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Cross-Linking of Vitreous Collagen and Degradation of Hyaluronic Acid Induced by Bilirubin-Sensitized Photochemical Reaction

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Abstract: To determine the mechanisms of vitreous liquefaction following vitreous hemorrhage, we investigated the effects of the free radicals produced by a bilirubin-sensitized photochemical reaction on collagen and hyaluronic acid (HA). Bovine vitreous collagen and HA were irradiated by visible light in the presence of bilirubin, which is produced from the degradation of hemoglobin, as a photosensitizer. Changes in molecular weight were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the collagen, and high-performance liquid chromatography for the HA. We found that free radicals caused an increase in high-molecular-weight components and insolubilization of the vitreous collagen, and a decrease in the molecular weight of HA. The changed molecular properties of the vitreous collagen could be attributed to the extensive cross-linking of the molecules. This cross-linking and the degradation of HA, both induced by the bilirubin-sensitized photochemical reaction, may contribute to vitreous liquefaction following vitreous hemorrhage. **Jpn J Ophthalmol 1997;41:154–159** © 1997 Japanese Ophthalmological Society

Key Words: Bilirubin, hyaluronic acid, photochemical reaction, vitreous collagen, vitreous hemorrhage, vitreous liquefaction.

Introduction

The vitreous is a transparent, avascular gel comprised of 99% water, 0.9% low-molecular-weight solutes, and 0.1% macromolecules such as collagen and hyaluronic acid (HA). The gel structure is maintained by a three-dimensional network of randomly organized, nonbranching, mainly type II collagen fibrils stabilized by interfibrillar HA molecules.[1] Destruction of this three-dimensional framework is believed to be the cause of the vitreous liquefaction that is clinically observed following vitreous hemorrhage.[2]

Bilirubin is produced from the heme-degradation pathway, which is regulated by heme-oxygenase.[3,4] The heme-oxygenase activity that is present in ocular tissues, such as the retina and ciliary body,[5] indicates that it is likely that bilirubin is also found in the vitreous cavity,[6] especially following vitreous hemorrhage.

Bilirubin is a known photosensitizer: visible light excites bilirubin and generates superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH) via the type I pathways, or singlet oxygen ($^1O_2$) via the type II pathways.[7,8] We have previously demonstrated that free radicals (such as O_2^-, H_2O_2, ·OH, or $^1O_2$), generated by photodynamic reaction and irradiated by visible light in the presence of photosensitizers, will cause vitreous liquefaction in vivo or in vitro.[9–14]

To enhance our understanding of the mechanisms of the vitreous liquefaction that follows vitreous hemorrhage, we investigated the effects of the free radicals that are produced by a bilirubin-sensitized photochemical reaction on collagen and HA.

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Materials and Methods

Preparation of Vitreous Collagen

Vitreous collagen was extracted from calf eyes, using a modification of the Seery and Davison method.[15] The calf eyes were obtained from a local abattoir and delivered in an ice-cooled container within 2–3 hours postmortem. The eyes were incised circumferentially about 5 mm posterior to the corneal limbus. The posterior portion, including the sclera, choroid, and retina, was separated cleanly from the vitreous body. The total vitreous was dissected carefully from the ciliary body, the zonules, and the lens with blunt forceps; it was then washed in sterile normal saline and any contaminated tissues were discarded. After mixing with protease inhibitors (0.5 mg/L leupeptin, 1 mmol/L ethylenediaminetetraacetic acid disodium, 0.7 mg/L pepstatin, and 0.2 mmol/L phenylmethane sulfonyl fluoride [PMSF]) (Boehringer Mannheim, Indianapolis, IN, USA), it was homogenized at 4°C in a Ten Broeck tissue grinder (Corning Glass Works, Corning, NY, USA). The dispersion was centrifuged (100 000 × *g*) for 45 minutes at 4°C in an ultracentrifuge (L3-50, Beckman, Palo Alto, CA, USA). The insoluble residue, which included the collagen, was resuspended in 20 volumes of 1 mol/L ethylenediaminedihydrochloride (Fluka, Ronkonkoma, NY, USA) with 0.2 mmol/L PMSF, and agitated overnight at 4°C. The suspension was centrifuged at 100 000 × *g* for 30 minutes and the insoluble residue was washed twice in sterile water and freeze-dried. This freeze-dried residue was resuspended in 0.5 mol/L acetic acid and treated with pepsin (2 × crystallized; Sigma, St Louis, MO, USA) (10 mg enzyme/100 mg sample) at 4°C for 8 days. This suspension was then centrifuged at 100 000 × *g* for 30 minutes at 4°C. The pH of the supernatant was adjusted to 7.2 by the addition of 1 mol/L sodium hydroxide (NaOH), and the collagen was precipitated by the addition of solid sodium chloride to a concentration of 4.4 mol/L. The precipitated collagen was separated by centrifugation (100 000 × *g*, 30 minutes, 4°C), dissolved in 0.5 mol/L acetic acid, dialyzed against distilled water at 4°C overnight, and freeze-dried to yield solubilized vitreous collagen.

