Protective Effect of Prostaglandin E1 on Radiation-Induced Proliferative Inhibition and Apoptosis in Keratinocytes and Healing of Radiation-Induced Skin Injury in Rats

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Radioprotection/Prostaglandin E1/Apoptosis/Keratinocytes/Healing-impaired wound.

We examined the effects of prostaglandin E1 (PGE1) on radiation-induced proliferation inhibition and apoptosis in keratinocytes and healing of radiation-induced skin injury in a rat model. PGE1 had a protective effect on radiation-induced growth inhibition in keratinocytes in vitro, but not in fibroblasts. Varying concentrations of PGE1 were subcutaneously administered into the posterior neck region. X-irradiation at a dose of 20 Gy was administrated to the lower part of the back using a lead sheet with two holes 30 min to 1 h before or after the administration of PGE1. Although X-irradiation induced epilation, minor erosions, or skin ulcers in almost all rats, PGE1 administration prior to irradiation reduced these irradiation injuries. Staining with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling showed that proportions of apoptotic keratinocytes in the X-irradiated skin of PGE1-administered rats were significantly lower than for those in the skin of rats which did not receive PGE1. Cutaneous full-thickness defective wounds were then formed in X-irradiated areas to examine the time course of wound healing. Wound healing was significantly delayed because of X-irradiation, but PGE1 administration prior to irradiation led to a significantly shorter delay in wound healing compared with controls. Decreasing delay in wound healing was correlated with concentration of PGE1 administrated. Thus, PGE1-administration may potentially alleviate the radiation-induced skin injury.

INTRODUCTION

Currently, radiation therapy is widely used for treatment and examination. However depending on the irradiation dose used, acute injurious effects of radiation on the skin, such as erythema, epilation, desquamation, hyperpigmentation, and erosion, referred to as radiodermatitis, are common side effects.1,2) Chronic radiation-induced skin ulcers are often observed in the region of radiodermatitis.3) These are characterized by poor healing and high relapse rate and are generally intractable. Radiation can result in chromosomal aberrations and a mitotic delay in the cell cycle in vitro and in vivo4–6) and can lead to cellular apoptosis in various tissues. Therefore, an agent that can prevent or alleviate radiation damage is strongly required for safety of radiation therapy.

Prostaglandin E1 (PGE1) is from a family of naturally occurring acidic lipids with various pharmacologic effects. Vasodilation, inhibition of platelet aggregation, and stimulation of intestinal and uterine smooth muscle are among the most notable of these effects.7) In particular, intravenous administration of PGE1 lowers the blood pressure by decreasing peripheral resistance and increases the cardiac output and rate by reflex. In addition, both intravenous and subcutaneous administrations of PGE1 are effective for treating ischemic leg ulcers.7–9) PGE1 also has a wide range of cytoprotective effects by preventing apoptotic cell death and has been shown to offer protection against injuries to hepatocytes,10,11) endothelial cells,12) cardiomyocytes13) and keratinocytes.14,15)

Apoptosis is a type of physiological cell death that can occur in response to a variety of physical stresses including X- or UV-irradiation, hyperthermia,16) and agents such as glucocorticoid hormones,17) calcium ionophores,18) and environmental contaminants.19) Many types of cells, including
keratinocytes, thymocytes, splenocytes, lymphocytes, and macrophages have been observed to undergo apoptosis after exposure to clinically relevant doses of ionizing radiation.

Apoptosis can be characterized by several biochemical and morphological changes, including DNA fragmentation, impaired ATP synthesis, shrinkage of cytoplasm, condensation of nuclear chromatin, endoplasmic reticulum-derived vacuoles, and cytoplasmic “bubbling.” Condensed nucleus is fragmented into membrane-enclosed “apoptotic bodies”. The ultimate biochemical characterization is DNA fragmentation into oligonucleosomal subunits that can be recognized from random cleavage observed in cells undergoing necrosis. An immunohistochemical method for detecting apoptosis, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining has been developed for in situ apoptotic cell labeling.

To the best of our knowledge, there are no previous studies which have examined the effects of PGE1 for alleviating radiation-induced skin injury with respect to preventing cellular apoptosis. The purpose of this study was to evaluate the effects of PGE1 administration on preventing radiation-induced apoptosis of skin cells and on stimulating the healing of skin injuries. A rat model and TUNEL staining was applied to detect cellular apoptosis.

