▣ PAPER ▣

SHRINKAGE OF VITREOUS BODY CAUSED BY HYDROXYL RADICAL

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In this study, we examined the effect of hydroxyl radical generated by γ-ray and UV irradiation on shrinkage of vitreous body. Change in gel ratio of vitreous body and change in the properties of its components (collagen, sodium hyaluronate) were analyzed. By comparing these results, the amount of hydroxyl radical, which induces the considerable shrinkage of vitreous body, was evaluated from theoretical calculation based on experimental condition and some reported kinetic parameters. It was concluded that the integrated amount of hydroxyl radical required to liquefy half of the vitreous body (Vitreous body gel ratio = 50%) was estimated as 140 $\mu molg^{-1}$ from γ-ray irradiation experiment. Also, from UV irradiation experiment result, it was confirmed that the effect of hydroxyl radical is larger than that of other reactive species. The causes of shrinkage of vitreous body are supposed as follows, 1) decrease in viscosity by cleavage of glycoside bond in sodium hyaluronate, 2) leaching of collagen from vitreous body and 3) leaching of crosslinked products and scission products of collagen.

Keywords : Vitreous body, Hydroxyl radical, γ-ray, UV irradiation, Collagen, Sodium hyaluronate

1. INTRODUCTION

Human being gets about 90% of the information *via* visual sense[1,2]. Therefore, it is generally thought that the sight greatly influences the QOL (quality of life) of people. It is a vitreous body to take an important role to obtain visual information[3]. The vitreous body, which holds about 80% of the volume of the eyeball, is located between the crystalline lens, a ciliary body and the retinas and maintains transparency to send light stimulation to the retina[4,5]. In addition, the vitreous body supports neighboring eyes organizations and takes a role to protect naked eye tissue from external force, for example to prevent wound-related detached retinas by its high dynamic strength and excellent viscoelasticity[1,2]. However, the function of the vitreous body deteriorates with aging; shrinkage of vitreous body causes detached retinas and there is a threat that loss of eyesight is induced depending on a case[6,7]. Elucidation of the mechanism on vitreous body shrinkage with the aging is yearned to prevent detached retinas.

A lot of researchers have extensively studied about the shrinkage of vitreous body. Paul *et al*. reported that the gel ratio of the vitreous body decreased with aging[8]. And, it was reported that the mechanisms of shrinkage of the vitreous body, which was composed of 3-dimensional network structure of collagen, hyaluronic acid and its derivative salts[9], were the main chain scission of hyaluronic acid or its derivative salts and resulting the liquefaction of the vitreous body[10-13]. On the other hand, Spoel reported that crosslinking of the collagen was a main cause of the shrinkage of the vitreous body[14,15]. The studies, such as above-mentioned, about the mechanisms of the shrinkage of vitreous body were performed on a premise that the accumulated reactive oxygen species (ROS) affected the shrinkage of the vitreous body by a depression of the antioxidant ability with the aging. The shrinkage of the vitreous body is influenced by not only such an internal factor but also external factors such as chemical additives in food, air pollution and the increasing ultraviolet (UV) irradiation caused by depletion

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of the ozone layer[16]. However, there are yet few studies that evaluated the quantitative threshold of ROS to induce the shrinkage of the vitreous body.

The ROS generated in the living body is usually removed by enzymes such as superoxide dismutase (SOD), catalase and peroxidase (POX). However, when the enzyme functions deteriorate with aging[8], ROS is not removed enough[17,18]. Among the above-mentioned enzymes, the function of catalase to decompose the hydrogen peroxide, is known to degrade with aging[19,20]. Furthermore, the remaining hydrogen peroxide which could not be removed is converted into hydroxyl radical by the factors such as Fenton reaction and UV. Especially, the hydroxyl radical is one of the most reactive chemical species among the ROS, and it reacts with an eyeball organization including the vitreous body and decreases those functions[21,22].

