Roum Biotechnol. Lett. Vol. 6, No. 2, 2001, pp.137 - 145

ORIGINAL PAPER

Corneal Collagen Interaction with Proteoglycans and Glycosaminoglycans

LUCIA MOLDOVAN*, OTILIA ZARNESCU**, ANCA OANCEA*, ANISOARA CIMPEAN**, LIDIA CONSTANTINESCU*

** National Institute for Biological Sciences, 296 Spl. Independentei, P.O. Box 17-16, 77748, Bucharest, Romania, Fax: +40-1-220.76.95*
*** Faculty of Biology, University of Bucharest, 91-95 Spl. Independentei, Bucharest, Romania*

Received: 28th February 2001, Accepted: 25th March, 2001

Abstract

This study investigated the effects of proteoglycans (decorin and lumican) and glycosaminoglycans (chondroitin/dermatan sulfate and keratan sulfate) on type I collagen fibrillogenesis using an in vitro assay for fibril formation. Proteoglycans, glycosaminoglycans and collagen were extracted and purified from pig cornea.

Our turbidimetric and electron microscopic studies indicate that both corneal proteoglycans and their glycosaminoglycan chains influence *in vitro* collagen fibril formation, diminishing their diameters, but the process of collagen fibril growth inhibition by glycosaminoglycans is low compared to that of intact proteoglycans. These data show that the interaction of pig corneal collagen with proteoglycans is one mechanism modulating fibril diameter and the activity of fibril size regulation is due mainly to proteoglycans protein core rather than their glycosaminoglycan chains.

Keywords: collagen, proteoglycans, glycosaminoglycans, cornea, fibrils, interaction

Introduction

Corneal transparency is dependent on the size and arrangement of collagen fibrils within the corneal stroma [1]. The corneal stroma is composed primarily of collagen type I fibrils and two proteoglycans (PGs): one with chondroitin/dermatan sulfate (CS/DS) side-chains (decorin) and one with keratan sulfate (KS) side-chains (lumican) [2]. Both of these proteoglycans have been localized to specific binding sites on collagen fibrils in the cornea as revealed by electron microscopy [3].

Corneal mature stroma consists of small-diameter (~25 nm) collagen fibrils arranged in orthogonal layers. The maintenance of constant fibril diameter, regular packing of fibrils and organization of these fibrils into orthogonal lamellae determine the integrity and transparency of the tissue.

Collagen fibril formation is a multistep process and there are numerous phases where fibril characteristics, such as diameter, may be regulated. A number of factors have been suggested as being important in the regulation of fibril diameter. These include hydroxylation and glycosylation of collagen molecules [4], procollagen processing [5,6], collagen-collagen interactions [7,8] and noncollagenous matrix molecules presence [9]. Cellular compartmentalization of the spatial and temporal events in collagen fibrillogenesis also is important in the regulation of fibril formation, allowing the fibroblast to exert control over fibril architecture by controlling the extracellular mixing and postdepositional processing of matrix components [10]. Determining the role of each factor is important in understanding the establishment of matrix architecture.

Previous studies have demonstrated that the mature stroma contains heterotypic fibrils of types I and V collagen and the amount of type V collagen is inversely proportional to fibril diameter [8]. It also was shown that the presence of type I collagen propeptides at either the carboxyl or the amino terminus, and changes in the relative amounts of specific proteoglycans altered fibril formation [11-13].

The aim of this study was to investigate the effects of the corneal proteoglycans (decorin and lumican) and glycosaminoglycans (CS/DS and KS) on *in vitro* corneal collagen fibrillogenesis, by using specific analysis methods.

Materials and Methods

Biological material. Pig eyes were provided from the slaughterhouse – Bucharest. Corneas were dissected from the globe at the limbus. The epithelium and the endothelium were removed by scraping with a scalpel.

Collagen extraction. Soluble type I collagen was extracted from mature pig corneas using diluted acetic acid in the presence of pepsin. Type I collagen was purified by differential salt precipitation from acid and neutral solutions by the method described in our previous paper [14].

