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Differential Expression of Mouse α5(IV) and α6(IV) Collagen Genes in Epithelial Basement Membranes[1]

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We first completed the primary structure of the mouse α5(IV) and α6(IV) chains, from which synthetic peptides were produced and α chain–specific monoclonal antibodies were raised. Expression of collagen IV genes in various basement membranes underlying specific organ epithelia was analyzed by immunohistochemical staining using these monoclonal antibodies and other antibodies from human and bovine sequences. It was possible to predict the presence of the three collagen IV molecules: $[\alpha1(IV)]_2\alpha2(IV)$, α3(IV)α4(IV)α5(IV), and $[\alpha5(IV)]_2\alpha6(IV)$. In skin basement membrane two of the three forms, $[\alpha1(IV)]_2\alpha2(IV)$ and $[\alpha5(IV)]_2\alpha6(IV)$, were detected. The α3(IV)α4(IV)α5(IV) molecule was observed as the major form in glomerulus, alveolus, and choroid plexus, where basement membranes function as filtering units. The molecular form $[\alpha5(IV)]_2\alpha6(IV)$ was present in basement membranes in tubular organs such as the epididymis, where the tubes need to expand in diameter. Thus, the distribution of the basement membranes with different molecular composition is consistent with tissue-specific function.

Key words: basement membrane, collagen IV, epithelium, gene expression, testis.

Epithelial cells specify tissues or organs. These cells, held together by intercellular junctions, cover the entire surface of multicellular organisms to protect the inside of the body from the outside. At their basal side a thin layer of extracellular matrix called the basement membrane serves to attach the epithelium to the underlying connective tissue. Basement membranes are composed mainly of collagen IV, glycoproteins called laminin, nidogen, and others, and heparan sulfate proteoglycans (*1–3*). Basement membranes are not only found at the surface of the body but also inside; the epithelial lining continues its structure into the inside of the body (*4*). For instance, the surface of the gastrointestinal tract and pulmonary tract are a continuation of the epithelial lining, underneath which the sheet-like structure of the basement membrane is always present. There are many parenchymal organs inside the body (*4*). Most of them are composed of organ-specific epithelial and mesenchymal cells and extracellular matrices. All organ-specific epithelial cells are attached to various types of basement membranes. Some basement membranes provide a bordering barrier for regulating the exchange of macromolecules between the extracellular matrix and the other tissues. Other basement membranes are also found between adjacent epithelial layers (*4*). Such examples are found in the renal glomeruli, lung alveoli, liver sinusoids, brain capillaries, astrocytes, *etc.* In these cases the basement membranes are thicker and more complex, presumably as a result of the fusion of two different types of basement membranes.

In humans six distinct genes have been identified as belonging to the collagen IV gene family (*3–5*). They are organized into three sets, COL4A1/COL4A2 on chromosome 13, COL4A3/COL4A4 on chromosome 2, and COL4A5/COL4A6 on X-chromosome (*3*, *5*, *6*). Within each set the genes are arranged head-to-head, and their expression is regulated by bidirectional promoters between the genes. Transcriptional regulation of the COL4A1/COL4A2 set has been well characterized (*7*, *8*). We reported that the transcription of COL4A4 and COL4A6 seems to be controlled by two alternative promoters (*9*, *10*). Whereas collagen IV molecules composed of α1/α2 chains are widely distributed, molecules comprising combinations of the other four chains, α3–α6, are important components of specialized basement membranes (*5*, *11*, *12*). We hypothesized that α3–α5 chains and α5 and α6 chains form heterotrimeric molecules (*5*). This helps explain the observation that the kidney and skin basement membranes from patients with Alport syndrome caused by mutations in the α5-coding gene, COL4A5, are

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; RACE, rapid amplification of cDNA ends; NC1, none-collagenous domain 1; COL, collagenous.

defective in the α3, α4, and α6 chains together with the α5 chain (*5*, *11*, *13*).

We isolated full-length cDNAs for the mouse α5(IV) and α6(IV) chains and determined their complete primary structures. Based on these primary structures we raised chain-specific monoclonal antibodies against synthetic peptides. We analyzed collagen IV gene expression in epithelial basement membranes from various mouse tissues using monoclonal antibodies against mouse, human, and bovine sequences. The results suggest that the composition of collagen IV molecules in various tissues is consistent with the biological function of various basement membranes.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones—To obtain full-length cDNAs for α5(IV) and α6(IV) chains, we screened a mouse lung library (Clontech, ML1046a) constructed by oligo(dT) and random primers and inserted into λgt10 vectors. Human cDNA (TM-51) previously cloned was used as the probe (*14*). For the initial screening we lowered the stringency of hybridization and washing for the cross-hybridization (*15*). Having obtained the initial mouse clones for both α5 and α6 chains, we used the regular conditions of hybridization to extend them in the 5′ direction (*15*). Fourteen overlapping cDNA clones for α5 and 12 for α6 were isolated to cover the coding regions. Nucleotide sequencing analysis was performed by the dideoxy chain termination technique on double stranded pBluescript II vectors (Stratagene, La Jolla, CA). We used the fluorescence-labeled dye-terminator method and an ABI automatic sequencer (373A).

