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Single high-dose irradiation aggravates eosinophil-mediated fibrosis through IL-33 secreted from impaired vessels in the skin compared to fractionated irradiation

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ABSTRACT

We have revealed in a porcine skin injury model that eosinophil recruitment was dose-dependently enhanced by a single high-dose irradiation. In this study, we investigated the underlying mechanism of eosinophil-associated skin fibrosis and the effect of high-dose-per-fraction radiation. The dorsal skin of a mini-pig was divided into two sections containing 4-cm^2 fields that were irradiated with 30 Gy in a single fraction or 5 fractions and biopsied regularly over 14 weeks. Eosinophil-related Th2 cytokines such as interleukin (IL)-4, IL-5, and C–C motif chemokine-11 (CCL11/eotaxin) were evaluated by quantitative real-time PCR. RNA-sequencing using 30 Gy-irradiated mouse skin and functional assays in a co-culture system of THP-1 and irradiated-human umbilical vein endothelial cells (HUVECs) were performed to investigate the mechanism of eosinophil-mediated radiation fibrosis. Single high-dose-per-fraction irradiation caused pronounced eosinophil accumulation, increased profibrotic factors collagen and transforming growth factor-β, enhanced production of eosinophil-related cytokines including IL-4, IL-5, CCL11, IL-13, and IL-33, and reduced vessels compared with 5-fraction irradiation. IL-33 notably increased in pig and mouse skin vessels after single high-dose irradiation of 30 Gy, as well as in irradiated HUVECs following 12 Gy. Blocking IL-33 suppressed the migration ability of THP-1 cells and cytokine secretion in a co-culture system of THP-1 cells and irradiated HUVECs. Hence, high-dose-per-fraction irradiation appears to enhance eosinophil-mediated fibrotic responses, and IL-33 may be a key molecule operating in eosinophil-mediated fibrosis in high-dose-per fraction irradiated skin.

1. Introduction

Skin is usually the first site of entry in radiation treatment, and various degrees of skin reactions can occur. Interest in hypofractionated radiotherapy, which may potentiate normal tissue injury, has increased lately with improvements in radiation delivery technology and image guidance, and its clinical applications are increasing for treating various tumor sites including the breast. For example, recent accelerated partial breast irradiation delivers a high-dose-per-fraction to the tumor cavity and adjacent breast tissue in 1–10 fractions. Breast cancer patients' skin is often in close proximity to or even within the irradiation field, and possible complications include delayed wound healing, ulceration, necrosis, telangiectasia, fibrosis, and poor cosmetic outcome [1]. Fibrosis is a common late complication caused by ionizing radiation. However, no effective treatment exists, and the mechanisms underlying fibrosis are not clearly understood.

Irradiation can induce cytokine and chemokine production that leads to fibrosis and polarized immune responses [2,3]. Abnormal immune reactions such as inflammation and polarized immune

