Type IV collagen alpha chains of the basement membrane in the rat bronchioalveolar transitional segment

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Summary. In the present study, we have analyzed the α (IV) chain distribution in the subepithelial basement membrane (BM) of the rat pulmonary airway from the bronchi to alveoli. We have furthermore analyzed the α (IV) chain distribution in the subepithelial BM of the bronchioalveolar duct junction (BADJ) using α (IV) chain specific monoclonal antibodies. Our results show that the BM of the bronchial and bronchiolar epithelium contains [α 1(IV)]₂ α 2(IV) and [α 5(IV)]₂ α 6(IV) molecules and confirmed that the alveolar BM consists of [α 1(IV)]₂ α 2(IV) and α 3(IV) α 4(IV) α 5(IV) molecules. There are also small regions in BADJ consisting of only [α 1(IV)]₂ α 2(IV) molecules without α 3(IV) α 4(IV) α 5(IV) and [α 5(IV)]₂ α 6(IV) molecules. Moreover, the bronchioalveolar stem cells (BASCs)—primordial cells for bronchiolar Clara cells and alveolar type II (AT2) cells—lie adjacent to such small regions. These findings suggest that [α 1(IV)]₂ α 2(IV) may be important for the BASCs to self-renew or to self-maintain themselves and that microenvironments produced by α (IV) chains may be important for cell differentiation.

Introduction

The epithelium of the pulmonary airway contains a wide variety of cellular populations, each of which resides in distinct anatomical locations. Basal, secretory, and ciliated cells line the conducting airways of the bronchial tree. The nonciliated columnar Clara cells constitute the majority of the terminal bronchiolar epithelium. The alveolar type I (AT1) and type II (AT2) cells constitute the alveolar epithelium (Kim *et al.*, 2005). These epithelial cells are underlaid by a continuous thin sheet-like extracellular matrix, the basement membrane (BM), which separates the epithelial cells from the stroma. In addition, BM in the lung is found in the vascular endothelium and the smooth muscle cells. BM plays important roles in biological functions such as cell adhesion, differentiation, and migration (Brauer *et al.*, 1989). Furthermore, in the renal glomerulus, BM contributes to the molecular sieve for the selective removal of small molecules from the blood (Hudson *et al.*, 1993).

Type IV collagen, known to be a major structural component of BM, provides a scaffolding for the other BM constituents (Timpl, 1989). Type IV collagen has six genetically different α (IV) chains: α 1(IV)- α 6(IV) (Ninomiya *et al.*, 1995; Sado *et al.*, 1998). The α (IV) chains are structurally divided into three parts: the C-terminal non-collagenous domain (NC1), the N-terminal 7S domain, and the central helical domain (Sado *et al.*, 1998). These assemble to form three different trimeric molecules: [α 1(IV)]₂ α 2(IV), α 3(IV) α 4(IV) α 5(IV), and [α 5(IV)]₂ α 6(IV) (Gunwar *et al.*, 1998; Sado *et al.*, 1998; Boutaud *et al.*, 2000; Borza *et al.*, 2001). Distribution patterns of these molecules in various BMs

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have been analyzed using specific monoclonal antibodies against α (IV) chains. For example, [α 1(IV)]₂ α 2(IV) molecule was detected in the BM of most tissues, and α 3(IV) α 4(IV) α 5(IV) molecule was confirmed in primordial and atretic ovarian follicles (Nakano *et al.*, 2007), a part of the subepithelial BM of the alimentary canal (Sato *et al.*, 2007), glomeruli (Ninomiya *et al.*, 1995), alveoli (Saito *et al.*, 2000) and the choroid plexus (Urabe *et al.*, 2002). Also [α 5(IV)]₂ α 6(IV) molecule was detected in the subepithelial BM of the alimentary canal (Sato *et al.*, 2007), epidermal BM (Hasegawa *et al.*, 2007) and all stages of the ovarian follicle (Nakano *et al.*, 2007).

