European Journal of Cell Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

Induction of immunogenic cell death by radiation-upregulated karyopherin alpha 2 in vitro

Kyung-Hee Song[a], Seung-Youn Jung[a], Seong-Mook Kang[a], Mi-Hyoung Kim[a], Jiyeon Ahn[a], Sang-Gu Hwang[a], Jun-Ho Lee[b], Dae-Seog Lim[b,**], Seon Young Nam[c], Jie-Young Song[a,*]

[a] Division of Radiation Cancer Research, Korea Institute of Radiological & Medical Sciences, Seoul 01812, Republic of Korea
[b] Department of Biotechnology, CHA University, Gyeonggi-do 11160, Republic of Korea
[c] Low-Dose Radiation Research Team, Radiation Health Institute, Korea Hydro & Nuclear Power Co., Ltd., Seoul 01450, Republic of Korea

ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 6 April 2016
Accepted 6 April 2016

Keywords:
Danger signals
Dendritic cells
Karyopherin α2
Immunogenic cell death
Ionizing radiation

ABSTRACT

Accumulating evidence suggests the potential for radiation therapy to generate antitumor immune responses against tumor cells by inducing immunogenic cell death and phenotypic changes. We recently found that ionizing radiation upregulated karyopherin α2 (KPNA2) in HT-29 colorectal tumor cells using quantitative proteomic analysis. To determine whether this increased KPNA2 could function as a damage-associated molecular pattern to induce antitumor immune responses, mouse bone-marrow-derived dendritic cells (BMDCs) were treated with KPNA2. KPNA2 enhanced the surface expression of CD40, CD54, CD80, CD86, and MHC class I/II on BMDCs. DCs treated with KPNA2 exhibited increased secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23, and TNF-α. Co-culture of $CD4^+$ T cells and KPNA2-treated DCs resulted in induction of Th1/17 cytokines (IFN-γ and IL-17) and reduction of TGF-β production. Moreover, KPNA2-treated DCs were capable of increasing granzyme B and perforin expression in cytotoxic T lymphocytes. These results demonstrated that radiation-induced dying colorectal cancer cells released considerable amounts of KPNA2 that induce the maturation and activation of DCs for synergistic antitumor effect of radiation.

1. Introduction

Radiation therapy (RT) is an effective cancer treatment modality that is administered in a manner that maximizes tumor damage and minimizes effects on normal adjacent tissues (Moran et al., 2005). Both direct and indirect effects of RT initiate a series of downstream signaling events that result in either the repair of damaged macromolecules or progress towards several types of cell death, such as apoptosis, necrosis, mitotic catastrophe, and autophagy (Baskar et al., 2012; Eriksson and Stigbrand, 2010). Radiation has been considered an immunosuppressive and tolerogenic stimulus to immune cells for a long time. Although exposure of tumor cells to radiation can result in the efficient release of immunostimulating factors, the immunosuppressive tumor microenvironment may hamper the development of therapeutically effective anti-tumor immune responses (Golden et al., 2012). However, preclinical studies in various tumor models have recently shifted attention to the fact that exposing tumor cells to lethal doses of radiation can elicit strong anti-tumor immunogenic cell death (ICD) (Obeid et al., 2007; Panaretakis et al., 2009). ICD can stimulate the immune system by emitting danger-associated molecular patterns (DAMPs) capable of acting as danger signals. Dying, stressed or injured cells release or expose surface molecules that can function as either adjuvants or danger signals for the innate immune system (Krysko et al., 2012). DAMPs include calreticulin, secreted ATP, high mobility group protein B1 (HMGB1), heat shock proteins (HSP) or chaperokines (Dudek et al., 2013). Sub-lethal irradiation (IR) of breast, lung, and prostate carcinoma *in vitro* was demonstrated to induce the secretion of ATP and HMGB1 by both dying and surviving tumor cells, thereby rendering them more susceptible to T cell-mediated killing (Gameiro et al., 2014).

