

Molecular Study of Collagen XVII Gene in Retina

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Abstract: Collagen XVII is known to be a structural component of hemidesmosomes, which mediate adhesion of epidermal keratinocytes and certain other epithelial cells to the underlying basement membrane. In this study, we demonstrate that the expression of collagen XVII in vitreoretinal border in adult human eyes, the cultured Müller cells of retina were analyzed for the presence of mRNA of types I-VII, IX, and XVII collagen by RT-PCR. In addition to, this Müller cells were analysed by immunocytochemical and western blot using anticollagen antibodies. The cultured Müller cells synthesized mRNA for types I-VII, IX, and XVII collagen. The immunocytochemical and western blot analysis confirmed the expression of all types I-VII, IX, and XVII collagen. This collagens secretion into the medium was established for types I-VII, IX, and XII collagen. We concluded that the cultured Müller cells of retina can synthesize internal limiting lamina and vitreous collagens. The collagen production by müller cells explains the previous morphological findings in the embryonic and postnatal conditions in vivo.

Keyword: XVII collagen, Müller cells of retina, RT-PCR, immunocytochemical and western blot analysis.

1. Introduction

Collagen XVII is known to be a structural component of hemidesmosomes, which mediate adhesion of epidermal keratinocytes and certain other epithelial cells to the underlying basement membrane. The intracellular component of collagen XVII interacts with the β 4-integrin subunit, plectin, and BPAG1 [Hopkinson and Jones, 2000] to form a stable attachment of hemidesmosomes to keratin intermediate filaments within the cell. Recently, turnover and remodeling of vitreous collagen was described in human donor eyes. Evidence for collagen breakdown in matrix areas bordering liquefied spaces was found in the human vitreous [Los et al., 2003]. In addition to collagen breakdown, a study on vitreous collagens and two studies on the vitreoretinal interface found evidence of postnatal collagen synthesis in the human eye. The first detected type II procollagens in the vitreous [Itakura et al., 2005] and the latter two described intraretinal fibers and isolated packages of vitreous collagen (type II) [Wang et al., 2003; Ponsioen et al., 2005]. On aging, intraretinal collagen fibers expanded under the ILL at the vitreous base into networks and made contact with the basal vitreous, leading to the formation of vitreoretinal collagen connections [Wang et al., 2003]. We observed comparable intraretinal packages of vitreous collagen, often associated with surrounding Müller cell processes, focal interruptions of the ILL, and the presence of macrophages and cell debris. These findings could be consistent with a process of interactive remodeling with a net synthesis of vitreous collagens. Because of their close proximity to collagen packages, Müller cells may be involved in this process of matrix remodeling [Ponsioen et al., 2005].

Müller cells are radially oriented macroglia that traverse the retina from its inner (vitreous) border to the outer limiting membrane. These cells have many local functions: they stabilize the retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space [Newman et al., 1996; Sarthy et al., 2001]. In vivo and in vitro, Müller cells can produce and express several cytokines, growth

factors, and receptors [Sarthy et al., 2001]. Other features of Müller cells are the expression of cellular retinaldehyde binding protein (CRALBP), vimentin, and, on activation, glial fibrillary acidic protein (GFAP) [Willbold et al., 1998; Limb et al., 2002]. A possible role in the production of vitreous macromolecules during growth and in adulthood has been suggested [Swann et al., 1980; Mann et al., 1928], but little is known about their capability to produce vitreous and internal limiting lamina (ILL) collagens postnatally. Whether Müller cells are capable of producing basement membrane components is a matter of debate. Some studies find evidence hereof [[Laqua et al., 1975; Holländer et al., 1991], while others fail to confirm it [Foos et al., 1975; Sarthy et al., 1993].

In vitro, capacity of the human Müller cell line [Limb et al., 2002], to synthesize vitreous collagens (i.e., types II, V/XI, VI, and IX) [Bishop et al., 1996], collagens (types IV and VI) [Ihanamaki et al., 2004], type VII collagen [Myllyharju et al., 2004], which appears to be present in the human retina by immunohistochemical staining), collagens described in epiretinal and vitreoretinal membranes (types I-V) [Scheiffarth et al., 1988; Okada et al., 1995], and a collagen not related to the vitreoretinal interface (the hemidesmosomal transmembrane type XVII collagen found in basement membranes of stratified and pseudostratified epithelia) [Claudepierre et al., 2005]. In the present study, we investigate the expression of Müller cells for collagen XVII, and their secretion into the cell medium.

2. Methods

2.1 Culture of cells

The Müller cells MIO-M1 [Limb et al., 2002] were cultured to confluence in Dulbecco's modification of Eagle's medium (DMEM) high glucose containing L-glutamax I (Life Technologies Inc.), 10% fetal bovine serum (FBS; Life Technologies Inc.) and 1% penicillin/streptomycin. For western blot (WB) analyses of the supernatant, Müller cells were cultured in DMEM high glucose containing L-glutamax I without FBS and supplemented with 1% G5 (Life Technologies Inc.), 0.2 mM β -aminopropionitrile

fumurate salt (β -APN; Sigma), 0.2 mM ascorbic acid (Sigma), and 1% penicillin/streptomycin, since 10% FBS caused clotting of the medium after our concentration procedure. The serum-free medium with supplements was introduced after 24 h to allow uniform attachment of the Müller cells. Ascorbic acid promotes the intracellular hydroxylation of prolyl and lysyl residues during collagen synthesis [Wright et al., 1988], whereas β -APN inhibits the enzyme lysyl oxidase in the extracellular space thus preventing collagen cross-link formation [Englard et al., 1986].

