

Evaluation of Stem Cell Components in Retrocorneal Membranes

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The purpose of this study was to elucidate the origin and cellular composition of retrocorneal membranes (RCMs) associated with chemical burns using immunohistochemical staining for primitive cell markers. Six cases of RCMs were collected during penetrating keratoplasty. We examined RCMs with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) staining and immunohistochemical analysis using monoclonal antibodies against hematopoietic stem cells (CD34, CD133, c-kit), mesenchymal stem cells (beta-1-integrin, TGF- β , vimentin, hSTRO-1), fibroblasts (FGF- β , α -smooth muscle actin), and corneal endothelial cells (type IV collagen, CD133, VEGF, VEGFR1). Histologic analysis of RCMs revealed an organized assembly of spindle-shaped cells, pigment-laden cells, and thin collagenous matrix structures. RCMs were positive for markers of mesenchymal stem cells including beta-1-integrin, TGF- β , vimentin, and hSTRO-1. Fibroblast markers were also positive, including FGF- β and α -smooth muscle actin (SMA). In contrast, immunohistochemical staining was negative for hematopoietic stem cell markers including CD34, CD133 and c-kit as well as corneal endothelial cell markers such as type IV collagen, CD133 except VEGF and VEGFR1. Pigment-laden cells did not stain with any antibodies. The results of this study suggest that RCMs consist of a thin collagen matrix and fibroblast-like cells and may be a possible neogenetic structure produced from a lineage of bone marrow-derived mesenchymal stem cells.

Keywords: Burns, Chemical; Cornea; Mesenchymal Stem Cell; Retrocorneal Membrane

INTRODUCTION

Retrocorneal membranes (RCMs) are translucent web-like structures which gradually grow into the posterior surface of the cornea (1). They have been observed in various clinical conditions associated with disease and damage of the corneal endothelium such as ocular chemical burns, perforating corneal injuries, and infectious diseases (2, 3). The production of RCM is thought to be an end-stage disease process of the cornea leading to corneal opacity and blindness (4).

The origin of RCM is not yet well understood. Previous studies of RCM have focused on endothelial mesenchymal transformation (1, 4-6), which suggested that translucent web-like structures gradually grow from the posterior surface of the cornea into the anterior chamber by perforating Descemet's membrane. In addition, light microscope examinations in previous studies revealed that RCMs resulted in the accumulation of an acellular, extracellular matrix. No cells could be identified in the early phases of corneal chemical burns (7); in the later stages following a corneal chemical burn, however, new types of cells, such as inflammatory and spindle cells, infiltrate the acellular cornea secondary to destruction of the ocular-blood barrier.

Primitive cells may promote wound healing through the activation of growth factors and cytokines, which may influence stem cell migration, proliferation, and differentiation during wound healing. In addition to this activation, pain has proven to be an important triggering factor for stem cell activation (8, 9).

A disrupted ocular blood barrier due to severe ocular surface damage, such as a chemical burn, may increase the serum component of the anterior chamber and alter the anterior chamber microenvironment. Given this background information, we hypothesized that the composition of a RCM might be derived from primitive cells such as mesenchymal stem cells rather than just an endothelial mesenchymal transition. To elucidate the types of cells that comprise a RCM, we analyzed the cell types in RCMs using various primitive cell markers.

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MATERIALS AND METHODS

Patients and specimens collection

Six patients (four males and two females), aged between 42 and 74 yr, (mean age, 53.2 \pm 11.0 yr) were recruited from the Department of Ophthalmology at Chung-Ang University Hospital. They all had a fibrous RCM behind the corneal endothelium which is clinically differentiated with epithelial downgrowth or thickened Descemet's membrane by overlying corneal edema and

Table 1. Patient demographics

Case No.	Age (yr)	Sex	Cause of corneal burn	Grade	Treatment prior to PKP	Duration to PKP, month
1	54	Female	Alkali burn	Severe	TAMP, artificial tears, autologous serum eyedrops, T-lens	12
2	42	Female	Alkali burn	Moderate	Artificial tears, autologous serum eyedrops, T-lens	10
3	74	Male	Alkali burn	Severe	TAMP, artificial tears, autologous serum eyedrops, T-lens	14
4	47	Male	Alkali burn	Severe	TAMP, artificial tears, autologous serum eyedrops, T-lens	13
5	48	Male	Alkali burn	Severe	TAMP, artificial tears, autologous serum eyedrops, T-lens	8
6	54	Male	Alkali burn	Moderate	TAMP, artificial tears, autologous serum eyedrops, T-lens	12

