Integrin $\alpha_1/\beta_1$ and $\alpha_2/\beta_1$ as a receptor for IgA1 in human glomerular mesangial cells in IgA nephropathy

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Received 14 May 2011, accepted 26 December 2011

Abstract

IgA nephropathy (IgAN) is characterized by mesangial deposition of IgA1 and galactose-deficient IgA1 is expected to play a pathogenic role. However, the identity of the receptor for IgA1 is still controversial. Hence, the aim of this study was to explore the receptor for galactose-deficient IgA1. Human monoclonal IgA1 was treated with exoglycosidase and FITC-conjugated control, asialo- and agalactosyl-IgA1 was used as a probe to detect the receptor in cultured human mesangial cells. Tumor necrosis factor-\(\alpha\) or transforming growth factor-\(\beta\)1 treatment accelerated IgA1-binding on mesangial cells, and these effects were diminished by the addition of dexamethasone, whereas these changes were not dependent on galactose-deficiency of IgA1. According to comprehensive gene expression analysis, we focused on integrin $\beta_1$. Pre-treatment by Mn$^{2+}$, which activates integrin by changing its structure, enhanced the binding of IgA1 in cultured mesangial cells. Furthermore, pre-incubation with collagens specifically enhanced binding of IgA1 in the cultured human mesangial cells without activation by Mn$^{2+}$. Collagen type IV distributed in the mesangial region of the glomeruli as well as Bowman’s capsule and tubular basal membrane in IgAN patients, and the IgA1 with collagen type IV induced proliferative signals on mesangial cells by phosphorylating extracellular signal-regulated kinase more effectively than the IgA1 alone. Immunoprecipitation assay revealed the binding of IgA1 and integrin $\alpha_1/\beta_1$ and $\alpha_2/\beta_1$ heterodimer and down-regulation of integrin $\alpha_1$, $\alpha_2$ and $\beta_1$ expression in human mesangial cells induced by each specific small interfering RNA diminished the ability to bind IgA1 probe. Integrin $\alpha_1/\beta_1$ and $\alpha_2/\beta_1$ would be a candidate receptor for IgA1.

Keywords: collagen, dexamethasone, O-glycan, transforming growth factor, tumor necrosis factor

Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis and is one of the main causes of end-stage renal disease throughout the world (1, 2). Typical features of IgAN consist of glomerular mesangial IgA deposition associated with focal or diffuse expansion of mesangial regions with cells and matrix and persistent hematuria and mild proteinuria over many years; furthermore, 20–30% of patients develop progressive renal insufficiency ≥20 years after initial discovery of the disease (1, 3). Several mechanisms of IgA deposition on mesangial cells have been proposed, including the formation of IgA-immunogen complexes derived from environmental or infectious antigens (4), or as the result of IgA-IgG complex formation (5, 6), IgA1-soluble Fc\(\alpha\) receptor (CD89) complex (7), or IgA self-aggregation (8, 9). Corticosteroids and renin-angiotensin system-blocking agents are used for the treatment of IgAN (10); however, no specific and fundamental therapy has yet been proposed since the precise mechanism of the development of IgAN is still to be elucidated (11).

Several recent studies reported the importance of aberrant O-linked glycosylation in the IgA1 hinge region for the pathogenesis of IgAN (12). Of the two subtypes of IgA, IgA1 and IgA2, the deposits in the glomerular mesangial region are considered to be composed of polymeric IgA1 (13, 14). IgA1 is characterized and distinguished from IgA2 by its proline-rich hinge region, which consists of 23 amino acids and 9 potential O-glycation sites possessing 4 or 6 O-glycans (15–17). These O-glycans starting from serine or threonine residues (17, 18) are considered to play a protective role against IgA1 self-aggregation and adhesion to
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extracellular matrix proteins (19). Recent studies demonstrated that reduced galactosylation of O-glycans in the IgA1 hinge region was observed both in the serum and in the glomeruli of IgAN patients (20-22). Galactose-deficient IgA1 exhibits enhanced binding capacity to human mesangial cells (23) as the result of accelerated self-aggregation or increased binding capacity to extracellular matrices (19), and thus, galactose-deficient IgA1 is expected to play a pathogenic role in the development of IgAN (24).

