

## The Protective Effect of Interleukin-11 on the Cell Death Induced by X-ray Irradiation in Cultured Intestinal Epithelial Cell

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### Interleukin-11/PI3K/X-ray Irradiation/Intestinal Epithelial Cell.

Interleukin-11 (IL-11) is a well known anti-inflammatory cytokine that is associated with cell growth, and also participates in limiting X-ray irradiation induced intestinal mucosal injury. The aim of this study was to evaluate the protective effect of IL-11 on the cell injury induced by X-ray irradiation in rat intestinal epithelial IEC-18 cells. Recombinant human IL-11 (rhIL-11) treated cells were irradiated and then examined for cell viability. To evaluate irradiation injury, trypan blue staining was used to detect the dead cells. The viability of irradiated cells was up-regulated by rhIL-11 treatment and also resulted in the activation of p90 ribosomal S6 kinase (p90RSK) and S6 ribosomal protein (S6Rp). Wortmannin, a specific inhibitor of PI3K, suppressed the activation of S6Rp in rhIL-11 treated cells, and decreased the up-regulation of viability by rhIL-11 treatment in irradiated cells. The TUNEL assay was also performed to estimate the rate of apoptosis in X-ray induced cell death. There was no difference in the results between trypan blue staining and the TUNEL assay. Further, rhIL-11 down-regulated the expression of cleaved caspase-3 in irradiated cells. These results suggest that rhIL-11 may play an important role in protection from radiation injury.

### INTRODUCTION

The intestine is an important dose-limiting organ during radiation therapy of tumors in the pelvis or rectum.<sup>1,2)</sup> Acute mucosal damage has been described to include the destruction of crypt cells, a decrease in villous height, and a decrease in the number, ulceration, and necrosis of the gastrointestinal epithelium<sup>3–5)</sup> with activated inflammatory cells. This view has been supplanted by the recognition that radiation-induced changes in cellular function and alterations secondary to cell death contribute substantially to the intestinal radiation response.<sup>6,7)</sup> In contrast, delayed radiation enteropathy is a chronic condition that may develop after several years and is characterized by progressive intestinal wall fibrosis and vascular sclerosis.<sup>8)</sup> It is presumed to

be an inflammatory process involving various mediators such as eicosanoids, cytokines, and reactive oxygen metabolites.<sup>5,9–11)</sup> However, the pathogenesis of radiation enteritis remains to be determined.

Interleukin-11 (IL-11) is well known among the anti-inflammatory cytokines associated with cell growth, differentiation and death. It is also a component of the IL-6 family, which includes leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotropic factor (CNTF), and cardiotrophin-1 (CT-1).<sup>12)</sup> IL-11 was initially cloned from immortalized primate bone marrow stromal cell lines.<sup>13)</sup> Recently, it was reported that IL-11 decreased acute injury to X-ray irradiated intestinal mucosa<sup>14,15)</sup> and reduced the clinical signs and histological lesions of inflammatory bowel disease.<sup>16,17)</sup>

IL-11 has a variety of biofunctions which overlap with those of IL-6. The overlapping biological activities of these two cytokines can be explained by their use of multi component receptors, including a common transducer gp130 unit.<sup>18)</sup> IL-11-induced signaling is mediated by the formation of a hexameric receptor complex, composed of two molecules each of IL-11, IL-11 receptor (IL-11R)  $\alpha$  and gp130.<sup>19)</sup> IL-11-induced signaling leads to activation of tyrosine phosphorylation and janus kinase (JAK) of gp130. These events

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activate phosphatidylinositol 3-kinase (PI3K), mitogen activated protein kinase (MAPK) and signal transducer and activator transcription (STAT).<sup>20-23</sup> Recent studies have demonstrated that PI3K activation was directly controlled with apoptosis and the apoptotic transcription response.<sup>24-27</sup>

This study sought to establish the protective effect of IL-11 on cell death induced by X-ray irradiation in cultured intestinal epithelial cells, and to elucidate the signaling pathway regulated by IL-11 in the protection from radiation injury.

