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Gamma irradiation alters the phenotype and function of CD4⁺CD25⁺ regulatory T cells

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Abstract

To examine the effects of gamma irradiation on Tregs, changes in phenotype and suppression function in Tregs treated with or without gamma ray were analyzed. Purified CD4⁺CD25⁺ regulatory T cells were irradiated at different dosages with a ¹³⁷Cs source gamma ray at 4.8 Gy/min. After culture, the phenotype and function changes were determined by flow cytometry and [³H]-thymidine incorporation, respectively. A dose-dependent reduction of Tregs proliferation in response to gamma irradiation was noted, which paralleled the apoptosis induction of Tregs. Gamma irradiation downregulated the Tregs expression of CD45RO, CD62L, FOXP3, membrane TGF- β , but upregulated Bax and GITR. High dose gamma irradiation (30Gy) significantly abolished the suppression of Tregs on CD4⁺CD25⁻ T cells proliferation. Thus Tregs not only influences the phenotype but also alters their suppressive capacities. Our findings suggest that radiotherapy may be an important strategy to alter the immunologic balance of Tregs and effector cells in cancer therapy.

Keywords

Gamma irradiation; Regulatory T cells

1. Introduction

CD4⁺ CD25⁺ regulatory T cells (Tregs) comprise 5–10% of the circulating CD4⁺ T cell population and powerfully suppress immune responses. A large body of experimental data suggests an essential role played by these cells in a host of clinically relevant areas, such as self-tolerance, transplantation, allergy and tumor/microbial immunity (Wing et al., 2006). Our previous work also showed that Tregs constitute an important element in the development and progression of hepatocellular carcinoma (HCC) by contributing to the dysfunction of anti-tumor immunity (Cao et al., 2007). Many investigators are pursuing strategies to either modulate the function of or the number of Tregs, which may offer a means to regulate host immunity for therapeutic effect in autoimmune diseases and cancers (Wolf et al., 2006; Zou,

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2006). In murine tumor models, either depletion of Tregs by anti-CD25 monoclonal antibody (mAb) administration (Li et al., 2003; Jones et al., 2002) or adoptive cell transfer after Tregs depletion (Shimizu et al., 1999; Tanaka et al., 2002) results in significant antitumor activity.

The efficacy of radiation therapy in the treatment of many tumor types is well established. While radiation induced tumor regression is largely the result of directed damage to the radiosensitive tumor cells, the accumulating evidences point to a number of additional immune-mediated mechanisms, such as influencing the phenotype and function of immune cells via radiation (Anton et al., 1998; Cao et al., 2004; Dunn and North, 1991; Merrick et al., 2005; North, 1984, 1986; Reuben et al., 2004; Rho et al., 2004; Roses et al., 2008; Shan et al., 2007; Takahashi et al., 1991; Yoshino et al., 2008). Among immune cells, susceptibility of lymphocytes to gamma radiation is well known. However, there is little information on the effects of gamma radiation on Tregs phenotype, survival and suppressive ability. To explore these questions, we monitored changes in Tregs phenotype and suppression function after gamma radiation. These studies show that gamma radiation impacts Tregs phenotype, proliferation and suppressive function.

2. Materials and methods

2.1. Antibodies and Reagents

The following monoclonal antibodies were used: CD4-APC, CD4-FITC, CD25-PE, CD25-CyC, CD28-Cyc, CD45RA-FITC, CD45RO-FITC, CD69-FITC, CD62L-FITC, CD152 (CTLA-4)-APC (all from BD Biosciences), GITR-FITC (R&D system); FOXP3-PE (eBioscience), Isotype -matched antibodies were used with all samples. All other reagents were of analytical grade.

2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte-enriched buffy coats obtained from healthy blood donors (LifeSouth) as previously described (Cao et al., 2007). Blood was diluted 1/1 with PBS (without calcium or magnesium), layered on Histopaque®-1077 (Sigma-Aldrich) and centrifuged for 30 min at 400×g. The mononuclear cell-rich layer was removed, washed twice with PBS, counted and resuspended in complete RPMI 1640 medium (Mediatech Inc.).