MATERIALS AND METHODS

Rats and feeding conditions
Six-week-old Fisher 344 male rats (CREA Japan Inc., Tokyo, Japan) were kept on a 12-h light–dark cycle in the center for laboratory animal science of National Defense Medical College until they were 10–14 weeks old. They were given standard Purina chow (CREA Japan Inc., Tokyo, Japan) and water ad libitum. These animal experiments were approved and conducted following the guidelines for animal experimentation of the National Defense Medical College, Tokorozawa, Saitama, Japan.

Drugs
PGE1, Alprostadil Alfadex (Ono Pharmaceutical, Osaka, Japan) was dissolved with saline to make 9 mg/ml PGE1 solution and diluted to the desired concentrations with saline.

Radiation conditions
Before irradiating a rat’s dorsal skin, its hair was shaved using electric clippers. The distance between the radiation source and a rat placed on a turntable was 35 cm. The X ray apparatus was an MBR-1505R2 (Hitachi Medical Corporation, Tokyo, Japan) and operated at 150 kV, 5 mA with 0.5 mm thick Al filtration, providing a dose-rate of 1 Gy/min. The rat skin was locally irradiated through 2 holes (diameter = 2.3 cm each) being made in 3-mm thick lead slab (Fig. 1).

A rat was anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital (50 mg/ml; Dainippon Pharmaceutical Co., Ltd. Osaka, Japan) and laid on its stomach on a turntable. A lead plate was placed so that the lower back was exposed and the head and body trunk were protected from the radiation. We prepared 3 groups of rats (6 rats/group) that received total single irradiation doses of 15, 20, and 30 Gy. The skin characteristics of each rat were observed visually at 3 days and at 1, 2, 4, and 8 weeks after irradiation for erythema, epilation, depigmentation, erosion, and ulceration.

Cell growth assays
Human dermal fibroblast cells (DFCs) and epidermal keratinocyte cells (EKCs) were purchased from Kurabo Industries Ltd. (Bio-Medical Department, Osaka, Japan). Cells used in this study were those between the fourth and eighth passages. DFCs were grown in DMEM (Life Technologies Oriental, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin). EKCs were grown in defined keratinocyte serum-free medium (KSFM) (HuMedia-KB2; Kurabo, Osaka, Japan).

For cell growth assays, both cell types were plated at an initial density of 2,000 cells/well in 96-well tissue culture plates (Sumitomo Bakelite Corp.) in culture medium for 1 day, after which PGE1 (10 μg/ml) was added. Cells were X-irradiated directly and then cultured for the indicated time periods. After incubation, 100 μl of the indicated medium that included 10 μl of WST-1 reagent (Cell counting kit, Dojindo, Kumamoto, Japan) was added to each well. Optical Density was read at 450 nm using an Immuno Mini Plate Reader after an 1 h incubation.

Immunohistochemistry
Cellular apoptosis assessed by DNA fragmentation was examined using an ApopTag® Plus Peroxidase In Situ Apoptosis Detection kit (TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, Millipore, USA). Skin sections were deparaffinized and exposed to proteinase K (20 μg/ml) for 15 min at room temperature to unmask fragmented DNA 3′-OH ends. Samples were washed with distilled H2O and incubated in 3% hydrogen peroxide for 15 min to suppress endogenous peroxidase activity. After 2 rinses in phosphate-buffered saline (PBS), sections were sequentially incubated in equilibration buffer at room temperature for 10 sec. After tapping off excess liquid from the samples, terminal deoxynucleotidyl transferase enzyme was applied and the samples were incubated at 37°C for 1 h. Stop buffer solution was applied to the samples at room temperature for 10 min to terminate the 3′-OH labeling reaction, and an anti-digoxigenin conjugate was applied at room temperature for 30 min. After washing the samples in
PBS, peroxidase substrate was added and stained for 6 min. The sections were counterstained with 0.5% methyl green to study tissue morphology. Between incubation steps, sections were rinsed in 3 changes of PBS.

