvate, and 1 % L-glutamine (all purchased at Gibco/ life Technologies, Paisley, Scotland). AMC11, a spontaneously growing mesangial cell line of adult human origin (kindly provided by Prof. Holthofer, Helsinki), was cultured in DMEM with 10 % FCS. Cells were harvested by trypsinization.

Flow cytometry

Cells were washed with FACS buffer (0.5 x PBS containing 1 % BSA/ 2.8 % glucose/ 0.01 % NaN_3) and incubated with monomeric and polymeric IgA. Following incubation for 1 hour at 4 º C, cells were washed and incubated for one hour at 4 º C with monoclonal anti-IgA mAb 4E8 (IgG1) (29). IgA binding was visualized using PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and fluorescence intensity was assessed by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

Cytokine analysis

Production of IL-8 and MCP-1 was measured in supernatants of cultured mesangial cells. Prior to stimulation, cells were transferred to 96-wells plates (Costar, Corning, NY) at a den-

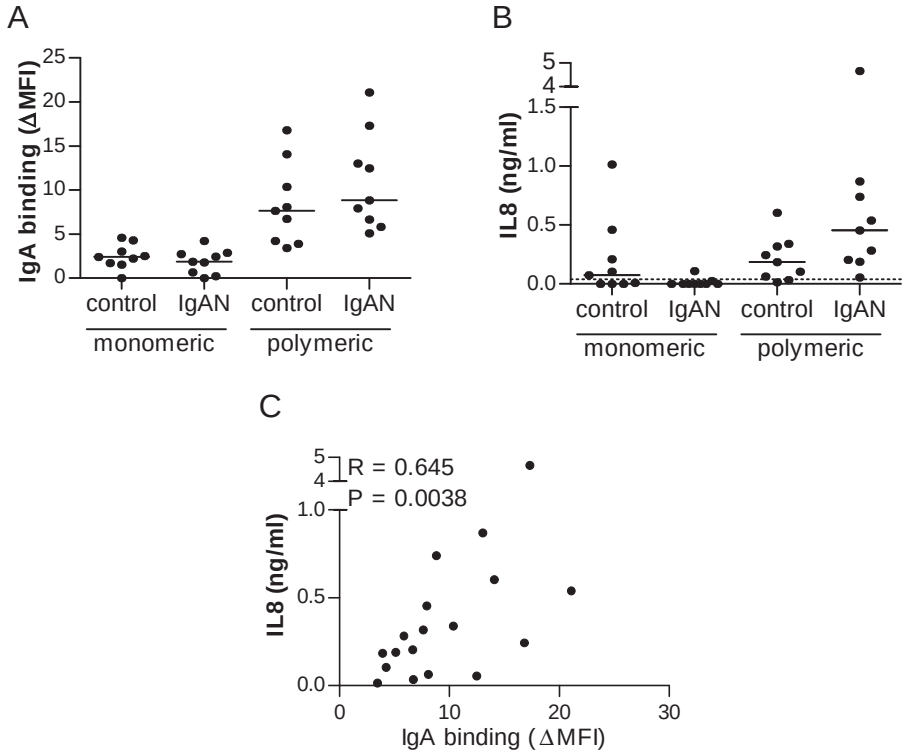

Figure 2. Increased binding and stimulation of mesangial cells with polymeric IgA. A) Normal human mesangial cells were incubated with different *molecular forms of IgA (200 µg/ml) from IgAN patients and controls, and assessed for IgA binding by flow cytometry. Depicted is the mean fluorescence intensity after subtracting the isotype control. B) Human mesangial cells (15 x 103 cells/well) were stimulated with different molecular forms of IgA (200 µg/ml). After 72 hours supernatants were harvested and tested for IL-8. Horizontal lines indicate the median, the dotted line represents the detection limit. IL-8 was undetectable in cultures without IgA. Polymeric IgA versus monomeric IgA (A, B): P < 0.01. C) Correlation between production of IL-8 after stimulation of mesangial cells with IgA and the binding of IgA to mesangial cells.*

sity of 15 x 10^3 cells per well and cultured overnight in culture medium with 0.5 % serum. Cells were cultured in the presence or absence of monomeric and polymeric IgA for 72 hours, in concentrations as indicated. The concentration of IL-8 and MCP-1 in culture supernatants was measured by ELISA as described (30,31).

N-glycan analysis

The IgA heavy chains were isolated on SDS-PAGE under reducing conditions and visualised by Coomassie staining. The N-Glycans were released from these excised gel bands by PNGase F, labelled with the fluorophore 2-aminobenzamide and analysed by normal phase (NP) HPLC with exoglycosidase sequencing as described (32).

Identification of Gel Bands by Mass Spectrometry.

The Coomassie-stained IgA heavy chain bands from an SDS-PAGE gel were excised and in-gel digested with trypsin (sequencing grade; Roche) as described (32).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test and the Wilcoxon signed rank test. The Spearman Rank correlation coefficient was used to analyze correlations. Differences in N-glycan composition were evaluated using the Student's T-test. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Polymeric IgA binds better to mesangial cells and induces more cytokine production after activation of mesangial cells

Human IgA was purified with an anti-IgA affinity column (Figure 1A). In the flow through no IgA was detectable whereas IgA was eluted by acid elution. The fractions containing IgA were pooled. Purified IgA was applied to a gel filtration column (Figure 1B), and fractions containing polymeric IgA (pIgA) and monomeric IgA (mIgA) were pooled as indicated.

To examine possible functional differences between monomeric and polymeric IgA from IgAN patients and controls, we investigated the binding of IgA to mesangial cells, as well as the cytokine response following stimulation. Polymeric IgA showed a 5.9-fold higher binding to mesangial cells than mIgA (P = 0.0003), but

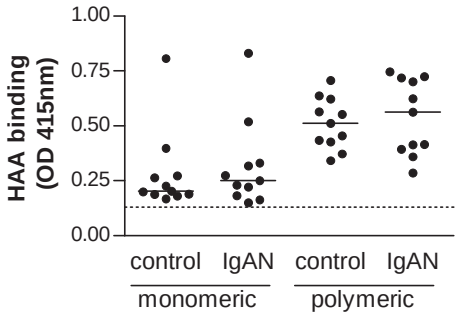

Figure 3. HAA binding to monomeric and polymeric IgA.
Monomeric IgA and polymeric IgA of IgAN patients and controls were coated in ELISA plates (5 µg/ml), followed by incubation with biotin-labeled HAA. Horizontal lines indicate the median, the dotted line represents binding to HSA. Polymeric IgA versus monomeric IgA: P = 0.0003.

there was no difference between IgAN patients and controls (Figure 2A).

Supernatants of the cells stimulated with IgA for 72 hours were tested for production of the chemokines IL-8 and MCP-1. The IL-8 production was significantly higher after stimulation of mesangial cells with polymeric IgA than after stimulation with mIgA (P = 0.010), whereas IL-8 production was undetectable in cultures without IgA (Figure 2B). Furthermore, IL-8 production tended to be higher upon stimulation with polymeric IgA from IgAN patients compared with that from controls (P = 0.077). A significant correlation was observed between binding of pIgA to mesangial cells and IL-8 production after co-culture (Figure 2C; R = 0.6450, P = 0.0038). Furthermore, stimulation with IgA also clearly enhanced production of MCP-1, and MCP-1 production correlated with IL-8 production after stimulation with different IgA samples (R = 0.59, P = 0.01). These functional data indicate intrinsic differences between monomeric and polymeric IgA.

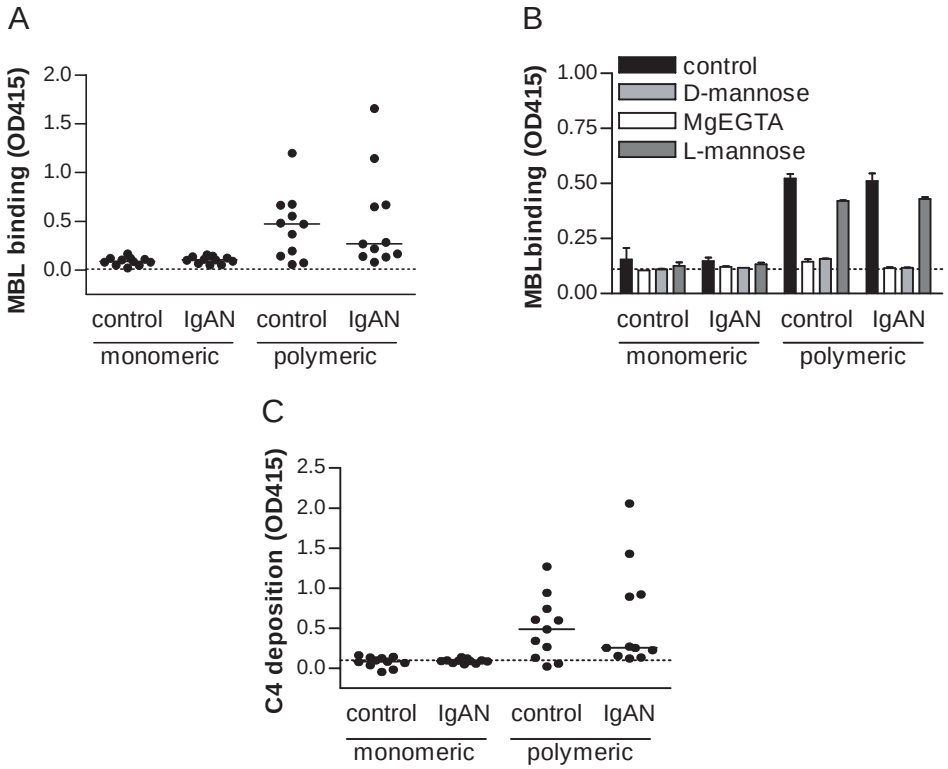

Figure 4. MBL binding to polymeric IgA from IgAN patients and controls.
Monomeric and polymeric IgA were coated in ELISA plates, followed by incubation with purified MBL-MASP complexes. A) Detection of MBL binding. B) MBL was pre-incubated with MgEGTA (10 mM), D-mannose (100 mM) or L-mannose (100 mM) before incubation on wells coated with IgA as indicated. MBL binding was detected. The horizontal dashed line represents the negative control C) Detection of C4 activation after incubation with purified C4. For each IgA sample, blanc values, obtained after incubation with C4 and/or detection antibodies without MBL, were subtracted. Dashed lines indicate OD values obtained with coating of HSA. Polymeric IgA versus monomeric IgA (A, C): P = 0.0001.

Interaction of polymeric IgA with HAA lectin

It has been reported that O-glycans of patients with IgAN contain more Tn antigen (GalNAc-Ser/Thr) compared to controls (18,33). Terminal GalNAc can be detected by specific lectins, including Helix Aspersa (HAA). We investigated the binding of HAA to IgA by ELISA (Figure 3). The binding of HAA to IgA from healthy individuals and IgAN patients was 4 fold higher for polymeric IgA than for monomeric IgA (P = 0.0003). However, we could not observe a difference in HAA binding between patients and controls.

MBL exclusively binds to polymeric IgA, resulting in C4 activation

We and others showed that IgAN is associated with complement activation via the lectin pathway (10,34). Therefore, the binding of MBL to purified IgA from patients with IgAN and controls was studied, showing that MBL binds to pIgA but not to mIgA with a high inter-individual variation, both for IgAN patients and controls (Figure 4A). Using parallel detection of immobilized IgA on plates, we confirmed that equal amounts of IgA were present in coated wells, indicating that differences in coating could not explain the observed differences (not shown). Binding of MBL to IgA was completely inhibitable by D-mannose and MgEGTA but not by L-mannose (Figure 4B), confirming that the C-type lectin domain of MBL was involved in binding to IgA.

Binding of MBL to pIgA from IgAN patients and from controls resulted in activation of purified C4 (Figure 4C), presumably involving C4 cleavage by MBL-associated MASP-2. This activation of C4 showed a strong correlation with MBL binding (R= 0.98, P < 0.0001 for pIgA).

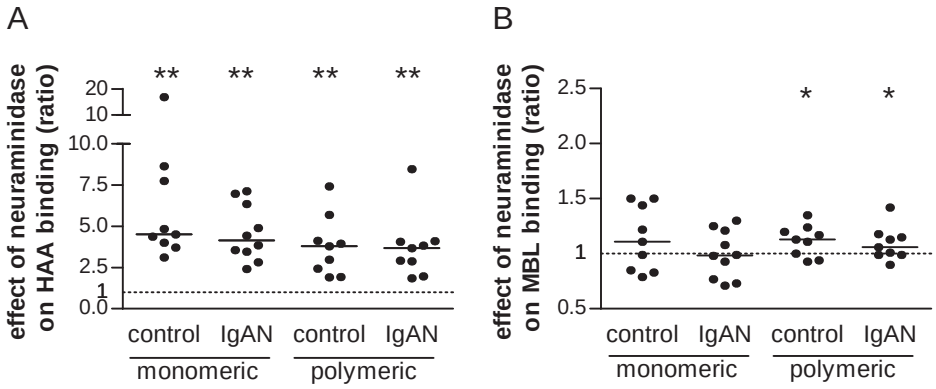

Figure 5: Increased interaction with HAA after treatment of IgA with neuraminidase.

*IgA (5 µg/ml) was coated in ELISA. After treatment with neuraminidase HAA binding (A) and MBL binding (B) were detected. Depicted is the ratio of non-treated and treated IgA after subtraction of background values, and the dotted line represents the non-treated IgA. * P < 0.05, ** P <0.01.*

Treatment of IgA with neuraminidase enhances its interaction with HAA

To examine whether sialic acids, commonly present on N-linked and O-linked glycans of IgA, might hamper the interaction of IgA with HAA and/or MBL we treated immobilized IgA with neuraminidase. After treatment of IgA the interaction with HAA increased significantly (3.8-fold for control mIgA, 3.1-fold for IgAN mIgA, 3.2-fold for control pIgA, 3.0-fold for IgAN pIgA; $P < 0.004$) (Figure 5A), suggesting the presence of sialylated Tn antigen, as the removal of the sialic acid exposes the GalNAc (Tn) epitope. In contrast, the binding of MBL to IgA was hardly affected by neuraminidase, only showing a minor increase after treatment of polymeric IgA (1.1-fold) (Figure 5B), consistent with the known specificity of MBL for glycans presenting 3,4 cis hydroxyls such as mannose to which sialic acids do not attach.

Molecular composition of monomeric and polymeric IgA from IgAN patients and controls

The results presented above indicate major functional differences between monomeric and polymeric IgA. We therefore investigated the molecular composition of monomeric and polymeric IgA from IgAN patients and controls. The size distribution of IgA from IgAN patients and controls was similar (mean % polymeric IgA, controls: 18.9 %, patients: 18.8%; $P = 0.89$). The IgA preparations were assessed for IgA1 and IgA2 content by ELISA. As described before (35), IgA2 is a minor constituent of human serum IgA. However, the relative amount of IgA2 was significantly higher in polymeric IgA (20 ± 4.1 %) as compared to monomeric IgA (9.2 ± 4.7 %) (Figure 6A) ($P< 0.0001$), suggesting that circulating IgA2 is more likely to be produced as polymeric complexes than circulating IgA1. The relative amount of IgA2 was similar in IgA from patients and controls.

Subsequently, we measured SIgA in monomeric and polymeric IgA from IgAN patients and controls. In agreement with the molecular size of SIgA, SIgA is exclu-

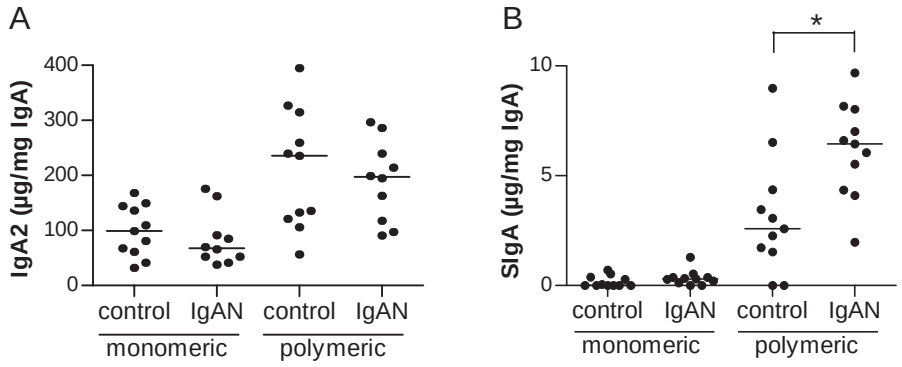

Figure 6. Increased concentration of SIgA and IgA2 in polymeric IgA.
*The relative content of IgA2 (A) and SIgA (B) in monomeric and polymeric IgA as assessed by ELISA. Horizontal lines indicate the median. Polymeric IgA versus monomeric IgA (A, B): $P < 0.0001$. SIgA content in pIgA of IgAN patients versus controls: * $P = 0.0152$.*

sively present in polymeric IgA (Figure 6B). SIgA comprised less than 1 % of total polymeric serum IgA. However, the proportion of SIgA present in polymeric IgA from IgAN patients is 2.5 times higher than in polymeric IgA from controls (P= 0.0152).

