

p53 Expression and Apoptosis in the Lens after Ultraviolet Radiation Exposure

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PURPOSE. To localize p53 protein and active caspase-3 in the albino rat lens and to compare p53 mRNA and active caspase-3 expression in ultraviolet radiation (UVR) 300 nm exposed lenses and their contralateral nonexposed controls.

METHODS. Ten Sprague-Dawley albino rats were unilaterally exposed to 8 kJ/m² UVR, and the contralateral eyes were left nonexposed. In total, four exposed lenses and their respective contralateral nonexposed lenses were analyzed by immunohistochemistry to localize p53 and active caspase-3. In addition, six exposed and contralateral nonexposed lenses were analyzed by real-time RT-PCR. Quantified p53 and caspase-3 expression were compared between the in vivo UVR 300 nm exposed lenses and the contralateral nonexposed lenses.

RESULTS. All lenses exposed to UVR developed cataract. Immunohistochemistry showed that p53 and active caspase-3 were localized in the lens epithelial cells. Quantified p53 and caspase-3 expression were significantly higher in lenses exposed to UVR than in nonexposed lenses.

CONCLUSIONS. p53 and caspase-3 expression increase in lens epithelial cells after UVR exposure. In the lens, apoptosis induced by UVR may be associated with increased p53 expression. (*Invest Ophthalmol Vis Sci.* 2007;48:4187–4191) DOI:10.1167/iovs.06-0660

The *p53* gene, situated on chromosome 17p13.1, is a well-defined cancer-suppressor gene. The gene produces a 53-kDa phosphoprotein that plays a role in regulating cell proliferation¹ and apoptosis in response to DNA injury.^{2,3} It also suppresses angiogenesis.⁴ The *p53* gene is the most frequently mutated gene in human cancer.⁵ The p53 protein exists as wild-type and various types of mutant forms. Wild-type p53 without mutation is found in normal cells and is critically important in the repair of DNA damage.

DNA damage activates p53, resulting in either cell-cycle arrest or apoptosis. Cell-cycle arrest allows time for the cell to repair the damage. Apoptosis permits the body to excrete cells with damaged DNA. Loss of p53 function, therefore, indirectly decreases genomic stability. The mutant form of p53 is frequently found in cancer cells. Overexpression of the *p53* gene

has been found in several types of human malignancies and in the eye in pterygia and pingueculae.⁶

Information is limited regarding the expression and the normal function of p53 protein in normal noncancerous tissues. Almon et al.⁷ were among the first to demonstrate significant expression of p53 protein in normal untransformed tissue. They showed increased expression of p53 protein in murine testicular tissue and suggested a role for p53 protein in meiotic spermatogenesis. Expression of p53 protein has also been demonstrated in animal central nervous system (CNS) neurons,⁸ human epithelial lesions,⁹ and several other tissues. Recently, high expression of p53 protein has been demonstrated in normal, adult rodent ocular tissues.¹⁰ Because many of these tissues consist of nondividing cells (neurons), it is likely that p53 protein has additional important functions in addition to regulating cell proliferation and apoptosis.

Ultraviolet radiation (UVR) and chemical or physical agents all may induce tissue stress. Low-dose stress to a tissue may not directly kill cells but may damage DNA, cell membranes, or other cell organelles. Such damage may be repaired if the cell survives or may lead to programmed cell death, or apoptosis.¹¹ Apoptosis may be associated with increased expression of p53 protein. The apoptosis pathway may also be p53 independent. Apoptosis after UVR exposure has been demonstrated in the lens epithelium in vitro.^{12,13} Takamura et al.¹⁴ demonstrated apoptosis in the lens epithelium of rat sugar cataract and speculated that apoptosis in this model was probably secondary to increased expression of p53 protein. There has been, to our knowledge, no observation of p53 protein-associated apoptosis in the albino rat in vivo model.