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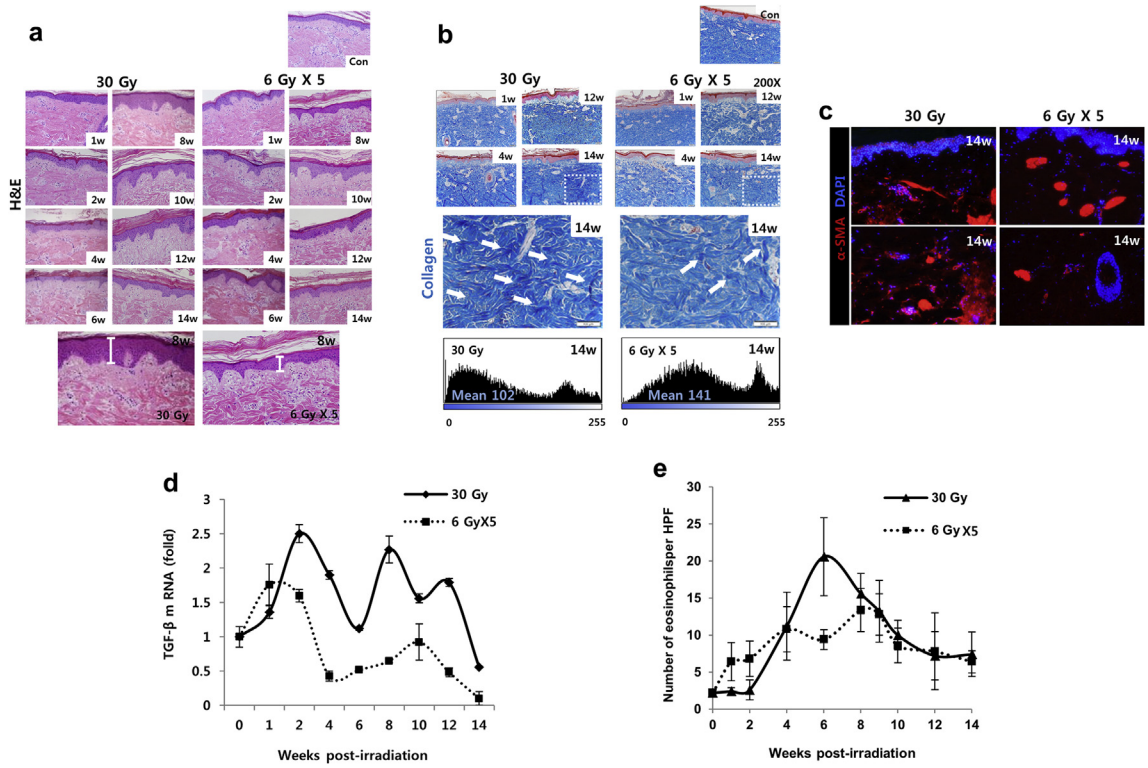


Fig. 1. Single high-dose radiation with 30 Gy induces enhanced production of profibrotic factors compared to 30 Gy in 5 fractions. Porcine skin subjected to 30 Gy single-fraction or 5-fraction irradiation was biopsied at the indicated times and stained with H&E, Masson's trichrome, and TGF-β and α-SMA antibodies. mRNA was isolated from biopsy specimens for qRT-PCR. (a) Histology, (b) collagen deposition, (c) α-SMA expression, (d) TGF-β mRNA and protein expression, and (e) the mean numbers of eosinophils in 5 high-power fields (magnification, ×400) from tissue sections irradiated with 30 Gy in a single fraction or 5 fractions are plotted against time, and in 30 Gy single-fraction- or 5-fraction-irradiated porcine skin.