The α (IV) chains of BM of the alveolar epithelium have been confirmed (Nakano *et al.*, 2001). However, little is known about the distribution patterns of the α (IV) chains in the bronchioles and the bronchioalveolar duct junction (BADJ). In this study, we investigated the distribution of the α (IV) chains in bronchioles and alveoli, using immunofluorescence microscopy with specific monoclonal antibodies, to find the special regions consisting of only [α 1(IV)]₂ α 2(IV) molecule in the BADJ, where bronchioalveolar stem cells (BASCs) exist (Kim *et al.*, 2005; Liu *et al.*, 2008).

Materials and Methods

Animals and tissues

Male Wistar rats of 6 weeks of age were purchased from Japan SLC, Inc.. Under ether anesthesia, their anterior thoracic walls were removed. In order to inflate the lungs, O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo) diluted to 1/2 with phosphate-buffered saline (PBS) was injected through the trachea. The lung tissues were then excised, embedded in O.C.T. compound, frozen in liquid nitrogen, and kept at -80°C . The frozen tissues were cut with a cryostat into 4–5 μm sections, which were kept under -30°C .

Antibodies

The α (IV) chain-specific monoclonal antibodies H11, H22, H31, RH42, H52, b14, and H65 were used in this study (Table 1) (Ninomiya *et al.*, 1995; Borza *et al.*, 2002; Heidet *et al.*, 2003). An affinity purified goat polyclonal antibody against Clara cell 10 protein (CC10 (T18)) (Santa Cruz Biotechnology, Inc., California, USA) and a rabbit polyclonal antibody against surfactant associated protein-C (SP-C (FL-197)) (Santa Cruz Biotechnology, Inc., California, USA) were used. BASCs have been reported to differentiate into Clara cells and AT2 cells and to express both CC10 and SP-C; thus, BASCs are termed

double-positive cells (DPCs) (Kim *et al.*, 2005). DAPI (4', 6-diamidino-2-phenylindole) (Molecular Probes, Oregon, USA) was used for nuclei staining.

Common procedures in immunofluorescent staining

Prior to immunostaining, frozen sections were dried and fixed with acetone for 10 min. Then 6M urea in a glycine-HCl buffer (pH3.5) was applied to the sections for 15 min at room temperature in order to expose the reactive epitopes of the antibodies of H11, H22, H31, H52, and b14. All the washing was performed with phosphate-buffered saline (PBS) for 5 min three times. Just before the first antibody was applied, any non-specific reaction was blocked with 2.5% skimmed milk in PBS for 10 min. After immunostaining as described below, the sections were mounted with Fluoromount (Diagnostic BioSystems).

Immunofluorescent staining for α 1(IV) through α 6(IV) chains

The sections were incubated with the chain-specific antibodies for 60 min at room temperature and washed. An indirect immunofluorescent method using FITC-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Inc., California, USA) was applied to detect the localization of primary antibodies.

Double immunofluorescent staining for α 4(IV) and α 6(IV)

To distinguish the localizations of α 3(IV) α 4(IV) α 5(IV) and [α 5(IV)]₂ α 6(IV) molecules, we performed a double

Table 1. List of type IV collagen α chain-specific monoclonal antibodies (MAbs) used in this study.

MAbs	Epitopes	Usage
H11	α 1(IV) chain NC1 domain	indirect, direct
H22	α 2(IV) chain NC1 domain	indirect
H31	α 3(IV) chain NC1 domain	indirect
RH42	α 4(IV) chain NC1 domain	direct
H52	α 5(IV) chain NC1 domain	indirect
b14	α 5(IV) chain NC1 domain	direct
H65	α 6(IV) chain helical domain	indirect

staining of $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ chains. The sections were incubated with H65 for 60 min at room temperature and washed. In order to detect the localization of H65, Rhodamine-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Inc., California, USA) was applied for 60 min at room temperature. After washing, the sections were incubated with FITC-conjugated RH42 for 60 min at room temperature.