PKP, Penetrating keratoplasty; TAMP, Temporary amniotic membrane patch graft; T-lens, therapeutic soft contact lens.

frayed and irregular edge of RCMs. Despite temporary amniotic membrane patch graft and medical treatment for several months, all of patients had eventually undergone penetrating keratoplasty (PKP) and amniotic membrane transplantation due to corneal opacities and corneal thinning caused by chemical burns (Table 1). During PKP, after trephination of the cornea, abnormal web formation characterized of an RCM was obtained. Surgeon tried to dissect and separate an RCM from corneal overlying corneal endothelium meticulously to prepare the fixation block for histologic examination.

Histologic examinations

All tissue specimens were fixed in 4% buffered paraformaldehyde in the operating room. Fixed RCMs were dehydrated and embedded in paraffin. Paraffin sections were deparaffinized in xylene, rehydrated, and quenched with endogenous peroxidase. Cryostat sections were placed on gelatinized slides and fixed in cold acetone. Tissue sections were equilibrated in Tris-buffered saline (TBS) and blocked in non-immune serum (Zymed Laboratories, South San Francisco, CA, USA). The 4- μ m-thick sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome stain. In addition, for immunohistochemical stains, tissue sections were incubated with mouse monoclonal antibody against each of human type IV collagen, CD34, CD133, FGF- β , TGF- β , VEGF, vascular endothelial growth factor receptor-1 (VEGFR1), c-kit (CD117), beta-1-integrin (CD29), vimentin, hSTRO-1, and α -smooth muscle actin (SMA) overnight at 4°C. Dilution rate and purchase companies of antibodies used are listed in Table 2. Sections were washed in TBS before adding the bionylated secondary antibody, rewashed, and incubated for 1 hr with peroxidase-conjugated streptavidin, and the presence of peroxidase was revealed by adding substrate-chromogen (3-amino-9-ethylcarbazole) solution. The sections then were counterstained with hematoxylin, examined under an optical microscope (BX51, Olympus America Inc, Center Valley, PA, USA) and photographs were taken using image capture software (Baumer Twain Ver. 1.0, Humin Tech Corp., Seoul, Korea).

We collected positive findings of photos stained against each target protein from different case samples not from the identical case to sum representative photos and eventually show well-organized results.

Table 2. Monoclonal and polyclonal antibodies used for immunohistochemical staining

Antibody	Immunoglobulin	Dilution	Source
Type IV collagen	Mouse monoclonal IgG	$\times 100$	Santa Cruz*
CD34	Mouse monoclonal IgG	$\times 100$	Santa Cruz
CD133	Rabbit polyclonal IgG	$\times 100$	Abcam [†]
FGF- β	Goat polyclonal IgG	$\times 100$	Santa Cruz
TGF- β	Mouse monoclonal IgG	$\times 100$	Santa Cruz
VEGF	Rabbit polyclonal IgG	$\times 50$	Abcam
VEGFR1	Rabbit monoclonal IgG	$\times 100$	Abcam
c-kit	Rabbit polyclonal IgG	$\times 50$	Enogene [‡]
$\beta 1$ -integrin	Mouse monoclonal IgG	$\times 50$	Dako [§]
Vimentin	Mouse monoclonal IgG	$\times 100$	Dako
hSTRO-1	Mouse monoclonal IgG	$\times 100$	Santa Cruz
α -SMA	Mouse monoclonal IgG	$\times 100$	Santa Cruz

*Santa Cruz, Santa Cruz, CA, USA; [†]Abcam, Cambridge, MA, USA; [‡]Enogene, New York, NY, USA; [§]Dako, Glostrup, Denmark.