Irradiation With Photosensitizer

The vitreous collagen was dissolved in 0.5 mol/L of acetic acid (4 mg/mL) and 0.125 mL of the solution was placed in each of several small plastic beakers (11 mm diameter, 22.5 mm deep; surface area of solution, 95 mm^2). This was mixed well with bilirubin conjugate (Sigma Calbiochemical, La Jolla, CA, USA) dissolved in 0.024 mL of 10× concentrated phosphate buffer and 0.1 mL of 0.65 N of NaOH to achieve a pH of 6.8. The beakers were covered with transparent polyethylene film to prevent evaporation.

HA (molecular weight, 2 × 10^6), prepared from rooster comb, was donated by Hoya, Tokyo. This was dissolved in distilled water (1 mg/mL) and 0.2 mmol/L of bilirubin was added. One mL of the resulting solution was placed on each of several small plastic dishes having a surface area of 200 mm^2.

There were three groups in the irradiation experiment: the experimental group (collagen or HA solution irradiated with bilirubin), the irradiation control group (solutions irradiated without bilirubin), and the dark control group (solutions with bilirubin but stored in the dark). The first two groups were placed on a white light irradiation system that had two fluorescent tubes (F15T8/CW) and a small cooling fan to counteract heat produced during irradiation. The light intensity was 22 000 lux, measure by a lux meter (LX-101, Edmund Scientific, Barrington, NJ, USA); this is approximately one-quarter the intensity of bright sunshine at noon during the spring in Boston, MA, USA. The temperature on the irradiation stage was controlled at 23–25°C by a thermocouple digital thermometer (BAT-12, Sensortek, Clifton, NJ, USA).

Sodium azide (Sigma), as an $^1O_2$ quencher, or mannitol (Sigma) as an ·OH quencher, was added to the sample solutions containing bilirubin in order to investigate the inhibitory effects of radical scavengers.

SDS-Polyacrylamide Gel Electrophoresis

After irradiation by white light, 0.25 mL of the electrophoresis sample buffer [2% SDS; 0.0625 mol/L Tris-hydrochloride, pH 6.8; 10% glycerol; 5% 2-mercaptoethanol; and 0.025% bromophenol blue] was added to the collagen solutions.[16] The mixture was placed in boiling water for 5 minutes and centrifuged at 2 000 × *g* for 5 minutes. The sample solution (20 μL) was loaded onto a polyacrylamide gradient gel (4–20%, Mini-Protean® II Ready Gels, Bio-Rad, Richmond, CA, USA). Constant voltage (200 mV/gel) electrophoresis with electrophoresis running buffer (0.124 mol/L Tris, 0.959 mol/L glycine, and 0.5% SDS) was done for 45 minutes. The gel was stained with coomassie blue (Rapid Coomassie Stain®, Diversified Biotech, Newton, MA, USA). After staining, the gel was dried with a gel dryer and scanned by a densitometer (DU-70 Spectrometer, Beckman, Fullerton, CA, USA) with a gel-holder attachment. The molecular size of the collagen peptide was calculated from the migration distance of the peptide band on SDS polyacrylamide gel electro-

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phoresis (SDS-PAGE). Bovine dermal type I collagen (VITROGEN 100®, 3.0 mg/mL, Celtrix, Palo Alto, CA, USA) was used as the molecular size standard for collagen.

HPLC Analysis

We used a Waters 600 Multisolvent Delivery System (Millipore, Milford, MA, USA) for HPLC analysis of the HA samples that had been irradiated for various times. The samples were separated at room temperature on a 30 cm size exclusion column Spherogel TSK G6000 PW with a 7.8 mm inner diameter (Beckman, San Ramon, CA, USA). The mobile phase used 0.1 mol/L sodium phosphate buffer (pH 6.8) with a flow rate of 0.5 mL/min. Peaks were detected at 210–600 nm with a Waters 990 Photodiode Array Detector (Millipore). The peak area was calculated by integrating at 210 nm; the average molecular weight of HA was estimated from the retention time of each sample and the calibration standard regression equation.

Results

Radical-Induced Changes in Vitreous Collagen

After white-light irradiation, the experimental solution clearly showed precipitation (Figure 1), presumably insoluble aggregated collagen, when the solution was boiled with 4% SDS electrophoresis sample buffer. The dark and irradiated control groups showed no precipitation.

