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Vitreous collagen metabolism before and after vitrectomy

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Abstract *Purpose:* To assess vitreous metabolism by measuring C-propeptide levels of type II procollagen (pCOL-II-C) and hyaluronan levels in the vitreous and in the vitreous fluid after vitrectomy for macular hole. *Methods:* We obtained 1-ml vitreous samples during vitrectomy from 34 patients (34 eyes) with a macular hole (age range 50–77 years, mean 64 years). After vitrectomy, we performed fluid–air exchange in six eyes because of unresolved macular holes and collected 4-ml fluid samples. Gel-filtration high-performance liquid chromatography (HPLC) was used to determine the molecular weight of pCOL-II-C in the samples. The pCOL-II-C level was measured by sandwich enzyme immunoassay and hyaluronan by sandwich binding protein assay. *Results:* HPLC showed that pCOL-II-C in the vitreous samples corresponded to purified pCOL-II-C from cartilage. The vitreous pCOL-II-C level (4.7±0.3 ng/ml) was similar to reported synovial fluid levels. In six eyes that underwent fluid–air exchange, pCOL-II-C in the fluid samples remained at a level similar to that in the vitreous samples, while hyaluronan levels in the fluid samples were significantly lower than in the vitreous samples. *Conclusions:* The molecular weight and concentrations of pCOL-II-C in the vitreous are similar to those in joint fluid. In patients with a macular hole, type II procollagen may be secreted persistently into the vitreous cavity before and after vitrectomy.

Introduction

The vitreous body, the largest structure in the eye, makes up 80% of its volume. The vitreous gel is comprised of a three-dimensional structure of collagen and high-molecular-weight hyaluronan [4]. Vitreous gel is homogeneous in infant eyes. Although the vitreous liquefies with aging [12] and plays a key role in many vitreoretinal diseases, not a great deal is known about its synthesis and metabolism. The vitreous collagen fibrils are comprised of collagen types II, V/XI, and IX, of which type II collagen predominates and accounts for approximately 75% of the vitreous collagen [3, 4]. Type II procollagen, which is a soluble precursor, is synthesized in the cell and released outside the cell and has amino-propeptide (N-propeptide) at one end and carboxy-propeptide (C-propeptide) at the other end (Fig. 1) [4]. When procollagen becomes collagen, both peptides are cleaved from the procollagen by specific proteases [15]. In humans, type II collagen is mainly present in the vitreous and joints. In Stickler syndrome, a mutation in the C-propeptide region of the type II collagen molecule results in ocular and joint diseases, such as vitreoretinal degeneration and chondrodysplasias [18]. Orthopedists use C-propeptide of type II procollagen (pCOL-II-C) as a marker of the production of type II collagen in the synovial fluid [13, 20, 21]. Bishop et al. detected type II procollagen and partially processed forms of type II collagen in adult bovine eyes [2]. They suggested that type II collagen may be synthesized postnatally.

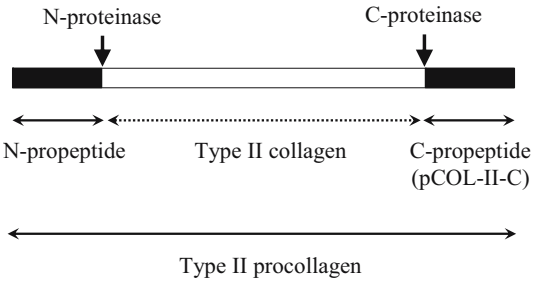

Fig. 1 Type II procollagen, the soluble precursor of type II collagen. It has amino-propeptide (*N-propeptide*) and carboxy-propeptide (*C-propeptide*). Both peptides are cleaved by proteinases and procollagen becomes a collagen molecule

To clarify the synthesis and metabolism of the vitreous components in human adult eyes, we measured the levels of pCOL-II-C and hyaluronan in vitreous gel obtained intraoperatively and vitreous fluid obtained after vitrectomy.

Patients and methods

We prospectively collected vitreous specimens from the eyes of 34 consecutive patients (16 men and 18 women) during pars plana vitrectomy for the treatment of a macular hole (mean patient age 64 years, range 50–77 years). All operations were performed by the same surgeon (S.K.) at Gunma University Hospital between October 2001 and April 2003. No patients had undergone previous vitreous surgery.