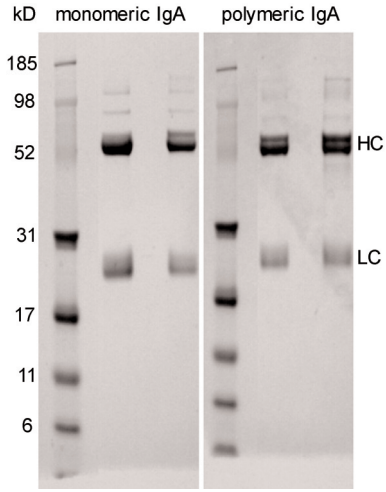

Figure 7. SDS-PAGE analysis of monomeric and polymeric IgA.
Separation of monomeric and polymeric IgA with 10% SDS-PAGE under reducing conditions shows heavy and light chains after staining with Coomassie. IgA heavy chains run as a doublet (upper and lower heavy chains).

Polymeric IgA shows a different composition of N-linked glycans compared to monomeric IgA

Recent studies showed that MBL is able to bind to N-linked glycans of IgG (36) and IgM (37). Based on the known structures of N-linked and O-linked glycans on IgA, it is more likely that MBL would bind to N-linked glycans than to the O-linked glycans. Furthermore, information on N-linked glycosylation of IgA in IgAN is not available. Therefore, we characterized the N-glycans of 6 monomeric and 6 polymeric IgA preparations in detail.

Heavy chains and light chains of monomeric and polymeric IgA were separated by SDS-PAGE (Figure 7). N-linked glycans were released via an in-gel digestion of the heavy chain and light chain bands of IgA using PNGase F. Isolated glycan samples were labeled with 2-aminobenzamide and run on normal phase HPLC. Consistent with earlier data (32), light chains of IgA were found not to be glycosylated (not shown). The elution pattern of heavy chains is shown in Figure 8A. The most prominent peaks, present between glucose units 8 and 10, represent complex glycans that are sialylated, as was demonstrated by a neuraminidase digestion (abs; Figure 8B). No obvious differences could be observed between N-glycans from patients and from controls. However, upon comparison of glycans from monomeric and polymeric IgA, a single peak at GU 6.2 was present in all polymeric IgA samples but absent in monomeric IgA. Digestion with mannosidase (jbm, Figure 8B) demonstrated that this is an oligomannose structure (Man 5), as schematically drawn in Figure 8A.

Using sequential enzyme digestions (Figure 8B), the glycan structures on these

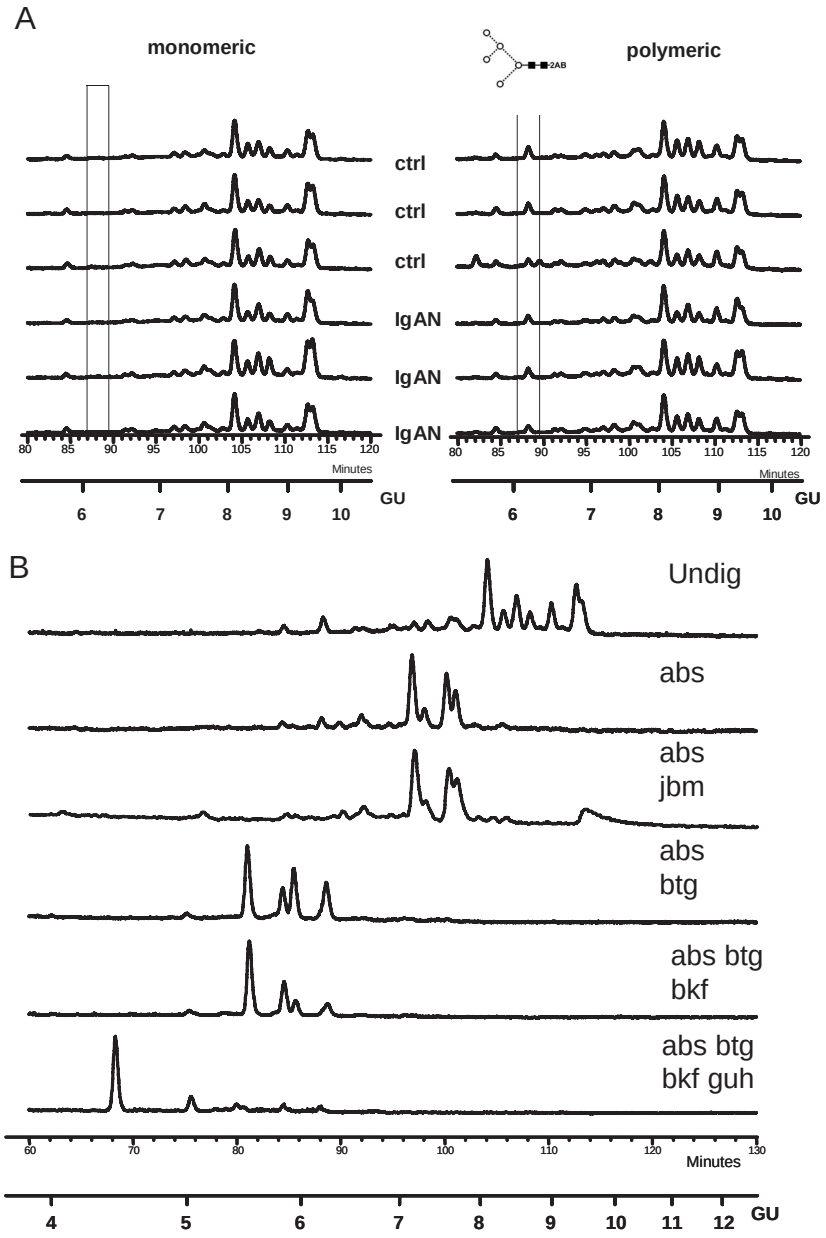

Figure 8. Analysis of N-linked glycans on IgA heavy chains.
A) Analysis of N-glycans of the heavy chain of polymeric IgA from controls and from patients with IgAN by NP-HPLC. The figures show the individual elution profiles following fluorescence detection. Retention times are standardized to a glucose oligomer ladder to give glucose units (GU). The boxed peaks are only present in polymeric IgA and are identified as oligomannose (Man 5), as indicated. B) Digestion of the N-glycans with A. ureafaciens sialidase (abs), jack bean α-mannosidase (jbm), bovine testes β-galactosidase (btg), bovine kidney α-fucosidase (bkf) and Streptococcus pneumoniae N-acetyl β-glucosaminidase (guh), followed by separation with NP-HPLC.

Table 2. Analysis of N-glycans of IgA heavy chains of monomeric and polymeric IgA

Major structure*	GU	monomeric							polymeric							
		Control			IgAN			Mean	Control			IgAN			Mean	P value
A2B	**5.80**	1.53	2.07	2.66	1.85	1.71	2.25	**2.01**	1.32	2.55	2.49	1.63	1.60	1.88	**1.91**	0.4469
Man 5	**6.17**	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	4.51	3.60	2.24	3.71	3.06	2.48	**3.27**	**0.0002**
A2G1[3] A2BG1[6]	**6.52**	0.66	1.02	0.84	1.03	1.21	1.67	**1.07**	0.95	1.72	1.39	1.11	1.14	1.24	**1.26**	0.3214
A2BG1[3] FA2G1[6]	**6.61**	1.48	1.68	1.89	1.21	1.67	2.28	**1.70**	1.23	1.09	1.88	1.06	1.16	1.66	**1.35**	**0.0187**
FA2G1[3] FA2BG1[6]	**6.92**	0.00	0.38	0.46	0.46	1.07	0.60	**0.50**	1.69	2.04	0.66	0.88	1.73	1.02	**1.34**	**0.0264**
FA2BG1[3] A2G2	**7.20**	2.10	1.89	2.23	1.87	1.37	2.08	**1.92**	2.16	2.65	2.75	1.32	2.09	1.09	**2.01**	0.7806
A2BG2	**7.37**	3.17	3.62	4.22	2.53	2.04	2.73	**3.05**	3.36	4.72	4.17	2.59	3.77	4.42	**3.84**	0.0663
FA2G2 A3G2	**7.65**	8.20	8.03	8.57	5.58	7.01	6.75	**7.36**	9.56	10.34	7.55	7.13	10.84	10.60	**9.33**	**0.0448**
FA2BG2	**7.73**															
A3BG2	**7.94**	2.51	1.52	2.28	1.66	1.61	1.12	**1.78**	2.00	2.32	3.64	1.69	2.74	2.96	**2.56**	0.0816
A2G2S1	**8.13**	20.22	21.15	20.65	21.64	16.24	20.75	**20.11**	17.43	17.28	19.15	21.81	15.89	17.83	**18.23**	**0.0338**
A3G3 A2BG2S1	**8.35**	7.81	7.20	5.56	5.66	6.65	6.29	**6.53**	8.40	8.64	6.50	6.44	7.64	8.04	**7.61**	**0.0017**
FA2G2S1 A3BG3	**8.50 8.52**	9.87	7.80	10.95	11.67	11.34	10.99	**10.44**	10.06	7.85	9.25	11.27	10.11	9.65	**9.70**	0.0709
FA2BG2S1	**8.72**	6.88	5.15	6.26	5.84	8.59	6.29	**6.50**	8.67	6.87	8.34	6.34	9.31	7.56	**7.85**	**0.0034**
A2G2S2	**9.03**	5.90	5.76	5.05	5.62	4.58	4.61	**5.25**	7.84	7.57	9.32	8.94	6.67	7.92	**8.04**	**0.0010**
A2BG2S2	**9.19**	2.80	2.28	2.06	3.03	2.51	2.55	**2.54**	1.96	2.14	1.79	1.93	1.67	2.68	**2.03**	**0.0493**
FA2G2S2	**9.40**	14.69	16.27	13.92	18.02	15.56	15.77	**15.70**	10.32	9.67	10.05	14.55	10.84	9.69	**10.85**	**0.0002**
FA2BG2S2	**9.48**	12.17	14.17	12.40	12.34	16.84	13.27	**13.53**	8.55	8.93	8.83	7.59	9.77	9.27	**8.82**	**0.0003**

N-glycans were identified on IgA heavy chains of monomeric and polymeric IgA from six individuals (3 IgAN patients and 3 controls, as indicated), using sequential exoglycosidase digestion as indicated in Fig. 8. For each structure, the percentage of total N-glycans is indicated. Differences between monomeric and polymeric IgA are evaluated by paired t-test. *Structural abbreviations: all N-glycans have two core GlcNAcs; F, core fucose linked α1-6 to inner GlcNAc; Man(x), number (x) of mannose on core GlcNAcs; A, number (x) of antenna (GlcNAc) on trimannosyl core; B, bisecting GlcNac linked β1-4 to inner mannose; Gx, number (x) of galactose on anntenne; Sx, number (x) of sialic acids on anntenne.

samples were identified and quantified (Table 2). Comparison of monomeric and polymeric IgA revealed that 3.3 % of the total glycan pool contained Man5 in polymeric IgA, whereas this structure is absent in monomeric IgA. Furthermore, the double sialylated glycans are under-represented in polymeric IgA as compared to monomeric IgA (29 % and 37 %, respectively, P = 0.001), resulting in a shift to smaller glycan structures on polymeric IgA. There were no significant differences between patients and controls. Together, the results indicate that N-linked glycosylation of the IgA heavy chain is significantly different between monomeric and polymeric IgA (Table 2).

DISCUSSION

Deposition of IgA in the renal mesangium is the primary characteristic of IgAN, responsible for glomerular inflammation and finally the development of renal failure. Based on earlier observations of mesangial IgA, this IgA is believed to be largely polymeric IgA1. In the present study we show that polymeric IgA, as opposed to monomeric IgA, from IgAN patients and from healthy controls shows increased binding to and activation of mesangial cells, and has a superior capacity to bind the complement-activating lectin MBL. These aspects of polymeric IgA are most likely to be involved in induction of glomerular deposition and inflammation. Furthermore, we provide evidence that polymeric IgA is differently glycosylated from monomeric IgA, as suggested by lectin binding studies and demonstrated by a direct identification of N-linked glycans. Noteworthy, the only obvious difference that was observed between IgA from IgAN patients and controls was a substantial increase in the fraction of SIgA in polymeric IgA from IgAN patients.

Comparisons between IgA isolated from patients and from healthy controls were reported previously (19,22,28,38-40). Most experiments were performed with either fractionated total serum without an IgA purification step (28,39,40) or with pooled serum IgA purified with Jacalin, a lectin that binds Gal 1-3GalNAc (39-41). In our study we purified IgA from individual patients and controls with an anti-IgA mAb. With this method we prepared monomeric and polymeric IgA that contains total and highly pure serum IgA without a preceding selection for certain IgA glycoforms. In contrast to methods using Jacalin, this method also enabled us to isolate the IgA2 present in serum.

Previous studies described that the binding of polymeric IgA to mesangial cells was higher than that of monomeric IgA (23,28) although this could not be reproduced by others (24). Moreover, the binding of patient IgA and that of in vitro degalactosylated IgA was higher than that of control IgA (24). In the present study, we confirm a prominent increase in mesangial cell binding of polymeric over monomeric IgA, but we did not detect a difference between patient and control IgA. Our studies further establish the pro-inflammatory properties of polymeric IgA, and demonstrate that IgA-induced chemokine production is correlates with the interaction of IgA with the mesangial cell surface. It is most likely that next to the increased binding of pIgA, also a more efficient receptor crosslinking will contribute to its proinflammatory action. In addition, also the observed biochemical properties of pIgA might contribute to this process. However, unlike previous investigations (27), this

property could not be specifically attributed to IgA derived from IgAN patients.

Next to the direct effects of IgA on mesangial cells, activation products of the complement system, involving both the alternative pathway and the lectin pathway, are likely to drive the local inflammatory process. Activation of the lectin pathway of complement via an interaction between MBL and IgA has been shown before (12), and in the present study we confirm and extend these data by showing that binding of MBL is a common feature of polymeric but not monomeric IgA isolated from different donors. Since ligand recognition by MBL requires multiply presented carbohydrates, MBL binding could be favored by the structure of polymeric IgA. Binding of MBL leads to activation of C4 presumably via activation of the C4-cleaving enzyme MASP-2. In a healthy situation, the binding of MBL to polymeric IgA could be involved in host defense. However, in IgAN, lectin pathway activation via polymeric IgA is unfavorable (10).

Many studies on IgA from IgAN patients focused on glycosylation. IgA is extensively glycosylated, both via N-linkages (IgA1 and IgA2) and O-linkages (IgA1) (21). It was consistently observed that serum IgA from IgAN patients contains smaller O-linked glycans, with less sialylation and galactosylation, than IgA from healthy controls (18). Previous investigations suggested that this was predominantly the case for monomeric IgA (40). Our experiments using HAA, a lectin that is commonly used to detect terminal GalNAc present on non-galactosylated O-linked glycans, suggested the presence of terminal GalNAc (Tn antigen) predominantly on polymeric IgA, both from patients and controls. This is in agreement with a previous study showing reactivity of HAA with high molecular weight serum proteins (28) and with data provided by Leung et al. (40). Binding of HAA was strongly increased by neuraminidase treatment, suggesting a high frequency of non-galactosylated O-linked glycans on IgA, that expose terminal GalNAc after enzymatic removal of sialic acid.

A detailed quantitative analysis of N-linked glycans on IgA heavy chains of monomeric and polymeric IgA revealed several significant differences between these molecular forms of IgA. In this respect, polymeric IgA consistently contained an oligomannose structure that was undetectable on all monomeric IgA preparations, and showed significantly less glycans with two terminal sialic acid residues. Recent studies showed that MBL can bind to certain glycoforms of human IgM, involving GlcNAc-terminated glycans and oligomannose structures on the IgM heavy chains (42). Therefore, the presence of specific glycans on polymeric but not monomeric IgA might also be involved in its recognition by MBL. At present it is unknown whether a specific glycosylation pattern of the heavy chain of polymeric IgA is involved in the polymerization of IgA, and/or whether this merely is related to the conditions present during production of the different forms of IgA. Earlier studies from our group indicated that polymeric serum IgA contains dimeric IgA linked with J chain, as well as complexes of monomeric IgA linked via other mechanisms (43). In the first case, polymerization takes place in the B cell, and the presence of oligomannose, which is a premature glycan structure, might suggest the ER as a possible location for polymerization, which prevents further synthesis of the glycan structure by steric hindrance. In the latter case, polymerization might take place outside the B cell.

Part of the observed differences in glycosylation between monomeric and poly-

meric IgA might also be explained by the presence of SIgA in the polymeric fraction of serum IgA, since the SIgA heavy chain N-glycosylation is very different from that of monomeric serum IgA (32). SIgA has about 8 % oligomannose structures and 60 % glycans with exposed GlcNAc with less than 15 % for all glycans sialylated (32), compared to monomeric serum IgA where most of the glycans are sialylated. However, SIgA comprises only less than 1 % of total polymeric serum IgA.