Proteoglycans extraction. The small pieces of stroma were extracted with 10 volumes of 4 M guanidine-HCl in 0.01 M sodium acetate buffer, pH 5.8, in the presence of protease inhibitors (0.01 EDTA, 0.1 M amino-caproic acid, 5 $\mu g \cdot mL^{-1}$ pepstatin A), at 4^oC, for two days. After centrifugation at 5000 rpm for 30 min, the obtained residue was reextracted with the same solvent, for one day. Both guanidine extracts were further purified by cesium chloride gradient ultracentrifugation (40000 rpm for 24 h) under dissociative conditions. Fractions

containing proteoglycans were pooled dialysed against 6 M urea containing 0.05 M Tris, pH 7.0 and 0.05 M sodium EDTA and applied to a 1x50 cm column of DEAE Sepharose CL-4B and then to a 1.1x75 cm column of Sepharose CL-4B [15]. The elution position of proteoglycans was obtained by determination of uronic acids (for decorin) and neutral sugars (for lumican) [16]. Proteoglycan fractions were pooled, dialysed and lyophilized.

Glycosaminoglycans (GAGs) were extracted from pig corneal stroma with papain and fractionated by Dowex 1x2 Cl^- chromatography and precipitated with ethanol, as described in our previous work [17].

Fibrillogenesis assay. Type I collagen was dissolved in 0.5 M acetic acid at 1 $mg \cdot mL^{-1}$ concentration, centrifuged at 10000 rpm for 20 min and dialysed against phosphate buffer pH 7.4, at 4^oC. Fibrils were formed by incubating collagen solutions at 35^oC, for 10h.

Purified lumican, decorin and their glycosaminoglycans (KS and CS) were used at a concentration of 0.05 $mg \cdot ml^{-1}$, based on uronic acid content as determined by orcinol method [18]. Fibrils formation was initiated by mixing type I collagen solution (0.5 $mg \cdot ml^{-1}$) with proteoglycan and glycosaminoglycan solutions and then dialysed against phosphate buffer pH 7.4. The samples were warmed at 35^oC for 10 h. Collagen fibrillogenesis was monitored by recording the absorbance at 315 nm at 10 min intervals, over a period of 10 h, using a *UV-spectrometer CECIL*.

Electron microscopy. The copper grids were floated on collagen samples for 5 min and then drained by contact with filter paper and stained with 1% phosphotungstic acid, pH 7.0 for 5 min. The grids were washed with 5 drops of distilled water, dried and examined using *a Philips transmission electron microscope*.

Results and Discussion

Turbidimetric study of PG and GAG effects on collagen fibrillogenesis

In order to investigate PG and GAG effects on *in vitro* fibrillogenesis of corneal collagen, the following materials were used: decorin (PG-CS/DS), lumican (PG-KS), CS/DS and KS extracted and purified from pig cornea. After every component mixing with collagen solution at a ratio of 1:10, dialysis against TFS and incubation at 35^oC, the formed precipitates were examined by measuring the absorbance at 315 nm.

The precipitation curves are constituted of three clearly defined regions. The first domain is a lag phase (t lag) without turbidity change and it corresponds to collagen fibril nucleation. The second domain is the phase of fibril growth with a rapid increase in turbidity. The third domain is a maturity phase with a final value of absorbance at 315 nm and includes the formation of three-dimensional networks

of fibrils. Fibrillogenesis curves show that the addition of CS and, respectively, KS had as effect the reduction in final absorbance (**Figure 1**). Compared to the kinetics of fibrillogenesis with collagen alone, the presence of CS diminished the final absorbance value by 3%, while KS by 7%. Moreover, both GAG addition resulted in an increase in the length of the nucleation phase (t lag) of fibrillogenesis.

The presence of decorin and lumican had as effect the increase in nucleation phase about 4 times and a reduction in final absorbance in a more obvious way compared to GAGs. By the addition of decorin, the absorbance maximal value decreased by 17% and in the case of lumican, by 22% (**Figure 1**).

