AB (TYPE V) AND BASEMENT MEMBRANE (TYPE IV) COLLAGENS IN THE BOVINE LUNG PARENCHYMA: ELECTRON MICROSCOPIC LOCALIZATION BY THE PEROXIDASE-LABELED ANTIBODY METHOD

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ABSTRACT

The microdistribution of AB collagen and basement membrane (type IV) collagen in the bovine lung parenchyma has been studied by the immunoelectron microscopic method.

AB collagen was isolated from the pepsin digests of bovine lung parenchyma and purified with differential salt fractionation. Identification and purity of the collagen were assessed by SDS-polyacrylamide gel electrophoresis, collagenase digestion, phosphocellulose chromatography, amino acid analysis and by electrophoresis of CNBr-cleavage products.

Type specific rabbit antibodies to AB and type IV collagens showing no cross-reaction with other types of collagen were prepared by the immunoadsorption technique.

Immunoelectron microscopic studies using the indirect peroxidase-labeled antibody method have shown that AB collagen is located on the surfaces of varied cells, such as bronchiolar and alveolar epithelial cells, vascular endothelial cells and alveolar septal cells. On the other hand, type IV collagen is located along basement membranes of alveoli and capillaries.

KEY WORDS antibody / basement membrane / collagen / immunoelectron microscopy / lung parenchyma

The molecular heterogeneity of collagen is now well established (7) and at least five genetically distinct collagens (type I-V) have been identified in mammalian tissues. Type I-III collagens are present in the interstitium, and the basement

membrane (type IV) collagen is present only in basement membranes.

AB (type V) collagen was first isolated from human placental tissues by Burgeson *et al.* in 1976 (4). Thereafter, AB collagen has been identified in many tissues such as vessel walls (5), bone and cartilage (22), skeletal muscle (6), cornea and skin (10), synovia (3), lungs (14), and tendons (12). However, its molecular structure and tissue localization still remain to be elucidated, although both would provide key items of information about the physiological function of this collagen.

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Burgeson *et al.* (4) first emphasized that AB collagen was extracted from basement membrane-rich tissues such as placenta. However, the contents in tissues of AB collagen may not be related to those of basement membranes *in situ*, since the collagen was also found in cartilage.

Another approach which may help to clarify the tissue localization of AB collagen is the use of the immunohistological technique. Furthmayr *et al.* reported the codistribution of type IV and AB collagens in the basement membranes of human lungs and murine kidneys, based on double label indirect immunofluorescence staining (15) and immunoferritin labeling of ultrathin frozen sections (23). In addition, McCullagh *et al.* (17) and Pöschl and von der Mark (21) reported on the basis of immunofluorescence studies that AB collagen might also form part of the interstitial extracellular matrix.

In investigating tissue localizations of collagen types by using antibodies to individual types of collagen, the purity of the antigens and the cross-reactivity of the antibodies with other types of collagen might affect the results obtained. Furthermore, analysis by the immunofluorescence light microscopic method may not give much information on the microdistribution in tissues of collagen types. Therefore, we aimed to elucidate the relation between AB collagen and type IV collagen in terms of tissue localizations by using an immunoelectron microscopic technique with antibodies type-specific to AB and type IV collagens, which were isolated and purified from bovine lung lobules and glomerular basement membranes, respectively.

MATERIALS AND METHODS

Isolation of Collagens

All procedures for collagen isolation were performed at 4–10°C otherwise stated. AB collagen was isolated from the bovine lung tissue. Fresh bovine lungs were obtained from a local slaughterhouse. Pulmonary lobules were separated manually from the tracheobronchial tissue on ice to minimize the contamination of bronchial cartilage. The collected lobules were washed extensively for one week with several changes of distilled water containing 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 10 mM *N*-ethylmaleimide and then homogenized three times in chilled distilled water with a VirTis homogenizer (Model No. 60 K, Gardiner, NY) at 30,000 rpm for 30 sec and again washed extensively with distilled water. The homogenate was then incubated for 24 hr with pepsin