We observed that polymeric serum IgA contains more IgA2 than monomeric IgA. This could be because IgA2 polymerises more easily than IgA1, or that IgA2-producing B-cells preferentially secrete polymeric IgA. Bone-marrow-derived IgA as present in serum is largely monomeric and of the IgA1 subclass, whereas mucosal IgA is largely polymeric, containing J chain and secretory component (44), and contains a substantial fraction of IgA2 (35). Therefore, an increased fraction of IgA2 and the presence of SIgA in circulating polymeric IgA may suggest its production by the mucosal immune system. Quantitative measurement of the presence of SIgA in polymeric serum IgA suggests that only a minor part of polymeric IgA contains secretory component. We hypothesize that this SIgA is derived from the mucosal surface. Circulating dimeric IgA without secretory component could be partially produced in mucosal lymphoid tissue and directly transported towards the circulation.

Although SIgA requires transepithelial transport for the attachment of secretory component to dimeric IgA, the presence of low concentrations of circulating SIgA has been described before (17,45,46). Moreover, increased serum levels of SIgA have been reported in various diseases (17,47-49). In contrast to previous studies, we now determined the SIgA concentration in highly purified polymeric serum IgA. Our data demonstrate a clear relative increase in SIgA in polymeric IgA from patients with IgAN compared with controls. We recently reported a preferential interaction of SIgA with mesangial cells, and showed glomerular accumulation of SIgA in IgAN (17). Therefore, our current results further support a role for SIgA in the pathogenesis of IgAN.

Taken together, the presented data suggest that a part of circulating polymeric IgA has a mucosal origin. There is accumulating evidence that the pathogenesis of IgAN is related to aberrant production of IgA. In this respect, in vivo studies indicated that IgAN patients have a disturbed mucosal immune response, which was restricted to production of antibodies of the IgA1 subclass (50). Our present observation that secretory IgA is increased in the polymeric IgA fraction of IgAN patients further supports a role for abnormal mucosal immunity. Since IgAN is a slowly progressive disease, it is well conceivable that only a minor subfraction of circulating IgA in IgAN patients is abnormal, and that this IgA gradually accumulates in the mesangial area. We hypothesize that this abnormal IgA is at least partially derived from the mucosal immune system. Since our data strongly indicate that large-sized IgA is especially able to interact with mesangial cells, and to induce complement activation, the gradual deposition of such pro-inflammatory IgA may eventually lead to renal disease.

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CHAPTER 6

A pathogenic role for Secretory IgA in IgA Nephropathy.

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Summary

IgA nephropathy (IgAN) is characterized by deposits of IgA in the renal mesangium. It is thought that deposits of IgA mainly involve high molecular weight (HMW) IgA1. However there is limited information on the exact composition of HMW IgA in these deposits. In this study we investigated the presence of secretory IgA (SIgA) in human serum and in the glomerular deposits of a patient with IgAN. Furthermore, we analysed the interaction of SIgA with mesangial cells. With ELISA SIgA concentrations in serum of IgAN patients and healthy controls was measured. Patients and controls both have circulating SIgA that was restricted to the HMW fractions. Patients tend to have higher levels of SIgA, but this difference was not significant. However, in patients with IgAN, high serum SIgA concentrations were associated with hematuria. Binding of size fractionated purified serum IgA and secretory IgA to mesangial cells was investigated with flow cytometry. These studies with mesangial cells showed stronger binding of SIgA to primary mesangial cells, compared to binding of serum IgA. Importantly, after isolation and elution of glomeruli from a nephrectomized transplanted kidney from a patient with recurrent IgAN, we demonstrated a 120-fold accumulation of SIgA compared to IgA1 in the eluate. In conclusion we have demonstrated that SIgA strongly binds to human mesangial cells, and is present in significant amounts in serum. Furthermore, we showed that SIgA is accumulated in the glomeruli of an IgAN patient. These data suggest an important role for SIgA in the pathogenesis of IgA nephropathy.

Kidney Int. 69: 1131, 2006

INTRODUCTION

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease shows a spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA in the glomerular mesangium (1-3). It is generally thought that deposits of IgA mainly involve IgA1 and for a large part consist of high molecular weight (HMW) IgA (4). The composition of HMW forms of IgA in serum is diverse and may include dimeric IgA, CD89/IgA complexes, IgA immune complexes and IgA-fibronectin complexes (5-9).

Several reports have shown that the glycosylation of IgA1 in patients is different from that in controls. Patient IgA1 contains more terminal GalNAc and this could play a role in the deposition of IgA1 in the mesangium (10-12). IgA from the glomeruli of IgAN patients consists at least partly of HMW IgA (13) and is under-O-glycosylated (10,14). In these studies no data was presented concerning the presence of secretory IgA (SIgA). When deposited in the kidney, the IgA1-containing complexes are linked to inflammation. Stimulation of mesangial cells with HMW IgA leads to enhanced production of cytokines and chemokines, including IL-6, TGF-β, TNF-α, MCP-1, IL-8 and MIF (15-18). These cytokines and chemokines might play a role in the development and progression of renal injury in IgAN patients.

SIgA is the dominant immunoglobulin in external mucosal secretions like in oral, respiratory, and intestinal cavities, and is often characterized as a component of the immune systems "first-line defence" against pathogenic micro-organisms (19). The SIgA molecule is composed of two IgA monomers, linked by a junction peptide called J chain, and the secretory component (SC) that wraps around the dimer (20). Next to its presence in mucosal secretions, small amounts of SIgA can also be found in human serum (21,22). Moreover, increased serum levels of SIgA have been reported in various diseases (23-25), indicating that SIgA may be a marker of clinical interest. A previous study has suggested that the serum concentrations of SIgA are not different in IgAN patients compared to healthy controls (23). The physiological roles of serum IgA and SIgA are quite different, and the presence of the highly glycosylated SC can have major effects on the biological functions of SIgA (26,27). In literature it is still controversial if SIgA is able to bind to mesangial cells (28,29). One study showed that SIgA is able to bind to mesangial cells (28), however this could not be confirmed in another study (29).

In the present study we investigated the presence of SIgA in sera of IgAN patients and healthy controls, and examined the binding of different molecular forms of IgA to human mesangial cells with special interest for SIgA. Finally we investigated the presence of SIgA in the glomerular eluate of an IgAN patient.

MATERIAL AND METHODS

Human subjects

In this study, we included 19 healthy volunteers and 47 patients with primary IgAN (30). The latter were defined by mesangial deposits of IgA. None of these patients had clinical or laboratory evidence of Henoch Schoenlein purpura, systemic lupus erythematosus, liver disease or received immunosuppressive therapy. Patients were included in the study between

October 1998 and February 1999. Blood and urine samples were collected, clinical characteristics and laboratory data of the patient group were obtained (Table 1) retrospectively (observation time 5.9 ± 0.5 years) and prospectively (follow-up time 3.7 ± 0.2 years).

Renal cortex was obtained from a transplant nephrectomy from a male patient (1977), with biopsy-proven recurrent IgAN. He was diagnosed with macroscopic hematuria (serum creatinine 146 µmol/l) in 1993. The renal biopsy of 1996 showed characteristic features of progressive IgAN, and dialysis was started. In April 2002 he received a cadaveric renal transplantation. Three months later a decline in renal function was observed and a renal biopsy showed the presence of interstitial nephritis and recurrent IgAN. In a biopsy taken 6 months following transplantation characteristics of the interstitial nephritis were disappeared, but IgA was still present. Due to decreased patient compliance there were two episodes of acute rejection in 2003 and 2004, which eventually led to graft loss. Informed consent was obtained from all subjects.

Table 1: Clinical characteristics of the patients with IgAN at the point of serum SIgA measurement.

	Number of patients	Median	Range
Male/ female	38/ 9		
ACE-inhibitor/AII antagonist	24/ 3		
Age (years)		47	19-69
Systolic blood pressure (mmHg)		130	100-160
Diastolic blood pressure (mmHg)		80	55-100
Proteinuria (g/day)		0.6	0.1-5.2
Glomerular filtration rate (ml/min)		71	15-137

IgA purification

Serum from healthy controls was used for IgA purification, according to methods described before (9). In brief, serum was applied to an anti-IgA (HisA 43, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) affinity column. IgA was eluted with 0.1 M glycine/ 0.3 M NaCl (pH 2.8). The eluted protein fractions containing IgA, as assessed by ELISA (31), were pooled and dialysed. The IgA was size-separated with a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands) into pIgA and mIgA.

Cell culture

NHMC (normal human mesangial cells, Cambrex, USA) were expanded according to the protocol provided by the manufacturer. Experiments with NHMC were performed in RPMI with 10 % FCS, 1 % non-essential amino acids, 0.5 % transferrin/insulin/selenium, 1 % sodium pyruvate, 1 % L-glutamine (all purchased at Gibco/ life Technologies, Paisley, Scotland). AMC11, a spontaneously growing adult human mesangial cell line (kindly provided by Prof. Holthofer, Helsinki), was cultured in DMEM with 10 % FCS. Cells were cultured in culture flasks (Greiner, Frickenhausen, Germany) at 37 ºC in a humidified incubator with 5 % CO2/ 95 % air. For passage the cells were harvested by trypsinization (0.02 % (w/v) EDTA/ 0.05 % (w/v) trypsin in PBS from Sigma (St. Louis, MO)).

Monocyte-derived dendritic cells were generated as described before (32).

Flow cytometry

Cells were harvested, washed with FACS buffer (0.5 x PBS containing 1 % BSA/ 2.8 % glucose/ 0.01 % NaN_3) and incubated with monomeric and polymeric serum IgA, and SIgA (Sigma). Following incubation for 1 hour at 4 ºC, cells were washed and incubated for one hour at 4 ºC with monoclonal anti-IgA Ab 4E8 (IgG1) (31). IgA binding was visualized using PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and assessed for fluorescence intensity by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

For analysing the presence of the mannose receptor, anti-mannose receptor antibody (D547.3; kindly provided by F. Koning, Leiden University Medical Center, Leiden, The Netherlands) was used followed by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark). For the detection of the two chains of MAC-1, anti-human CD11b (leu-15-PE, BD Biosciences) and anti-CD18 (IB4, ATCC) were used.

For inhibition of the binding of SIgA to mesangial cells, the cells were pre-incubated with purified free SC (100 µg/ml) (33), 10 mM EDTA or 10 mM $CaCl_2$ for 1 hour at 4 ºC. Subsequently, without washing, SIgA was added and this binding was visualized as described above. Inhibition of binding of SIgA was also detected with a pre-incubation of IgA for one hour at 4 ºC; subsequently Alexa-conjugated SIgA was added. After one hour the binding of Alexa-conjugated SIgA was measured.

Cytokine analysis

Production of IL-6 was measured in supernatants of mesangial cells after 72 hr stimulation. Prior to stimulation, cells were transferred to 48-wells plates (Costar, Corning, NY) at a density of 25 x 10^3 cells per well and cultured overnight in culture medium with 0.5 % serum. Cells were stimulated with IL-1, serum IgA, or SIgA. The concentration of IL-6 in culture supernatants was measured by specific ELISA as described previously (34).

RNA extraction and RT-PCR

Total RNA was extracted from mesangial cells using RNeasy mini kit (Qiagen, Valencia, CA). OD260/280 ratio was measured to determine the quantity and purity of RNA preparations. Fixed amounts of total cellular RNA (1 µg) were reverse transcribed into cDNA by oligo(dT) priming, using M-MLV reverse transcriptase (Gibco/Life Technologies, Breda, The Netherlands). PCR to detect the human mannose receptor was performed with specific primers (sense 5'-TTG AGT GGA GTG ATG GGA CC-3'; antisense 5'-TTT CTG GAC CTT GGC TTC GT-3') using AmpliTaq DNA polymerase (Applied Biosystems, Roche, Mannheim, Germany). The PCR reaction was performed under standard conditions (35). The cDNA samples were also subjected to PCR for GAPDH as an internal control (35), PCR products were resolved on 1 % agarose gels and bands were visualized by ethidium bromide staining.

Preparation of glomerular eluate

Glomeruli were isolated from a nephrectomized kidney from a transplanted IgAN patient with recurrent disease. For this purpose the renal cortex was separated from the medulla. After slicing the cortex in little pieces, the glomeruli were collected on a 150-mesh sieve and stored at -70ºC with protease inhibitors (Complete, Mini, and Roche). The glomeruli were washed with PBS and the final pellet was resuspended in 5 ml of elution buffer (2 M KSCN in 0.01 M phosphate buffer, pH 7.6) as described before (36). After stirring at room tempera-

ture (RT) for 60 min, the suspension was centrifuged at 8,000 g for 15 min at 4ºC in a high-speed centrifuge (Beckman, Avanti J25-1). The supernatant was collected and dialysed overnight against PBS. The precipitate that was formed during dialysis was removed by centrifugation for 15 min at 17,000 g and was negative for immunoglobulins. The remaining supernatant was concentrated to one-third of the original volume.

ELISA for human SIgA, IgA1, IgA2, IgG and IgM

To test the specificity of the antibodies used for the SIgA ELISA, Ninety-six well Nunc Maxisorp microtitre plate (Gibco/Invitrogen) was coated with 2 µg/ml IgA and BSA in carbonate buffer (pH 9.6) overnight at RT. After washing, the plate was incubated with monoclonal antibodies (2 µg/ml) specific for secretory component: NI194-4 (IgG1-k; Nordic Immunology, Tilburg, The Netherlands) (37) or 3F8 (kindly provided by dr R.M. Goldblum, University of Texas Medical Branch, Galveston) (38) in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. Bound antibody was detected with goat anti-mouse Ig HRP (Dako). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, USA).

In order to quantify SIgA levels in isolated IgA and serum, a sandwich ELISA specific for SIgA was developed. Plates were coated with 2 µg/ml monoclonal antibody specific for secretory component (NI194-4; 3F8) in carbonate buffer (pH 9.6) (100 µl/well) overnight at RT. Subsequently, the plates were washed with PBS/ 0.05 % Tween. Plates were incubated with IgA or with serum from patients and controls in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgA was detected using mouse anti-human IgA (4E8) conjugated to digoxigenin (Dig), followed by $F(ab)_2$ anti-Dig antibodies conjugated to HRP (Roche). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured. A calibration line was produced using purified SIgA (Sigma).

Concentrations of IgA1, IgA2, IgG and IgM in sera and glomerular eluate were determined using specific ELISAs (39).

Western blot analysis

IgA preparations were subjected to 10% SDS-PAGE under reducing conditions, followed by semi-dry blotting to PVDF (immobilin-P, Millipore, Bedford, MA). Blots were blocked for 2 hours at RT in TBS/ 0.1 % Tween/ 5 % skim milk powder (Fluka, Buchs, Switzerland). Blots were subsequently incubated with 2 µg/ml monoclonal antibody specific for secretory component (NI194-4) in TBS/ 0.1 % Tween/ 2.5 % skim milk powder overnight at 4 ºC. After washing with TBS/ 0.1 % Tween, blots were incubated with HRP conjugated goat anti-mouse immunoglobulin (Dako) for 2 hours at RT. After washing bands were visualized with Supersignal (Pierce Chemical Co., Rockford, IL) and exposure to HyperfilmTM films (Amersham Pharmacia).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test. Differences were considered statistically significant when p values are less than 0.05.

RESULTS

Specific detection of SIgA in human serum

An ELISA system was developed to specifically measure the amounts of secretory IgA in serum. In this system anti-SC antibodies were coated, samples were applied and SIgA was detected with anti-IgA antibodies. Purified SIgA was readily detected by this ELISA with a detection limit of 100 ng/ml (Figure 1A). In contrast, purified monomeric serum IgA is not recognized in the ELISA, even when applied at high concentrations (Figure 1A). In accordance with previous publications (23), a specific signal for SIgA could be detected in serum of healthy individuals (Figure 1B). The specificity of this assay is especially dependent on the specificity of the anti-SC antibody. Using Western blot, we showed that this monoclonal antibody only recognized the 75 kDa SC (Figure 2A). Furthermore comparison of the NI194-4 antibody with another anti-SC antibody 3F8 (38) showed specificity for SIgA both in a direct ELISA (Figure 2B) as well as in a sandwich ELISA (Figure 2C).

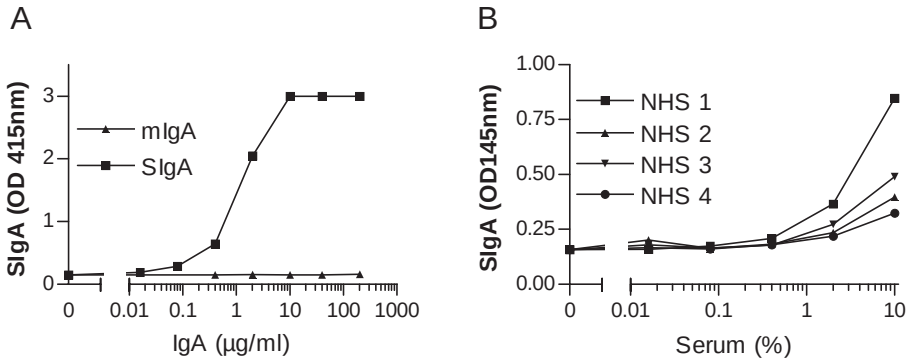

Figure 1: Specific detection of SIgA in human serum.
A) Purified SIgA and monomeric serum IgA were measured in a sandwich ELISA, as detailed in the Material and Methods. B) Measurement of SIgA in serum from healthy individuals in different serum dilutions. NHS, normal human serum.