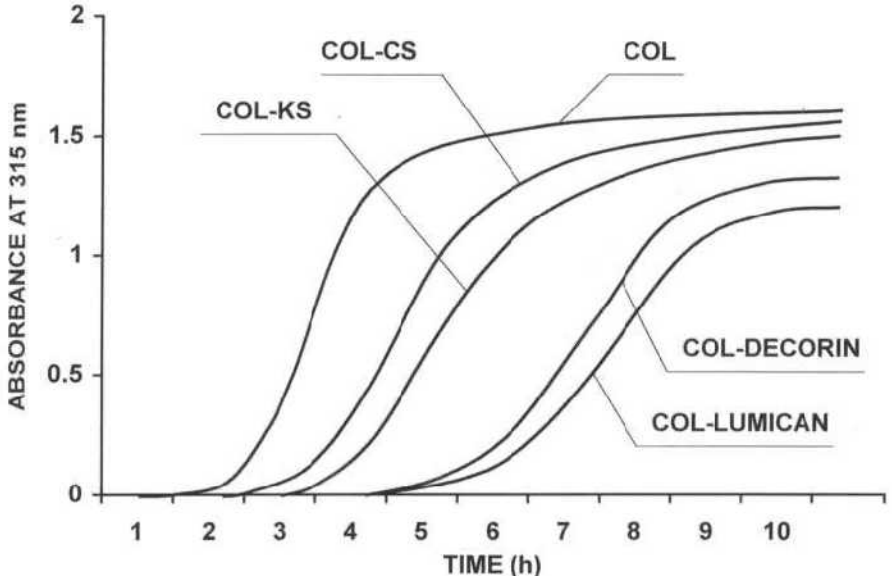

Figure 1 – Corneal proteoglycans' (deorin and lumican) and component glycosaminoglycans' (CS/DS and KS) effects on collagen fibril formation. *500 μg type I collagen (COL) was successively mixed with 50 μg (uronic acids) of PG and the same amount of GAGs. Absorbance was monitored at 315 nm, at 10 min intervals.*

Morphological analysis of collagen fibrils formed in the presence of PG and GAG.

The following experiment was performed in order to reveal the morphology of fibrils formed in the presence of PGs and GAGs. There were studied samples of fibrils at growth stage, following 10h incubation. In controls, representing type I collagen, all the fibrils showed 65 nm banding period (**Figure 2A**). Collagen fibril diameter was of 148±90 nm (**Figure 3A**). Samples containing collagen and PGs as well as those with GAGs were examined also after 10h

incubation, when precipitates' formation was found. In all the variants the resulted fibrils exhibited a typical banding pattern, but the fibrils were thinner than those formed exclusively of collagen (**Figures 2B, C, D, E**). Thus, fibrils formed in the presence of CS were of 138±55 nm diameter (**Figure 3B**) and those with KS, were of 125±50 nm (**Figure 3C**). Decorin and lumican presence had as effect the formation of fibrils with thickness of about 110±24 nm (**Figure 3D**) and 90±18 nm, respectively (**Figure 3E**). These data show that both corneal PGs and their GAG chains influence *in vitro* collagen fibril formation, diminishing their diameters, but the process of collagen fibril growth inhibition by CS and KS is low compared to that of intact PGs.