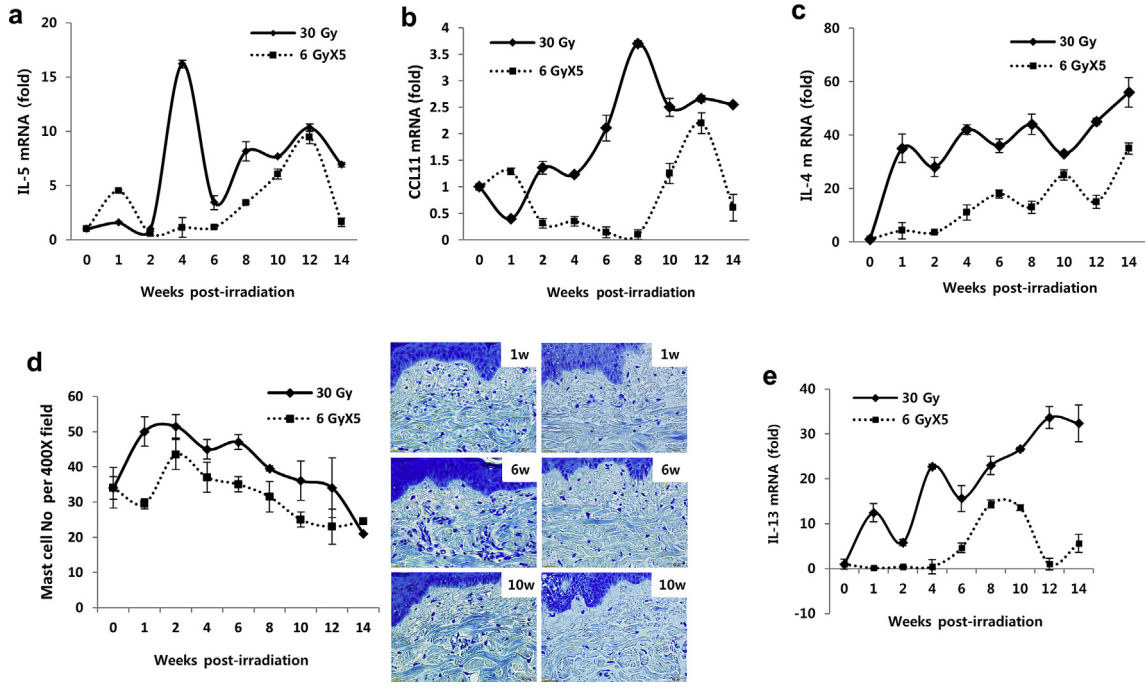


Fig. 2. Upregulation of eosinophil-related factors by 30 Gy single-fraction vs. 5-fraction irradiation of porcine skin. mRNA was isolated from biopsy specimens, and qRT-PCR was performed with target primers. β-actin-normalized mRNA levels of (a) IL-5, (b) CCL11, and (c) IL-4 in porcine skin after 30 Gy single-fraction or 5-fraction irradiation. (d) Number of mast cells in porcine skin after 30 Gy single-fraction or 5-fraction irradiation. Mast cells were stained with toluidine blue and counted per 400 × field. (e) IL-13 mRNA level in porcine skin after 30 Gy single-fraction or 5-fraction irradiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responses may in turn be involved in fibrosis [4]. Eosinophils were reported to play a major role in pulmonary fibrosis [5] by triggering Th2-polarized immune reactions [6]. Th2 immune response has been associated with upregulation of Th2-type cytokines including IL-4, IL-5, IL-10, and IL-13 and induction of mast cells [7,8]. Highly polarized Th2 cytokine responses are also closely related to fibrosis progression [9]. The cytokine IL-33 potently stimulates secretion of Th2 cytokines such as IL-5 and IL-13 by ST2-expressing immune cells, which contribute to the development of Th2 immune responses [10,11]. Administration of IL-33 to mice resulted in increased serum levels of Th2 cytokines including IL-4, IL-5, and IL-13, as well as IgG1 and IgE, and inflammation was accompanied by eosinophil accumulation in the lung and gut [12]. IL-33 also induces cutaneous fibrosis via eosinophil-derived IL-13 and lung fibrosis through type-2 macrophage-induced IL-13 and transforming growth factor (TGF)-β [13,14].

We previously developed a porcine skin injury model and characterized the dose–response relationship in high-dose single-fraction irradiation. In these experiments, eosinophil recruitment was dose-dependently enhanced by single high-dose irradiation [15]. In this study, we investigated the underlying mechanism of eosinophil-associated skin fibrosis and the effects of high-dose-per-fraction radiation.

2. Materials and methods

2.1. Porcine skin irradiation and biopsy

The radiation-induced porcine skin injury model using a female mini-pig (Medi Kinetics Co., Ltd., Pyeongtaek, Korea) was established as described previously with minor modification [15]. The study design was approved by the Institutional Animal Care and Use Committees. Briefly, the dorsal skin was divided into two sections. A lead shield containing 11 cut-out 2 cm × 2 cm squares separated from each other by at least 2.5 cm, was placed over one section of the dorsal skin, and either 30 Gy in a single fraction or in 5 fractions was delivered with a 6-MeV electron beam. The pig was housed and observed for 14 weeks to allow acute and late effects of radiation to develop.

2.2. Mouse and cell irradiation

Radiation was delivered with an X-RAD 320 X-ray unit (Precision, CT) equipped with fixed and adjustable collimation fixtures. For mouse skin irradiation, the collimators produced a beam with a 1 cm × 1 cm coverage area. The percentage depth doses were determined after absolute dosimetric measurements with Gafchromic EBT3 film, and acrylic and water-equivalent RW3 slab phantoms (PTW, RW3). Radiation dosimetry for cultured cells was carried out using a cell culture dish, water, an RW3 phantom slab, and Gafchromic EBT3 film. Further details of mouse and cell irradiation were described by Yoo et al. [16].

