Involvement of Interleukin-1β–Converting Enzyme in Apoptosis of Irradiated Retinoblastomas

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Purpose. To investigate whether interleukin-1β-converting enzyme (ICE), a mammalian homologue of the *Caenorhabditis elegans* cell death gene *ced-3*, is involved in γ-irradiation-induced apoptosis (programmed cell death) of human retinoblastoma cells.

Methods. The induction of apoptotic cell death in human retinoblastoma cell lines WERI-Rb-1 and Y79 by γ-irradiation was determined with a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide colorimetric assay and the DNA-binding fluorochrome bis (benzimide) trihydro-chloride (Hoechst 33258) staining. The change of ICE protein level in tumor cells during apoptosis was determined by immunoblotting assay. Whether the specific tetrapeptide ICE inhibitor Ac-YVAD-CMK affected γ-irradiation-induced apoptosis in tumor cells was also examined. The effect of ICE overexpression on tumor cells was evaluated by a transient transfection assay using ICE expression vector.

Results. γ-Irradiation inhibited the cell viability of WERI-Rb-1 and Y79 cells in a dose-dependent manner and induced apoptosis. The protein level of ICE was remarkably enhanced after the treatment. The apoptotic cell death induced by γ-irradiation was suppressed by the tetrapeptide ICE inhibitor Ac-YVAD-CMK. Moreover, overexpression of ICE induced apoptosis in tumor cells.

Conclusions. These findings suggest that ICE may play an important role in γ-irradiation-induced apoptosis in retinoblastoma cells. Transfer of the ICE gene induces apoptosis in these cells without γ-irradiation. (*Invest Ophthalmol Vis Sci.* 1998;39:2769-2774)

Retinoblastoma is the most common intraocular malignancy of childhood, thought to arise from primitive neuroectodermal cells.[1] Surgical enucleation and γ-irradiation are the two standard modalities of treatment most frequently used.[2-4] However, the precise mechanisms of tumor cell death after γ-irradiation remain unknown.

In many tumor cells, apoptosis (programmed cell death) is induced after γ-irradiation or the application of DNA-damaging drugs.[5-7] Apoptosis is a genetically encoded cell death program defined by characteristic changes in morphology and biochemistry[8,9] and is a pathway that may be disrupted in tumor cells, conferring a survival advantage.[9] In some cell lines, apoptosis is mediated by tumor suppressor p53 gene.[10] On the other hand, there are some cell lines in which apoptosis is observed even in the presence of mutant p53.[11] However, the molecular mechanisms regulating apoptosis are unknown.

Previously, we have demonstrated that interleukin-1β-converting enzyme (ICE) may mediate cisplatin-induced apoptosis in malignant glioma cells regardless of p53 status.[12] ICE gene, a mammalian homologue of the *Caenorhabditis elegans* cell death gene *ced-3*,[13] has been identified as the regulator of apoptosis in several cells.[14,15] Therefore, we wished to determine whether ICE is involved in the cell death of human retinoblastoma cell lines WERI-Rb-1 and Y79 after γ-irradiation. Here we report that γ-irradiation increased the expression of ICE protein and induced apoptosis in tumor cells. We also demonstrate that this apoptosis was suppressed by the tetrapeptide ICE inhibitor Ac-YVAD-CMK. Moreover, overexpression of ICE induced apoptosis in tumor cells without γ-irradiation. We suggest that ICE may play a key role in γ-irradiation-induced apoptosis.

Materials and Methods

Cell Culture

Human retinoblastoma WERI-Rb-1 and Y79 cells were used in this study. Tumor cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO-BRL), 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin as described previously.[16]

γ-Irradiation and Cell Viability Assay

The cytotoxic effects of γ-irradiation on WERI-Rb-1 and Y79 cells were quantified using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT; Boehringer-Mannheim Biochemicals, Indianapolis, IN) colorimetric assay as described previously.[12,16] Briefly, tumor cells were seeded at 1×10^5 cells/well (0.1 ml) in 96-well flat-bottomed plates (Corning, NY) and incubated overnight at 37°C. Then, cells were irradiated using a ^{137}Cesium source as described previously.[16] The ^{137}Cesium γ-irradiation source was applied for dosages of 2.5 Gy to 10.0 Gy. The similarly positioned samples rotated within the source chamber. The irradiation dosage was calculated with consideration to the decay factor of the source. After incubation for 3 days, MTT assay was performed. The statistical significance of findings was assessed using the unpaired Student's *t*-test.

