Specific Changes in the Basement Membrane of the Proximal Tubules in the Murine Polycystic Kidney Detected by the Novel Anti-basement Membrane Monoclonal Antibody D28

Shizuko NAGAO, Yoh-ichi KOYAMA***, and Hisahide TAKAHASHI

Laboratory Animal Center, *Division of Cell Biology, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake-shi, Aichi 470-11, Japan

(Received 25 March 1994/Accepted 31 May 1994)

In order to analyze renal abnormalities in mice with polycystic kidney disease (PKD), we produced a series of monoclonal antibodies reactive with the murine kidney by hybridizing P3U1 myeloma cells with spleen cells from DBA/2 mice immunized with the kidney of adult-type PKD mice, DBA/2FG-*pcy*. One clone, D28, reacted specifically with the basement membrane of the proximal tubules of DBA/2 mice and DBA/2FG-*pcy* mice. It did not react with other parts of the murine kidney nor with other tissues such as the skin, ovary, fallopian tube, testis, lung and small intestine. While other components such as collagen IV, laminin and the core protein of proteoglycan could be found, the D28 epitope could not be found in the basement membrane of renal cysts formed in adult-type (DBA/2FG-*pcy*) and infant-type (C57BL/6J-*cpk*) PKD mice. The D28 epitope did not, however, disappear from the basement membrane of proximal tubules in other types of renal abnormalities. These results suggest that the formation of renal cysts in the proximal tubules is associated with an alteration to the proximal tubule-specific structure of the basement membrane. The D28 monoclonal antibody should prove a useful tool with which to analyze basement membrane-associated abnormalities in genetic PKD.—KEY WORDS : basement membrane, monoclonal antibody, mouse, polycystic kidney

Human hereditary polycystic kidney disease (PKD) is a disorder caused by an autosomal recessive (infant-type) or dominant mutation (adult-type) and characterized by the progressive enlargement of the kidneys, which become filled with numerous expanding cysts [14]. A renal cyst is formed by dilation of the nephron and the collecting ducts [10] and it may be the result of an abnormal susceptibility to the epidermal growth factor (EGF) or the transforming growth factor β (TGF- β) [31,33], a high expression of the *c-erb-2* oncogene [18], an altered localization of Na⁺, K⁺-ATPase [33,34], or changes in the turnover or the expression of basement membrane (BM) components [32]. However, precise mechanisms of the renal abnormalities in PKD remain unclear.

Several animal models of human PKD have been studied. A murine model of infant-type PKD, the C57BL/6J-*cpk* (B6-*cpk*) mouse strain, carries an autosomal recessive mutation on chromosome 12 [7]. B6-*cpk* mice have abnormalities in the expression of the BM components [8], preproEGF mRNA [11], sulfated glycoprotein-2 [16], some oncogenes [6,17] and Na⁺, K⁺-ATPase [2]. Takahashi *et al.* [26,27] presented an adult-type murine model of PKD, DBA/2FG-*pcy* (D2-*pcy*) mouse strain, which carries the mutant gene, *pcy*, in the genetic background of DBA/2 (D2) mouse. Pathogenesis caused by the *pcy* gene is influenced by the genetic background, and renal abnormalities are far more severe in D2-*pcy* mice than in C57BL/6FG-*pcy* mice [23]. The Han:SPRD rat is another animal model of PKD

***Present address : Nippi Research Institute of Biomatrix, 1-1 Senju-Midori-cho, Adachi-ku, Tokyo 120, Japan

and is remarkable in that the homozygous mutant rats exhibit infant-type PKD while the heterozygous animals display an adult-type PKD phenotype [22].

For this study, we produced a series of anti-kidney monoclonal antibodies (mAbs) and carried out an immunohistochemical analyses of the abnormalities in the renal basement membrane of PKD mice. We show that the BM of the proximal tubules contains a highly specific epitope which is detected by the novel mAb but disappears upon the formation of renal cysts.

Materials and Methods

Animals : Production of the D2-*pcy* mice has elsewhere been described [23,26,27]. B6-*cpk* mice [12] and NC mice [28] were obtained from Drs. Grantham and Gattone (University of Kansas Medical Center, U.S.A.) and from Dr. Kondo (Nagoya University, Japan). Daunomycin was i. v. injected into 8-week-old D2 mice at 20mg/kg body weight and the mice were sacrificed 20 days after the treatment.

Production of anti-kidney mAbs : Two series of immunization were carried out to produce anti-kidney mAbs ; both D2 and D2-*pcy* mice were immunized with kidney from 1-day-old mice of the counterpart strain by subcutaneous injection of homogenized kidney mixed with Freund's complete adjuvant. Every 28 days for the next 6 months the mice were then given subcutaneous injections of kidney homogenate mixed with Freund's incomplete adjuvant. Production of anti-kidney mAb was monitored by indirect immunofluorescence staining of frozen sections of kidney from 1-day-old mice. Dissociated spleen cells from immunized mice were fused with P3U1 myeloma cells by a standard method employing polyethylene glycol. Ascites were obtained from BALB/c-*nu/nu* mice, which were intraperitoneally inoculated with hybridoma cells. Class and subclass of the mAbs were determined with an Ouchterlony double diffusion test.