On the other hand, the identity of the receptor for IgA1 in mesangial cells is still controversial. Three known IgA receptors, CD89, polymeric Ig receptor (pIgR) and asialoglycoprotein receptor (ASGP-R), have been proposed as candidates for IgA1 (25). However, pIgR is localized primarily in human secretory epithelia (26), and its expression has not been confirmed in human mesangial cells (27). ASGP-R is a C-type lectin and binds desialylated glycoproteins. Some groups have reported on the existence of ASGP-R in human mesangial cells (28, 29), but others could not identify it (4, 27). In human mesangial cells, the expression of CD89, which is mainly expressed in blood cells and binds to the Fc region of monomeric and polymeric IgA, is also still controversial. Several groups detected CD89 in human mesangial cells (30, 31), while others failed to detect its expression (4, 27, 28). Fc α/β receptor was proposed as a candidate IgA receptor (33), but its pathogenic role is still to be determined. Transferrin receptor was originally identified from the lysate of monocytemesangial cell line as a monomeric IgA1 receptor (34) and then polymeric IgA with galactose deficiency was found to interact with transferrin receptor more efficiently (35). Polymeric IgA1 was reported to induce up-regulation of transferrin receptor expression, resulting in activation of human mesangial cells (36). Moura et al. reported that IgA-transferrin receptor interaction in cultured human mesangial cells was inhibited by monoclonal anti-transferrin receptor antibody or transferrin itself by ~70% (34, 36) or with soluble transferrin receptor by ~50% (35, 36). These data suggest that human mesangial cells could still possess alternative galactose-deficient IgA1 receptors besides transferrin receptor. Hence, the aim of the present study is to identify the receptor for galactose-deficient IgA1 in mesangial cells using IgA1 probe with galactose-deficient O-glycans in its hinge region.

Methods

Study design

The protocol of the present study adhered to the Declaration of Helsinki and was approved by the ethics committee of our institute. Written informed consent for using kidney biopsy specimens was obtained from all the participants.

Cells and reagents

Normal human mesangial cells were purchased from DS Pharma Biomedical (Osaka, Japan). Mouse monoclonal anti-human integrin β1 (clone HUTS-21 for flow cytometry and MAR4 for immunohistochemistry), anti-human integrin α1 (SR84), α2 (12F1), α3 (C3 II.1), α5 (IIA1), αv (13C2), anti-human transferrin receptor (M-A712) and anti-human CD36 (CB38) antibodies as well as mouse IgG1 and IgG2a isotype controls were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA) and used for immunohistochemistry. For flow cytometry, each mAb with PE conjugation was used. Collagen types I, III, IV and V, laminin and fibronectin extracted from human placenta were purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse monoclonal anti-collagen type I (COL-1), type III (FH-7A), type IV (COL-94), laminin (LAM-89) and fibronectin (IST-4) antibodies (Sigma–Aldrich) were used for immunohistochemistry.

FITC-conjugated galactose-deficient IgA

One milligram of monoclonal human IgAκ derived from myeloma cells (Acris Antibodies, Herford, Germany) was incubated with 0.25 U of neuraminidase with or without 0.1 U of β-galactosidase (Sigma–Aldrich) in 0.05 M acetic acid, pH 5.0, for 72 h at 37°C. In vitro desialylation and degalactosylation were confirmed by lectin ELISA using biotinylated Jacalin lectin, Sambucus Nigra lectin, Peanut Agglutinin lectin and Vicia Villosa lectin (Vector Laboratories, Burlingame, CA, USA) as described (23). FITC was conjugated using FITC conjugation kit according to the manufacturer’s instructions (Sigma–Aldrich) with monoclonal IgA1 or polyclonal IgA1 purified from the sera of IgAN patients by agarose-bound Jacalin (Vector laboratories) and used as a probe for receptor in cultured mesangial cells.

Cell culture

Human mesangial cells characterized by α-smooth muscle actin (+), E-selectin (−), CD 14 (−), CD 16 (−), CD 62E (−) and podocalyxin (−) were cultured in CS-C complete medium (DS Pharma Biomedical) in 5% CO2 incubator at 37°C. For in vitro cytokine stimulation, 100 ng ml−1 recombinant human tumor necrosis factor (TNF)-α or transforming growth factor (TGF)-β1 (R & D Systems, Minneapolis, MN, USA) with or without 200 ng ml−1 dexamethasone (Sigma–Aldrich) was added in the culture medium and incubated for 72 h. Comprehensive gene expression analysis of cultured human mesangial cells treated with TNF-α, TGF-β1, TNF-α with dexamethasone, TGF-β1 with dexamethasone and control cells was conducted using GeneChip Human Genome U133 Plus 2.0 (Affimetrix, Santa Clara, CA, USA). Among the 9094 genes, 1455 genes up-regulated by TNF-α and TGF-β1 and down-regulated by addition of dexamethasone and in control condition were selected by template matching method with Pearson correlation coefficient of ≥0.6.

