

## LABORATORY SCIENCE

## Distribution of the collagen IV isoforms in human Bruch's membrane

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*Br J Ophthalmol* 2003;**87**:212–215

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Accepted for publication  
22 August 2002

**Aims:** To determine the distribution of the  $\alpha 1$  to  $\alpha 6$  chains of type IV collagen in Bruch's membrane of the human posterior pole.

**Methods:** Cryosections (10  $\mu\text{m}$ ) from 18 human eyes (20 months to 83 years old) were acid treated, blocked with 10% normal goat serum, incubated for 1 hour with monoclonal antibodies against type IV collagen isoform specific peptides at 1:75 dilution, and visualised with an ABC staining kit.

**Results:** In Bruch's membrane, the  $\alpha 1$ (IV) and  $\alpha 2$ (IV) chains were identified in retinal pigment epithelial (10/18 = 55%) and choriocapillaris basement membranes (18/18 = 100%); the  $\alpha 3$ (IV),  $\alpha 4$ (IV), and  $\alpha 5$ (IV) chains were also found in the retinal pigment epithelial basement membrane (13/18 = 72%). In the choroid, the  $\alpha 1$ (IV) and  $\alpha 2$ (IV) chains were detected in the blood vessels (18/18=100%). The  $\alpha 6$ (IV) chain was not identified in any sections.

**Conclusion:** The heterogeneous distribution of  $\alpha 1$ –2(IV) and  $\alpha 3$ –5(IV) in Bruch's membrane could give insights into the function of this structure in health, ageing, and diseases such as age related macular degeneration.

The basement membrane is a specialised extracellular matrix produced by several cell types that provides mechanical support, divides tissues into compartments, serves as a selective molecular sieve, and influences cell phenotype.<sup>1</sup> Type IV collagen is one of the major components of the basement membrane. Six genetically distinct type IV collagen chains,  $\alpha 1$ (IV)– $\alpha 6$ (IV), have been identified. Each type IV collagen molecule is a trimer composed of three  $\alpha$  chains which form heterotrimeric molecules of  $\alpha 1$ –2(IV),  $\alpha 3$ –5(IV), and  $\alpha 5$ –6(IV) chains.<sup>2–3</sup> The different collagen IV molecules have distinct topographical locations and functions. For example,  $\alpha 1$ –2(IV) collagen is found in blood vessels with ubiquitous distribution where it functions to maintain the integrity of the vascular wall during exposure to varying blood pressures.<sup>2</sup>  $\alpha 3$ –5(IV) collagen has a more limited distribution. In the kidney, it is confined to the glomerular basement membrane where it is essential for the long term maintenance of the structure and filtration of the glomerulus.<sup>4–7</sup> Finally,  $\alpha 5$ –6(IV) collagen has recently been found in smooth muscle basement membranes.<sup>8</sup>

Bruch's membrane is a pentalaminar structure composed of the RPE basement membrane on the inner surface of the membrane, two collagenous zones divided by a middle elastic layer, and the choriocapillaris basement membrane on the outer surface of the membrane. The collagen IV isoform distribution in the neurosensory retina has been previously well characterised.<sup>9</sup> While a number of studies have investigated the collagen IV expression pattern in Bruch's membrane, these studies for the most part have been limited by the use of anti-collagen IV type 1–2 antibodies.<sup>10–14</sup> Owing to the autofluorescence of Bruch's membrane which limits using immunofluorescent techniques, the inherent structural complexity of Bruch's membrane, and previously unrecognised isoforms of collagen IV, the pattern profile of the six type IV isoforms in Bruch's membrane remains largely unknown.<sup>9–13–15</sup> Identifying the specific collagen IV isoforms expressed in Bruch's membrane could provide functional insights into this structure as well as determine a role in ageing and disease. For example,  $\alpha 3$ –5(IV) collagen is associated with cell differentiation since it is expressed by highly differ-

entiated central corneal epithelium, but not by undifferentiated limbal stem cells.<sup>16</sup> Alternatively, Petitclerc *et al* demonstrated that  $\alpha 3$ (IV) collagen contains anti-angiogenic properties.<sup>17</sup> Thus, regions of Bruch's membrane that are devoid of  $\alpha 3$ (IV) collagen could be susceptible to developing choroidal neovascular membranes. The purpose of this study was to define the expression pattern of the collagen IV isoforms in Bruch's membrane of the human globe by using a collection of isoform specific monoclonal antibodies which recognise epitopes localised to the C terminal non-collagenous globular domains of  $\alpha 1$ –6(IV) chains.<sup>18</sup>

