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Chronic retinal effects by ultraviolet irradiation, with special reference to superoxide dismutases

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Summary. Recently ultraviolet light (UV) reaching the Earth's surface has been gradually increasing in amounts by the destruction of the ozone layers. Large parts of UV are absorbed in the cornea and lens, and only a few amounts reach the retina; however, the effect on the retina is not fully elucidated. 38 rats were irradiated 0.5-5.0 J/cm^2 UV from 6 to 50 times every 24 hours, and their retinal effects were investigated morphologically, immunohistochemically and immunochemically for superoxide dismutases (SOD). Morphologically, the destruction of rod outer segments (ROS) and dissociation of cell membranes between the pigment epithelial cells (PE) were already observed by 6 times 0.5 J/cm^2 UV irradiations. As the doses of UV increased, heterochromatins and lipid droplets increased in the PE. Significant damage was not observed, except in ROS and PE. In normal retina, Cu/Zn SOD were mainly distributed from the inner limiting membrane (ILM) to the ganglion cell layer, and the PE; however, after 6 times 0.5 J/cm^2 UV irradiations, the distribution became widened from inner to outer plexiform layer (OPL). At that time, the concentrations of Cu/Zn and Mn SOD increased in the retina. The present study reveals that the morphological damage caused by UV irradiation is observed in the ROS and PE, where no immunoreactivities could be detected to Cu/Zn and Mn SOD. However, morphological damage was not from the ILM to OPL, where the immunoreactivities to both Cu/Zn and Mn SOD were observed.

Key words: Enzyme immunoassay, Immunohistochemistry, Retina, Superoxide dismutase, Ultrastructure, Ultraviolet irradiation

Introduction

Although some solar ultraviolet light (UV) from 280 to 400 nm (UV-A: 320-400 nm; UV-B: 280-320 nm) penetrates the stratospheric ozone and reaches the Earth's surface, most of it is absorbed in the stratospheric ozone layers (Cutchis, 1974; DeLuisi and Harris, 1983). However, due to the recent destruction of the ozone layers, increasingly large amounts of UV have gradually begun reaching the Earth's surface. The threshold for UV-A to produce retinal lesions is about six times lower than that of visible light (Ham et al., 1980). In addition, without the anterior segments of the eye, such as the cornea and lens, which protect the retina from much light damage, the retina is susceptible to damage by UV-A or short-wavelength visible light (Ham et al., 1980, 1982). Therefore, retinal effects caused by UV have been mainly investigated, using aphakic eyes (Ham et al., 1980, 1982). However, in the present study, the retinal lesions by UV were investigated in the natural situation, using phakic eyes. In addition, it has been thought that UV causes retinal lesions through oxygen-free radicals (Handelman and Dratz, 1986). One of the free radicals, superoxide anion radical, generated during the monovalent metabolic reduction of oxygen, to O_2 and H_2O_2 (Fridovich, 1974; MaCord and Fridovich, 1969) is catalyzed by the superoxide dismutase. Therefore, the distribution and concentration of Cu/Zn and Mn SOD in UV-irradiated retinas were studied immunohistochemically and immunochemically, and compared with those in the normal retinas.

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Materials and methods

Forty Wistar rats (albino) about 10 weeks of age purchased from Japan Clea were used in the present study. The rats were housed with a 12-hour light (visible light) and 12-hour dark cycle for 1-2 weeks. Before the beginning of the dark cycle, thirty-eight rats were UV-A or UV-B irradiated from the 40 cm upper side of the cage, using a Dermaray (M-DMR-1, Eizai). Four non UV-irradiated retinas were used as controls. UV doses (watts) were measured by a UV Radiometer (UV-R 305/365D (II), Topcon), and the doses of 0.5, 1.0, 3.0 and 5.0 J/cm^2 were adjusted by the irradiation time of UV-A or UV-B. UV irradiations were repeated 6, 10, 12, 15, 25, 30, 35 and 50 times every 24 hours (Tables 1, 2).

Table 1. The number of the UV-irradiated retinas for morphological study.

NUMBER OF	0.5 J/cm²		1.0 J/cm²		3.0 J/cm²		5.0 J/cm²	
IRRADIATIONS	UV-A	UV-B	UV-A	UV-B	UV-A	UV-B	UV-A	UV-B
6-10	2	2	2	2	1	1	1	
11-20	1	1	2	2	1	1		
21-30			2	2				
31-50			1	1				1

Table 2. The number of the UV-irradiated retinas for immunohistochemical study.

NUMBER OF	0.5 J/cm²		1.0 J/cm²		3.0 J/cm²		5.0 J/cm²	
IRRADIATIONS	UV-A	UV-B	UV-A	UV-B	UV-A	UV-B	UV-A	UV-B
6-10	3	3	2	2	1	1	1	
11-20	3	3	2	2				
21-30	2	2	2	2	1	1		
31-50	2	2	1	1				1

Morphological procedure

Immediately after the last UV irradiation, 26 retinas were anesthetized with pentobarbital solution (Table 1). They were briefly perfused with physiological saline by inserting a needle into the left ventricle, followed by perfusion with Schmechel's fixative (Schmechel et al., 1980), composed of 4% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid and 2% sucrose in 0.1M sodium acetate buffer, pH 6.0. After perfusion, the eyes were enucleated and immersed in the same fixative. After fixation for half a day, the retinas were dissected and washed overnight in 50 mM Tris-HCl (pH 7.6) containing 150mM NaCl. Specimens were then postfixed with 2% osmium tetroxide in 50mM Tris-HCl buffer (pH 7.6) for 1 hour, dehydrated in a series of graded ethyl alcohols and embedded in Epon 812. Ultrathin sections (70 nm) of the retinas were obtained using an ultramicrotome (MT-5000, Sorval) with glass or diamond knives. The sections for electron microscopy were stained with uranyl acetate followed by lead citrate (Reynolds, 1963), then examined with an electron microscope (JEM, 1200 EX, JEOL) at 80 kV.

