Preliminary Technical Report

Epitope-defined monoclonal antibodies against type-IV collagen for diagnosis of Alport’s syndrome

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Abstract

Background. Alport’s syndrome can be diagnosed by staining the α5 chain of type IV collagen in kidney biopsy specimens with a monoclonal antibody. Because antibodies already established against the α5 chain require denaturation treatment of cryostat sections to expose their epitopes. To save time and effort for staining, a new epitope-defined monoclonal antibody whose epitope is initially exposed on the surface of the molecule was established.

Methods. Two monoclonal antibodies against the triple-helical domains of the type IV collagen α2 and α5 chains were established with synthetic peptides as immunogens by the rat lymph node method. Their epitope were EAIQP at the positions of 675–679 of the α2 chain, and IDVEF at the positions of 251–255 of the α5 chain, respectively. They were purified with synthetic peptide-coupled affinity columns, and then conjugated with Texas red and FITC, respectively.

Results. The mixture of fluorochrome-conjugated antibodies was able to detect the distribution of the α2 and α5 chains in the normal and Alport kidney and skin by direct immunofluorescence staining with and without denaturation treatment of the sections.

Conclusions. The direct double immunofluorescence staining of kidney and skin cryostat sections with the fluorochrome-conjugated antibodies is useful, reliable, and convenient for diagnosis of Alport’s syndrome.

Key words: Alport’s syndrome; diagnosis; kidney biopsy; monoclonal antibodies; skin biopsy; type IV collagen

Introduction

Alport’s syndrome is a hereditary nephritis caused by gene mutation of the α3(IV), α4(IV), and α5(IV) chains of type-IV collagen [1–3]. The genes of the α3(IV) and α4(IV) chains are on chromosome 2 in a head-to-head fashion [4], whereas the gene for the α5(IV) chain resides on chromosome X [2]. Staining of these α chains in the kidney and skin basement membranes (BM) with monoclonal antibodies has made it possible to diagnose Alport’s syndrome at the protein level [5,6]. However, the staining procedure with these antibodies involves acid–urea treatment, incubation of sections with diluted animal serum to avoid non-specific adsorption of secondary antibody to the sections, incubation with primary antibody, and incubation with secondary antibody. The authors thought that this complicated, time-consuming and labour-intensive staining was not a good method for the first step of examination at the time of a kidney biopsy even when there is a suspicion of Alport’s syndrome. In place of the indirect staining, direct immunofluorescence staining with fluorochrome-conjugated antibodies would be more suitable for this purpose.

The α5(IV) chain is the key chain in the diagnosis of Alport’s syndrome. First, in this syndrome, the X chromosome-linked type is the major one, and is induced by gene mutation of the α5(IV) chain [7]. Second, abnormal distribution of the α5(IV) chain can be detected in the kidney of Alport’s patients at the protein level in both X chromosome-linked Alport’s syndrome [5,6] and the autosomal recessive type [8], because the glomerular BM and part of the tubular BM contain type-IV collagen molecules composed of the α3(IV), α4(IV), and α5(IV) chains [9,10]. Third, the α5(IV) chain is also present in the normal skin epidermal BM in combination with the α6 chain [9,10]. Detection of an abnormal distribution such as protein deletion and a discontinuous (mosaic) pattern in the epidermal BM of patients with X chromosome-linked Alport’s syndrome is of diagnostic value.

In the present study epitope-defined monoclonal antibodies against the triple-helical domain of the α2(IV) and α5(IV) chains were established by the rat lymph node method originally developed by the
Staining of α2(IV) and α5(IV) chains

authors [10,11], with synthetic peptides as immunogens. The antibodies were purified with affinity columns coupled with the synthetic peptides, and conjugated with Texas red and fluorescein isothiocyanate (FITC), respectively. These fluorochrome-conjugated antibodies proved useful and reliable in the staining of kidney and skin biopsy specimens for diagnostic purposes.