2.3. RNA-Seq analysis using mouse skin tissue

To reveal what factors promote eosinophil recruitment in irradiated skin, we performed RNA-Seq using irradiated- and non-irradiated mouse skin samples. Mouse flank skin was pulled aside, followed by irradiation with 30 Gy. Total RNAs were isolated using TRIzol (Invitrogen), and mRNA was isolated from total RNAs using oligo-dT beads. Construction and sequencing of an RNA-Seq library were performed based on Illumina HiSeq2000 protocols to generate 101 paired-end RNA-Seq reads. The quality of raw data was checked using FastQC [17], and the adaptor sequences of the Illumina sequencing platform were trimmed using Trimmomatic (ILLUMINACLIP:2:30:10 MINLEN:75) before read alignment. All quality-filtered reads were aligned to the Mus_musculus genome (GRCm38) from the Ensembl database (release 73) using Tophat [18]. Aligned reads were sorted using Samtools [19], and the read count for each gene was calculated using HTseq [20]. To identify differentially expressed genes between the two conditions, we used the R package DESeq [21], which is based on a negative binomial model. Based on the DEGs identified using DESeq ($P < 0.01$, Bonferroni corrected), we conducted Ingenuity Pathway Analysis (Qiagen). Endothelial cells, epithelial cells, dermis, and epidermis were selected to conduct the core analysis in Ingenuity Pathway Analysis, and other options were set to default.

2.4. Statistical analysis

Data are presented as mean ± SD, and groups were statistically compared using the unpaired 2-sided Student's *t*-test. All experiments were performed at least three times. $P < 0.05$ (*) and $P < 0.01$ (**) were considered statistically significant.

All other materials and methods are described in the Supplementary materials.

3. Results

3.1. Single-fraction irradiation with 30 Gy induced higher expression of fibrosis-related factors and eosinophils recruitment in porcine skin than 5-fraction irradiation

The maximum tolerable dose without overt ulceration of porcine skin after single-fraction irradiation with 15, 30, 50, or 75 Gy was 30 Gy in our previous study [15]. Here, we investigated the effect of single-fraction 30 Gy on fibrosis-related factors and eosinophils compared with a fractionated scheme (6 Gy, 5 times) using a radiation-induced porcine skin injury model (Fig. S1). Fig. 1 shows irradiation-induced histological changes of the skin. Histological examination of biopsy specimens indicated that 30 Gy single high-dose irradiation induced more epidermal hyperplasia, collagen accumulation, and α-SMA expression than 5-fraction irradiation at 14 weeks (Fig. 1a–c). Additionally, TGF-β mRNA and protein expression was more enhanced by single high-dose irradiation than fractionated irradiation (Fig. 1d). In our previous study, we demonstrated that inflammatory responses including IL-6 expression and eosinophil infiltration were increased in irradiated porcine skin [15]. IL-6 expression was enhanced in both fractionation schemes, with no significant difference between the two schemes (Fig. S2). The average number of eosinophils was determined in five high-power fields (magnification, ×400) in irradiated tissue sections, and the rate of increase and peak eosinophil count were more pronounced after 30 Gy single-fraction irradiation compared to 5 fractions (Fig. 1e). The average number of eosinophils increased sharply after 2 weeks, peaked at 6 weeks, and subsided to baseline levels at 12 weeks after 30 Gy single-fraction irradiation. Induction of eosinophils appeared to coincide with the acute and subacute skin response to radiation.

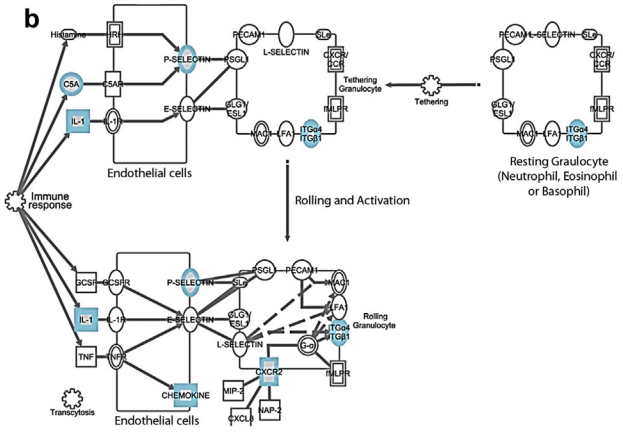
3.2. Upregulation of eosinophil-related factors following irradiation