Flow cytometry analysis

To detect the binding of probe IgA1, cultured human mesangial cells were incubated with FITC-conjugated galactose-deficient IgA1 (100 μg ml−1) for 30 min on ice in PBS with 3% BSA and 0.02% NaN3 and then analyzed by FACSCalibur (Becton, Dickinson and Company). Manganese chloride, calcium chloride or magnesium chloride was added to the cells at each concentration 30 min before
incubation with FITC-conjugated probe IgA. Collagen, laminin and fibronectin were incubated with FITC-conjugated probe IgA for 30 min on ice in advance and used for flow cytometric analysis.

Immunohistochemistry of kidney biopsy specimen
For the detection of each integrin or extracellular matrix, 4 μm of frozen section of kidney biopsy specimen was fixed in cold methanol. After incubation with 5% goat serum for blocking of non-specific reaction, mouse mAb specific for each integrin or extracellular matrix was incubated on the specimen for 90 min at room temperature, followed by incubation with tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG polyclonal antibody or FITC-conjugated goat anti-mouse IgM polyclonal antibody (Sigma–Aldrich). Deposited IgA was based on kidney biopsy that revealed the presence of dominant glomerular mesangial deposits of IgA as assessed by immunofluorescence examination. Biopsy specimens of the patients with systemic disease that would cause IgA deposition in glomeruli, such as hepatic glomerulonephritis, Henoch-Schönlein purpura, carcinoma or rheumatoid arthritis, were excluded.

Western blotting
Cultured human mesangial cells (3 × 10^5) were incubated with galactose-deficient IgA1 (100 μg ml^{-1}) or galactose-deficient IgA1 pre-incubated with type IV collagen (25 μg ml^{-1}) at 37°C for the indicated time and were lysed and electrophoresed in polyacrylamide gel in a reduced condition. The cell lysate was then transferred to polyvinylidene difluoride membrane, blocked with blocking reagent, reacted with peroxidase-conjugated goat anti-biotin antibody (Sigma–Aldrich) for 2 h, incubated with tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG1 (Sigma–Aldrich) and reacted with peroxidase-conjugated goat anti-biotin antibody (Sigma–Aldrich) for detection of co-precipitated biotinylated IgA1.

Statistical analysis
Statistical differences were calculated with one-way analysis of variance followed by Fisher’s protected least significant difference. P < 0.05 was considered significant.

Results

Desialylation and degalactosylation of IgA1 enhanced binding affinity to cultured human mesangial cells
Human monoclonal IgA1 κ generated from myeloma cells was treated with neuraminidase with or without β-galactosidase in order to investigate the role of terminal N-acetyleneuraminic acid or galactose of O-glycans in the hinge region of IgA1 (Fig. 1A). In vitro desialylation and degalactosylation were confirmed by lectin ELISA using biotinylated Jacalin, Sambucus nigra lectin, Peanut agglutinin and Vicia villosa lectin, which specifically recognize N-acetylgalactosamine, terminal sialic acid, terminal galactose and exposed N-acetylgalactosamine, respectively, and then control IgA, desialylated (deSia) IgA and desialylated and degalactosylated (deSia/deGal) IgA was conjugated with FITC to be used as a probe for the receptor in mesangial cells. In vitro desialylation alone produced significant amounts of O-glycans with exposed N-acetylgalactosamine (Fig. 1B) and also enhanced binding activity of IgA1 in mesangial cells (Fig. 1C and D). No substantial increase in IgA1-binding capacity was observed by additional degalactosylation of IgA1.

