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Altered Distribution of the α1 Chain of Collagen IV in the Chronic Allograft Nephropathy

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Previously, we demonstrated the altered formation of collagen IV, which is the main constituent of the basement membrane, in renal allografts by staining with two monoclonal antibodies against the α1 chain of collagen IV. In the present study, we investigated the alteration of collagen IV in chronic allograft nephropathy (CAN), which is an irreversible change that can occur in renal allografts. Biopsy specimens of normal kidneys (Group A: n = 5) and acute rejection (Group B: n = 10) were studied as controls. Fifty biopsy specimens from 41 patients who had been diagnosed as having CAN were divided into two groups, according to renal function: Group C (n = 35), sCr 2～4 mg/dl, and Group D (n = 15), sCr > 4 mg/dl. Two monoclonal antibodies, JK199 and JK132, those recognize the α1 chain of collagen IV were used. In Group A, JK199 reacted with the glomerular basement membrane (GBM), the mesangial matrix (MM), the basement membrane of Bowman's capsule (BBM) and the tubular basement membrane (TBM). JK132 only reacted with the MM, BBM and TBM. In Group B, JK199 reacted with GBM, MM, BBM, TBM and the interstitium (INS). JK132 only reacted with MM, BBM and TBM. In Group C and Group D, JK199 and JK132 reacted universally with GBM and INS in addition to MM, BBM and TBM. The intensity of the reaction was higher in Group D than in Group C. Thus, the reactivity of JK132 with GBM and INS was a unique finding for the CAN specimens. These results suggest that collagen IV is upregulated in CAN and the reactivity of JK132 in GBM and INS may represent the point of irreversible dysfunction of renal allografts.

Introduction

Kidney transplantation is now widely accepted as a treatment for end-stage renal diseases. One-year graft survival is more than 90%, and the occurrence of complications resulting from the transplant has decreased in the last decade[1]. However, the number of renal allografts lost after the first year of transplantation has not decreased[2]. Approximately 40% of grafts suffer a loss of function within 10 years[3]. This type of renal allografts' deterioration, occurring late after the kidney transplantation is known as chronic rejection or chronic allograft nephropathy (CAN)[4][5]. CAN is a major obstacle that must be overcome to improve the long-term outcome of renal allografts. Some studies have suggested that CAN may be caused not only by antigen-dependent or immunological cause[6], but also by

antigen-independent or non-immunological causes[7)8)].

Irrespective of the cause, CAN is clinically characterized by a decline in allograft function associated with persistent and progressive proteinuria after the first three post-transplant months, resulting in the fatal loss of graft function. Histologically, CAN is characterized by atherosclerosis, tubular atrophy, and progressive fibrosis in the glomeruli and interstitium[5)]. Although many risk factors for CAN have been identified, the precise mechanisms remain unknown.

The basement membrane is a specialized extracellular matrix (ECM) whose main role is to separate epithelia or endothelia from the surrounding tissues[9)]. The basement membrane is composed of different molecules, such as collagen IV, nidogens and other molecules, and collagen IV is the main constituent. Collagen IV consists of three α chains, and to date, 6α chains have been cloned[10)]. An isoform consisting of two α1 and one α2 chains, designated as $[\alpha 1\,(IV)]_2\,\alpha 2\,(IV)$, is the major constituent[11)]. Alteration in the structure of collagen IV are thought to play an important role in some diseases[12)~14)].

JK199 and JK132 are monoclonal antibodies for collagen IV that originate from human placenta[15)]. JK199 recognizes $[\alpha 1\,(IV)]_2\,\alpha 2\,(IV)$ in a triple helical conformation of collagen IV. In contrast, the epitope that JK 132 recognizes is hidden within the triple helical conformation in the normal glomerular basement membrane (GBM). It is the amino-acid sequence of residues 1165～1179 of α1 (IV)[16)]. In the normal kidney, JK199 reacts with GBM, the mesangial matrix (MM), the basement membrane of Bowman's capsule (BBM), and the tubular basement membrane (TBM). On the other hand, JK132 reacts with MM, BBM and TBM, but not with GBM[17)]. Previous studies have shown that combined staining with these 2 monoclonal antibodies was useful in revealing the mechanisms of several renal diseases[14)18)]. Especially, the emergence of the reactivity or the increased reactivity of JK 132 has been demonstrated in pathological alteration in several diseases.