Morphometric analyses

The total number of epidermal cells was counted in 4 adjacent fields: for (1) non-irradiated skin sites, (2) 20-Gy irradiated sites, (3) untreated skin, and (4) 20-Gy irradiated, PGE₁-administrated skin sites using a light microscope (40× objective and 10× photo eyepiece). Each field represented a total length of approximately 100 μm per skin sample (control or experimental). The mean numbers of total cells and apoptotic cells at each post-irradiation time were calculated by averaging the total number of cells and apoptotic cells in each of the eight skin sites harvested from each animal (N = 8). Significant differences in the mean number of apoptotic cells between control and experimental groups were determined by paired t-test.
Effect of PGE₁ in wound healing of non-irradiated and irradiated skin

Four groups of rats were prepared. A control group was administered 1 ml of saline, and 3 groups administered with 25, 50, or 100 μg of PGE₁ in 1 ml of saline solution. PGE₁ was subcutaneously injected into the posterior neck region. Each group comprised 12 rats, and these were further divided into 2 sub-groups (6 rats each). One sub-group was post-irradiated in which the rats were irradiated with 20 GY 1 h after administering either saline or PGE₁ using a lead plate with 2 holes (2.3 cm in diameter each). This was immediately followed by forming a cutaneous full-thickness defect wound, including the dermal muscle, with an 8 mm biopsy punch (Fig. 1C). The size of the wound was measured and wound healing rate was determined immediately after forming the wound and at 3, 5, 7, 10, 14, and 20 days. Healing included a combination of wound constriction and epithelialization. The other sub-group was non-irradiated in which a skin defect was formed but without irradiation, after which similar observations were made.

Another group was created: a pre-irradiation group, in which the rats were irradiated before the administration of 100 μg of PGE₁. A skin defect was formed after irradiation and similar observations were made (Fig. 1C).

Statistical analysis

Results are given as mean ± SD. Comparisons of mean number of apoptotic cells between control and experimental groups were determined by Student’s t-test. Otherwise, a Kruskal-Wallis (KW) test was used for comparisons among groups and Dunn’s test was used for multiple comparisons. Statistical analysis used StatMate III for Windows (ATMS Co., Ltd., Tokyo, Japan). P-values of < 0.05 were considered significant.

RESULT

Radiation dose and radiodermatitis

As shown in Table 1, for the irradiated groups that received more than 15 Gy, epilation and depigmentation were observed at approximately 1 week post irradiation. In the 30-Gy irradiation group, erosion and ulcers appeared at 4 weeks. Observations were made for 23 weeks; however, many rat wounds had not healed by then. In the 20-Gy irradiation group, mild erosion appeared at 4 weeks and remained partially for 8 weeks. In the 15-Gy irradiation, only mild epilation was observed without the formation of any erosion. Mild erythema was observed in the 10-Gy irradiation group on day 3 with no formation of any epilation, depigmentation, erosion, or ulceration. Although the results are not shown, with 100-μg PGE₁ pre-administration in the 20-Gy irradiation group, only mild erythema was observed on day 3.

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<th>Time</th>
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<td>3 days</td>
<td>Erythema</td>
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<td>1 week</td>
<td>Epilation, depigmentation</td>
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<td>2 weeks</td>
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<td>4 weeks</td>
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<td>8 weeks</td>
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Fig. 2. | Effects of PGE₁ on cell growth of 20-Gy irradiated DFCs (A) and EKCs (B). PGE₁ (10 μg/ml) was added to culture medium at different times: at 1 h before X-irradiation (●), 1 h after X-irradiation (●), just before X-irradiation (■), and just after X-irradiation (□). Other conditions were: no addition of PGE₁ with 20-Gy irradiated (●) and non-irradiated (○) cells. Significant radioprotection by PGE₁ for 20-Gy irradiated EKCs was observed on days 1 and 7.

<table>
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<th>Table 1.</th>
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<td>2 weeks</td>
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<td>8 weeks</td>
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Cell growth

The growth of both EKC’s and DFC’s was significantly inhibited by 20-Gy irradiation (Fig. 2). The addition of PGE₁ had little effect on stimulating growth in cells that were not X-irradiated (data not shown). Although X-irradiated DFC growth inhibition was little reversed by the addition of 10
μg/ml of PGE₁ (Fig. 2A), the 20-Gy irradiated EKC growth inhibition was significantly reversed when KSFM was supplemented with 10 μg/ml of PGE₁ at 1 h before 20-Gy irradiation (Fig. 2B). However, the irradiated EKC growth inhibition was little reversed by the addition of less than 5 μg/ml of PGE₁ (data not shown).