## MATERIALS AND METHODS

### *Cell culture*

The IEC-18 cell line of rat ileum epithelial cells was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained/passaged in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal calf serum (FCS) (Invitrogen), and 0.1 U/ml insulin from bovine pancreas (Sigma, St. Louis, MO, USA) at 37°C in a humidified 90% air-10% CO<sub>2</sub> atmosphere. Cells were always used below passage 15. Subconfluent cells were washed and replaced by DMEM with 0.1% FCS one day before the start of experiments.

### *X-ray irradiation*

A single dose 10 Gy of X-ray irradiation was used in all experiments. Serum starved IEC-18 cells were pretreated before X-ray irradiation for 6 hours with recombinant human (rh) IL-11 (0, 25, 50 and 100 ng/ml), which was provided by Astellas Pharma Inc. (Tokyo, Japan). The cells were irradiated with Toshiba EXS-300 (Tokyo, Japan) at a rate of 0.798 Gy/minute. After irradiation, all cells were transferred to fresh culture medium and incubated. In some experiments, the inhibitor wortmannin and PD98059 (Sigma) were added one hour before rhIL-11 treatment.

### *Trypan blue staining*

Cell viability was calculated as a ratio between viable cells and total cells. Six hours after irradiation, supernatants were removed from the incubated monolayers. The monolayers were trypsinized and combined with the supernatants. After centrifugation, the supernatants were discarded, the cell pellet was resuspended and incubated with 0.1% trypan blue solutions (Muto Chemicals Co., Ltd., Tokyo, Japan), for 2 minutes at room temperature, and from each dish samples were taken for counts in four fields of a hemocytometer. Each experiment was performed four times in duplicate.

### *TUNEL assay*

Six hours after treatment, the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed using the Apoptag peroxidase in situ apoptosis detection kit,

which was purchased from Chemicon International (Temecula, CA, USA). The cells were trypsinized and fixed in 1% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan), in PBS, pH 7.4, for 10 minutes at room temperature. Further, the cells were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme for 1 hour at 37°C, and anti-digoxigenin conjugate for 30 minutes at room temperature. The reaction sites for the TUNEL assay were visualized using Histofine Sample Stain kit (Nichirei, Tokyo, Japan). Cell nuclei were stained with 0.5% methyl green, and apoptotic and total cells were then counted under  $\times 400$  magnification. Eight representative microscopic fields were analyzed. The results were expressed as a percentage of the surviving cells.

### *Western blot analysis*

Cell lysates were prepared with lysis buffer containing 50 mM Tris-HCl (pH 7.2), 15mM NaCl, 1% Igepal CA-630, 1% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 14,000 rpm, 0°C for 30 minutes, and total protein was determined in the supernatant by the Bradford method with a protein assay reagent (Bio-Rad, Hercules, CA, USA). Samples were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Biotinylated protein molecular weight standards were applied in one lane of each gel. The nonspecific proteins were blocked with 5% non-fat milk in Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl [pH 7.6]) plus 0.1% Tween-20 for 1 hour and then incubated separately for 1 hour at room temperature or overnight at 4°C with various antibodies such as anti-phospho-p90 RSK antibody (p-p90 RSK), anti-phospho-p38 MAPK antibody (p-p38 MAPK), anti-phospho-S6 Ribosomal protein antibody (p-S6Rp) and anti-cleaved caspase-3 antibody, which were obtained from Cell Signaling Technology (Beverly, MA, USA). The membranes were washed three times with TBS plus 0.1% Tween-20 and further incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using chemiluminescence detection reagents (ECL plus, Amersham Biosciences) and exposed to Hyperfilm (Amersham Biosciences).