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Apoptotic Features by γ-Irradiation

To determine whether irradiated WERI-Rb-1 and Y79 cells displayed an apoptotic morphology, cells were stained with the DNA-binding fluorochrome bis (benzimide) trihydro-chloride (Hoechst 33258) as described previously.[12] Briefly, harvested tumor cells (1×10^5) were suspended for fixation in 200 μl 1.0% formaldehyde and 0.2% glutaraldehyde and then incubated for 5 minutes at room temperature. The fixative was then removed, the cells were washed with phosphate-buffered saline (PBS, pH 7.4; GIBCO-BRL), resuspended in 20 μl PBS containing 8 μg/ml of Hoechst 33258, and incubated at room temperature for 15 minutes. Aliquots of 10 μl were then placed on glass slides coated with 3-aminopropyltriethoxysilane. Two hundred cells were counted and scored for the incidence of apoptotic chromatin changes under fluorescence microscopy. The statistical significance of findings was assessed using the unpaired Student's *t*-test.

Immunoblotting Assay

Irradiated WERI-Rb-1 and Y79 cells were rinsed three times with PBS, pelletized at 3000*g* for 5 minutes, and lysed in 500 μl freshly prepared extraction buffer (10 mM Tris-HCl, pH 7, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 5 mM dithiothreitol) for 20 minutes on ice as described previously.[12] The extracts were cleared by centrifugation for 30 minutes at 10,000*g*. Equal amounts of protein estimated by the Bio-Rad Protein Assay (Richmond, CA) were separated by electrophoresis on a 10% polyacrylamide gel in sodium dodecyl sulfate and thereafter subjected to electrotransfer to nitrocellulose that was saturated with PBS (pH 7.4), supplemented with 2.5% skimmed milk powder and 0.1% Tween-20 (PMT) buffer for 1 hour at room temperature. The antibodies to ICE (Santa Cruz Technologies, Santa Cruz, CA) and actin (Boehringer-Mannheim) were incubated for 1 hour at room temperature with the nitrocellulose membranes, respectively. After being washed in PMT, the membrane was incubated with anti-IgG-horseradish peroxidase conjugate (1:1500 dilution) for 1 hour at room temperature. The membrane was incubated with the Enhanced Chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) for 1 minute and exposed to a Hyperfilm-ECL for 1 to 10 minutes. The intensity of each band was quantitated by a densitometer.

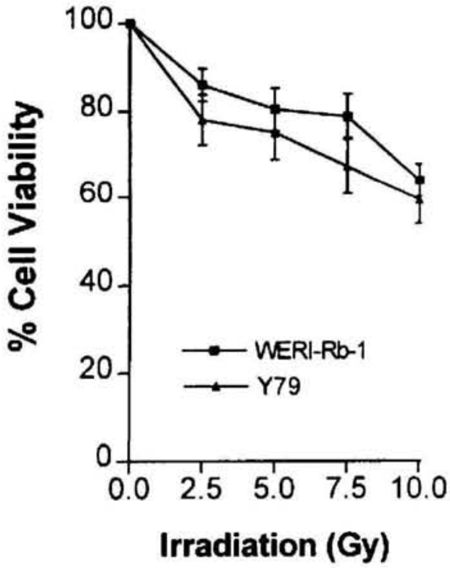
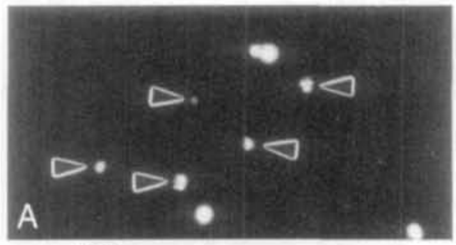


FIGURE 1. Effects of γ-irradiation on cell viability in WERI-Rb-1 or Y79 cells. Three days after γ-irradiation, viability was determined by a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay. The viability of the untreated cells was regarded as 100%. Values represent the mean ± SD of results from four independent experiments.