**TUNEL-staining of 20-Gy irradiated skin**

TUNEL staining showed a significantly large number of apoptotic DFCs in dermal tissues of non-PGE₁-administered rat skin compared with those of 100-μg PGE₁-administered group only on day 0 (5 h), while there are not significant differences in apoptotic DFCs between PGE₁-pre-administered

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**Fig. 3.** TUNEL staining results for apoptotic cells. TUNEL staining was used to evaluate the effects of 100-μg PGE₁-pre-administration to inhibit apoptosis of skin cells after 20-Gy radiation on days 0, 3, 7, 14, and 30. Although PGE₁-pre-administrated DFCs were significantly reduced on day 0 (after 5 h), significant differences between PGE₁-pre-administrated and control DFCs were not observed on days 3, 7, 14, and 30 (n = 6; 3 rats each condition). Black arrows indicate apoptotic keratinocytes and white arrows indicate apoptotic fibroblasts. Double arrows indicate thickness of the epidermis.
and non-PGE₁-administered skin on day 3, day 7, day 14, and day 30. In contrast, the proportion of apoptotic cells per total EKCs in epidermal tissue of non-PGE₁-administered group was significantly higher compared with proportions of PGE₁-pre-administered group on day 0 (about 5 h), day 3, day 7, and day 14 (Fig. 3 and 4B). Hypertrophic epidermis was observed in the skin of both PGE₁-pre-administered group and non-PGE₁-administered group on day 14. While the hypertrophic epidermis in skin of PGE₁-pre-administered group subsequently decreased in 30 days, the hypertrophic epidermis in skin increased significantly in non-PGE₁-administered group (Fig. 3 and 4C). Furthermore, TUNEL staining showed a significantly greater number of apoptotic nuclei in hair follicles of non-PGE₁-administered group compared with those of PGE₁-pre-administered group on day 30 (Fig. 4A).

**Effects of PGE₁ in wound healing of non-irradiated skin and 20-Gy irradiated skin**

When the irradiated and non-irradiated groups were com-

![Fig. 4. Effects of PGE₁-pre-administration on apoptotic nuclei in hair follicles (A), epidermis (B), and hypertrophic epidermis (C). Black arrows indicate apoptotic follicle cells and double arrow indicates thickness of the epidermis (A).](image-url)
pared in the non-PGE$_1$-administered group, significantly delayed wound healing was observed in the irradiated group. For more than half of the rats in the irradiated group, incomplete healing was observed at 2 weeks, and complete wound healing took 30 days.

Regarding differences in wound healing rate based on the dose of PGE$_1$ in non-irradiated groups (Figs. 5A and 6A), the 100-μg PGE$_1$-pre-administration group had good healing rates at 3 and 5 days compared with the non-PGE$_1$-administration group ($P < 0.05$). There were no significant differences in the other groups. However, after 7 days, both the control and the PGE$_1$-pre-administered groups had equivalent healing rates, and all of these cases were healed at 12 days.

Regarding differences in wound healing rate based on the dose of PGE$_1$ in 20-Gy irradiated groups (Figs. 5B and 6B), the 100-μg PGE$_1$-pre-administration group had good healing rates at 3, 5, and 7 days ($P < 0.05$). At 14 days, the 100-μg and 50-μg PGE$_1$-pre-administration groups had better healing rates compared with the control ($P < 0.001$). Although the 25-μg PGE$_1$-pre-administration group exhibited no difference from the control, the 100-μg and 50-μg PGE$_1$-pre-administration groups had statistically significant differences from the control on day 20 ($P < 0.05$).

Regarding differences in wound healing rate based on time of administration in the irradiated groups (Fig. 7), both
the pre- and post-administration groups had good healing rates from 3 days to 20 days compared with the control group \( (P < 0.01) \). Also, the pre-administration group had significantly higher healing rates at all time points compared with the post-administration group \( (P < 0.01) \).

The 200-μg PGE\(_1\)-pre-administration group was also observed, but wound healing tendency was almost the same as for the 100-μg PGE\(_1\)-pre-administration group. However, side effects such as diarrhea and weight loss were frequently observed in the 200-μg PGE\(_1\)-pre-administration group compared with the groups that received lower concentrations of PGE\(_1\) (data not shown).