Therefore, in this study, we focused attention to the hydroxyl radical which was considered to be a main cause of the vitreous body shrinkage with the aging. The effects of formation and accumulation of hydroxyl radical *via* γ-ray or UV-irradiations on the shrinkage of vitreous body have been quantitatively studied. First, in order to examine the irradiation-induced change in macroscopic properties of the vitreous body, the γ-ray was used which could evoke spatially homogeneous reactions in the body tissue; the effects of radiation-generated radical species including hydroxyl radical on the vitreous body and its model compounds, i.e. sodium hyaluronate and collagen, were studied from the observations of the gel ratio of the vitreous body, change in viscosity of aqueous solution including sodium hyaluronate, and change in thickness of collagen gel. Next, in order to pick out the effects of hydroxyl radical, aqueous solution including sodium hyaluronate was exposed to monochromatic UV-C light with wavelength of 250 nm in the presence of hydrogen peroxide. And the effects of formation of hydroxyl radical on the viscosity of aqueous solution including sodium hyaluronate were studied. By comparing these results, the amount of hydroxyl radical, which induces the considerable shrinkage of vitreous body, was evaluated from theoretical calculation based on experimental condition and some reported kinetic parameters.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Vitreous bodies were used eyes of pigs (purchased from Osaka Meat Centar, transportation temperature was 277 K or below), not exceeding 6 months old. Collagen gel was prepared using collagen gel cultivation kit (Nitta Gelatin). Sodium hyaluronate was used sample derived from microbe (molecular weight; 10^6, Nacalai Tesque, Inc.). Hydrogen peroxide was prepared from 30% concentration (Wako Pure Chemical Industries, Ltd.). The electrophoresis was used with 15 – 25% of polyacrylamide gel (Daiichi Pure Chemicals Co.,Ltd.), and SDS-PAGE molecular weight standards-low (Bio Rad) was used as a molecular weight marker.

2.2 Irradiation

γ-ray and UV were irradiated in this experiment in order to induce radical. γ-ray irradiation was performed using a ^{60}Co source at room temperature under air atmosphere. The absorbed dose was calculated from radioactivity of ^{60}Co source, distance between source and irradiation time, based on the point-source approximation. UV-irradiation was perfumed using monochromatic light source (Bunkoh-Keiki Co.,LTD.; MRL-100). Vitreous body, collagen gel and sodium hyaluronate solution were irradiated with γ-ray, and sodium hyaluronate solutions containing hydrogen peroxide were irradiated with UV.

3. ANALYSIS PROCEDURE

3.1 Vitreous Body Gel Ratio and Change in Molecular Weight of Collagen in Vitreous Body

Vitreous bodies were prepared in order to estimate vitreous body gel ratio and change in molecular weight of collagen in vitreous body by γ-ray irradiation. Vitreous bodies were isolated by incising sclerotic coats with a surgical knife. 6 months old in pigs were approximately equivalent to three years old in human, and the effect of ageing was negligible in the initial state. To avoid the effect of individual difference, a pair of eyes (1 cm^3, pH 7.4) derived from one pig was used for one set of experiment; one side was used for γ-ray irradiation and another side was used as a control sample. The samples for measurement of gel ratio were irradiated in the range from 50 to 1000 kGy in absorbed dose (dose rate; 2.1 to 42 $kGyh^{-1}$). The samples for measurement of molecular weight of collagen were irradiated in the range from 10 to 500 kGy (dose rate; 0.41 to 21 $kGyh^{-1}$). After irradiation vitreous humor was separated from vitreous body, then the weight of remaining vitreous body was measured. As shown in Equation 1, the weight of irradiated vitreous body divided by that of non-irradiated vitreous body was defined as gel ratio of vitreous body[10]. Vitreous body gel ratio was calculated from the average weights of 10 samples for each dose.

$$\text{Vitreous body gel ratio} = \text{gel weight of irradiated vitreous body (g)} / \text{gel weight of non-irradiated vitreous body (g)} \quad (1)$$

Change in molecular weight of collagen included in vitreous body and leaching vitreous humor after γ-ray irradiation was estimated by electrophoresis. Vitreous body and vitreous humor after γ-ray irradiation were centrifuged (800g ×5 min) in order to precipitate impurities such as retina. After centrifugation, 8 µl of supernatant liquid was mixed with 8 µl of SDS protein denaturant, and heated at 368 K for 5 min. The electrophoresis was conducted with polyacrylamide gel (15-25%, Daiichi Pure Chemicals Co.,Ltd.) at 120 V for 120 min. SDS-PAGE molecular weight standards-low (Bio Rad, 5 µl) was used as a molecular weight marker.