Double immunofluorescent staining for $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$

The $\alpha 1(\text{IV})$ chain comprises [$\alpha 1(\text{IV})$] $_2$ $\alpha 2(\text{IV})$ molecule, while $\alpha 5(\text{IV})$ chain comprises both $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ and [$\alpha 5(\text{IV})$] $_2$ $\alpha 6(\text{IV})$ molecules. We performed a double staining of $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains to detect the area composed of only [$\alpha 1(\text{IV})$] $_2$ $\alpha 2(\text{IV})$ molecules. The sections were incubated with FITC-conjugated H11 for 60 min at room temperature and washed. They were then incubated with HiLyte555-conjugated b14 for 60 min at room temperature.

Double immunofluorescent staining for CC10 and SP-C

The sections were incubated with a mixture of antibodies against CC10 and SP-C for 60 min and washed. Then they were incubated with a mixture of FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.) and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 60 min at room temperature.

Fluorescence microscopy

Immunostained sections were observed with a fluorescence microscope (IX71, Olympus, Tokyo) equipped with a digital camera (DP-70, Olympus).

Results

Basement membranes of bronchi, bronchioles and alveoli

Frozen sections of the rat inflated lung were morphologically well preserved without deformity and showed continuous linear images of the BM after the immunofluorescent staining. The subepithelial BM of the pulmonary airway contained all six α chains of the type IV collagen, the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains (Table 2). The $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains were detected continuously from the bronchi to alveoli. From the main bronchi to the terminal bronchioles, the BM beneath the epithelium showed positive immunofluorescence against the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains, but no signal against the $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains. While the alveolar subepithelial BM showed positive immunofluorescence against the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ and $\alpha 5(\text{IV})$ chains, there was no signal against the $\alpha 6(\text{IV})$ chain (Fig. 1). This staining pattern meant that the BM beneath the bronchial and bronchiolar epithelium contained [$\alpha 1(\text{IV})$] $_2$ $\alpha 2(\text{IV})$ and [$\alpha 5(\text{IV})$] $_2$ $\alpha 6(\text{IV})$ molecules, and the BM beneath the alveolar epithelium contained [$\alpha 1(\text{IV})$] $_2$ $\alpha 2(\text{IV})$ and $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ molecules. In addition to that, the BM around smooth muscle fibers spirally arranged in the bronchiolar wall showed positive

Table 2. Immunohistochemical expression of type IV collagen α chains in the normal rat lung basement membrane (BM).

BM	Type IV collagen α chains					
	$\alpha 1(\text{IV})$	$\alpha 2(\text{IV})$	$\alpha 3(\text{IV})$	$\alpha 4(\text{IV})$	$\alpha 5(\text{IV})$	$\alpha 6(\text{IV})$
Alveolar epithelium	+	+	+	+	+	—
Bronchiolar epithelium	+	+	—	—	+	+
Blood vascular endothelium	+	+	—	—	—	—
Smooth muscle cells	+	+	—	—	+	+

+ : positive — : negative

Fig. 1. Light micrographs of cryosections of rat terminal bronchioles (Br) and alveoli (Al), stained with hematoxylin and eosin (A) and immunostained with monoclonal antibodies, specific for the $\alpha 1$ (IV) chain (B), $\alpha 2$ (IV) chain (C), $\alpha 3$ (IV) chain (D), $\alpha 4$ (IV) chain (E), $\alpha 5$ (IV) chain (F), and $\alpha 6$ (IV) chain (G). The subepithelial BM of terminal bronchiole and the BM around smooth muscle fibers react positively to anti- $\alpha 1$ (IV), anti- $\alpha 2$ (IV), anti- $\alpha 5$ (IV), and anti- $\alpha 6$ (IV) antibodies. The alveolar BM reacts positively to anti- $\alpha 1$ (IV) through anti- $\alpha 5$ (IV) antibodies. The subendothelial BM of blood vessels reacts positively to anti- $\alpha 1$ (IV) and anti- $\alpha 2$ (IV) antibodies. These results are summarized in Table 2. White arrows: subepithelial BM of terminal bronchioles, red arrows: alveolar BM, SM: BM around smooth muscle fibers, arrowheads: subendothelial BM of blood vessels. Bars: 10 μ m