We used type IV collagen, FGF- β , TGF- β , vimentin, and α -SMA to identify fibroblasts; CD34, CD133, vimentin, and beta-1-integrin to identify keratocytes; type IV collagen, CD133, VEGF, VEGFR1, and vimentin to identify corneal endothelial cells; beta-1-integrin, vimentin, TGF- β , and hSTRO-1 to identify mesenchymal stem cells; and CD34, CD133, and c-kit to identify hematopoietic stem cells.

Ethics statement

The institutional review board of Chung-Ang University approved this study (C2013143[1103]). Informed consent was obtained from all participants for the use of their tissues; and all procedures were performed according to the principles of the Declaration of Helsinki.

RESULTS

Histopathologic analysis

Routine histological sections with H&E staining revealed that the RCMs were composed of multiple layers of connective tissue. The membranes exhibited different features in terms of thickness, extent, vascularity, and cellularity. Spindle-shaped cells were widely spread in the stroma of the RCM tissue. Pigment-laden cells, which appeared to be limbal pigment epithelium, ciliary bodies, and/or iris pigment epithelium were also present in the tissue (Fig. 1A and B). RCMs consisted of multi-

ple collagen layers and did not show evidence of an epithelial or endothelial layer. PAS staining (Fig. 1B) did not reveal any specific findings and Masson's trichrome staining (Fig. 1C) showed that the membranes were composed of collagenous tissue.

Immunohistochemical analysis

The six cases of RCMs had similar staining patterns after immunohistochemical staining. A summary of the immunohistochemical staining results for cells within the RCMs is displayed in Table 3. FGF- β -positive (Fig. 2A) and TGF- β -positive (Fig. 2B) spindle-shaped cells were observed in the RCMs. A marker representative of fibroblasts, α -SMA, reacted strongly with the spindle-shaped cells (Fig. 2C). Cells that stained positive for mesenchymal stem cell markers such as beta-1-integrin and vimentin as well as hSTRO-1-positive spindle-shaped cells (8, 10) were

Table 3. Results of immunohistochemical staining for cells in retrocorneal membranes in comparison with those of mesenchymal stem cells, fibroblasts, and corneal endothelial cells

	Cells on RCM	MSC	Fibroblast	CEC
Type IV collagen	-		+	+
CD34	-	-		-
CD133	-			+
FGF- β	+		+	
TGF- β	+	+	+	
VEGF	+			+
VEGFR1	++			+
c-kit	-	-		
β 1-integrin	++	+		
Vimentin	++	+	+	+
hSTRO-1	+	+		
α -SMA	+++		+	

RCM, retrocorneal membrane; MSC, mesenchymal stem cell; CEC, corneal endothelial cell.

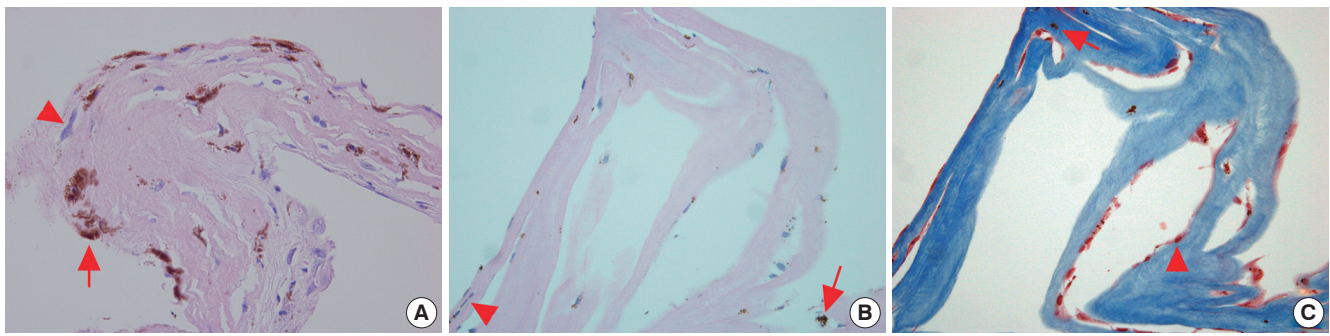


Fig. 1. Histochemical findings of the retrocorneal membranes. (A) H&E staining of the Case 1 demonstrates avascular connective tissue with spindle-shaped cells (arrow head) and pigment-laden cells (red arrow). (B) PAS staining of the Case 3 reveals no specific findings. (C) Masson's trichrome staining detects a collagen fiber structure within the retrocorneal membranes (Case 3). (B & C) In Case 3, there are also spindle-shaped cells (arrow head) and pigment-laden cells (red arrow) in both of PAS staining and Masson's trichrome staining photos (A, B, C: \times 400).