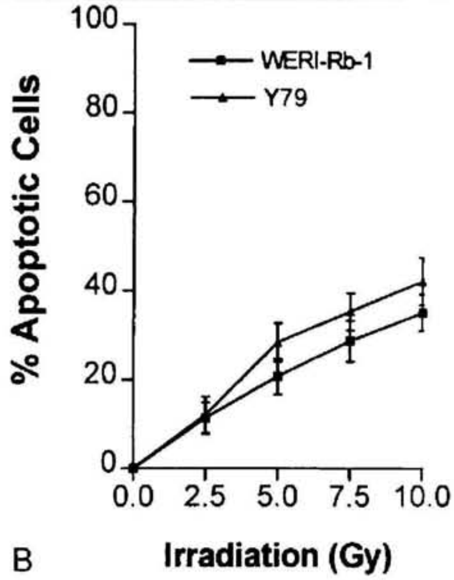


FIGURE 2. Apoptotic cell death in retinoblastoma cells 3 days after γ-irradiation. (A) Morphologic changes associated with apoptosis in WERI-Rb-1 cells 3 days after 10.0-Gy irradiation. Nuclei of the cells were stained with Hoechst 33258 (magnification, ×200). *Arrowheads* indicate apoptosis. (B) The percentage of tumor cells exhibiting morphologic changes of apoptosis. A total of 200 cells were counted for each dose point. Values represent the means ± SD of results from four independent experiments.

ICE Inhibition Assay

We determined whether a specific tetrapeptide ICE inhibitor, Ac-YVAD-CMK (BACHEM, Torrance, CA), affected γ-irradiation-induced apoptosis in retinoblastoma cells. This inhibitor was added to culture media 12 hours before γ-irradiation. Then, MTT assay was performed. The statistical significance of findings was assessed using the unpaired Student's *t*-test.

ICE Transfection

To determine whether overexpression of ICE induced apoptosis in retinoblastoma cells, the ICE-lac Z fusion gene (pβactM10Z containing the intact murine ICE cDNA fused to the *Escherichia coli* lacZ gene) was used.[14] The day before ICE

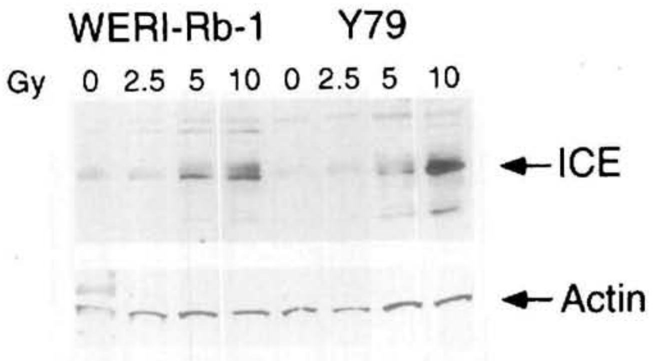


FIGURE 3. Expression of interleukin-1β-converting enzyme (ICE) protein in WERI-Rb-1 and Y79 cells treated with 0-, 2.5-, 5.0-, and 10.0-Gy irradiation. Cell samples were taken 3 days after the treatment. Immunoblotting assay using anti-ICE protein polyclonal antibody was performed with equal amounts of proteins. The anti-actin monoclonal antibody was used for protein-loading equivalence. Data shown are representative of three independent experiments.

transfection, tumor cells were seeded at 5×10^5 cells/ml in each of six-well dishes. For each well, 5 μg of ICE-lacZ or the control gene (pactβgal') construct was transfected into tumor cells by lipofectamine-mediated gene transfer (GIBCO-BRL) as described previously.[12] The cells were incubated for 5 hours in OPTI-MEM medium (GIBCO-BRL) containing each plasmid, then an equal volume of culture medium containing 20% fetal calf serum was added without removing the transfer mixture. To detect the expression of chimeric gene in transfected cells, 24 hours later cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 minutes, rinsed three times with PBS, and stained in X-Gal buffer (0.4 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 4 mM $K_3Fe[CN]_6$, 4 mM $K_4Fe[CN]_6$-$3H_2O$, and 2 mM $MgCl_2$ in 0.1 M sodium phosphate buffer [pH 7.5]) at 37°C for 5 hours. Tumor cells were then stained with Hoechst 33258 (8 μg/ml) as described above to detect apoptotic morphology. Although the transfection efficiency was low (10%-12%), it was possible to determine the representative sample size from which 100 cells were counted for each treatment group.