5′ RACE—To determine the transcriptional start site we performed 5′ RACE, for which 5′ RACE System version 2 kit (GIBCO BRL) and accompanying protocol were used (*16*). We isolated total RNAs from the lung, the kidney, and the mouse transformed keratinocyte, PAM cells by the guanidinium thiocyanate extraction method (*15*). Two primers were synthesized for this purpose: primer MA5-12 (5′-ATCCTGGTAAACCTGGATGACC-3′) and primer MA5-13 (5′-CTGGATGACCTTCTAAACCTGG-3′). The first-strand cDNA was synthesized by SuperScript II RT (GIBCO BRL) from primer MA5-12 by use of total RNAs. The synthesized cDNA was purified by Prep A-gene (Bio-Rad). This material was used for PCR between primer MA5-13 and the hybrid primer under the following conditions: 94°C for 60 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. At the end of the last cycle, the sample was further incubated at 72°C for 5 min. The products were electrophoresed on 2% agarose gels, blotted onto Hybond N^+ (Amersham), hybridized with ^{32}P-labeled specific probe TSP8E2 (*15*). For cloning the PCR product, the TA cloning system (InVitrogen) was used (*17*).

Isolation of Genomic DNA Fragments—When overlapping cDNAs for α6 were sequenced and characterized, a stretch of nucleotides was missing in one cDNA clone but not in the other, suggesting the possibility of alternative splicing. To confirm this possibility, we screened a genomic library, 129SVJ Mouse Genomic Library (Stratagene, #946309), inserted in Lambda FIXII vector with TK2-4 Eco-Bam probe (*15*). Two mouse genomic clones, gSY1 and gSY2, were isolated and characterized by Southern blotting and sequencing.

Production of Monoclonal Antibodies—Complete amino acid sequences for α5 and α6 chains were deduced from the overlapping cDNAs. We synthesized two peptides, ATVDM-SDMFNKPQSETLC (residue numbers 1737–1754 in the α5 chain) and TTVEERGQFREEPVSETLC (1737–1754 in the α6 chain) within the NC1 domains of the α5(IV) and α6(IV) chains (locations indicated in Figs. 1 and 2B), respectively, and used them as antigens to raise monoclonal antibodies. We recently developed a new efficient procedure, the rat lymph node method, for raising monoclonal antibodies (*18*). Briefly, WKY/NCrj rats (Charles River Japan, Yokohama) were immunized in the hind foodpads with 100 μg of hemocyanin-coupled synthetic peptide emulsified with Freund's complete adjuvant. Three or four weeks later the rats were sacrificed; and lymphocytes, obtained from the medial iliac lymph nodes of the rats, were fused with mouse myeloma cells (SP2/0-Ag14). Supernatants from hybridoma cultures were screened by ELISA, using hemocyanin-free peptides from individual α(IV) chains.

The initial screening was performed by ELISA with the synthetic peptides themselves and other peptides of the same region from all α(IV) NC1 domains. More than two thousand clones were screened, and we picked 245 positive clones that reacted specifically with the α6(IV) peptide. Out of almost the same number, 191 clones were positive for the α5(IV) peptide; and a second screening was performed by the indirect immunofluorescence method using mouse tissue sections. Thus, two clones, M54 and M69, were established as α5 chain–specific and α6 chain–specific monoclonal antibodies, respectively, out of the respective 5 and 13 positive clones for both ELISA and immunofluorescence. Other monoclonal antibodies for the human α1(IV), α2(IV), α3(IV), and α4(IV) chains, previously characterized (*11*, *12*), also reacted with mouse tissues, and therefore we used them in the experiments.

Tissue Samples—Tissue samples from 129Sv mice of embryonic day 16, and those from the mice at 0–10 days and 2–72 weeks after birth were obtained. Under deep anesthesia the mice were killed, and various organs and tissues were dissected out. For immunostaining for collagen IV chains, tissue samples were put into a cryomold filled with OTC compound (Miles) and frozen instantly by adding liquid nitrogen (*19*). Others were fixed in formalin or Bouin's solution and embedded in paraffin.

Immunohistochemistry of Type IV Collagen—The tissue distribution of collagen IV chains was examined by indirect immunofluorescence method (*19*). Serial frozen sections of approximately 4 μm thickness were mounted onto silanized slide glasses. After drying for 1 h, the sections were fixed with acetone for 10 min. To expose epitopes for antibodies, we denatured the tissue sections with 6 M urea in 0.1 M glycine-HCl buffer (pH 3.5) for 20 min at room temperature for staining using H12 [specific for α1(IV) chain], H22 [specific for α2(IV) chain], H31 [α3(IV) chain], H43 [α4(IV) chain], H53, M54 [α5(IV) chain], and M69 [α6(IV) chain] antibodies, and for 0–10 min for B66 [specific for α6(IV) chain] staining. After washing with PBS for 15 min, nonspecific staining was blocked by incubating the sections with 5% skim milk for 10 min. Monoclonal antibodies were added onto the sections, which were then incubated for 1 h at room temperature. PBS was added to wash out excess antibodies, after which the sections were reacted with

FITC-labeled goat anti-mouse IgG (Organon Teknika-Cappel) for 60 min at room temperature. They were then washed with PBS for 15 min, coverslipped, and observed by fluorescent microscopy. To distinguish the testis from the epididymis, we applied a monoclonal antibody (1A4, Sigma) that reacts specifically with smooth muscle cell actin for staining by the direct fluorescence antibody method (*20*). Thereby it was possible to tell the difference between the seminiferous tubules and the ductus epididymidis. Cells involved in spermatogenesis and extracellular matrix were differentially stained with hematoxylin eosin and Azan.