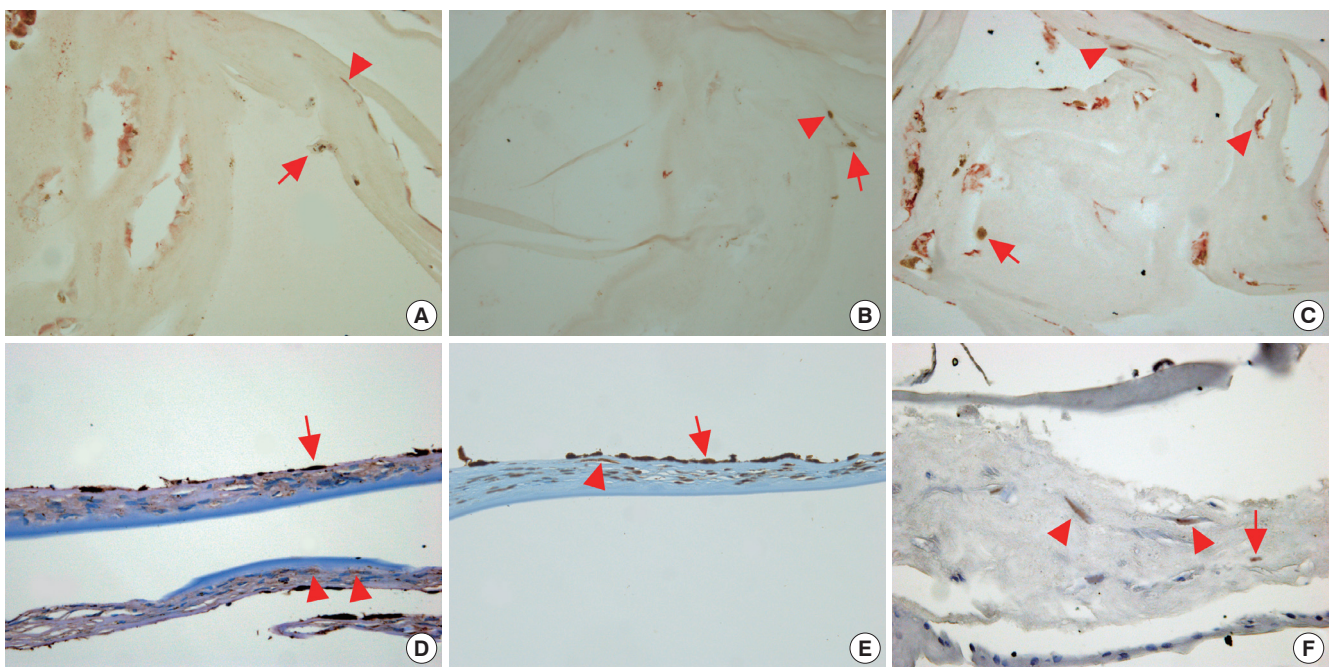


Fig. 2. Spindle-shaped cells in the retrocorneal membranes were positive for FGF- β (Case 4, A), TGF- β (Case 4, B), α -SMA (Case 4, C), β 1-integrin (Case 5, D), vimentin (Case 5, E) and hSTRO-1 (Case 6, F) (arrow heads). Pigment-laden cells do not react with any of the antibodies (red arrows) (A-F: \times 400).