To determine the molecular size of SIgA in serum, IgA was isolated from serum using affinity chromatography. Size-fractionation revealed that SIgA was specifically present in the HMW fractions (Figure 3A). Using the same procedure, IgA was isolated from 8 healthy controls followed by gel filtration. Pools containing pIgA and mIgA, respectively, were obtained and assessed for the amount of SIgA (Figure 3B). In all cases, SIgA was demonstrated exclusively in the pIgA pool.

Next we assessed concentrations of SIgA in serum of 47 IgAN patients and 19 healthy controls (Figure 4A). Both in controls and in patients significant serum concentrations of SIgA were detected. There was no significant difference (p= 0.159) in the SIgA concentrations in serum of patients (3.3 ± 3.0 µg/ml) compared to controls (2.2 ± 1.2 µg/ml). After comparison the SIgA concentration in serum of IgAN patients with different clinical parameters there were no correlations found. However concentrations of SIgA in IgAN patients were more heterogeneous than in controls. Therefore we divided the IgAN group in patients with elevated levels of SIgA (> 5 µg/ml, mean ± 2 SD of control sera), and patients with normal levels (<5 µg/ml).

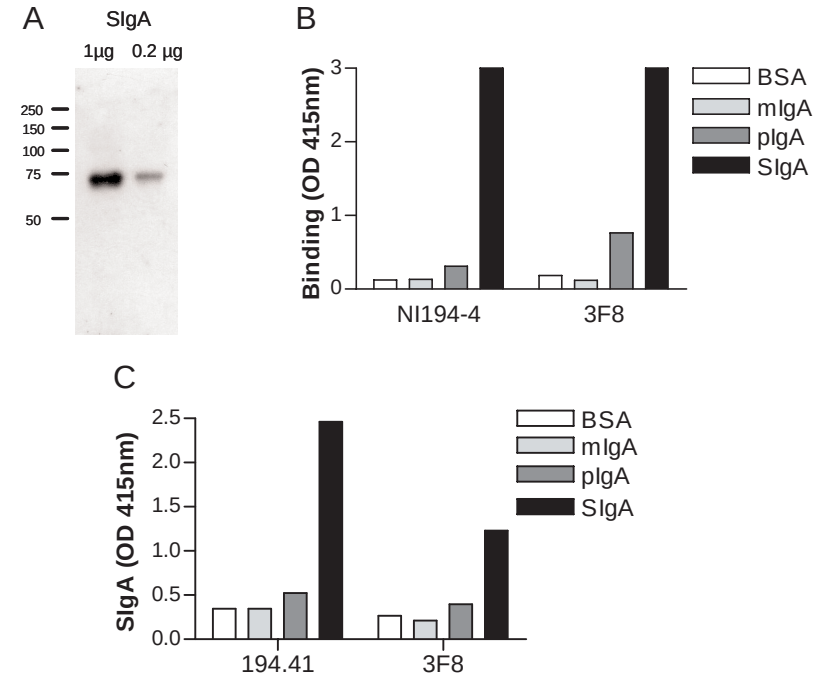

Figure 2: Specific detection of SIgA and secretory component.
A) Detection of secretory component in SIgA, 1 and 0.2 µg SIgA were loaded on 10 % SDS-PAGE gel under reducing conditions after blotting secretory component was detected with 2µg/ml NI194-4. B) Detection of secretory component with NI194-4 and 3F8 (2 µg/ml) in different forms of IgA (2 µg/ml) coated on ninety-six well Nunc maxisorp microtitre plate. C) Different forms of IgA measured with sandwich ELISA using two different coating antibodies (NI194-4 and 3F8).

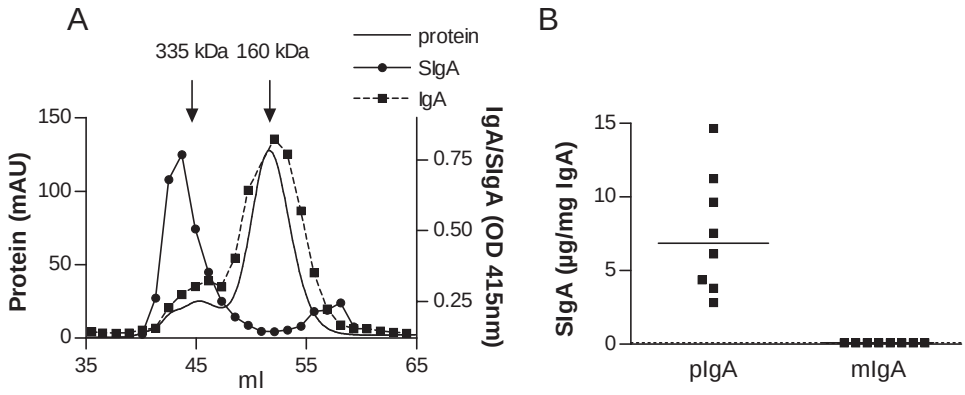

Figure 3: SIgA is present in high molecular weight fractions of serum IgA.
A) IgA was affinity purified and size-fractionated on a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA and SIgA by ELISA. B) From 8 healthy controls, IgA was purified and size fractionated as above. IgA was pooled in pIgA fraction (39-47.5ml) and mIgA fraction (47.5-57ml). Both IgA and SIgA concentrations were determined, and depicted is the amount of SIgA corrected for the amount of total IgA The horizontal dashed line represents the detection limit. The horizontal solid lines indicate the median.

There were no significant correlations with the degree of proteinuria or creatinine clearance and the serum concentration of SIgA (Figure 4B). However, there was significantly more pronounced hematuria in the group with higher levels of SIgA (p= 0.04) (Figure 4B).

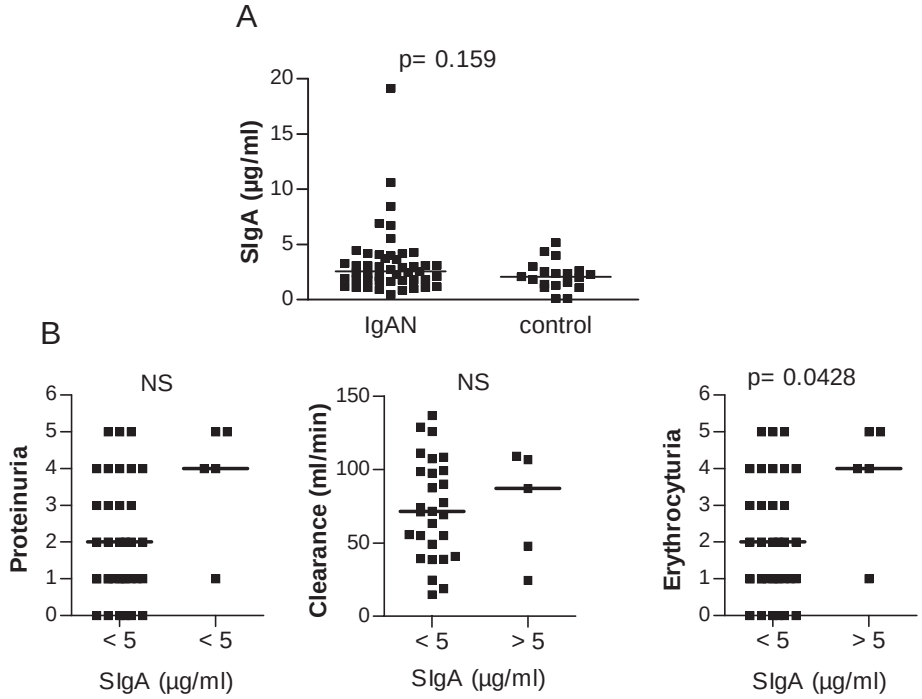

Figure 4: Measurement of SIgA in serum of IgAN patients and controls and relation to clinical parameters.

A) The concentrations of SIgA were determined in serum from patients (n= 47) and controls (n=19). The horizontal dashed line represents the detection limit and the horizontal solid lines indicate the median B) Based on SIgA concentrations IgAN patients were divided in two groups: normal SIgA levels (<5µg/ml; mean ± 2 SD of control sera) and increased SIgA levels (≥ 5µg/ml). These groups were analyzed for proteinuria, creatinin clearance (700 creatinin in urine (mmol/ 24 hours)/ creatinin in serum (µmol/l) and erythrocyturia at the time of sampling. The horizontal solid lines indicate the median. Statistics were performed using the Mann-Whitney test (ns= not significant)*

SIgA binds to mesangial cells and induces cell activation

After demonstrating the presence of SIgA in the circulation, we investigated its capacity to interact with mesangial cells. After incubation of the mesangial cell-line AMC11 or normal human mesangial cells (NHMC) with 200 µg/ml of different molecular forms of IgA, the binding was examined by FACS analysis. Binding of monomeric serum IgA to NHMC is very low (Figure 5A). In contrast, the polymeric form of serum IgA showed a clear binding to mesangial cells. However the best binding was observed with similar concentrations of SIgA, which occurred in a dose-dependent fashion, present over a wide range of concentrations (Figure 5B). Although the mean fluorescence intensity of IgA binding to NHMC, compared with

AMC11, was overall higher for all forms of IgA, the relative differences between the different forms of IgA were the same.

The different molecular forms of IgA were compared for their capacity to induce IL-6 production by mesangial cells. Stimulation of NHMC for 72 hours with IgA resulted in an increased IL-6 production. This increase was most prominent following stimulation with SIgA (Figure 6A). The induction of IL-6 production by mesangial cells was dose dependent: a 17-fold increase was observed compared to the negative control upon stimulation with 200 µg/ml SIgA (Figure 6B).

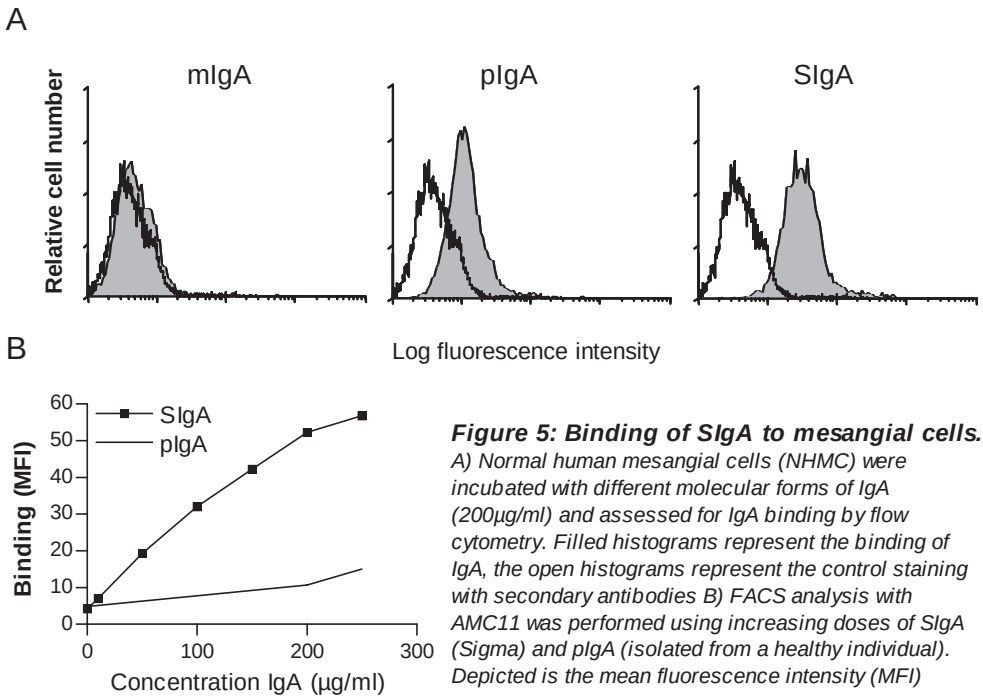

Figure 5: Binding of SIgA to mesangial cells. *A) Normal human mesangial cells (NHMC) were incubated with different molecular forms of IgA (200µg/ml) and assessed for IgA binding by flow cytometry. Filled histograms represent the binding of IgA, the open histograms represent the control staining with secondary antibodies B) FACS analysis with AMC11 was performed using increasing doses of SIgA (Sigma) and pIgA (isolated from a healthy individual). Depicted is the mean fluorescence intensity (MFI)*

Mesangial cells do not express the mannose receptor (CD206) or MAC-1 (CD11b/CD18)

Recently we showed that dendritic cells are able to bind SIgA via the mannose receptor (CD206) (40). Therefore we investigated the presence of the mannose receptor as a potential SIgA receptor on mesangium cells. However both using FACS analysis (Figure 7A) and RT-PCR (Figure 7B) we were not able to demonstrate the presence of the mannose receptor on mesangial cells. In both cases, DC served as a positive control. Similarly, we were not able to demonstrate the presence of CD11b/CD18 (Figure 7C), recently identified as a co-receptor for SIgA binding (41).

Binding of SIgA to mesangial cells is not inhibited by secretory component, EDTA and calcium

To investigate in more detail the mechanism of binding of SIgA to mesangial

cells, the cells were pre-incubated with free SC. After pre-incubation with SC the binding of SIgA to mesangial cells was not affected (Figure 8A). Binding of SIgA was slightly inhibited with SIgA and pIgA but not with mIgA (Figure 8A), suggesting interaction with the IgA part of the molecule.

To investigate the potential contribution of C-type lectins, a family of cell surface molecules including the mannose receptor, the role of calcium in SIgA binding to human mesangial cells was examined. However, neither the addition of extra calcium, nor the removal of calcium using EDTA showed a significant effect on the binding of SIgA to mesangial cells (Figure 8B).

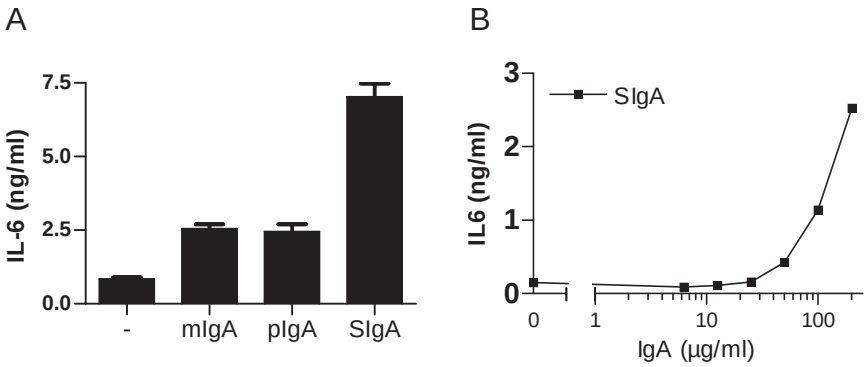

Figure 6: SIgA increase IL-6 production by mesangial cells.

A) Normal human mesangial cells (NHMC) (25x10^3 cells/well) were stimulated with different molecular forms of IgA (200µg/ml). After 72 hours supernatants were harvested and tested for IL-6 using ELISA. Depicted is the mean ± SD B) NHMC was stimulated with different concentrations of SIgA (Sigma) as described above and IL-6 production was assessed.

SIgA is present in glomerular eluate from a kidney of an IgAN patient.

To determine the potential role of SIgA in the pathogenesis of IgAN, we had the unique opportunity to analyse the glomerular eluate of a nephrectomized specimen derived from a patient with recurrent IgAN. After elution, concentrations of specific immunoglobulin isotypes were determined. In the glomerular eluate, all immunoglobulin classes measured were detectable (Table 2), including SIgA in a concentration of 2 µg/ml. To exclude that the immunoglobulins in the eluate were the result of aspecific trapping from the circulation, serum immunoglobulin levels were determined in the serum of this patient at the time of nephrectomy (Table 2). The ratio of the immunoglobulin concentrations in the eluate and the serum can be used as a measure of the specific accumulation in the glomerular deposit (Figure 9). In this analysis we observed a ratio for SIgA which was 120 fold higher that the ratio for IgA1.

DISCUSSION

This is the first study to support a role for SIgA in the pathogenesis of IgAN. We show that SIgA is present in low concentrations in serum of healthy individuals as well as in IgAN patients. In patients with higher SIgA serum concentrations hematuria is more pronounced. Furthermore we show that SIgA exhibits the strongest

binding to mesangial cells compared to serum IgA. Finally, in the eluate of glomeruli from a kidney of an IgAN patient a strong accumulation of SIgA was detected. Taken together these data suggest an important role for SIgA in the pathogenesis of IgAN.

The high incidence of IgAN recurrence after renal transplantation, and the disappearance of deposits of IgA from accidentally transplanted kidneys clearly suggests that intrinsic alterations and/or structural characteristics of IgA contribute to the process of deposition (42,43). The predominance of IgA1 deposits and the specific hinge region of IgA1 with potential O-linked glycosylation sites, has initiated a directed search for alterations in glycosylation. Indeed, both in serum but, more importantly, also in the eluate of renal deposits (14), a specific reduction of O-linked galactosylation has been observed (10,12,14). Furthermore, with size fractionation of eluted proteins from kidney sections, it was shown that deposited IgA was mostly HMW of nature (13). In addition based on different staining methods, it has been proposed that a large part of the deposited IgA is high molecular weight of nature (44-46).