RESULTS AND DISCUSSION

cDNA Cloning for Mouse α5(IV) and α6(IV) Chains—Northern-blot hybridization using total RNA from the mouse lung showed that mRNAs for the α5(IV) and α6(IV) chains were both approximately 6.8 kb (data not shown). Previously isolated and characterized cDNA, TM-51, coding for the COL1 domain of the human α6(IV) chain, was used for screening mouse DNAs from the lung library (*14*). Clone TK1 was isolated first, and the remaining 11 clones, TK2, 4, 6, 8, 14, 15, 22, 27, 29, 30, and 31, were obtained by overlapping. There was still a gap in composite cDNAs, which was covered by RT-PCR between the two primers, TKSP5 (5′-TCATTGCGTCTAGCATAGTG-3′) and TKSP7 (5′-TACAAGGACCTCCAGGATTT-3′). Thus, TK32 and TK33 were subcloned and sequenced.

During the screening of α6 cDNAs we accidentally identified a cDNA, TSP2, for the α5(IV) chain, which was used for isolating the remaining 11 clones, TSP5, 7, 8, EK1, 2, 5, 6, 7, 8, TKRC10, TK12, to cover most of the entire coding region of α5. A missing sequence was covered by RT-PCR between the two primers MA5-4 (5′-AATAGGCCAACCTGGCTTAC-3′) and MA5-5 (5′-CCAGGTTGTCCAGGAAATCC-3′); and two subclones, TK35 and TK36, were isolated and sequenced.

Primary Structure of Mouse α5(IV) and α6(IV) Chains—The twelve isolated cDNA clones encompassing 6.8 kb mRNA provided an open reading frame that encoded the mouse α5(IV) polypeptide of 1,691 amino acid residues. As shown in Fig. 1, we found a tentative 26-residue hydrophobic leucine-rich signal sequence located at the amino end of the α5 chain. The signal peptide cleavage site was predicted by use of the computer program PSORT II (*21*). The deduced amino acid sequence of the α5(IV) chain started with a 15-residue noncollagenous segment with 6 cysteines at the amino terminus, followed by a 1,421-residue collagenous domain and a 229-residue carboxyl terminal NC1 domain. The collagenous G-X-Y repeat sequence was interrupted by short noncollagenous regions at 22 locations. The interruptions differed in length from 1 to 13 residues. The carboxy terminal NC1 domain had 229 residues including 12 cysteines. When the mouse α5(IV) was compared with the human α5(IV) (*22*), the mouse α5(IV) showed an insert of 6 amino acid residues, GPPGFQ (amino acid numbers 1336–1341 in Fig. 1). The overall amino acid identity was 90.4%. Further, the location and the length of the 22 imperfections of the collagenous G-X-Y repeat sequence were exactly the same as those of the human α5. Of the 23 cysteinyl residues in the mouse α5 chain, 19 were located exactly at the same positions as those of the human sequence.

The predicted α6(IV) chain also contained 1,691 amino acid residues, which included 15 residues of the tentative signal peptide (*14*). The central COL1 domain contained 1,417 residues, and the collagenous sequence was interrupted 24 times by various noncollagenous segments. Most of the interruptions were at the same locations as those in the human α6 chain. When the domain structures of the mouse and human α6 chains were compared, the mouse α6 showed insertional amino acid residues at two locations, one at amino acid number 40 (Fig. 1) and the other at numbers 461–463, but three residues were missing between 314 and 315. The carboxyl terminal NC1 domain contained 228 residues with 12 cysteines. Comparison of the amino acid sequence of the mouse and human α6 chains showed 79.6% identity. Most of the imperfections of the collagenous G-X-Y repeat were observed at the same locations. The locations of the 21 cysteinyl residues were also exactly the same as those of the human sequence.

Monoclonal Antibodies—To raise mouse-specific monoclonal antibodies for tissue staining, we adopted the strategy of using synthetic peptides to make monoclonal antibodies by the rat lymph node method, and of screening by ELISA and tissue staining (*18*). Locations of the two synthetic peptides for the mouse α5 and α6 chains are indicated in Fig. 2, A and B. ELISA assay first and tissue staining second using frozen sections were used for screening clones. The two positive clones, M54 and M69, of the antibodies showed a specific staining pattern for α5(IV) and α6(IV) chains, respectively in basement membranes in mouse kidney sections. However, neither of them reacted with human tissues at all, which could be due to the sequence differences as shown in Fig. 2B; and the staining activity toward the mouse tissues was not so strong. Monoclonal antibody H53 (*19*, *23*), which was raised against a peptide within the human α5COL1 domain (Fig. 2A), reacted with the mouse tissues exactly as M54. We previously used native bovine α(IV) NC1 domains as antigens and screened by tissue staining for the α6 chain. A positive monoclonal antibody showed specific staining for the α6 chain such as in basement membranes in Bowman's capsules, and we named the clone B66 (*12*). It reacted with bovine and human tissues, and fortunately also reacted with mouse sections as well. Therefore, we decided to use H53 and B66 antibodies for α5 and α6, respectively, for further experiments. Rat antibodies H53, M54, M69, and B66 were of the IgG 2a, κ, IgG 2a, κ, IgG1, κ, and IgG2b, κ type, respectively.