## MATERIALS AND METHODS

## Tissue

Human eyes (20 months to 83 years old) were obtained from the Sierra Eye and Tissue Bank (Sacramento, CA, USA) within 21 hours after death and placed in phosphate buffered saline (PBS) pH 7.4 in 4% paraformaldehyde at 4°C. The anterior segment was removed and the posterior pole was examined under a dissecting microscope and categorised according to the protocol in the ALARM study.<sup>19</sup> The macular region, which was a 6 × 6 mm calotte centred around the fovea, was cryoprotected using the technique of Barthel and Raymond.<sup>20</sup> Briefly, calottes were rinsed three times in 1X PBS containing 5% sucrose (w/v) for 10 minutes at 4°C and were progressively infiltrated with sucrose by 30 minute incubations at 4°C in PBS containing 10%, 12.5%, and 15% sucrose (w/v). Finally, calottes were infiltrated overnight in 1X PBS containing 20% sucrose (w/v) at 4°C.

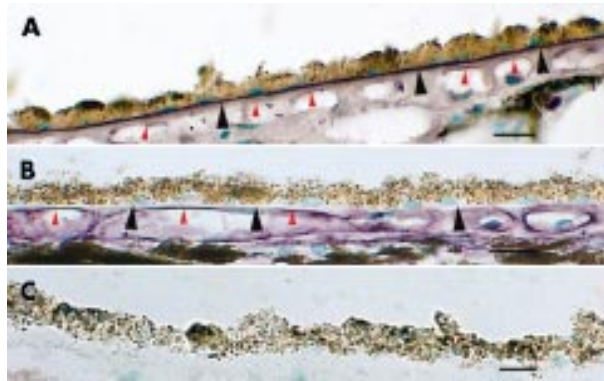
## Tissue embedding

Calottes were first infiltrated in a 2:1 sucrose 20% (w/v):OCT compound (Miles, Inc, Elkhart, IN, USA) mixture for 30 minutes at room temperature according to the method of Barthel and Raymond.<sup>20</sup> Each calotte was then embedded in fresh 2:1 sucrose 20% (w/v):OCT mixture and frozen by immersion in isopentane (Aldrich Chemical Co, Inc, Milwaukee, WI, USA) chilled with dry ice. All tissue blocks were stored at –80°C for later use.

**Table 1** Distribution of  $\alpha 1-6(IV)$  collagen in Bruch's membrane and choroid of human globes detected with isoform specific anti-collagen IV monoclonal antibodies

Isoform	$\alpha 1(IV)$	$\alpha 2(IV)$	$\alpha 3(IV)$	$\alpha 4(IV)$	$\alpha 5(IV)$	$\alpha 6(IV)$
RPE BM	10	10	13	13	13	0
CC BM	18	18	5	5	5	0
BV (choroid)	18	18	0	0	0	0

Using Fisher's exact test, when seen, the expression of the collagen IV isoforms was significant, with  $p < 0.00001$  except  $\alpha 3(IV)-\alpha 5(IV)$  in the choriocapillaris BM where  $p = 0.045$



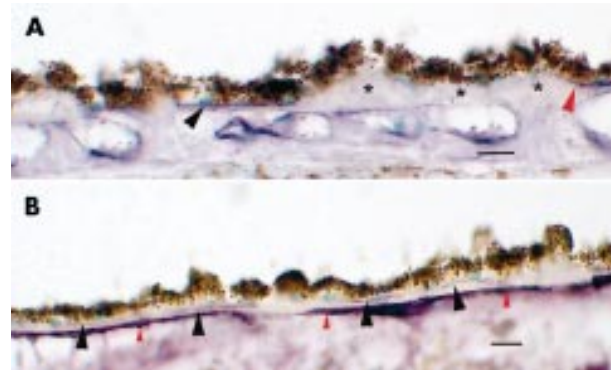
**Figure 1** Distribution of collagen IV isoforms in Bruch's membrane and choroid. (A) From an 83 year old female showing immunostaining for  $\alpha 3(IV)$  in the RPE basement membrane (black arrowheads) and minimal staining in the choriocapillaris basement membrane (red arrowheads). Similar staining patterns were seen for  $\alpha 4-5(IV)$  (data not shown). (B) From a 64 year old male with staining for  $\alpha 1(IV)$  predominantly in the choriocapillaris basement membrane (red arrowheads) and no staining in the RPE basement membrane (black arrowheads). Choroidal blood vessels also stain for  $\alpha 1(IV)$ . Similar staining was seen for  $\alpha 2(IV)$  (data not shown). (C) In contrast, no staining in the same 64 year old male is seen with the rat IgG control. Bar = 10  $\mu m$ .