2.2 Reverse transcriptase-polymerase chain reaction

Total RNA from the Müller cells was extracted by RNeasy Mini kit method (Qiagen) according to the manufacturer's instructions. To eliminate DNA contamination, we treated RNA samples with DNase treatment Ambion-kit (DNA-free). RNA concentration and purity were determined on a spectrophotometer by calculating the ratio of optical density at wavelengths of 260 and 280 nm. Two μ g RNA was

reverse transcribed into cDNA using reverse transcriptase according to manufacturer's protocol for a total reaction of 20 μ l.

For the PCR reaction, 1 μ l cDNA was added to 23 μ l "master mix" consisting of 2.5 μ l 10 \times PCR buffer, 2.5 μ l 2 mM dNTP mix, 1.5 μ l 25 mM MgCl₂, 0.25 μ l (5 U/ μ l) Taq DNA polymerase (Fermentas) and 16.25 μ l milli-Q water. Finally, a total of 1 μ l of the two specific flanking primers (50 μ M) was added (Table 1). The mixtures were initially denatured at 94 °C for 5 min. The PCR consisted of 35 cycles at the following conditions: denaturation at 94 °C for 0.5 min, annealing at 55 °C (for types I, II, III, V, and IX collagen) and at 58 °C (for types IV, VI, VII, XI, and XVII collagen) for 1 min, and an extension period at 72 °C for 1 min. These cycles were followed by a final extension period at 72 °C of 10 min. PCR products were analyzed by agarose gel electrophoresis (1%) with 500 ng/ml ethidium bromide. Keratinocytes were used as positive control for type XVII collagen.

Table 1: The primers used in the RT-PCR analysis

Collagen mRNA	Forward primer 5'→3'	Reverse primer 5'→3'	Size (bp)
<i>COL1A1</i>	TCG GCG AGA GCA TGA CCG ATG GAT	GAC GCT GTA GGT GAA GCG GCT GTT	254
<i>COL2A1</i>	GTG GAA GAG TGG AGA CTA CTG	TGT ACG TGA ACC TGC TAT TG	419
<i>COL3A1</i>	ACC GAT GAG ATT ATG ACT TCA CT	CTG CAC ATC AAG GAC ATC TTC AG	369
<i>COL4A2</i>	ATC GGC TAC CTC CTG GTG AA	GCT GAT GTG TGT GCG GAT GA	648
<i>COL5A1</i>	GAC TAC GCG GAC GGC ATG GAA	CCT GCC AGG CCA CTG ACT GGT A	454
<i>COL6A1</i>	GGA GCT CAA GGA AGC CAT CAA G	TCC TCC AGC AGC TCT GCA TAG T	342
<i>COL7A1</i>	CCG AGG ACG AGA TGG TGA AGT TG	CTG GCT CCA GGT CCT GTG TCT AC	261
<i>COL9A1</i>	GCC TCT GGT GAA GAA GGT GAA	TGC TGA TCT GTC GGT GCT CTA	245
<i>COL17A1</i>	ATG GAG CTG CTC ATC ATG AC	AGG AGT AGC AGC CAG GTG AG	460

2.3 Immunocytochemistry

By light microscopy (LM), Müller cells were identified by their morphology and by their expression of CRALBP, vimentin, and GFAP. The expression of cellular characteristics was measured in at least three microscopic areas at a magnification of 10 times. To determine the intracellular expression of collagens, we specifically stained Müller cells with antibodies against human types I-VII, IX, and XVII collagen.

For immunocytochemical staining, cells were seeded for 48 h in glass chamber slides. After fixation with 1:1 acetone/methanol for 10 min at -20 °C, the slides were washed with phosphate buffered saline (PBS) and pre-incubated for 30 min with 3% serum of the producer of the secondary antibody in PBS with 2% BSA (BSA) (Sanquin, Amsterdam, the Netherlands), followed by incubation for 1 h with primary antibodies diluted 1:50 in PBS with 1% BSA. In the case of types VI, VII, IX, and XI collagen, the latter step was preceded by blocking steps with avidin and biotin. The primary antibodies included the following: rabbit polyclonal antibodies against CRALBP (UW55, a kind gift from J.C. Saari, University of Washington, Seattle, WA) and human types I, III, V (Abcam, Cambridge, UK), and XI collagen (a kind gift from J. Oxford, Boise State University, Boise, Idaho); a biotinylated rabbit polyclonal antibody against human type VI collagen (Abcam); goat polyclonal

antihuman antibodies against types II and IV collagen (Southern Biotechnology Associates, Birmingham, AL); and mouse monoclonal antihuman antibodies against vimentin (DAKO, Glostrup, Denmark), GFAP (Sigma), and types VII (Abcam), IX (USBiological, Swampscott, MA) and XVII collagen (1A8C [Nishizawa et al., 1993]; a kind gift from K. Owaribe, Nagoya University, Japan). Subsequently, cells were washed with PBS and endogenous peroxidases were blocked. Secondary antibodies included a swine-antirabbit peroxidase (DAKO), a biotinylated goat-antirabbit (GARbio, DAKO), a rabbit-antigoat peroxidase (DAKO), a rabbit-antimouse peroxidase (DAKO), a biotinylated goat-antimouse IgG1 (GAMbio, SBA), and a biotinylated goat-antimouse IgG2a (GAMbio, SBA). Secondary antibodies were diluted 1:100 with PBS containing 2% human serum was derived from a pool of human volunteers and added for 1 h at room temperature. Biotinylated type VI collagen, GARbio, and GAMbio (IgG1 and IgG2) were followed by incubation with ABC complex horse radish peroxidase (DAKO) for 20 min. Finally, cells were stained with 3-amino-9-ethylcarbazole (AEC; Sigma) and hematoxylin. Negative controls underwent the entire procedure, except for the substitution of the primary antibody.