We previously reported that, in acute rejection, the intensity of JK199 increased in GBM, MM, BBM, TBM, and the interstitium (INS). However, the intensity of JK132 increased in MM, BBM and TBM, but not in GBM or INS[19)]. Our results suggested that the differential staining pattern of JK 199 and JK132 in GBM and INS might reflect functional differences between acute rejection and CAN.

In the present study, we investigated the reactivity of JK199 and JK132 in CAN specimens, and discovered distinctive staining patterns for JK199 and JK132. These patterns were different from those seen in acute rejection.

Patients

Materials and methods

The patients' characteristics are summarized in Table 1. Diabetic patients in pre- and post-transplantation were not enrolled. All kidney transplants were living-related. Generally, the warm ischemic time was less than 5 minutes, and the total ischemic time was around 1～2 hours. Biopsy specimens of normal kidneys (Group A: n = 5) and acute rejection kidneys (Group B: n = 10) were examined as control groups. Fifty biopsy specimens from 41 patients who had been diagnosed as CAN were divided into two groups, according to renal function. Group C consisted of patients whose serum creatinine level was between 2.0～4.0 mg/ml (n = 35), and Group D consisted of patients whose serum creatinine level was more than 4.0 mg/ml (n = 15). Renal biopsies were performed, percutaneously, using an 18 G needle under local anesthesia, after obtaining informed consent. The histological diagnosis of CAN was made based on the Banff working clas-

Table 1 Patients' characteristics

	Group A (n= 5)	Group B (n= 10)	Group C (n= 35)	Group D (n= 15)
Mean age (year)	41.5	45.1	44.3	45.6
Sex Male	3	4	19	8
Female	2		16	7
Mean donor age (year)		57.5	59.7	56.8
Average serum creatinine (mg/dl)	0.8 ± 0.3	1.2 ± 0.8	2.8 ± 1.1	5.4 ± 1.4

Table 2 Diagnosis of biopsy specimens of Group C and Group D

	Group C	Group D
Grade I	22	6
Grade II	5	7
Grade III	0	2
Others	8	5

Diagnosis of CR was performed based on Banff 97 diagnostic categories[5]. Grade I: mild. Grade II: moderate. Grade III: severe. Others: Changes not considered to be due to rejection.

sification criteria[12]. A detailed histological diagnosis of the biopsy specimens in Group C and Group D is presented in Table 2.

Immunohistochemistry

To detect collagen IV in renal allografts, formalin-fixed paraffin-embedded tissue sections were stained with goat anti-type IV collagen polyclonal antibody (Southern Biotechnology, Birmingham, AL), using the standard avidin-biotin-peroxidase complex (ABC) technique. Sections were incubated with polyclonal anti-type IV collagen antibody followed by a biotin-conjugated rabbit anti-goat IgG (DAKO, Carpintena, CA), by ABC (Vector, Burlingame, CA), and by hydrogen peroxide containing 3,3′-diaminobendizine.