### Immunohistochemistry

Serial cryosections (10  $\mu m$ ) oriented parallel to the optic nerve-fovea plane were fixed with 4% paraformaldehyde for 10 minutes, acetone for 10 minutes, and 0.1 M KCl-HCl (pH 1.5) for 10 minutes. Sections were treated with 3%  $H_2O_2$  in methanol for 5 minutes followed by blocking with 10% normal goat serum (Vector Laboratories, Inc, Burlingame, CA, USA) for 10 minutes. Sections were incubated with rat monoclonal antibodies against type IV collagen isoform specific peptides ( $\alpha 1$ , H11;  $\alpha 2$ , H22;  $\alpha 3$ , H31;  $\alpha 4$ , H43;  $\alpha 5$ , H53;  $\alpha 6$ , H63)<sup>18</sup> at 1:75 dilution for 1 hour followed by goat anti-rat biotinylated secondary antibody (1:500; ICN Pharmaceuticals, Inc, Aurora, OH, USA) for 30 minutes. The reaction was amplified using an ABC kit for 30 minutes (Vector Laboratories, Inc), and visualised with VIP reagent (Vector Laboratories, Inc) for 5 minutes. The sections were counterstained with methyl green (Vector Laboratories, Inc). A purified rat IgG (ICN Pharmaceuticals, Inc) was used for controls, and results were confirmed with a second set of monoclonal anti-collagen IV isoform antibodies ( $\alpha 1$ , H12;  $\alpha 2$ , H25;  $\alpha 3$ , H32;  $\alpha 4$ , H44;  $\alpha 5$ , H52;  $\alpha 6$ , H66).<sup>18</sup>

### Photography

Specimens were examined with an Olympus BH-2 microscope (Olympus Optical Co, Ltd, Tokyo, Japan) with a Kontron Elektronik ProgRes 3012 charged coupled device camera (Kontron Elektronik GmbH, Eching, Germany). Digitalised images were captured through the ProgRes digital camera plug-in directly to Adobe Photoshop 5.0 (Adobe Systems, Inc, Mountain View, CA, USA).



**Figure 2** Expression pattern of collagen IV in Bruch's membrane containing basal deposits. (A) The RPE basement membrane (black arrowhead) which stains for  $\alpha 3(IV)$ , is interrupted in an area of a basal deposit (\*\*\*) in an 83 year old female donor. The RPE basement membrane is also identified adjacent to the RPE cell overlying the basal deposit (red arrowhead). (B) In a different section from the same donor, the RPE basement membrane (black arrowheads) is lightly stained in a basal deposit that spans the entire section. The choriocapillaris basement membrane (red arrowheads) is prominently stained with the anti- $\alpha 1(IV)$  antibody. Bar = 10  $\mu m$ .

### Statistical analysis

A specific isoform was designated "expressed" when specific labelling was either intermittently or uniformly distributed in the sections examined. The Fisher's exact test was used to test the null hypothesis that the expression of the individual isoform in the fundus was due to chance. The McNemar's test was used to determine the probability that differences in expression between different collagen isoforms were due to chance. The expression patterns observed in this study were considered significant when the probability calculated from the above tests was  $p < 0.05$ .

### RESULTS

Eighteen human eyes were obtained from donors ranging in age from 20 months to 83 years old. One eye was from a 20 month old, three eyes were from donors 50-59 years old, 11 eyes were from donors 60-69 years old, and three eyes were from donors 70-83 years old.

Table 1 shows the isoform specific expression profile of collagen IV in the human posterior pole. Strong immunostaining for the  $\alpha 1-2(IV)$  and  $\alpha 3-5(IV)$  chains was observed in Bruch's membrane. In the RPE basement membrane, there was a slight predominance of immunostaining for  $\alpha 3-5(IV)$  (13/18 eyes = 72%) compared to  $\alpha 1-2(IV)$  (10/18 eyes = 55%), although this difference was not significant ( $p = 0.24$ , McNemar's test) (Fig 1). No age related distribution of either  $\alpha 1-2(IV)$  or  $\alpha 3-5(IV)$  isoforms was identified.  $\alpha 1-2(IV)$  were identified in the choriocapillaris basement membrane in 100% (18/18 eyes) while  $\alpha 3-5(IV)$  were observed in 28% (5/18 eyes;  $p = 0.001$ , McNemar's test). Mild immunostaining for the collagen  $\alpha 1-5(IV)$  isoforms was observed in the middle layers of Bruch's membrane. In the choroid,  $\alpha 1(IV)$  and  $\alpha 2(IV)$  were detected in the blood vessels (18/18 eyes = 100%). Mild staining was seen in the choroidal stroma with antibodies to  $\alpha 1(IV)$  to  $\alpha 5(IV)$ .

Collagen IV  $\alpha 6$  expression was not seen anywhere in the posterior segment with either H63 or H66 antibody.