Abbreviations: APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; DAMP, damage-associated molecular pattern; DC, dendritic cell; ICD, immunogenic cell death; KPNA2, karyopherin alpha 2; MHC, major histocompatibility complex; RT, radiation therapy; SILAC, stable isotope labeling by amino acids.

* Corresponding authors at: Division of Radiation Cancer Research, Korea Institute of Radiological & Medical Sciences, 75 Nowon-ro, Nowon-gu, Seoul 01812, Republic of Korea.
** Corresponding authors at: Department of Biotechnology, CHA University, CHA Bio Complex 335 Pangyo ro, Bundang gu, Seongnam, Gyeonggi do 11160, Republic of Korea.
E-mail addresses: dslim@cha.ac.kr (D.-S. Lim), immu@kirams.re.kr (J.-Y. Song).

http://dx.doi.org/10.1016/j.ejcb.2016.04.002
0171-9335/

It is increasingly recognized that local radiation can evoke inflammation at tumor sites under the proper circumstances. Moreover, radiation can induce the generation of a microenvironment that favors the recruitment of immune cells, particularly dendritic cells (DCs) (Green et al., 2009). DCs act as professional dying cell scavengers and are the most potent antigen-presenting cells (APCs) in the body, showing clear superiority in inducing primary immune responses to a variety of infections or tumors (Steinman and Inaba, 1999). Additionally, DCs are capable of cross-presenting antigens from the tumor cells killed by IR to stimulate a specific T-cell response.

The interplay between immune cells and tumor cells tends to be complex, and the ultimate success of RT may depend on a number of factors, such as the cell type, the dose and quality of radiation, the type of cell death, the type of immune cells that phagocytize the tumor cells, and the redox state. Therefore, we investigated novel intracellular proteins released by irradiated tumor cells and evaluated whether they could serve as ICD inducers.

To identify radiation-induced secretory molecules, we conducted a quantitative proteomic approach in irradiated human colorectal cancer HT-29 cells based on stable isotope labeling by amino acids in cell culture (SILAC) and isolated an interesting protein (karyopherin α2 or KPNA2). KPNA2 (also called importin α) is a member of the karyopherin superfamily that is involved in transporting molecules between the cytoplasm and the nucleus of eukaryotic cells. Because KPNA2 expression has been demonstrated to be significantly increased in breast cancer (Dahl et al., 2006; Noetzel et al., 2012), melanoma (Winnepenninckx et al., 2006), cervical cancer (van der Watt et al., 2009), esophageal cancer (Ma and Zhao, 2014), lung cancer (Wang et al., 2015), ovarian cancer (Huang et al., 2013), endometrial cancer (Ikenberg et al., 2014), prostate cancer (Mortezavi et al., 2011; Grupp et al., 2014), brain cancer (Gousias et al., 2014), liver cancer (Hu et al., 2014) and bladder cancer (Jensen et al., 2011), the value of KPNA2 as a prognostic maker has been highly considered. KPNA2 is also known to promote angiogenesis and motility, resulting in carcinogenesis and the production of oncogenic factors. However, whether RT-induced KPNA2 can modulate the immune response surrounding tumor cells into anti-tumorigenic or pro-tumorigenic immune states is questionable. Thus, we investigated the effect of KPNA2 on DC functions and subsequent immune responses. The obtained data are presented in this study, and the importance of these findings is discussed.

2. Materials and methods

2.1. Mice

Female BALB/c mice (6–8 weeks of age) were purchased from Samtako Bio Korea (Gyeonggi, Korea). The animals were housed in a controlled environment (22 ± 3 °C and 50% ± 20% humidity) and maintained with access to food and water *ad libitum*. All animal experiments were conducted according to the Korean National Guidelines of Laboratory Animal Experiments based on the protocol approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS 13-056).