Immunohistochemical staining : Kidneys were obtained from D2 and D2-*pcy* mice at 1 day, 3 and 8 weeks of age and from B6-*cpk* mice at 1, 2 and 3 weeks of age. Eight-week-old D2 mice were the source of other tissues such as the lung, small intestine, skin, ovary,

fallopian tube and testis. Tissue fragments were embedded in O.C.T. compound, and 6- μ m-thick frozen sections were cut. After being fixed in ethanol, the sections were allowed to react overnight at 4°C either with the D28 ascites diluted to 1/500 with phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA) or with an isotype-matched control anti-Thy-1.1 mAb (Serotec, Kidlington, Oxford) diluted to 1/200 with PBS/BSA. In order to detect BM components, frozen sections were first treated with anti-collagen IV rabbit antiserum (LSL, Nerima, Tokyo), anti-laminin rabbit antiserum (LSL), or anti-core protein of heparan sulfate proteoglycan (HSPG) rabbit antiserum (a kind gift from Dr. Yamada, National Institute of Health, Bethesda, MD). Then thin sections were treated with fluorescein isothiocyanate (FITC)-conjugated goat IgG anti-mouse IgM (Cappel, West Chester, Pennsylvania), which was used as a second antibody. Normal rabbit serum was used as a negative control.

The precise location of the D28 epitope in the kidney was determined by double-staining sections with the D28 ascites as described above and with either rhodamine-conjugated Lotus tetragonolobus (LTA) lectin (E-Y Laboratories, San Mateo, California), rhodamine-labeled Peanut agglutinin (PNA) lectin (E-Y Laboratories), or rhodamine-labeled Dolichos biflorus agglutinin (DBA) lectin (E-Y Laboratories). LTA lectin binds specifically to the proximal tubules, PNA lectin to the distal tubules and collecting ducts, and DBA lectin only to the collecting ducts of the murine kidney [9,19].

Results

The D28 mAb detects a specific epitope found in the BM of the proximal tubules : In 1-day-old D2 mice, only the proximal tubules but not the distal tubules and the collecting ducts of the kidney were found to be dilated. In older D2 mice, 3 and 8 weeks of age, no dilation was found in any region of the tubules nor the collecting ducts. One of the anti-kidney mAbs, D28, produced from spleen cells of immunized D2 mice reacted with the BM of at least some renal tubules in mice of all ages while it did not bind to any other parts of the kidney such as the Bowman's capsule, loop of Henle, or collecting

Table 1. The distribution of the D28 epitope in the tubular BM of D2, B6, D2-*pcy* and B6-*cpk* mice

strain	region		proximal region (LTA-positive)	distal region (PNA- or DBA-positive)
	age			
D2 and B6	the examined all ages		+	—
D2- <i>pcy</i>	1-day		+	—
	3-week		+	—
	8-week		—	—
B6- <i>cpk</i>	1-week		±	—
	2-week		—	—
	3-week		—	—

+ : show the presence of D28 epitope
± : show the reduced D28 epitope

— : show the absence of D28 epitope

ducts. Three BM components, collagen IV, laminin, and the core protein of HSPG, were detected in the BM of all tubules, as well as in the collecting ducts, Bowman's capsules, mesangial matrix and the capillary walls of glomeruli (not shown). The control mAb against the subclass, anti-Thy1.1 IgM, bound to only intraglomerular BM but did not react with tubules and vessels (not shown). In order to determine the precise location of the D28 epitope in the tubules, double-staining was carried out on frozen sections of the kidney employing the D28 mAb and either LTA, PNA or DBA lectin. As shown in Fig. 1 for 8-week-old mice, the D28 mAb bound to the BM of all LTA-positive proximal tubules (Figs. 1 A, B) but not to any PNA-positive distal tubules (Figs. 1 C, D) or DBA-positive collecting ducts. This staining pattern occurred in mice of all ages, and it demonstrated that the D28 mAb binds specifically to the BM of the proximal tubules in the kidney.

We then examined the tissue distribution of the D28 epitope in 8-week-old D2 mice. The three BM components were detected in the BM of the skin, ovary, fallopian tube, testis, lung and small intestine (not shown). However, the D28 mAb did not react with these tissues, nor did it react with rat kidney (not shown). These findings show that the D28 mAb recognizes an epitope in the BM of the proximal tubules that is specific to the above described region of the tissue of special species.