(1 g/20 g dry weight tissue, Worthington Biochemical Corp., Freehold, New Jersey). The undigested residue was removed by centrifugation, then AB collagen was isolated and purified from the supernatant, as described by Rhodes and Miller (22), with the exception that at the final step of purification, AB collagen was precipitated by dialysis against 0.1 M NaCl/0.05 M Tris-HCl buffer, pH 7.4, instead of dialyzing against 0.01 M Tris-HCl buffer, pH 8.6, containing 0.02 M NaCl and 2.0 M urea. The precipitate was collected by centrifugation, redissolved in 0.5 M acetic acid, dialyzed extensively against distilled water and lyophilized.

Type IV collagen was isolated from bovine glomerular basement membranes as described previously (19). Type I and type III collagens (from fetal calf skin) and type II collagen (from bovine articular cartilage) were prepared in a recent study (13).

Analytical Methods

SDS-polyacrylamide gel electrophoresis of collagen chains and their cyanogen bromide cleavage-peptides was carried out in 5% and 15% separating gels, respectively, as reported (8).

Bacterial and animal collagenase digestion was performed using purified *Cl. histolyticum* collagenase (Amano Pharmaceutical Co., Ltd., Nagoya) (20) and tadpole skin collagenase (11). Phosphocellulose chromatography of AB collagen was carried out as described by Rhodes and Miller (22). Amino acid analysis of AB collagen and its α -chains was performed using a JEOL model 6AS amino acid autoanalyzer after hydrolysis with 6 M HCl in an evacuated sealed tube at 110°C for 24 hr.

Preparation of Antisera and Type-Specific Antibodies

Rabbits were immunized with the AB collagen isolated from bovine lung parenchyma. The initial injection was given intradermally with 2 mg of the antigen which was dissolved in 5 mM acetic acid and emulsified with an equal volume of Freund's complete adjuvant (DIFCO Laboratories, Detroit, Michigan). Four and six weeks after the initial injection, intradermal booster injections of 1 mg of the antigen in 5 mM acetic acid were administered. Blood was collected and serum was separated 10 weeks after the initial immunization. The antibodies against AB collagen were purified by immunoadsorption on an affinity column composed of the antigen

(AB collagen) conjugated to CNBr-activated Sepharose 4B (16). The specific anti-AB collagen antibodies were eluted with 3 M NaSCN/0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl followed by 0.1 M glycine-HCl buffer, pH 2.8, then dialyzed against 0.15 M NaCl/0.05 M Tris-HCl buffer, pH 7.5 (2), and concentrated to a 1/6 volume of the antiserum loaded on the column by ultrafiltration using an Amicon Diaflo UM-10 membrane (Amicon Co., Lexington, Mass.). The antibodies to type IV collagen were prepared in a recent study (13).

Passive Hemagglutination Assay and Double Diffusion Test

The titers of purified antibodies and cross reactivity against other types of collagen were determined using the passive hemagglutination assay as described by Herbert (9). A suspension of fresh sheep red blood cells coated with bovine Type I, II, III, IV or AB collagen and of cells with an α A-chain or an α B-chain (0.5%, 25 μ l) was incubated overnight at room temperature with 25 μ l of a series of dilution of anti-AB collagen antibody. The antibodies to type IV and AB collagens were examined by means of the double diffusion test using goat antibodies to rabbit serum, IgG or IgM.