3.2 Change in Thickness of Collagen Gel

As model material, collagen gel (pH 7.4) of 0.24 wt% was prepared using collagen gel cultivation kit[23]. 2.5 ml of concentrated culture medium (10 × MEM Hanks culture solution) and 2.5 ml of reconstruction buffer solution (50 mM NaOH solution containing 260 mM $NaHCO_3$ or 200 mM HEPES) were added into 20 ml of Cellmatrix TypeII-A solution (3 $mgdm^{-1}$, pH 3.0)with continuous stirring. The mixed collagen solutions were sealed in a Petri dish to prevent water evaporation, and were warmed at 310 K for 30 min until gelation was completed. Collagen gel was cut into 1 cm^3 cube the cube with a surgical knife and sealed again in the Petri dish. Collagen gel sample were irradiated in the range from 10 to 100 kGy in absorbed dose (dose rate; 5 to 50 $kGyh^{-1}$). The thickness of each collagen sample was estimated using the optical microscope (BX 51, Olympus), in which the position resolution along the optical axis was 1 µm. The average value of measured thickness of ten arbitrary points was calculated as thickness of a collagen gel.

3.3 Change in Viscosity of Sodium Hyaluronate Solution

Both of the γ-ray and UV were irradiated inside each sodium hyaluronate solution. As γ-ray irradiation sample, aqueous solution of 0.04 wt% of sodium hyaluronate (pH 6.0) was prepared by dissolving sodium hyaluronate into milli-Q water. The sample was sealed in the vials and irradiated in the range from 50 to 500 Gy in absorbed dose (dose rate; 50 to 500 Gyh^{-1}).

UV-irradiated samples were prepared by addition of hydrogen peroxide to adjust the concentration of hydrogen peroxide in the samples to 0.1 wt%. 2 ml of the sample was poured into a quartz cell (10 mm × 10 mm × 43 mm) for UV-visible absorption spectrometer, irradiated with UV. The samples were irradiated with monochromatic light (wavelength: 250 nm, beam shape: 6.0 mm × 18.0 mm, beam intensity: 2.6 $mWcm^{-2}$) for 12 hours. Viscosity of γ-ray and UV irradiated sodium hyaluronate solution with and without hydrogen peroxide (0.1 wt%) before and after irradiation was measured by using viscometer (VM-1G, Yamaichi Electronics co).

4. RESULTS AND DISCUSSION

4. 1 Change in Gel Ratio of Vitreous Body by γ-ray Irradiation

From a view of macroscopic change, we investigated the effect of free radical formed by γ-ray irradiation on the vitreous body gel ratio. Vitreous body gel ratio was estimated with Equation 1. Fig. 1 shows vitreous body gel ratio as a function of absorbed dose.

Since the vitreous body contains about 99% of water, irradiation effect of γ-ray on the vitreous body is mainly considered to be indirect effect through water radiolysis. Hydroxyl radical, hydrogen radical and electron as middle active species are formed by water radiolysis. An electron turns into hydrated electron which is strong reducing agent reacts with water to form hydrogen radical[24,25]. The G value of water radiolysis is 2.7 and 0.6 for G(·OH) and G(H·), respectively[26]. It is presumed from G values that hydroxyl radical is relatively easy to generate. The horizontal axis of Fig. 1 shows integrated amount of hydroxyl radical calculated from G value along with absorbed dose.

The vitreous body gel ratio decreased with increase in absorbed dose, which indicates that increase in hydroxyl radical generated by irradiation promotes shrinkage of vitreous body. It is reported that enzyme potency that decomposes hydrogen peroxide decreases with ageing in human eyes[27,28]. It is also reported that decrease in enzyme potency by ageing was the common trend among