Immunohistochemical procedure

The 40 retinas were fixed by the same fixatives as in the morphological experiments (Table 2). Half a day after the fixation, they were transferred to 50mM Tris-HCl (pH 7.6) containing 150 mM NaCl and kept at 4 °C. After dehydration in a series of graded ethyl alcohols, the specimens were placed in 0.3% H_2O_2 in absolute methanol for 30 minutes to block the endogenous peroxidase activity and then embedded in paraffin. 5 µm serial sections were prepared and were immunostained by using Cu/Zn or Mn SOD antibody and the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. (1981). The primary Cu/Zn or Mn SOD antibodies used in the present study are described elsewhere (Kurobe et al., 1990; Kurope and Kato, 1991). As controls, sections were incubated with normal rabbit serum or with preabsorbed antibody instead of the primary antibody, which gave no positive stainings.

Immunoassay procedure

The 5 retinas of 6 times 0.5 J/cm² UV-A or UV-B irradiations were used for immunohistochemical study. Immediately after the last UV irradiation, the rats were anesthetized with pentobarbital solution and the retinas were dissected under the binocular microscope. All samples were kept frozen at -80 °C until analysis. The tissue was homogenized at 0 °C in a 10-volume of 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA with a Physcotron homogenizer (NS-50, Niti-On, Chiba, Japan). The homogenates were centrifuged at 45,000 rpm for 40 minutes, and soluble fraction was used for the assay of immunoreactive SOD. Polystyrene balls with immobilized antibodies were incubated in duplicate with shaking at 30 °C for 4 hours with 10 µl of the standard SOD or samples in a final volume of 0.5 ml with 10mM sodium phosphate buffer (pH 7.0) containing 0.3M NaCl, 5mM $MgCl_2$, 0.1% bovine serum albumin, 0.5% protease treated gelatin (Kato et al., 1980) and 0.1% NaN_3 (Buffer G). After aspirating reaction mixture, each polystyrene ball was washed twice in each tube with 2 ml of cold buffer A: 10mM sodium phosphate buffer (pH 7.0) containing 0.1M NaCl, 5mM $MgCl_2$, 0.1% bovine serum albumin (demineralized, Organon Teknika), and 0.1% NaN_3, transferred into a fresh tube with 0.2 ml of buffer G containing 1 milliunit of the galactosidase-labelled antibody Fab', and incubated at 4 °C overnight with shaking. Then each polystyrene ball was washed as described above and transferred to a fresh tube containing 0.1 ml of buffer A. The galactosidase activity bound to the ball was assayed with a fluorogenic substrate, 4-methylumbelliferyl-ß-galactoside (Sigma Chemical), at 30 °C for 20 minutes with shaking. The reaction was terminated by adding 2.5 ml of 0.1M glycine-NaOH buffer (pH 10.3), and the fluorescence intensity of the 4-methylumbelliferone released was measured against a freshly prepared standard solution of 1 µM 4-methylumbelliferone in the glycine NaOH buffer. The wave lengths used for excitation and emission analysis were 360 and 450 nm. respectively. Protein concentrations in extracts were determined by the Bio-Rad Protein Assay kit (Bio-Rad) which utilized the protein-dye binding method (Bradford, 1976).

Results

Morphological study

In the present study, the destruction of rod outer segments (ROS) and the dissociations of the cell

membranes between the pigment epithelial cells (PE) were already observed by 6 times 0.5 J/cm^2 UV-A or UV-B irradiations, especially by the UV-B range (Fig. 1A-C). In non-UV irradiated rat retina, the destruction of ROS and the dissociation of the cell membranes between the PE were not observed (Fig. 1D). As the doses and numbers of UV-A exposures increased, the lipid droplets in the PE increased in volume (Figs. 2A, 3A). The destruction of the ROS by UV-A irradiation became more severe than that by UV-B irradiation (Figs. 2, 3); however the destruction of ROS by 50 times 0.5 or 1.0 J/cm^2 UV-B irradiations was not so severe (Fig. 3B). In the nucleus of PE, the heterochromatin increased (Figs. 2A,B, 3A), except for the irradiation by 50 times 0.5 or 1.0 J/cm^2 (Fig. 3B). By 35 times 5.0 J/cm^2 UV-B irradiations, not only was there an increase of heterochromatins but also irregularity in the shape of the nucleus of PE was observed (Fig. 2B). Other than the effects on ROS and PE, no significant UV irradiation-induced damage was observed in the retina.