The study was conducted according to the tenets of the Declaration of Helsinki. The institutional Review Board Ethics Committee of our institution approved the study protocol. After an explanation of the purpose of the experiment had been given to each patient, all patients provided written informed consent.

After creation of three ports, a core vitrectomy was performed with infusion occluded. The central vitreous was resected with a cutter until 1 ml of vitreous gel accumulated in the sterile tube. The vitreous samples were centrifuged for 10 min at 3,000 rpm (1,630 *g*). The liquid component then was aspirated without the sediment and stored at −80°C. Vitrectomy was performed with infusion of BSS (balanced salt solution; BSS Plus, Alcon Laboratories, Ft. Worth, TX). At the end of vitrectomy, the fluid in the vitreous cavity was exchanged with 30% SF_6 gas in all cases. At 8–9 days after vitrectomy, fluid samples of approximately 4 ml were collected during fluid–air exchange from six eyes with an unresolved macular hole. The fluid samples were collected in a sterile syringe and processed in the same manner as the vitreous samples.

Five samples of BSS served as controls. On the day of vitrectomy, we collected serum samples from ten of 34 patients with a macular hole. The specimens were centrifuged for 10 min at 3,000 rpm and the serum was stored at −80°C.

According to a previously described method [20], pCOL-II-C of one vitreous sample was fractionated on gel-filtration high-performance liquid chromatography (HPLC; TSK-gel G3000 SW, Tohso, Tokyo, Japan) and eluted at a flow rate of 0.5 ml/min. Fractions of 0.1 ml were collected and assayed using the pCOL-II-C kit, the details of which are described below. As a control, purified pCOL-II-C in the kit was fractionated and the pCOL-II-C assay was performed as follows.

According to the manufacturer's instructions, the pCOL-II-C level was analyzed using the sandwich enzyme immunoassay (EIA) kit (Teijin, Tokyo, Japan), purchased from Sysmex (Hyogo, Japan). The details of the EIA kit were reported by Shinmei et al. [20]. The kit includes purified pCOL-II-C from bovine cartilage by the method of Choi et al. [8]. The intra-assay coefficient of variation is 3.25–5.33%; the minimum level of detection is 0.2 ng/ml.

According to the manufacturer's instructions, the levels of hyaluronan in the vitreous were determined by sandwich binding protein assay (Chugai Pharmaceutical, Tokyo, Japan) [7]. The intra-assay coefficient of variation is 5.2–10.2%; the minimum level of detection is 0.01 μg/ml.

All values are expressed as the mean ± standard error. The significance of the differences between the groups was evaluated by the Wilcoxon signed-rank test. Correlations between groups were studied by Pearson's correlation coefficient test. *P* values less than 0.05 were considered significant.

Results

The HPLC profiles of the vitreous sample showed that immunoreactive materials eluted as a main peak, which was the same as purified pCOL-II-C (Fig. 2).

In the vitreous samples obtained from 34 patients with a macular hole during surgery, the mean pCOL-II-C level was 4.7±0.3 ng/ml (range 2.5–5.7 ng/ml) and the mean hyaluronan level was 91.7±5.6 μg/ml (range 45.1–127.0

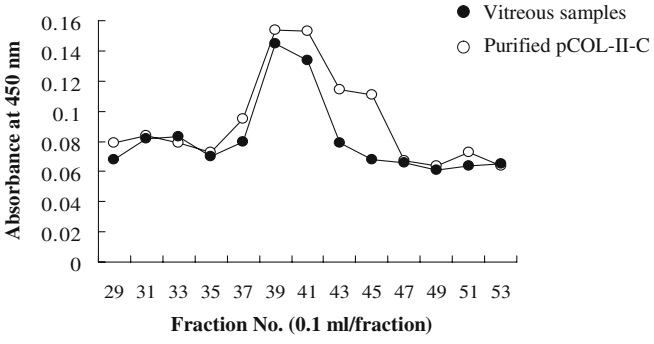

Fig. 2 Elution profiles of C-propeptide of type II procollagen (pCOL-II-C) in vitreous samples. The vitreous samples (*filled circles*) have a similar distribution to that of pCOL-II-C purified from bovine cartilage (*open circles*)

Table 1 Levels of pCOL-II-C and hyaluronan

Samples	pCOL-II-C (ng/ml)	Hyaluronan (μg/ml)
Vitreous samples (*n*=34)	4.7±0.3	91.7±5.6
Fluid samples (*n*=6)	4.1±0.4	28.5±7.5
Serum samples (*n*=10)	<0.2	0.05±0.01
BSS (*n*=5)	<0.2	<0.01