Since eosinophils can regulate Th2 immunity [6], eosinophil-related Th2 cytokines such as IL-4, IL-5, and CCL11 (eotaxin) were evaluated by quantitative real-time PCR (qRT-PCR). Increases in IL-4, CCL11, and IL-5 mRNA levels were more pronounced after 30 Gy single-fraction irradiation (Fig. 2a–c) compared with fractionated irradiation. Expression of CCL11 and IL-5, which are significant factors in eosinophil recruitment, began increasing at 4 weeks and peaked at 6 weeks after irradiation, paralleling the pattern of

a

Function	p-value	Genes
Adhesion of eosinophil	0.003	C5,ITGA5,SELP,THBD
Adhesion of immune cells	0.001	C5,CD4,CXCR2,ITGA5,SELP,THBD,TXN
Chemotaxis of vascular endothelial cells	0.0001	CXCR2,PTGS2,THBD,THBS1,THBS2,FOXC1, SEMA3E,THBD

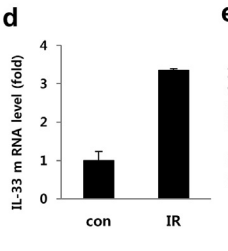
b



c

ID	Fold Change	P-value	Associated Gene Name
ENSMUSG00000 044103	4.24	3.66E-15	interleukin 1 family, member 9 [Source:MGI Symbol;Acc:MGI:2449929]
ENSMUSG00000 039217	3.53	6.37E-08	interleukin 18 [Source:MGI Symbol;Acc:MGI:107936]
ENSMUSG00000 024810	7.07	5.28E-20	interleukin 33 [Source:MGI Symbol;Acc:MGI:1924375]

d



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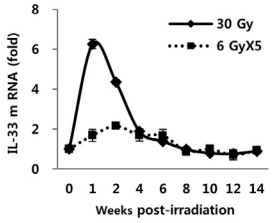

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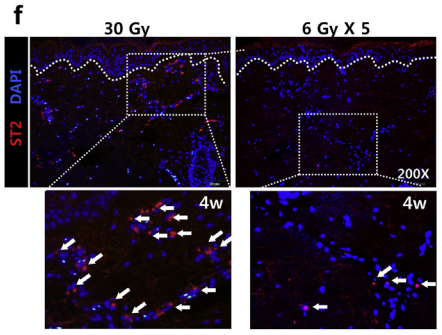

Fig. 3. Enrichment of granulocyte adhesion and upregulation of IL-33. Ingenuity Pathway Analysis was conducted using differentially expressed genes (DEGs; $P < 0.01$, Bonferroni corrected) in mouse skin irradiated with 30 Gy (single dose) and non-irradiated. (a) Enrichment of granulocyte adhesion molecules and (b) diapedesis pathways by canonical pathway analysis and (c) IL-1 family genes including IL-1, 18, and 33 among upregulated DEGs. (d) IL-33 mRNA levels determined by qRT-PCR in 30 Gy irradiated mouse skin. Porcine skin was irradiated with 30 Gy in a single fraction or 5 fractions and biopsied at the indicated times. (e) IL-33 mRNA levels determined by qRT-PCR in irradiated porcine skin and (f) ST2 receptor expression in irradiated porcine skin biopsied at 4 weeks after irradiation and stained with ST2 antibody (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eosinophil recruitment. The induction of mast cells, which also play an important role in eosinophil-mediated Th2 immunity [22], was quantified with toluidine blue. The number of mast cells per 400 × microscopic field in the infiltrated dermal area remained higher following single high-dose irradiation vs. 5-fraction irradiation with 30 Gy (Fig. 2d). Expression of IL-13, which is known to be capable of driving tissue fibrosis [14], increased over time and was also more pronounced after single-fraction compared to 5-fraction irradiation with 30 Gy (Fig. 2e).