2.4 Western Blot

Isolation of cell extract and supernatant concentration

Cells were harvested in sodium dodecyl sulfate PAGE (SDS-PAGE) denaturation buffer (10 mM Tris-HCl, pH 7 containing 1 mM EDTA, 2.5% SDS, 5% 2-mercaptoethanol, and 10% glycerol). The extract was heated for 5 min at 100 °C to unfold the collagen helices into separate α -chains.

After dead cells and cellular debris were removed by centrifuging at 1,600 rpm for 5 min, the supernatant was concentrated by ultrafiltration with an Amicon membrane (100,000 kDa cut-off; Millipore, Billerica, MA) and, in the case of collagen XI, with a Vivaspin 0.5 ml concentrator (30,000 kDa cut-off; Vivascience, Hanover, Germany).

Immunoblotting procedure

Polyacrylamide SDS electrophoresis was performed according to the method of Laemmli, 1970, using 3.9% and 5% slab gels and a 72-mm wide 2D gel comb in the Bio-Rad Mini Protean II electrophoresis apparatus (Bio-Rad, Hercules, CA). After separation, the gel was blotted to nitrocellulose using the Mini Protean II blotting unit (Bio-Rad) with 22 mM Tris, 168 mM Glycine, 0.05% SDS, and 20% methanol as a transfer buffer. After the transfer, the nitrocellulose was blocked for 1 h in TBS-buffer (20 mM Tris-HCl and 500 mM NaCl; pH 7.5) containing 3% BSA. Primary antibodies (Table 2) were diluted 1:500 in TBS and added to the blot. After incubation overnight, the blot was washed with TBS containing 0.05% Tween-20 (TTBS), and then secondary antibodies (Table 2) diluted 1:500 in TTBS were added. After 1 h incubation, the blot was washed with TTBS and incubated with alkaline phosphatase (AP)-conjugated tertiary antibody (Table 2) diluted 1:250 in TTBS for another hour. After washing with TTBS and AP buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM MgCl₂, pH 9.5), the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in AP buffer. All

incubation and washing steps were performed at room temperature.

2.5 Collagenase digestion

Cell extracts were mixed with CaCl₂ to a final concentration of 10 mM to inactivate EDTA. Type VII collagenase (high purity grade, Sigma) was added in increasing concentrations (0–30 units/ml) to 60 μ l cell extract and incubated for 1 h at 37 °C. Previously, the absence of nonspecific proteases in this collagenase batch had been confirmed [Pas et al., 1997]. To confirm that type VII collagenase specifically cleaves collagen, we exposed Müller cell extracts to this collagenase, after which vimentin and CRALBP expressions were checked. After incubation, samples were mixed with SDS-PAGE sample buffer to inactivate collagenase activity.

2.6 Cell viability

To determine cell viability, Müller cells and their medium supplemented with 1% G5, 0.2 mM β -APN, and 0.2 mM ascorbic acid were harvested and compared to a well with 10% FBS, which served as a reference, after an incubation period of 48 h. Dead cells were stained with trypan blue, counted in a Bürker-Türker counter (W. Schreck, Hofheim, Germany), and compared to viable cells.

3. Results

3.1 Reverse transcriptase polymerase chain reaction

Müller cells expressed mRNA of all tested collagen types, in addition to, type XVII collagen (Figure 1). Amplimers were seen at the expected positions, which are for *COL1A1* at 254 bps, for *COL2A1* at 419 bps, for *COL3A1* at 369 bps, for *COL4A2* at 648 bps, for *COL5A1* at 454 bps, for *COL6A1* at 342 bps, for *COL7A1* at 261 bps, for *COL9A1* at 245 bps, and for *COL17A1* at 460 bps.

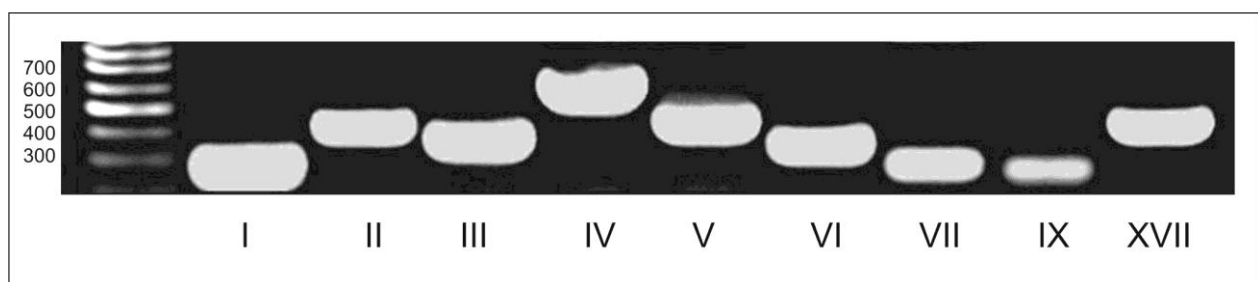


Figure 1: mRNA of types collagen by RT-PCR on Müller cell extracts. From left to right, bands indicating the positions of types I, II, III, IV, V, VI, VII, IX, and XVII collagen are depicted. At the left margin, a 100 bp DNA ladder has been added.