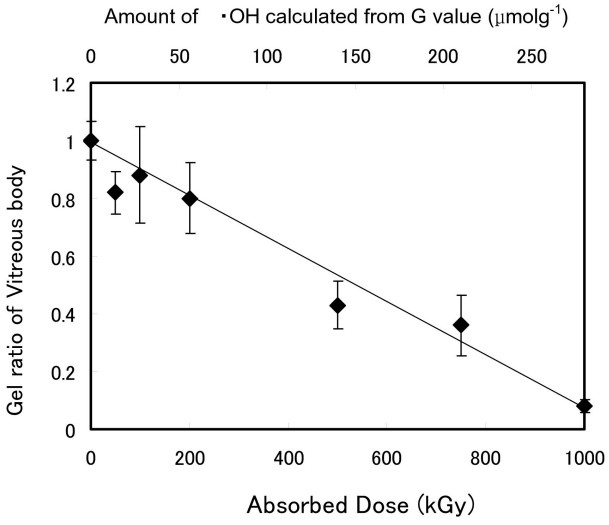

Fig. 1. Change in gel ratio of vitreous body by γ-ray irradiation.

the mammals such as the rat, pig and monkey besides human[17,20]. On the other hand, Bishop et al examined for liquefaction degree of vitreous body by ageing in human, and reported that liquefaction degree of vitreous body progressed with gel shrinkage after 40's, and more than half of vitreous body was liquefied in 80's~`90's[8]. There is possibility that the excessive amount of hydrogen peroxide which could not be removed by enzyme turns into hydroxyl radical by Fenton reaction or UV irradiation, and causes shrinkage of vitreous body. From integrated amount of hydroxyl radical calculated from G value, the amount of hydroxyl radical required to liquefy half of the vitreous body (Vitreous body gel ratio = 50%) was estimated as 140 μmolg^{-1}.

From these results, we confirmed that the vitreous body gel ratio decreased with increase in absorbed dose, which indicates that increase in hydroxyl radical generated by irradiation promotes shrinkage of vitreous body. The vitreous body is composed 99% of water and about 1% of solid component. Most of the solid components are collagen fiber and hyaluronic acid. Viscosity and elasticity of vitreous body can be supported and structure of the vitreous body is maintained by collagen fiber and hyaluronic acid[4,29,30]. Therefore, in order to check which of components is influenced more sensitively by hydroxyl radical resulting shrinkage reaction inside vitreous body, the model gel and/or solution containing each of collagen and sodium hyaluronate was prepared and studied.

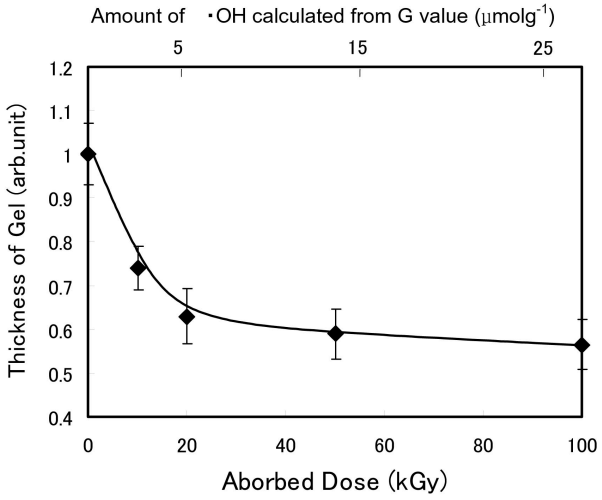

Fig. 2. Dependence of the thickness of collagen hydrogel film on γ-ray irradiation dose.

4. 2 Result of model experiment

4.2.1 Change in Thickness of Collagen Gel by γ-ray Irradiation

The vitreous body has a structure of fiber construction. Collagen fiber is an important substance to maintain the elastic strength of gel structure since about 80% of the vitreous body fibers are being regarded as the collagen fiber[9]. In order to confirm whether collagen fiber holds gel condition in spite of after γ-irradiation, collagen gel sample with a concentration of 0.24 wt% was prepared and γ-irradiation test was done. Since the concentration of collagen in actual vitreous body has been reported as 0.001-0.015 wt%[29-31], 0.24 wt% is some larger than in vitreous body. However, in this study, high concentration was selected in order to detect the gel shrinkage sensitively.

Thickness of irradiated collagen gel normalized by that of non-irradiated gel was shown in Fig. 2. Thickness of collagen gel decreased with increase in absorbed dose. Collagen gel contains large amount of water as well as vitreous body, and hydroxyl radical is easy to generate through water radiolysis in the gel.