The monoclonal antibodies, JK199 and JK132, (a kind gift from Dr. Jun Kido, Pharmaceutical Research Laboratory, Shiseido Research Center, Tokyo, Japan), were prepared as described previously[15)20)]. Sp2/O culture medium containing 2.5 μg/ml of normal, mouse IgG (Chemicon International, Temecula, CA), was used as a control antibody for JK199 and JK132[18)]. Needle biopsy specimens were immediately snap-frozen using liquid nitrogen. Unfixed cryostat sections, 6～8 μm thick, were prepared on albumin-coated glass and immersed in acetone for 5 min at 4 ℃, and rehydrated in PBS. The sections were incubated with normal goat serum for 30 min at room temperature. Primary antibodies (2.0 μg/ml) were then added, and the mixture was incubated for 1 hr at 37 ℃. After being washed with PBS, sections were incubated with 2.0 μg/ml of FITC conjugated rabbit anti-mouse IgG for 1 hr at room temperature, then washed with PBS.

One well-trained pathologist analyzed samples in a blinded manner. The intensity of JK199 and JK132 was scored as follows: +1 was the intensity of Group A. As the intensity increased, scores were graded from +1 to +4. Score 0 indicated the absence of the signal.

Immunoelectron microscopy

Cryostat sections (6 μm) of the biopsy specimens were mounted on glass slides. The sections were incubated with JK199 and with a secondary antibody conjugated with 0.8 nm gold (Aurion Co. Ltd., Netherlands). The sections were rinsed with 0.1 M phosphate buffer (pH 7.4), soaked in 2.5% glutaraldehyde and 0.2% tannic acid in 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer. The sections were immersed in a silver enhancement solution (Aurion, Co. Ltd., Netherlands) for

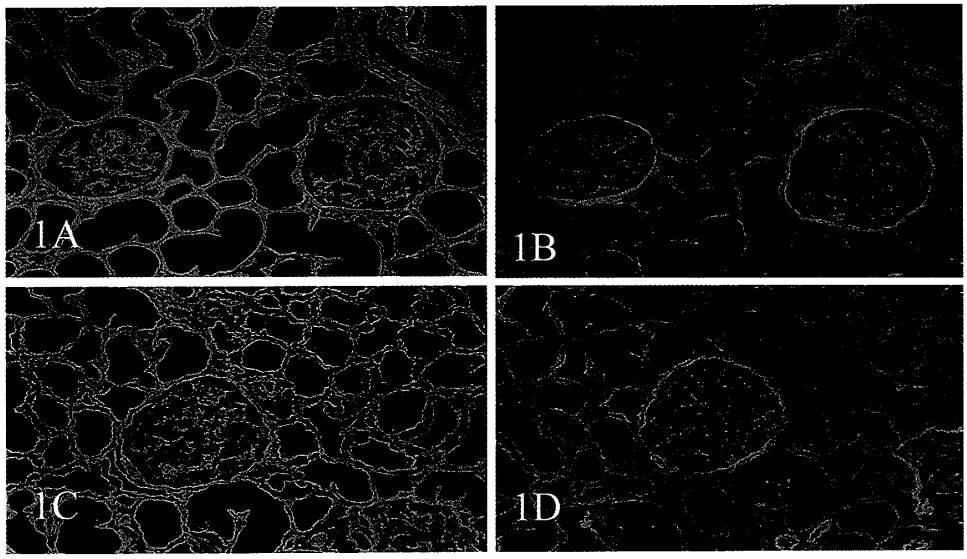

Fig. 1 A representative case of Group A and Group B
In normal kidney, JK199 reacted with the glomerular basement membrane (GBM), the mesangial matrix (MM), the basement membrane of Bowman's capsule (BBM) and the tubular basement membrane (TBM) (1A : ×100), and JK132 reacted with MM, BBM and TBM (1B : ×100). In acute rejections, an increased intensity of JK199 staining was observed in GBM, MM, BBM, TBM and the interstitium (INS) (1C : ×100), and an increased intensity of JK132 staining was observed in the MM, TBM and BBM (1D : ×100).