TNF-α and TGF-β1 up-regulated IgA1 receptors, whereas additional dexamethasone quenched their effect
To reproduce the inflammatory circumstances in the patients with IgAN, the mesangial cells were cultured with or without TNF-α or TGF-β1, and dexamethasone was added in the same culture medium to investigate the effect of corticosteroid, which was commonly employed as a therapy for IgAN. FITC-conjugated deSia/deGal IgA1 probe bound ~4.5% of the cultured human mesangial cells. TNF-α or TGF-β1 treatment increased the expression of the receptor for deSia/deGal IgA1, and more interestingly, these effects were clearly diminished by addition of dexamethasone in the same culture medium with TNF-α or TGF-β1 (Figs. 2A and B). To verify the universality of the regulatory effect of TNF-α
Fig. 1. Desialylated or desialylated and degalactosylated IgA1 showed enhanced binding activity in cultured human mesangial cells. (A) O-glycans in the hinge region of human IgA1 consist of six different kinds of structures. NANA, N-acetylneuraminic acid; Gal, galactose; GalNAc, N-acetylgalactosamine. (B) Control IgA1, desialylated (deSial) IgA1 or desialylated and degalactosylated (deSial/deGal) IgA1 were characterized for terminal sialic acid (Sambucus Nigra), galactose (Peanut agglutinin) or exposed N-acetylgalactosamine content (Vicia Villosa) by lectin ELISA. Jacalin, which has affinity toward N-acetylgalactosamine, was employed as a quantitative control. Cut-off value is shown as a dotted line determined as the value of absorbance without IgA1 samples. (C) FITC-conjugated deSial or deSial/deGal IgA1 probe bound cultured human mesangial cells more efficiently than control IgA1 probe. (D) Desialylation alone caused statistically significant increase of the number of IgA1-binding cultured mesangial cells; however, additional degalactosylation caused no increased binding capacity (n = 3). These are the representative of three independent experiments. †P < 0.05, *P < 0.01, **P < 0.001, ‡not significant.
or TGF-β1 and dexamethasone on the binding of IgA1 in mesangial cells, polyclonal IgA1 was purified from the sera of IgAN patients and also used as the probe after FITC conjugation. This polyclonal IgA1 probe also detected the same regulatory effects of the receptor expression (Fig. 2B). Furthermore, control IgA1, deSial IgA1 and deSial/deGal IgA1 probes were also tested to the mesangial cells with each treatment, and again, although regulatory effect of TNF-α or TGF-β1 and dexamethasone were observed in the same way, deSial IgA1 and deSial/deGal IgA1 demonstrated the same level of binding capacity in mesangial cells in each treatment (Fig. 2C).

Integrin β1 as a candidate receptor for galactose-deficient IgA1

According to these data, we conjectured that these two different types of cytokines, TNF-α and TGF-β1, would commonly induce galactose-deficient IgA1 receptor gene expression and additional dexamethasone would suppress it. Following this hypothesis, we extracted total RNA from the mesangial cells treated with TNF-α, TGF-β1, TNF-α with dexamethasone, TGF-β1 with dexamethasone or without any treatment as a control to conduct comprehensive gene expression analysis using DNA array (Table 1). Among the candidate genes of the IgA1 receptor, we focused on integrin β1, whose mRNA expression was up-regulated by both TNF-α and TGF-β1 and down-regulated by additional dexamethasone treatment. Integrin β1 forms a heterodimer with an integrin α subunit (37). Among the variety of integrin α subunits capable of forming a heterodimer with β1 subunit, α1, α2, α3, α5, α8 and αv were reported to be expressed on human mesangial cells (38–40). We examined the cell surface expression of integrin β1 by flow cytometry to confirm whether this molecule was actually regulated by TNF-α, TGF-β1 and dexamethasone treatment. Figure 2(D) shows, in accordance with the results of DNA array analysis, the cell surface expression of integrin β1 was induced in the cultured mesangial cells stimulated with TNF-α or TGF-β1 and additional dexamethasone down-regulated its expression.

Mn2+ activated IgA1 receptor

Integrin α and β subunits have Mg2+- and Ca2+-binding sites, and an integrin heterodimer changes its structure from ‘bent...
**Table 1.** Gene expression analysis in cultured human mesangial cells regulated by TGF-β1, TNF-α and dexamethasone

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>TGF-β1</th>
<th>TGF-β1 + dex</th>
<th>TNF-α</th>
<th>TNF-α + dex</th>
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<tr>
<td>C1q and TNF-related protein 2</td>
<td>122.6</td>
<td>248.8</td>
<td>44.6</td>
<td>444.4</td>
<td>19.3</td>
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<tr>
<td>Cadherin 11, type 2</td>
<td>322.2</td>
<td>962.2</td>
<td>544.9</td>
<td>600.4</td>
<td>291.1</td>
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<tr>
<td>CD274</td>
<td>2174.9</td>
<td>3679.8</td>
<td>1503.9</td>
<td>6263.2</td>
<td>2693.5</td>
</tr>
<tr>
<td>Filamin A interacting protein 1-like</td>
<td>2009.6</td>
<td>2923.0</td>
<td>1397.7</td>
<td>2264.0</td>
<td>1107.7</td>
</tr>
<tr>
<td>Glutamate receptor interacting protein 1</td>
<td>69.5</td>
<td>153.5</td>
<td>52.8</td>
<td>134.6</td>
<td>11.1</td>
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<tr>
<td>Integrin β1</td>
<td>134.1</td>
<td>220.2</td>
<td>58.8</td>
<td>143.9</td>
<td>23.0</td>
</tr>
<tr>
<td>IL 1 receptor antagonist</td>
<td>226.5</td>
<td>400.1</td>
<td>116.1</td>
<td>799.2</td>
<td>174.6</td>
</tr>
<tr>
<td>LDL receptor-related protein 12</td>
<td>943.2</td>
<td>1062.6</td>
<td>891.6</td>
<td>1173.3</td>
<td>582.7</td>
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<tr>
<td>Myosin X</td>
<td>274.8</td>
<td>570.1</td>
<td>435.6</td>
<td>510.3</td>
<td>319.6</td>
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<tr>
<td>Protocadherin 10</td>
<td>90.5</td>
<td>238.1</td>
<td>137.1</td>
<td>164.4</td>
<td>93.9</td>
</tr>
<tr>
<td>Tensin 1</td>
<td>101.1</td>
<td>227.5</td>
<td>181.2</td>
<td>216.7</td>
<td>111.0</td>
</tr>
</tbody>
</table>