Distribution of the D28 epitope in the kidney of adult-type PKD mice : In 1-day-old D2-*pcy* mice, segmental dilation was found in the DBA-positive collecting ducts and also in the

tubular regions, the LTA-positive proximal and the PNA-positive distal region. The distribution of the D28 epitope was the same between D2 and D2-*pcy* mice ; it was found in the BM of the proximal tubules, which were identified by the presence of LTA-binding sites (Figs. 2 A, B) and the absence of PNA-binding sites (Figs. 2 C, D) and DBA-binding sites (not shown).

In 3-week-old D2-*pcy* mice, renal tubules were more enlarged than in 1-day-old mice. The D28 mAb bound only to some of the BM of the proximal tubules, which were again LTA-positive (Figs. 3 A, B) and PNA-negative (Figs. 3 C, D) and DBA-negative (not shown). The D28 mAb bound to some of the BM of the proximal tubules, which were again LTA-positive (Figs. 3 A, B) and PNA- and DBA-negative (not shown) but not to the tubular BM of the distal region, PNA- (Figs. 3 C, D) and DBA-positive (not shown).

In 8-week-old D2-*pcy* mice, numerous large renal cysts were found, and some Bowman's capsules had also become enlarged. It was difficult to identify the origin of these cysts because none of them bound LTA lectin while some of them bound PNA lectin and DBA lectin. Although the epithelial cells of the cysts were flatter than those of normal tubules, the three BM components, collagen IV, laminin and the core protein of HSPG, mentioned above were detected in the BM of the large cysts by specific antibodies (Figs. 4 A, B, C). The D28 epitope (Fig. 4 D) could not be detected in the BM which were supposed to be the proximal tubules since these larger cysts did not react with PNA lectin and DBA lectin (not

shown). We concluded that the distribution of the D28 epitope along the tubules was not altered by the *pcy* mutation and, in particular, that the epitope disappears from the BM of the bigger cysts.

Distribution of the D28 epitope in the kidney of infant-type PKD mice: In B6-*cpk* mice, cyst formation starts before birth in the proximal tubules, and numerous cysts emerging from proximal tubules and collecting ducts were found in 1-, 2- and 3-week-old mice [12]. Distribution of the D28 epitope in the kidney of B6-*cpk* mice was essentially the same as in D2 and D2-*pcy* mice (not shown). The reactivity to the D28 mAb was already reduced in the LTA-positive proximal cysts of 1-week-old B6-*cpk* mice (not shown), but almost completely lost in those of 2- and 3-week-old mice (Figs. 5 A, B) while collagen IV, laminin and HSPG were present in the BM of the cysts of mice of all ages (not shown). Then the D28 epitope never be observed in the BM of either PNA-positive (Figs. 5 C, D) or DBA-positive (not shown) cysts.

Distribution of the D28 epitope in the kidney of mice with other renal abnormalities: We studied the distribution of the D28 epitope in the kidney of Daunomycin-treated mice to see whether its disappearance was specific to expanding cysts. As had been reported on rats [15], treatment with Daunomycin resulted in a dilation of the collecting ducts and the tubules in both the proximal and the distal region (not shown). The D28 epitope persisted in the BM of the dilated proximal tubules whereas the PNA-positive distal tubules did not react with the D28 mAb (not shown). The D28 epitope was also found with its usual distribution pattern in the kidney of NC mice in which deposits of the immune complexes are present in the glomeruli (not shown).

Discussion

The BM of the kidney has several important functions: it is an adhesive substrate in the glomeruli and tubules, plays a pivotal role in embryonic development and organogenesis of the kidney and is involved in permselective functions [1].

Befitting the complex structure and function of the kidney, the composition of the renal BM is heterogeneous and has been identified

with the help of specific monoclonal and polyclonal antibodies [1,30]. Among these mAbs and polyclonal antibodies, the LAM-2 [21] and Lam-2 [20] mAbs, the 3M-1glycoprotein polyclonal antibody [5,24], and our novel D28 mAb have a similar specificity in that they do not react with the BM of the distal tubules. Unlike the other antibodies, however, the D28 mAb does not react with the BM of the Bowman's capsule [5,20,21,24]. It is therefore highly specific to BM of the proximal tubules. Thus, the D28 mAb revealed the unique BM structure of the proximal tubules.