Immunohistochemical study

In normal adult rat, Cu/Zn SOD was mainly distributed in the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL) and the PE, whereas Mn SOD was present in the GCL, the inner plexiform layer (IPL) and outer plexiform layer (OPL) and inner granular layer (IGL) (Fig. 4). In the UV-irradiated retina, Cu/Zn SOD was also present in the IPL, IGL and OPL (Figs. 5-9). Hence the distribution in UV-irradiated retina was similar to that of Mn SOD in normal and UV-irradiated rat retina, except that Cu/Zn SOD was also present in the PE (Figs. 5B, 6B). The change in distribution of Cu/Zn SOD was already observed by 6 times 0.5 J/cm^2 UV-A or UV-B irradiations (Figs. 5A, 6A), while that of Mn SOD was not observed between the normal and UV irradiated rat retina (Fig. 4B, 5B, 6B). As the doses and numbers of UV irradiations increased, the immunoreactivity to Cu/Zn SOD in the IPL strikingly increased (Figs. 5A, 6A, 7-9). Cu/Zn and Mn SOD were not immuno-

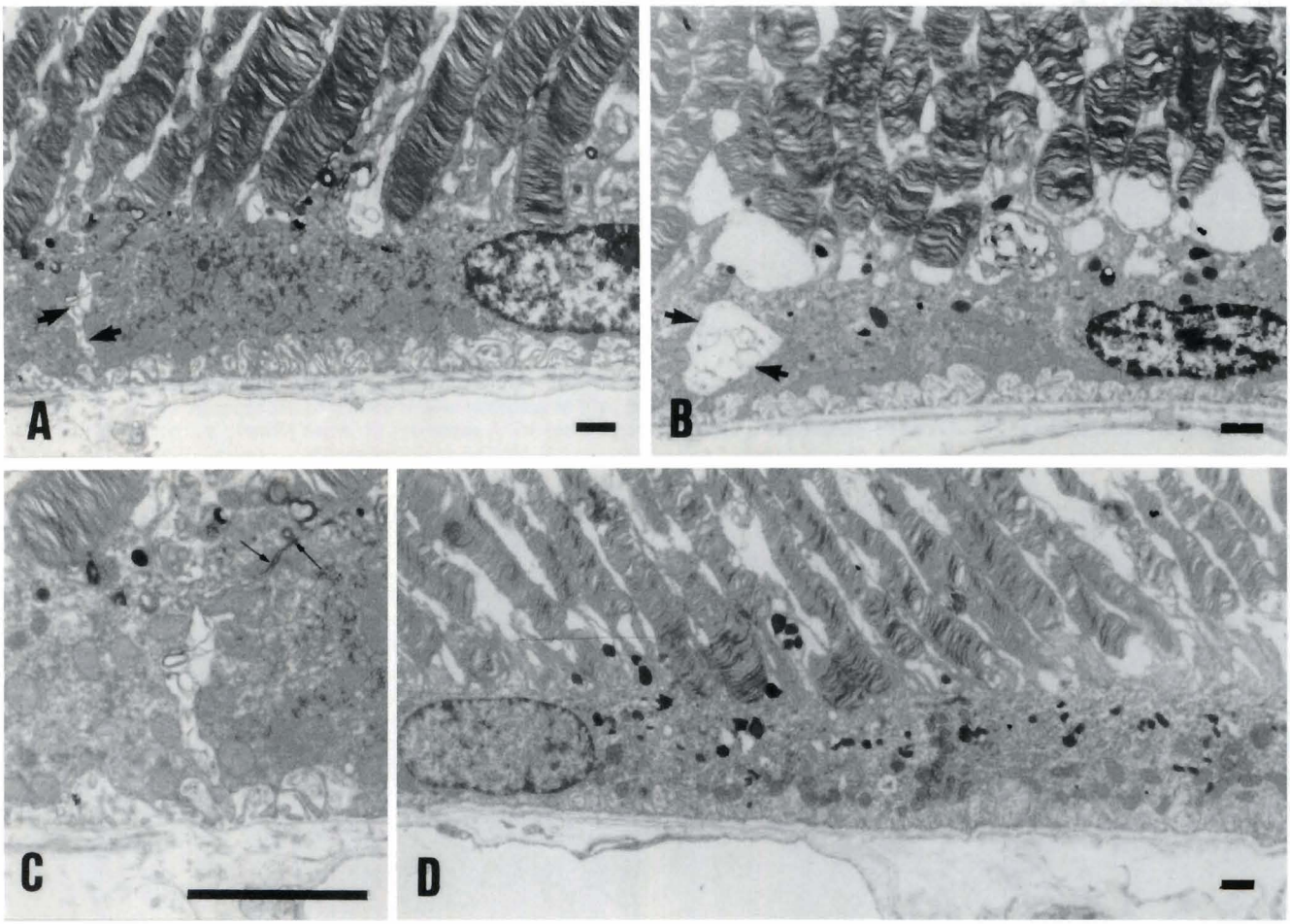

Fig. 1. Electron microscopic views of the ROS and PE by 6 times 0.5 J/cm^2 UV-A (**A,C**) or UV-B (**B**) irradiations, and that of ROS and PE in normal rat retina (**D**). The dissociations of the cell membranes are observed in the basal side of the PE between the neighboring PE (A,B: arrows), especially by the exposure to UV-B (B), although the junctional complexes in the apical side of the PE are not damaged by 6 times UV-A or UV-B exposures (C: small arrows). D: in normal rat retina, the damage to the cell membranes in the ROS and PE is not observed. Bar: 1 μm.