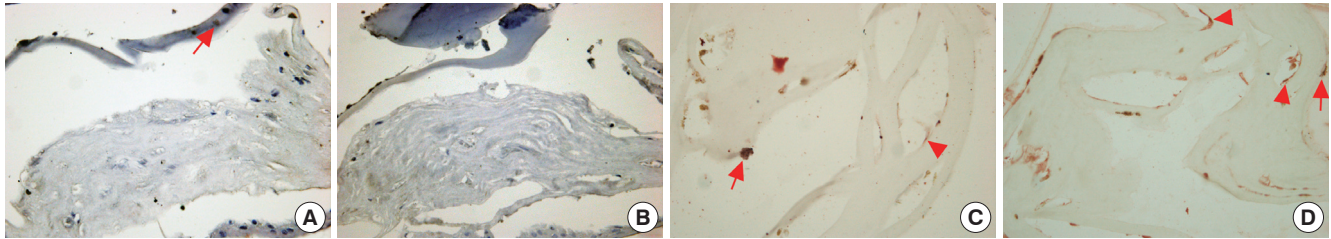


Fig. 3. CD34 (Case 6, **A**) and c-kit (Case 6, **B**) are not detected in any cells within the retrocorneal membrane. In contrast, VEGF (Case 4, **C**) and VEGF receptor 1 (Case 4, **D**) are slightly expressed (arrow heads). Red arrows indicate pigment-laden cells (**A-D**: $\times 400$).

Table 4. Comparison of several studies regarding retrocorneal membranes

	Marker for	Our study	Kawaguchi et al. (1)	Leung et al. (4)	Cockerham and Hidayat (5)	Dogru et al. (14)	Jakobiec and Bhat (16)	Wunderlich et al. (25)
CD34, CD133, c-kit	Hematopoietic stem cell	-	Not checked	Not checked	Not checked	Not checked	-CD34*	Not checked
Type IV collagen CD133, VEGF, VEGFR1	Corneal endothelial cell	-	Type IV collagen [†]	Not checked	Not checked	Type IV collagen [†]	Not checked	Not checked
$\beta 1$ -integrin, vimentin, TGF- β hSTRO-1	Mesenchymal stem cell	+	Vimentin [‡]	Not checked	Vimentin [‡]	Not checked	Vimentin [‡]	Vimentin [‡]
α -SMA	Fibroblast	+	+	Not checked	+	Not checked	+	+

*CD34 was negative and the rest were not tested; [†]Type IV collagen was positive, and the rest were not tested; [‡]Vimentin was positive, and the rest were not tested.

scattered widely throughout all RCMs. Additionally, beta-1-integrin (Fig. 2D), vimentin (Fig. 2E) and hSTRO-1 (Fig. 2F) were expressed in the spindle-shaped cells. However, all cells in the RCMs were negative for type IV collagen, CD133, and endothelial progenitor cell markers such as CD34 (Fig. 3A) and c-kit (Fig. 3B) except VEGF (Fig. 3C) and VEGFR1 (Fig. 3D). Pigment-laden cells did not react with any of the tested antibodies.

DISCUSSION

The incidence of RCM formation is known to be between 0%-7% after a perforating injury of the cornea and up to 54% after failed perforating keratoplasty (11). Risk factors that have been identified for RCM formation include chemical trauma, perforating injury, surgical trauma, and infectious disease. Many studies have been conducted to identify the pathogenesis of RCMs (1-6); however, the results have been largely inconclusive. These past studies have suggested that RCMs are connected to damaged portions of Descemet's membrane and that RCMs grow from the corneal endothelium into the anterior chamber (4, 5, 12-15). However, these hypotheses are based on the prerequisite that damaged corneas have functional keratocytes and endothelia.

We reasoned that endothelial cells of a damaged cornea, especially a chemically damaged cornea, would lack viability because cells are not present in the early stages following corneal damage, but as time progresses inflammatory cells begin to invade the corneal stroma. Additionally, Jakobiec and Bhat (16) reported that RCMs can be divided into five subtypes based on the results of immunohistochemical staining; however, their

study analyzed only RCMs that were adherent to the posterior surface of the cornea and, in contrast, our study examined RCMs that were separated from the cornea. As such, we undertook this study based on the assumption that RCMs are composed of cells from other sites that substitute for the non-functional corneal cells rather than from an endothelial mesenchymal transformation.