**DISCUSSION**

Cellular apoptosis is critical in many physiological contexts, including embryogenesis, immune cell maturation and response, tissue homeostasis, and in the cellular response to injury.\(^{22}\) In pathological states, excessive apoptosis may contribute to organ and tissue injury. Thus, selective preservation of cell modulation of apoptotic processes could have an impact on the treatment of many diseases. Previous studies have indicated that apoptosis played an important role in radiation-induced injuries on epidermal keratinocytes,\(^{14,15}\) thymus lymphocytes,\(^{20}\) hepatocytes,\(^{10,11}\) and bone marrow cells.\(^{26}\) Our study showed that X-irradiation induced apoptosis of EKCs and DFCs in mouse skin \( \text{in vivo} \) and inhibited these cell proliferation \( \text{in vitro} \). Pre-treatment with PGE\(_1\) reduced EKC’s apoptosis \( \text{in vivo} \) and reversed proliferation inhibition \( \text{in vitro} \) by X-irradiation, although PGE\(_1\) did not affect these responses in DFCs.

X-irradiation induced radiodermatitis, such as erythema, epilation, depigmentation, and erosion. When cutaneous full-thickness defective wounds were formed in the irradiated areas to examine the time course of wound healing, it was significantly delayed because of skin injuries induced by irradiation. The administration of PGE\(_1\) prior to irradiation led to significantly shorter delays in wound healing compared with controls in a concentration-dependent manner. These results suggest that PGE\(_1\) is effective for alleviating radiation damage to the skin itself as well as for promoting wound healing.

Regarding the dosage of prostaglandin, Walden \textit{et al.}\(^{27}\) reported that the radioprotective effect increased in a concentration-dependent manner with PGE\(_2\). Hanson \textit{et al.}\(^{28}\) also described that the effect remained unchanged or was
slightly decreased when a certain concentration was exceeded. In our experiments, a positive correlation between concentration and efficacy in the PGE1-pre-administration groups was observed. However, in the 200-μg PGE1-pre-administration group, wound healing rates were almost the same as in the 100-μg PGE1-pre-administration group and side effects, such as diarrhea and weight loss, were frequently observed (data not shown). These results suggest that there is an optimum concentration for PGE1.

The radioprotective and anti-apoptotic mechanisms for PGE1 remain to be studied both in vitro and in vivo. This in vitro study showed that over 10 μg/ml of PGE1 was required for the reversion of the 20-Gy irradiated EKC growth inhibition. As one possibility for requirement of such high concentrations, PGE1 may play as anti-reactive oxygen species, which the inhibitory effect of PGE1 on reactive oxygen species may in turn result in anti-apoptotic activity. PGE1 has in fact been found to have an inhibitory effect on reactive oxygen species, although this was an in vitro effect. It is believed that this inhibitory effect may be because of inhibition of reactive oxygen species generated by X-irradiation, which could ameliorate cell apoptosis and skin damage.\(^{29,30}\) In addition, PGE1 actions for increasing blood flow may also positively affect wound healing.\(^{7–9}\) In either case, multiple mechanisms may contribute to its protective effects. Based on our present study, skin damage can be alleviated by administering PGE1 before irradiation; hence, radiation-induced skin injury caused by radiation therapy may potentially be alleviated by PGE1-pre-administration.

With regard to treating skin damage caused by irradiation, mild cases can be healed with ointments, but severe cases cannot be healed with conservative therapy. In those cases, some type of surgical procedure may be required. For example, when performing a skin graft, if the graft bed is irradiated, the formation of granulation and blood vessels and skin graft survival rate should be decreased. Moreover, a skin flap that is damaged by radiation may result in a poor flap survival rate, i.e., if the area surrounding a lesion is irradiated, treatment methods will encounter difficulties because of the effects of radiation. Therefore, a simple method that can reduce such damage as much as possible is required.

Prospective studies by Hanson et al.\(^{31}\) and Khan et al.\(^{32}\) described that PGE1-administration reduced radiation damage, such as oral ulcers and radiation colitis. In our experiments, both the pre- and post-PGE1-administrations promoted wound healing of irradiated skin. Furthermore, PGE1-pre-administration resulted in a better improvement in wound healing than did post-administration. Thus, PGE1-pre-administration not only promoted wound healing, but also had a radioprotective effect on the skin.

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