RESULTS AND DISCUSSION

Cytotoxic Effects of γ-Irradiation on Retinoblastoma Cells

To determine the effects of γ-irradiation on WERI-Rb-1 and Y79 cells, cell viability was measured in treated and untreated cells using the MTT colorimetric assay. Three days after a single exposure to various doses of γ-irradiation, the viability of tumor cells decreased in a dose-dependent manner (Fig. 1). The viabilities of WERI-Rb-1 and Y79 cells were 64% and 60%, respectively, 3 days after 10.0-Gy irradiation.

Induction of Apoptosis by γ-Irradiation

Characteristic features of apoptotic cells include DNA fragmentation and condensed or fragmented nuclei. Hoechst 33258 staining was performed on tumor cells to detect apoptotic morphology 3 days after γ-irradiation. As shown Figure 2A, a significant number of WERI-Rb-1 cells cultured 3 days after 10.0-Gy irradiation displayed apoptotic morphology, including

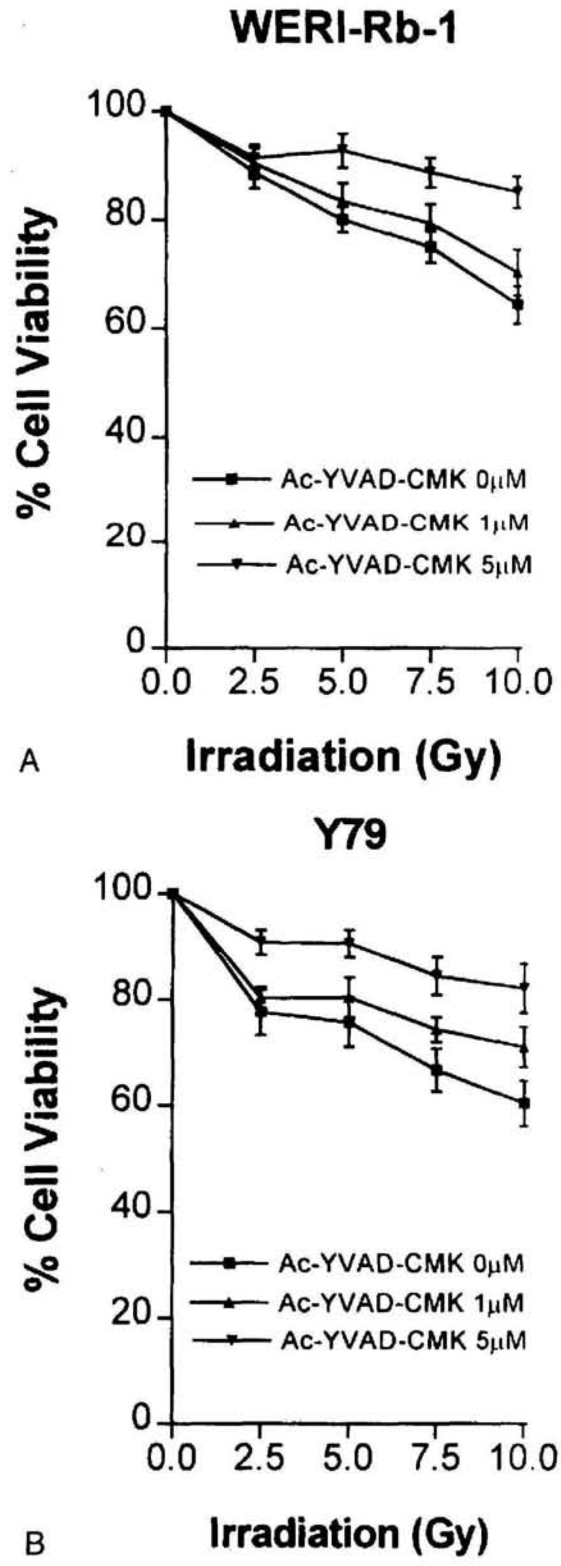


FIGURE 4. Effect of the tetrapeptide interleukin-1β-converting enzyme (ICE) inhibitor (Ac-YVAD-CMK) on γ-irradiation–induced cell death. The cell viability of WERI-Rb-1 (A) and Y79 (B) cells 3 days after γ-irradiation was determined by a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay. Ac-YVAD-CMK was added to aliquots 4 hours before γ-irradiation. Values represent the mean ± SD of results from four independent experiments.