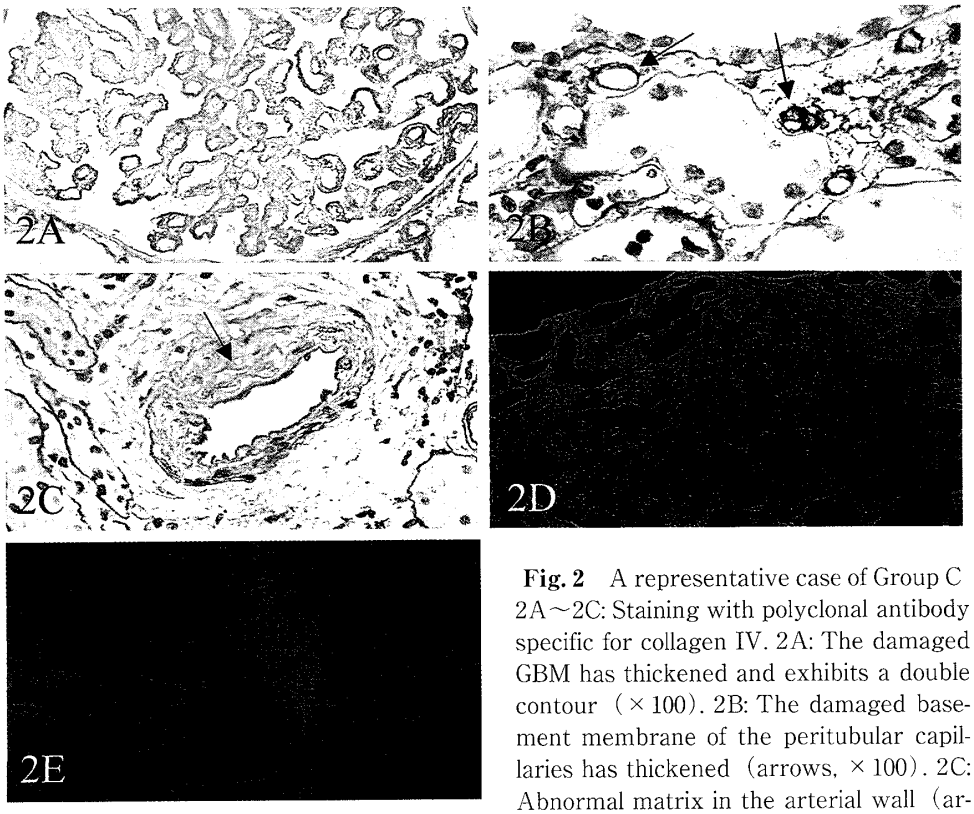

Fig. 2 A representative case of Group C
2A～2C: Staining with polyclonal antibody specific for collagen IV. 2A: The damaged GBM has thickened and exhibits a double contour (×100). 2B: The damaged basement membrane of the peritubular capillaries has thickened (arrows, ×100). 2C: Abnormal matrix in the arterial wall (arrow, ×100). 2D: JK199 (×50) and 2E: JK 132 (×50) reacted with GBM, MM, BBM, TBM and INS.