The aims of the present work were to localize p53 wild type and caspase-3 and to study p53 and caspase-3 expression after in vivo UVR 300 nm lens exposure in albino rats.

METHODS

Sprague-Dawley rats were exposed unilaterally in vivo to UVR 300 nm. One week after exposure, the animal was killed, and the lenses were removed for analysis of macroscopic appearance, intensity of forward light scattering, immunohistochemical localization of p53 wild-type protein and active caspase-3 expression, and quantification of p53 mRNA and caspase-3 expression by amplification with real-time reverse transcription-polymerase chain reaction (RT-PCR) for final quantitative measurement of the amplified p53 and caspase-3 expression.

Experimental Animals

The 6-week-old female albino Sprague-Dawley rat was the experimental animal. Animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ethical permission was obtained from Stockholms Norra Djurförsöksetiska nämnd (N 135/02).

Exposure to Ultraviolet Radiation

Ten minutes before exposure, the animal was anesthetized with ketamine 94 mg/kg plus xylazine 14 mg/kg intraperitoneally.¹⁵ Tropicamide was instilled in both eyes. Five minutes after pupillary dilation, the animal was unilaterally exposed for 15 minutes to a total dose of 8 kJ/m² UVR 300 nm.

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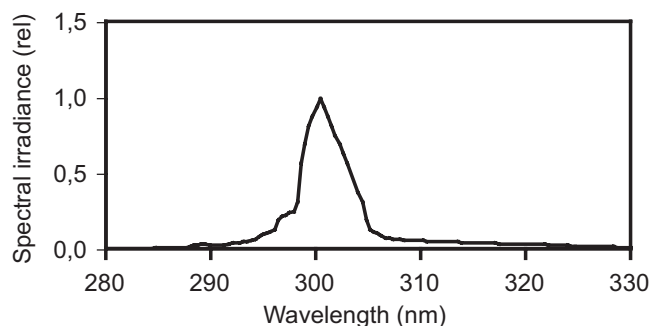


FIGURE 1. Spectral radiance of UVR at the cornea.

UVR was generated with a high-pressure mercury lamp. The radiation was collimated, passed through a water filter and an interference filter ($\lambda_{\text{max}} = 300 \text{ nm}$, $\lambda_{0.5} = 10 \text{ nm}$), and finally projected on the cornea of the exposed eye.¹⁶ The spectral radiance (Fig. 1) of UVR was recorded with a spectrometer (PC 2000; Ocean Optics, Dunedin, FL), and the total UVR dose at the corneal plane was measured with a thermopile (7104; Oriel, Irvine, CA) that had been calibrated to a National Institute of Standard and Technology (NIST) traceable standard.

Recording of Macroscopic Structure

One week after exposure to UVR, the rat was killed with carbon dioxide (CO_2) overdose, followed by cervical dislocation and enucleation of both eyes. From each eye, the lens was extracted and placed in balanced salt solution (BSS). Vestiges of the ciliary body were removed from the lens equator under a microscope. Photographs were taken of each lens against a dark background with a white grid. During photography, the anterior surface of the lens faced the camera, and the intensity of forward light scattering of each lens in BSS was measured.

Light-Scattering Measurement

The intensity of forward light scattering was measured with a light dissemination meter,¹⁷ which uses the principle of dark-field illumination. The illuminating light transilluminates the object measured at a 45° angle against the horizontal plane. At this angle, light cannot enter the objective aperture. If the object scatters in the forward direction, a defined fraction of light reaches the objective and is measured by a photodiode.

The opacity standard was a lipid emulsion of diazepam (Diazemuls; Kabi Vitrum, Stockholm, Sweden) and the unit was expressed as transformed equivalent diazepam concentration (μEDC).¹⁷ A typical value for a normal rat lens is approximately $0.1 \mu\text{EDC}$, and for a very

opaque lens it is approximately $1 \mu\text{EDC}$. Between 0 and $1 \mu\text{EDC}$, the intensity of forward light scattering increases approximately linearly with the concentration of the light-scattering solution in the measurement cuvette.

