Blocking of programmed death ligand 1 enhances natural killer cell-mediated immunity against malignant melanoma cells

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Abstract

Background Since ionizing radiation has showed the dramatic effect to kill the cancer cells by direct DNA damage as well as triggering anti-cancer immune responses through release of various tumor antigens and induction of NK activating molecules, it has been used for long time to treat many cancer patients including patients with melanoma. However, it has been known that radiotherapy might promote the remnant cancer cells to escape immune system. One of the suggested ways is induction of a ligand for programmed death-1 (PD-L1) after radiotherapy in head and neck cancer, bladder cancer and lung cancer cells which engages the receptor, programmed death-1 (PD-1) in immune cells. PD-1/PD-L1 axis transduces the inhibitory signal and suppresses the adaptive immunity in T cells. However, their role in innate immunity remains poorly understood. Therefore, we investigated whether ionizing radiation could change the expression of PD-L1/2 in malignant melanoma cells and the receptor, PD-1, in NK-92 cells. **Results** Surface PD-L1/2 levels on melanoma cells were increased by ionizing radiation in a dose-dependent manner but the level of PD-L1 was not changed significantly in NK-92 cells. Radiation-induced PD-L1/2 suppressed the activity of the NK-92 cells against melanoma cells despite of upregulation of NKG2D ligands. Furthermore, activated NK cells had high level of PD-1 and could not kill PD-L1/2+ melanoma cells effectively. When we used PD-L1 inhibitor or silenced PD-L1 gene to inhibit PD-1/PD-L1 axis, they reversed the activity of the suppressed NK cells. **Conclusions** Through these results, we supposed that PD-1/PD-L1 blockade could enhance the immune responses of NK cells against melanoma cells after radiotherapy and might overcome the PD-L1 mediated radioresistance of cancer cells.

Background

Radiotherapy is a major modality in treatment of most common cancers including melanoma. Both pro- and anti-cancer immune responses could be induced in cancer microenvironment after radiation. The anti-cancer immune responses are observed in some cancers. Several immune stimulation genes such as TNF- α were upregulated and antigenic proteins such as HSPs were released after radiotherapy in bulky cancers and glioblastoma[1-3]. However, it was known more recently that radiation promotes the remnant cancer cells to escape immune system and distant metastasis through the increased expression of TGF- β , PD-L1 and MMP-2 in cancer cells [4-6]. Furthermore, ionizing radiation may alter the anti-cancer activity of lymphocytes through dysregulation of immune check points molecules such as PD-1 and CTLA-4[7, 8]. Therefore, these adverse effects of radiotherapy should be considered and managed to treat the cancer patients. Since it was known that radiotherapy could induce the PD-L1 in several cancer cells including Head and Neck Squamous Cell Carcinoma, bladder cancer and Non-Small Cell Lung Cancer [9-11], it was supposed that PD-1/PD-L1 axis blockade was required to inhibit the adverse effect of radiotherapy and may be benefit to treat cancer patients.

NK cells are critical innate immune lymphocytes which destroy virally infected or cancerous cells through targeted cytotoxicity [12]. Interestingly, we found that NK cells expressed PD-1 on cell surface and the level of PD-1 increased significantly during their activation. Therefore, it was supposed that NK cell-

mediated immune responses were controlled by the level of PD-1 as if the cancer reactive T cells did and its blockade might be required to obtain the sufficient anti-cancer immunity. In this study, we evaluated the efficacy on NK cell-mediated anticancer immune responses after irradiation and investigated the role of PD-1/PD-L1 axis in NK cells.

Results

Surface PD-L1 and PD-L2 were increased by ionizing radiation with dose-dependent manner in melanoma cells

The surface expression of PD-L1/2 in melanoma cells was detected using PE-conjugated anti-PD-L1/2 antibodies and analyzed by flow cytometry. The expression level of PD-L1 was relatively higher than PD-L2 in A375 cells and SK-MEL-28 cells. The expression of PD-L1 and PD-L2 were increased following 8 Gy and 16 Gy irradiation in a dose-dependent manner in two melanoma cells (Fig.1). It was suggested that ionizing radiation may be a potent inducer of two ligands of PD-1 and high dose irradiation may have adverse effects in anti-cancer immunity.