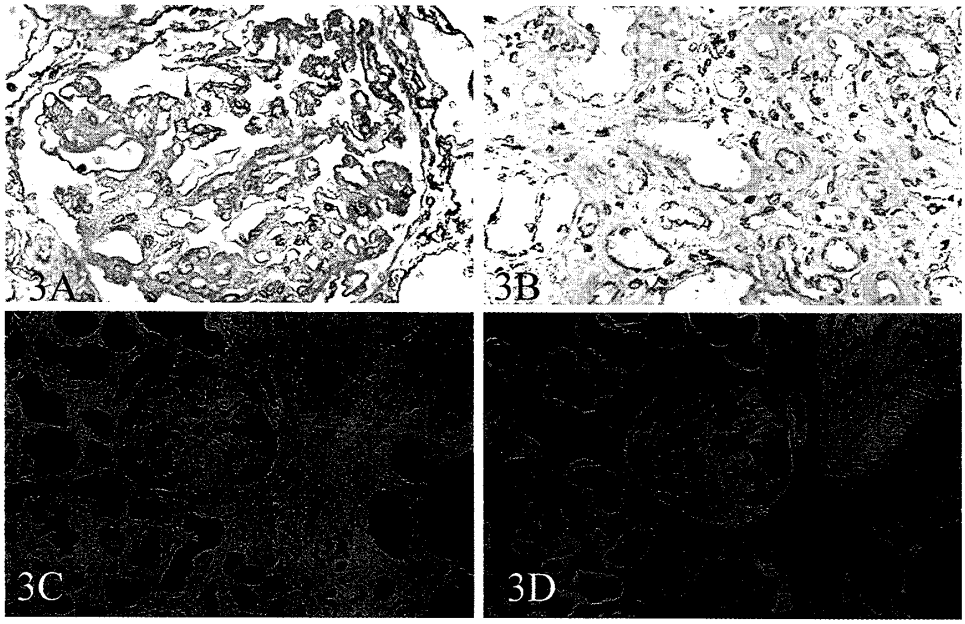

Fig. 3 A representative case of Group D
3A, 3B: Staining with polyclonal antibody specific for collagen IV (×100). An increased staining intensity of JK199 (3C) and JK132 (3D) is visible in the GBM, MM, BBM, TBM and INS (×50).

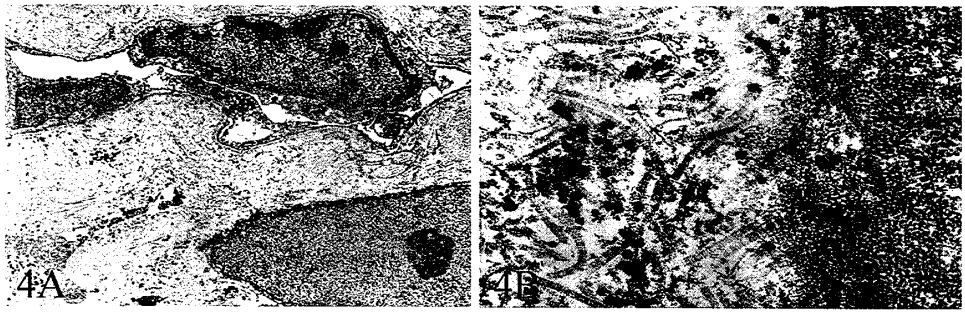

Fig. 4 Immunoelectron micrographs showing the localization of collagen IV in INS of CAN specimens
Collagen IV was detected by the nano gold-silver enhancement method (4A) and the anti-collagen IV monoclonal antibody, JK199 (4B). Silver-enhanced gold particles, approximately 0.6 μm in diameter, are deposited in INS.

10～20 min at room temperature and covered with a drop of 1% chitosan solution in 0.5% acetic acid to reinforce the sections. After chitosan infiltration, the sections were immersed in 2.5% glutaraldehyde, dehydrated with a graded series of alcohol, and embedded in epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate.

Results

Figure 1 shows a representative case from Groups A and B. In Group A, JK199 reacted with GBM, MM, BBM and TBM (Fig. 1A). JK132 reacts with MM, BBM and TBM (Fig. 1B). In Group B, JK199 reacted with INS, in addition to GBM, MM, BBM and TBM (Fig. 1C). JK132 reacted with MM, BBM and TBM (Fig. 1D).