Distribution of Mouse α5(IV) and α6(IV) Chains in Epithelial Basement Membranes (Fig. 3)—The distribution of the α5(IV) and α6(IV) chains in epithelial basement membranes was examined extensively throughout the mouse body. Basement membranes under stratified squamous epithelial cells in the skin were stained by the α5(IV) and α6(IV) antibodies, as was the case with α1(IV) and α2(IV) antibodies (Fig. 3, *11*). However, α3(IV) and α4(IV) chains were not expressed in general here. This staining pattern did not change much in the skin covering the entire surface of the body. However, when the keratinocytes in the sweat glands and hair roots were examined, the molecular composition was different. Interestingly, α5 and α6 chains had disappeared from the basement membranes along the sweat glands; therefore, α1 and α2 chains were the major chains (Fig. 3, arrowhead). Unexpectedly, in basement membranes in hair root, the major molecule comprised α3,

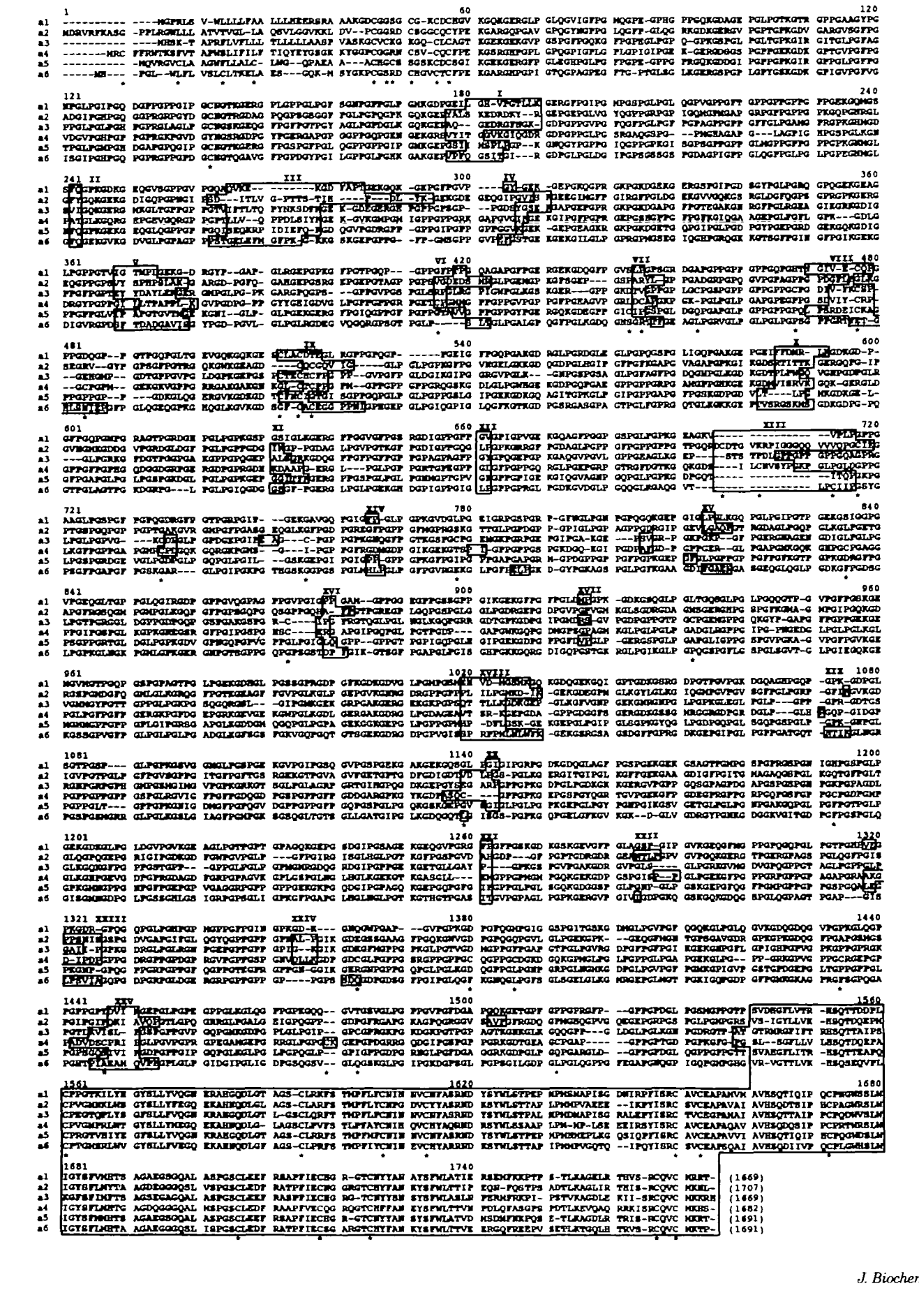

Fig. 1. **Comparison of amino acid sequences of the six α(IV) polypeptides of the mouse.** Sequences for α5(IV) and α6(IV) chains deduced from the overlapping cDNAs in this paper are compared with those of the mouse α1 (*31*), α2 (*32*), α3 (*33*), and α4 (*33*) chains previously reported. Locations of the interruptions of Gly-X-Y repeat sequence and the carboxyl NC1 domain are indicated by boxed-in areas. Representative interruptions are numbered from I to XXV. Amino acid sequences were aligned, and the locations that were not best aligned are indicated by short bars; therefore, the numbers above the sequences do not show the amino acid numbers counted from the amino terminal ends. The figures in parentheses appearing at the end of the individual sequences indicate total amino acid residues. Locations of cysteinyl residues in at least one of the α chains are indicated by asterisks underneath the sequences.

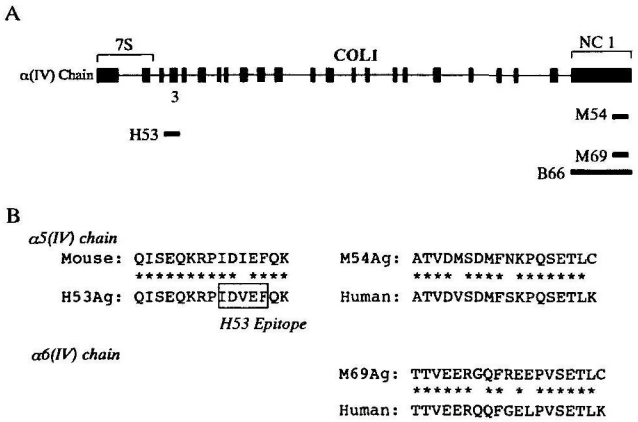

Fig. 2. **Relative location of the antigens within the α5(IV) and α6(IV) chains.** A: Schematic representation of the α(IV) chain by a bar. Filled boxes indicate the location of imperfections and NC domains located at both ends, whereas the thin lines represent the Gly-X-Y repeat sequences. Approximate positions of the peptide sequences used for monoclonal antibodies M54, H53, and M69 are shown by short bars. The NC1 fraction of bovine kidney basement membrane was used for monoclonal antibodies B66 and screened by immunofluorescence to obtain α6 antibody; therefore the bar indicates the NC1 region. B: Amino acid sequences of antigen peptides and comparison of mouse and human sequences in the corresponding regions. For α5 and α6 chains, we used M54 and M69 mouse sequences, respectively, for antibody production, which are compared with the human sequence. A peptide of the human α5COL1 domain was used for the production rat antibody H53, and its epitope was determined by Geysen's method (*12*).