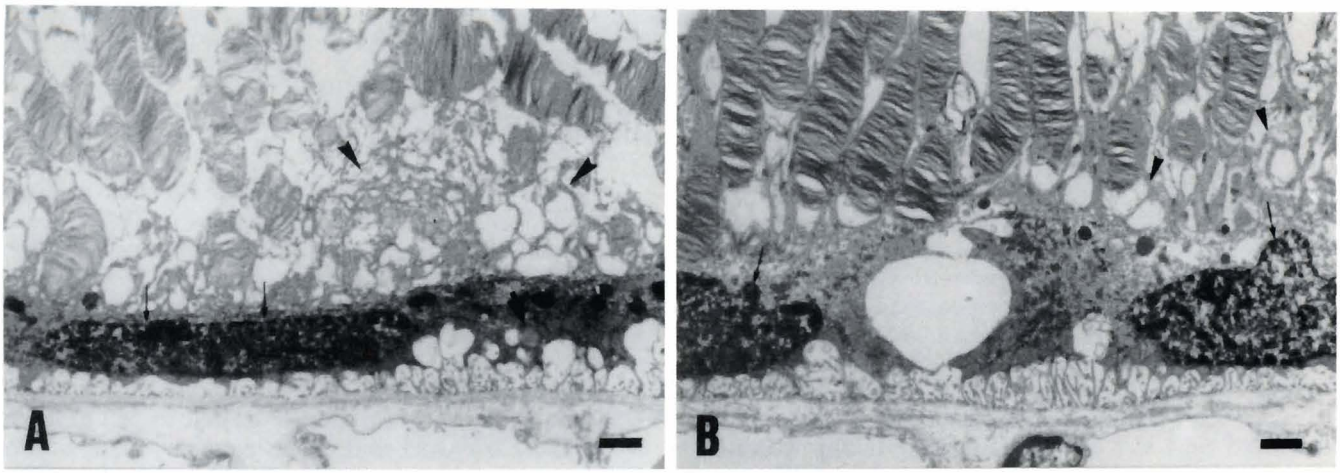

Fig. 2. Electron micrograph views of the ROS and PE by 10 times 5.0 J/cm^2 UV-A (**A**) and 35 times (**B**) UV-B irradiations. A small lipid droplet is observed in the PE by the exposure of 5.0 J/cm^2 UV-A (A: large arrow). The cell membranes of ROS neighboring the PE are damaged (A,B: arrowheads), especially by UV-A irradiation (A). The heterochromatins in the cell nucleus of PE increase by the exposure of 5.0 J/cm^2 UV-A or UV-B (A,B: small arrows). In 5.0 J/cm^2 UV-B irradiation, the cell nucleus is also irregular in shape (B: small arrows). Bar: 1 μm.

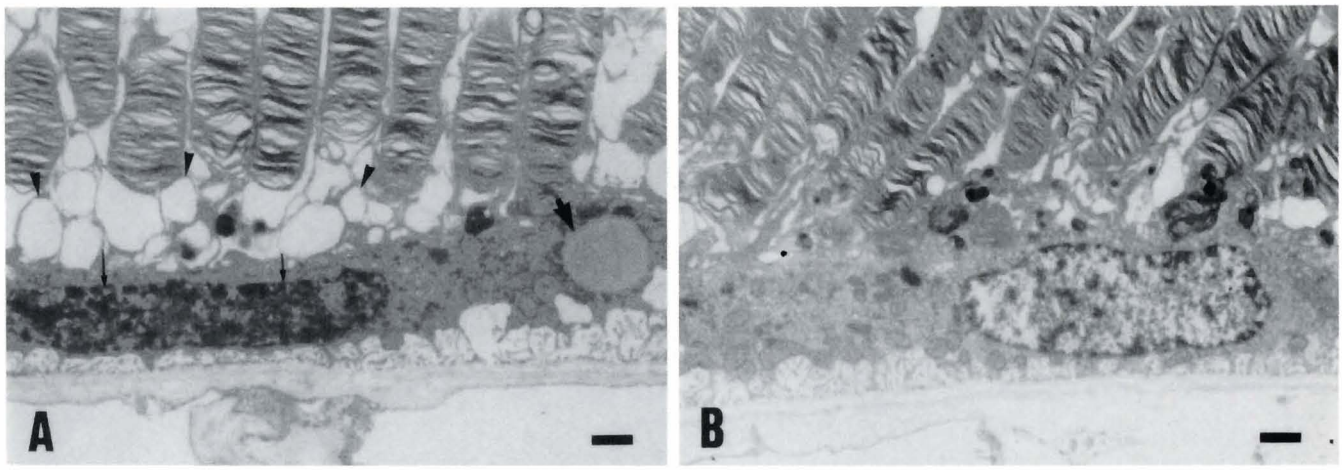

Fig. 3. Electron microscopic views of the ROS and PE by 50 times 1.0 J/cm^2 UV-A (**A**) or UV-B (**B**) irradiations. ROS are destroyed by the exposure of UV-A (A) more significantly (arrowheads) than by that of UV-B (B). In the PE, lipid droplets increase in volume by the exposure of UV-A (A: arrows). The increase of heterochromatins in the cell nucleus of PE is observed by 50 times UV-A exposures (A: small arrows), but not by that of UV-B. Bar: 1 μm.