In Figure 2, the SDS-PAGE, the dark and irradiated control samples show an intense band of collagen peptide typical of vitreous collagen. Our previous studies, using immunoblotting, have linked this band to the α-chain of type II collagen.[10,11] A less intense band representing the β-component of type II collagen is also visible in the middle. The γ-component in the control samples remains indistinct. A faint band, presumably the α1-chain of type V collagen, appeared above the band of the α-chain. SDS-PAGE analysis also gave the estimated molecular weights of the α-chain type II collagen (103 000), the α1-chain type V collagen (123 000), and the β-component of type II collagen (230 000). These weights conform reasonably well with reported values.[17]

In the experimental solutions, the bands of α-chain type II collagen in the experimental solutions faded following irradiation at various times because most of the irradiated collagen precipitated out of the solution. The bands of both the β- and the γ-components appeared darker than in the controls after irradiation, indicating the formation of more high-molecular-weight (cross-linked) products in the vitreous collagen.

Radical-Induced Changes in HA

HPLC analysis during the HA experiment is shown in Figure 3. The estimated molecular weight of HA decreased to 60% (1 250 000) of the original after 12 hours of irradiation in the presence of 0.2 mg/mL bilirubin. It continued to decrease to 25% (480 000) at 48 hours of irradiation. The HA in the control

Figure 1. After boiling with 4% sodium dodecyl sulfate electrophoresis sample buffer, vitreous collagen solutions were centrifuged. (**D**) Collagen solution stored in the dark for 48 hours with bilirubin (0.2 mmol/L) (dark control). (**I**) Collagen solution irradiated for 48 hours without bilirubin (irradiation control). (**E**) Collagen solution irradiated for 48 hours with bilirubin. Arrow indicates precipitation.

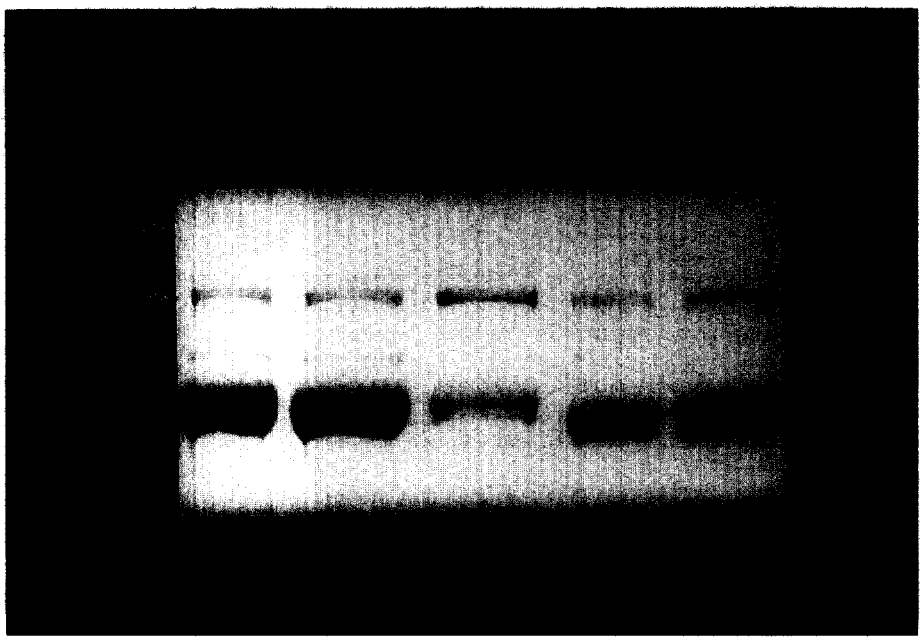

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of collagen sample. Column D, collagen stored in the dark for 48 hours with bilirubin (0.2 mmol/L) (dark control). Column I, collagen irradiated for 48 hours without bilirubin (irradiation control). Column E, collagen irradiated for 48 hours with bilirubin. Column Na, collagen irradiated for 48 hours with bilirubin and sodium azide (100 mmol/L). Column Ma, collagen irradiated for 48 hours with bilirubin and mannitol (100 mmol/L). Arrows indicate α-, β-, γ-components of vitreous collagen, respectively.

groups showed virtually no change after 24 hours (1 900 000) or 48 hours (1 800 000) of irradiation.

Inhibitory Effects of Radical Scavengers

The addition of sodium azide (1 mmol/L), $^1O_2$ quencher, or mannitol (10–100 mmol/L), an ·OH scavenger, significantly reduced the changes in molecular weight of both collagen (Figure 2) and HA (Table 1) when treated with bilirubin and white light.