CD4⁺ T cells were purified from PBMC using the human EasySep™ CD4⁺ T Cell Enrichment Cocktail (Stemcell Technologies Inc.). The negatively selected CD4⁺T cells were incubated with anti-CD25 microbeads (30 µg per 10⁸ cells) and subjected to magnetic separation with the MS column (all from Miltenyi Biotec) to obtain the purified Tregs from the column and the eluted negative CD4⁺CD25⁻ T cells fraction as previously described (Cao et al., 2007). The purity for Tregs and CD4⁺CD25⁻ T cells was >90 and 95%, respectively, by flow cytometry. Monocytes were positively selected from PBMC by using anti-CD14 microbeads (Miltenyi Biotec) following the manufacture's instruction and irradiated by gamma ray (30Gy) for further use. The purity for CD14⁺ monocytes monitored by flow cytometry was more than 90%.

2.3. Gamma irradiation of cells

Purified Tregs were divided into several equal parts. One part was not irradiated; the others were γ-irradiated at different dosages with a ¹³⁷Cs source (Gammacell 200; Energy Atomic of Canada) at 4.8 Gy/min. After irradiation, the medium was immediately removed and the cells were resuspended at 1 × 10⁶/mL in complete RPMI 1640 medium. Cell viability of 100% was determined by trypan blue exclusion staining for all experiments.

2.4. Phenotype analysis

After gamma irradiation, Tregs ($1 \times 10^6/\text{mL}$) were cultured in fresh complete RPMI 1640 medium overnight, washed twice with PBS, blocked with 2% human AB serum in PBS for 7 min at room temperature, and incubated with different combinations of CD4, CD25, CD28, CD45RA, CD45RO, CD62L and GITR for 30 min at 4°C for extracellular surface staining. After surface staining, intracellular staining with CD152 and FOXP3, was performed. Acquisition and data analysis were performed with FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). The results were expressed as percentages of cells that were positively stained.

2.5. Membrane TGF- β assay

Tregs (irradiated and non-irradiated) were cultured overnight, washed, blocked with anti-CD16/anti-CD32 and stained with primary mouse anti-human LAP (TGF- β 1) mAb (R&D System) or control mouse IgG, followed by secondary goat anti-mouse IgG -FITC (Santa Cruz). After washes, cells were subjected to flow cytometry.

2.6. Suppression Assays

CD4⁺CD25⁻ T cells ($1 \times 10^5/\text{well}$) and monocytes (γ -irradiated, $1 \times 10^5/\text{well}$) were cultured in triplicate without or with 1×10^5 irradiated or non-irradiated Tregs (at ratio of 1:1 or 1:1:1) in 96-well U-bottom plate in the presence of PHA (5 $\mu\text{g}/\text{mL}$) for 2 days. Cells were pulsed for another 18 h with 1 μCi ³H-thymidine, and the uptake of radioactivity was measured using a liquid scintillation counter (Perkin Elmer). Results were expressed as the mean counts per min (cpm).

2.7. Blockade of Tregs suppression by anti-TGF- β and gamma irradiation

Tregs were co-cultured with or without CD4⁺CD25⁻ T cells plus irradiated autologous monocytes in the absence or presence of anti-TGF- β (500 ng/mL) with the stimulation of PHA (5 $\mu\text{g}/\text{mL}$) in the 96-well U-bottom plate for 2 days, and 1 μCi ³H-thymidine was added to each well for another 18 h incorporation. The radioactivity was measured and expressed as cpm $\pm$ SD. CFSE-labeled CD4⁺CD25⁻ T cells were co-cultured with or without non-irradiated or irradiated (30Gy) Tregs or irradiated (30Gy) monocytes (at ratio of 1:1 or 1:1:1) in the absence or presence of anti-TGF- β (500 ng/mL) in 12-well plate. On day 3, cells were harvested and cells divisions were measured by flow cytometry.

2.8. Measurement of apoptosis and proapoptotic protein Bax expression

Apoptosis was measured with an Annexin V-FITC Apoptosis detection Kit (BD Biosciences Pharmingen). Briefly, after overnight culture, washed cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's instructions. Apoptotic cells were detected using flow cytometry performed on a FACSCalibur. Thirty thousand gated events were acquired for each condition, and data were analyzed using CellQuest software. PE-conjugated -Bcl-2 or -Bax monoclonal antibody (Santa Cruz) were used to determine whether Bax and Bcl-2 were involved in apoptosis induced by gamma irradiation. Following extracellular staining with CD4 and CD25, cells were washed twice with cold PBS and fixed and permeabilized in Cytotfix/Cytoperm buffer (BD Biosciences), incubated with Bax-PE and Bcl-2-PE antibodies for 30 min. Isotype controls were included in all the experiments. Cells were washed and analyzed with a FACSCalibur flow cytometer. Results were expressed as percentage of positive cells.