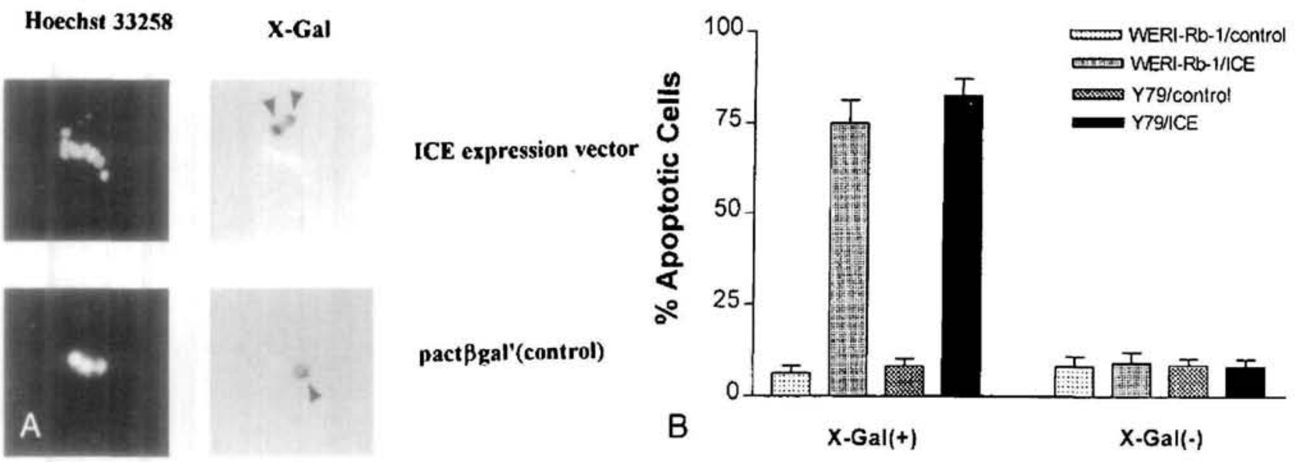


Figure 5. Induction of apoptosis in retinoblastoma cells by overexpression of interleukin-1β-converting enzyme (ICE). WERI-Rb-1 and Y79 cells were transiently transfected with the ICE-lacZ and control β-galactosidase gene and, 24 hours later, fixed, and stained with Hoechst 33258 after X-Gal (0.4 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 4 mM $K_3Fe[CN]_6$, 4 mM $K_4Fe[CN]_6$-$3H_2O$, and 2 mM $MgCl_2$ in 0.1 M sodium phosphate buffer [pH 7.5]) staining. (**A**) The staining of the nucleus with Hoechst 33258 and X-Gal staining of WERI-Rb-1 cells are shown in the *left* and *right* panels, respectively (magnification, ×200). *Arrowheads* indicate X-Gal–positive cells. (**B**) The percentage of tumor cells (X-Gal–positive or X-Gal–negative) exhibiting morphologic changes of apoptosis 1 day after transfection. A total of 100 cells were counted for each treatment group. The percentage of apoptotic cells was determined by dividing the number of apoptotic cells by the total number of X-Gal–positive cells counted. Values represent the mean ± SD of results from four independent experiments.

nuclear condensation and fragmentation. Similar staining was observed in Y79 cells (data not shown). To quantify apoptotic cells, the ratio of cells with fragmented or condensed nuclei was determined. The percentage of tumor cells that exhibit apoptotic features in WERI-Rb-1 cells was 10%, 22%, 30%, and 37% 3 days after 2.5-, 5.0-, 7.5-, and 10.0-Gy irradiation, respectively (Fig. 2B). The percentage of apoptotic cells in Y79 was 12%, 28%, 36%, and 42% 3 days after 2.5-, 5.0-, 7.5-, and 10.0-Gy irradiation, respectively. There was a dose-dependent increase in the proportion of tumor cells undergoing apoptosis in both cell lines (Fig. 2B).

Accumulation of ICE Protein by γ-Irradiation

To examine whether γ-irradiation affected the expression of ICE protein in WERI-Rb-1 and Y79 cells during apoptosis, immunoblotting assay was performed 3 days after γ-irradiation. Untreated tumor cells expressed very low levels of ICE protein (Fig. 3). After γ-irradiation, ICE expression increased in both tumor cells in a dose-dependent manner. ICE protein level became significantly higher (fivefold and eightfold, respectively) in WERI-Rb-1 and Y79 cells 3 days after 10.0-Gy γ-irradiation when compared with untreated controls. These results suggested that γ-irradiation enhanced the expression of ICE protein and induced apoptosis in WERI-Rb-1 and Y79 cells.