Discussion

The present study demonstrated that free radicals generated by a bilirubin-sensitized photoreaction cause increases in the amount of insoluble material

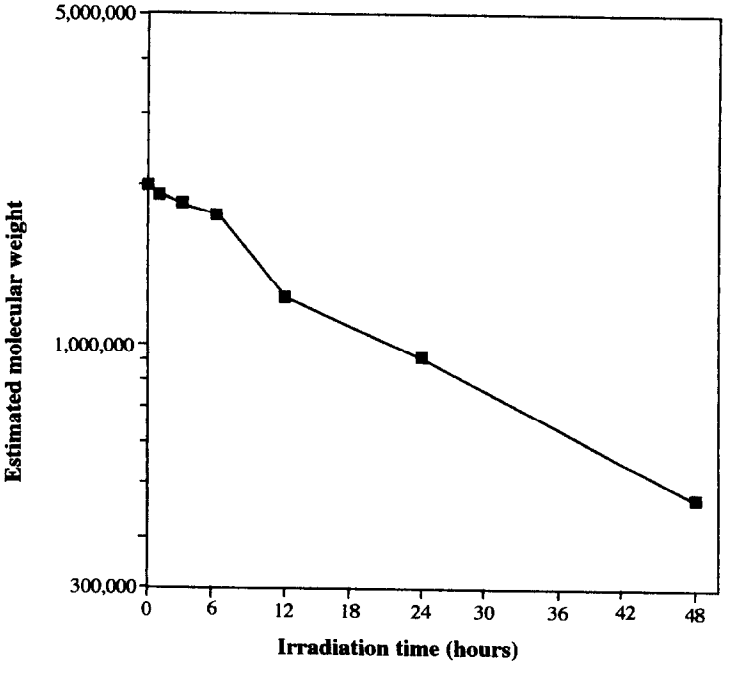

Figure 3. Changes in estimated molecular weight of hyaluronic acid irradiated in the presence of bilirubin (0.2 mmol/L).

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Table 1. HPLC of HA After White Light Irradiation (48 hours) in the Presence of Bilirubin and Radical Scavengers

Samples	Estimated Molecular Weight ($\times 10^4$)	
	Mean	Range
HA	200	205–195
HA + Bil	48	44–49
HA + Bil + NaN_3 (1 mmol/L)	118	113–123
HA + Bil + NaN_3 (100 mmol/L)	128	122–135
HA + Bil + Mannitol (1 mmol/L)	92	88–96
HA + Bil + Mannitol (100 mmol/L)	155	149–160

Concentrations of HA and bilirubin are 1 mg/mL and 0.2 mmol/L, respectively. HPLC, high-performance liquid chromatography; HA, hyaluronic acid; Bil, bilirubin; NaN_3, sodium azide.

and high-molecular-weight components of the vitreous collagen, apparently due to extensive cross-linking of the molecules. The bilirubin-sensitized photoreaction is known largely to follow the type II pathways that generate $^1O_2$ rather than the type I pathways that produce ·OH, O_2^- and H_2O_2.[7,8] In this study, inhibition of the phenomenon by sodium azide, a $^1O_2$ quencher, indicated the involvement of $^1O_2$ in photo-induced vitreous collagen cross-linking. The role of $^1O_2$ in this cross-linking was earlier investigated in our laboratory: $^1O_2$ generated by a riboflavin- or hematoporphyrin-sensitized photoreaction was shown to cause cross-links and aggregation of both types I and II collagen.[9,10,12] Mannitol, an ·OH quencher, also inhibited the cross-linking of vitreous collagen in the current study, indicating that ·OH also contributes to the cross-linking of vitreous collagen. Chemically-generated ·OH has been shown to cause cross-linking of type I collagen.[18]

This present study demonstrated that photodynamically-generated free radicals also cause a decrease in the molecular weight of HA, due to the degradation of the polysaccharide. Significant inhibition of the phenomenon was found when sodium azide or mannitol was added, suggesting that $^1O_2$ and ·OH are involved in the degradation of HA.

Our earlier studies have shown that free radicals generated by a photoreaction can cause vitreous liquefaction in vivo and in vitro.[9,10,12–14] It is conceivable that, in photo-induced vitreous liquefaction, the degradation of HA and the cross-linking of collagen by free radicals may occur simultaneously. The combination of these changes in collagen and HA may alter the mechanochemical interactions between these two molecules and lead to destruction of the three-dimensional framework of the vitreous gel, resulting in liquefaction.

We do not think, however, that free radicals generated from bilirubin-sensitized photochemical reactions are the only cause of vitreous liquefaction in eyes with a history of vitreous hemorrhage. There are a number of other factors that may be involved: serum components, such as fibronectin, are also reportedly implicated.[19] Also, the metal ion released from hemoglobin in the red blood cell has been reported as inducing HA degradation[20] leading to vitreous liquefaction.

The present study has demonstrated that bilirubin-sensitized, photodynamically-generated free radicals cause insolubilization of vitreous collagen, apparently due to extensive cross-linking of collagen molecules and the degradation of HA. Both of these changes may, therefore, contribute to the vitreous liquefaction seen following vitreous hemorrhage.

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