In vitro fibrillogenesis of collagen II from pig vitreous humour

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Collagen from pig vitreous humour was fractionated into a soluble and an insoluble fraction by centrifugation. Most of the collagen II in the soluble fraction was present as pN-collagen II (procollagen II without the C-terminal propeptide), besides smaller quantities of procollagen II, collagen II and two as yet unidentified α-chains of collagen II. Other collagen types may be present only in trace amounts. Collagen II of the insoluble fraction, which is mostly deposited in fibrillar aggregates, consists of both pN-collagen II and collagen II. To determine the possible role of collagen II precursors in the formation of the extracellular matrix of the vitreous humour these collagen molecules were purified and *in vitro* fibrillogenesis was used to demonstrate that pN-collagen II could form fibrils in mixtures with collagen II. These fibrils have a reduced mass per unit length depending on the content of pN-collagen in the mixture. Cross-sections of the newly formed fibrillar aggregates revealed a flattened shape. The incomplete processing of the precursors of collagen II may be part of regulatory mechanisms possibly controlling the formation of a translucent scaffold as is required in the vitreous humour.

INTRODUCTION

Only a small number of cells synthesize the extracellular matrix components of the vitreous body, which contains less than 0.2% w/v scaffolding material of an otherwise water-filled interior of the eye and which constitutes approximately two-thirds of the entire volume of the eye [1]. Collagen types II, V, VI, IX and XI have been identified as structural constituents of the collagenous matrix of this highly specialized tissue [2]. Similar to hyaline cartilage, collagen II is the predominant collagen type. Remarkably, collagen IX in vitreous humour is different from that found in hyaline cartilage as it contains an extended chondroitin sulphate chain attached to the NC3 domain of the α2(IX) chain [3]. Distinctly different from hyaline cartilage, however, is the observation that collagen V or heterotypic collagen XI/V molecules co-exist in the fibrils of the vitreous humour along with collagens II and IX [4,5].

Collagen II is synthesized as a precursor form which is converted during secretion into functional collagen II by both a pN-propeptidase and a pC-propeptidase [6]. Collagen fibrils in the vitreous humour are much thinner and have a uniform diameter compared with collagen II fibrils in cartilage [3]. In a previous study it has been shown by segment long spacing (SLS)-crystallites that pN-collagen II is present in the soluble as well as in the insoluble fraction of the vitreous humour of rabbits [7–9]. It has been argued that pN-collagen II (procollagen II without the C-terminal propeptide) could play a role in the regulation of the diameter of collagen fibrils, comparable with that demonstrated for pN-collagen I during fibril formation in chicken embryos [10].

In the present study, we show the presence of pN- and procollagen II in the soluble fraction as well as in collagen fibrils of the vitreous humour, and examine the effect of purified pN-collagen on fibrils formed *in vitro* to describe a possible functional role of the propeptides still attached to the collagen II molecule.

MATERIALS AND METHODS

Extraction of collagen from vitreous humour

Freshly removed pig eyes (300 eyes for one preparation) were obtained from a local slaughterhouse. The eyes were opened by incision with a dissecting knife and the vitreous humour was squeezed out of the outer ocular membranous containment and centrifuged at 90000 *g* at 4 °C. Furthermore, the pellet was washed with 0.05 M Tris, pH 7.4, 1 M NaCl and subsequently dialysed extensively against 0.05% acetic acid. The supernatant was adjusted to 30% $(NH_4)_2SO_4$ and stirred at 4 °C overnight. The precipitate was collected by centrifugation and subsequently dissolved in 0.05% acetic acid followed by dialysis against the same solution. After the insoluble material had been removed by centrifugation, the supernatant was deep frozen (−20 °C) and used for further biochemical characterization.

Partial purification of native collagenous components

The collagen from the soluble fraction of the vitreous humour was dialysed against 0.2 M NaCl and 2 M urea in 0.03 M Tris buffer, pH 7.4. The sample was loaded on to a DEAE-Sephacel resin (Pharmacia, Freiburg, FRG) which was equilibrated and eluted with 0.2 M NaCl and 2 M urea in 0.05 M Tris buffer, pH 7.4. Collagenous proteins which were not retained on the resin were eluted in the eluate front, pooled and then extensively dialysed against 0.1 M acetic acid and lyophilized.