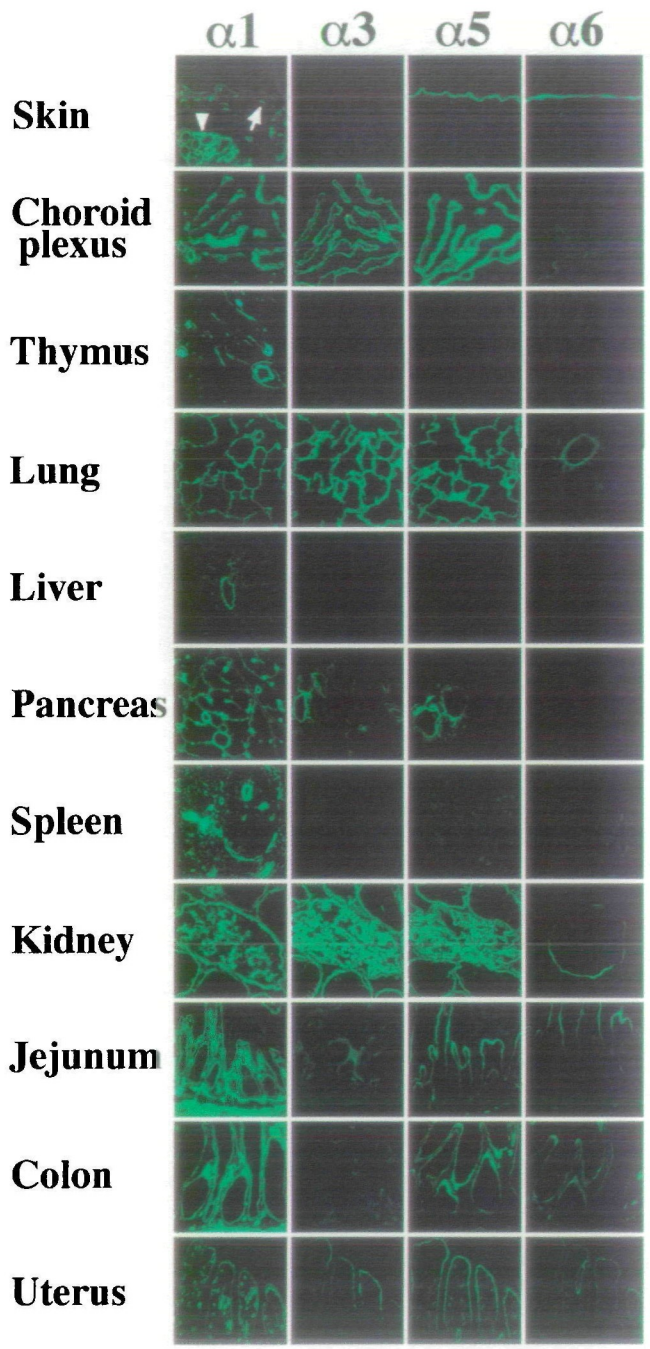

Fig. 3. **Differential distribution of the six α(IV) chains in epithelial basement membranes of various tissues.** Monoclonal antibodies for the six chains were used for the individual tissue staining. Since the staining pattern was always the same with α1 and α2 monoclonals and with α3 and α4 monoclonal antibodies, only α1 and α3 staining patterns are shown in the figures. Note that when the staining patterns for α3 and α5 appear to be the same, for instance, in brain (choroid plexus), lung (alveoli), pancreas (interlobular ducts), kidney (glomeruli), and uterus (uterine glands), it indicates the presence of the α3α4α5 molecule; whereas a similar staining pattern between α5 and α6 as in the skin, jejunum, colon, and uterus, shows the presence of the $[\alpha5]_2\alpha6$ molecule.

α4, and α5 chains (data not shown). Thus, α(IV) chain composition was switched along the skin basement membranes in various places. The epithelia and basement membranes of the exterior continue into the gastrointestinal and respiratory tract through the mouth. Basement membranes under stratified squamous epithelial cells within the oral cavity and pseudostatified columnar cells in the bronchi had a similar pattern to that of the skin. At the blind end of the airway, where gasses and various materials are transported and exchanged, α3, α4, and α5 chains were expressed in the alveolar basement membranes under the lung epithelial cells. However, in basement membranes along capillaries we detected only the common molecular form of α1/α2 chains (Fig. 3, arrow).

The inner side of the gastrointestinal tract is covered by a lining of columnar epithelial cells, underneath which basement membranes are attached. They were reactive with monoclonal antibodies specific for α1, α2, α5, and α6. This pattern did not change in the basement membranes in the gastrointestinal tract in general. But closer and more precise observation showed variation in the chain composi-