The Expression NKG2D ligands increased by ionizing radiation in melanoma cells

The surface expression of NKG2D ligands including MICA, MICB and ULBP1-3 were detected using mouse anti-human specific antibodies and goat PE-conjugated anti-mouse IgG secondary antibodies. The expression of five NKG2D ligands were increased following 8 Gy irradiation in two melanoma cells (Fig.2). It was well known that the DNA damage agents including ionizing radiation could increase the NKG2D ligands by facilitation protein translation through ATM-ATR pathway[13]. We confirmed the same radiation effects on melanoma cells.

The expression of PD-1 increased dramatically during activation of NK-92 cells

Although NK-92 cells in steady state expressed a little amount of PD-1 (Fig.3A), the expression of PD-1 was dramatically increased when they are stimulated by K562 cells (Fig.3B). It seems that active NK cells might be intrinsically suppressed to prevent uncontrolled immune responses by induction of PD-1 and it makes be hard to maintain the potent cytotoxic activity for sufficient time against cancer cells physiologically. Therefore, it was supposed that PD-L1^{high} cancer cells might survive we through escape from NK cell-mediated immune responses.

The susceptibility of melanoma cells to NK cells is increased by PD-L1 inhibitor.

The high expression of PD-L1 in melanoma cells and increased PD-1 in NK cells might repress the activity of NK cells after short period of action and it seems insufficient time to eliminate melanoma cells. We investigated whether anti-cancer immune responses against melanoma cells could be affected by using PD-L1 or PD-1 inhibitor. Under the various experimental conditions, susceptibility of melanoma cells to NK-92 cells was significantly enhanced by inhibition of PD-1/PD-L1 axis (Fig.4).

Discussion

The immune checkpoint PD-1 is expressed on many cancer infiltrating lymphocytes in response to inflammation. The engagement of PD-1 on the lymphocyte by PD-L1 on melanoma cells downregulates T-cell function and might promote exhaustion of cancer reactive T cells [14, 15]. The usage of anti-PD-1 and anti-PD-L1 antibodies has been remarkably successful, both in terms of response rates (30%–45%) and durability (2–3 years) in melanoma[16-24], even after discontinuation of the treatments[24, 25]. These enthusiastic studies about PD-1/PD-L1 have been focused on adaptive immune cells, especially T cells. The role and regulation of PD-1/PD-L1 in innate immunity remains poorly understood until now.

Recent emerging data indicate that combining PD-L1 inhibitors with other therapies including chemotherapy, radiotherapy and immunotherapy might be more beneficial to cure cancer patients[26-28] and we already have been studied that ionizing radiation was efficient modality to trigger NK cell-mediated immune responses[29]. Therefore, we evaluated the efficacy on NK cell-mediated anticancer immune responses by the combination with PD-L1 inhibitor.

Activation of NK cells rapidly induced PD-1 receptor on their surface and irradiated melanoma cells also expressed high level of ligands of PD-1. Therefore, it was supposed that the blockade of PD-1/PD-L1 was required to maintain the NK cell-mediated anti-cancer immunity against melanoma cells. We used the ionizing radiation to trigger the immune responses against melanoma cells by induction of NKG2D ligands, a kind of NK cell activating molecules. It was known that radiotherapy could induce the PD-L1 in several cancer cells including HNSCC, bladder cancer and NSCLC[9-11]. However, the mechanisms to induce the PD-L1 is not understood yet. It required more sophisticated strategies to find clues to resolve it.

Taking into account these facts, we hypothesized that blocking of programmed death ligand 1 could enhance natural killer cell-mediated anticancer immunity to melanoma cell lines.

Conclusions

We have examined the level of PD-1/PD-L1 expression on NK cells and melanoma cells by ionizing radiation, respectively. Ionizing radiation could increase the expression of surface PD-L1 and it suppressed the susceptibility of melanoma cells to NK cells though NKG2D ligands expression was increased by ionizing radiation. However, PD-1 or PD-L1 inhibitor enhanced susceptibility to NK cells by inhibition of PD-1/PD-L1 axis. Taken together these results, the blockade of PD-1 could enhance NK cell-mediated anticancer immunity to melanoma cells.