Purification of α-chains of pN-collagen II

The collagen from the soluble fraction was subjected first to chromatography on a molecular-sieve column (Pharmacia, Superose TM6, Freiburg, FRG). The column was equilibrated with 0.15 M NaCl, 0.05 M NaH_2PO_4, 4 M urea, pH 6.5, at a flow rate of 0.1 ml/min. The fractions containing monomers were directly loaded on to a C_{18} reverse-phase column (Waters, U.S.A.). The separation of pN-α1(II) chains from other components was performed using a linear gradient of 21–32% acetonitrile in water with 0.1% trifluoroacetic acid (v/v) over 37 min at a flow rate of 1 ml/min. Individual fractions were analysed by SDS/PAGE and immunoblotting.

SDS/PAGE

For the separation of collagen chains, SDS/PAGE was performed on slab gels, using 6% (w/v) polyacrylamide for the

Abbreviation used: SLS, segment long spacing; PN-collagen II, procollagen II without the C-terminal propeptide.

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running and 4% for the stacking gels [11]. The separated collagen chains were stained by either Coomassie Blue staining or immunoblotting using collagen-type-specific antibodies [12].

Preparation of antisera against collagen

Antisera against mouse collagen II were raised in rabbits. The antibody titre was determined by a direct e.l.i.s.a. and the specificity of the antisera were checked by immunohistological staining of suitable tissue sections and by immunoblotting against highly purified collagen [12].

Immunoblotting

Following electrophoretic separation, protein bands were blotted on to a nitrocellulose membrane by wet or semidry electrotransfer. Filters were blocked with 15% (w/v) non-fat dry milk in distilled water and incubated overnight at room temperature with the primary antiserum (1:50 in 0.1% BSA). The filter was then incubated with alkaline phosphatase-conjugated pig immunoglobulins against rabbit immunoglobulins (Dako Diagnostika, Denmark; diluted 1:3000) for 1 h at room temperature. 5-Bromo-4-chloro-3-indolyl phosphate (7.5 mg/50 ml; Serva, Heidelberg, FRG) and NitroBlue Tetrazolium (15 mg/50 ml; Sigma, Deisenhofen, FRG) were used as substrates [12].

Circular dichroism

C.d.-spectra were recorded on a Jasco J-500 A spectropolarimeter, equipped with a quartz cell of 1 cm pathlength. The molar ellipticity was calculated on the basis of a mean residue molar mass of 98 g/mol.

In vitro fibril formation

Stock solutions were prepared from collagen solutions in 0.05% acetic acid, which were ultracentrifuged prior to use (200000 *g*, 1 h, 4 °C). Only the upper half-segment of the supernatant was collected and used for *in vitro* assays of fibrillogenesis. The fibrillogenesis of collagen II with increasing amounts of pN-collagen II was carried out under self-assembly conditions at 32 °C in 0.5 cm quartz cuvettes following the method described by Williams et al. [13]. Fibril formation was monitored by turbidity measurements and the structure of the fibrils was observed by electron microscopy using methods described previously [14]. The cuvette contents were transferred to 1.5-ml Eppendorf caps and fibrillar collagen was removed by centrifugation at 14000 *g* for 30 min in the cold. The remaining concentration of collagen in the supernatant was measured spectropolarimetrically and the registered maximum turbidity was then normalized by dividing the absorbance by the concentration of collagen deposited in the fibrils. Incorporation of pN-collagen into fibrils was analysed by immunoblotting after the fibrils had been separated from soluble collagen by centrifugation at 100000 *g* for 1 h.

Electron microscopy

For electron microscopy, aliquots of assembly mixtures were incubated in parallel to the turbidity–time assay. After 1000 min four samples, each of 3 μl, were transferred to Formvar-coated copper grids using a micropipette with a widened tip diameter. Fibrils were allowed to settle for 30 min. Then the buffer was drained cautiously and three washing steps were performed. Subsequently, the fibrils were stained with freshly prepared phosphotungstate (1%, w/v; pH 7.4) for 2 min, washed another three times, and dried. Grids were examined using a Zeiss EM 109 electron microscope. Fibril width was measured directly during observations by use of a manual optical picture analysis device, MOP AM-03 (Kontron, Eching, FRG).