Figure 2 shows a representative case from

Table 3 Summary of reactivity of JK199 and JK132

	A	B	C	D
Reactivity of JK199				
GBM	1 ± 0	2 ± 0	3 ± 0.3	4 ± 0.4
MM	1 ± 0	2 ± 0	3 ± 0.1	4 ± 0.1
BBM	1 ± 0	2 ± 0	3 ± 0.2	4 ± 0.1
TBM	1 ± 0	2 ± 0	3 ± 0.1	4 ± 0.1
INS	0	1 ± 0	3 ± 0.2	4 ± 0.2
Reactivity of JK132				
GBM	0	0	1 ± 0	2 ± 0.1
MM	1 ± 0	2 ± 0.1	3 ± 0.2	4 ± 0.2
BBM	1 ± 0	2 ± 0.5	3 ± 0.4	4 ± 0.4
TBM	1 ± 0	2 ± 0.2	3 ± 0.3	4 ± 0.4
INS	0	0	1 ± 0.1	2 ± 0.2

Group C. The patient underwent a graft biopsy 8 years after kidney transplantation. His serum creatinine level at the time of the biopsy was 2.5 mg/dl. The biopsy specimen showed chronic allograft nephropathy (Ia) with cyclosporine-associated arteriolopathy. After staining the biopsy specimens with a polyclonal antibody against collagen IV, the damaged GBM appeared to be thickened, with a double contour (Fig. 2A). The basement membrane of the peritubular capillary was also thickened and interrupted (Fig. 2B). Accumulation of abnormal matrix in the arterial wall, which is suggestive of cyclosporine-associated arteriopathy, was observed (Fig. 2C). The intensity of both JK199 (Fig. 2D) and JK132 (Fig. 2E) had increased in the GBM, MM, BBM, the atrophic TBM and INS.

Figure 3 shows a representative case from Group D. The patient underwent a graft biopsy 6 years after kidney transplantation. His serum creatinine level was 4.1 mg/dl. Examination of the biopsy specimen revealed chronic allograft nephropathy (IIa, Figs. 3A, B). JK199 (Fig. 3C) and JK132 (Fig. 3D) reacted with GBM, MM, BBM, TBM and INS. The intensity of JK199 and JK132 was higher in Group D, compared to Group C, suggesting that the intensity of JK199 and JK 132 is correlated with the functional impairment of the renal allografts.

To further clarify the location of collagen IV in INS, biopsy specimens of CAN were examined by immunoelectron microscopy. Silver-enhanced gold particles, which were collagen IV, were deposited in INS and surrounded by newly formed collagen fibrils (Fig. 4).

The results of staining with JK199 and JK132 are summarized in Table 3. JK132 reacted with GBM and INS only in CAN.

Discussion

The present study revealed that collagen IV was upregulated in CAN specimens. In CAN, the polyclonal antibody against collagen IV reacted with the GBM, MM, BBM, TBM and INS. The reactivity of JK199 and JK132 showed the same distribution as the polyclonal antibody. This upregulation of collagen IV was uniformly observed in CAN irrelevant of different risk factors and was correlated with the dysfunction of the renal allografts.

The GBM has a distinctive structural feature; the basement membrane of the glomerular endothelium and that of the urinary epithelium is fused, with very little ECM. ECM is located only in the mesangial area[21]. In the present study, the reactivity of JK132 in the GBM suggested that the mesangial matrix had expanded into the

space between the glomerular endothelium and the urinary epithelium, producing impairment in filtration[9]. In several previous studies, the mesangial matrix expansion was observed in the glomerulosclerotic area of the kidney[8)20)]. In the present study, the increased intensity of JK132 in the GBM of CAN specimens, but not of acute rejection specimens, suggests that this microscopic change in the GBM is unique to CAN. Thus, staining with JK132 may provide a hallmark for distinguishing progressive and irreversible renal graft dysfunction from reversible acute graft dysfunction.

The interstitium of normal kidneys contains only small amount of collagen IV. We previously reported that an increased intensity of JK 199 staining was observed in the INS of acute rejection specimens[19]. In the present study, both JK 132 and JK199 showed an increased staining intensity in the INS of CAN specimens. The immunoelectron microscopic study revealed that newly formed collagen IV was observed surrounded by collagen fibrils in the interstitial space in CAN specimens. This structure consisted of collagen IV and other collagens and resembled a very immature basement membrane.