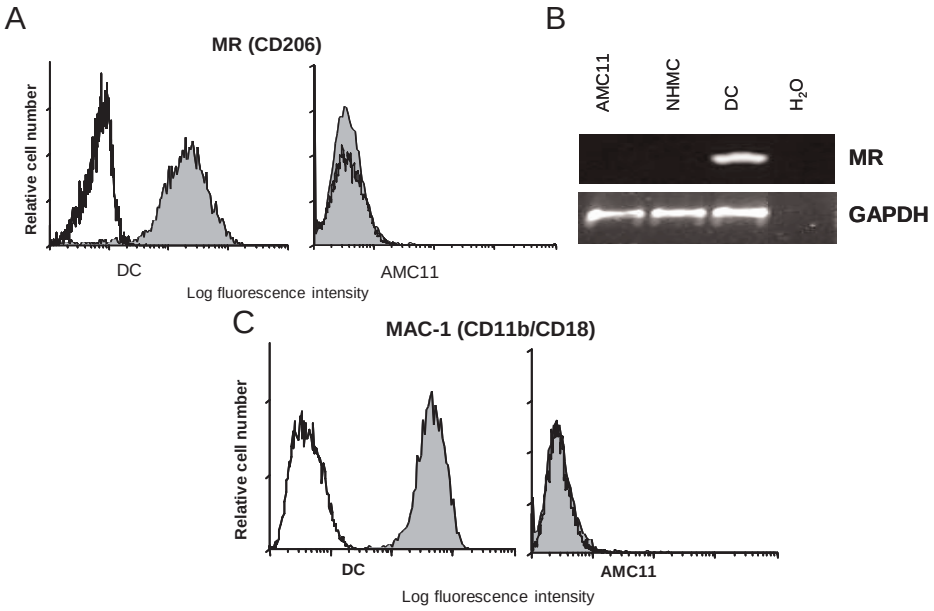

Figure 7: Mannose receptor and MAC-1 are not present on mesangial cells.
A) FACS analysis of mannose receptor on DC and AMC11. Filled histograms represent expression of mannose receptor; open histograms represent the control staining with secondary antibodies B) Mannose receptor mRNA expression was analyzed by RT-PCR as described in the material and methods C) Presence of MAC-1 on mesangial cells and DC was tested with FACS analysis. Filled histograms represent expression of MAC-1. Open histograms represent the control staining with secondary antibodies.

The results from all these methods provide indirect indications for the composition of the IgA1 deposits. We now show by eluting glomeruli, that glomeruli show a strong and specific accumulation of SIgA compared to other serum immunoglobulins. However, this technique can only be applied in limited cases of situations. We have tried to demonstrate the presence of SIgA deposits using traditional immunofluorescense on cryosections. Until now, we were not able to show SIgA in renal

sections, even not in cryosections of the kidney used for our elution study. This might be due to inappropriate reagents or conformational changes of the deposited SIgA, thereby masking the SC epitope. Therefore it will be necessary to generate other reagents for the detection of deposited SIgA, and a more thorough analysis of renal biopsies.

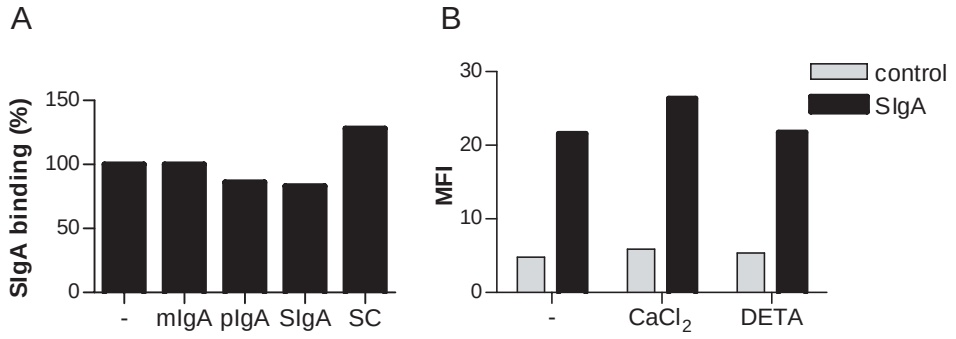

Figure 8: Binding of SIgA to mesangial cells is not affect by free secretory component or calcium but is affected by IgA.

A) Mesangial cells were pre-incubated with mIgA, pIgA, SIgA (400 µg/ml) or free secretory component (SC) (100 µg/ml). After one hour SIgA (200 µg/ml) was added and the binding of SIgA to mesangial cells was examined with flow cytometry. Depicted is the percentage of SIgA binding of a representative experiment of 2 experiments. B) Cells were incubated with SIgA (200 µg/ml) in the presence of absence of EDTA (10 mM) or calcium (10 mM) followed by detection of SIgA binding as described in the Material and Methods.

As described above, it is assumed that glycosylation of IgA is an important factor in IgAN. Previous studies have shown the role of the glycosylation of IgA on the activation of mesangial cells (47,48), which could be partially explained by altered interaction with mesangial cells (49). The glycosylation of SIgA is different compared to that of serum IgA in several aspects. First, SIgA is a tetra molecular complex consisting of two IgA molecules, a J chain and the SC wrapped around the H chain. Modelling of SIgA suggests that the N-glycans on the heavy chain can be masked by the SC (50). This may also result in a different exposure of the O-glycans. Moreover, specific analysis of the glycosylation of the IgA heavy chain present in SIgA, demonstrated different N-glycan structures compared to that of serum IgA, with terminal GlcNAc residues on the majority of the N-glycans (50). The O-glycans on the hinge region of the heavy chain of SIgA1 presented a wide range of glycan structures, of which the major part is now characterized (50). Finally, also the SC itself is heavily glycosylated. However we were not able to inhibit the binding of SIgA to mesangial cells with SC, suggesting that the SC part is not important for interaction with mesangial cells. It will be a major challenge to isolate SIgA from serum of IgAN patients and to determine specific alterations in glycosylation.

Having shown that SIgA strongly binds to mesangial cells, an important question is which receptor is involved in this binding and whether this receptor is different from that of serum IgA. Several IgA receptors have been described in the literature. The best known receptors, the polymeric Ig receptor (pIgR), the asialoglycoprotein receptor and CD89, have already been described to be absent on mesangial cells (28,51,52). The transferrin receptor is described as an IgA receptor present on

mesangial cells but it has been reported that this receptor is not able to bind SIgA (29). We have previously reported the binding of SIgA to the mannose receptor on dendritic cells (40), but we were not able to demonstrate the presence of the mannose receptor on mesangial cells. Similarly we were not able to demonstrate the presence of CD11b/CD18 (MAC1), a co-receptor for CD89 specifically involved in recognizing SIgA or free SC (41). Therefore until now we have no indication for the mesangial IgA receptor involved in SIgA binding. Our inhibition experiments suggest that IgA rather than SC is recognized and that no C-type lectin is involved. Importantly we found that the putative receptor is able to transmit proinflammatory signals, since SIgA induced a strong dose-dependent increase in IL-6 production by mesangial cells. This seems in contrast with the proposed anti-inflammatory role of SIgA (53).

Table 2: Immunoglobulin concentrations in serum and glomerular eluate of IgAN patient.

	IgA1	IgA2	SIgA	IgM	IgG
Serum (mg/ml)	5.9	0.59	0.016	0.74	10
Glomerular eluate (µg/ml)	6.1	0.65	2	2.2	6.8

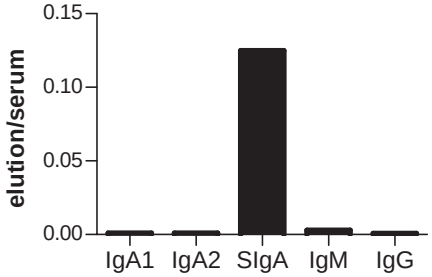

Figure 9: SIgA is accumulated in the glomerular eluate of an IgAN kidney
To make an estimate for the specific accumulation in the glomerular deposit, immunoglobulin concentrations in the eluate and serum were compared. Depicted is the ratio of concentration of different immunoglobulins in the eluate and the serum.

Generation of SIgA, i.e. production of dIgA followed by transcytosis using the pIgR, is a specific process taking place at mucosal surfaces (54). Interestingly, IgAN patients often present macroscopic hematuria following upper respiratory tract infections. Mucosal challenge also leads to an increased production of IgA in the systemic compartment, probably based on the migration of B cells (the mucosa-bone marrow axis) (55). This mucosa-bone marrow traffic has been confirmed by challenging healthy individuals intranasally with the neoantigen cholera toxin subunit B (CTB) (56). In patients with IgAN we observed a reduced mucosal IgA response to mucosal immunization with CTB (56). At present it is not clear whether a mucosal challenge also regulates levels of circulating SIgA. Still our finding of glomerular accumulation of SIgA provides a link between the mucosal immune system and renal deposits.

In summary, we have shown that SIgA is able to bind to mesangial cells in a

dose-dependent manner and that this binding is calcium-independent and cannot be inhibited with free SC. Furthermore low concentrations of SIgA can be detected in serum. Further research is needed to determine to which receptor SIgA can bind and what the mechanism of cell activation induced by SIgA is. SIgA is strongly accumulated in the glomeruli of a kidney from an IgAN patient. Therefore we conclude that our data support a role for SIgA in the pathogenesis of IgAN, and further research to define such a pathogenic role is warranted.

Acknowledgements

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CHAPTER 7

Demonstration of secretory IgA in kidneys of patients with IgA nephropathy

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Summary

Recently we reported a possible role for secretory IgA (SIgA) in IgA nephropathy (IgAN), as suggested by increased serum levels in patients with active disease and accumulation of SIgA in a glomerular eluate. Therefore, we attempted to find support for these findings by analysis of the presence of SIgA in biopsies of IgAN patients. Renal biopsies of 26 patients with biopsy-proven IgAN were analyzed for the presence of SIgA. In 15 % clear deposition of SIgA was demonstrable. The presence of SIgA in these biopsies showed a strong correlation with deposition of MBL and C4d. It has been previously documented that patients with MBL deposits have more severe renal injury. Therefore, these data provide additional evidence for a pathogenic role for SIgA in IgA nephropathy.

Submitted

INTRODUCTION

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide. The hallmark of this disease is deposition of IgA in the glomerular mesangium, together with markers of complement activation (1-3). It is generally thought that deposits of IgA consist of IgA1 which is mostly polymeric (4). The composition of polymeric IgA in serum is highly diverse and may include CD89/IgA complexes, dimeric IgA, IgA immune complexes and secretory IgA (SIgA) (5-7).

SIgA is the dominant immunoglobulin in external mucosal secretions like oral, respiratory and intestinal cavities, and is often characterized as a component of the immune systems' first line defense against pathogens (8). Next to its presence in mucosal secretions, small amounts of SIgA can also be found in human serum (7,9,10). Noteworthy, increased serum levels of SIgA have been reported in various diseases (11-13), and are associated with more hematuria in IgAN patients (7). Moreover, polymeric serum IgA of patients with IgAN contains higher SIgA concentrations as compared to healthy controls (14).

Glomerular IgA deposition is associated with activation of the complement system (15), involving the alternative pathway and the lectin pathway of complement (16). Recent studies indicate that deposition of MBL, one of the recognition molecules of the lectin pathway of complement, in a subpopulation of IgAN patients is associated with a more severe renal injury (16,17), compatible with the observation that MBL bind to polymeric IgA (18).

The aim of the present study was to investigate whether SIgA can be demonstrated in biopsies from patients with IgA nephropathy. The results show that SIgA can be found in a subpopulation of IgAN patients, and that presence of SIgA is associated with the presence of MBL and C4d.

MATERIAL AND METHODS

Patients and biopsies

Renal biopsies were selected from patients with IgA nephropathy of whom a renal biopsy was taken between January 2001 and December 2003. Patients were selected when adequate tissue was obtained for diagnostics (at least 8 glomeruli in light microscopy sections; complete immunohistology and electron microscopy examination), and when sufficient frozen material was available for additional staining after immunodiagnosis (at least 6 glomeruli in at least 15 (5µm thick) tissue sections). Cases with Henoch-Schönlein purpura, systemic lupus erythematosus, liver cirrhosis or other systemic diseases were excluded. In total, 26 biopsies were selected for evaluation.

Among selected patients, 77 % were male and 23 % females. Creatinine clearance was calculated according to the Cockroft formula (range 16-130 ml/min).

Immunofluorescence

For immunofluorescence stainings, unfixed renal tissue was embedded in OCT compound (Sakura Tissue-tek, Bayer), snap-frozen in a mixture of isopentane and dry-ice and stored at -80°C. Subsequently, 5µm sections were placed on slides and stored at -20°C until

immunostaining.

We used mouse monoclonal antibodies directed against the following molecules: MBL (mAb 3E7, kindly provided by Prof. Fujita, Fukushima, Japan (27)), and secretory component (SC) (mAb NI194-4 from Nordic (7)). Rabbit polyclonal antibodies were applied for detection of IgA (FITC-labeled anti-human IgA, Dako), C3 (FITC-labeled anti-human C3c, Dako) and C4d (Biomedica (28)). For indirect immunofluorescence, after fixation in cold acetone, tissues were incubated sequentially with the primary antibody and the proper fluorescently labeled secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse Ig or Alexa Fluor 546-conjugated goat anti-mouse Ig, Molecular probes). Slides were finally mounted with anti-fading aqueous mounting medium (Fluorsave, Calbiochem).

Statistical analysis

Data were compared between IgAN patients showing positive and negative glomerular staining of SIgA, respectively. Frequency analysis was performed using the Fisher exact test. Other comparisons were evaluated using the Mann Whitney U test. Differences were considered statistically significant when P was below 0.05.

Table 1: Histological data from IgA nephropathy patients

Parameter	SIgA neg (N= 22)	SIgA pos (N= 4)	P
Intense mesangial proliferation (% of cases)	32	75	0.264
Extracapillary proliferation present (% of cases)	27	50	0.543
Global sclerosis (% of glomeruli ; median)	13	23	0.943
Segmental sclerosis (% of glomeruli ; median)	3.5	8	0.972
Interstitial infiltration (0-3 scale scoring: median)	1	2	0.319
Interstitial fibrosis (0-3 scale scoring: median)	1	1	0.831
Vessel lesions present (% of cases)	45	25	0.614
C3 present (% of cases)	91	50	0.0987
C4d present (% of cases)	4.5	100	0.0003
Glomerular MBL staining present (% of cases)	4.5	100	0.0003

SIgA-negative cases and SIgA-positive cases are defined on basis of glomerular staining.

RESULTS

Immunofluorescence staining for SIgA was performed in renal biopsies from 26 IgAN patients. Glomerular SIgA positivity was observed in a mesangial pattern in 4 biopsies (15 %, Figure 1), whereas glomeruli in 22 biopsies were negative.

Next we examined the presence of molecules of the complement system (Table 1). In line with our previous study we observed that 19 % were positive for MBL and C4d (16). We observed a strong association between the presence of SIgA and the presence of MBL (P = 0.0003) and C4d, respectively (P = 0.0003).

Based on the presence (15 %) or absence (85 %) of glomerular SIgA Two IgAN

patient groups were defined and further characterized. SIgA-positive and negative cases had a similar male/female distribution and no difference in renal function (Table 2). There was a clear trend towards a younger age at time of renal biopsy for patients with positive glomerular SIgA staining as compared to negative SIgA staining (p= 0.0596, Table 2). There was also a trend towards more severe mesangial proliferation in SIgA- positive biopsies as compared to SIgA-negative biopsies (Table 1). This is in line with our previous observation that deposition of MBL is associated with more severe renal injury, characterized by more intense mesangial and extracapillary proliferation, glomerular sclerosis and interstitial damage (16).

Table 2: Clinical and laboratory data from IgA nephropathy patients

Parameter	SIgA neg (N= 22)	SIgA pos (N= 4)	P
Age at renal biopsy (Years; median)(range)	33 (21-57)	26.5 (24-31)	
Female gender (%)	27	0	0.5425
Proteinuria (gram/24hr; median)	1.4	2.1	0.2864
Macroscopic hematuria present (% of cases)	37	25	0.6603
Serum creatinine (ì mol/l; median)	1.3	1.4	0.4553
Creatinine clearance (ml/min; median)	53	71.5	0.9151
Serum IgA (mg/ml)	2.4	2.49	0.6698

SIgA-negative cases and SIgA-positive cases are defined on basis of glomerular staining. All data were obtained at the time of renal biopsy

DISCUSSION

The present study provides further evidence for a possible pathogenic role for SIgA in IgAN. We demonstrate glomerular deposition of SIgA in biopsies of a subpopulation of IgAN patients. Furthermore, there was a strong association between glomerular SIgA staining and the presence of MBL and C4d, suggesting activation of the lectin pathway of complement in cases with SIgA deposition.

Deposition of IgA in the mesangial area is the hallmark of patients with IgAN and it is generally thought that this deposition drives a local inflammatory response. Previous research has concentrated on quantitative and qualitative differences of IgA deposited in the kidney. It has been proposed that IgA in renal deposits is mostly high MW of nature and might contain differences in glycosylation which might affect receptor interaction or effector functions. We now show that in a subset of patients SIgA can be demonstrated in the renal biopsies, in line with our previous investigation of a renal eluate (7). Deposition of SIgA in patients with IgAN has not been widely documented, although it was observed in a recent study (19).