Immunohistochemical Detection of p53 Protein and Caspase 3

The lens was dehydrated through a series of ethanol steps (95%; 90%; 70%) finishing with xylene and finally paraffin embedded at 60°C . The block was cut into $4 \mu\text{m}$ sections with a microtome. The section was floated on distilled water in a bath at room temperature and then heat-stretched on distilled water at 50°C in another bath. Finally, the section was mounted on a positively charged slide (Superfrost plus, Menzel-Gläser, Braunschweig, Germany).

Before analysis, the section was heated in an oven at 60°C for 30 minutes and then cooled to room temperature. Subsequently, paraffin was removed in xylene, followed by rehydration using a passage through steps of increasing concentrations of ethanol (95%, 90%, and 70% ethanol), and was put into phosphate-buffered saline (PBS), pH adjusted to 7.4.

Slides used for p53 detection were placed in 10 mM citrate buffer at pH 6.0 and allowed to boil for 15 minutes in a microwave oven at 650 W. The slides used for caspase 3 detection were placed in TE buffer, pH 9.0 (10 mM Tris base, 0.5 mM EDTA) for 30 minutes in a microwave oven at 650 W. All the slides were cooled to room temperature and subsequently were placed in 0.1 M PBS. Tissues were blocked with a blocking buffer (3% horse serum; 2.5% fat-free milk; PBS) and were incubated for 1 hour at room temperature. Primary mouse monoclonal p53 (FITC) antibodies (mouse monoclonal [B20.1 (BP 53.122)] to p53 protein; Abcam Ltd., Cambridge, UK) were diluted to $5 \mu\text{g}/\text{mL}$ with blocking buffer (3% horse serum; 2.5% fat-free milk; PBS) and primary rabbit polyclonal caspase 3 (rabbit polyclonal [ab2302 to 50] to caspase 3 protein; Abcam Ltd., Cambridge, UK) were diluted to $20 \mu\text{g}/\text{mL}$ with blocking buffer (3% horse serum; 2.5% fat-free milk; PBS). The slides were incubated for 1 hour at room temperature and thereafter were washed in PBS. Sections incubated with primary rabbit polyclonal caspase 3 were thereafter incubated with anti rabbit secondary antibodies for 1 hour at room temperature, and the sections were treated with diaminobenzidine (DAB). Finally, the sections were mounted with mounting medium containing DAPI (Vectashield; Vector Laboratories, CA). All control sections were processed in the absence of primary antibody. The slides were washed, mounted, and photographed within a few hours under a fluorescence digital microscope camera (BX 51, U-TV0.5XC-2 microscope; Olympus, Tokyo, Japan; DC 200, CH-9435 camera; Leica, Heerbrugg, Germany).

Exposed to UVR Non-exposed to UVR

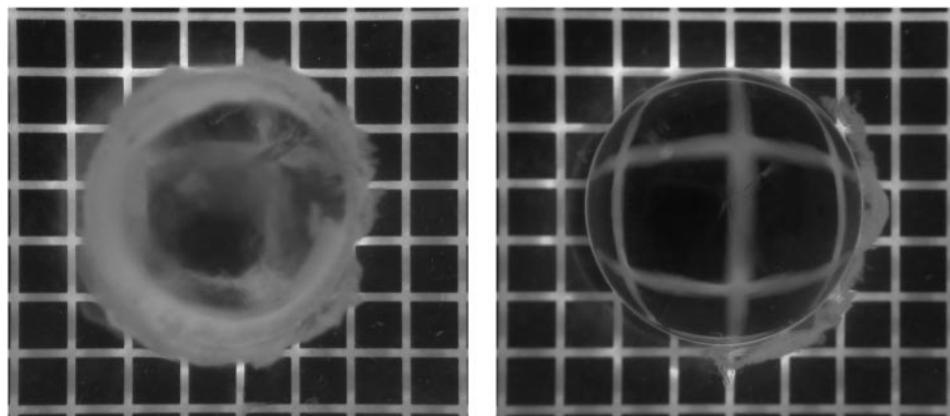


FIGURE 2. Macroscopic appearance of UVR 300 nm exposed lenses and contralateral nonexposed lenses.