Immunoelectron Microscopy

Immunoelectron microscopy by the indirect peroxidase-labeled antibody method was performed as described by Yamada and Nakane (26). Fresh bovine lung specimens obtained from a local slaughterhouse were immediately fixed with periodate-lysine-paraformaldehyde fixative (PLP) (0.01 M NaIO₄, 0.075 M lysine, 0.0375 M sodium phosphate buffer, 2% paraformaldehyde) for 6 hr. They were then washed overnight in 0.05 M sodium phosphate buffer, pH 7.4, (PBS) with 10% sucrose, PBS with 15% sucrose for 12 hr and PBS with 15% sucrose and 10% glycerol for 2 hr. The resulting tissues were embedded in OCT compounds (Lab-Tek Products, Naperville, Illinois) with 10% glycerol, quick-frozen in liquid nitrogen and stored at -70°C. The 4 μ m frozen sections cut in a cryostat were mounted on glass slides coated with egg albumin and washed three times in PBS with 10% sucrose. For indirect immunohistochemical staining, the tissue sections were incubated at 4°C overnight with antibodies to type IV collagen or AB collagen.

The tissue sections were washed with PBS

containing 10% sucrose eight times at 4°C for 12 hr, then incubated at 4°C overnight with horseradish peroxidase (HRPO)-labeled anti-rabbit IgG goat antibodies (Miles Laboratories, Inc., Elkhart, Indiana) diluted 1:16 with the buffer. The tissue sections were washed eight times at 4°C for 12 hr with the buffer. After postfixation in 2% glutaraldehyde for 20 min, they were reacted with 0.02% diaminobenzidine (DAB)/0.05 M Tris-HCl buffer, pH 7.6, for 1 hr and with DAB with 0.005% peroxide for several min. They were then fixed in 2% osmium tetroxide for 30 min, dehydrated in graded series of ethanol to 100% and finally embedded in a capsule of SPUR (Polysciences, Inc., Warrington, Pennsylvania). The blocks were examined by light microscopy to select positively stained areas. Ultrathin sections of these areas were cut on a LKB ultramicrotome and observed without additional staining or with uranyl acetate staining under a Hitachi 12A electron microscope. As controls, tissue sections were incubated with antibodies preincubated with AB collagen (2 mg/ml) at 4°C overnight or incubated with normal rabbit serum diluted 1:16 with PBS containing 10% sucrose.

RESULTS AND DISCUSSION

Chemical Studies

AB collagen was identified and its purity assessed by SDS-polyacrylamide gel electrophoresis, collagenase digestion, phosphocellulose chromatography, amino acid analysis and by gel electrophoresis patterns of its CNBr-cleavage products. The collagen preparation precipitated at 0.1 M NaCl concentration/Tris-HCl buffer, pH 7.4, (see Materials and Methods) contained only AB collagen. Typical electrophoretic profiles of the 0.1 M NaCl precipitate before and after reduction with dithiothreitol are shown in Fig. 1, channels 2 and 3, respectively; as described by Burgeson *et al.* (4), its mobility was unaffected by the presence of disulfide-reducing reagent. Digestion of the material with *Cl. histolyticum* collagenase resulted in the complete hydrolysis (Fig. 1, channel 4).

As reported (24), the collagenous material was resistant to the action of tadpole collagenase under the conditions where Type I and II collagens were digested about 95% and 80%, respectively (data not shown). When the collagen was heat-denatured and chromatographed on a phosphocellulose column, it was resolved into two components which were eluted out in the fractions corresponding to α A- and α B-chains as

Fig. 1 SDS-polyacrylamide gel electrophoresis patterns of AB collagen and its bacterial collagenase digests and CNBr-cleavage products. 5% gel (1-5) and 15% gel (6-8). Calf skin type I collagen as control (1), AB collagen (0.1M NaCl precipitate, see text) before (2) and after (3) reduction with dithiothreitol, AB collagen after bacterial collagenase treatment (4) and the enzyme alone (5) and CNBr-cleavage products of rat $\alpha_1(I)$ - (6), bovine αA - (7) and αB -chains (8). The arrowheads indicate the protein components derived from collagenase preparation. CB: CNBr

described by Rhodes and Miller (22) (Fig. 2). SDS-polyacrylamide gel electrophoresis patterns of these components were quite similar to those of αA - and αB -chains, respectively (Fig. 2b, channels 1-3), although a minor component corresponding to an αC -chain (24) was coeluted in the first half of the αA -chain fraction. No effluent fractions contained either type IV collagen or its subcomponents, based on SDS-polyacrylamide gel electrophoresis.