### *Statistical analysis*

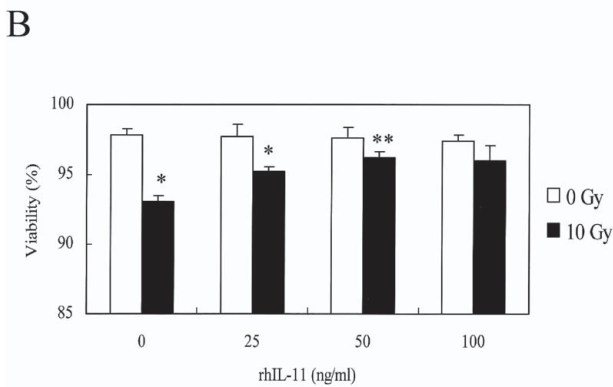
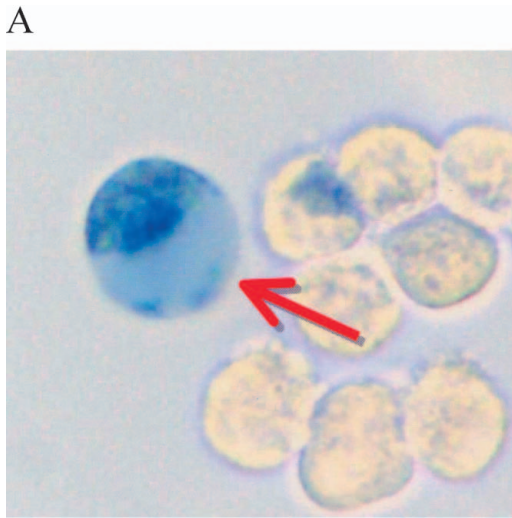
The data were expressed as mean  $\pm$  S.D. Mann-Whitney's U test was performed for statistical analysis. A *p*-value less than 0.05 was considered statistically significant.

## RESULTS

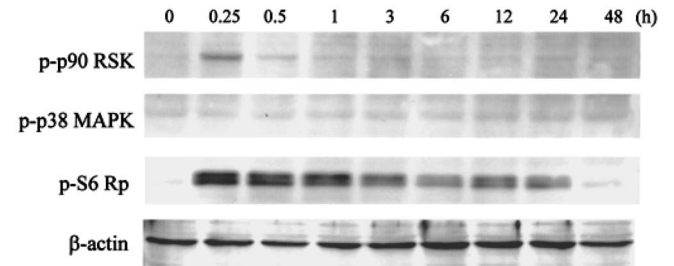
The difference in viability of rhIL-11 treated and non-treated cells was checked by trypan blue staining (Fig. 1A). RhIL-11 treated cells showed a much higher viability than

the non-treated cells, and the maximum effect of rhIL-11 was noted at 50 ng/ml (Fig.1B). Next, western blot analyses were performed to elucidate the protective activity of rhIL-

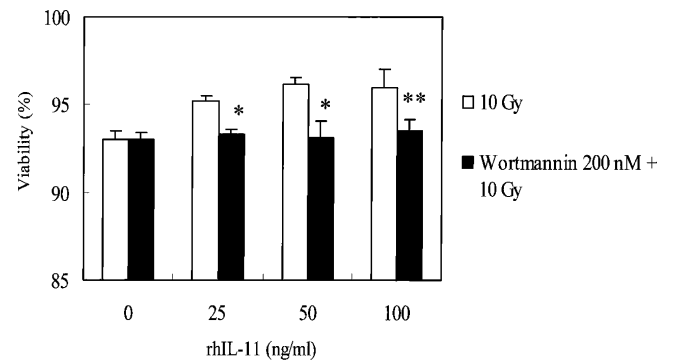
11 on IEC-18 cells. RhIL-11 induced the phosphorylation of p90 RSK and S6Rp at 15 minutes after the treatment, but failed to induce the phosphorylation of p38 MAPK (Fig. 2). Our data also showed that the activation of S6Rp was at a higher level than p-p90 RSK. These results implicated p-S6Rp and p-p90 RSK in having key roles in the up-regulation of viability in X-ray irradiated IEC-18 cells.