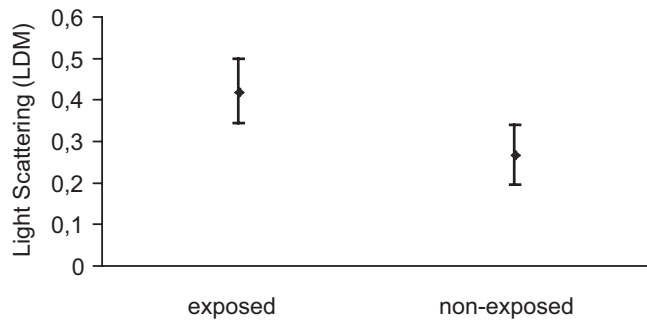


FIGURE 3. Intensity of forward light scattering in in vivo UVR 300 nm exposed lenses and their contralateral nonexposed lenses. The bars show the 95% confidence interval for the mean.

Real-Time RT-PCR of p53 and Caspase 3

RNA was isolated with extraction reagent (TRIzol; Invitrogen, Sweden), according to manufacturers guidelines and 1 μ g of total RNA was reverse-transcribed with a first-strand synthesis system for PCR. Bioanalyzer (Agilent, Palo Alto, CA) was used for quality and concentration determination of RNA samples. An oligo-peptide (dT)₁₅ and AMV reverse transcriptase (Roche, Basel, Switzerland) were used for first-strand synthesis of complementary DNA (cDNA). The cDNA made from lens RNA was amplified by real-time PCR (Light-Cycler; Roche) by using p53-primers (RN00755717m1) and caspase 3-primers (RN00580568-g1) and as reference gene, rRNA 18S was used (Hs99999901-s1) all primers were applied (Assays-on-Demand; Applied Biosystems, Foster City, CA). The mathematical model used for calculation of differentiated mRNA expression is based on PCR efficiencies and the mean crossing point. Finally, p53 mRNA and caspase 3 expression ratios were compared between in vivo UVR-exposed and contralateral nonexposed lenses.

Experimental Design

Altogether, 10 rats were exposed unilaterally to the same dose of UVR. The intensity of forward light scattering was measured three times in both lenses from each animal. Thereafter, the lens was photographed for macroscopic anatomy. Then the lenses from four randomly chosen rats were processed for immunohistochemistry. From each lens, four sections were stained and observed. The lenses from the remaining six rats were processed for quantitative measurement of specific p53 and caspase-3 expression by transcription to cDNA and PCR amplification. For the measurement, the transcription to cDNA and the PCR amplification was repeated 2 times per lens.

RESULTS

Macroscopic Appearance

Photographs of UVR-exposed and nonexposed lenses are shown in Figure 2. Nuclear cataract and vacuoles developed in UVR-exposed lenses. No cataract was found in the contralateral

Real time RT-PCR p53

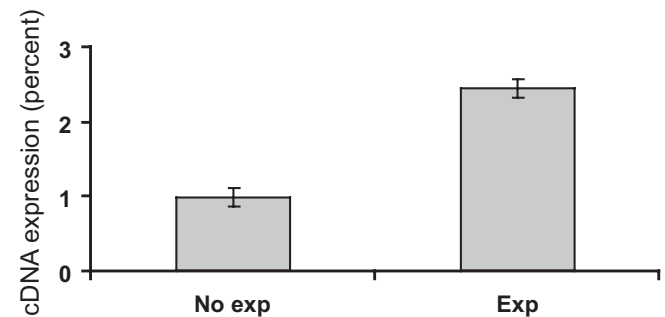


FIGURE 5. p53 mRNA expression in UVR 300 nm exposed lenses and their contralateral nonexposed lenses.

nonexposed lenses. None of the groups showed dense nuclear cataracts.