2.9. Statistical Analysis

Paired comparisons (irradiated Tregs versus non-irradiated Tregs) were done by Wilcoxon's signed rank test; the other data were compared using the non-parametric Wilcoxon's test. The *P*-values quoted are two-tailed and differences are considered statistically significant at $P \leq 0.05$. Throughout the text, data are expressed as median and range.

3. Results

3.1. Irradiation downregulates CD45RO, CD62L, FOXP3 and upregulates GITR expression on Tregs

To investigate the effect of gamma irradiation on Tregs phenotype, purified Tregs were exposed to different dosages of γ -ray. Compared to the untreated group, the irradiated Tregs showed reduced expression of CD62L, FOXP3, CD45RO and enhanced expression of GITR in a dose-dependent manner (Fig. 1). In contrast, CD152 expression was upregulated by low dose of γ -ray (1.8 Gy) and downregulated by high dose of γ -ray (30 Gy). There was no difference in expression of CD4, CD28, CD45RA between irradiated Tregs and untreated Tregs (data not shown). These results also showed that CD4⁺CD25⁺FOXP3⁺T cells decreased in a dose-dependent manner.

3.2. High dose irradiation abolishes the suppressive capacity of Tregs on autologous T cells proliferation

To study the irradiation effect on the suppressive function of Tregs on autologous naïve T cells, Tregs were co-cultured with CD4⁺CD25⁻ T cells and irradiated CD14⁺ monocytes (at ratio of 1:1:1) in the presence of PHA at 5 μ g/ml following exposure to 0, 1.875, 3.75, 7.5, 15 and 30Gy of γ -ray. In comparison to the untreated Tregs (0 Gy, Treg0), suppressive abilities of Tregs was downregulated by gamma irradiation in a dose-dependent way, but only the high dose irradiated Tregs (30Gy, Treg30) lost its suppression on autologous T cells proliferation ($p < 0.05$). There was no significant difference between the untreated Tregs and other doses of γ -ray treated Tregs (Fig. 2).

Due to deficiency in specific marker reliable for CD4⁺CD25⁺ Tregs, it is very difficult to isolate human CD4⁺CD25⁺ Tregs with high purity. The identification of CD4⁺CD25⁺ Tregs still depends on documenting their suppressive ability. The purity of Tregs in our experiments was >90%. However, we cannot exclude the possibility of contamination of small numbers of activated CD4⁺CD25⁺ T effector cells, but the purified Tregs display suppressive ability (suppress >50% of CD4⁺CD25⁻ T cells proliferation, $P = 0.0155$) (Fig. 2).

3.3. Gamma irradiation leads to reduction of membrane TGF- β expression and loss of suppressive function in Tregs

It was previously demonstrated that membrane TGF- β mediates Tregs suppression on T cells in cell-to-cell contact way (Nakamura et al., 2001, 2004; Oida et al., 2006). Based on the above results that high dose irradiation inhibited Treg suppressive ability, we investigated the impact of γ -ray (30Gy) on the expression of membrane TGF- β in Tregs. Compared with untreated Tregs ($40.5 \pm 4.9\%$), the irradiated Tregs significantly displayed decreased membrane TGF- β expression ($15.5 \pm 1.4\%$, $P = 0.0001$) (Fig. 3A). To further evaluate the relationship of TGF- β and Tregs function, we used anti-TGF- β to block Tregs suppression function. Compared with the suppression of non-irradiated Tregs (40230 ± 2231 cpm), anti-TGF- β can significantly block the suppression of Tregs on autologous T cells (52680 ± 2557 cpm, $P = 0.0049$) (Fig. 3B).

To further evaluate the impact of gamma irradiation on Tregs suppressor function, a responder T cell proliferation assay was performed with CFSE-labeled CD4⁺CD25⁻ T cells. Flow

cytometry was used to determine the percentage of cells with reduced CFSE fluorescence, which would be indicative of proliferation. High-dose irradiated Tregs (30Gy) lose their capacity to suppress T cell proliferation and has a similar effect of anti-TGF- β (1000 ng/mL) on Tregs suppression (Fig. 3C). These results suggest that membrane TGF- β expression is very important for Tregs suppression function and can also be impacted by gamma irradiation.