Generation of secretory IgA (SIgA) is a specific process taking place at mucosal surfaces (20). It has been reported that SIgA adheres selectively to microfold (M) cells irrespective of their antigen-binding specificity (21,22), followed by its transport across the epithelium and targeting of dendritic cells (DC) (23,24). In vitro it has been demonstrated that DC can bind and endocytose SIgA (25). Probably not all

SIgA is internalized by DC and this SIgA may end up in the circulation. Indeed, small amounts of SIgA have also been found in human serum (9,10). Moreover, increased serum levels of SIgA have been reported in various diseases (11-13) indicating that SIgA may be a marker of clinical interest. We hypothesize that after mucosal challenge the production of SIgA at mucosal sites is increased. This could potentially lead to increased serum SIgA concentrations (7) and thereby, via a presently undefined mechanism, lead to glomerular deposition of SIgA.

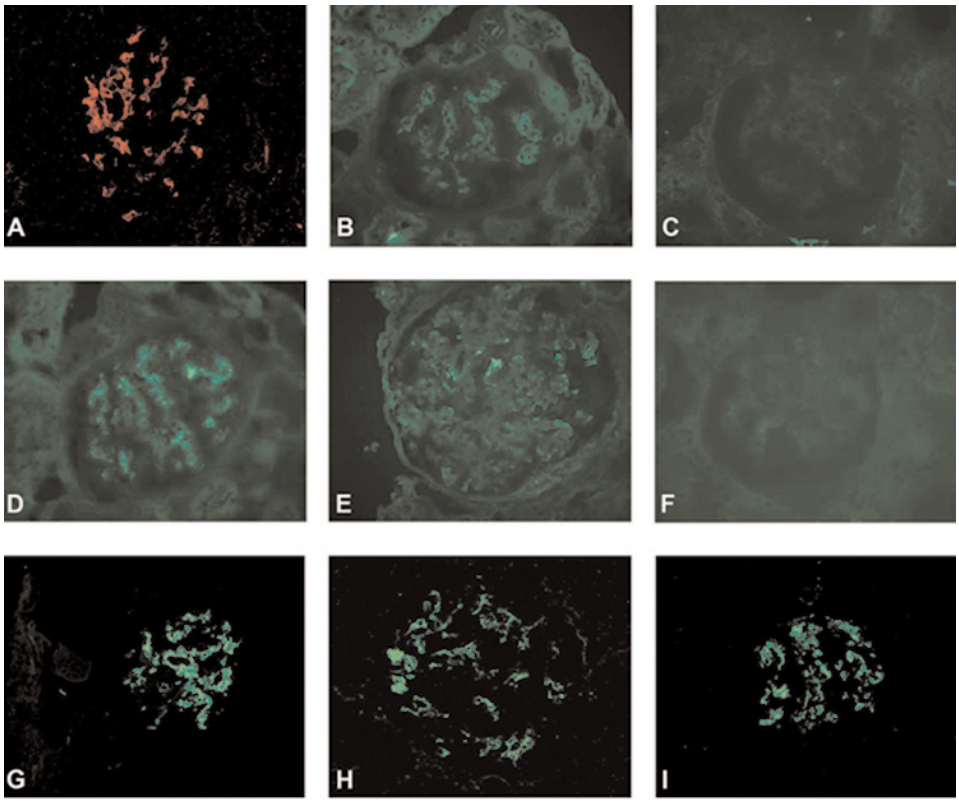

Figure 1. Glomerular SIgA deposition in IgAN patients.
Renal tissue from patients with IgAN was stained for the presence of SIgA (A, B and C), MBL (D, E and F), IgA (G), C4d (H) and C3 (I). Representative images are shown. Renal tissues are derived from different patients who showed positive (A and B) and negative staining (C) for SIgA and positive (D and E) and negative staining (F) for MBL. All patients were positive for IgA (G). A subpopulation of patients was positive for C4d (H) and C3 (I).

Earlier studies have shown that the carbohydrate moieties on SIgA are different as compared to serum IgA (26). Furthermore, it was shown that MBL can interact with SIgA upon conformational change under acid conditions. This suggests that disruption of the non-covalent interactions between secretory component and the IgA heavy chain can lead to MBL binding and subsequently complement activation via the lectin pathway. The data from the present study suggest that in the glomeruli of a subpopulation of IgAN patients, the deposition of SIgA may lead to unmasking

the heavy chain of IgA of SIgA leading to MBL binding and complement activation.

Recently, it has been described that MBL deposition in glomeruli is associated with more severe renal disease (16). In the present study we show a strong co-deposition of SIgA and MBL, suggesting SIgA as a strong cofactor. Furthermore, more hematuria was observed in patients with higher concentrations of SIgA in serum (7). Moreover, after elution of isolated glomeruli from a patient with IgAN a 120-fold accumulation of SIgA in the glomeruli was observed. In the present study we provide additional evidence for a pathogenic role of SIgA in a subpopulation of patients with IgA nephropathy.

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General Discussion and Summary

CHAPTER 8

Introduction

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease is characterized by mesangial deposits of IgA (1-3). These deposits are thought to be composed mostly of high molecular weight IgA1, sometimes together with IgM or complement components like C3 (4,5). The disease shows a broad spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. Case reports have shown that IgA deposits disappear after transplantation of a kidney with IgA deposits into a non-IgAN patient (6). Furthermore, after renal transplantation recurrent mesangial IgA deposition is observed in about 50 % of the patients (7). These results strongly suggest that the basic abnormality of the disease lies within the IgA immune system rather than in the kidney.

In serum of IgAN patients, elevated levels of IgA and IgA immune complexes were observed (8,9). Furthermore, aberrantly glycosylated IgA, due to a reduced activity of the β1-3 galactosyltransferase, has been described in patients with IgAN. This aberrantly glycosylated IgA is also demonstrated in glomerular IgA deposits, suggesting a better binding of this IgA as compared to normal glycosylated IgA.

Another important factor is the binding of IgA to mesangial cells. Several findings point to an IgA receptor on mesangial cells (10,11). For most of the IgA receptors it has already been demonstrated that they are not present on mesangial cells (12,13). There are some new candidates like Fcα/μR (14) or transferrin receptor (15) but, there is only limited information available about these receptors and further research has to confirm their possible role in IgAN.

The present thesis focuses on different aspects of IgAN, namely IgA and the binding of IgA to its receptors. In the first part of this thesis (chapter 2,3) we focused on possible receptor-mediated mechanisms underlying mesangial IgA deposition. In the second part of the thesis (chapter 4 to 7) we focused our attention on the characterization of IgA and associated molecules present in the mesangium of IgAN patients and on the characterization of serum IgA from patients and controls.

Binding of IgA to IgA receptors

Several IgA receptors have been identified and characterized. One of these receptors is FcαRI/CD89. A number of studies already showed that CD89 is not present on mesangial cells. Still, there are suggestions for a role of this receptor in IgAN. Human CD89 transgenic mice spontaneously develop at old age renal disease with characteristics of IgAN (16). On the other hand, our group showed that injection of soluble human CD89 into mice does not induce deposition of IgA in kidneys (17). It remains unclear how human CD89 could affect IgA deposition, since we and others showed that human CD89 cannot react with murine IgA (17,18).

Complexes of CD89 with monomeric IgA are present in the circulation (19). However, it is also shown that especially high molecular weight IgA can interact with CD89 (20-22). Therefore, we investigated the binding of different molecular forms of IgA with CD89 with different methods. In chapter 2 we confirm data from literature (20-22), that high molecular weight IgA can interact with CD89. We however, using

surface plasmon resonance (SPR), a method in which the interaction in time can be measured, found that the initial interaction of monomeric IgA and CD89 is comparable to polymeric IgA-CD89 interactions. However, the interaction of polymeric IgA with CD89 is more stable as compared to monomeric IgA ultimately resulting in a higher final binding of polymeric IgA to CD89 than monomeric IgA. A recently published crystal structure of the IgA1Fc-CD89 complex showed that one IgA molecule is in complex with two CD89 molecules, confirming interactions of mIgA with CD89 (23). Furthermore, the initial interaction of mIgA with CD89 might also be sufficient for internalization of mIgA via CD89, thereby creating intracellular CD89-IgA complexes which then can be secreted in the circulation (19).

After cleavage of the N-glycans present on IgA we demonstrated enhanced association of IgA to CD89, suggesting an important role for the N-glycans on IgA in the initial interaction. In literature, it is still controversial whether N-glycans present on IgA do influence the interaction with CD89 (24,25). In the crystal structure of the IgA1Fc-CD89 complexes the observed N-glycans are close but do not directly contact the receptor (23). Some amino acid mutations in the CD89 molecule, which are not in direct contact with IgA, also hamper the binding of IgA-CD89. Because of the presence of the N-glycans close to the contact sites it could be that the N-glycans hamper the IgA/CD89 interaction.

Another recently identified IgA receptor is Fcα/µR. This receptor has been described to be present on B cells and macrophages (26). Furthermore, high expression in the kidney is observed (26). The Fcα/µR is also suggested to be present on mesangial cells, and thereby could play a role in IgAN. However there are no suitable reagents available to study this receptor. Therefore we made chimeric proteins to study IgA and IgM binding to this receptor. We demonstrated that IgA binds to Fcα/µR, likely through the proximal immunoglobulin like domain. The binding of IgA to Fcα/µR was equal for monomeric and polymeric IgA and was glycan dependent. Therefore these findings do not fit in the observations that it is mostly pIgA that is present in the renal mesangium of IgAN patients (27). Moreover, the glycan composition of IgA described in patients with IgAN (28-30), does not have any effect on the interaction of IgA with Fcα/µR. After removing the N-glycans and the sialic acids from the IgA molecule, the interaction with the Fcα/µR is hampered, whereas deglycosylation of IgA leads to an increase in binding to mesangial cells (31). We confirmed the presence of the Fcα/µR on mesangial cells. Furthermore, we showed an upregulation of Fcα/µR transcript in mesangial cells after IL-1 stimulation. However, high molecular weight IgA binds better to mesangial cells as compared to monomeric IgA and we could not detect differences in IgA binding to IL-1 stimulated mesangial cells. In conclusion, the Fcα/µR could play a role in the clearance of IgA in IgAN, but its in vitro characteristics do not favor a significant role for this receptor in IgAN.

Complement in IgA nephropathy

In IgAN deposits of IgA are commonly associated with complement components, most often C3, the membrane attack complex (C5b-9) and properdin (32,33). Furthermore, increased levels of split products of activated C3 have been observed in the circulation of IgAN patients (34-36). These findings suggest involvement of the

alternative pathway in IgAN. Indeed, in vitro as well as in vivo studies, have indicated that IgA can directly activate the alternative pathway of complement (37-39).

Recently, the lectin pathway of complement, with their recognition molecules MBL, H-ficolin and L-ficolin, has been described (40-43). MBL is able to bind directly to a number of microorganisms, via carbohydrates expressed on their surface (44,45). Upon binding, MBL activates the complement cascade via the lectin pathway, which plays a critical role in the first line of host defense against these pathogens. Furthermore, genetic mutations in the MBL gene, leading to low serum MBL levels and non functional MBL, have a negative impact on several chronic diseases (46-48). However evidence is increasing that MBL and the lectin pathway of complement can also be unfavorable for disease progression. This is proposed for rheumatoid arthritis, and is suggested by the presence of MBL in renal biopsies from patients with IgAN (49-51). Furthermore, it was shown that MBL is able to bind to polymeric IgA which leads to the activation of the lectin pathway in vitro (52).

In chapter 4 we investigated the presence of MBL in glomerular IgA deposits of IgA nephropathy patients. We showed that in 25 % of the cases co-deposition of IgA with MBL was present (Figure 1). Furthermore, patients with MBL deposition suffered from more severe renal injury as compared to the MBL negative cases, suggesting an important role for MBL in disease progression. In a recent Japanese study it has been suggested that MBL deposition was restricted to biopsies with IgA2 deposition (53). However, in the biopsies that we investigated we were not able to detect any IgA2. The difference with an earlier report might be due to ethnical differences of the patients.

In chapter 5 we investigated the differences between monomeric and polymeric IgA purified from patient and control. We confirmed that specifically polymeric IgA is able to bind to MBL and induce C4 activation. However, no differences between patients and controls were observed. Furthermore, we showed that on the heavy chain of polymeric IgA oligomannose structures are present which are absent on monomeric IgA. The carbohydrate recognition domain of MBL is able to bind in a calcium-dependent way to a number of saccharides, such as D-mannose, L-fucose, and N-acetylglucosamine (GlcNAc) (44). Therefore, the oligomannose structure present on the heavy chain of IgA could be a likely candidate to bind MBL, but this needs further confirmation

Serum IgA in IgA nephropathy

In IgAN, the glycosylation of the IgA1 molecule plays an important role. It has been described that the IgA1 molecule is aberrantly glycosylated leading to increased Tn antigen (GalNAcα1-Ser/Thr) residues (28,30). Allen et al. have reported that the β1-3 galactosyltransferase synthesis activity was remarkably lower in peripheral B lymphocytes of IgAN patients as compared to healthy controls (54). The β1-3 galactosyltransferase activity is dependent on the coexistence of a specific chaperone, Cosmc (core 1 β1-3-Gal-T-specific molecular chaperone) (55), and in IgAN patients it is suggested that downregulation of Cosmc is important for the aberrant O-glycosylation of IgA1 (56).

Serum IgA from IgAN patients was shown to have an increased binding to

mesangial cells (57), however this could not be confirmed by others (58). Furthermore, increased production of cytokines and chemokines was shown after stimulation of mesangial cells with serum IgA from IgAN patients (59-61). To mimic this aberrantly glycosylated IgA, IgA was treated with enzymes. In vitro studies with deglycosylated IgA suggested that the undergalactosylated IgA in IgAN patients might be involved in increased interaction with mesangial cells (31). Noteworthy, these in vitro studies made use of the lectin Jacalin (which specifically recognizes Galβ1-3GalNAc) for IgA purification, possibly leading to a selection of only a subpopulation of serum IgA1.

In chapter 5 we purified IgA from serum of patients and controls with an anti-human IgA affinity column and separated these IgA preparations in monomeric and polymeric IgA. With this method we did not select for typical IgA glycosylation structures. With these highly purified monomeric and polymeric IgA preparations we studied the interaction with mesangial cells and lectins. The interaction with mesangial cells, and the induction of IL-8 production was better for polymeric IgA than for monomeric IgA, but there was no difference between patients and controls. Stimulation of mesangial cells with IgA and IgA binding to mesangial cells has been studied before (31,60,62,63), but this is one of the first studies that a correlation between binding of IgA to mesangial cells and chemokine production after stimulation of mesangial cells with the same IgA preparations shows.

Another interesting finding in this chapter is the binding of Helix aspersa (HAA) to the IgA preparations. HAA is a lectin which recognizes terminal GalNAc. In an earlier report, size fractionation of total serum of an IgAN patient showed HAA reactivity in the high molecular weight fractions suggesting interaction with high molecular weight IgA (31). In our study, we confirmed this better binding of polymeric IgA to HAA as compared to monomeric IgA. Binding of HAA was strongly increased by neuraminidase treatment, suggesting a high frequency of non-galactosylated O-linked glycans on IgA, that expose terminal GalNAc after enzymatic removal of sialic acid.

Polymeric serum IgA contains more IgA2 as compared to monomeric serum IgA (chapter 5). Mucosal IgA is largely polymeric IgA whereas serum IgA contains mainly monomeric IgA. Furthermore, the IgA subclass distribution varies with different mucosal locations (5) depending on the site, the total IgA amount consists of 15 to 65 % IgA2. Therefore, an increased polymeric IgA2 fraction may suggest its production at the mucosal sites. Another explanation could be the glycosylation of the IgA molecule. IgA2 contains additional N-glycans as compared to IgA1. At present it is unknown whether the glycans present on IgA are important for the polymerization process. The observation of a difference in N-glycosylation of polymeric IgA versus monomeric IgA, together with the presence of two to three additional N-glycans on IgA2 as compared to IgA1, may suggest a more efficient polymerization of IgA2, and therefore could explain that more IgA2 is found in the polymeric IgA fraction.

Secretory IgA in IgA nephropathy

About 40% of patients with IgAN have recurrent episodes of macroscopic hematuria frequently preceded one or two days earlier by infections. Upper respiratory

tract infections occur most frequently (64), but also occasionally other infections have been implicated, including gastrointestinal and urinary tract infections (65,66). Furthermore, mucosal immunization with a neo-antigen in healthy individuals leads to an antigen-specific immune response in plasma, suggesting a relation between mucosa and bone marrow. However, this response is reduced after immunization of patients with IgAN (67). SIgA is the major immunoglobulin responsible for protecting the mucosal surfaces. However, at present it is not clear whether a mucosal challenge also regulates levels of circulating SIgA.

Generation of secretory IgA (SIgA) is a specific process taking place at mucosal surfaces (68), after binding of dimeric IgA (dIgA) to the polymeric Ig receptor (pIgR) followed by a process of transcytosis. In addition also a process of transepithelial transport has been described in which SIgA adheres selectively to microfold (M) cells irrespective of their antigen-binding specificity (69,70), and that SIgA is subsequently transported across the epithelium and targeted to dendritic cells (DC) (71,72). In vitro it has been demonstrated that DC can bind and endocytose SIgA (73). It could be hypothesized that not all SIgA is internalized by DC and this SIgA may end up in the circulation. Indeed, small amounts of SIgA can also be found in human serum (74,75). Moreover, increased serum levels of SIgA have been reported in various diseases (76-78) indicating that SIgA may be a marker of clinical interest.