Fig. 2. Double immunofluorescent staining images for $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ chains in the BM of bronchioalveolar duct junction show two patterns. Arrows show the end of the BM, so that the areas between two arrows have neither $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ nor $[\alpha 5(\text{IV})]_2 \alpha 6(\text{IV})$ (A–D). Arrowheads indicate bronchioalveolar duct junctions. The BMs possessing the $\alpha 4(\text{IV})$ or $\alpha 6(\text{IV})$ chain directly connect (E–H). A and E: the images of the $\alpha 4(\text{IV})$ chain (green), B and F: the images of the $\alpha 6(\text{IV})$ chain (red), C and G: merged images, D and H: merged images and DAPI (blue). Br: terminal bronchioles, Al: alveoli. Bar: 10 μm

Fig. 3. Double immunofluorescent staining for $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ in the bronchioalveolar duct junction. Two patterns of immunostaining have been confirmed. The BM containing $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ molecule and that containing [$\alpha 5(\text{IV})$] $_2$ $\alpha 6(\text{IV})$ molecule connect directly (**F–J**) or insert a short area of BM containing [$\alpha 1(\text{IV})$] $_2$ $\alpha 2(\text{IV})$ molecule only (**A–E**). The width of the area is about a cell size (**E**). Arrows show the end of $\alpha 5(\text{IV})$. Arrowheads show bronchioalveolar duct junctions. **A** and **F**: the images of $\alpha 1(\text{IV})$ chain (green), **B** and **G**: the images of $\alpha 5(\text{IV})$ chain (red), **C** and **H**: merged images, **D** and **I**: merged images and phase contrast images, **E** and **J**: merged images and DAPI (blue). Br: terminal bronchioles, Al: alveoli. Bars: 10 μm

Fig. 4. Double immunostaining images using antibodies against CC10 (**A**, green) and SP-C (**C**, red) and double immunostaining images of the adjoining section using antibodies against $\alpha 1(\text{IV})$ (**E**, green) and $\alpha 5(\text{IV})$ (**G**, red) in the bronchioalveolar duct junction of the rat. **B** and **F**: merged images, **D** and **H**: merged images and phase contrast images. Br: terminal bronchioles, Al: alveoli. **A–D** and **E–H** are serial sections. Double positive cells for CC10 and SP-C are found at the end of columnar epithelial cells (**A–D**, arrows). In the same area of the adjoining section, the subepithelial BM has the $\alpha 1(\text{IV})$ chain but not the $\alpha 5(\text{IV})$ chain, indicating that the area has only $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ (**E–H**). Bars: 10 μm

immunofluorescence against the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains. The subendothelial BM of blood vessels showed positive immunofluorescence against the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains (Fig. 1).

Alpha (IV) chains in the bronchioalveolar duct junction (BADJ)

The double staining against the $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ chains showed two patterns of a transitional zone between the $\alpha 4$ -positive alveolar type BM and $\alpha 6$ -positive bronchiolar type BM: a separate pattern with an $\alpha 4$ -negative/ $\alpha 6$ -negative region (50%), and a continuous pattern without an $\alpha 4$ -negative/ $\alpha 6$ -negative region (50%) (Fig. 2). We carried out a double staining of the $\alpha 1$ and $\alpha 5$ chains to detect the $\alpha 1$ -positive/ $\alpha 5$ -negative areas that contained neither $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ nor $[\alpha 5(\text{IV})]_2 \alpha 6(\text{IV})$ molecules at continuous BM in the BADJ. In sections observed, 10 out of 14 junctions showed continuously positive labeling for both $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains (71%), and 4 junctions contained $\alpha 1$ -positive/ $\alpha 5$ -negative parts (29%) (Fig. 3). We have noted cells adjacent to the small region—about a single cell dimension—where only $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ molecules existed. To determine whether these cells were bronchially or alveolarly derived, we used antibodies CC10 and SP-C to identify bronchial cells and alveolar type II cells, respectively. We observed 17 CC10-positive/SP-C-positive cells. Fourteen (82.4%) of them were positioned as the first or second cell from the bronchioalveolar epithelial transition end of bronchiolar epithelial cells, and the remaining 3 (17.6%) were at the position of the third or fourth cell. To analyze the relationship between CC10-positive/SP-C-positive cells and the $\alpha(\text{IV})$ chains, we performed a double staining of $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains on the adjacent sections (Fig. 4). In 8 out of 9 pairs of junctions, CC10-positive/SP-C-positive cells were situated at the position of the first or the second cell from the bronchioalveolar transition end point of the bronchiolar epithelial cells. Four CC10-positive/SP-C-positive cells out of them were on the BM containing only the $\alpha 1(\text{IV})$ chain. The remaining 4 cells were on the BM containing both $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains. In 1 out of 9 pairs of junctions, the CC10-positive/SP-C-positive cell was located at the position of the fourth cell from the end of the bronchiolar epithelial cell and was on the BM containing both $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains.