RESULTS

Precursors of collagen II in the soluble fraction of vitreous humour

The vitreous humour was fractionated into an insoluble and a soluble fraction by centrifugation. The collagen in the soluble fraction was precipitated by $(NH_4)_2SO_4$, dissolved in 0.05% acetic acid and further dialysed against the same solution. An aliquot of this material showed, after SDS/PAGE under reducing conditions, three bands which migrated slower than the α1(II) chains (Figure 1a, lane A). Two of these bands could also be observed under non-reducing conditions (lane B). After pepsin digestion and electrophoretic separation under reducing conditions only a single band could be observed, which migrated in the same position as pepsin-digested collagen II from whole vitreous humour (Figure 1a, lanes C and D). Upon immunoblotting of the gels shown in Figure 1(a), the three bands visible under reducing conditions and migrating slower than α1(II) collagen chains showed a strong reaction with antibodies against collagen II, as did the single collagenous band following limited pepsin digestion (Figure 1b, labels of lanes are the same as in Figure 1a). These results suggest that the three bands represent precursors of collagen II with additional pepsin-sensitive peptides attached to the ends of the triple-helical domain of collagen II.

Another aliquot of the same material was fractionated by f.p.l.c. on a molecular-sieve column under non-reducing conditions. The chromatographic fractions with a molecular mass in the range of collagen α-chains were collected and applied to a reverse-phase column (Figure 2). The purified fraction, which represented the major component of collagen II in the soluble fraction of the vitreous humour, showed a single band both in SDS/PAGE and immunoblotting (Figure 2 insert, right lane; collagen II (left lane) for comparison). Referring to the electrophoretic profile of the α1-chains of procollagen II, pC-collagen II and pN-collagen II one can deduce that the α1-chain with the highest molecular mass is pro-α1(II). The protein band migrating between α1(II) and pN-α1(II) has not yet been identified. Apparently, pC-collagen molecules are not present in the soluble fraction of the vitreous humour.

The electrophoretic separation of the cyanogen bromide-cleaved peptides exhibited two bands for the purified pN-collagen

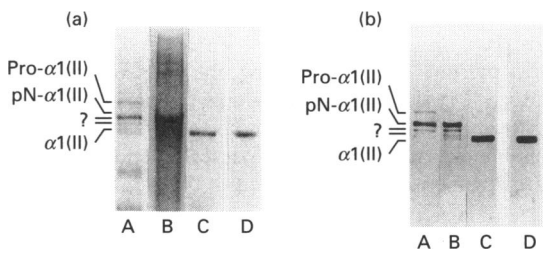

Figure 1 Identification of the precursors of collagen II in the soluble fraction of the vitreous humour by SDS/PAGE (a) and immunoblotting (b)

The collagen from the soluble fraction of the vitreous humour prior to (lanes A and B) and after (lane C) pepsin digestion was identified by SDS/PAGE and immunoblotting with antisera against collagen II under reducing condition (lanes A and C) and under non-reducing conditions (lane B). Lane D is the pepsin-digested and purified collagen II from unfractionated vitreous humour.

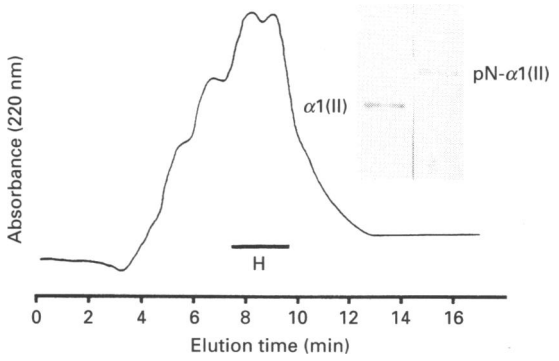

Figure 2 Identification of pN-collagen II in the soluble fraction of the vitreous humour

Collagen in the soluble fraction of the vitreous humour was collected by precipitation with $(NH_4)_2SO_4$. The pellet was diluted in 0.05% acetic acid and further purified by ion-exchange chromatography. After molecular-sieve chromatography a fraction containing the α-chains was applied to a reverse-phase column C_{18}. Insert: SDS/PAGE of fraction H (right) and α1(II) collagen for comparison (left).