3.3. IL-33 induction in mouse skin following 30 Gy single high-dose irradiation

To identify factors that promote eosinophil recruitment in irradiated skin, we performed RNA sequencing (RNA-Seq) in irradiated mouse skin. RNA-Seq analysis using DESeq identified 679 differentially expressed genes (DEGs) (333 upregulated, 346 downregulated) in irradiated mouse skin samples. Ingenuity Pathway Analysis of diseases and functions for the 333 upregulated DEGs showed that these were significantly related with eosinophil adherence ($P < 0.003$), immune cell adherence ($P < 0.001$), or vascular endothelial cell chemotaxis ($P < 0.0001$). Related genes and functions are summarized in Supplementary Table 1. Consistent with phenotypic changes, fifth complement component (C5), chemokine (C-X-C motif) receptor 2 (CXCR2), alpha 5-integrin (ITGA5), P-selectin (SELP), and IL-1, which are involved in immune response; eosinophil recruitment; and vascular endothelial cell chemotaxis, were upregulated, and canonical pathway analysis revealed enrichment of the granulocyte adhesion and diapedesis pathways in the DEGs of 30-Gy-irradiated mouse skin (Fig. 3a).

IL-1R signaling plays a central role in the regulation of immune and inflammatory responses. IL-1, IL-18/IL-37, IL-33, and IL-36/IL-38 are among the IL-1 family members that share the IL-1Rα chain [23]. Therefore, we searched the DEGs for IL-1 family genes and found that IL-1, IL-18, and IL-33 were upregulated DEGs in 30-Gy-irradiated mouse skin (Fig. 3b). IL-33 had the highest fold-change (>7-fold) among IL-1 family genes, consistent with recent reports that IL-33 is expressed in endothelial and epithelial barrier tissues, such as skin and vessels, that play important roles in the response to damage and infection [24,25]. In mice, IL-33 is not expressed constitutively in normal blood vessels [26]. However, damaged vascular endothelial cells and tissue in an ApoE (−/−) atherosclerosis mouse model produce high IL-33 levels [27].

We validated the IL-33 mRNA upregulation using qRT-PCR, which indicated 4-fold higher IL-33 mRNA levels in 30 Gy-irradiated mouse skin than non-irradiated skin (Fig. 3c). Moreover, expression of IL-33 mRNA and the IL-33 receptor ST2 was more significantly elevated in porcine skin 1 week after 30 Gy single high-dose irradiation compared with 5-fraction irradiation (Fig. 3d, e). These results indicate that high-dose radiation induces IL-33 production in the early period (1–2 weeks) after irradiation and may be accompanied by vascular endothelial cell damage.

