Effects of Gamma Irradiation on BCL2 and TPR53BP2 Expression in the Porcine Ciliary Body

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Abstract: Background. When dissection of porcine eyes from a living body results in the cessation of aqueous humor production and blood flow, programmed cell death regulated by TPR53BP2 and BCL2 genes may occur in the pigmented epithelium (PE) and non-pigmented epithelium (NPE) of the ciliary body. Blood products are subjected to gamma irradiation in order to prevent cellular damage resulting from transfusion-associated graft-versus-host disease. We investigated whether gamma irradiation influenced BCL2 or TPR53BP2 genes as well as the morphology of the porcine ciliary body. Methods. We irradiated the anterior segments of porcine eyes by using 60Co gamma-rays (20 Gy). To study BCL2 and TPR53BP2 expression, the irradiated specimens were fixed in formalin and embedded in paraffin and then incubated with mouse monoclonal anti-human BCL2 or TPR53BP2 antibody. Results. Following dissection, an imbalance in homeostasis began with positive BCL2 and TPR53BP2 expression in the edematous ciliary processes, and resulted in atrophy of the NPE. Increased BCL2 and TPR53BP2 expression were evident just after gamma irradiation. Decreased TPR53BP2 expression occurred after 8 h of incubation, and thereby suppressed apoptosis in the NPE; hence, the structure of the ciliary body that was incubated for 8 h after gamma irradiation was well preserved. Conclusions. Irradiation renders the ciliary body in enucleated porcine eyes less vulnerable to apoptosis, and thereby exerts a profound preservative effect.

Key words: BCL2, gamma irradiation, organ culture, porcine ciliary body, TPR53BP2

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Introduction

In the absence of compensatory changes in the rate of cell proliferation, an increase in the rate of cell death can result in cell loss [15]. BCL2 is an inner mitochondrial membrane protein that inhibits programmed cell death [9]. Coincident expression of human BCL2 protein with TPR53BP2 prolongs the survival of murine erythroleukemia cells [12].

Gamma irradiation of blood products is considered to be the mainstay of transfusion-associated graft-versus-host disease prevention because irradiated blood leukocytes show less responsiveness and are therefore less effective in graft-versus-host disease. A significantly lower expression of HLA class II antigens was observed on uveal melanomas that were irradiated before enucleation, and in contrast to non-irradiated tumors, these tumors lacked lymphocytic infiltration [10]. There is evidence of intercellular variability with regard to the production of cytokines that were modified by irradiation activity, and this might influence the repair of cellular damage in blood components after irradiation [8].

Berggren [5] observed the distention and swelling of the ciliary processes that had been removed from the eye and were maintained in a chamber containing chilled buffer with little oxygen. Warming of the bathing solution and raising the oxygen tension caused a shrinkage of the processes, presumably resulting from the restoration of the normal pumping action of the epithelium. The shrinkage was dependent on the presence of sodium and potassium in the bathing solution and was inhibited by blocking sodium potassium ATPase with ouabain. We attempted to identify which part of the ciliary body is damaged first, due to the cessation of blood flow at the initial phase of the organ culture, an experimental model for ocular hypotony and phthisis, and to determine whether gamma irradiation influences genes such as BCL2 or TPR53BP2 that are related to programmed cell death as well as the morphology of the porcine ciliary body.

Materials and Methods

In total, 8 crossbred pigs obtained from a breeding farm were used in the experiment. At the slaughterhouse, an electric shock was applied to the head using a pole to render the animal unconscious. Then, the pigs were euthanized by exsanguination from the carotid artery, and the eyes were enucleated. Specimens from 16 eyes of 8 porcines were allocated to the irradiated and non-irradiated groups, and were examined after 0, 4, 8, and 24 h of incubation for the products of the genes related to apoptosis.

Following the aseptic dissection of the anterior segments from porcine eyes, we irradiated the anterior segments consisting of the ciliary body, iris, and cornea with 60Co gamma-rays for 30 min; the total gamma-ray irradiation was 20 Gy. Following irradiation, the anterior segments were cultured in a humidified incubator at 37°C with 95% air/5% CO2, and maintained in Ham F12 medium supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY, USA) and kanamycin (41.5 units/ml).