JK199 and JK132 also react with normal TBM. As shown in the present study as well as the previous study[3], the intensity of JK199 and JK132 staining increased in atrophic TBM, and the reactivity was correlated with the level of tubular atrophy.

In the interstitium of the kidney, the peritubular capillaries are located adjacent to the tubuli play a pivotal role in maintaining renal function. Thus, damage to the peritubular capillaries is directly reflected as renal dysfunction. Indeed, an experimental model of glomerulitis in rats revealed that the number of peritubular capillaries was clearly correlated with renal dysfunction[4]. The current study showed that the staining of CAN specimens with a polyclonal antibody against collagen IV could provide valuable information about the degree of damage to the peritubular capillaries. Electron microscopic studies have revealed circumferential and multilamellation of the peritubular capillary basement membrane in CAN specimens[5)19)]. In the present study, staining with polyclonal antibody against collagen IV revealed a thickened and interrupted basement membrane in the peritubular capillaries. These findings correspond with those of previous reports. Since peritubular capillaries are a functionally important architecture in the kidney, any damage to these structures is likely to be directly reflected in the functioning of the renal allograft. Thus, changes in collagen IV in the peritubular capillaries may provide information about the extent of the damage to the renal allograft.

In conclusion, the increased intensity of JK199 and JK132 indicates that collagen IV is upregulated in the glomeruli and the interstitium of CAN biopsy specimens. The increased intensity of JK132 in the GBM and the INS in the CAN specimens suggests the uncontrolled accumulation of altered $[\alpha 1\ (IV)\ ]_2\ \alpha 2\ (IV)$. Thus, the combined staining of biopsy specimens with JK199 and JK132 may be useful for distinguishing CAN from acute cellular rejection. Furthermore, a greater understanding of the alterations in collagen IV may help to elucidate the mechanisms leading to CAN.

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References

1) **Ota K**: Asian transplant registry. Transplant Proc **31**: 205–209, 1999
2) **Gjertson DW**: Survival trends in long-term first cadaver-donor kidney transplants. *In* Clinical