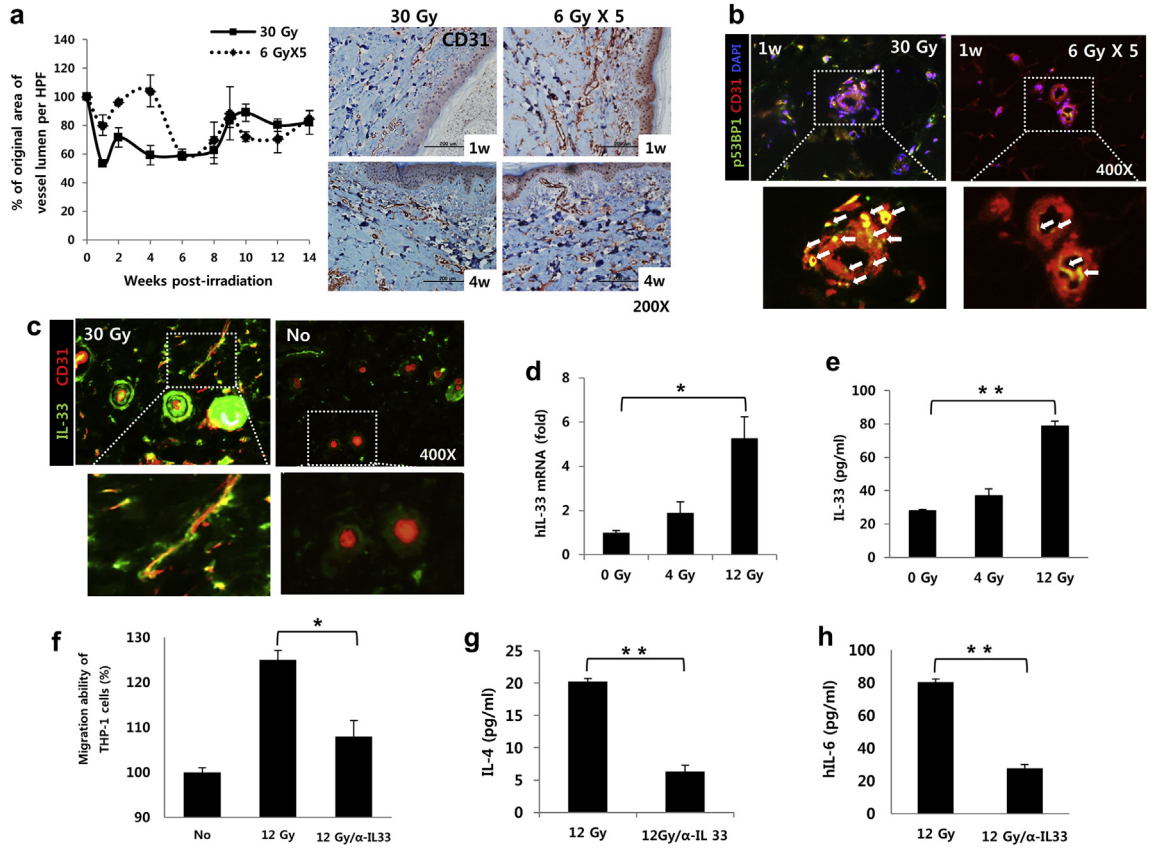

Fig. 4. Production of IL-33 and blood vessel damage by high single-dose irradiation. (a) Mean density of CD31-stained small arterioles, determined by summing the luminal areas of microvessels, in 5 high-power fields (× 400) from sections of each biopsy specimen, expressed as a percentage of the baseline value obtained on Day 0 and (b) co-staining with p53-binding protein 1 (p53BP1, green) and CD31 (red) antibodies to demonstrate blood vessel damage at 1-week biopsy (blue, DAPI nuclear stain; original magnification, × 400) in porcine skin irradiated with 30 Gy in a single- or 5-fraction schedule. (c) IL-33 secretion from blood vessels in 30 Gy-irradiated or non-irradiated mouse skin co-stained with IL-33 (green) and CD31 (red) antibodies (original magnification, × 400). IL-33 (d) mRNA and (e) production in HUVECs irradiated with 0, 4, or 12 Gy and incubated for 48 h. IL-33 inhibition by anti-IL-33 antibody led to (f) downregulation of THP-1 cell migration in transwell plates after 36-h incubation with 12 Gy-irradiated HUVEC-conditioned culture medium and decreased levels of (g) IL-4 and (h) IL-6 in culture medium after 48-h co-culture of 12-Gy-irradiated HUVECs and THP-1 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Eosinophil-mediated Th2 immune reaction by IL-33 secreted from impaired vascular endothelial cells in the skin

Since ablative-hypofractionated radiotherapy methods, which deliver over 10 Gy per fraction, have been reported to induce vascular damage [28], microvessel density was assessed by CD31 staining. The density of dermal microvessels in porcine skin was lower during the early period (0–6 weeks) after 30 Gy single high-dose irradiation (Fig. 4a). To identify vascular damage induced by irradiation, biopsy specimens from irradiated porcine skin were co-stained with p53-binding protein 1 (P53BP1), which is a classic DNA damage response marker, and CD31 antibodies. The number of p53BP1 and CD31 double-positive cells was greater in skin irradiated with 30 Gy in a single fraction (Fig. 4b).

Next, we investigated whether IL-33 is secreted from dermal blood vessels upon irradiation. Since no IL-33 antibody was available for immunofluorescence staining of porcine skin, we used irradiated mouse skin for IL-33 and CD31 co-staining. IL-33 expression was significantly increased in dermal vessels after 30 Gy single-fraction irradiation (Fig. 4c). To identify whether IL-33 is released from damaged vascular endothelial cells by radiation *in vitro*, we used HUVECs cells and set 12 Gy and 4 Gy cell irradiation as the counterparts of single high-dose and 5-fraction irradiation with 30 Gy *in vivo*. Culture media were collected 48 h after irradiation to measure IL-33 synthesis. The mRNA and protein levels of IL-33 were increased by radiation in a dose-dependent manner (Fig. 4d, e). We also evaluated immune cell recruitment and cytokine release via IL-33-mediated immune responses in a human monocyte co-culture system. The migration of THP-1 cells co-cultured with 12 Gy-irradiated HUVECs was enhanced compared to those cultured with non-irradiated HUVECs (Fig. 4f). This enhanced THP-1 cell migration and production of IL-4 and IL-6 were impaired by anti-IL-33 antibody treatment (Fig. 4g–h), suggesting that endothelial cells impaired by single high-dose radiation could produce IL-33, which might encourage immune responses such as inflammation and immune cell recruitment.