3.2 Immunocytochemistry

Light microscopy results revealed that Müller cells preserved their morphology and characteristics under the

culture method with FBS. They were all positive for vimentin and CRALBP, and less than 5% of the cells were GFAP positive. In the case of collagen staining, the cytoplasm of all Müller cells was positive for all collagens (Table 2; Figures 2A-F). The pattern of the cytoplasmic staining varied in intensity and had a granular to fibrillary aspect. Types V, VI, and XVII collagen also stained positively outside the cell, e.g., as small granules and fibers. All negative controls showed no staining.

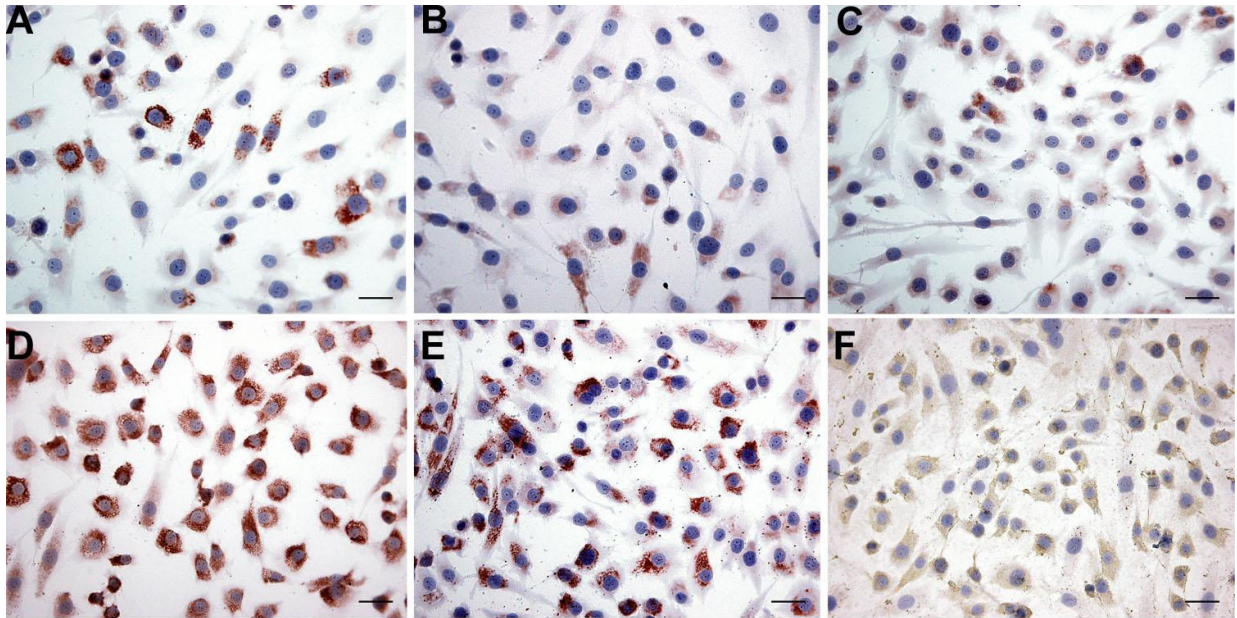


Figure 2: Immunocytochemical analyses of cultured Müller cells. A: Type XVII collagen shows a granular staining with a variable intensity around cells. B: Type II collagen is seen as a faint staining in the cytoplasm. C: Type III collagen is positive in all cells. D: Types IV and IX collagen is visible as a strong granular cytoplasmic staining. E: Type V collagen shows mainly staining in the cytoplasm. F: In the case of type VI collagen, the cells are predominantly stained in the cytoplasm. Bars in all panels equal 50 µm.

Type XVII	++	+	Granules-fibers	++	Granular-fibrillar	Diffuse
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Table 2: Immunocytochemical analysis for retinal Müller cells. Müller cells were fixed on glass chamber slides and stained with specific antibodies against types I-VII, IX, and XVII collagen. LM confirmed the intracellular expression of all the above-mentioned collagens. The aspect of the collagen staining differed in aspect (diffuse, granular, or fibrillar) and intensity. In the table, the following symbols and abbreviations are used: strongly positive (++), positive (+), negative (-), not detected (n.d.), medium with 10% fetal bovine serum (FBS), and medium with 1% G5.

Table 2:

Collagen Types	Cytoplasm staining		Extracellular staining		Cytoplasm aspect	
	FBS	G5	FBS	G5	FBS	G5
Type I	++	+	n.d.	++	Granular	Granular
Type II	+	+	n.d.	+	Diffuse	Diffuse - granular
Type III	+	+	n.d.	n.d.	Diffuse	Diffuse
Type IV	++	++	n.d.	n.d.	Granular	Granular
Type V	++	+	Granules	++	Granular - fibrillar	Diffuse
Type VI	+	++	Granules - fibers	n.d.	Granular	Granular
Type VII	+	++	n.d.	n.d.	Diffuse	Granular
Type IX	++	++	n.d.	n.d.	Diffuse	Diffuse

3.3 Western blot

The cell extracts of Müller cells and medium with G5 were immunoblotted after SDS-PAGE gel for the presence of types I-VII, IX, and XVII collagen. The gels showed specific collagen bands (Table 3; Figure, 3A-C), which disappeared the intensity of bands on treatment with increasing doses of collagenase. In the case of types IV, VI, and XVII collagens (Figure 3B-C), new product bands were detected after collagenase treatment. The collagenase had no effect on the bands of CRALBP and vimentin as control.