Integrated amount of hydroxyl radical for each absorbed dose was calculated from G value, and shown in Fig. 2. Thickness of collagen gel decreased with increase in absorbed dose, and it means that hydroxyl radical generated by irradiation reacts with collagen to cause dehydration and shrinkage of collagen gel.

One of the causes of shrinkage of collagen gel is crosslinking caused by hydroxyl radical[14,32] . It is supposed that crosslinking by disulfide bond was formed by hydrogen elimination by hydroxyl radical in cystine region[20].

4.2.2 Change in Viscosity of Sodium Hyaluronate Solution by γ-ray Irradiation

About 0.04wt% of the vitreous body is hyaluronic acid and sodium hyaluronate. Hyaluronic acid and sodium hyaluronate strongly influence the viscosity of vitreous body[10]. Therefore, model aqueous solution containing 0.04 wt% of sodium hyaluronate was irradiated with γ-ray and change in viscosity was estimated. Fig. 3 shows the result.

Concentration of sodium hyaluronate was set to 0.04 wt%, which was as same as the sodium hyaluronate content in vitreous body. In this case, direct effect of γ-ray irradiation on sodium hyaluronate is negligible, because the samples are diluted solutions. Integrated amount of hydroxyl radical corresponding to each absorbed dose was calculated from G value, and shown as the horizontal axis in Fig. 3.

Viscosity of sodium hyaluronate solution decreased with increase in absorbed dose, and approached to 1. The integrated amount of hydroxyl radical required to decrease viscosity to 1 is 0.14 μmolg^{-1}. It means that hydroxyl radical generated by γ-ray irradiation decreases viscosity of sodium hyaluronate.

Shimada et. al. showed that viscosity of polymer solution decreases with decrease in molecular weight of

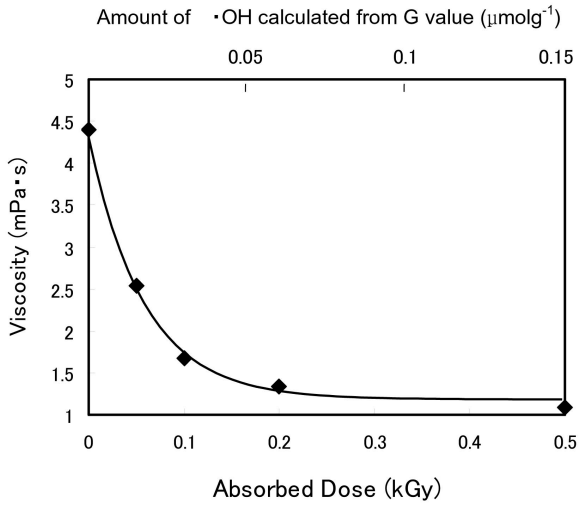

Fig. 3. Viscosity change of sodium hyaluronate solution as a function of γ- ray irradiation dose.

polymer[30]. It is supposed that hydroxyl radical reacts with sodium hyaluronate which causes main chain scission and decreases molecular weight. It was generally known that hyaluronic acid and sodium hyaluronate consists of glucuronic acid and N-acetylglucosamine conbined with glycoside bond[10]. Ueno et.al. reported that C5 hydrogen of glucuronic acid tends to eliminate by reaction with hydroxyl radical, and causes scission of glycoside bond, which results in depolymerization of hyaluronic acid[1,34,35].

Based on this report, it is supposed that hydroxyl radical generated by γ-ray irradiation reacts with sodium hyaluronate, eliminates the hydrogen atom, and generates sodium hyaluronate radical. It is also supposed that formation of sodium hyaluronate radical causes binding cleavage of glycoside bond which results in decrease in molecular weight.

From the model experiment shown above, we confirmed that crosslinking and shrinkage of collagen gel and, decrease in molecular weight of hyaluronate solution were induced by formation of hydroxyl radial. The integrated amount of hydroxyl radical required to decrease viscosity to 1 is estimated as 0.14 μmolg^{-1}.

On the other hand, 14.0 μmolg^{-1} of integrated amount of hydroxyl radical decreases thickness of collagen gel to 0.6 fold of initial thickness. From the results of model experiment, we confirmed that the amount of hydroxyl radical necessary to change the properties of sodium hyaluronate was lower than that of collagen gel.