The formation of renal cysts is accompanied by increased epithelial cell proliferation and altered transtubular fluid transport [13,14]. BM abnormalities play an important role in cyst formation and bring forth changes in the functioning of the kidney. An increased turnover and a decrease in HSPG was observed in the renal BM of PKD patients and drug-induced PKD rats [3,4,32]. In infant-type PKD mice, B6-*cpk*, abnormalities of the BM in the cyst were found [8,12,25]. The gene expression of collagen IV and laminin seemed likewise abnormal [8]. We showed that the D28 epitope, which is the specific structure in the BM of the proximal tubule, disappears from that of the cysts as they grow bigger although BM components such as collagen IV, laminin and proteoglycan do not disappear. Our results provide further evidence that the formation of cysts is accompanied by a structural change in the BM, particularly in the proximal region of the tubules. These changes in D28 epitope and components occur similarly in the two murine models of PKD, B6-*cpk* and D2-*pcy*. Our study also showed that the D28 mAb detected in the BM structure was specific to the formation of cysts, as the D28 epitope did not disappear from the tubules even when they became dilated through the drug treatment. The solubilized BM components, collagen IV and laminin, did not contain the D28 epitope since an enzyme-linked immunosorbent assay could not demonstrate a specific binding of the D28 mAb to these molecules. Although the exact molecular change is not clear at present, the cyst-related change in the BM of the proximal tubules seems to be specifically localized, since the adjoining BM remained positive for the D28 epitope. The D28 mAb will be a useful tool to investigate the cause and the

process of renal cyst formation in the mouse.

The authors gratefully acknowledge the technical contributions of Noboru Ogiso. This work was supported in part by two grants-in-aid for scientific research from Fujita Health University and from the Ministry of Education, Science and Culture of Japan.

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新らたな抗基底膜モノクローナル抗体であるD28によって検出された マウス多発性嚢胞腎症の近位尿細管基底膜の特異的な変化

長尾 静子・小山 洋一*・高橋 久英

藤田保健衛生大学総合医科学研究所実験動物センター

*藤田保健衛生大学総合医科学研究所応用細胞研究部門

我々は、マウス多発性嚢胞腎症 (PKD) における腎臓の異常を解析するために、成人型 PKD マウスである DBA/2FG-*pcy* の腎臓を免疫した DBA/2マウスから得た脾臓と、P3U1ミエローマ細胞を融合し、マウスの腎臓に反応するモノクローナル抗体を作製した。得られたクローンのひとつであるD28は、DBA/2マウスとDBA/2FG-*pcy* マウスの近位尿細管基底膜に特異的に反応した。しかし、D28抗体はマウスの腎臓の他の部位や、皮膚、卵巣、卵管、精巣、肺および小腸等の腎臓以外の組織には、反応しなかった。基底膜構成物質であるコラーゲン IV、ラミニンおよびプロテオグリカ

ンのコア蛋白は、成人型PKDマウスであるDBA/2FG-*pcy* と乳幼児型 PKD マウスである C57BL/6J-*cpk* に形成された腎嚢胞の基底膜に、検出されたが、D28エピトープは認められなかった。また、他の腎臓疾患では、D28エピトープは近位尿細管基底膜から消失しなかった。これらの結果は、近位尿細管に形成される腎嚢胞が、近位尿細管に特異的な構造の異常に関与することを示唆している。この様に、D28モノクローナル抗体は、遺伝性 PKD における基底膜に関連した異常を解析するために有益な道具となるであろう。

Explanation of Figures

Fig. 1. Location of the D28 epitope in the kidney of 8-week-old D2 mice. Frozen sections were double-stained either with LTA lectin (A) and D28 mAb (B) or for PNA lectin (C) and D28 mAb (D). Arrows in (A) and (B) indicate the LTA-positive proximal tubules and arrowheads in (C) and (D) indicate the PNA-positive distal tubules.

Fig. 2. Location of the D28 epitope in the kidney of 1-day-old D2-*pcy* mice. Frozen sections were double-stained either with LTA lectin (A) and D28 mAb (B) or for PNA lectin (C) and D28 mAb (D). In (A) and (B), arrows indicate LTA-positive proximal tubules. In (C) and (D), arrowheads indicate PNA-positive distal tubules, and asterisks indicate PNA-negative tubules.

Fig. 3. Location of the D28 epitope in the kidney of 3-week-old D2-*pcy* mice. Frozen sections were double-stained either with LTA lectin (A) and D28

mAb (B) or for PNA lectin (C) and D28 mAb (D). Arrows in (A) and (B) indicate the LTA-positive proximal tubules and arrowheads in (C) and (D) indicate the PNA-positive distal tubules.

Fig. 4. Indirect immunofluorescence staining of BM components in the kidney of 8-week-old D2-*pcy* mice. (A) collagen IV, (B) laminin, (C) core protein of HSPG, (D) D28. Arrows indicate the BM.

Fig. 5. Indirect immunofluorescence staining of the D28 epitope in the kidney of 2-week-old B6-*cpk* mice. Frozen sections were double-stained either with LTA lectin (A) and D28 mAb (B) or for PNA lectin (C) and D28 mAb (D). Arrows in (A) and (B) indicate the LTA-positive proximal tubules and arrowheads in (C) and (D) indicate the PNA-positive distal tubules.

Fig. 1.

Fig. 2.

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