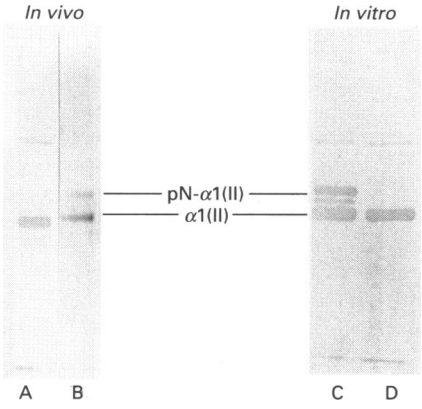

Figure 3 Immunoblotting of pN-collagen II present in fibrils formed either *in vivo* or *in vitro*

Lane A, purified collagen II; lane B, collagen solubilized from fibrils formed *in vivo*; lane C, collagen contained in fibrils precipitated *in vitro* out of a mixture of collagen II with 10% pN-collagen II; lane D, fibrils formed by collagen II only.

α-chains, in addition to those seen in the pepsin-digested collagen II from the vitreous humour (results not shown). Since neither the number of methionine residues nor the amino acid sequence of pig collagen II are known at present we can only assume that the additional peptides are derived from the N-terminal procollagen II extension peptides.

pN-collagen II in native and *in vitro*-formed fibrils

The insoluble fraction contains the polymerized extracellular matrix components. This material was dissolved in sample buffer of SDS/PAGE overnight under reducing conditions and was separated electrophoretically. Immunoblotting with collagen II antibodies demonstrated that pN-collagen II is solubilized in significant amounts in relation to collagen II (Figure 3, lane B).

To study the influence of pN-collagen II on *in vitro* fibrillogenesis, native pN-collagen II was prepared from the soluble supernatant of the vitreous humour using DEAE-cellulose

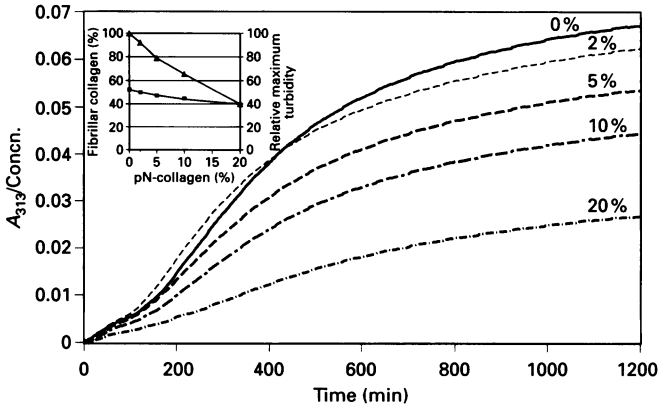

Figure 4 Kinetics of fibril formation

Turbidimetric measurements of pepsin-digested collagen II and pN-collagen II from pig vitreous humour. Numbers indicate the relative content of pN-collagen in the incubation mixture. Insert: plot of the relative maximum turbidity (pure collagen II is 100%) and remaining percentage fibrillar collagen versus percentage pN-collagen II in the incubation mixture.

chromatography to remove proteoglycans and other acidic components. The solubilized collagen was examined spectropolarimetrically and a normal collagen spectrum was measured. This is an indication that hyaluronan or other components of the vitreous are only present in very small amounts. The densitometrically determined relative proportion of pN-collagen on SDS-gels was about 90% compared with collagen II (results not shown). It can not be excluded that other collagen types may be present in trace amounts.

In vitro fibrillogenesis in mixtures of pepsin-extracted collagen II and pN-collagen II from pig vitreous humour was monitored by time-dependent turbidity measurements (Figure 4a). From the data obtained it is possible to deduce that the mass per unit length of the fibrils decreased with increasing amounts of pN-collagen in the starting solution. This notion is supported by two observations: (i) the decrease of the maximum turbidity due to rising amounts of pN-collagen in the starting mixture is larger than the decrease brought about by the amount of collagen molecules which are not incorporated into the fibrils (insert, Figure 4); and (ii) lane C in Figure 3 shows that both pN-collagen II along with collagen II molecules were incorporated into fibrils formed *in vitro* as this material had been analysed after the fibrils were collected by centrifugation.

In another set of experiments morphometric analysis was used to determine the diameters of the fibrils, which were spread on grids for electron microscopy. The apparent fibre size of the spread fibrils increased with increasing amounts of pN-collagen II incorporated into the fibrils (Figure 5b).

DISCUSSION

The extracellular matrix of the vitreous humour has a number of properties which are probably associated with the presence of precursors of collagen II in a mature tissue. The liquid phase of the vitreous humour accounts for more than 99.8% of the entire volume and there are only a small number of cells which metabolize the extracellular matrix, mostly in the periphery of the vitreous body. Therefore it is concluded that this tissue once formed during embryogenesis remains in this state with only a little metabolic activity [15].