Signal strength of several typical genes up-regulated by TGF-β1 and TNF-α and down-regulated by dexamethasone treatment were shown in alphabetical order. TNF; tumor necrosis factor, LDL; low-density lipoprotein.

form’ to ‘extended form’ to acquire an activated state in the presence of Mn$^{2+}$ (41). To confirm the contribution of integrins to IgA1 binding, cultured human mesangial cells were treated with Mn$^{2+}$ for integrin activation and Ca$^{2+}$ or Mg$^{2+}$ for inactivation before incubation with FITC-conjugated deSial/deGal IgA1 probe. Figure 3 showed that Mn$^{2+}$ dramatically activated the receptor for IgA1 in a dose-dependent manner, whereas Ca$^{2+}$ or Mg$^{2+}$ induced only slight changes.

**Distribution of integrin α subunits in the glomeruli of IgAN patients**

To identify the α subunit involved in IgA1 binding, α1, α2, α3, α5 and αv expressions on kidney biopsy specimens of IgAN patients were examined by immunohistochemical study and their localizations were compared with the distribution of IgA1 deposition. The integrin α1 and α3 expression was observed globally in mesangial cells and all tubule epithelial cells. The integrin α2 subunit also existed globally in mesangial cells and the part of the tubule epithelial cells, and the distribution of α1, α2 or α3 was closely overlapped that of IgA1 deposition. Expression of the integrin α5 was absent both in the glomeruli and in the tubule epithelial cells, and the integrin αv subunit was weakly and segmentally expressed in the mesangial region alone (Fig. 4).

deSial/deGal IgA1–collagen complex facilitated IgA1 deposition and proliferative response in cultured human mesangial cells

Considering the close interaction between the integrin heterodimers and the extracellular matrices, we speculated that IgA1 might be able to deposit as a complex with extracellular matrix. To examine the contribution of the extracellular matrices in IgA1–integrin interaction, several types of collagen, laminin and fibronectin were pre-incubated with FITC-conjugated deSial/deGal IgA1 probe and then applied on cultured mesangial cells. Surprisingly, pre-incubation of deSial/deGal IgA1 with collagen types I, III, IV and V enhanced the binding of IgA1 in cultured mesangial cells dose dependently without any activation by Mn$^{2+}$. On the other hand, the enhancing effect of fibronectin, a ligand of integrin α5/β1, α8/β1 or αv/β1 heterodimer, and laminin, a ligand of integrin α3/β1 heterodimer (42), was minimal (Fig. 5). Following these results, we examined the distributions of each extracellular matrix in the glomeruli of kidney biopsy specimens of IgAN patients. In accordance with previous reports (39, 43, 44), collagen type IV and fibronectin were detected in the mesangial region, as well as in capillary walls, Bowman’s capsule and basal membrane of tubular cells, and the localization of these two extracellular matrices overlapped the distribution of IgA deposits in the glomerular region. On the other hand, collagen type I, type III and laminin were absent in the mesangial region and were observed in Bowman’s capsule and basal membrane of tubular cells (Fig. 6). To clarify the biological effect of IgA1–collagen type IV complex in mesangial cells, deSial/deGal IgA1 pre-incubated with collagen type IV or deSial/deGal IgA1 alone was added in cultured mesangial cells, and ERK phosphorylation was examined by western blotting. Figure 7 indicates that deSial/deGal IgA1 pre-incubated with collagen type IV induced more prominent phosphorylation of ERK in cultured human mesangial cells than deSial/deGal IgA1 alone did.