The anterior segments were fixed in a combined fixative of 5% formalin and 2.5% glutaraldehyde after 0, 4, 8, and 24 h of incubation, embedded in paraffin, and cut into 3 µm thick sections using a microtome. After deparaffinizing the sections with xylene and ethanol, the specimens were stained with hematoxylin and eosin, dehydrated in a graded series of ethanol and xylene solutions, and mounted. For studying the BCL2 and TPR53BP2 expression, formalin-fixed and paraffin-embedded specimens were immunohistochemically stained using a streptavidin-biotin-peroxidase kit (Histofine, SAB-PO (M) kit, NICHIREI BIOSCIENCE INC., Tokyo, Japan). Excess serum was drained and the sections were incubated with a mouse monoclonal anti-human BCL2 (clone:124, Dako Co., Tokyo, Japan) or TPR53BP2 antibody (clone: DO-7, Dako, Co., Tokyo, Japan). We scanned the black-and-white photographs of the eye specimens with a scanner and chose three different points in the NPE (BCL2 gene in the cytoplasm and TPR53BP2 gene in the nucleus) using SigmaScan (Jandel Scientific Co., San Rafael, CA, USA). We used a method involving the measurement of intensity of individual pixels in the scanned image to demonstrate the changes in BCL2 and TPR53BP2 genes expression in the gray scale images. The intensity indicates how dark or light a pixel is, and the higher the gray scale level is, the brighter the pixel is. SigmaScan is designed to scan eight bit images. Each pixel is assigned a value between 0 and 255 for gray scale images. The intensity of each pixel is also reported as a
value between 0 and 255 in gray scale levels that refer to the ordered ranking of gray levels.

We used an unpaired t-test to see if the means of two different samples between the gamma-ray irradiated and non-irradiated groups were significantly different, because our samples were drawn from normally distributed populations with the same variances. The unpaired t-test is a parametric test based on estimates of the mean and standard deviation parameters of normally distributed populations.

BCL2 and TPR53BP2 genes expressions were analyzed separately, and the data were examined by analysis of variance (ANOVA; two-way ANOVA using testing data classified by two characteristics, such as time and irradiation) (Tukey test) [14]. The statistical package used in the analysis was SigmaStat (Jandel Scientific Co., San Rafael, CA, USA). A level of \( P \leq 0.05 \) was considered as statistically significant.

**Results**

**Effects of gamma irradiation and organ culture on the morphology of the ciliary body**

The ciliary body is composed of the ciliary muscle, stroma with capillary vessels, and the epithelial layer with the external and internal basement membranes (Fig. 1a). In the normal ciliary processes, these components appear to be arranged close together when viewed under high magnification (Fig. 1b). Both stroma with capillary vessels and the NPE were detached from the PE after 4 h of incubation (Fig. 2a), whereas they were still close to the PE in the ciliary processes after irradiation of gamma-rays (Fig. 2b). The ciliary processes became remarkably edematous after 8 h of incubation. A wide space was observed between the PE and stroma, and the NPE was detached from the PE (Fig. 3a). In sharp contrast, the structure of the ciliary body was well preserved on incubation for 8 h after gamma irradiation: separation of the NPE and the stroma was minimized, and the NPE remained apposed to the PE (Fig. 3b). Similarly, in the ciliary processes that were incubated for 24 h, the stroma was separated from the PE, and the structure of the NPE was distorted (Fig. 4a). However, in the ciliary processes irradiated by gamma-rays, the NPE and stroma were closer to the PE than in the non-irradiated processes (Fig. 4b).