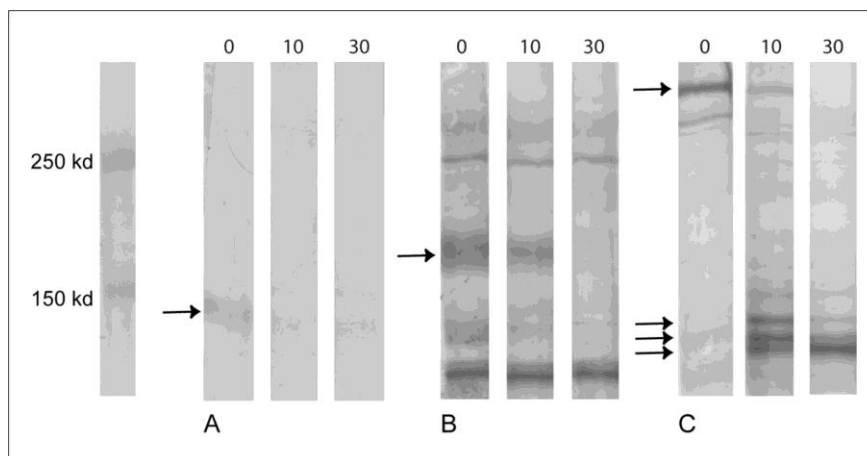


Figure 3: Western blot analyses of Müller cell extracts with the addition of collagenase (0, 10, and 30 units/ml). **A:** The specific band of type II collagen is shown at 150 kDa and disappears without formation of new bands when collagenase is added. **B:** At 180 kDa, the specific band of type XVII collagen and formation of new bands when collagenase is added. **C:** Addition of collagenase to type VII collagen results in the gradual disappearance of the specific band at 270 kDa and the appearance of breakdown products at 130, 135, and 140 kDa. Specific bands are indicated with arrows.

Table 3: The SDS-PAGE and western blot analysis: Collagen secretion into the medium was established for types I-VII, IX, and XVII collagen. The specific collagen antibodies detected the collagen bands (in kDa) obtained before and after treatment with collagenase. The results of both Müller cell extracts and medium with G5 are shown the same bands. The results after collagenase concern Müller cell extracts and confirm the collagen nature of the bands as seen by WB. "None" indicates no collagen bands were detected.

Table 3:

Collagen	Molecular weight of collagen bands in Müller cells	Molecular weight of collagen bands in culture medium	Molecular weight of collagen bands after collagenase
Type I	140 kDa	140 kDa	None
Type II	150 kDa	150 kDa	None
Type III	180 kDa	180 kDa	None
Type IV	130 and 210 kDa	130 and 210 kDa	75, 90, 110, and 250 kDa
Type V	260 kDa	260 kDa	None
Type VI	120 and 230 kDa	120 and 230 kDa	150 kDa
Type VII	270 kDa	270 kDa	130, 135, and 140 kDa
Type IX	200 kDa	150 and 200 kDa	None
Type XI	100, 150, and 200 kDa	150 kDa	None
Type XVII	180 kDa	180 kDa	35, and 20 kDa

3.4 Analysis of Conditioned Growth Medium

Without FBS but with G5, Müller cells preserved their immunocytochemical characteristics, although, morphologically, they appeared a little stretched. Cell viability remained above 95% with a slightly diminished proliferation rate compared to conditions with 10% FBS. In comparison with cells grown in the medium with FBS, the RT-PCR results were similar. LM results were comparable but showed less intense intracellular and more extracellular staining for types I, II, V, and XVII collagen. Types I and V collagen were visible outside the cell as fibrillar threads and types II and XVII collagen also, but to a lesser extent and they had a more granular aspect (Figures 4A-D). In the medium, we found specific collagen bands (Table 3) similar to those detected in the cell extracts except for type IX collagen where in addition to the 200 kDa band a weak band at 150 kDa was present. For type IX collagen, only the 150 kDa band was detected in the medium.