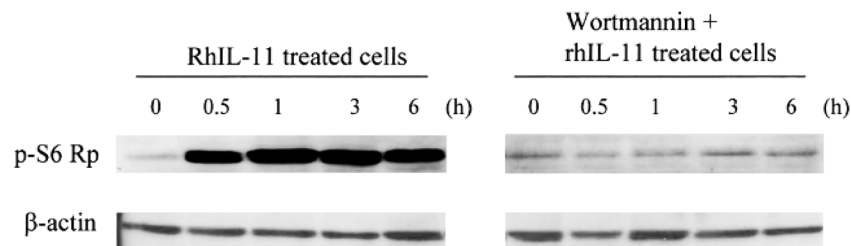
**Fig. 1.** Effect of rhIL-11 in X-ray irradiated IEC-18 cell. **A:** Cells were in serum starvation for one day and then irradiated with 10 Gy X-rays. Six hours after irradiation, trypan blue staining assays were performed. Arrow mark indicates death of cells. **B:** Cells were pre-incubated for 6 hours with rhIL-11, and then irradiated. At 6 hours after irradiation, cell viability was determined by trypan blue staining. Symbols above the bars indicate S.D. between X-ray irradiated and non-irradiated cells (n = 4, \*, p < 0.01, \*\*, p < 0.05).



**Fig. 2.** Analysis of protein tyrosine phosphorylation on rhIL-11 treated IEC-18 cell. Cells were in serum starved for 24 hours and then treated with 50 ng/ml rhIL-11 for 5 minutes. After the treatment, cells were incubated and cell lysates were collected at the indicated times. Phosphorylation of p90 RSK, p38 MAPK and S6 ribosomal protein were detected by western blot analysis.



**Fig. 3.** Effect of wortmannin on rhIL-11 treated and non-treated IEC-18 cell. Cells were preincubated for one hour with 200 nM wortmannin before rhIL-11 treatment, and then irradiated. At 6 hours after irradiation, cell viability was determined by trypan blue staining. Symbols above the bars indicate S.D. between wortmannin treated and non-treated cells (n = 4, \*, p < 0.01, \*\*, p < 0.05).



**Fig. 4.** Suppression of p-S6Rp by wortmannin on rhIL-11 treated IEC-18 cell. Western blot analyses were performed on wortmannin treated or non-treated cells. RhIL-11 activated S6Rp at 15 minutes, but this activation was suppressed by wortmannin.

Wortmannin, a specific inhibitor of PI3K, was used to confirm the effect of the PI3K signaling pathway on S6Rp activation. RhIL-11 up-regulated the viability in irradiated cells, but cells preincubated with wortmannin showed a significant suppression of the up-regulation of viability in irradiated rhIL-11 treated cells ( $n = 8$ ,  $p < 0.001$ ) (Fig. 3). These

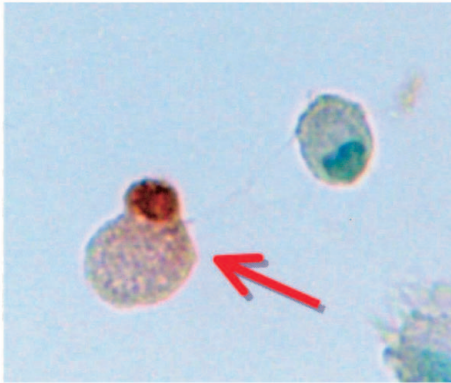
findings showed that PI3K was involved in the up-regulation of viability in rhIL-11 treated cells.

Western blot analysis was used to examine the effect of wortmannin on the induction of phosphorylation of S6Rp in rhIL-11 treated cells. The results showed that wortmannin suppressed the phosphorylation of S6Rp in rhIL-11 treated cells (Fig. 4).