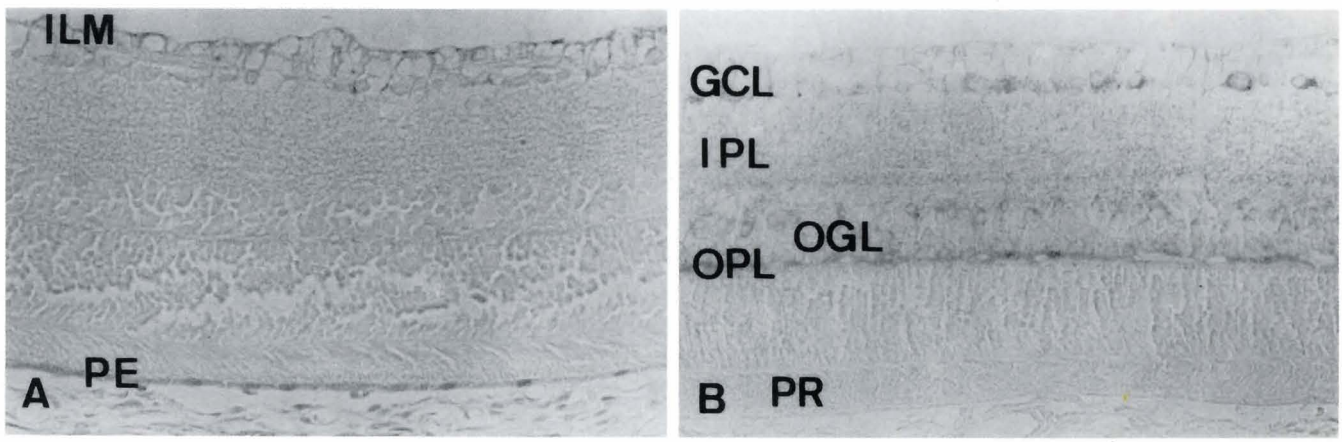

Fig. 4. Cross sections of the posterior portion of normal rat retina immunostained with the antibody to Cu/Zn (**A**) or Mn SOD (**B**). Cu/Zn SOD is mainly distributed in the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), and the PE, while Mn SOD is in the GCL, the inner (IPL), and outer plexiform layer (OPL) and inner granular layer (IGL). The immunoreactivity to Cu/Zn or Mn SOD is not observed in the photoreceptor cells (PR). x 200

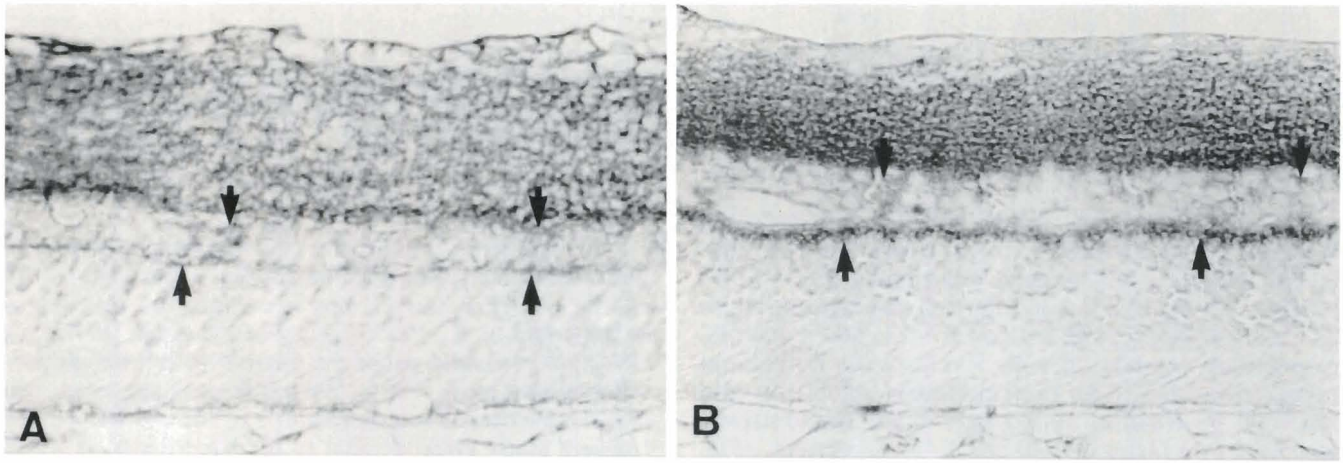

Fig. 5. Cross sections of the posterior portion of the retina by 6 times 0.5 J/cm^2 UV-A exposures immunostained with the antibody to Cu/Zn (**A**) or Mn SOD (**B**). Cu/Zn SOD is distributed in the deeper layers, such as the IPL, IGL and OPL (A: arrows); however, Mn SOD remains constant (B). The immunoreactivity to Cu/Zn SOD is not observed in the PR but in the PE. x 200