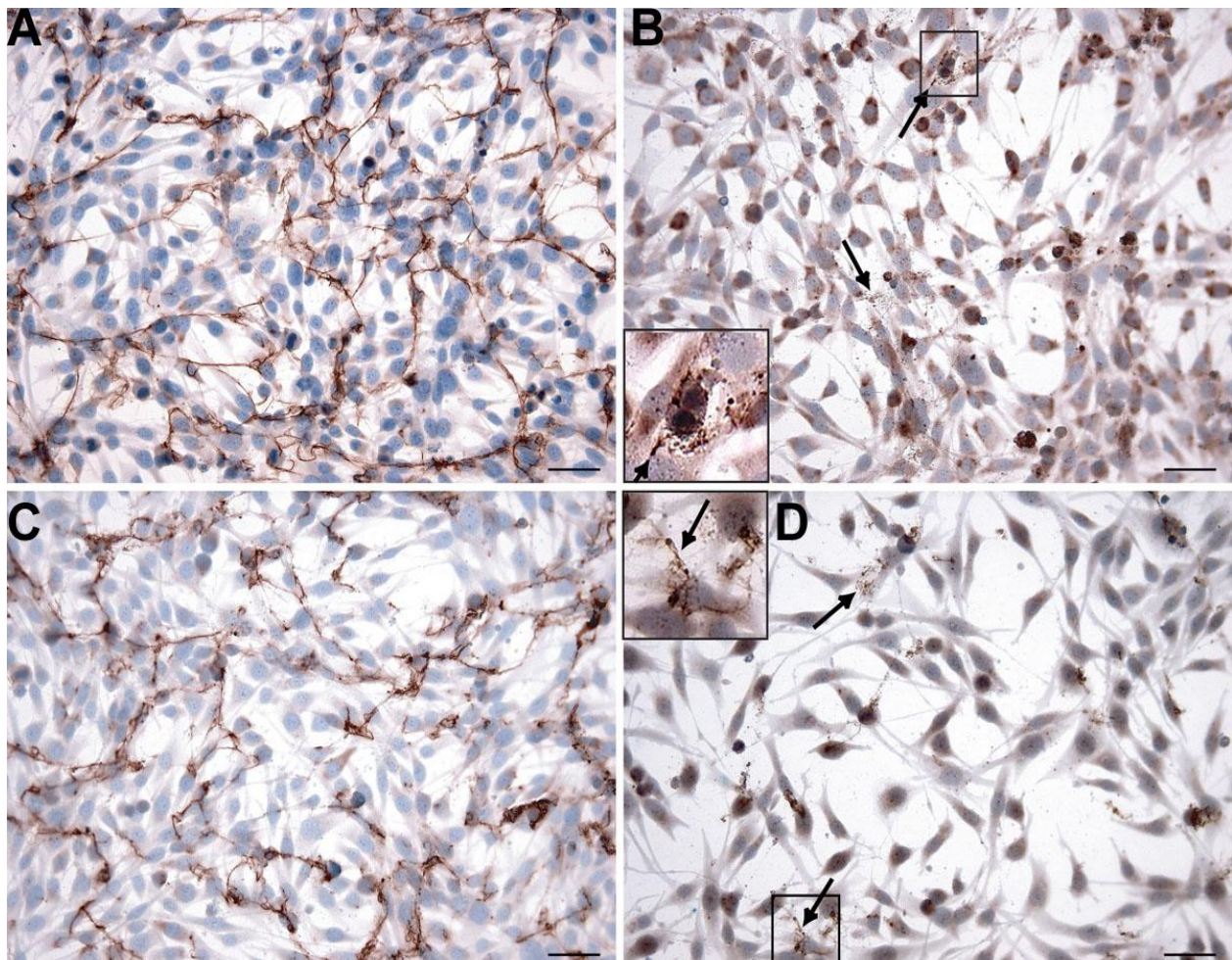


Figure 4: Immunocytochemical analyses of cultured Müller cells in medium. Types I (A) and V (C) collagen show clear extracellular fibrillar threads and less in intracellular staining. Types XVII (B) and II (D) collagen show some fine extracellular threads and small granules (arrows) in intracellular staining. In the inlays of Figures 4B and 4D, the extracellular collagen is magnified two times. Bars in each panel equal 50 μm .

4. Discussion

This study shows collagen synthesis by retinal Müller cells in vitro. Müller cells expressed mRNAs coding for types I-VII, IX, and XVII collagen. At the protein level, these collagens were demonstrated by immunocytochemical staining and shown to be present in the cytoplasm with LM. WB analysis of the cell extracts and of the medium in which the cells had been cultured confirmed the intracellular production and demonstrated that types I-VII, IX, and XVII collagen were also secreted into the medium. The detected collagen bands could be procollagen chains as well as collagen chains. Müller cells did express type XVII collagen, a basement membrane protein that was recently demonstrated near photoreceptor synapses and its outer segments [Claudepierre et al., 2005; Bishop, 2011]. Apparently, Müller cells synthesize those collagens that are found in their natural vicinity (vitreous, and retina).

Although the spontaneously immortalized Müller cells have been well characterized [Limb et al., 2002] and shown to keep their main characteristics, cell models in general have as a major limitation that they are artificial in vitro systems unlike an in vivo model. Cultured Müller cells therefore might display somewhat deviant behavior because the cells are not in their natural surrounding, since they are growing on medium and moreover have shown spontaneous immortalization. To confirm our findings, primary isolated Müller cells could provide additional information, but in vivo data would be preferable.

For the WB experiments with Müller cells, ascorbic acid and β -APN were added to stimulate collagen synthesis and prevent extracellular collagen cross-linking, respectively [Geesin et al., 1988; Eyre et al., 1984]. The differences in collagen staining observed by LM the increased extracellular staining for types I, II, V, and XVII collagen paralleled by a decreased intracellular staining were most likely the effect of ascorbic acid. The digestion experiments with collagenase confirmed the collagen nature of the bands as seen by WB.

We hypothesize that the in vitro capability of Müller cells to produce the aforementioned collagens might (1) adduce support to previously described morphological findings in the embryonic period [Swann et al., 1980], (2) contribute to the stable level of postnatal vitreous collagen [Balazs et al., 1982], and (3) explain, in part, the origin of epiretinal membranes in pathology (see below) [Rentsch et al., 1977].

In the embryonic vitreous, the neural retina and sometimes specifically Müller cells are indicated as possible sources of vitreous and collagens. In chicken embryos, retina was involved in collagen (e.g., type II) synthesis [Smith et al., 1976]. In the developing mouse neural retina, mRNA of

types II and IX collagen has been detected [Savontaus et al., 1998]. In the human embryo, Müller cells seemed continuous with the vitreous fibrils (primarily collagen type II) present at the vitreal side [Sebag et al., 1989]. The production of vitreous fibrils during embryonic growth of the eye was ascribed to Müller cells and other cell types Swann et al., 1980]. Also, müller cells were supposed to contribute to the formation in the human embryo.