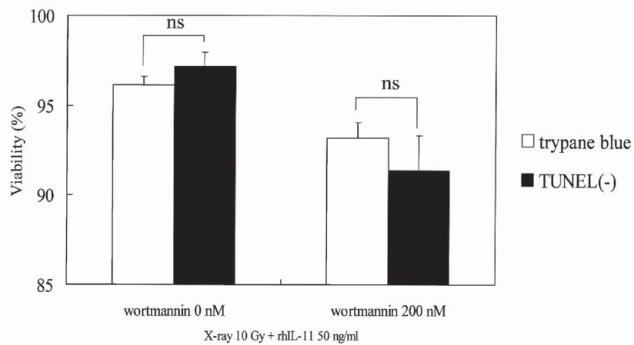
RhIL-11 also induced the phosphorylation of p90 RSK. PD98059, a specific inhibitor of MEK, was used to determine the role of p-p90 RSK in the up-regulation of cell viability by rhIL-11 treatment. There was no statistical difference in the viability between PD98059 treated and non-treated cells (data not shown).

Apoptotic cell death was examined by the TUNEL assay. Positive staining was observed in the cell nuclei (Fig. 5A). No significant difference could be demonstrated by this method for the two groups of irradiated cells that were treated and non-treated with rhIL-11 (Fig. 5B). Western blot analyses showed that rhIL-11 (50ng/ml) also suppressed the expression of cleaved caspase-3 in irradiated IEC-18 cells (Fig. 6).

A



B

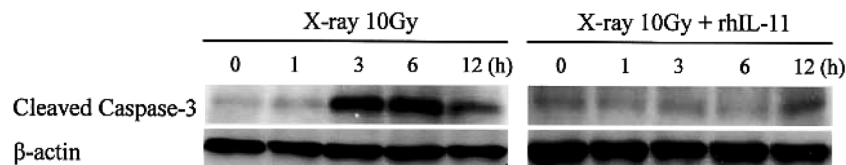


**Fig. 5.** Cell viability comparison of trypan blue staining and TUNEL assay. **A:** Cells were in serum starvation for one day and then irradiated with 10 Gy X-rays. At 6 hours after irradiation, TUNEL assays were performed. Apoptotic cell is indicated by the arrow. **B:** These two assays showed no statistical difference between them ( $n = 4$ ).

## DISCUSSION

One of the most important functions of the intestinal epithelial cell is the formation of a physiological barrier against antigens and potentially pathogenic organisms in the gut lumen.<sup>28</sup> X-rays have frequently caused an impairment of function in these cells. RhIL-11 has appeared to limit X-ray induced intestinal mucosal injury *in vivo*, which suggested its use in avoiding cell death.<sup>14,15</sup> This study showed that rhIL-11 up-regulated the cell viability of irradiated cultured intestinal epithelial cells (Fig. 1B). This suggests that IL-11 can protect intestinal epithelial cells from radiation injury.

IL-11 is known to activate PI3K, MAPK and Jak/STAT signaling pathways.<sup>20-23</sup> Also, it has already been reported that PI3K and MAPK contribute to cell survival or cell death following radiation injury in many cultured cell lines<sup>24-27</sup> except for cultures of normal intestinal epithelial cells. In this study, IEC-18, a cell line of normal intestinal epithelial cells was used to check the signaling pathways of PI3K and MAPK. RhIL-11 induced the activation of PI3K pathway in IEC-18 cells, but only slightly for the MAPK pathway. Wortmannin completely eliminated the up-regulation of cell



**Fig. 6.** Suppression of cleaved caspase-3 by rhIL-11 on X-ray irradiated IEC-18 cells. Western blot analyses were performed on rhIL-11 (50ng/ml) treated X-ray irradiated IEC-18 cells. RhIL-11 suppressed the expression of cleaved caspase-3 on X-ray irradiated IEC-18 cells.

viability and the activation of PI3K pathway induced by rhIL-11 (Fig. 3,4). These findings are consistent with the results of previous studies.<sup>20-23</sup> The activation of these kinases promotes cell survival and prevents cell death.