3.4. Gamma irradiation induces apoptosis and up-regulates Bax expression in Tregs

To clarify the effect of high dose of gamma ray irradiation on the viability of Tregs, a full apoptosis profile was done using the dual stains PI and annexin V as well as a series of trypan blue exclusion stains. Gamma irradiation can induce apoptosis in irradiated Tregs (Fig. 4A). The majority of these apoptotic cells were single positive for Annexin V (63.9%) indicating early apoptosis, only very small part of high dose γ -ray irradiated Tregs cells (2.8%) was dead (necrotic, PI positive). This apoptosis profile of Tregs shows that the major effect of 30 Gy is induction of apoptosis and not necrosis. Further, trypan blue exclusion staining showed Treg cells viability to be 100% after irradiation and greater than 95% after overnight culture. Also, the data in Fig. 4B show that the gamma irradiation-induced apoptosis in Tregs increased in a dose-dependent way. In addition, the pro-apoptotic Bax protein expression was enhanced corresponding to apoptosis induction (Fig. 4C), but no changes were observed for anti-apoptotic protein Bcl-2 expression (data not shown).

4. Discussion

Radiation therapy (RT) plays an important role in the treatment of many human malignancies including solid tumors, leukemia and lymphoma. It is believed to directly impact and eradicate tumor cells and, to a certain extent, trigger immune response (Friedman et al., 2003), but its impact on regulatory elements of immune system such as Tregs is not known.

The role of CD4⁺CD25⁺ Tregs in suppressing anti-tumor immune activity is subject to considerable interest. Tregs maintain immunological tolerance by suppression of autoreactive T cells. Tregs express the forkhead transcription factor FOXP3 and maintain immunological tolerance by suppression of autoreactive T cells. Accumulating evidence indicates that adaptive Tregs are enriched in tumor tissue, draining lymph nodes, malignant effusions and peripheral blood from patients with various cancers (Zou, 2005), and that depletion of Tregs results in significant anti-tumor activity (Li et al., 2003; Jones et al., 2002). Based on these findings, enhancement of anti-tumor immunity by the removal of suppressor cells such as Tregs has become an attractive strategy for augmenting more traditional cancer treatment (surgery and chemotherapy). We found here by systematic investigation into the effects of gamma radiation on Tregs that gamma irradiation has significant impact on CD4⁺CD25⁺FOXP3⁺Tregs. Gamma irradiation not only alters the phenotype by down-regulating CD45RO, FOXP3, CD62L, and upregulating GITR expression on CD4⁺CD25⁺ Tregs, but also inhibits the suppression function of CD4⁺CD25⁺ Tregs.

Membrane TGF- β mediates much of Tregs suppression on T cells in a cell-to-cell contact mechanism (Chen et al., 2003; Faria and Weiner, 2006; Nakamura et al., 2001, 2004; Oida et al., 2006). Gamma irradiation significantly decreased membrane associated TGF- β expression on Tregs, which had the same functional effects as that of anti-TGF- β antibody blocking Tregs suppression on CD4⁺CD25⁺ T cells. High-dose gamma irradiation (30Gy) abolished Tregs suppressive abilities via significantly decreasing its membrane-associated TGF- β expression (Fig. 3A). Although one cannot exclude the possibility that other doses of gamma ray may have similar effects, this finding confirms that TGF- β membrane expression is one of the mechanisms involved in Tregs-mediated suppression in this system, which is further supported by the blocking experiments in Fig. 3B and 3C. It is commonly accepted that FOXP3 is a key factor to maintain Tregs function (Fontenot et al., 2003; Hori et al., 2003; Yagi et al., 2004).

We also found that gamma irradiation downregulated FOXP3 expression on Tregs in a dose-dependent manner (Fig. 1).

The immune system responds to ionizing radiation with distinct characteristics depending on multiple factors such as dose and dose rate, tissue and cell types (James et al., 1988). Overall, immune cells are susceptible to radiation-induced damage and readily undergo apoptosis in response to small doses of radiation. Gamma irradiation induces apoptosis in human monocyte-derived DCs (Liao et al., 2004). Here, our data also show that the gamma ray-induced apoptosis increased in irradiated-Tregs in a dose-dependent manner (Fig. 4A and 4B).