Foulds et. al. reported about of the shrinking mechanism of vitreous body that depolymerization of hyaluronic acid plays an important role on liquefaction of vitreous body [10-13]. It corresponds to our result that the change in property of sodium hyaluronate was more sensitive than that of collagen.

However, other radicals except for hydroxyl radical such as hydrogen radicals generated by γ-ray irradiation is also not negligible. In order to extract the effect of hydroxyl radical, hydrogen peroxide (0.1 wt%) was added to sodium hyaluronate solution and then irradiated with UV, and viscosity of sodium hyaluronate solution before and after irradiation was measured. Comparing the results of change in viscosity of sodium hyaluronate by UV irradiation with that by γ-ray irradiation, we estimated the amount of hydroxyl radical which induces shrinkage of vitreous body.

4.2.3 Change in Viscosity of Sodium Hyaluronate Solution by UV Irradiation

As mentioned in 2.1, other radicals generated by water radiolysis by γ-ray irradiation are not negligible. Since we focused attention on the effect of hydroxyl radical, sodium hyaluronate solutions containing 0.1 wt% of hydrogen peroxide were irradiated with UV. Fig. 4 shows the change in viscosity of sodium hyaluronate solution containing 0.1 wt% of hydrogen peroxide with (a) and without (b) UV irradiation.

Decreasing rate of viscosity of UV-irradiated sample was larger than that of non-irradiated sample. In comparison with the slope of fitted curve in Fig. 4, the reaction efficiency of irradiated sample was about 2 times of that of non-irradiated sample. Hydrogen peroxide degrades into hydroxyl radical with almost 1 quantum yield by UV irradiation[36]. Concentration of hydroxyl radical generated by UV energy was calculated using following equation.

$$H_2O_2 \xrightarrow{h\nu} 2 \cdot OH \qquad (2)$$

Energy of one photon with wavelength of 250 nm was

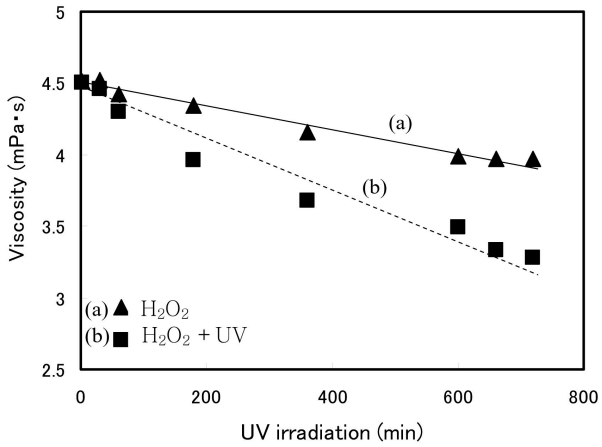

Fig. 4. Viscosity change of sodium hyaluronate by UV irradiation.

calculated by equation 3, 4. Total photon irradiated to the sample was calculated as 3.27×10^{15} per second, since the dose rate was 2.6 mWcm^{-2} as shown in equation 4.

$$E = h \times \frac{c}{250 \times 10^{-9}} = 7.951 \times 10^{-19}[J] \quad (3)$$

$$\frac{2.6 \times 10^{-3}[J\,\mathrm{sec}^{-1}]}{7.951 \times 10^{-19}[J]} = 3.27 \times 10^{15}(\mathrm{sec}^{-1}) \quad (4)$$

Absorbance of UV with wavelength of 250 nm by 0.1 wt% hydrogen peroxide solution is 0.237. In contrast, UV absorption by sodium hyaluronate is negligible. Based on this, 42 % of incident photon was absorbed in hydrogen peroxide. In case the reaction of equation 2 occurs with 1 quantum yield, generation rate of hydroxyl radical by UV irradiation is 2.75×10^{15} per second. Increasing rate of the concentration of hydroxyl radical was 2.28 μmol dm^{-3}sec^{-1}, because solution volume in this experiment was 2.0 ml. Hydroxyl radical generated by UV irradiation disappears by the reaction shown in Equation 5[36], because of its high reactivity, with the exception of reaction with sodium hyaluronate. The reaction speed of hydroxyl radical in Equation 5 is shown in equation 6.