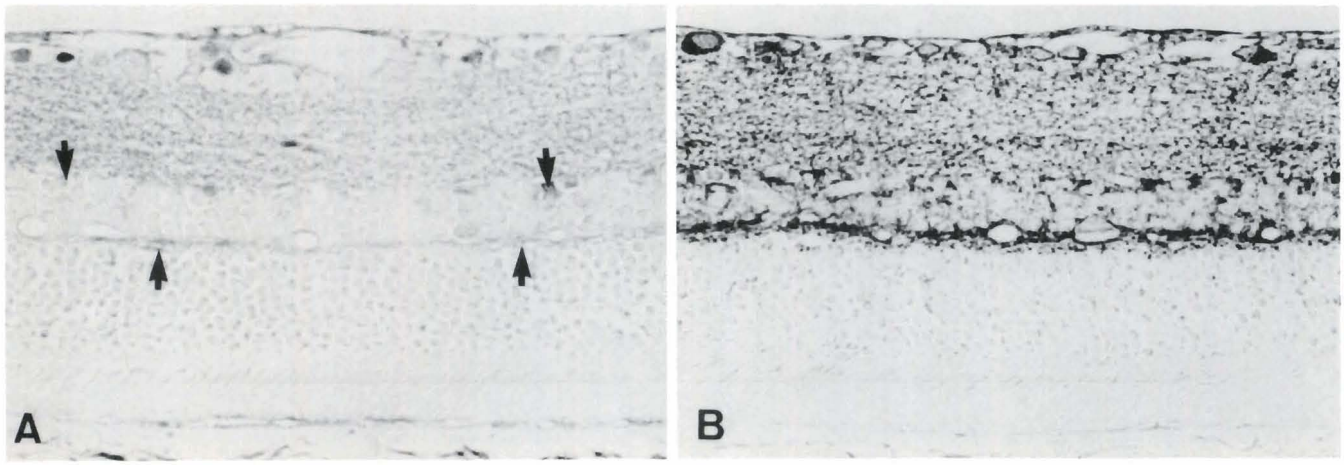

Fig. 6. Cross sections of the posterior portion of the retina by 6 times 0.5 J/cm^2 UV-B exposures immunostained with the antibody to Cu/Zn (**A**) or Mn SOD (**B**). Cu/Zn SOD is distributed in the deeper layers, such as the IPL, IGL and OPL (A: arrows); however, Mn SOD remains constant (B). The immunoreactivity to Cu/Zn SOD is not observed in the PR but in the PE. x 200

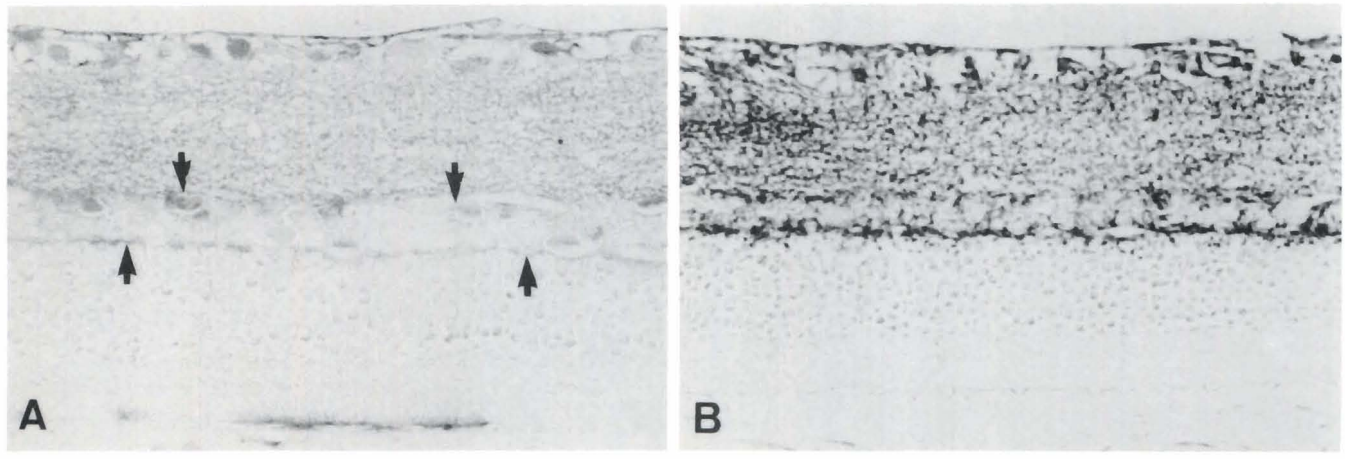

Fig. 7. Cross sections of the posterior portion of the retina by 50 times 0.5 J/cm^2 UV-A (**A**) or UV-B (**B**) exposures immunostained with the antibody to Cu/Zn SOD. Cu/Zn SOD is distributed in the deeper layers, such as the IPL, IGL and OPL (A, B: arrows). The immunoreactivity to Cu/Zn SOD is not observed in the PR but in the PE. x 200

histochemically detected in the photoreceptor cells (PR) in either normal or UV-irradiated rat retina (Figs. 4-9).

Immunochemical study

In normal rat retina, the concentrations of Cu/Zn and MnSOD were 3.98±0.31 μg/mg total protein and 0.96±0.14 μg/mg total protein, respectively (Fig. 10). By 6 times 0.5 J/cm^2 UV-A irradiations, the concentrations of Cu/Zn and Mn SOD increased 1.13- and 1.20-fold, respectively, compared with levels in the normal rat retina (Fig. 10). The concentration of Cu/Zn and MnSOD by 6 times 0.5 J/cm^2 UV-B irradiations increased by a factor of 1.40 and 1.41, respectively (Fig. 10). Compared with the concentrations of Cu/Zn and Mn SOD in normal rat retina, there was a significant increase of Cu/Zn and Mn SOD in the retina observed by 6 times 0.5 J/cm^2 UV-B irradiations (Cu/Zn SOD: $p<0.01$, Mn SOD: $p<0.05$).