Two eyes contained basal deposits and drusen in Bruch's membrane. In both eyes, two labelling patterns for collagen IV were observed. The more common pattern was an interruption of staining for all of the  $\alpha$ (IV) isoforms in basal deposits and drusen (Fig 2A). Less common was a continuation of the RPE basement membrane, which stained for both  $\alpha 1-2$ (IV) and  $\alpha 3-5$ (IV), through the deposits (Fig 2B).

## DISCUSSION

Our immunohistochemical studies identified a heterogeneous distribution of the collagen IV isoforms in the posterior fundus. In Bruch's membrane, the RPE basement membrane stained for both  $\alpha 1-2$ (IV) and  $\alpha 3-5$ (IV) while the choriocapillaris basement membrane stained primarily for  $\alpha 1-2$ (IV). Choroidal blood vessels, like those in the neurosensory retina,<sup>9</sup> stained for  $\alpha 1-2$ (IV) which is typical for endothelial basement membranes.<sup>2</sup> An unusual finding was the presence, on a limited basis, of  $\alpha 3-5$ (IV) in the choriocapillaris basement membrane.

The co-expression of  $\alpha 1-2$ (IV) and  $\alpha 3-5$ (IV) collagen at least partially explains the inconsistent results obtained from previous immunohistochemistry studies of Bruch's membrane with different type IV collagen antibodies. Previous studies have used polyclonal antibodies raised against type IV collagen isolated from human placenta or EHS sarcoma, both of which contain  $\alpha 1-2$ (IV) collagen.<sup>2</sup> Using an anti-collagen IV antibody raised against placenta, Marshall *et al* showed that type IV collagen was absent from the RPE basement membrane, but was present in the choriocapillaris basement membrane.<sup>13-21</sup> Likewise, in the rat, Lin and Essner found greater expression of collagen IV in the choriocapillaris than the RPE basement membrane with their polyclonal anti-collagen IV antibodies.<sup>15</sup> Our study suggests that these different staining characteristics could be explained by the mixture of both  $\alpha 1-2$ (IV) and  $\alpha 3-5$ (IV) collagen in the RPE basement membrane compared to the choriocapillaris basement membrane. Kleppel *et al* identified a signal in the inner portion of Bruch's membrane using a monoclonal antibody generated against the non-collagenous domain of type IV collagen isolated from human kidney glomerular basement membranes, which probably represents  $\alpha 3-5$ (IV).<sup>10-11</sup> In their study, the exact inner layer of Bruch's membrane was not identified. Our results extend the observations by Kleppel *et al* by precisely identifying both the specific  $\alpha 3-5$ (IV) isoforms and localising their distribution to the RPE basement membrane.

The limited sample size and elderly donor age prevent us from drawing any conclusions regarding the isoform distribution as a function of age. However, two eyes contained age related basal deposits in Bruch's membrane for which two unique staining patterns were observed. The more common finding was an interruption of labelling for any of the collagen IV isoforms. These interruptions in staining could represent degraded areas of RPE basement membrane, an artefact related to tissue processing, or epitope masking from macromolecules within basal deposits. Alternatively, basement membrane proteins develop, with age, an increase in the number of cross links either from oxidative stress or advanced glycation end products, which could shield the epitopes from our antibody set.<sup>22-24</sup>

The patchy distribution of the  $\alpha 1-2$ (IV) and (3-5)IV isoform staining pattern could influence changes that develop to Bruch's membrane. For example,  $\alpha 3-5$ (IV) is more resistant to proteolysis than other collagen IV isoforms.<sup>22-25</sup> Regions that contain  $\alpha 3-5$ (IV)—for example, by being relatively resistant to proteolysis, could promote matrix protein expansion from reduced degradation to promote early basal deposit formation during ageing.<sup>13-21-26</sup> Alternatively, Petitclerc *et al* demonstrated that  $\alpha 3$ (IV) contains anti-angiogenic properties.<sup>17</sup> Focal loss of

$\alpha 3$ (IV) or regions of relatively less  $\alpha 3-5$ (IV) collagen could increase susceptibility to choroidal neovascular membrane formation. With the potential differences in function among the different isoforms, further study is necessary to define its role in health and disease associated with Bruch's membrane.

## ACKNOWLEDGEMENTS

We thank Yoshikazu Sado, PhD, for his generous donation of the collagen IV antibodies and The Sierra Eye and Tissue Bank, Sacramento, CA, USA.

Grant support: National Eye Institute EY14005 (JTH), RPB Manpower Award (JTH), an unrestricted RPB grant, and a gift from the Friends of Macular Degeneration Society (JTH), grant in aid for International Scientific Research (Joint Research, 11694280) of Ministry of Education, Science, Sports, and Culture of Japan (YN).

Proprietary interest: None.

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*Br J Ophthalmol* 2003 87: 212-215

doi: 10.1136/bjo.87.2.212

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