Effects of ICE Inhibitor Ac-YVAD-CMK on γ-Irradiation–Induced Apoptosis

From the results of immunoblotting, we further determined whether a specific inhibitor of ICE, Ac-YVAD-CMK, suppressed γ-irradiation-induced apoptosis in retinoblastoma cells. As shown in Figures 4A and 4B, the administration of 5 μM Ac-YVAD-CMK inhibited γ-irradiation-induced cell death in WERI-Rb-1 and Y79 cells ($P < 0.01$ and $P < 0.01$, respectively). These results supported the hypothesis that ICE may mediate γ-irradiation-induced apoptosis in retinoblastoma cells.

Induction of Apoptosis by ICE Overexpression

ICE expression vector was transfected into retinoblastoma cell lines to investigate whether ICE itself induced apoptosis in such cells without γ-irradiation. As shown in Figure 5A, we found that X-Gal–positive WERI-Rb-1 cells transfected with the ICE expression vector displayed apoptotic features. In contrast, WERI-Rb-1 cells transfected with the control gene retained normal nuclear morphology. When the ICE gene was transfected into WERI-Rb-1 or Y79 cells, 75% or 80%, respectively, of X-Gal–positive cells displayed a typical apoptotic morphology (Fig. 5B). Fewer than 12% of X-Gal–negative cells showed apoptosis. In contrast, when β-galactosidase expression vector (pactβgal') was transfected to WERI-Rb-1 or Y79 cells, 6% or 8%, respectively, of X-Gal–positive cells exhibited apoptosis. These results indicated that overexpression of ICE induced apoptosis in retinoblastoma cells without γ-irradiation.

This study showed that ICE was involved in γ-irradiation–induced apoptosis in WERI-Rb-1 and Y79 cells. Moreover, the transfer of the ICE gene into retinoblastomas induced apoptosis without γ-irradiation. ICE was originally described as the cysteine protease required for the cleavage of pro-interleukin-1β at Asp^{116}-Ala^{117} to generate the active cytokine.[17,18] ICE or ICE-like proteases have been thought to be Asp-specific,[19,20] and there may be a common substrate present in cells that when cleaved by Asp-specific protease can cause apoptosis.[21] Overexpression of ICE has been shown to result in apoptosis in fibroblasts[14] and in ganglion neurons.[15] Recently, we also have demonstrated that ICE induces apoptosis in malignant glioma cells[12] and aortic endothelial cells.[22] However, ICE is not the only protease in the apoptotic pathway, because thymocytes and macrophages from ICE-deficient mice undergo apoptosis normally.[21] The serine protease granzyme B also induces cytotoxic lymphocyte-induced apoptosis.[23-25] Two or more pro-

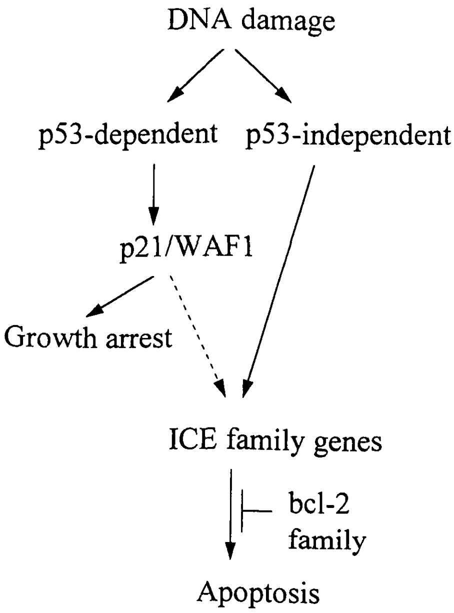


FIGURE 6. A diagram illustrating the model of apoptosis induced by DNA damage.

teases, therefore, may function redundantly in the induction of apoptosis. To date, more than 10 homologues of the ICE gene, such as Nedd-2/Ich-1_L, CPP32β, Mch2α, and Mch3α have been reported and designated the ICE family (caspase family).[26-30] Recent studies have demonstrated that γ-irradiation-induced apoptosis is associated with proteolytic activation of protein kinase C δ by an ICE-like protease.[31] Further studies, therefore, are necessary to investigate whether ICE may activate protein kinase C δ and induce apoptosis in retinoblastoma cells.