Discussion

In the present study, the cell membranes of the PE and ROS were severely damaged. By UV irradiation, the level of lipid peroxides increases in the retina (Ottolenghi et al., 1955; Feeney and Berman, 1976) and the lipid peroxides cause the damage to cell membranes (Yamakawa et al., 1991). Therefore, it is suggested that the destruction of cell membranes of PE and ROS in the present study could be due to lipid peroxidation which can be induced by UV irradiation. The lipid peroxides are synthesized from polyunsaturated fatty acids by

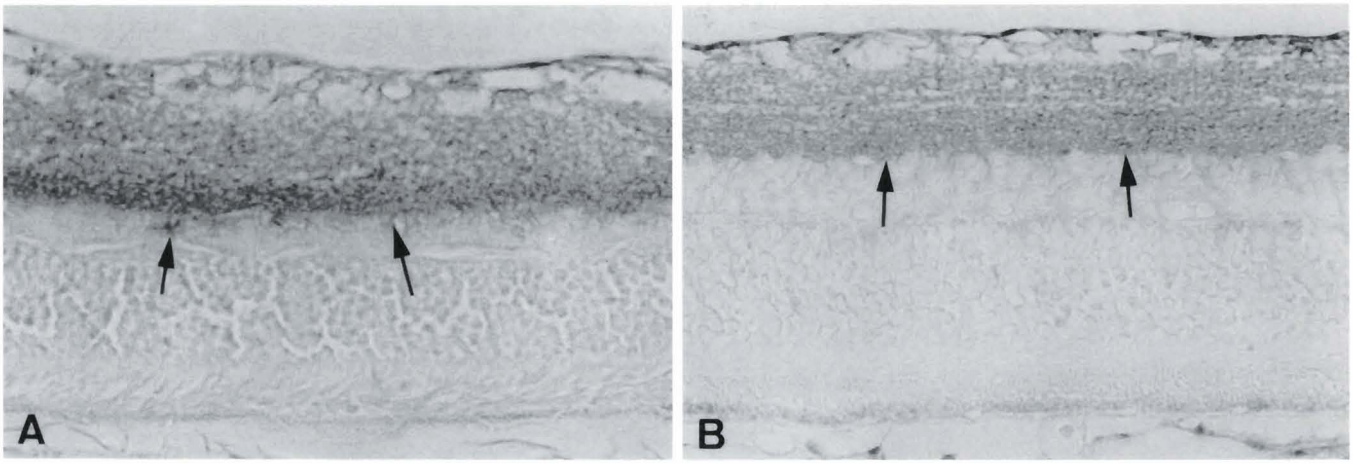

Fig. 8. Cross sections of the posterior portion of the retina by 10 times 1.0 J/cm^2 UV-A (**A**) or UV-B (**B**) immunostained with the antibody to Cu/Zn SOD. Cu/Zn SOD is distributed in the deeper layers, such as the IPL, and the inner portion of IGL (A,B: arrows). The immunoreactivity to Cu/Zn SOD is not observed in the PR but in the PE. x 200

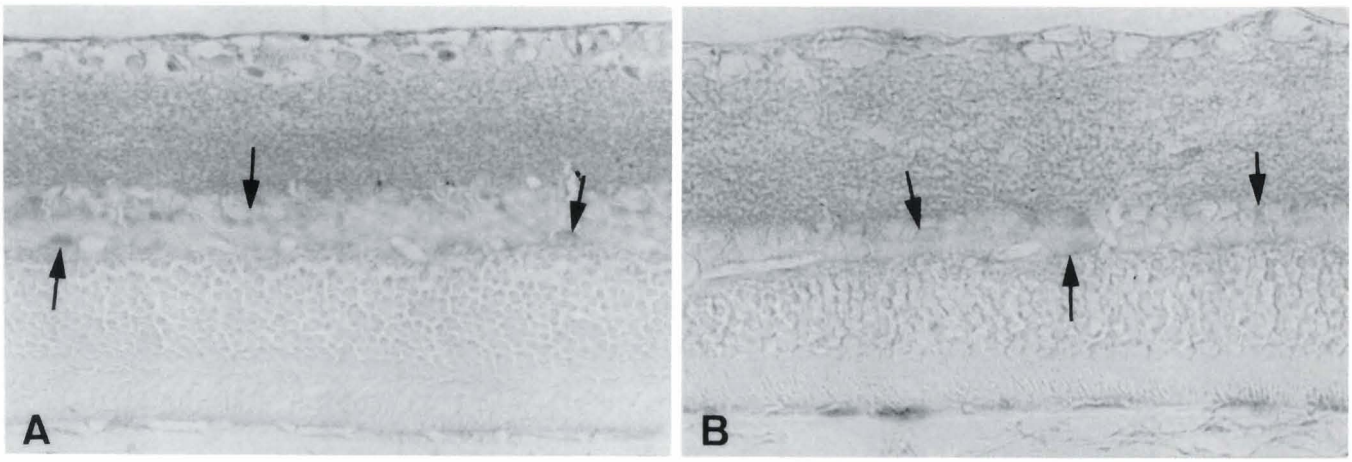

Fig. 9. Cross sections of the posterior portion of the retina by 10 times 5.0 J/cm^2 UV-A (**A**) or 35 times 5.0 J/cm^2 UV-B exposures (**B**) immunostained with the antibody to cu/Zn SOD. Cu/Zn SOD is distributed in the deeper layers, such as the IPL, IGL and OPL (A,B: arrows). The immunoreactivity to Cu/Zn SOD is not observed in the PR but in the PE. x 200