Table 1 shows the amino acid composition of the collagen and its two components isolated by chromatography. All the samples contained high amounts of glycine, proline, hydroxyproline and hydroxylysine which were typical of collagen, but showed a difference in the amounts of alanine, arginine, valine, leucine and isoleucine from all other types of collagen. The results were quite similar to those reported by Burgeson *et al.* (4). Gel electrophoresis patterns of the CNBr-cleavage products of the chromatographically isolated components (Fig. 1, channels 7 and 8) were quite similar to those of αA - and αB -chains reported (24), but different from those of type IV collagen (data not shown). The results described above indicate that the

Table 1 *The Amino Acid Composition of AB Collagen and its α -Chains from Bovine Lung (Residues/1000 Residues)*

Amino acid	AB collagen	αA	αB
3-Hyp	4	4	5
4-Hyp	113	103	103
Asp	48	50	51
Thr	23	30	23
Ser	26	43	32
Glu	93	89	100
Pro	115	97	114
Gly	333	321	323
Ala	41	57	41
$\frac{1}{2}$ Cys	ND	1	ND
Val	24	35	21
Met	12	14	12
Ile	17	13	20
Leu	38	36	39
Tyr	2	2	3
Phe	12	11	12
His	6	10	6
Hyl	35	19	40
Lys	14	16	16
Arg	44	49	39

ND: not detected

Fig. 2 Phosphocellulose chromatogram of AB collagen (0.1 M NaCl precipitate). a) Heat-denatured AB collagen (16 mg) was applied on a column (1.5×10 cm). The arrows indicate the elution positions of $\alpha_1(I)$ - and α_2 -chains. b) SDS-polyacrylamide gel (5%) electrophoresis of four different effluent fractions. Gel numbers correspond to the fractions indicated on the elution curve (a). The migration position of αC chain is designated by the arrowhead.

collagen recovered in the 0.1 M NaCl precipitate at neutral pH (see Materials and Methods) is AB collagen with little contamination of type IV collagen.

Immunochemical Analysis

Antibodies to AB collagen were raised in immunized rabbits. The anti-AB collagen antibody purified by affinity chromatography showed a titer of 1/256 with the corresponding antigen according to the passive hemagglutination assay, but titers of less than 1/2 with type I, II, III and IV collagens. The anti-AB collagen antibody maintained cross-reactions with αA - and αB -chains with titers of 1/32 and 1/64, respectively (Table 2) by hemagglutination test which is contrary to a recent report by Madri and Furthmayr (14). Anti-type IV collagen antibodies used in this study showed a titer of 1/1024

with the corresponding antigen and titers of less than 1/2 with type I, II, III and AB collagens, according to the passive hemagglutination assay (13). The antibodies to AB collagen and type IV collagen were confirmed to be IgG by the double diffusion method (data not shown).

Immunoelectron Microscopic Studies

Localization of AB collagen in the bovine lung parenchyma was studied by immunoelectron microscopy using antibodies specific to AB collagen. No electron-dense reaction products were found in the basement membrane or interstitial matrix of the lung parenchyma. The reaction products were found frequently but exclusively on the surface of varied cells such as endothelial cells of alveolar capillaries (Fig. 3) and pulmonary arterioles (Fig. 4), alveolar epithelial cells (Fig. 5) and ciliated epithelial

Table 2 *Passive Hemagglutination Titers of Purified Anti-AB Collagen Rabbit Antibodies for Bovine Type I, II, III, IV and AB Collagens*

Antibody to	Reciprocal titer ($-\log_2$) against						
	AB	A-chain	B-chain	Type I	Type II	Type III	Type IV
AB collagen	8	5	6	*	*	*	*