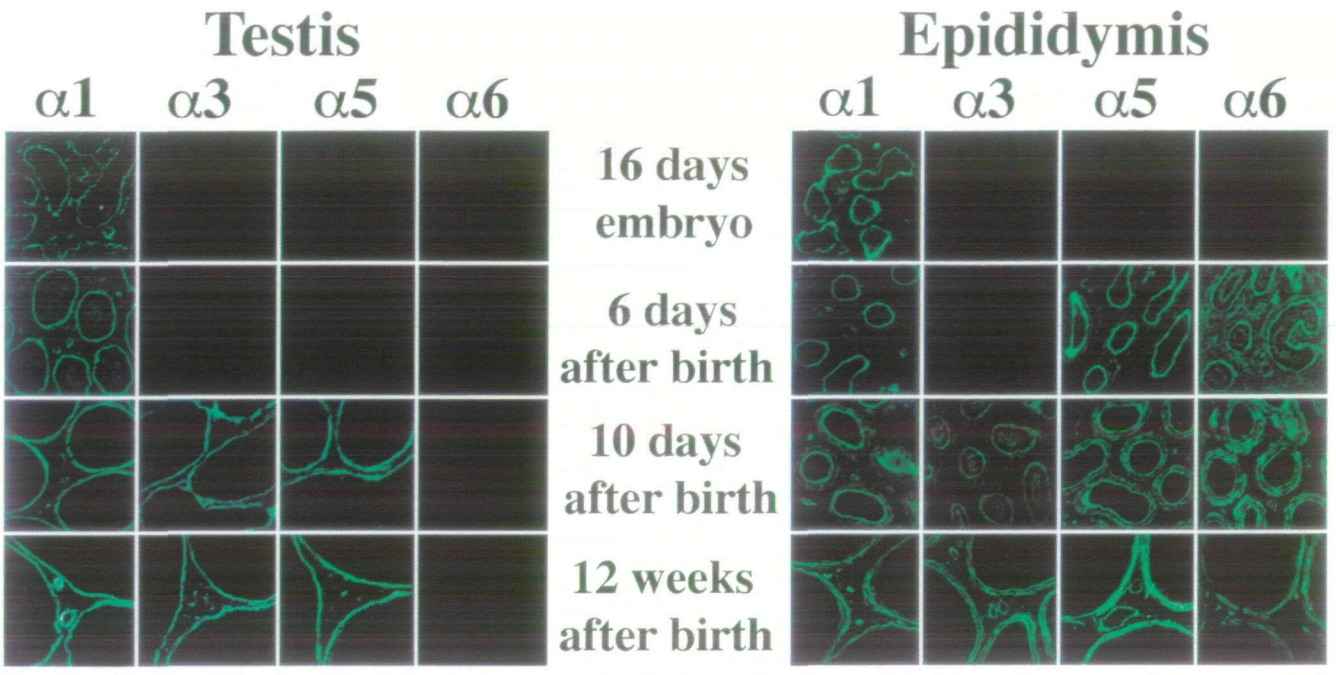

Fig. 4. **Distribution of the six α(IV) chains during mouse development in testis and epididymis.** In the specimens from 12-week-old mice, basement membranes in the testis were stained heavily by α1–α5 antibodies, but α6 antibody did not stain them, as shown in the bottom four figures of the left panel, suggesting the presence of only two molecular forms, α1/α1/α2, α3/α4/α5. The epididymis of the same stage was stained by α6 antibody as well, indicating the existence of an additional molecule, $[\alpha5]_2\alpha6$, in the epididymis of this stage.

tion of the collagen IV molecules in these basement membranes: the basement membranes beneath the villi were positive for α3 and α4 chains as well as for α1, α2, α5, and α6, suggesting the presence of the three molecular species of α3/α4/α5, α1/α1/α2, and α5/α5/α6 (*5*), whereas in the crypts α1 and α2 chains became the major components as α5 and α6 staining became weaker. This difference in distribution could be related to the different functions of the epithelium, *i.e.*, absorption in the villi and secretion of various enzymes and other molecules in the crypts (*4*).

In organs derived from the gastrointestinal tract, such as the liver, α1 and α2 were stained in Disse's space; but other α chains were not stained. Basement membranes under pancreatic acinar cells and islets of Langerhans, and splenic pulp showed the pattern just described. Thus, the epithelial cells in the parenchymal organs, which have their specific functions, did not express α5 and α6 chains. But basement membranes of the interlobular ducts in the pancreas and bile ducts in the liver, for instance, which might have a common function, were composed of α3, α4, and α5 chains.

Basement membranes under the transitional epithelia in urinary bladder showed the same α1/α2/α5/α6 pattern as the skin basement membranes. Ovarian follicles showed the unique pattern of being positive for all six α(IV) chains. As we reported before (*11*), glomerular basement membranes in human kidney showed an α1/α2/α3/α4/α5 positive pattern, suggesting that they have molecules of α1/α1/α2 and α3/α4/α5. We think that the filtering function of human glomerular basement membranes could be due to the ultrastructure meshwork composed of collagen IV molecules with α3/α4/α5. But basement membranes in Bowman's capsule demonstrated an α1/α2/α5/α6 pattern, which indicated that there were two types of molecules, α1/α1/α2 and α5/α5/α6. The molecular distribution in the mouse kidney appeared to be the same as that in the human. Nevertheless, the basement membranes close to the urinary pole