The postnatal vitreous has long been regarded as an almost inert extracellular matrix, in which hardly any production or breakdown of its macromolecular components occurs [Eyre 1984]. Recent studies question the inertness of the vitreous body and suggested turnover of vitreous components [Bertazolli et al., 1996]. Currently, two hypotheses on vitreous aging are postulated. The first is a concept of vitreous destabilization on aging because synchysis (liquefaction) and syneresis (aggregation of vitreous matrix components) lead to the formation of spaces and aggregated collagens; the second is a view that extracellular breakdown of vitreous matrix [Los et al., 2003] (synchysis) would coincide with production of vitreous collagen [Ihanamaki et al., 2004], leading to an increase in optically dense structures over time. In both views, the total amount of collagen in the human vitreous appears to remain stable during life [Balazs et al., 1984], which, for the latter theory, may indicate that collagen synthesis and collagen breakdown are in equilibrium. The in vitro capacity of the Müller cell to synthesize vitreous collagens suggests a possible role in postnatal vitreous collagen synthesis. In addition, our in vitro results support the possible role of Müller cells in the formation of sublamellar vitreoretinal collagen complexes expanding on aging [Wang et al., 2003].

Müller cells are found in epiretinal membranes in pathological circumstances such as massive retinal gliosis, preretinal macular fibrosis, idiopathic epiretinal membranes, and retinal injuries or degeneration [Nork et al., 1986]. These membranes can contain types I-V collagen [Okada et al., 1996]. In morphological studies, Müller cells appear to contribute to the formation of pathological membranes [Okada et al., 1990]. However, they may not be the only type of cell involved since glial cells; astrocytes, fibrocytes, and retinal pigment epithelium have also been observed in epiretinal membranes [Claudepierre et al., 2005].

In summary, the finding that immortalized human Müller cells synthesize collagens in vitro indicates that they might also be involved in this process in vivo. Collagen synthesis by Müller cells could explain and expand on previous morphological findings in the embryonic and postnatal period as well as in pathologic conditions.

References

- [1] Balazs EA, Denlinger J. L. Aging Changes in the Vitreous. In: Sekuler R, Kline D, Dismukes K, editors. Aging and Human Visual Function. New York: Alan R. Liss, Inc.; 1982. p. 45–58.
- [2] Bertazolli Filho R, Laicine EM, Haddad A. Biochemical studies on the secretion of glycoproteins by isolated ciliary body of rabbits. Acta Ophthalmol Scand. 1996; 74:343-7.