Intensity of Forward Light Scattering

Forward light scattering was significantly higher in lenses exposed to UVR 300 nm than in contralateral nonexposed lenses, as tested with a paired *t*-test: test statistic = 16.27, $t_{0.05(2)} \cdot 9 = 1.833$ (Fig. 3).

Localization of p53 Protein

Immunolocalization of the p53 protein using monoclonal antibody against p53 is shown in Figure 4. Lens epithelial cells exhibited staining with the monoclonal antibody used. Slightly increased staining was found in UVR-exposed lenses than in the contralateral nonexposed lenses.

p53 mRNA Expression

p53 mRNA expression in lenses exposed to UVR was 147% higher in UVR 300 nm exposed lenses than in contralateral nonexposed lenses (Fig. 5). The result was significant, as tested with a paired *t*-test: test statistic = 3.526, $t_{0.05(2)} \cdot 5 = 2.015$.

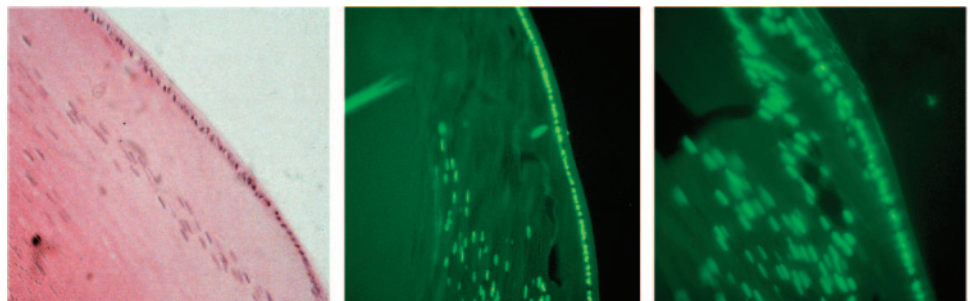
Localization of Active Caspase-3

Immunolocalization of active caspase-3 using antibodies against caspase-3 is shown in Figure 6. Positive staining is shown as brown (arrows). In the UVR-exposed lenses, the lens epithelial cells exhibited staining with the antibodies used. Immunostaining was high in the central part of the epithelium but decreased toward the equator. No staining of the epithelial cells was found in the UVR-nonexposed lenses (control lenses).

Caspase-3 Expression

Caspase-3 expression was 294% higher in UVR 300 nm exposed lenses than in contralateral nonexposed lenses (Fig. 7).

FIGURE 4. Immunolocalization of p53 protein in albino rat lens. *Left:* normal lens stained with hematoxylin-eosin to illustrate the normal anatomy of the lens. *Middle:* immunolocalization of p53 with monoclonal (FITC) antibodies against wild-type p53 in the UVR-exposed lenses. *Right:* immunolocalization of p53 with monoclonal (FITC) antibodies against wild-type p53 in the nonexposed lenses (control lenses).