In the final section of this thesis we addressed the role of SIgA in IgA nephropathy (Figure 1). First, we investigated the presence of SIgA in serum (chapter 6) and in purified IgA preparations (chapter 5). We showed that in purified IgA preparations SIgA is restricted to the polymeric IgA fractions and that the relative concentration of SIgA is higher in patients with IgAN as compared to controls. In serum we measured low concentrations of SIgA, but we could not observe differences in SIgA concentrations between patients and controls. However, we did find a relation between hematuria in patients with IgAN and the SIgA concentration in serum.

Tonsils in IgA nephropathy

Tonsils are located at the gateway of the respiratory and alimentary tract and belong to the mucosa-associated lymphoid tissue. The major function of the tonsils is a first line of defence against viral, bacterial and food antigens. Tonsils of IgAN patients contain more IgA secreting B cells as compared to other diseases (79). This increase is paralleled by an increase of the number of dimeric IgA secreting cells (79). Furthermore, SIgA can adhere to M cells and transported into the lymphoid tissues. Stimulation, by ultra short wave, of the tonsils of IgAN patients leads to deterioration in renal function as compared to patients with other kidney diseases (80). In addition, previous episodes of gross hematuria following upper respiratory tract infections and the level of serum secretory IgA were higher in IgAN patients with deterioration of renal function after tonsil stimulation that those without deterioration (80). Tonsillar IgA1 from IgAN patients contains more asialo IgA1 as compared to serum IgA1 (81,82), suggesting that underglycosylated tonsillar IgA is produced in this lymphoid organ. This abnormally produced IgA reach the peripheral blood, and because of their size or composition, fails to be properly filtered by the kidneys but rather accumulate in the mesangium. In this respect the role of SIgA in this process

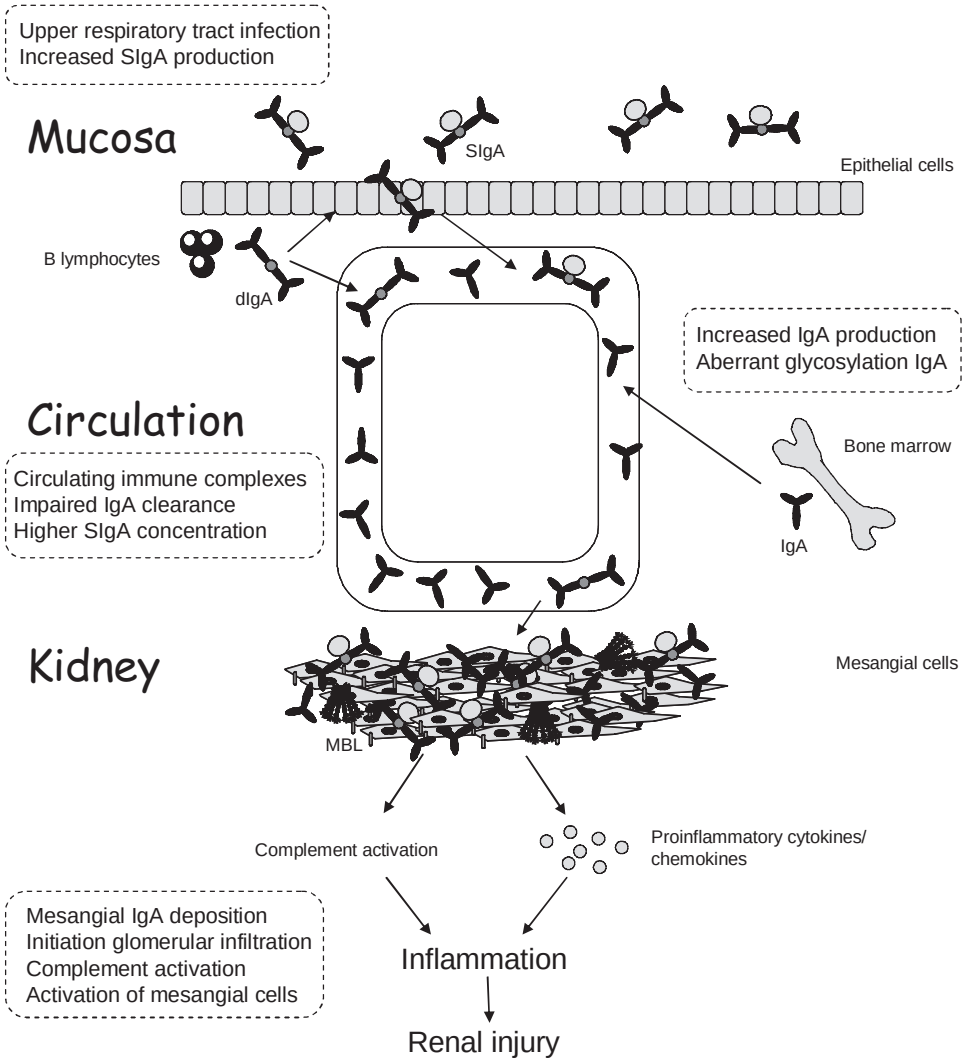

Figure 1: Hypothetical overview of different factors involved in IgA Nephropathy.
In IgAN three compartments are important, the mucosa, the circulation and the kidney. After upper respiratory tract infection 40 % of the IgAN patients have recurrent episodes of hematuria, suggesting a role for the mucosal compartment, whereby may be some SIgA is leaking in the circulation. Furthermore, the B lymphocytes present at the mucosal surface will produce dIgA which will be present in the circulation and via transcytosis in the epithelial cells secreted as SIgA in the mucosa. Via the circulation the SIgA is in contact with the mesangial cells to which it can bind. At the same time, the bone marrow is producing increasing amounts and aberrantly glycosylated IgA which is also present in the circulation. The binding of IgA to mesangial cells will lead to activation of the mesangial cells and the complement pathways. This mesangial cell activation will attract immune cells, and thereby inducing inflammation leading to renal injury.

was not studied. Tonsillectomy can improve the renal function and keep the renal function stable in some IgAN patients (83,84). Tonsillectomy had a favorable effect on long term renal survival in IgAN patients (85).

SIgA and mesangial cells

The activation of mesangial cells by IgA1 immune complexes is considered as an initiating event in the pathogenesis of IgA nephropathy (Figure 1). Therefore, we analyzed the interaction of serum IgA and SIgA with mesangial cells (chapter 6). We demonstrated a higher binding of SIgA to mesangial cells as compared to the binding of serum IgA. Passive protection by SIgA, secreted by the mucosal immune system, plays a central role in the protection of mucosal surfaces in general. However, after stimulation of mesangial cells with SIgA, the mesangial cells start to produce even more IL-6 than after exposure to serum IgA. Finally, we demonstrate in chapter 6 that immunoglobulins eluted from glomeruli of a patient show a strong accumulation of SIgA in the renal mesangium as compared to other immunoglobulins. These data suggest a pathogenic role for SIgA in IgA nephropathy.

SIgA glycosylation in IgAN

As mentioned before, it is assumed that glycosylation of IgA is an important factor in IgAN (Figure 1). Previous studies have shown a role for glycosylation of IgA and its subsequent interaction with mesangial cells (31,60,62,63). The glycosylation of SIgA is different as compared to serum IgA. Secretory IgA consists of two IgA molecules together with the J chain and the secretory component. Modeling SIgA has shown that secretory component masks the heavy chain of IgA and thereby prevents interaction with glycan structures (86). Furthermore, it was shown that N-glycans present on SIgA contain other structures as compared to serum IgA (86). In IgAN, especially the O-glycosylation of IgA was studied. The O-glycan structure on the hinge region of SIgA1 contains many different carbohydrate structures. These structures might be differently exposed because of the presence of secretory component. Taken together, this may lead to increased interaction of SIgA with mesangial cells. In this respect, the specific alterations in glycosylation of SIgA from IgAN patients would be very interesting to study.

SIgA in IgAN biopsies

To confirm the presence of SIgA in glomeruli of a patient with IgAN, we stained kidney biopsies from IgA nephropathy patients for SIgA (chapter 7). In 15 % of the cases positive staining of SIgA in biopsies was observed. In a Japanese study all IgAN biopsies studied were positive for secretory component, whereas normal kidneys were negative (87). However, the relation with the presence of secretory component and other molecules in the glomeruli or clinical parameters of the patients were not studied. Interestingly, this study (87) showed an association with single nucleotide polymorphisms (SNPs) in the pIgR and the presence of IgAN.

In chapter 7 we demonstrate a clear correlation between the presence of SIgA and MBL in renal biopsies. Earlier studies had already demonstrated an interaction of SIgA with MBL (86). Royle et al. suggested that disruption of the non-covalent interactions between secretory component and the IgA heavy chain may, for instance at low pH, lead to MBL binding and subsequent complement activation via the lectin pathway (86). The presence of SIgA with MBL in glomeruli of a subpopulation of IgAN patients suggests unmasking of the IgA heavy chain leading to MBL deposition. However, MBL was found in 20 % of the cases in the glomeruli, where-

as glomerular SIgA is detected in 15 % of the cases. This indicates that MBL most probably is not only binding to deposited SIgA but also to other high molecular weight forms of IgA.

Immunization studies in IgAN patients

Several immunization studies, both after systemic and oral secondary immunization, showed conflicting results with respect to the specific serum and mucosal IgA concentrations in IgAN patients (88-94). After mucosal immunization with CTB, IgAN patients exhibit a decreased mucosal and systemic antigen specific IgA response, whereas simultaneous systemic immunization with KLH did not lead to a difference in antigen specific IgA response between patients and controls (67). Unpublished results from our group showed the size distribution of the antigen specific IgA response in serum and nasal washes after immunization with CTB and KLH. The nasal washes contain mainly SIgA whereas serum IgA showed a typical serum IgA profile (mainly mIgA). Furthermore, SIgA is restricted to fractions containing high molecular weight IgA. The antigen specific IgA response is mostly of high molecular weight. Unfortunately, with the available reagents, we were not able to detect antigen specific SIgA. However, the partly overlapping profile would be in line with a role for antigen specific SIgA.

The systemic antigen specific IgA response we found was mainly associated with high molecular weight IgA. We hypothesize that this IgA may be partly derived from mucosal sites via production of dimeric IgA by mucosal B cells and leakage of SIgA from the mucosa (Figure 1). However, also in the bone marrow antigen specific IgA1 antibody producing cells were demonstrated (67). IgA from the circulation is mainly produced in the bone marrow and is monomeric, whereas the response we tested was mainly high molecular weight IgA. Therefore it would be very interesting to investigate the size distribution of bone marrow synthesized antigen specific IgA. In literature, up-regulation of pIgA synthesis in the bone marrow and an increase of pIgA-producing plasma cells have been described in IgAN patients (79,95). However, after intramuscular immunization with inactivated influenza virus mainly a monomeric IgA response is observed (88). It seems that the route of immunization is important for the size distribution of the antigen specific IgA response. Therefore, it would be very interesting to investigate the size of the antigen specific IgA after different immunization routes.

Conclusion

In summary, we have demonstrated in the first part of this thesis that monomeric IgA is able to interact with different IgA receptors and that this interaction is glycosylation dependent. Furthermore, we investigated the binding of IgA to a recently identified IgA receptor, thereby showing clear IgA binding to this receptor. However, the binding of monomeric and polymeric IgA were similar, suggesting that this receptor is probably not the mesangial receptor responsible for the deposition of IgA in the mesangium of IgAN patients.

In the second part of this thesis we showed clear differences between polymeric and monomeric IgA, which could finally lead to deposition of IgA (Figure 1). We

demonstrated a better interaction of polymeric IgA with mesangial cells than that of monomeric IgA. Furthermore, polymeric IgA is able to interact with MBL and thereby activates the lectin pathway of complement, in addition to the alternative pathway. We also showed that binding of MBL to IgA can lead to MBL deposition in the glomeruli of a subpopulation of IgAN patients. Moreover, we showed that this deposition of MBL is associated with more severe renal injury (Figure 1).

IgAN patients often have macroscopic hematuria following upper respiratory tract infections. Although, we could not observe differences in SIgA concentrations in serum of IgAN patients and controls, we showed a clear relation with the SIgA concentration in serum and hematuria. Furthermore, significantly more SIgA was present in affinity purified IgA preparations from IgAN patients as compared to controls. Moreover, we showed a strong accumulation of SIgA, as compared to other immunoglobulins, in the glomeruli, and this was in agreement with the positive SIgA staining in kidney biopsies of a subpopulation of IgAN patients (Figure 1). Altogether, the data presented in this thesis support a role for SIgA in the pathogenesis of a subpopulation of IgAN patients. Further research to define such a pathogenic role is warranted.

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Nederlandse Samenvatting

IgA nefropathie

Primaire IgA nefropathie (IgAN) is een veel voorkomende nierziekte, waarbij IgA neerslaat in het mesangium van de glomeruli (zeeflichaampjes) van de nier. Door de binding van IgA aan mesangium cellen kan dit leiden tot een lokale ontstekingsreactie. Deze ontsteking veroorzaakt schade met als gevolg uitscheiding van bloed en eiwit in de urine en uiteindelijk, in een groot deel van de patiënten tot nierfunctieverlies. De mechanismen die IgAN veroorzaken zijn nog onduidelijk. Er zijn aanwijzingen dat het primaire probleem niet de nier is, maar het IgA. Bijvoorbeeld; na transplantatie van een nier in een IgAN patiënt is er 50 % kans dat er opnieuw IgA neerslaat in de nier. Ook is er aangetoond dat als een nier van een IgAN patiënt in een niet IgAN patiënt wordt geplaatst de IgA neerslag verdwijnt. Deze data suggereren dat in IgAN niet alleen de nier belangrijk is maar dat de circulatie ook een rol speelt.

Immunoglobuline A (IgA) is een van de moleculen van het afweersysteem. Zodra er vreemde eiwitten (antigenen) in het lichaam aanwezig zijn, zullen er verschillende eiwitten binden aan deze antigenen waardoor de antigenen worden opgeruimd. In eerste instantie zullen dat niet specifieke eiwitten zijn waaronder eiwitten van het complement systeem. Daarnaast worden er specifieke antistoffen gemaakt en zullen er verschillende antistoffen aan deze antigenen binden, waaronder IgA. IgA is in hoge concentraties aanwezig in de slijmvliezen van bijvoorbeeld longen en darmen, maar is ook aanwezig in het bloed.

Er zijn verschillende vormen van IgA beschreven, waaronder monomeer IgA (mIgA)(1 IgA molecuul) en dimeer IgA (dIgA)(twee IgA moleculen die bij elkaar worden gehouden door een ander eiwit (J chain)). Polymeer IgA (pIgA) bestaat uit verschillende IgA moleculen aan elkaar, maar ook bijvoorbeeld complexen van IgA met andere moleculen. In het menselijke lichaam is vooral monomeer IgA aanwezig in het bloed terwijl in de slijmvliezen voornamelijk SIgA aanwezig is. Om SIgA te genereren, wordt dIgA door cellen van het weefsel dicht bij de slijmvliezen getransporteerd. Tijdens dit transport blijft het extracellulaire deel van de receptor (secretoir component) dat het dIgA bindt, aan het dIgA zitten. Het complex van dIgA met secretoir component, wordt SIgA genoemd.

Verder zijn er ook twee verschillende isotypen beschreven, IgA1 en IgA2. Een belangrijk verschil tussen IgA1 en IgA2 zijn de suikergroepen (glycosylering) die aanwezig zijn op het eiwit. IgA1 bevat namelijk O-glycanen die niet aanwezig zijn op IgA2, terwijl IgA2 meer N-glycanen bevat dan IgA1. In de literatuur is beschreven dat in patiënten met IgAN de O-glycosylering van IgA1 veranderd is en dat dit IgA een belangrijke rol in IgAN speelt.

Het mechanisme van IgA binding aan mesangiumcellen en welke IgA vormen een belangrijke rol spelen in IgAN is nog onduidelijk. Daarom hebben we in dit proefschrift gekeken naar IgA receptoren die een rol zouden kunnen spelen in IgAN en hebben we vervolgens de verschillende vormen van IgA bestudeerd in relatie met IgAN.