**Direct interaction between IgA1 and integrin α1, α2/β1 heterodimers**

Considering the results that IgA1 bound mesangial cells all by itself and IgA1 pre-incubated with collagen facilitated both binding affinity and proliferative signals, we speculated that receptor for IgA1 would be an integrin heterodimer which possesses binding affinity with type IV collagen, namely, integrin α1/β1 or α2/β1. To confirm the direct interaction between IgA1 and integrin α1/β1 or α2/β1 heterodimer, cultured mesangial cell lysate was incubated with biotinylated deSial/deGal IgA1 and then integrin α1/β1 or α2/β1–IgA1 complex was immunoprecipitated with anti-integrin α1-, α2- and β1-specific antibodies, respectively. Figure 8A demonstrates that biotinylated IgA1 was co-precipitated as a complex with integrin heterodimer. In the cultured human mesangial cells, the integrin α1 expression was also enhanced by TNF-α or TGF-β1 treatment, whereas the integrin α2 expression was down-regulated by dexamethasone (Figure 8B). Finally, to verify the interaction between IgA1 and integrin α1/β1 or α2/β1 heterodimer in
Fig. 3. Manganese ion enhanced IgA1 binding in mesangial cells. (A) Cultured mesangial cells were incubated with the indicated concentrations of Mn²⁺, Ca²⁺ or Mg²⁺ for 30 min on ice prior to exposure to FITC-conjugated galactose-deficient IgA1 probe. (B) Histogram represents each fluorescence peak of FITC-conjugated IgA1 probe bound with cultured human mesangial cells after pre-incubation with indicated concentrations of Mn²⁺, Ca²⁺ or Mg²⁺. Fluorescence peaks of the histogram were positively shifted dose dependently by Mn²⁺ (upper) but not by Ca²⁺ (middle) or Mg²⁺ (lower). Shaded histogram represents negative control (FITC-IgA1 not added). These are the representative of three independent experiments.
Expression of integrin β and α subunits in mesangial cells. Frozen kidney biopsy specimens from IgAN patient were reacted with mAb specific for each integrin α or β subunit (red) to examine its localization in glomerulus and tubular cells and for human IgA (green) to compare the distribution of IgA deposit with that of integrin subunits in the mesangial region (×100 magnification for the left column and ×200 magnification for the rest). These are the representative of five independent experiments.
mesangial cells, siRNA specific for integrin α1, α2, β1 or transferrin receptor gene had been administered to cultured human mesangial cells. Down-regulative effect of cell surface expression was in accordance with the specificity of administered siRNA, and siRNA specific for integrin β1 induced down-regulation of integrin α1, α2 and α3 simultaneously. Cell surface expression of transferrin receptor was not affected by siRNA specific for integrin α1 and α2, although integrin β1-specific siRNA rather up-regulated transferrin receptor expression in cell surface (Supplementary Figure 1, available at International Immunology Online). Binding ability with deSial/deGal IgA1 was significantly decreased by down-regulation of integrin α1, α2 and β1 but not by that of transferrin receptor (Fig. 8C). These specific losses in the ability to bind deSial/deGal IgA1 were also demonstrated after pre-treatment with Mn2+ (Fig. 8D); however, pretreatment with collagen type IV diminished the losses of binding ability, presumably due to far higher affinity of IgA1–collagen complex compared with IgA1 by itself (Fig. 8E). According to these findings, we conclude that integrin α1/β1 and α2/β1 heterodimers are involved in IgA1 deposition in mesangial cells.

**Discussion**

We have identified integrin α1/β1 and α2/β1 heterodimer as a new candidate IgA1 receptor in human mesangial cells and have shown the possibility of its involvement in the development of IgAN through the induction of proliferative stimulation as a complex with collagen in mesangial cells.

Integrins are cell adhesion molecules that mediate cell–cell, cell–extracellular matrix and cell–pathogen interactions with non-covalently associated α and β subunits (37, 45). They function as signal transmitters through a variety of signaling pathways including phosphatidylinositol 3-kinase-Akt pathway, mitogen-activated protein kinase/ERK pathway or Rho family GTPase pathway to modulate many aspects of cell behavior such as proliferation, survival/apoptosis, cytoskeletal organization, gene expression and differentiation (42). In the glomerulus, among the 19 different integrin α subunits and 8 different integrin β subunits forming at least 25 kinds of αβ heterodimers (41), integrin β1 is the most prominent and widely distributed integrin subunit detected in mesangial, endothelial and epithelial cells, especially in areas where these cells attach to glomerular basement membrane or extracellular matrix in normal and diseased kidney (46–48). The α1 subunit is expressed widely in both mesangial cells and endothelial cells, whereas the α2 subunit is restricted in mesangial cells and distal tubular cells, and both α1/β1 and α2/β1 heterodimers serve as collagen receptors (42). The α3 subunit is abundantly distributed in mesangial, endothelial and epithelial cells and α3/β1 heterodimer functions as a laminin receptor (42). The α5 subunit localizes in blood vessels, and some investigators report its distribution in mesangial cells, whereas αv subunit is detected in mesangial cells and tubular cells (38–40, 47), and the α2 subunit is exclusively expressed in mesangial cells (40). The α5/β1, αv/β1 and α8/β1 heterodimers are receptors for extracellular matrices that contain RGD sequences such as fibronectin (42). We examined the localization of these α subunits in human glomerulus with monoclonal antibodies, with the exception of α8, and our data were in accordance with previously reported findings except for α5 (Fig. 4).