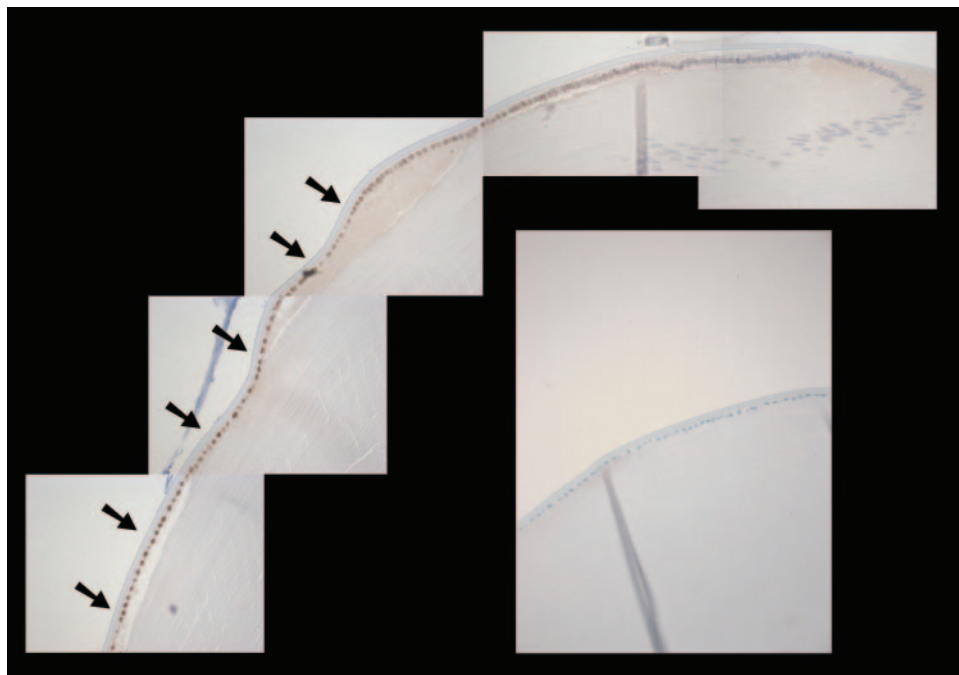


FIGURE 6. Immunolocalization of caspase-3 in the lens. *Left:* UVR exposed lens stained with antibodies against active caspase-3. Positive cells stained brown (arrows). *Right:* UVR nonexposed lens (control lens).

The result was significant as tested with a paired *t*-test: test statistic = 6.526, $t_{0.05(2)} \cdot s = 2.015$.

DISCUSSION

The aim of the present study was to demonstrate the localization of p53 and caspase-3 and of p53 and caspase-3 mRNA expression in rat lenses 1 week after in vivo exposure to UVR 300 nm.

The albino rat model was selected because the in vivo response to UVR 300 nm is well characterized for this model.^{18–21} The dose was selected to be just above threshold.²² Time to analysis after UVR exposure of the lens was set to 1 week. This time point was chosen because lens opacification in 6-week-old albino rats is maximal 1 week after UVR exposure.¹⁹

The macroscopic appearance of the UVR 300 nm exposed lenses in the present study was consistent with previous observations.²¹ The recorded difference of intensity of forward light scattering between exposed and contralateral nonexposed lenses was also consistent with previous observations.^{19,21}

The p53 protein is a nuclear transcription factor that plays a role in apoptosis and cell proliferation. An inactive isoform of p53 protein found in the cytoplasm has been reported.²³ In the present study, we found p53 protein predominantly located in the cell nucleus in the lens epithelium. The finding agrees with previous results described by Pokroy et al.¹⁰ Nuclear and cytoplasmic localization of p53 protein may represent active or inactive p53 protein. High cellular turnover predisposes for cellular nucleus localization of p53 protein. In the present study, most of the p53 antigenicity found was localized in the cell nucleus. The finding is consistent with the fact that the rats used in the experiment were young (6 weeks old) and were expected to express high cellular turnover.

p53 Immunostaining was slightly higher in the UVR-exposed lenses than in the nonexposed lenses. However, p53-positive immunostaining was found even in the nonexposed lenses. This finding is consistent with that of Pokroy et al.,¹⁰ which described p53 activity in nonexposed normal epithelial cells in the murine lens. We tried p53 immunostaining with several concentrations of antibodies and found the same results, a correlation in staining between exposed and nonexposed lenses and slightly increased staining in the UVR-exposed lenses compared with the nonexposed lenses. Immunohistochemistry was not sensitive enough to detect a difference between exposed and nonexposed lenses. Results were confirmed by quantifying p53 expression through real-time RT-PCR.