The method of trypan blue staining to detect dead cells<sup>29</sup> was used to evaluate the irradiation injury to IEC-18 cell. The protective effect of IL-11 against radiation injury on intestinal epithelial cells was clearly shown by using this staining method. However, trypan blue staining could not distinguish apoptotic cells from dead cells. It is well known that X-rays induce cell death due to apoptosis.<sup>30,31</sup> To evaluate the rate of apoptosis in X-ray induced cell death, the TUNEL assay was performed to detect DNA fragmentation.<sup>32,33</sup> In our data, there was no difference in the results between trypan blue staining and the TUNEL assay. This indicated that the dead cells detected by trypan blue staining consisted mainly of apoptotic cells. Further, rhIL-11 suppressed the activation of caspase-3 in irradiated IEC-18 cells. These results suggested that the apoptotic pathway plays an important role in the induction of cell death by irradiation of intestinal epithelial cells.

Akt, an up-stream protein of S6Rp, has been shown to promote cell survival via its ability to phosphorylate Bad.<sup>34,35</sup> Deng *et al*<sup>36</sup> reported that lysophosphatidic acid (LPA), a normal component of blood plasma, protects intestinal epithelial cells from radiation- and chemotherapy-induced apoptosis. They also showed that LPA prevented the activation of caspase-3 via suppression of Akt and ERK1/2 activation.<sup>37</sup> In this study, we demonstrated that rhIL-11 up-regulated the activation of S6Rp. We also showed that rhIL-11 suppressed the activation of caspase-3 by X-ray irradiation. Our data suggests a possible interaction between rhIL-11 and PI3K, although the precise relationship of these proteins remains to be clarified. To elucidate the effect of rhIL-11 on intestinal epithelial cells, further studies will be required. Our data suggest that the p-S6Rp may play a more critical role for the up-regulation of cell viability in rhIL-11 treated irradiated IEC-18 cells than the MAPK pathway.

A high dose of irradiation, 10Gy, was needed to induce apoptosis in the cultured cell line.<sup>38</sup> A dose of 2Gy or less was not enough to induce apoptosis or cell death. It is known that high dose irradiation is not used in the radiation therapy of malignancies. However, the patient of radiation accident or the survivors of atomic bomb explore or the Chernobyl accident received much high dose of irradiation. There are some reports that IL-11 promotes survival in mice or cultured cell lines.<sup>39-41</sup> Conceivably, pre-treatment with IL-11 might suppress the cell death of intestinal epithelial cells and reduce the mortality of patients exposed to high-dose irradiation.

IL-11 has exhibited a potent anti-inflammatory property in a number of animal models of gastrointestinal inflammation.<sup>16,17</sup> RhIL-11 acts to reduce inflammation through modulation of multiple proinflammatory mediators including

products of activated T cells.<sup>42</sup> Treatment with rhIL-11 down-regulated expression of proinflammatory cytokines including TNF-alpha, IL-1beta, and IFN-gamma. RhIL-11 also reduced the level of myeloperoxidase activity in the cecum indicating reduced inflammation.<sup>42</sup> After stimulation *in vitro* with anti-CD3 antibody, spleen cell cultures derived from rhIL-11-treated rats produced less IFN-gamma, TNF-alpha, and IL-2 than cultures derived from vehicle-treated rats.<sup>42</sup> Pro-inflammatory cytokines were not examined in this study. However, it is known that cell death induces inflammation in every organ and the expression of many pro-inflammatory cytokines.<sup>43,44</sup> Our results showed that rhIL-11 acts directly on intestinal epithelial cells and suppresses cell death of intestinal epithelial cells following X-ray irradiation. Thus, pre-treatment with IL-11 may be an effective strategy in preventing cell death of intestinal epithelial cells and in reducing the inflammation of the intestinal tract after radiation exposure.

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