**Effects of gamma irradiation on proteins regulating apoptosis (BCL2 and TPR53BP2) in the npe of the ciliary processes**

Gamma irradiation increased the expression of BCL2 and TPR53BP2 genes in the NPE at the start of culture (0 h), and a statistically significant difference was observed in the expression of BCL2 (\( P \leq 0.005 \)) (Table 1) and TPR53BP2 genes (\( P \leq 0.001 \)) (Table 2) between the gamma-ray irradiated and non-irradiated groups. BCL2 and TPR53BP2 gene expressions at 4 h of culture increased in comparison with those at the start of culture, but there were no significant differences between the gamma-ray irradiated and non-irradiated groups (Tables 1 and 2). At 8 h of organ culture, BCL2 gene expression remained similar to that at 4 h of culture (Table 1); however, TPR53BP2 gene expression was remarkably increased. Gamma irradiation did not alter BCL2 gene

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![Fig. 1. Light microscopic observations of the ciliary body at low magnification (a, the ciliary processes (closed arrows) 40X) and at higher magnification (b, scale bar: 100 μm). NPE (open arrows) and stroma (arrow head) are shown.](image-url)
expression whereas it significantly decreased TPR53BP2 gene expression ($P \leq 0.001$) (Table 2). At 24 h of culture, both BCL2 and TPR53BP2 gene expressions were at levels similar to those at the start of culture, and there were no differences in the BCL2 and TPR53BP2 gene products between the gamma-ray irradiated and non-irradiated groups (Tables 1 and 2).

The results of ANOVA with regard to BCL2 expression in the NPE showed a significant difference in the mean values of intensity between both the gamma-ray irradiated and non-irradiated groups ($P \leq 0.04$); a significant difference among different incubation periods was also observed ($P \leq 0.001$). However, in the case of TPR53BP2 expression, no significant difference was observed in the mean values of intensity between both the gamma-ray irradiated and non-irradiated groups, but a significant difference among the different incubation periods was observed ($P \leq 0.001$).

**Discussion**

The ciliary processes underwent remarkable edema 8 h after organ culture, and we found that cessation of blood flow due to dissection from living tissue strongly influenced the morphology of the ciliary processes, which were rich in blood supplies from the capillary vessels. In this study, both the stroma and NPE began detaching from the intact PE after incubation, but were still arranged close together in the ciliary processes that were irradiated by gamma-rays. Irradiation produced the reactive oxygen species, and we earlier concluded that L-dopa, one of the melanin precursors such as epi-
nephrine, acts as a free radical under high oxidative stress conditions, such as hyperoxia [1]. To determine the effects of L-dopa on the ciliary body, we conducted an animal experiment using the rat ciliary body [3]. The nitric oxide (NO) concentration generated in the vitreous of the melanotic rats was more than that in the amelanotic rats after L-dopa injection. Hematoxylin-eosin staining of the sections revealed vascular dilatation in the ciliary bodies after L-dopa injection and the dilation was greater in the melanotic rats than in the amelanotic rats. L-dopa, the precursor of melanin synthesis, caused vasodilatation in the vascular endothelium of the ciliary bodies of rats, and the vasodilatation was easily influenced by NO as well as by superoxides, which are regulated by presence of melanin. In this study, we found that the PE was more resistant than the NPE to an imbalance in homeostasis due to the cessation of blood flow, which resulted in the atrophy of the NPE and stroma.

In the present study, gamma irradiation first increased both BCL2 and TPR53BP2 gene expressions in the NPE of the ciliary processes immediately after irradiation. After 8 h of organ culture, the BCL2 gene expression had decreased in comparison with that after 4 h of incubation; however, the TPR53BP2 gene expression was remarkably increased to the highest level among all the time intervals examined. We observed that gamma irradiation inhibited TPR53BP2 expression in the ciliary processes after 8 h of incubation. A similar finding

Table 1. Gray scale levels of the NPE in the monochrome images of ciliary processes stained with BCL2 antibodies

<table>
<thead>
<tr>
<th>Time</th>
<th>0h</th>
<th>4h</th>
<th>8h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irradiated group</td>
<td>165.3 ± 7.5</td>
<td>147.0 ± 5.6</td>
<td>160.3 ± 9.0</td>
<td>187.3 ± 5.1</td>
</tr>
<tr>
<td>Irradiated group</td>
<td>126.7 ± 5.1</td>
<td>149.7 ± 5.5</td>
<td>159.7 ± 1.5</td>
<td>200.7 ± 8.6</td>
</tr>
<tr>
<td>p values (t-test)</td>
<td>*</td>
<td>N. S.</td>
<td>N. S.</td>
<td>N. S.</td>
</tr>
</tbody>
</table>

*: $P \leq 0.005$, N. S.; no significant differences.