Subjects and methods

The amino acid sequence of synthetic peptide used for the α2(IV) chain was CDDTDRVAGGDRQEAIQPG in imperfection XIII (Figure 1) [12,13]. That for the α5(IV) chain was CQISEKRPIDVEFQK, a cysteine for conjugation and a sequence in imperfection III (Figure 1) [14]. They were conjugated to keyhole limpet haemocyanin by the maleimido benzoyl-N-hydroxysuccinimide-ester method [15] for use as immunogens. Candidate hybridomas obtained from cell fusion were screened first with ELISA using the synthetic peptides and then with indirect immunofluorescence staining of human cryostat sections with and without acid-urea denaturation [10].

Established hybridoma cells were cultured in a modular minifermenter (Heraeus Instruments, Osterode, Germany) to obtain a culture supernatant containing a high concentration of the antibody. Antibodies were purified with synthetic peptide-coupled affinity columns according to the method described previously [16]. Texas red (Molecular Probes, Inc., Eugene, OR, USA) and FITC (Sigma, St. Louis, MO) were conjugated to the purified antibodies [17].

The epitope of the monoclonal antibody was mapped with overlapping peptides on the surface of different rods (multipin-peptide scanning method) [18]. The peptides were custom ordered from Chiron-Mimotopes, Inc., Australia. Overlapping 10-residue peptides homologous with the sequences of imperfection XIII of the α2(IV) chain and imperfection III of the α5(IV) chain were used.

Kidney and skin tissues from human subjects were placed in OCT compound (Miles Inc., Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. They were then cut into 4-μm sections in a cryostat and air dried.

Direct immunofluorescence staining was done as follows:

![Fig. 1. Schematic representation of the α2(IV) and α5(IV) chains.](image)

Table 1. Epitope scanning of rat monoclonal antibodies against human α2(IV) and α5(IV) chains of type-IV collagen by the method of Geysen et al. [18]

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Absorbance</th>
</tr>
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<tbody>
<tr>
<td>H25</td>
<td>AVGGDRQEA1</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>VGGDRAEQOJ</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td>GGDRAEQ1OJP</td>
<td>2.202</td>
</tr>
<tr>
<td></td>
<td>GDRQAAIQPG</td>
<td>2.129</td>
</tr>
<tr>
<td></td>
<td>DRQAIQPGC</td>
<td>2.225</td>
</tr>
<tr>
<td></td>
<td>RQAIQPGCG</td>
<td>&gt;2.500</td>
</tr>
<tr>
<td></td>
<td>QEAIQPGCGG</td>
<td>&gt;2.500</td>
</tr>
<tr>
<td></td>
<td>EAIQPGCGGG</td>
<td>2.201</td>
</tr>
<tr>
<td></td>
<td>AIQPGCGGGG</td>
<td>1.185</td>
</tr>
<tr>
<td>H53</td>
<td>ISEQKRPIDV</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>SEKRPIDVE</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>EKRPIDVEF</td>
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</tr>
<tr>
<td></td>
<td>QKRPIDVEFQ</td>
<td>&gt;2.500</td>
</tr>
<tr>
<td></td>
<td>KRPIDVEFQK</td>
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</tr>
<tr>
<td></td>
<td>RPIDVEFQKG</td>
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<td>DVEFQKGDDQ</td>
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<tr>
<td></td>
<td>VEFQKGDDQ</td>
<td>0.060</td>
</tr>
</tbody>
</table>

after the sections had been rinsed three times with PBS, they were incubated with a mixture of fluorochrome-conjugated antibodies for 1 h. After having been washed with PBS, they were mounted and viewed with a Zeiss Axioshot microscope. Indirect immunofluorescence staining were done as previously described [10].

Results

Two monoclonal antibodies were established. H25 was a rat IgG1, κ subclass antibody, and specific for the α2(IV) chain, and the epitope sequence was determined by the multipin-peptide scanning method and found to be EAIQP (Table 1) at the positions of 675–679, which were located in imperfection XIII (Figure 1). H53 was a rat IgG2a, κ subclass antibody, and against the α5(IV) chain, and the epitope sequence was IDVEF (Table 1) at the positions of 251–255 located in imperfection III (Figure 1). These antibodies could stain normal kidney and skin tissues both with and without acid-urea treatment of the sections, which indicates that the epitopes were exposed on the surface of the molecule and resistant to denaturation treatment. The staining patterns of H25 and H53 were the same as those of monoclonal antibody H21 and H22, and H51 and H52 respectively, which were epitope-defined and against sequences near the carboxyl terminal of the NC1 domain [10].