$$H_2O_2 + \cdot OH \xrightarrow{k_1} H_2O + HO_2$$

$$\cdot OH + \cdot OH \xrightarrow{k_2} H_2O_2 \quad (5)$$

Where, $k_1 = 4.5 \times 10^7$[M^{-1}sec^{-1}], $k_2 = 6 \times 10^9$[M^{-1}sec^{-1}]

$$\frac{d[\cdot OH]}{dt} = 2\eta\Phi \times \left(1 - 10^{-\varepsilon[H_2O_2]\ell}\right) - k_1[H_2O_2] \times [\cdot OH] - k_2[\cdot OH]^2 \quad (6)$$

The first term in the right-hand side corresponds to generation of hydroxyl radical by UV irradiation, and the value is 2.28 μmol dm^{-3}sec^{-1}. The second term corresponds to disappearance by the reaction with hydrogen peroxide. The third term corresponds to deactivation and disappearance by collision between generated hydroxyl radical. The third term is especially important in case of high radiation intensity corresponding to high concentration of hydroxyl radical.

The equilibrium concentration of hydroxyl radial was calculated as 1.72 pmoldm^{-3} by equation 7, assuming that the concentration of hydrogen peroxide [H_2O_2] is constant at the initial concentration of 0.1 wt%=29.4 mmoldm^{-3}, because initial amount of hydrogen peroxide is higher enough than that consumed by the reaction induced by UV irradiation.

$$2\eta\Phi\left(1 - 10^{-\varepsilon[H_2O_2]\ell}\right) = k_1[H_2O_2] \times [\cdot OH] + k_2[\cdot OH]^2 \quad (7)$$

The calculated equilibrium concentration of hydroxyl radical generated from hydrogen peroxide by UV irradiation is 10^{-10} times lower than that of initial concentration of hydrogen peroxide (29.4 mmoldm^{-3}). It means that decrease in viscosity of the sodium hyaluronate which was shown in Fig. 4 was doubly promoted by generation of extremely low concentration of hydroxyl radical.

It suggests that hydroxyl radical is notably reactive among the active oxygens which is the middle active species generated by water radiolysis. From this result, it was confirmed that the effect of hydroxyl radical is larger than that of other reactive species generated by UV irradiation.

From the above-mentioned, we confirmed that chain scission of sodium hyaluronate undergoes in relatively low dose, while no significant changes are induced in collagen gel in such a low dose region. However, in relatively high dose, collagen gel was decomposed. Next, we will discuss about such a decomposition of collagen gel based on the molecular weight analysis by means of gel electrophoresis.

4.3 Measurement of Molecular Weight of Ollagen in Vitreous Body and Vitreous Humor by Electrophoresis after γ-ray Irradiation

Change in molecular weight of collagen included in vitreous body and vitreous humor seeped out after γ-ray irradiation was estimated by electrophoresis. Fig. 5 and 6 shows the results on vitreous body and vitreous humor. Since most of the protein included in vitreous body and vitreous humor is collagen, it is supposed that most of the

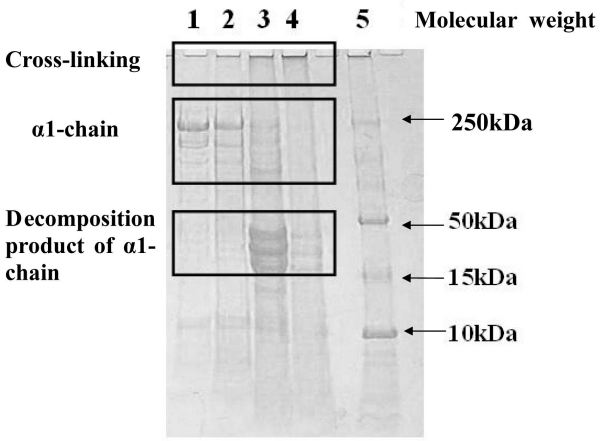

Fig. 5. Electrophoretic separation of vitreous body by γ- ray irradiation.Lane 1: control; lanes 2-4: absorbed dose (10 kGy, 100 kGy, 500 kGy); Lane 5: protein maker. SDS polyacrylamide gel concentration: 15-25%.