The processing of procollagen molecules occurs during ex-

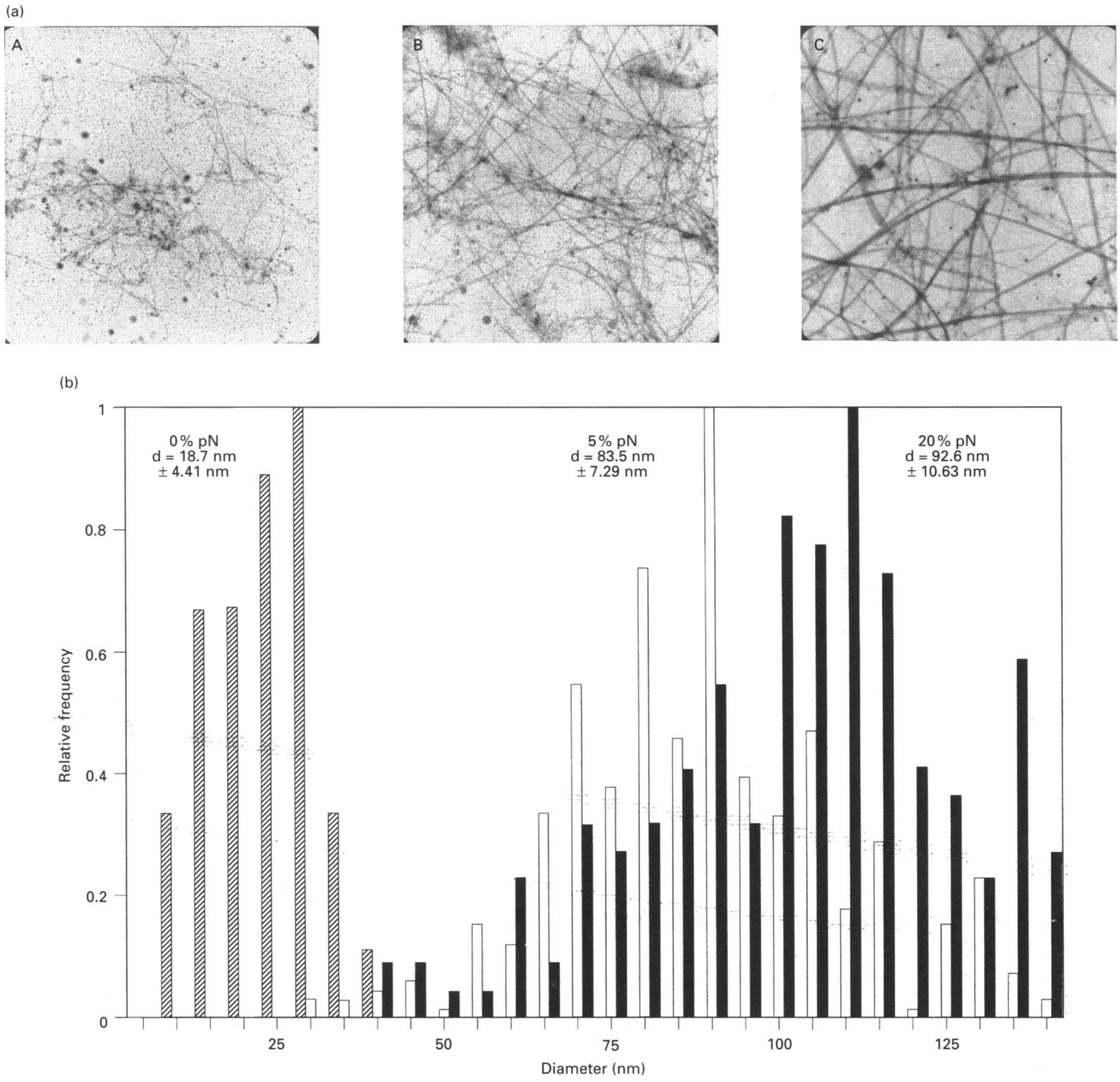

Figure 5 Electron micrographs (a) of *in vitro*-formed fibrils of pepsin-digested collagen II mixed with pN-collagen II extracted from pig vitreous humour

(**a**) Panel A, 0% pN-collagen II; panel B, 5% pN-collagen; panel C, 20% pN-collagen II. (**b**) Morphometric determination of collagen fibre sizes from electron micrographs (n = 100).

cretion into the extracellular space and is executed in such a way that in most tissues virtually no precursor molecules are found in the mature tissue [16]. Surprisingly, mature vitreous humour contains substantial amounts of collagen II precursor molecules, as shown by electrophoretic separation and immunodetection.