Caspase-3 staining was found predominantly in the central part of the epithelium of the lens and decreased toward the nuclear bow, possibly because the rats were young and the apoptotic cells from the nuclear bow had migrated to the lens nucleus. The central part of the epithelium consisted of quiescent cells, and the apoptotic cells remained there and were detected. The UVR radiation source was placed in front of the eye. It is possible that the central part of the lens received a higher UVR dose than the equator. Increased UVR dose may explain the increased apoptosis found in the central part of the lens. Another possible explanation to this finding may be iris protection against UVR exposure in the nuclear bow, though

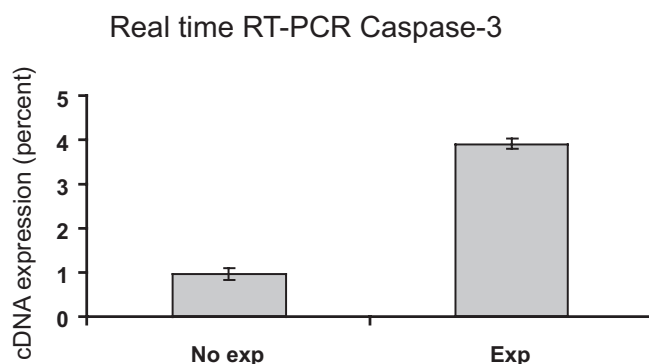


FIGURE 7. Caspase-3 expression in in vivo UVR 300 nm exposed lenses and their contralateral nonexposed lenses.

the rats used were albino and had no iris pigmentation, and their pupils were dilated before exposure.

The current finding that p53 and caspase-3 mRNA expression in the UVR-exposed lenses was significantly higher than in the contralateral nonexposed lenses is consistent with previous observations that in vivo UVR 300 nm exposure to the lens induces apoptosis.¹³ Apoptosis may or may not be associated with the p53 protein. Apoptosis in the lens after UVR exposure seems to be mediated through increased expression of p53 protein. Takamura et al.¹⁴ report that apoptosis occurs in the lens epithelium in the sugar-induced cataract model, and they postulated that apoptosis is mediated by the p53 protein. However, Takamura et al.¹⁴ used a qualitative method to determine apoptosis. Further studies to corroborate the relationship between p53 mRNA expression and apoptosis in the lens are required.

Antioxidant nutrients have been found to enhance the expression of the wild-type p53 gene, considered to be a cancer-suppressor gene,²⁴ and to diminish the expression of the mutant p53 gene, an oncogene expressed in a large number of tumors.²⁵ It appears that the antioxidant nutrients may inhibit, prevent, or regress experimental cancer through control of the p53 gene. The wild-type cancer-suppressor p53 gene is stimulated and enhanced in expression by antioxidant nutrients, whereas antioxidant nutrients diminish mutant p53 gene expression. Schwartz et al.²⁶ have shown that vitamin E significantly inhibits the development of squamous cell cancer of hamsters and that the inhibition is mediated by p53 protein. We have demonstrated that vitamin E protects the lens against cataract induced by in vivo exposure to UVR 300 nm.²⁷ We speculated that the protection mechanism was attributed to the antioxidant properties of vitamin E. Further investigations are needed to study the role of the p53 protein in vitamin E cataract protection.

The involvement of p53 protein in pathologic reactions is well established. Less is known about its function in normal tissues. To clearly understand how p53 protein functions in the regulation of the cell cycle, it is important to identify all its possible functions and all p53 interacting proteins. The present study investigated p53 expression and, thus, a possible function of p53 protein in lenses exposed in vivo to UVR 300 nm and the corresponding UVR-nonexposed contralateral controls. p53 mRNA expression increases in lenses exposed in vivo to UVR 300 nm compared with contralateral controls. Further investigations regarding p53 regulation, the role of p53 in normal cell physiology, and the response of p53 to UVR exposure are necessary.

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