4. Discussion

Vascular change has been reported to stimulate fibrosis through release of various cytokines including TGF-β and IL-6 [29]. High-dose irradiation decreases tumor microvascular density and $CD68^{+}$ tumor-associated macrophages in irradiated tumors [30]. Vascular endothelial cells appear to be one of the main targets of hypofractionated radiotherapy in the ablative dose range used in stereotactic body radiotherapy or radiosurgery (>10 Gy per fraction) [28]. However, changes in the tumor micro-environment after hypofractionated radiotherapy and the impact of varying fraction size are not well understood. In our previous study, single high-dose radiation of porcine skin induced loss of vessel density and

eosinophil infiltration of the irradiated site [15]. In this study, 30 Gy single high-dose irradiation exhibited more pronounced reduction of vessels and increase of eosinophil infiltration compared to fractionated radiation. RNA-Seq analysis of irradiated mouse skin showed elevation of chemotaxis-associated factors of vascular endothelial cells.

Expression of the IL-1 cytokine family member IL-33 (also known as IL-1F11 and NF-HEV) markedly increased. Furthermore, increases in IL-33 mRNA and the IL-33 receptor ST2 were more pronounced in pig skin during the early period (1 week) after 30 Gy delivered in a single fraction compared with in 5 fractions. In the present study, we demonstrated that blocking IL-33 inhibited IL-4 and IL-6 secretion in a co-culture system with THP-1 cells and irradiated HUVECs. Since IL-33 is expressed in various cell types [24,31,32], it may have been produced in other skin cells such as dermal fibroblasts, mast cells, or lymphocytes in this study. However, IL-33 mRNA and ST-2 expression were sharply increased at 1 week after high-dose irradiation of porcine skin, indicating that IL-33 induction was increased as an early response. The acute effect of radiation on blood vessels is an important trigger, and vascular endothelial cells are a primary target for killing tumor cells in hypofractionated ablative radiotherapy (stereotactic body radiotherapy or radiosurgery) compared with conventionally fractionated radiotherapy. Therefore, IL-33 induction in vascular endothelial cells impaired by high-dose radiation may act as an immune modulator in recruiting eosinophils to the irradiated site in the current study. Additionally, since IL-33 is a chemo-attractant of Th2 cells, enhancing production of IL-4, IL-5, and IL-13, which can stimulate Th2 immune responses such as mast cell activation via IgE-dependent immune reactions. Accordingly, IL-33 could also act on mast cells that in turn induce Th2 environment, resulting in eosinophil recruitment in this study.

Eosinophils release Th2-type cytokines and act as direct regulators of fibroblast proliferation and collagen synthesis, in part through TGF-β [5]. Such eosinophil-produced soluble factors can act as autocrine signals through engagement of cytokine receptors in eosinophils [33]. In the present study, upregulation of eosinophil-mediated Th2 cytokines was more pronounced with single high-dose irradiation compared with a multiple-fraction schedule, which might affect eosinophil recruitment. Especially, eosinophil mediated-profibrotic factors IL-13 and TGF-β might affect fibrotic responses such as collagen accumulation and α-SMA expression.

In conclusion, single high-dose-per-fraction irradiation can induce more pronounced disruption of vascular endothelial cell function compared with fractionated irradiation. Vascular endothelial cells damaged by high-dose radiation secrete IL-33, which may stimulate fibrotic responses via eosinophil recruitment and eosinophil-mediated Th2 immune responses in irradiated skin (Fig. S3). Thus, IL-33 can be a key factor in eosinophil-mediated fibrosis in hypofractionated radiotherapy, and IL-33 may be a useful factor to predict common complications such as fibrosis in cancer patients treated with radiotherapy.

Conflict of interest

The authors state no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.081.

Transparency document

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