Binding van IgA aan IgA receptoren

In IgAN bindt IgA aan een IgA receptor op de mesangiumcellen, waardoor het IgA neerslaat in de nier. Verschillende IgA receptoren zijn beschreven, maar van de

meest beschreven IgA receptoren is al aangetoond dat deze niet aanwezig zijn op de mesangiumcellen. In hoofdstuk 2 is de binding van verschillende vormen van IgA aan een bekende IgA receptor (FcαRI/CD89) met verschillende methodes bestudeerd. CD89 is een IgA receptor dat beschreven is op myeloide cellen en waarvan onze groep al eerder heeft aangetoond dat deze receptor niet aanwezig is op mesangiumcellen. Toch kan deze receptor wel belangrijk zijn in IgAN, doordat de klaring van het IgA via de IgA receptor verminderd kan zijn. Dit zou een hogere concentratie van het IgA in de circulatie tot gevolg kunnen hebben wat kan leiden tot binding van IgA aan mesangiumcellen. In dit hoofdstuk worden de binding verschillen, zoals beschreven in de literatuur, tussen monomeer IgA en polymeer IgA aan CD89 bevestigd. In dit hoofdstuk is gebruikt gemaakt van twee verschillende methodes om de binding van IgA aan CD89 te meten. Met deze methodes laten we zien dat er geen verschillen waren in de initiële fase van de binding van monomeer en polymeer IgA aan CD89, maar dat de uiteindelijke binding van polymeer IgA aan CD89 sterker was dan de binding van monomeer IgA aan CD89. Verder werd aangetoond dat de N-glycanen aanwezig op IgA van belang waren voor de binding van IgA met CD89, terwijl de O-glycosylering van IgA geen rol lijkt te spelen.

Recent is een nieuwe IgA receptor beschreven (Fcα/μR) die wel aanwezig zou zijn op mesangium cellen en ook verhoogd tot expressie wordt gebracht na stimulatie van de mesangiumcellen. Omdat er nog geen reagentia aanwezig zijn om deze receptor in detail te bestuderen hebben we een recombinante vorm van de receptor gemaakt (hoofdstuk 3). Deze recombinante receptor was in staat om IgA en IgM dosis afhankelijk te binden. De binding van monomeer en polymeer IgA aan Fcα/μR waren gelijk terwijl de binding van IgM aan Fcα/μR lager dan de binding van monomeer en polymeer IgA was. Ook hier waren de N-glycanen van belang voor de binding aan de receptor. Een ander aspect van de receptor, bestudeerd in dit hoofdstuk, was de expressie van de receptor in mesangiumcellen in relatie met de IgA binding aan mesangiumcellen. De receptor expressie was hoger na stimulatie van mesangium cellen terwijl er geen verschil in binding van IgA aan de mesangiumcellen was na stimulatie. Deze resultaten suggereren dat de Fcα/μR op mesangiumcellen geen directe rol speelt in IgAN.

Serum IgA in IgA nefropathie

Er zijn aanwijzingen in de literatuur dat er verschillen zijn tussen IgA van IgAN patiënten en controles, voornamelijk met betrekking tot de glycosylering van IgA. De neerslag van IgA in de nier van patiënten is door verschillende onderzoeksgroepen bestudeerd. De resultaten laten zien dat het IgA in de nier voornamelijk bestaat uit hoog moleculair IgA met een abnormale glycosylering. Verder is al aangetoond dat IgA in de nier het complement systeem kan activeren. In hoofdstuk 4 is de neerslag van IgA met moleculen van het complementsysteem in de nier bestudeerd. Hierin werd duidelijk aangetoond dat de aanwezigheid van MBL, een molecuul van de lectine route van het complement systeem, in de nierneerslag leidt tot ernstigere nierontsteking.

In de literatuur zijn er ook glycosylerings verschillen beschreven tussen het serum IgA van patiënten en controles, In hoofdstuk 5 hebben we IgA geïsoleerd uit serum van patiënten en controles en bestudeerd op verschillende aspecten, zoals

interactie met lectines en mesangiumcellen. Verder hebben we ook gekeken of er verschillen in IgA2 en SIgA concentratie tussen IgA van patiënten en controles waren te detecteren. Het opvallende van deze studie was dat er grote functionele en moleculaire verschillen zijn tussen monomeer en polymeer IgA maar niet tussen patiënten en controles. Het enige verschil tussen patiënten en controles was een verhoogde concentratie SIgA in patiënten.

SIgA in IgA nefropathie

Naast allerlei andere functionele en moleculaire veranderingen is de SIgA concentratie in de gezuiverde IgA preparaten van patiënten en controles ook gemeten. Zoals verwacht was SIgA alleen aanwezig in polymeer IgA, daarbij was er een significante verhoging van de SIgA concentratie in de patiënten ten opzichte van de controles. Deze verhoging van SIgA in patiënten was niet te meten in serum van patiënten en controles (hoofdstuk 6), hoewel patiënten met een hogere SIgA concentratie in het serum ook meer hematurie (bloed in urine) hadden. Verder is er in hoofdstuk 6 ook aangetoond dat SIgA kan binden aan mesangiumcellen en dat deze binding in vergelijking met serum IgA superieur is. Voor SIgA is beschreven dat het in de slijmvliezen een bescherming biedt tegen antigenen zonder een ontstekingsreactie te initiëren. Na stimulatie van mesangiumcellen met SIgA een verhoging van de IL-6 productie (een cytokine dat belangrijk is voor een ontstekingsreactie) was gemeten, suggererend dat er na binding van SIgA aan mesangiumcellen wel een ontstekingsreactie kan ontstaan. Om aan te tonen dat SIgA ook daadwerkelijk aanwezig is in een nier van een IgAN patiënt hebben we de glomeruli uit de nier van deze patiënt geïsoleerd. Na elutie van de eiwitten uit deze glomeruli was er een duidelijke accumulatie van SIgA in de neerslag aanwezig. Dit suggereert dat SIgA aanwezig kan zijn in de IgA neerslag van patiënten met IgAN. Deze neerslag is in hoofdstuk 7 bevestigd met kleuringen van nierweefsel van IgAN patiënten en controles. In 15% van de patiënten was SIgA aanwezig terwijl alle controles negatief voor SIgA waren.

Conclusie

Bij IgAN patiënten is er vaak een verergering van de ziekte, met bloed in de urine, na een ontsteking van de bovenste luchtwegen. Een activatie op de slijmvliezen zal resulteren in een verhoogde IgA productie en met name het genereren van SIgA. In dit proefschrift laten we zien dat als patiënten bloed in de urine hebben er ook meer SIgA in het bloed aanwezig is. Verder laten we zien dat SIgA in staat is aan mesangiumcellen te binden en er dan voor te zorgen dat er IL-6 door de mesangiumcellen wordt geproduceerd. Uiteindelijk laten we zien dat er een accumulatie van SIgA in de nier is en dit wordt bevestigd met een kleuring van SIgA in nierweefsel. Alles bij elkaar suggereren bovenstaande data dat SIgA een rol speelt in de pathogenese bij een subpopulatie van de IgAN patiënten. Verder onderzoek is nodig om de rol van SIgA in IgAN te bevestigen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 21 juli 1979 geboren in Purmerend. In 1997 behaalde zij het VWO diploma aan het Jan van Egmond College te Purmerend. Aansluitend begon zij aan de studie medische biologie aan de Vrije Universiteit (VU) te Amsterdam. De wetenschappelijke stages werden gelopen op de afdeling Medische Chemie van de Vrije Universiteit (dr I.M. van Die) en de afdeling Parasitologie van het Leids Universitair Medisch Centrum (LUMC) (dr. A. van Remoortere). Na het behalen van het doctoraal examen in 2002, trad zij in dienst bij de afdeling Nierziekten van het LUMC als assistent-in-opleiding. Hier werkte zij onder begeleiding van dr C. van Kooten en Prof. dr. M.R. Daha aan het in dit proefschrift beschreven onderzoek. Vanaf oktober 2006 is zij werkzaam als post-doc bij de afdeling Klinische Chemie en Hematologie van het Universitair Medisch Centrum Utrecht (dr. P.J. Lenting).

PUBLICATIONS

P.J.M. van der Boog, C. van Kooten, G. van Zandbergen, N. Klar-Mohamad, B.D. Oortwijn, N.A. Bos, A. van Remoortere, C.H. Hokke, J.W. de Fijter, M.R. Daha. Injection of recombinant FcαRI/CD89 in mice does not induce mesangial IgA deposition. 2004. *Nephrology Dialysis Transplantation 19:2729-36.*

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B.D. Oortwijn, A. Roos, D.J. van Gijlswijk-Janssen, M.C. Faber-Krol, J.W. Eijgenraam, M.R. Daha, C van Kooten. Differential glycosylation of polymeric and monomeric IgA: a possible role in glomerular inflammation in IgA nephropathy. *Journal of the American Society of Nephrology in press*

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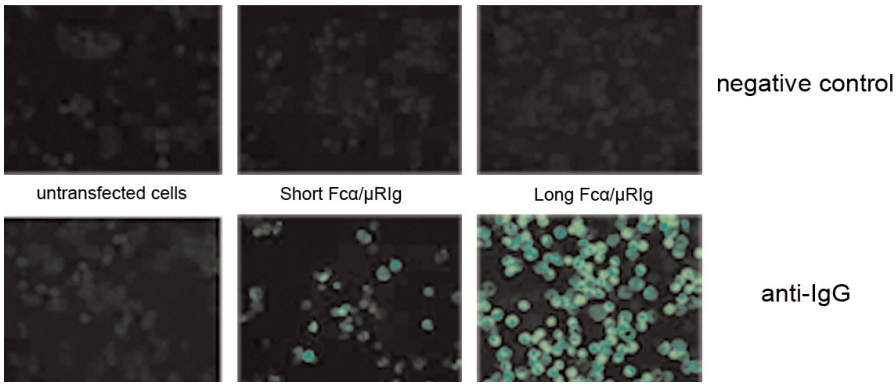
COLOR FIGURES



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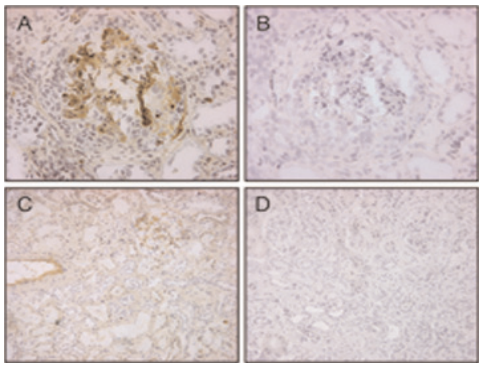

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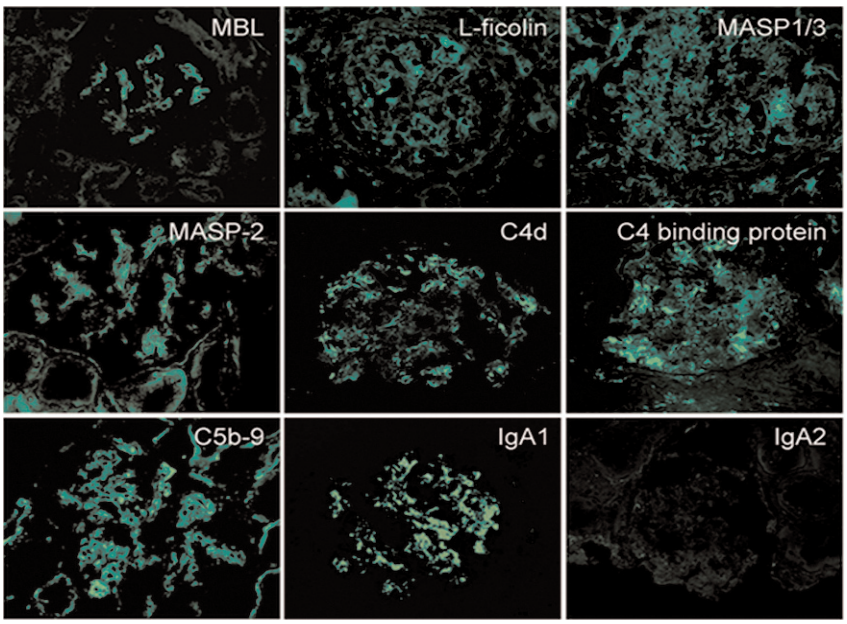

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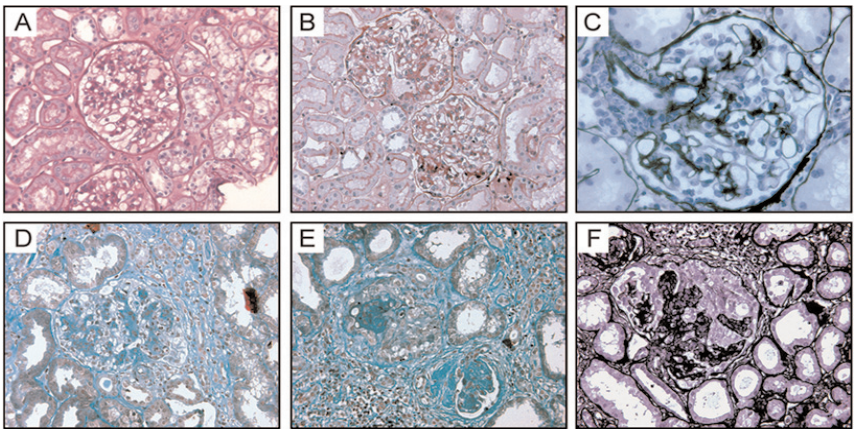

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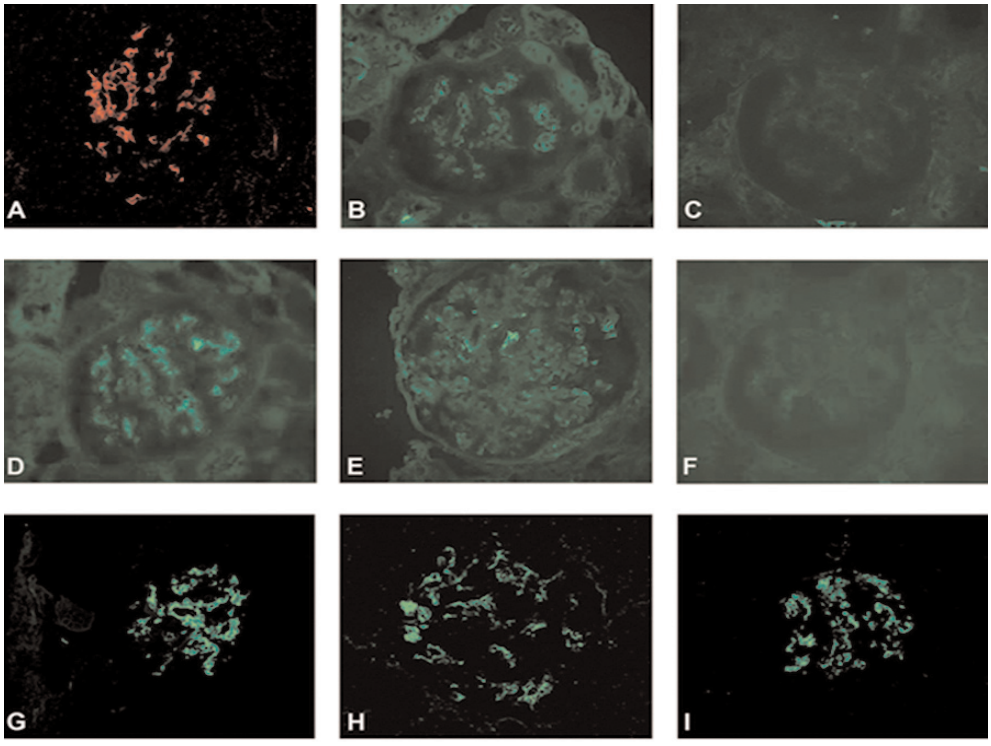

Chapter 7 Figure 1 page 113

Chapter 3, Figure 1B, page 46

Fcα/μRIg fusion proteins.
B) IgG staining of transfected cells after cytospin centrifugation.

Chapter 4, Figure 1, page 60

Glomerular MBL deposition in IgAN patients.
Biopsies from patients with IgAN were stained with mAb 1C10 (A), mAb 3E7 (C, D) or an isotype control mAb (B). Figures A, C, and D are derived from different patients who showed positive (A, C) or negative (D) staining for MBL. Figures A and B are from the same patient. Please note tubular and vascular staining for MBL in C, in addition to glomerular staining.

Chapter 4, Figure 2, page 61

Lectin pathway activation in IgAN.
Renal tissue from IgAN patients was stained for the presence of MBL (mAb 3E7), L-ficolin (mAb GN4), MASP-1/3 (mAb 1E2), MASP-2, C4d, C4 binding protein, C5b-9, IgA1 and IgA2 (mAb NI512), as indicated. Representative images are shown.

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Renal histological damage in IgAN patients.
Renal histology from three patients without glomerular MBL deposition (A-C) shows glomeruli affected by mild mesangial proliferation and mild mesangial matrix expansion. No major tubulo-interstitial lesions are visible. Images D-F represent patients with glomerular MBL deposition (N = 3). Glomerular injury is characterized by intense mesangial proliferation and mesangial matrix expansion (D), segmental and global sclerosis (E), and extracapillary proliferation (F), whereas tubular dilations, interstitial infiltration and fibrosis are evident in the interstitium (D-F). Sections were stained with PAS (A, B), PASM (C, F) or trichrome (D, E) techniques.

Chapter 7, Figure 1, page 113

Glomerular SIgA deposition in IgAN patients.
Renal tissue from patients with IgAN was stained for the presence of SIgA (A, B and C), MBL (D, E and F), IgA (G), C4d (H) and C3 (I). Representative images are shown. Renal tissues are derived from different patients who showed positive (A and B) and negative staining (C) for SIgA and positive (D and E) and negative staining (F) for MBL. All patients were positive for IgA (G). A subpopulation of patients was positive for C4d (H) and C3 (I).