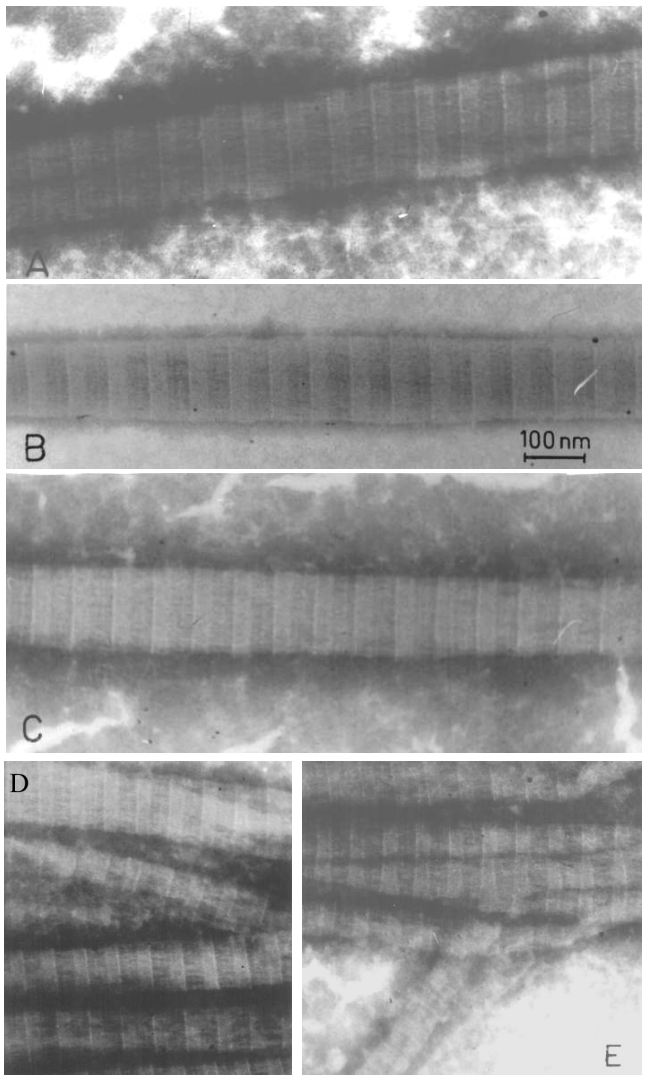

Figure 2 *– Electron micrographs of collagen fibrils formed in vitro, in the presence of proteoglycans and glycosaminoglycans. Fibrils formed from type I collagen (**A**) as well as those formed in the presence of CS/DS (**B**), KS (**C**), decorin (**D**) and lumican (**E**) exhibited a 65 nm typical banding pattern.*