Integrin heterodimers are activated in the presence of Mn2+ (41). Our data in Fig. 3 indicate the possibility that IgA1 receptors in cultured human mesangial cells are...
usually in an inactivated state and are activated in the presence of Mn$^{2+}$. While the activation by Mn$^{2+}$ strongly supports the involvement of integrin heterodimers in IgA1 binding, these findings could also explain why only several percent of cultured mesangial cells could capture IgA1 probe even though each mesangial cell expressed integrin $\alpha_1$, $\alpha_2$, and $\beta$1 subunits on its cell surface. We speculate that this is because integrin heterodimers in the cultured mesangial cells are normally in an inactivated state and the inactivated integrin $\alpha_1/\beta$1 or $\alpha_2/\beta$1 heterodimer could possess very weak affinity toward IgA1.

More interestingly, IgA1 bound mesangial cells much efficiently by pre-incubation with various types of collagens. Even though co-localization of collagen type IV and IgA deposits in the mesangial region of kidney biopsy specimens from IgAN patients did not necessarily provide us with direct evidence to prove that IgA was deposited as a complex with collagen type IV, considering the increased tendency of galactose-deficient IgA1 to adhere the extracellular matrices (19, 49, 50), complex formation would have increased the affinity to the integrin $\alpha_1/\beta$1 and $\alpha_2/\beta$1 heterodimer resulting in facilitation of the binding in

Fig. 6. Distribution of extracellular matrices in the kidney of IgAN patients. Frozen kidney biopsy specimens of IgAN patient were reacted with mAb specific for each extracellular matrix (red) to examine its distribution in glomerulus and tubulointerstitial regions and for human IgA (green) to compare the distribution of IgA deposit with that of extracellular matrices in the mesangial region ($\times$100 magnification for the left column and $\times$200 magnification for the rest). These are the representative of five independent experiments.
mesangial cells even in its inactivated state. We also speculated that this collagen–IgA complex would have possessed far higher affinity toward integrin α1/β1 and α2/β1 heterodimers than IgA1 alone and would have overcome the down-regulation of these heterodimers induced by siRNA treatment (Fig. 8E). Similarly, proliferative stimulation would be induced more efficiently by IgA1–collagen type IV complex than by IgA1 alone, probably due to originally lower affinity of IgA1 to the integrin α1/β1 or α2/β1 heterodimer compared with that of IgA1–collagen complexes, even though the existence of other different and specific receptor for IgA1–collagen complex has not been completely excluded. Alternative collagen receptor, CD36, was absent in both native mesangial cells in IgAN patients and cultured mesangial cells (Supplementary Figure 2, available at International Immunology Online).

Collagen type IV is a constituent of mesangial matrix and is reported to be increased in amount in various forms of glomerular injury (51–53), and its production is induced by TGF-β or platelet-derived growth factor (PDGF) in diseased kidney (54–57). We are tempted to conjecture that IgA1 generated in an IgAN patient might filter into the mesangial region to form complexes in situ with collagen type IV produced in the glomerulus under TGF-β or PDGF stimulation, otherwise circulating IgA1-collagen complexes, if present, could bind to any cells that express integrin α1/β1 or α2/β1 heterodimers. And, thus, IgA1–collagen type IV complexes generated in situ or polymeric IgA1 might be deposited in mesangial cells via integrin α1/β1 heterodimers possibly up-regulated by pro-inflammatory cytokines, such as TNF-α, or by growth factors such as TGF-β as well as via integrin α2/β1 heterodimers. Enhanced binding affinity of IgA in the sera of IgAN patients toward collagen type IV (58) would strongly support this conjecture. We also speculate that steroid therapy might directly reduce the expression of integrin α2/β1 heterodimers in mesangial cells as well as the inhibition of TGF-β1 production (59), resulting in a curative effect in IgAN patients.