The mean pCOL-II-C levels in vitreous samples and in vitreous fluid samples after vitrectomy (*fluid samples*) are similar to the reported level of pCOL-II-C in synovial fluid (0.2–19.1 ng/ml) [20]. Although the mean level of hyaluronan in the vitreous samples is similar to the reported level [10], that in the fluid samples is lower. The levels of pCOL-II-C and hyaluronan in the serum and BSS samples are undetectable or very low

μg/ml) (Table 1). In the six eyes that underwent postoperative fluid–air exchange, the mean pCOL-II-C level in the fluid samples was 4.1±0.4 ng/ml (range 2.8–5.4 ng/ml) (Table 1). There was no significant difference in pCOL-II-C levels between the vitreous samples and the fluid samples in the six eyes (Fig. 3). In three eyes, the pCOL-II-C levels were even higher in the fluid samples than the vitreous samples. In contrast, the hyaluronan level in the fluid samples from the six eyes was significantly lower (28.5±7.5 μg/ml, range 5.8–46.7 μg/ml) than that in the vitreous samples (91.7±5.6 μg/ml) (P<0.05) (Fig. 3).

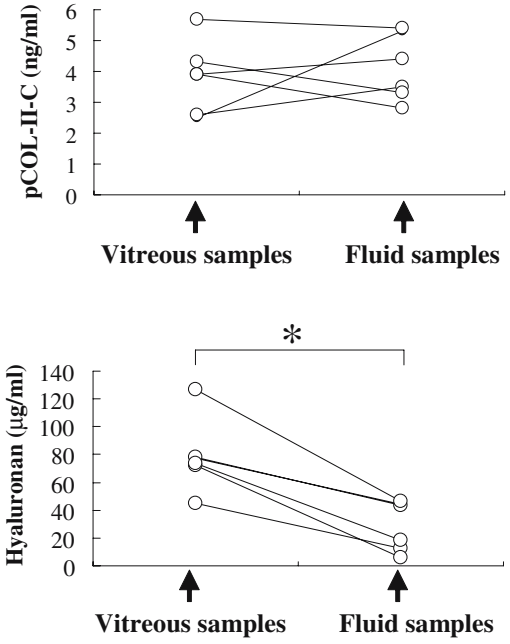

Fig. 3 In six eyes with a macular hole, there is no significant difference in the mean level of pCOL-II-C between the vitreous samples (4.7±0.3 ng/ml) and the fluid samples (4.1±0.4 ng/ml). In three eyes, the pCOL-II-C levels are even higher in the fluid samples than in the vitreous samples. The mean level of hyaluronan in the fluid samples (28.5±7.5 μg/ml) is significantly lower than in the vitreous samples (91.7±5.6 μg/ml; *P<0.05)

In the five control BSS samples, both the pCOL-II-C levels (<0.2 ng/ml) and the hyaluronan levels (<0.01 μg/ml) were under the minimum levels of detection. In the serum samples from 10 of the 34 patients, the mean pCOL-II-C level was less than 0.2 ng/ml and the mean hyaluronan level was 0.05±0.01 μg/ml (Table 1).

Discussion

In this study, we measured pCOL-II-C but not type II collagen itself. pCOL-II-C was first isolated from bovine fetal epiphyseal cartilage [8] and identified by amino acid analysis [24]. In cartilage, type II collagen is synthesized by chondrocytes as a procollagen. After procollagen is secreted into the extracellular matrix, C- and N-propeptide are rapidly cleaved from the ends of procollagen by specific enzymes [23]. Each newly formed type II collagen molecule links to another at the ends, and the collagen molecules form a long chain of collagen fibril. Thus, concentrations of C-propeptide in the fluid may reflect newly synthesized type II collagen, and pCOL-II-C is useful for orthopedists as a productive marker of type II collagen in the joint. The levels of pCOL-II-C in joint fluid are significantly higher in osteoarthritis and traumatic arthritis than in rheumatoid arthritis, and the measurement of pCOL-II-C contributes to the diagnose of joint diseases [13, 20, 21].