Derived from neuroectodermal cells, retinoblastoma is the most common intraocular malignancy of childhood.[1] The standard treatment modalities for the tumor include enucleation, radiotherapy, photocoagulation, cryotherapy, and chemotherapy. Retinoblastomas are very radiosensitive, but complications of γ-irradiation such as cataract, radiation-induced retinopathy, optic neuropathy, and radiation-induced tumors have been reported.[4] Because little is presently known about the mechanism of γ-irradiation therapy, understanding the molecular function of γ-irradiation-induced cell death in retinoblastomas is helpful to circumvent the complications. We have provided evidence here that ICE is closely involved in γ-irradiation-induced apoptosis in retinoblastoma cells. On the other hand, we have recently demonstrated that p53 and its associated protein WAF1/CIP1 are enhanced in retinoblastoma cells by γ-irradiation.[16] Overexpression of WAF1/CIP1 induced apoptosis in tumors without γ-irradiation. The p53 protein is elevated and can lead to apoptosis in some cells by DNA-damaging agents such as γ-irradiation.[7] The cascade of the p53 pathway leads to activation of a downstream protein, WAF1/CIP1. WAF1/CIP1 functions as an inhibitor of cyclin-dependent kinases and induces cell cycle arrest or apoptosis.[32,33] Judging from the fact that cells without the WAF1/CIP1 gene still undergo apoptosis after γ-irradiation,[34] there may be apoptosis pathways other than p53/WAF1/CIP1. Recently, we have demonstrated that ICE induces p53-dependent or p53-independent apoptosis, which is suppressed by the bcl-2 family.[12] On the other hand, p53 is shown to trigger ICE-induced apoptosis.[35] Taken together, we speculate that ICE family genes may be the final common pathway to apoptosis induced by DNA damage via the p53-dependent or p53-independent pathway (Fig. 6), although the association between p21/WAF1 and the ICE family remains to be determined. More recently, Yu et al.[36] and our group[37] demonstrated that the retroviral transfer of ICE or ICE-related genes significantly suppressed the growth of malignant glioma cells in vitro and in vivo through the induction of apoptosis. Therefore, the transfer of ICE family genes as an apoptosis-inducer may have potential as the novel treatment of human tumors such as retinoblastomas, although we have the enormous difficulty in delivering those vectors in gene therapy.

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Ascorbate in the Corneal Epithelium of Diurnal and Nocturnal Species

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Purpose. To compare the amount of ascorbic acid in the corneal epithelium of various species to unveil possible differences between diurnal and nocturnal mammals.

Methods. Ascorbic acid was determined by high-performance liquid chromatography, using an LC-10 system (Shimadzu, Kyoto, Japan).

Results. Diurnal animals show a higher ascorbate concentration in the corneal epithelium than nocturnal animals. Ascorbate concentration is higher in the corneal epithelium than in the matching aqueous humor in diurnal and nocturnal species. The highest ascorbate concentration is found in the corneal epithelium of the reindeer.

Conclusions. Ascorbate level in the corneal epithelium seems to vary in accordance with ambient radiation exposure of the respective species, just as in the aqueous humor. Both phenomena are regarded as environmental adaptations, and the ascorbic acid is suggested as protecting against photokeratitis and as acting as an ultraviolet filter for internal eye structures. (*Invest Ophthalmol Vis Sci.* 1998;39:2774-2777)

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Eye tissues are constantly exposed to light, but the capacity of several photoprotective systems present in this organ is not unlimited, because increased exposure to ambient radiation enhances age-related changes in the lens and retina.[1,2] A detailed knowledge of the different protective systems is therefore much needed.

Protection from solar radiation is given by pigmented substances such as melanin and lutein and by enzymes like superoxide dismutase. Important, too, are quenchers that deactivate intermediates from photoreactive processes. Glutathione, α-tocopherol, and ascorbate are efficient quenchers and abundant in many eye tissues.

It has long been assumed that the high ascorbate concentration in the aqueous humor of humans[3] and that the low ascorbate levels observed in the aqueous humors from cataractous eyes[4] indicate that shortage of this substance may be an important pathogenetic factor. It now seems clear that ascorbate levels are low in cataractous eyes because of an insufficient intake of ascorbate in many elderly people.[5] The aqueous level largely depends on serum values,[6] and normal aqueous concentration is restored in cataractous patients on oral ascor-