* not detected

Fig. 3

Fig. 4

Fig. 5 Electron micrograph of immunoperoxidase reaction in the alveolar wall with uranyl acetate staining. Reaction products with specific anti-AB collagen antibodies are present on the alveolar epithelial cell surface (arrow). Ep, alveolar epithelial cell; Ed, alveolar endothelial cell; Alv, alveolar lumen; BM, basement membrane. $\times 26,000$

cells of bronchioli (Fig. 6), although in most cases, the reaction products were observed on the luminal side of the cell surfaces but scarcely on the basal side. Furthermore, the cell surface of lymphocytes (Fig. 3) and alveolar septal cells (data not shown) as well as the membrane of pinocytotic vesicles (Fig. 4) were also stained.

Fig. 3 Electron micrographs of immunoperoxidase reaction in the alveolar wall of bovine lung with uranyl acetate staining. Electron dense reaction products with specific anti-AB collagen antibodies are present on the cell surfaces of endothelial cells (arrow) and lymphocyte (arrowhead). Ed, capillary endothelial cell; Ep, alveolar epithelial cell; Cp, capillary lumen; Alv, alveolar lumen; BM, basement membrane; Ly, lymphocyte. $\times 11,000$; inset, $\times 19,000$

Fig. 4 Electron micrograph of immunoperoxidase reaction in the intima of the pulmonary arteriole with uranyl acetate staining. Electron dense reaction products with specific anti-AB collagen antibodies are present on the endothelial cell surface (arrowhead) and the membrane of pinocytotic vesicle (arrow). Ed, endothelial cell of arteriole; BM, basement membrane. $\times 39,000$

In control studies, no significant reaction products were detected in the bovine lung tissue incubated with normal rabbit serum or with anti-AB collagen antibodies preincubated with the antigen collagen (data not shown).

When localization of type IV collagen was examined using antibodies specific to the collagen, the electron-dense reaction products were exclusively located along basement membranes of alveoli and capillaries (Figs. 7 and 8), but not on alveolar cells or in the interstitium.

Fig. 6 Electron micrograph of immunoperoxidase reaction in the bronchiolar epithelium with uranyl acetate staining. Reaction products with specific anti-AB collagen antibodies are present on the ciliated epithelial cell surface. Ep, ciliated epithelial cell of bronchiole; Br, bronchiolar lumen. $\times 28,000$

Fig. 7 Electron micrograph of immunoperoxidase reaction in the alveolar wall of bovine lung with uranyl acetate staining. Reaction products with specific anti-type IV collagen antibodies are present along the epithelial and capillary basement membranes. Alv, alveolar lumen; Ed, endothelial cell; Cp, capillary; BM, basement membrane. $\times 7,500$

Fig. 6

Fig. 7

Fig. 8 Electron micrograph of a high magnification of Fig. 7. The reaction products with specific anti-type IV collagen antibodies are exclusively present on the basement membrane. $\times 15,000$

The results described here clearly indicate that the tissue localization of AB collagen is distinct from that of type IV collagen. Recent reports by Madri *et al.* described that both AB and type IV collagens are codistributed along basement membranes of human lungs (14, 15) and murine kidneys (23). The discrepancy between their data and ours is difficult to explain. One of the reasons for it might be due to poor permeability of the anti-AB collagen antibodies into the tissue specimens prepared in this study. However, it seems unlikely, since under similar experimental conditions basement membranes were stained with anti-type IV collagen antibodies which showed a titer of the same order as that of the anti-AB collagen antibodies employed. Both antibodies were prepared with the collagens from bovine organs and applied to the tissues from the same animal species.

Taking all the results into account, AB collagen may not be a basement membrane component but a cell-surface-associated component and its frequent but restricted distributions on the outer surface of epithelial and endothelial cells suggest that this collagen may play important roles in cytoskeletal organization and cell migration (25) and differentiation, although

the relationship between cell types and physiological functions of AB collagen remains to be elucidated.

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