There are four protein bands (Figure 1) migrating slower than the collagen II bands on SDS/PAGE. Since there are no interchain disulphide bonds between the precursor molecules, it is fair to assume that the precursor extension resides at the N-terminal end of the collagen II molecule. In conclusion, some 90% of the collagenous proteins in the soluble fraction of the vitreous consist of pN-collagen molecules as well as of smaller quantities of procollagen II and two as yet unidentified collagen II chains. The SLS-crystallites produced from these collagen molecules reported by Hong and Davision [8] and cleavage of these molecules by N-procollagen N-protease reported by Bishop et al. [17] are further support for the occurrence of pN-collagen in the vitreous humour. Recently, Bishop et al. [18] investigated the supernatant after centrifugation of the bovine vitreous and found substantial amounts of collagen IX and negligible amounts of collagen V/XI. Although these authors used a different method to precipitate collagen, it is obvious that the

composition of the supernatant after centrifugation of pig vitreous is different from bovine vitreous.

From these data the questions arise (a) as to how precursors of collagen II can be retained under physiological conditions in a mature tissue and (b) whether they play a functional role in the organization and maintenance of the extracellular matrix in vitreous humour.

(a) Although there is no experimental evidence from our studies to explain the maintenance of precursor collagen molecules, the low metabolic rate in the vitreous humour may have the consequence that this tissue is locked in an embryonic state [15]. In a situation such as the early development of chicken tendon, pN-collagen I is present in the tissue [10]. It was suggested that pN-collagen I down-regulates collagen fibril diameters and reduces the tendency of the fibrils to fuse into bundles [10].

The as yet unidentified forms of collagen II, migrating between pN-collagen II and collagen II, could possibly represent a degraded or further processed pN-collagen II. Considering the embryonic state of the vitreous humour it could also be argued that the bands are alternative splice products such as pN-α(II)a, which is as yet only identified on the mRNA level in embryonic tissue [19].

(b) In the fibrils formed both *in vivo* and *in vitro*, we demonstrated that pN-collagen was part of the collagen aggregates. Comparing collagen fibrils from hyaline cartilage with those of vitreous humour the latter are much thinner and have a more uniform diameter [4]. From the turbidity data it is obvious that pN-collagen slows down fibril formation and one can assume that the amino propeptide of pN-collagen protrudes from the fibrillar surface, interferes with the lateral alignment of collagen II molecules and down-regulates the fibril diameter. The morphometric analysis of fibrils formed *in vitro* from mixtures of collagen II and pN-collagen II on one side and the light-scattering data on the other side seem to contradict each other. The increase of the apparent fibril diameter may, however, arise from the propensity of pN-collagen to induce the formation of ribbon-like rather than circular cross-sections for the collagen II/pN-collagen II aggregates [20,21]. Ribbon-like aggregates in turn appear broader when spread on an electron-microscopic grid, although they have a lower mass per unit length. Furthermore it is possible that the cross-section of the fibrils has a hieroglyphic shape with a low mass per unit length, as reported by Watson et al. [22] and Holmes et al. [23] for fibrils formed in the skin of patients suffering from Ehlers–Danlos type VII.

One can conclude that the involvement of pN-collagen II in the fibril formation in the vitreous humour interferes with the lateral aggregation and therefore may contribute to the formation of the extremely thin collagen fibrils and is thus maintaining transparency beside of the main regulating factors collagens IX and V/XI and non-collagenous components as proteoglycans and hyaluronan.

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Vitreous humour is a unique example for the occurrence of precursors of collagen in normal mature tissue, while impaired pN-collagen I processing is the molecular basis of diseases such as Ehlers–Danlos Syndrome type VII or dermatosparaxis with severe biomechanical consequences as a result of impaired fibril formation [22–26]. Occurrence of pN-collagen II in Kashin–Beck disease is the reason for the dysfunction of articular cartilage [12]. It will therefore be interesting to investigate whether pathological aggregates, frequently observed during aging (e.g. 'mouche volante') in the vitreous humour, are the result of a decrease of the physiologically required pN-collagen II content in this tissue.

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