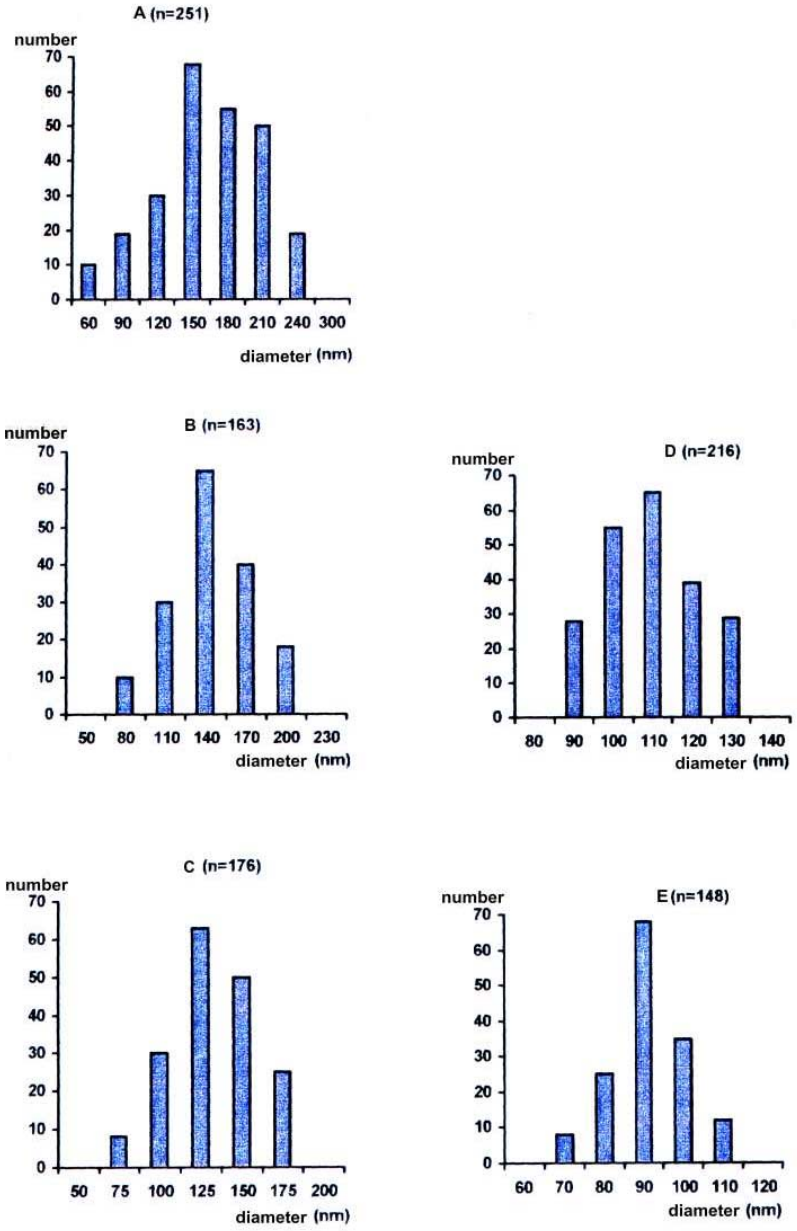

Figure 3 *– Diameter distribution of collagen fibrils formed in the presence of proteoglycans and glycosaminoglycans (n-number of measured fibrils). Fibrils constituted of type I collagen exhibited larger diameters (148±90 nm) (**A**) compared to those formed in the presence of CS (138±55 nm) (**B**), KS (125±50 nm) (**C**), decorin (110±24 nm) (**D**) and lumican (90±18 nm) (**E**).*

Like most dense stromal connective tissues, the cornea contains predominantly type I collagen. Nevertheless, unlike other tissues containing type I collagen in large quantities, such as sclera, the corneal fibrils have small, uniform diameters (about 25 nm). Because these fibrillar properties are thought to be required for corneal transparency, the elucidation of the mechanism by which they are controlled is very important for the understanding of the development and growth of a functional cornea.

In order to study the process of corneal collagen fibrillogenesis, we used type I collagen extracted and purified from mature pig corneal stroma. Type I collagen solutions were redissolved and reprecipitated under medium conditions close to those of tissue. Both our turbidimetric and electron microscopic studies demonstrated that type I corneal collagen assembled in order to form fibrils, morphologically similar to the tissue ones, with a periodicity of 65 nm. For the study of this mechanism kinetics, the collagen fibrils' formation was originally compared with proteins' precipitation in supersaturated solutions, and then with precipitation of inorganic salts, soluble in crystalline form [19]. Yet the mechanism of collagen types' precipitation is much more complicate than that of inorganic salts or proteins, but the basic principles are generally the same. Thus, it is thought that collagen fibrils' formation occurs in three stages: nucleation, i.e. aggregation of soluble collagen molecules resulting in formation of nuclei which are the smallest aggregates able to exist as separate phases; nuclei development into fibrils by adding other soluble collagen molecules, forming visible precipitates and maturation of fibrils [20].

The two major PGs in the pig corneal stroma were identified as lumican, corneal keratan sulfate proteoglycan and decorin, a chondroitin/dermatan sulfate proteoglycan. Previous studies demonstrated that noncollagenous matrix components, including PGs and glycoproteins, exert a major influence upon fibril assembly and tissue organization [9]. Our observations concerning matrix macromolecules in pig cornea indicate that collagen fibrils formed *in vitro* in the presence of PGs and GAGs are thinner than those formed in their absence. Although, when we used PGs (decorin and lumican), fibril diameters decreased by about 1.5 times compared to only 1.1 times in case of using GAGs. The obtained results demonstrate that activity of fibril size regulation is due mainly to PG protein core composition and in a lesser extent to glycan chains. Similar data were reported for bovine corneal PGs [20]. Moreover, it is possible that in case of using GAGs, the inhibition activity could be given by peptides attached to them, obtained following the extraction with papain. It was also reported that GAGs only temporarily interact with collagen monomers during fibril formation [21,22].

The mechanism through which corneal PGs inhibit collagen fibrillogenesis is unknown. It is possible that they might influence the ratio of different collagen types, which co-polymerize, thus indirectly affecting the fibril sizes. PGs and GAGs presence also had as effect the length of fibrillogenesis nucleation period, a

fact that demonstrates that these components interfere in the fibrillar process even from the beginning, when nuclei or subfibrillar intermediate aggregates are formed. On the other hand, fibrillation curves' profiles are little different for the two PGs, meaning that they have different effects on fibrillogenesis. Thus, lumican diminished type I collagen fibril diameter 1.64 times, while decorin, 1.35 times. In all cases, the fibrils had diameters much larger than those of 25 nm, characteristic to the corneal stroma *in vivo*. The difference is due to the role of keratocytes in fibrils formation and their structure. Cellular control of the mixing of different macromolecules and post-depositional processing has an important role in the control of fibril formation.