- [3] Bishop PN. The biochemical structure of mammalian vitreous. *Eye*. 2011; 10:664-70.
- [4] Cheah KS, Lau ET, Au PK, Tam PP. Expression of the mouse alpha 1(II) collagen gene is not restricted to cartilage during development. *Development*. 1991; 111:945-53.
- [5] Claudepierre T, Manglapus MK, Marengi N, Radner S, Champlaud MF, Tasanen K, Bruckner-Tuderman L, Hunter DD, Brunken WJ. Collagen XVII and BPAG1 expression in the retina: evidence for an anchoring complex in the central nervous system. *J Comp Neurol*. 2005; 487:190-203.
- [6] Englard S, Seifter S. The biochemical functions of ascorbic acid. *Annu Rev Nutr*. 1986; 6:365-406.
- [7] Eyre DR, Paz MA, Gallop PM. Cross-linking in collagen and elastin. *Annu Rev Biochem*. 1984; 53:717-48.
- [8] Foos RY, Gloor BP. Vitreoretinal juncture; healing of experimental wounds. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1975; 196:213-20.
- [9] Gartner J. Pathology of the basal lamina at the peripheral vitreoretinal junction. *Dev Ophthalmol*. 1981; 2:353-62.
- [10] Geesin JC, Darr D, Kaufman R, Murad S, Pinnell SR. Ascorbic acid specifically increases type I and type III procollagen messenger RNA levels in human skin fibroblast. *J Invest Dermatol*. 1988; 90:420-4.
- [11] Hogan MJ, Alvarado JA, Weddell JE. *Retina. Histology of the human eye*. Philadelphia: W.B. Saunders Company; 1971. p. 393-522.
- [12] Holländer H, Makarov F, Dreher Z, van Driel D, Chan-Ling TL, Stone J. Structure of the macroglia of the retina: sharing and division of labour between astrocytes and Müller cells *J Comp Neurol*. 1991; 313:587-603.
- [13] Hopkinson S. and J. C. R. Jones, "The N terminus of the transmembrane protein BP180 interacts with the N-terminal domain of BP230, thereby mediating keratin cytoskeleton anchorage to the cell surface at the site of the hemidesmosome," *Molecular Biology of the Cell*, vol. 11, no. 1, pp. 277-286, 2000.
- [14] Ihanamaki T, Pelliniemi LJ, Vuorio E. Collagens and collagen-related matrix components in the human and mouse eye. *Prog Retin Eye Res*. 2004; 23:403-34.
- [15] Itakura H, Kishi S, Kotajima N, Murakami M. Vitreous collagen metabolism before and after vitrectomy. *Graefes Arch Clin Exp Ophthalmol*. 2005; 243:994-8.
- [16] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680-5.
- [17] Laqua H, Machemer R. Glial cell proliferation in retinal detachment (massive periretinal proliferation). *Am J Ophthalmol*. 1975; 80:602-18.
- [18] Limb GA, Salt TE, Munro PM, Moss SE, Khaw PT. In vitro characterization of a spontaneously immortalized human Muller cell line (MIO-M1). *Invest Ophthalmol Vis Sci*. 2002; 43:864-9.
- [19] Los LI, van der Worp RJ, van Luyn MJ, Hooymans JM. Age-Related Liquefaction of the Human Vitreous Body: LM and TEM Evaluation of the Role of Proteoglycans and Collagen. *Invest Ophthalmol Vis Sci*. 2003; 44:2828-33.
- [20] Mann IC. The vitreous and suspensory ligament of the lens. The development of the human eye. London: Cambridge University Press; 1928. p. 151-89.
- [21] Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet*. 2004; 20:33-43.
- [22] Newman E, Reichenbach A. The Muller cell: a functional element of the retina. *Trends Neurosci*. 1996; 19:307-12.
- [23] Nishizawa Y, Uematsu J, Owaribe K. HD4, a 180 kDa bullous pemphigoid antigen, is a major transmembrane glycoprotein of the hemidesmosome. *J Biochem*. 1993; 113:493-501.
- [24] Nork TM, Ghobrial MW, Peyman GA, Tso MO. Massive retinal gliosis. A reactive proliferation of Muller cells. *Arch Ophthalmol*. 1986; 104:1383-9.
- [25] Okada M, Matsumura M, Ogino N, Honda Y. Müller cells in detached human retina express glial fibrillary acidic protein and vimentin. *Graefes Arch Clin Exp Ophthalmol*. 1990; 228:467-74.
- [26] Okada M, Ogino N, Matsumura M, Honda Y, Nagai Y. Histological and immunohistochemical study of idiopathic epiretinal membrane. *Ophthalmic Res*. 1995; 27:118-28.
- [27] Pas HH, Kloosterhuis GJ, Heeres K, van der Meer JB, Jonkman MF. Bullous pemphigoid and linear IgA dermatosis sera recognize a similar 120-kDa keratinocyte collagenous glycoprotein with antigenic cross-reactivity to BP180. *J Invest Dermatol*. 1997; 108:423-9.
- [28] Ponsioen TL, van der Worp RJ, van Luyn MJ, Hooymans JM, Los LI. Packages of vitreous collagen (type II) in the human retina: an indication of postnatal collagen turnover? *Exp Eye Res*. 2005; 80:643-50.
- [29] Ramirez JM, Trivino A, Ramirez AI, Salazar JJ, Garcia-Sanchez J. Structural specializations of human retinal glial cells. *Vision Res*. 1996; 36:2029-36.
- [30] Rentsch FJ. The ultrastructure of preretinal macular fibrosis. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1977; 203:321-37.
- [31] Sarthy V. Collagen IV mRNA expression during development of the mouse retina: an in situ hybridization study. *Invest Ophthalmol Vis Sci*. 1993; 34:145-52.
- [32] Sarthy V, Ripps H. *The Retinal Müller Cell, Structure and Function*. New York: Kluwer Academic / Plenum; 2001. p. 1-65.
- [33] Savontaus M, Ihanamaki T, Metsaranta M, Vuorio E, Sandberg-Lall M. Localization of type II collagen mRNA isoforms in the developing eyes of normal and transgenic mice with a mutation in type II collagen gene. *Invest Ophthalmol Vis Sci*. 1997; 38:930-42.
- [34] Savontaus M, Ihanamaki T, Perala M, Metsaranta M, Sandberg-Lall M, Vuorio E. Expression of type II and IX collagen isoforms during normal and pathological cartilage and eye development. *Histochem Cell Biol*. 1998; 110:149-59.
- [35] Scheiffarth OF, Kampik A, Gunther H. von der MK. Proteins of the extracellular matrix in vitreoretinal membranes. *Graefes Arch Clin Exp Ophthalmol*. 1988; 226:357-61.

- [36] Sebag J. Embryology of the Vitreous. In: Arcata Graphics/Halliday, editor. The Vitreous. New York: Springer-Verlag; 1989. p. 7–16.
- [37] Smith GN, Linsenmayer TF, Newsome DA. Synthesis of type II collagen in vitro by embryonic chick neural retina tissue. Proc Natl Acad Sci USA. 1976; 73:4420-3.
- [38] Swann DA. Chemistry and biology of the vitreous body. Int Rev Exp Pathol. 1980; 22:1-64.
- [39] Wang J, McLeod D, Henson DB, Bishop PN. Age-dependent changes in the basal retinovitreal adhesion. Invest Ophthalmol Vis Sci. 2003; 44:1793-800.
- [40] Willbold E, Layer PG. Muller glia cells and their possible roles during retina differentiation in vivo and in vitro. Histochem J. 1998; 30:531-52.
- [41] Wolburg H, Willbold E, Layer PG. Muller glia endfeet, a basal lamina and the polarity of retinal layers form properly in vitro only in the presence of marginal pigmented epithelium. Cell Tissue Res. 1991; 264:437-51.
- [42] Wright GC, Jr, Wei XQ, McDevitt CA, Lane BP, Sokoloff L. Stimulation of matrix formation in rabbit chondrocyte cultures by ascorbate. 1. Effect of ascorbate analogs and beta-aminopropionitrile. J Orthop Res. 1988; 6:397-407.