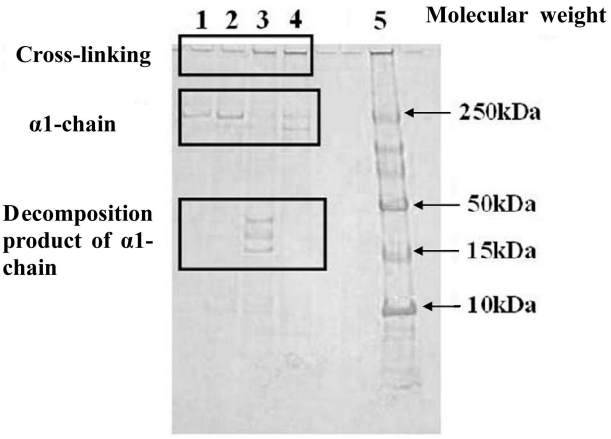

Fig. 6. Electrophoretic separation of vitreous humor by γ- ray irradiation. Lane 1: control; lanes 2-4: absorbed dose (10 kGy, 100 kGy, 500 kGy); Lane 5: protein maker. SDS polyacrylamide gel concentration: 15-25%.

bands observed in electrophoresis is collagen and its reaction products[2-4].

Non-irradiated vitreous body (lane 1) showed an obvious band at 250 kDa identified by protein marker (lane 5). Another band was also observed over 250 kDa. Collagen is the protein with triple helix structure with 300 nm in length and 1.5 nm in diameter, in which α-chain was combined mutually with repetition of Gly-Pro-X arrangement[37-39]. α-chain is roughly divided into α1- and α2-chain by difference of amino-acid sequence, and the types of collagen is classified by the combination of α-chain[40]. Most of the collagen included in vitreous body of pig are the TypeII collagen consisting of three α1-chains [1,38]. It is reported by Jongjareonrak et. al. that TypeII collagen shows obvious two bands with difference over 100 kDa on SDS-PAGE pattern[32,41,42].

Based on this report, it is supposed that the band observed in the range from 100 kDa to 250 kDa is α1-chain, and the band over 250 kDa is the components with relatively high molecular weight including crosslinked product and ß-structure on this experiment. It is supposed that the observed band below 50 kDa corresponds to the products of α1-chain dissociation.

From the results, following two possibilities is considered. First, from the results of SDS-PAGE (Fig. 5) of vitreous body, we confirmed that the amount of α1-chain decreased with increase in absorbance dose. Crosslinked product was under detection limit in the collagen in lane 1 (control; non-irradiated), collagen in vitreous body was crosslinked with increase in absorbed dose in lane 2 (10 kGy), 3 (100 kGy) and 4 (500 kGy). On the other hand, the decomposition product of α1-chain was observed in lane 3 and 4. It means that hydroxyl radical generated by γ-ray irradiation reacted with collagen to cause both cross-linking and main chain scission. The trend was also confirmed in case of collagen in vitreous humor.

Second, comparing figures 5 and 6, the molecular weight distribution in vitreous body in gel state and vitreous humor showed similar trend. It means that collagen which contributes to stabilize the vitreous body[7] seeps out from vitreous body by γ-ray irradiation. It is also supposed that leaching of crosslinked collagen and scission products is one of the important factors that causes shrinkage of vitreous body.

5. CONCLUSION

In this study, we examined the effect of hydroxyl radical generated by γ-ray and UV irradiation on shrinkage of vitreous body. Change in gel ratio of vitreous body and change in the properties of its components were analyzed. The amount of hydroxyl radical in these experiments were estimated by calculation. It was concluded that the integrated amount of hydroxyl radical required to liquefy half of the vitreous body (Vitreous body gel ratio = 50%) was estimated as 140 $\mu molg^{-1}$.

The causes of shrinkage of vitreous body are supposed as follows, 1) decrease in viscosity by cleavage of glycoside bond in sodium hyaluronate, 2) leaching of collagen from vitreous body and 3) leaching of crosslinked products and scission products of collagen. To prevent shrinkage of vitreous body, it is necessary to control generation and accumulation of hydroxyl radical and its precursors. The techniques are needed to control radicals, especially hydroxyl radical using antioxidant agent with biocompatibility.

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