2.2. SILAC-based proteomic analysis

HT-29 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in l-lysine-depleted RPMI 1640 (Invitrogen, Grand Island, NY, USA) supplemented with 10% dialyzed FBS (Invitrogen) and 0.1 mg/mL heavy [U-$^{13}C_6$] or light l-lysine (Invitrogen). Every 3–4 days, the cells were split and the media replaced with the corresponding light or heavy labeling medium. After approximately six doubling times, the cells achieved almost 100% incorporation of the amino acid isotopes. Cells grown with light l-lysine (1×10^6 cells/100 mm dish) were exposed to 8 Gy ^{137}Cs γ-radiation (Gammacell 3000 Elan, MDS Nordion, Canada) and harvested after 48 h. An equal ratio (1:1) of irradiated and non-irradiated HT-29 cells were mixed and fractionated using the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit K0311 (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. The efficacy of fractionation was determined via western blotting using GAPDH and Lamin A/C as the cytosolic and nuclear control proteins, respectively. BIOCON (Suwon, Gyeonggi, Korea) was conducted to identify proteins altered by IR. The database search and analysis were performed as previously described (Kim et al., 2015).

2.3. Generation of bone marrow-derived DCs

Bone-marrow-derived DCs (BMDCs) were harvested from the femur and tibia of the hind legs. Then, the cells were depleted of red blood cells with RBC lysis buffer (Lonza, Basel, Switzerland). Cells were cultured for 6 days in RPMI 1640 containing 10% FBS, 10,000 units/mL penicillin (Gibco), 20 ng/mL rmGM-CSF (JW CreaGene, Seoul, Korea) and 10 ng/mL rmIL-4 (JW CreaGene). Every 2 days, the supernatant was discarded and replaced with media supplemented with 20 ng/mL rmGM-CSF and 10 ng/mL rmIL-4. After 7 days, DCs were incubated for 24 h with 1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) to generate fully mature DCs (mDCs). At the same time, the cells were treated with 1 μg/mL rhKPNA2 (ATGP1389, ATGen, Gyeonggi, Korea) to differentiate DCs and investigate the effect of KPNA2. To avoid endotoxin (LPS) contamination in rhKPNA2, the limulus ameobocyte lysate (LAL) test was performed using the LAL-QCL-1000™ kit from Lonza (Walkersville, MD, USA) according to the manufacturer's instructions. The purity of the BMDC population was assessed by flow cytometry after CD11c labeling (greater than 90% CD11c$^+$ cells).

2.4. Western blot analysis

Cell lysates were prepared using previously reported methods [22] and subjected to SDS-PAGE. The resolved proteins were electrotransferred to nitrocellulose membranes. The membranes were incubated with a blocking buffer for 1 h and probed with anti-KPNA2 (Santa Cruz Biotechnology, Paso Robles, CA, USA) or anti-β-actin antibodies (Sigma-Aldrich) overnight at 4 °C. After incubation with secondary antibodies, peroxidase activity was assessed using a chemiluminescence-based detection (LAS-3000, FujiFilm, Tokyo, Japan) according to the manufacturer's recommendations.

2.5. Co-culture of DCs and T cells

DCs (2×10^5 cells/mL) were co-cultured with CD4$^+$ T cells (1×10^6 cells/mL) isolated from splenocytes obtained from naïve BALB/c mice using a CD4 isolation kit (pluriSelect, Leipzig, Germany). The cells were co-cultured in RPMI1640 medium supplemented with 10% FBS for 72 h at 37 °C with 5% CO_2. To evaluate T-cell proliferation, the isolated CD4$^+$ T cells were stained using a CellTrace™ CFSE Cell Proliferation Kit (Life technologies, Seoul, Korea) and analyzed by flow cytometry. The DCs (2×10^5 cells/mL) were transferred to a 96-well plate and then co-cultured with CFSE-labeled T cells (2×10^6 cells/mL) for 5 days at 37 °C with 5% CO_2.

2.6. Flow cytometry

Immunostaining was performed as previously described (Karlsen et al., 2011). Briefly, after 10 min of incubation at 4 °C with

Please cite this article in press as: Song, K.-H., et al., Induction of immunogenic cell death by radiation-upregulated karyopherin alpha 2 in vitro. Eur. J. Cell Biol. (2016), http://dx.doi.org/10.1016/j.ejcb.2016.04.002