IgA–fibronectin complex has also been proposed by several investigators as playing a role in the pathogenesis of IgAN on the basis of the elevated serum level of circulating IgA–fibronectin complexes (60) or the findings for a mouse model of IgAN in which IgA–fibronectin complex formation was not prevented by genetically lacked uteroglobin through interaction with the heparin binding site of fibronectin (61) resulting in the glomerular mesangial deposition of IgA along with fibronectin and collagen type IV (57, 62). However, serum uteroglobin levels incorporated in IgA–fibronectin complexes were rather elevated in the patients with IgAN (58), and IgA–fibronectin complexes themselves were also increased in patients with Henoch-Schönlein

Fig. 7. deSial/deGal IgA1-collagen type IV complex-induced proliferative signals more prominently in mesangial cells. (A) deSial/deGal IgA1 or a mixture of deSial/deGal IgA1 and collagen type IV was co-incubated with cultured mesangial cells for the indicated times, and the phosphorylation of ERK (P-ERK) was determined by western blotting. (B) Relative density of P-ERK1 and P-ERK2 band against ERK1 and ERK2 band was quantified by densitometry in each condition (IgA, white and IgA with collagen type IV, black) and compared with the relative density at 0 min as 1.0 (striped). These are the representative of three independent experiments.
purpura without renal involvement and cirrhotic patients without urinary abnormalities (63). Thus, the role of IgA–fibronectin complex in the pathogenesis of IgAN is still controversial.

In the present study, we employed deSial/deGal IgA1 probe for the exploration of IgA1 receptor in mesangial cells except for Figs. 1 and 2. Absence of galactose in the hinge region of IgA1 was reported to be crucial for the development of IgA–IgG complex formation (5, 6) or IgA self-aggregation (8, 9, 19), but in this in vitro experimental conditions, desialylation alone could enhance binding affinity to mesangial cells and additional degalactosylation did not trigger extra binding affinity. We would rather conjecture that integrin α1/β1 and α2/β1 heterodimers are not specific receptors limited to deSial/deGal IgA1 but for IgA1 with variety of O-glycans in its hinge region, and binding affinity would be different according to the different structure of O-glycans. These subjects are still to be clarified in further investigations; however, this study provides us with a new candidate of IgA1 receptor in mesangial cells and proposes an innovative mechanism of IgA1 deposition in the pathogenesis of IgAN. We believe that these findings will lead us to a new fundamental therapeutic strategy against this disease.

Supplementary data
Supplementary data are available at International Immunology Online.

Funding
This work was supported by a grant-in-aid for Young Scientists (B) (No. 21790800) to Y. K. and a grant-in-aid for Scientific Research (B) (No. 20390234) to I.N. from the Japan Society for the Promotion of Science, Ministry of Education, Culture, Sports, Science and Technology, the Kanae Foundation for the Promotion of Medical Science to Y.K and the Study Group on IgAN to Y.K.

Acknowledgements
We thank Naofumi Imai, Katsuru Minowa, Takae Watanabe and Hiroko Aita for expert technical assistance. Preliminary data from this study were presented in a poster session at the World Congress of Nephrology, April, 2011, Vancouver, Canada.

Disclosure
All the authors have no conflicting interests.

Fig. 8. Integrin α1 and α2 down-regulation induced loss in the ability to bind IgA1 probe. (A) Cultured human mesangial cell lysate co-incubated with biotinylated deSial/deGal IgA1 was reacted with each specific anti-integrin or control antibody for immunoprecipitation, and IgA1 in immune complexes were visualized by western blot using anti-biotin antibody. (B) Cultured human mesangial cells were co-cultured with TNF-α (upper) or TGF-β1 (lower) with or without the addition of dexamethasone (Dex). Expression of integrin α1 (left) was enhanced with TNF-α or TGF-β1 (bold line), whereas integrin α2 expression (right) was down-regulated by dexamethasone (dotted line). Shaded histogram represents isotype antibody control. (C) Cultured human mesangial cells treated with siRNA specific for integrin α1, α2, β1, transferrin receptor (TIR) or control siRNA were reacted with FITC-conjugated deSial/deGal IgA1 probe. Bar graph shows the percentage of probe IgA1-binding cells determined by flow cytometry. (D) Cultured mesangial cells treated with those siRNA were reacted with probe IgA1 with 10 mM Mn²⁺. (E) Cultured mesangial cells treated with those siRNA were reacted with probe IgA1 pre-incubated with 25 μg ml⁻¹ collagen type IV. These are the representative of three independent experiments. *P < 0.05, †P < 0.01, ‡not significant compared with control siRNA treatment.
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