showed a different molecular form, having α3/α4/α5, although the rest of the basement membranes in the mouse Bowman's capsule were composed of α5/α5/α6. Intriguingly, the presence of α3/α4/α5 molecules is consistent with the appearance of the cuboidal epithelial cells, which were not identified in Bowman's capsules in humans. The subperitoneal basement membrane was stained with all six antibodies, suggesting that the three molecular forms are present in the peritoneum. Endometrium is covered with simple columnar epithelial cells, and the epithelial lining invaginates deep into the endometrium to form glands. Basement membranes close to the surface were reactive with α3, α4, and α5 antibodies; but α1, α2, α5, and α6 chains were present in those in the uterine glands. This distribution seems to be similar to that in the gastrointestinal tract. Basement membranes within the brain tissue are mostly vascular basement membranes, and all of them were positive only with α1 and α2 antibodies. Basement membranes in the choroid plexus (Fig. 3), where cerebrospinal fluid is secreted, appeared to be composed of α3, α4, and α5 chains.

The present staining pattern of the mouse tissues suggests that there are three molecular forms of type IV collagen, *i.e.*, α1/α1/α2, α3/α4/α5, and α5/α5/α6. Our previous data from human tissues (*23*) and Alport syndrome cases (*19*), where in the latter the α5 chain is missing due to mutations in COL4A5, also support this suggestion. For instance, whereas there are α3, α4, and α5 chains in glomerular basement membranes in the normal kidney, which function as filtering units between plasma and urine, when COL4A5 is mutated, α3 and α4 chains are missing as well as the α5 chain in the Alport glomerular basement mem-

branes, which results in the typical phenotypic changes seen in Alport Syndrome, *i.e.*, diffuse proliferative changes and lamellation in glomeruli (*5*, *19*). In the uterus, α3/α4/α5 chains appeared in basement membranes in glandulae uterinae. Thus, we suspect that basement membranes incorporating α3/α4/α5 molecules would have some functions related to permeability of tissue fluid, because these molecules were identified not in all basement membranes but only in glomerulus, alveolus, and choroid plexus, and peritoneum. Earlier biochemical analysis using transfection experiments proved that the α1(IV) and α2(IV) chains form a collagen molecule of $[\alpha1(IV)]_2\alpha2$ (*24*). The primary structure of the individual α(IV) chains suggests the molecular formation of α3/α4/α5 and α5/α5/α6 (*5*), but proof will be only obtained by biochemistry and/or transfection experiments.