Conclusions

- Under medium conditions similar to those *in vivo*, type I collagen extracted and purified from pig cornea was reconstituted as fibrils, morphologically similar to the tissue ones, with a 65 nm periodicity.
- In the presence of corneal PGs (lumican and decorin), type I collagen fibril diameter decreased about 1.5 times compared to only 1.1 times in the case of using corresponding glycosaminoglycans (CS/DS and KS). These data indicate that the interaction of type I collagen with proteoglycans is one mechanism modulating fibril diameter. The activity of fibril size regulation is due mainly to PG protein core composition rather than of their GAG chains.

References

1. D.M. MAURICE, *The cornea and sclera*, in *The eye*, vol. 1B, 3rd ed., DAVSON, H. ed., Academic Press, New York, 1984, pp.1-34.
2. J.L. FUNDERBURGH, G.W. CONRAD, *J. Biol. Chem.,* **265**(14), 8297-8303 (1990).
3. J.E. SCOTT, M. HAIGH, *Biochem. J.*, **253**, 607-610 (1988).
4. J. IBRAHIM, J.J. HARDING, *Biochim. Biophys. Acta.*, **992**, 9-22 (1989).
5. K.E. KADLER, D.J.S. HULMES, Y. HOJIMA, D.J. PROCKOP, *Ann. NY Acad. Sci.*, **580**, 214-224 (1990).
6. A. ROMANIC, E. ADACHI, K.E. KADLER, Y. HOJIMA, D.J. PROCKOP, *J. Biol. Chem.,* **266**, 12703-12709 (1991).
7. E. ADACHI, T. HAYASHI, *Connect. Tiss. Res.,* **14**, 257-264 (1986).
8. D.E. BIRK, J.M. FITCH, J.P. BABIARZ, K.J. DOANE, T.F. LINSENMAYER, *J. Cell Science*, **95**, 649-657 (1990).
9. K.J. DOANE, J.P. BABIARZ, J.M. FITCH, T.F. LINSENMAYER, D.E. BIRK, *Exp. Cell Research*, **202**, 113-124 (1992).
10. D.E. BIRK, R.L. TRELSTAD, *J.Cell Biol.,* **99**, 2024-2033 (1984).

11. M. MIYAHARA, K. HAYASHI, J. BERGER, F.K. TANZAWA, R.L. TRELSTAD, D.J. PROCKOP, *J. Biol. Chem.,* **259**, 9891-9898 (1984).
12. A.P. MOULD, D.J. HULMES, D.F. HOLMES, C. CUMMINGS, C.H. SEAR, J.A. CHAPMAN, *J. Mol. Biol.,* **211**, 581-594 (1990).
13. J.E. SCOTT, *Biochem. J.*, **195**, 229-233 (1984).
14. G. NEGROIU, L MOLDOVAN, M. CALOIANU, N. MIRANCEA, D. MIRANCEA, *Rev. Roum. Biochim*., **25**, 2, 143 (1988).
15. L. MOLDOVAN, A. OANCEA, O. CRACIUNESCU, L. CONSTANTINESCU, *Rom. J. Biol. Sci.*, III, 1-3, 10-15 (1999).
16. M.A. JERMIN, *Anal. Biochem.*, **68**, 332-335 (1975).
17. L. MOLDOVAN, O. GHEORGHIU, F. CUTAS, M. CALOIANU, *Anal. Univ. Buc.,* **XLIII**, 28-32 (1994).
18. T. BITTER, H. MUIR, *Anal. Biochem.,* **4**, 330-334 (1962).
19. D.E. BIRK, F.H. SILVER, R.L. TRELSTAD, *Matrix Assembly*, in *Cell Biology of Extracellular Matrix*, 2nd ed., ed. by E.D. HAY, Plenum Press, New York, 1991, pp. 221-254.
20. J.A. RADA, P.K. CORNUET, J.R. HASSEL, *Exp. Eye Res.,* **56**, 635-648 (1993).
21. J.E. SCOTT, *Biochem Eye,* **19**, 877-881 (1991).
22. I.M. RAWE, S.J. TUFT, K.M. MEEK, *Histochem. J.,* **24**, 311-318 (1992).