Cellular apoptosis is critically regulated by various intracellular and extracellular signaling mechanisms. The Bcl-2 family of proteins comprising both anti- and pro-apoptotic members plays pivotal roles in regulating cell apoptosis. In this family, the balance of Bcl-2/Bax in a cell is regarded as one of the crucial factors determining whether or not the cell will undergo apoptosis. Bcl-x_L overexpression inhibited gamma radiation-induced apoptosis (Wang et al., 2006). Interestingly, we found a dose-dependent enhancement in the expression of pro-apoptotic protein Bax (Fig. 4C) and no change in the expression of anti-apoptotic protein Bcl-2 (data not shown). This suggests that the Bcl-2/Bax balance shifted towards apoptosis induction in Tregs after gamma irradiation.

In summary, gamma irradiation not only alters the phenotype, induces apoptosis, but also inhibits proliferation and suppression function of CD4⁺CD25⁺ Tregs. Manipulation of Tregs in terms of their frequency and functional activity via gamma irradiation could be one of the therapeutic measures for enhancing antitumor immunity. These results may provide insight into the mechanism of gamma irradiation and offer new strategies for radiotherapy of cancer patients.

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Abbreviations

Tregs	CD4 ⁺ CD25 ⁺ regulatory T cells
HCC	hepatocellular carcinoma
PI	propidium iodide

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Figure 1.

Phenotypic characterization of CD4⁺CD25⁺ Tregs. Purified human CD4⁺CD25⁺ regulatory T cells from health subjects PBMC were cultured overnight and incubated with different antibodies for extracellular or intracellular staining after sham irradiation or irradiation by different doses of gamma ray. The gated viable cells were analyzed. Numbers in the figures represent the percentage of fluorescence-positive cells in corresponding areas. One representative data of 3 independent experiments.

Figure 2.

The effects of gamma irradiation on the suppression function of CD4⁺CD25⁺ Tregs. After sham irradiation or irradiation by different doses of gamma ray, CD4⁺CD25⁺ T cells (Tregs) suppressive abilities were evaluated by coculturing with or without CD4⁺CD25⁻ cells (T) in the presence of γ -irradiated autologous monocytes and PHA (5 μ g/mL) for 2 days. [³H]-thymidine was added to cultures for last 18 hours and the incorporation was measured as mentioned in Materials and Methods. Values are representative examples of 3 separate experiments. Treg: CD4⁺CD25⁺ T cells; T: CD4⁺CD25⁻ T cells

Figure 3.

High dose gamma irradiation abolishes the suppression function of CD4⁺CD25⁺ Tregs via reducing the membrane TGF-β expression. (A) Membrane TGF-β expression was determined by flow cytometry in Tregs after sham irradiation or irradiation by 30Gy of gamma ray and overnight culture. Values are the average ± SD of three independent experiments each performed in triplicate. (B) Anti-TGF-β (500 ng/mL) attenuates the suppressor ability of Tregs. Tregs were cocultured with or without CD4⁺CD25⁺ T cells (1:1) in the absence or presence of anti-TGF-β (500ng/mL) for three days. Cells proliferation was determined by [³H]-thymidine incorporation. (C) High dose (30 Gy) gamma ray possesses the same inhibitor effect as that of anti-TGF-β (1000 ng/mL) on the suppressor capacity of Tregss. CFSE-labeled CD4⁺CD25⁺ cells (CFSE-T) were cocultured without or with Tregs (irradiated at 30Gy or non-

irradiated) in the absence or presence anti-TGF- β (1000 ng/mL) for 3 days. The dye dilution was analyzed by flow cytometry. Values are representative examples of 3 separate experiments. Treg0 and Treg30: irradiated Tregs at dose of 0 Gy and 30 Gy, respectively; T: CD4⁺CD25⁻ T cells; M: gamma-irradiated autologous monocytes (30Gy). * p <0.05

Figure 4.

The effects of gamma irradiation on apoptosis induction and pro-apoptotic protein Bax expression in Tregs. After sham irradiation or irradiation by different doses of gamma ray, Tregs cultured overnight and then incubated with anti-Annexin V (A and B) or anti-Bax (C) antibodies, respectively. Thirty thousand-gated events were collected on FACSCalibur with CellQuest software. Numbers in the figures represent the percentage of fluorescence-positive cells in corresponding areas. Values are representative examples of 3 separate experiments.