Developmental Changes of the α5 and α6 Chains in Testis and Epididymis—Biochemical analyses of basement membranes of testis and epididymis revealed the presence of various α chain combinations of collagen IV molecules (*25–27*). As the testis and epididymis are parenchymal organs derived from the urogenitary tract, we examined the expression of col4a genes in these organs of adult mice. In specimens from 12-week-old mice, the basement membranes underneath the spermatogonia in the testis were stained heavily by α1–α5 antibodies but not by α6 antibody (Fig. 4). This staining pattern suggests that the basement membranes contained two molecular forms, α1/α1/α2, α3/α4/α5. Within the interstitial space, the arteries, veins and capillaries were obviously stained by α1 and α2 chain antibodies. In contrast, basement membranes in the epididymis in this stage were dually positive for all α(IV) chains, indicating the presence of the three molecular forms of α1/α1/α2, α3/α4/α5, and α5/α5/α6 at this stage. This additional staining could be due to the myoid cells that are preferentially present near the basement membrane zone (*27*).

Next we questioned how these three different molecules are formed in developing testis and epididymis. In early mouse development (16-day embryo), only α1 and α2 chains were detected in both testis and epididymis. However, α3, α4, α5 antibodies (and α6 as well) gave slight staining in the ductuli efferentes (data not shown). A similar staining pattern was recognized in a part of the rete testis and ductus epididymis, suggesting that the genes for α3–6 seem to be expressed in these regions ahead of those in other regions (data not shown).

In the epididymis of 2-day-old mice, expression of α5 and α6 genes was already detected (data not shown); but α3 and α4 expression was recognized only slightly in 6-day-old animals and definitely after 8 days, indicating that α5/α6 expression preceded α3/α4 expression. Further, in 10-day-old mice, myoid cells surrounding the epididymis were expressing α1/α2 and α5/α6 genes, as shown in Fig. 4. Antibodies for α3 and α4 gave positive staining at 10 days, but the pattern was not dually positive, *i.e.*, basement membranes covering epithelial cells were positive but those surrounding myoid cells were negative. In basement membranes in the testis, α6 chain was not detected at any stage; but α3, α4, and α5 chains were all expressed at day 8 after birth and at later stages. This was the time when the dual staining pattern was recognized in basement membranes next to the myoid cells at the periphery of the seminiferous tubules.

As described above, in 8-day-old mice, α3/α4/α5 chains appeared in basement membranes in both testis and epididymis. Meiotic divisions in the testes begin about 8 days after birth and spermatogenesis in the mouse actively starts by 9 days (*28*). It was reported that the blood-testis barrier formation is completed before sperm cells are produced (*29*). The function of the blood-testis barrier is to prevent germ cells that have a haploid gamete, *i.e.*, secondary spermatocytes, spermatids and others from being an antigenic stimulus for production of autoantibodies. It is intriguing that the appearance of α3/α4/α5 molecules in basement membranes in the testis is consistent with the stage when the blood-testis barrier is formed.

In the testis, Leydig cells, which elaborate androgens, chiefly testosterone, increase in population approximately 10 days after birth in male rats (*28*). This is the stage when maturing spermatogonia embark on meiosis to become primary spermatocytes in the rat. This stage corresponds to day 8 after birth in the mouse. Since there must be interactions between Leydig cells and Sertoli cells directly or indirectly through basement membranes, the appearance of a collagen IV molecule of α3/α4/α5 chain composition in the testis at day 8 after birth can be seen as a step in preparing for spermatogenesis through meiotic cell division by remodeling the basement membrane, changing its composition and thus its permeability.

Epithelial cells in the epididymis take charge of the maturation of sperm cells and absorb materials secreted from the testis. Sperm cells attain fertility from the exudate from the epididymis. Furthermore, more than 90% of the secretion from the testis is absorbed by the vas deferens and epididymis (*30*). Therefore, it is possible to think that there might be some functional relationship between a basement membrane integrating α3/α4/α5 chains and filtration of the secretion. This idea can be reasonably adapted to several locations of basement membranes where α3/α4/α5 chains exist, *e.g.*, glomerulus, lung, and cerebral ventricles.

The α5/α5/α6 chain molecular form was not detected in the testis but was observed in basement membranes in the epididymis at day 6 after birth as described above. Seminiferous tubules are furnished with many compartments firmly partitioned individually by septulae. On the other hand, the function of the epididymis is to accumulate and store sperm cells; therefore, the ductus must expand in diameter. We propose that the basement membranes reinforced with molecules of α5/α5/α6 chains serve to support tubular organs needing to expand, such as the epididymis. Similar basement membranes surrounding smooth muscle cells and containing α5/α5/α6 molecules were observed in other tissues such as the gastrointestinal tract and vasculature in tubular organs (*12*). Basement membranes underneath keratinocytes contain α5/α5/α6 molecules and need to expand so as to protect against physical force.

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