Table 2. Gray scale levels of the NPE in the monochrome images of ciliary processes stained with TPR53BP2 antibodies

<table>
<thead>
<tr>
<th>Time</th>
<th>0h</th>
<th>4h</th>
<th>8h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irradiated group</td>
<td>182.7 ± 0.6</td>
<td>152.0 ± 10.8</td>
<td>102.3 ± 0.6</td>
<td>211.3 ± 4.0</td>
</tr>
<tr>
<td>Irradiated group</td>
<td>147.3 ± 3.8</td>
<td>154.3 ± 4.5</td>
<td>137.3 ± 4.2</td>
<td>215.7 ± 3.8</td>
</tr>
<tr>
<td>p values (t-test)</td>
<td>**</td>
<td>N. S.</td>
<td>**</td>
<td>N. S.</td>
</tr>
</tbody>
</table>

**: $P \leq 0.001$, N. S.; no significant differences.
was noted for BCL2, the BCL2 gene expressions becoming similar at 4 h of culture between the irradiated and non-irradiated groups, following the induction of BCL2 expression by gamma irradiation at start of culture. Results of the ANOVA of BCL2 expression in the NPE revealed a significant difference in the mean values of the intensity between the gamma-ray irradiated and non-irradiated groups. This finding together with the fact that gamma irradiation prevented detachment of the NPE and the stroma from the PE after organ culture strongly suggests that gamma irradiation inhibited apoptosis in the NPE of the ciliary body. Sentman et al. [13] reported that BCL2 may act as an antioxidant preventing cell death induced by gamma irradiation. We speculate that cessation of blood flow induced mitochondrial damage in the NPE as the first event, when edema appeared between the PE and the stroma of the ciliary process. Okisaka et al. [11] showed that active secretion of sodium and potassium primarily occurs in the NPE and that the NPE is primarily responsible for the maintenance and production of aqueous humor. Since numerous mitochondria present in the NPE serve as the major energy source for active transport, the NPE can be easily damaged. We designed the present experiment to investigate if gamma irradiation could ameliorate the damage to the NPE induced by reducing active transport due to the cessation of blood flow.

Previously, we reported that hypoxia affected phagocytosis in the bovine retinal pigment epithelial (RPE) cells and resulted in damage to mitochondria [2]. We would like to elucidate the mechanism by which gamma irradiation, which produced reactive oxygen species, influenced the BCL2 gene expression; i.e., we would like to determine antioxidants in mitochondria [13] and the NPE of the ciliary processes, and the TPR53BP2 genes related to apoptosis. BCL2 is an inner mitochondrial membrane protein [9], and a loss of mitochondria from the RPE cells cultured under the condition of hypoxia with oxygen levels as low as 1% induces malfunction of phagocytosis and a decrease in antioxidants such as glutathione-containing sulfur [2]. Choi et al. [7] stated that apoptosis of rat ovarian granulosa cells tended to increase depending on the stage of follicular development, and that among the mitochondria-dependent genes (TPR53BP2, bax, and BCL2), TPR53BP2 closely correlated with granulosa cell apoptosis during follicular development.

Borges et al. [6] reported that gamma irradiation at a small dose (2 Gy) induced apoptosis in the proliferative zone (neuroblastic layer) of the developing retina. We speculated that the dose would determine whether irradiation is one of the inducers of apoptosis. The dose of gamma irradiation (20 Gy) used in our study is considerably higher than that used by Borges et al. [6]. Anderson et al. [4] reported that high-dose irradiation (1,000 rad) prevented neurodegeneration in inherited glaucoma. Similarly, in the present study, a high-dose of gamma irradiation had an effect on programmed cell death regulated by TPR53BP2 and BCL2 genes in the porcine ciliary body dissected from a living body. Thus, it is concluded that a high dose of gamma irradiation is useful for the preservation of organs in culture, most likely through suppression of apoptotic cell death. We refer to an article on the low frequency of glaucoma among Hiroshima survivors [16]; it would be useful to reexamine this population in this regard.

Acknowledgments
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References