Bishop et al. reported the presence of type II procollagen in the vitreous [2]. Ihanamaki et al. reported that new synthesis of vitreal type II collagen decreases with aging but continues at a very slow rate in the adult mouse eye [9]. In addition, N-propeptides of type II procollagen were reported to be retained on vitreous collagen fibrils [16]. On the other hand, C-propeptides tend to exist in free form in the fluid [17]. To study the synthesis and metabolism of vitreous type II procollagen, we chose to measure pCOL-II-C.

The HPLC profile of pCOL-II-C in the vitreous samples was superimposed on that in the cartilage (Fig. 2). The vitreous levels of pCOL-II-C in patients with a macular hole (2.5–5.7 ng/ml) were similar to the reported levels in the knee joints in patients with osteoarthritis (0.2–19.1 ng/ml), which indicates accelerated production of type II collagen in the joints [20].

In the present study, there was no significant difference in the pCOL-II-C levels between the vitreous samples and the fluid samples obtained during postoperative fluid–air exchange (Fig. 3). Occasionally, the pCOL-II-C levels were even higher in the fluid sample than in the vitreous sample, suggesting that pCOL-II-C is persistently secreted into the vitreous cavity at a similar level even after vitrectomy. After vitrectomy, the vitreous cavity is refilled by aqueous. Since the rate of aqueous production from the ciliary body is 2.5 μl/min, it takes 27 h to fill the vitreous cavity (4,000 μl) with new aqueous. In this study, fluid

samples were obtained 8 or 9 days after vitrectomy. Thus, the vitreous cavity appeared to be totally filled with new vitreous fluid that contained newly secreted pCOL-II-C. The level of pCOL-II-C in the vitreous may reflect the production of type II procollagen in the eye. Although the intravitreal pCOL-II-C level was similar to that in the synovial fluid, there was too little to detect by Western blotting (data not shown) and only EIA detected pCOL-II-C in the vitreous samples. In the adult human eye, the amount of type II procollagen produced may be very small, which also may be the reason why vitreous gel does not form clinically after vitrectomy.

In the vitreous, hyaluronan is the major substance that fills the spaces between the collagen fibrils [4]. We measured hyaluronan by sandwich binding protein assay. The vitreous levels of hyaluronan in our data were similar to those in autopsy eyes measured by Larsson and Osterlin [10]. The levels of hyaluronan in the fluid samples were much lower, for which there are two possible explanations. First, hyaluronan is not produced in the retina in adult eyes. We detected it in the vitreous gel but not in the vitreous fluid after vitrectomy. Another possibility is that the three-dimensional structure of the collagen fibers serves as the scaffold for hyaluronan to form the vitreous gel. Hyaluronan may escape from the vitreous cavity without becoming integrated into the gel structure.

pCOL-II-C was undetectable in serum samples from all ten patients using the EIA kit. In contrast, Nelson et al. [13] and Carey et al. [6] reported that pCOL-II-C in the blood was detected by radioimmunoassay. The reason we did not detect it is unclear. Even if blood contains pCOL-II-C molecules, normal retinas and ciliary bodies have tight junctions that prevent diffusion of small molecules such as fluorescein (376 Da) [1]. Because we obtained vitreous samples from patients with a macular hole without inflammatory diseases, the integrity of the tight junctions was maintained and the large pCOL-II-C molecules could not diffuse into the vitreous cavity from the blood. The hyaluronan level was 0.05±0.01 μg/ml in serum, similar to the reported level (0.01–0.1 μg/ml) [11], which is below 1/1,000 of the level in the vitreous samples. In the present study, the effect of pCOL-II-C and hyaluronan in the blood may be negligible.

The origin of newly formed procollagen is unknown. Newsome et al. suggested that embryonic chick vitreous collagen originates from the neural retina [14]. In situ hybridization studies have shown that mRNA of type II collagen is widely expressed not only in the retina but also in the sclera, cornea, lens and ciliary body of fetal chicks and mice [19, 22]. Bishop et al. suggested that the ciliary body, and in particular the nonpigmented ciliary epithelium, is the source of the vitreous collagen [5].

In the current study, we demonstrated for the first time the pCOL-II-C level in the vitreous. Secretion of pCOL-II-C into the vitreous cavity takes place at a similar level before and after vitrectomy. We speculate that the vitreous level of pCOL-II-C may reflect new synthesis of type II procollagen in the eye. Because there is no clinical evidence of newly formed vitreous gel after vitrectomy, other factors appear to be necessary to form a three-dimensional collagen framework entangled with hyaluronan.

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