Double immunofluorescence staining using Texas-red-conjugated H25 and FITC-conjugated H53 was carried out with 54 human kidney specimens from normal subjects (Figure 2, A–C) and patients with non-hereditary nephritis. H25 (anti-α2) stained the endothelial side of the glomerular BM, the tubular BM, the Bowman’s capsular BM, the mesangial matrix, and the capillary BM of all specimens. This staining...
Fig. 2. Staining of the α2(IV) and α5(IV) chains in kidney and skin. A–C, kidney from a normal subject; D–F, kidney from a male patient with X chromosome-linked Alport syndrome; G–I, skin from a female with X chromosome-linked Alport syndrome. Direct double immunofluorescence staining was used. A, D, G, staining with Texas-red-conjugated H25 for α2(IV); B, E, H, staining with FITC-conjugated H53 for α5(IV); C, F, I, dual exposure of both staining. No staining of the α5(IV) chain was observed in the glomerular and tubular BMs in the kidney of the male patient (E). A discontinuous (mosaic) pattern of the α5(IV) chain was observed in the epidermal BM of the female patient (H). × 90.

pattern was the same as that of H21 and of H22. H53 (anti-α5) stained the glomerular BM, Bowman’s capsular BM and the part of the tubular BM of all specimens, and this staining pattern was the same as that of H51 and of H52 [9,10]. Staining of two kidney biopsy specimens from male patients with X chromosome-linked Alport’s syndrome was carried out (Figure 2, D–F). Screening for gene mutations in all the exons (1–51) of the COL4A5 gene of the patients was carried out by PCR-SSCP analysis [19]. One patient had a deletion of adenine at nucleotide 1274 in exon 19 of the COL4A5 gene and the predicted mutation at the protein level was a protein deletion according to a frameshift. The gene mutation of the other patients was not defined by PCR-SSCP analysis. No staining of the α5(IV) chain was observed on the glomerular or tubular BMs of both patients (Figure 2, E). Positive staining of the α2(IV) chain was observed in the Alport sections, but the staining of glomerular BM was stronger than that of glomerular BM in the normal kidneys (Figure 2, D).

Staining of the α2(IV) and α5(IV) chains in skin tissues from two female patients with X chromosome-linked Alport’s syndrome was also done (Figure 2, G–I). The antibodies to the α2(IV) chain stained the epidermal and vascular BMs in a linear pattern. This pattern was the same as that seen in the normal skin. In contrast, the antibodies against the α5(IV) chain stained only the epidermal BM in a discontinuous (mosaic) pattern, although the staining was weaker than that stained by indirect staining. This discontinuous pattern is different from that of the normal skin, where H53 stained the epidermal BM in a continuous pattern.
Staining of α2(IV) and α5(IV) chains

Discussion

Epitope-defined monoclonal antibodies against the NC1 domains of α1–α6(IV) chains of type-IV collagen have already been established [10]. The monoclonal antibodies established in the present study were against the triple-helical domain. These epitope-defined monoclonal antibodies against different sites of the collagen molecule are useful for research on type-IV collagen and diagnosis of hereditary diseases related to this collagen because of their reliability. In addition, because the epitopes on the α2(IV) and α5(IV) chains in the present study were originally exposed and resistant to denaturation treatment of acid–urea, the antibodies were able to react with them even without denaturation treatment of the sections. This nature of the antibodies is good for immunostaining for a diagnostic purpose.

The results of the present study indicate that the mixture of fluorochrome-conjugated antibodies can be used as reliable reagents for the kidney and skin biopsy specimens. This direct immunofluorescence staining does not require much skill because the staining procedure is very simple. It reduces not only time and labour of the staining but also individual staining differences usually produced by the indirect immunofluorescence staining, which consists of several complicated steps. The use of direct staining would be particularly recommended at the time of a kidney biopsy when there is a suspicion of Alport’s syndrome, because it can be used in the same way as the usual immunofluorescence staining of IgG or IgA. It is useful to use the double staining of the α2(IV) and α5(IV) chains because the α2(IV) chain staining can help to check the staining conditions and to confirm the distribution of basement membranes in tissues.

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References


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