Discussion

The present study has shown the distribution of type IV collagen α chains in the subepithelial basement membrane (BM) of the rat lung by immunofluorescence microscopy using α chain-specific monoclonal antibodies. It has confirmed that the $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ molecule continuously exists along the subepithelial BM from the bronchi to alveoli, and that the alveolar BM contains the $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ molecule (Nakano *et al.*, 2001). In contrast, the bronchial and bronchiolar BM contain the $[\alpha 5(\text{IV})]_2 \alpha 6(\text{IV})$ molecule instead of the $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ molecule. This bronchial type BM, containing both the $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ and $[\alpha 5(\text{IV})]_2 \alpha 6(\text{IV})$ molecules, shows the same pattern of the collagen IV molecules seen in the epidermis (Hasegawa *et al.*, 2007) as well as in the esophagus and other gastro-intestinal epithelia (Sato *et al.*, 2007).

This bronchiolar type BM is common to the BM delineating epithelium facing both the outside and inside of the human body. The transitional segment from the bronchiolar type BM to the alveolar type BM was found at the point where columnar epithelial cells changed to flat ones. Because it is suggested that the BM is made by the adjoining cells (Ninomiya *et al.*, 1995), we suppose that there is a close relationship between the epithelial cell type and collagen type IV molecules in the subepithelial BM.

In the transitional segment between the bronchiolar type BM and the alveolar type BM, a small part of the BM consisting of the $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ molecule was confirmed to be aligned just beneath the CC10-positive/SP-C-positive cells. This result suggests that CC10-positive/SP-C-positive cells may produce only $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains but not $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains. The CC10-positive/SP-C-positive cells are suggested to be bronchioalveolar stem cells (BASCs) (Kim *et al.*, 2005), which self-renew themselves, and give rise to Clara cells, AT2 cells, or AT1 cells (either directly or via BASC-derived AT2 cells) (Giangreco *et al.*, 2002; Kim *et al.*, 2007). Thus, BASCs play an important role in both the maintenance and repair of bronchiolar and alveolar cells (Nolen-Walston *et al.*, 2008). We presume that the BASCs, producing only the $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$ molecule in order to self-renew and self-maintain, obtain the ability to produce the $\alpha 3(\text{IV})$ $\alpha 4(\text{IV})$ $\alpha 5(\text{IV})$ or $[\alpha 5(\text{IV})]_2 \alpha 6(\text{IV})$ molecule prior to the differentiation to bronchiolar or alveolar epithelial cells, respectively.

As shown in the present study, some BASCs were detected in the regions away from the BADJ, and the BM beneath such BASCs was positive to the $\alpha 5(\text{IV})$

antibody. These cells might be an intermediate form or under differentiation and migrating from the BADJ to alveoli or bronchioles because they were positive against CC10 and SP-C and formed the $\alpha 5(\text{IV})$ chain. Recently, type IV collagen has been shown to regulate bone morphogenetic protein (BMP) signaling by binding to BMP molecules (Wang *et al.*, 2008). High local BMP signaling is observed in the germ line stem cell niche in *Drosophila* ovary. Thus, the variety of $\alpha(\text{IV})$ chains may be important modulators for the BMP signaling that provides differentiation signals to the adjacent cells.

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