Methods

Cell Lines and Reagents

A375P and SK-MEL-28 are human melanoma cell lines was used in this study which was obtained from the American Type Culture Collection (Rockville, MD, USA). These cell lines were maintained in DMEM

media supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. the NK-92 cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in α -Minimum Essential Modified medium supplemented with 12.5% (v/v) fetal bovine serum, 12.5% (v/v) horse serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 200 U/mL of recombinant human interleukin-2, 100 mg/mL streptomycin, and 100 U/mL penicillin. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Flow cytometry.

Mouse anti-human CD273(PD-L2; #345505), CD274(PD-L1; #329709), CD279(PD-1; #329911) antibodies were purchased from BioLegend. Cell-surface staining was performed by incubating tumor-derived cell populations with selected antibodies on ice in the dark for 30 minutes in PBS. To determine the surface PD-L1/2, PD-1 on cancer cells, the analysis was performed using BD FACSCanto™ II (Becton Dickinson, San Jose, CA USA). The data was analyzed by the Flowjo software (TreeStar, OR, USA) and the expression levels were determined by the Mean Fluorescence Intensity (MFI).

Irradiation and combination with PD-L1 inhibitor

To irradiate the cancer cells, ClinaciX Linear Accelerator (Varian Medical Systems Inc., Palo Alto, CA, USA) was used under consults of Dr. Jiho Nam and Yong Gan Ki (Pusan National University Yangsan Hospital). The cells were irradiated with rate of 8 Gy/min under 10 mm depth-coverage of medium. Irradiated melanoma cells were recovered for 6 hours and then, PD-L1 inhibitor were treated. After further incubation for 18 hours, the cells were collected to other assays.

NK cell-mediated cytotoxicity.

NK cell-mediated cytotoxicity was determined using flow cytometry. The melanoma cells were stained with 50 mM carboxyfluorescein succinimidyl ester (CFSE) for 30 min at 37°C and washed three times. NK-92 cells and CFSE-stained melanoma cells were co-cultured for 4 hours. Propidium iodide (PI) was added to the co-cultured samples for identification of the dead cells. Cytotoxicity were calculated by formula of $(CFSE^{+}PI^{+} \text{ cells} / CFSE^{+} \text{ cells}) \times 100 (\%)$.

Abbreviations

ATM: Ataxia telangiectasia mutated protein; ATR: Ataxia telangiectasia and Rad3-related protein; CTLA-4: Cytotoxic T lymphocyte associated protein 4; HNSCC: Head and neck squamous cell carcinoma; HSP: Heat shock protein; IgG: Immunoglobulin G; MIC: MHC class 1 chain-related protein; MMP: Matrix metalloproteinase; NK: Natural killer; NKG2D: Natural killer group 2, member D; NSCLC: Non-small cell lung cancer; PD-1: Programmed death receptor-1; PD-L1: Programmed death ligand-1; PE: Phycoerythrin; TNF- α : Tumor necrosis factor-alpha; TGF- β : Tumor growth factor-beta; ULBP: UL16 binding protein

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YSL performed experiments and wrote the manuscript. HJC, and HRC performed experiments, including flow cytometry and cytotoxicity assay. WH performed statistical analysis. JHN and YGK performed ionizing radiation. CDK developed the platform of multiplex PCR for NKG2D ligands. JB designed and evaluated the study. All the authors read and approved the final version.

Competing interests

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

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Consent for publication

Not applicable

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Figures

Figure 1

Induced surface expression of programmed death ligand 1/2(PD-L1/2) in (A)A375P and (B)SK-MEL-28 myeloma cells by ionizing radiation. (Green – Control Purple – 8Gy Blue – 16Gy)

Figure 2

Induced surface expression of NKG2D ligands in (A)A375P and (B)SK-MEL-28 myeloma cells by ionizing radiation.

A **PD-1****B** **PD-1**

Figure 3

(A) Surface expression levels of programmed death 1 (PD-1) in steady state NK cells. (B) Surface expression of PD-1 in activated NK cells following co-culture with K562 cells. (E : T, Green – 2:1, Purple – 5:1, blue – 10:1)

Figure 4

Enhanced susceptibility of (A)A375P and (B)SK-MEL-28 human melanoma cancer cells to NK cells by treatment with PD-L1 inhibitor or siRNA PD-L1. Light brown – Control, Light green – control siRNA, Dark green – siRNA PD-L1 and Dark brown – PD-L1 inhibitor.