Transplants (Terasaki PI ed) p225, UCLA Tissue Typing Laboratory, Cecka JM, Los Angeles (1991)
3) **Paul LC**: Chronic renal transplant loss. Kidney Int **47**: 1491–1499, 1995
4) **Paul LC**: Chronic transplant nephropathy. Kidney Int **56**: 783–793, 1999
5) **Racusen LC, Solez K, Yamaguchi Y**: The Banff 97 working classification of renal allograft pathology. Kidney Int **55**: 713–723, 1999
6) **Humar A, Hassoun A, Kandaswamy R et al**: Immunologic factors: the major risk for decreased long-term renal allograft survival. Transplantation **68**: 1842–1846, 1999
7) **Dimény E, Wahlberg J, Fellström B**: Hyperlipidaemia in renal transplantation-risk factor for long-term graft outcome. Eur J Clin Invest **25**: 574–583, 1995
8) **Opelz G, Wujiac T, Ritz E**: Collaborative transplant study: Association of chronic graft failure with recipient blood pressure. Kidney Int **53**: 217–222, 1998
9) **Adachi E, Hopkinson I, Hayashi T**: Basement-membrane stromal relationships: Interactions between collagen fibrils and the lamina densa. Int Rev Cytol **173**: 73–156, 1997
10) **Oohashi T, Sugimoto M, Ninomiya M**: Identification of a new collagen IV chain, α6(IV), by cDNA isolation and assignment of the gene to chromosome Xq22, which is the same locus for COL4A5. J Biol Chem : **269**: 7520–7526, 1994
11) **Iwata M, Imamura Y, Hayashi T**: Evidence for a short form of α1(IV) as a major polypeptide in bovine lens capsule. J Biochem **117**: 1298–1304, 1995
12) **Brainwood D, Kashtan C, Turner AN**: Targets of alloantibodies in Alport anti-glomerular basement membrane disease after renal transplantation. Kidney Int **53**: 762–766, 1998
13) **Hudson BG, Reeders ST, Tryggvason K**: Type IV collagen. Structure, gene organization, and role in human disease. J Biol Chem **268**: 26033–26036, 1993
14) **Makino H, Haramoto T, Shikata T**: Distribution of a novel chain of basement membrane associated-collagen in the renal tissue. Connect Tissue **24**: 163–168, 1992
15) **Kino J, Adachi E, Hayashi T**: A novel chain of basement membrane-associated collagen as revealed by biochemical and immunohistochemical characterization of the epitope recognized by a monoclonal antibody against human placenta basement membrane collagen. Am J Pathol **138**: 911–920, 1991
16) **Kino J, Adachi E, Hayashi T**: Characterization of a monoclonal antibody against human placenta type IV collagen by immunoelectroblotting, antibody-coupled affinity chromatography, and immunohistochemical localization. J Biochem **103**: 829–835, 1988
17) **Makino H, Shikata K, Ota Z**: Immunoreactivity of the JK-132 monoclonal antibody directed against basement membrane collagen in normal and diabetic glomeruli. Virchow Arch **424**: 235–241, 1994
18) **Yoshida T, Matsubara O, Adachi E et al**: Increased deposition and altered distribution of basement membrane-related collagen in human fibrotic or cirrhotic liver. Connect Tissue **29**: 189–198, 1997
19) **Utsumi K, Simizu A, Yamato M et al**: Alteration of collagen IV in acutely deteriorated renal allografts. Transplantation **71**: 1757–1765, 2001
20) **Ohashi R, Kitamura H, Yamanaka N**: Peritubular capillary injury during the progression of experimental glomerulonephritis. J Am Soc Nephrol **11**: 47–45, 2000
21) **Monga G, Mazzucco G, Novara R**: Intertubular capillary changes in kidney allografts: A morphologic investigation on 61 renal specimens. Mod Pathol **5**: 125–130, 1992

慢性期移植腎機能低下例におけるIV型コラーゲンの分布の変化に関する検討

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IV型コラーゲンは基底膜の主要構成成分であり，生体内に広く分布している．移植後急性期腎機能低下例においては，腎臓のIV型コラーゲン分布に変化が認められる．今回我々は移植腎機能廃絶の主原因である慢性期移植腎機能低下症（chronic allograft nephropathy: CAN）におけるIV型コラーゲンの変化について検討した．当院において施行された腎移植患者を，移植腎機能に基づいて以下の群に分類し，組織所見について検討した．なおCAN症例は，光学顕微鏡所見で診断された症例を用いた．Group A（n＝5）：腎機能正常群，Group B（n＝10）：急性期移植腎機能低下群，Group C（n＝35）：CAN症例で血清クレアチニン値2～4 mg/dl，Group D（n＝15）：CANで血清クレアチニン値＞4 mg/dl．各症例におけるIV型コラーゲン分布について，IV型コラーゲンのα1鎖に対するモノクローナル抗体であるJK199, JK132を用いて，蛍光顕微鏡により検討した．Group AではJK199, JK132ともにメサンギウム基質（MM），ボーマン嚢基底膜（BBM），尿細管基底膜（TBM）に対する反応のみを認めた．Group Bでは，JK199はMM，BBM，TBMに加え，糸球体基底膜（GBM）および腎間質（INS）に対する反応を認めた．JK132の反応性はGroup Aと同様であった．一方，Group CおよびGroup Dでは，JK199においてはMM，GBM，BBM，TBM，INSに対する反応性が増強していた．JK132についてはMM，BBM，TBMに加え，GBMおよびINSにおいても反応が認められた．また，JK199およびJK132の反応性はGroup CよりもGroup Dにおいて強い傾向が認められた．GBMおよびINSにおけるJK132の反応陽性化はCANに特異的な所見であることが示唆された．