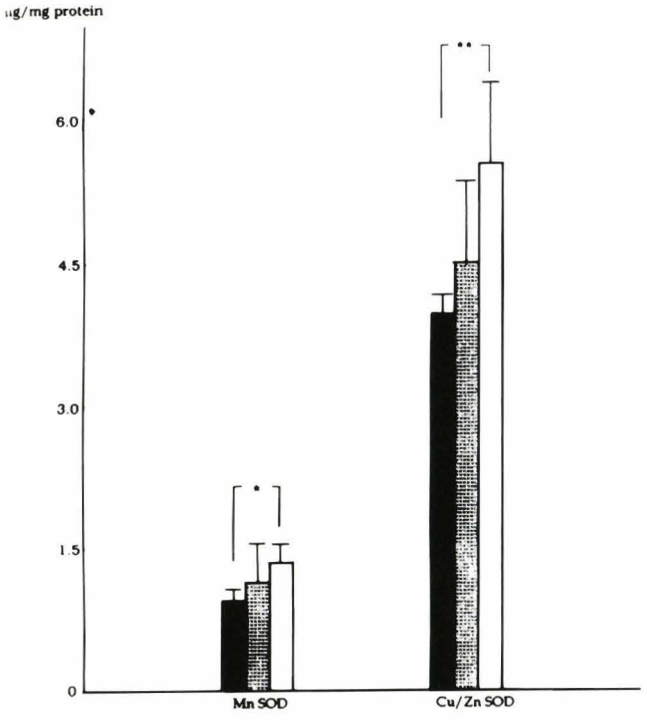

Fig. 10 The concentration of Cu/Zn or Mn SOD in the normal rat retina (black bars), and the retina by the exposure of 6 times 0.5 J/cm^2 UV-A (dotted bars) or UV-B irradiations (white bars). Both by 6 times exposures of UV-A or UV-B, the concentrations of Cu/Zn and Mn SOD increase compared with those in normal rat retina, especially by the exposures of UV-B. Each point represents the mean ± SE of the results from 4-5 specimens. *: $p<0.01$; **: $p<0.05$.

oxygen-free radicals (Anderson et al., 1974). In the present study, Cu/Zn SOD, which is thought to protect against damage by oxygen-free radicals, was present in the PE. However, the cell membranes of the PE were damaged. Therefore, it seems that only the presence of Cu/Zn SOD may not protect against damage to the cell membranes of PE by UV-induced oxygen-free radicals.

It has been reported that PE is not damaged by the UV-A irradiation of the eyes (Henton and Sykes, 1984; Rapp et al., 1990). However, the present study revealed that the cell membranes between the PE were damaged in an earlier period of 0.5 J/cm^2 UV irradiation. In addition, the damage was more severe by UV-B irradiation than by UV-A, although UV-A reached deeper regions of the retina than UV-B because of the longer wave length of UV-A. Therefore, these findings show that UV-B generates more severe retinal damages than UV-A in he same doses of UV irradiation. However, as the numbers of 0.5 or 1.0 J/cm^2 UV-B irradiation increased, the damage to the ROS and PE became less significant, compared with those by 0.5 or 1.0 J/cm^2 UV-A irradiation. Since the cornea gradually became opaque after 6-8 times UV-B irradiations in the present study, the findings are probably due to the fact that the smaller doses of UV-B reach the ROS and PE by the corneal opacity. On the contrary, as the numbers of 3.0 or 5.0 J/cm^2 UV-B irradiations increased, nuclei of the PE were severely damaged. Therefore, this finding supports the previous report that UV-B can be transmitted through the anterior segment of the eyes in sufficient intensity to damage the retinal DNA (Rapp et al., 1985) in the case where large doses of UV-B are repeatedly irradiated.

We previously reported that Cu/Zn SOD is observed in the ILM, NFL and GCL and PE of adult rat retina (Oguni et al., 1995). However, in this UV-irradiated retina, Cu/Zn SOD was observed not only in the ILM, NFL and GCL but also in the IPL, OGL and OPL. Immunochemically, we found that the concentration of both Cu/Zn and Mn SOD increased by UV irradiation. The retina is known to be nurtured from two sources, that is the inner portion of the retina from the ILM to the OPL is nurtured by retinal vessels, while the outer portions of the retina from the OGL to the PE are nurtured by choroidal vessels. The retinal regions by UV irradiation, where both Cu/Zn and Mn SOD were immunohistochemically detected, are nurtured by retinal vessels, not by choroidal vessels. In addition, significant morphological damage was not observed in these regions. Therefore, in these regions, Cu/Zn and Mn SOD seem to play an important role in the protection against the superoxide radical anions, which are generated by UV irradiation.

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