Type IV collagen is found in basal lamina, extracellular matrix proteins, mature stroma, and corneal endothelium (17). Some studies have found that type IV collagen was expressed in RCMs in syphilitic RCMs, which led the investigators to suggest that the endothelium may possibly be transformed into mesenchymal cells (1, 14). However, some researchers have asserted that type IV collagen antibodies do not react with all RCMs (4). Similarly, our study revealed no reaction of type IV collagen antibodies with the RCMs (Table 4). This implies that RCM can possibly be a kind of new creation unconnected with adjacent corneal endothelium or Descemet's membrane which expresses type IV collagen. However, RCMs also expressed several corneal endothelium marker including VEGF and VEGFR1. This partial expression pattern of corneal endothelial markers suggests the possible involvement of both of endothelial mesenchymal transition in RCM pathogenesis as previously reported (1, 14) and progenitor cell recruitment from other sites as a new concept of pathogenesis of RCM. This finding is supportive of the argument that RCMs do not originate from corneal endothelia. Instead, RCMs created by the process of neogenesis may originate from stem cells, such as hematopoietic stem cells or bone marrow-derived mesenchymal stem cells.

CD34 is a glycosylated transmembrane protein expressed in

both hematopoietic stem cells and normal corneal cells and is therefore not expressed in injured corneas (18). C-kit, also called CD117, is a cytokine receptor expressed on the surface of hematopoietic stem cells. In this study, cells of the RCMs did not react with hematopoietic stem cell markers CD34, CD133, and c-kit, suggesting that RCMs do not originate from hematopoietic stem cells.

Beta-1-integrin, also known as CD29, is a cell surface receptor that interacts with the extracellular matrix and mediates various intracellular signals. It plays important roles in cell adhesion and other various processes including embryogenesis, hemostasis, tissue repair, and immune response (19). In addition, Vimentin is an intermediate filament protein that is found in various non-epithelial cells, including mesenchymal cells and fibroblasts (1, 4). hSTRO-1 is a cell surface protein expressed by bone marrow stromal cells and erythroid precursors (20, 21). These three proteins are markers of mesenchymal stem cells, and, in this study, were shown to interact with RCM cells. Specifically, reactions for beta-1-integrin and vimentin were uniformly positive in the spindle-shaped cells.

Based on the vascularized cornea expressing copious CD34-positive cells (data not shown) adjacent to RCMs, we hypothesized that RCMs are created by bone marrow-derived mesenchymal stem cells. Mesenchymal stem cells migrate from bone marrow via vascular routes; causes of these migrations include pain from hypoxia, tissue damage, and chemotaxis induced by cytokines (8). Thus, we speculated that RCMs were created by bone marrow-derived mesenchymal stem cells that migrate via the vasculature in injured and inflamed tissues.

RCMs were not only composed of fibroblast-like spindle-shaped cells, but also pigment-laden cells. These pigment-laden cells did not stain with PAS, Masson's trichrome, or any of the immunohistochemical stains. Pigment cells are often found in the same environments as stem cells. For example, embryonic stem cells are found in pigmented epithelium, retinal stem cells originate from ciliary body or iris pigment epithelium, and limbal epithelial stem cells are pigmented in themselves (10, 22-24). Therefore, in these locations, pigmented cells might form a stem cell niche. In this way, pigment-laden cells in RCMs may create an environment for primitive cell differentiation resulting in newly formed structures.

Our study has a limitation which we could not perform all types of staining in all of cases. Instead, we collected positive findings of photos stained against each target protein from different case samples to sum representative photos. Because the RCM tissues are extremely thin and so very hard to handle, although such a summation of results might reduce the potency of generalization of our results, we could not perform all of marker staining in all of cases.

To clarify results of this study, evaluation of marker expression at the RNA level is also needed. In this study, we were able

to analyze only a very small amount of RCM tissue. As such, we only evaluated the protein levels within RCMs and did not use RT-PCR to evaluate RNA levels. Although the origin of cells in RCMs cannot be confirmed to be derived from endothelial or bone marrow stem cells in this study, the results of immunohistochemical staining suggested that RCM cells may originate from progenitor cells or mesenchymal stem cell-like cells.

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DISCLOSURE

The authors indicate no financial support or potential conflicts of interest.

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