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Characterization of direct radiation-induced immune function and molecular signaling changes in an antigen presenting cell line

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Abstract Radiation therapy is a widely used cancer treatment and pre-transplantation conditioning regimen that has the potential to influence anti-tumor and post-transplantation immune responses. Although conventionally fractionated radiation doses can suppress immune responses by depleting lymphocytes, single high doses of local tumor radiation can enhance immune responses. Using phospho-flow cytometry analysis of a human monocytic cell line, we identified novel radiation-induced changes in the phosphorylation state of NFκB family members known in other cell types to maintain and regulate immune function. These phosphorylation changes were p53 independent, but were strongly dependent upon ATM activation due to DNA damage. We found that radiation promotes the activation and APC functional maturation through phosphorylation of NFκB Essential Modulator (NEMO). Our results and the analytic methods are especially well suited to the study of functional changes in APC when radiation is used for immune modulation in clinical protocols.
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1. Introduction

It is well known that irradiation of tumors can directly kill cancer cells, as well as tumor endothelial cells and other cells within the tumor stroma, with associated secondary effects on tumor cell viability [1,2]. The effects on tumor infiltrating lymphocytes and antigen presenting cells (APC) within tumors are not well characterized [1–12]. Radiation is known to induce innate immune stimulatory signals [12,13], including the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-2) [14–17], and upregulation of co-stimulatory molecules, heat shock proteins, death receptors, and major histocompatibility complex molecules [1,12,18,19]. These "danger signals" can lead to maturation and activation of APC, which allow APC to process and present ingested antigen to T cells [20,21] released by damaged and dead cells in irradiated tumors.

Therefore, the goal of the study was to identify early radiation-induced signaling changes in APC and elucidate the effects of these signaling changes on APC receptor expression

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and function. We used the U937 cell line [22] derived from a histiocytic lymphoma, that exhibits monocyte morphology and functions as an APC. The model was selected because it provides a homogenous population of cells, arrested in a pliant state of maturation [23].

We focused on the NFκB pathway because this pathway is activated by radiation in tumors and modulates the expression of various apoptotic and anti-apoptotic genes [25], and it is a critical regulator in the development and maintenance of T cells, B cells, and APC [26]. The NFκB pathway regulates immune events, such as the cytokine transcription, microbial phagocytosis, cell differentiation, and cell proliferation [24,27]. In the classical or canonical pathway, NFκB complexes are inactive and reside in the cytoplasm in a complex with an inhibitory protein, IκBα. NFκB activation is mediated by the IkB kinase complex (IKK), with its two catalytically active kinases (IKKα and IKKβ), by the regulatory scaffold protein, NFκB Essential Modulator (NEMO). Phopshorylation and activation of NEMO are critical to phosphorylation and activation of IKKα and IKKβ, and activation of this complex leads to phosphorylation of IκBα, and release of NFκB for phosphorylation and entrance into the nucleus to modulate target gene expression [27,28]. We hypothesize that radiation-induced effects on early events in the NFκB pathway may activate or functionally modify certain immune cells, particularly radioresistant APC. The experiments described here were designed to elucidate the poorly understood role of the NFκB pathway in radiation-induced changes in APC, which are relevant to better understanding of the mechanism by which radiation can enhance tumor immunity.

2. Materials and methods

2.1. Cells and culture

Human monocytic cells U937 (obtained from Garry Nolan, Stanford University, Stanford, California, originally from ATCC repository) were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 1 mM glutamine, penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C with normal oxygen content or under hypoxic conditions (2% O_2).

2.2. Irradiation

Cells were irradiated with γ-rays by using a ^{137}Cs source with a fixed dose rate of 148 or 531 cGy/min.

2.3. Monoclonal antibodies and chemical reagents

Anti-CD3-FITC (UCHT1), anti-CD4-PE, anti-CD80-PE-Cy5, anti-CD86-PE, anti-CD40-FITC, anti-FACS stain buffer, and anti-Mouse IgK/Negative Control (FBS) Compensation Particles Set were purchased from BD Pharmingen. Anti p-IKKγ (NEMO) (S376)-FITC, anti-pNFkB p65 (S536)-Alexa Fluor 488, and anti-pIκBα (Ser32) Rabbit mAb, were purchased from Cell Signaling Technology. LIVE/DEAD fixable dead cell stain kit in Aqua was purchased from Invitrogen.

2.4. Flow cytometry analysis

U937 cells were harvested and washed with PBS and stained for viable cell populations using LIVE/DEAD Fixable Green Dead Cell Stain or Ethidium Monoazide (EMA) (5 μg/ml; Invitrogen). U937 cells were processed for flow cytometry as previously described [29].

2.5. Wildtype p53 transfection

pCMV-Neo-Bam p53 wt and pCMV-Neo-Bam empty control plasmids were purchased from Addgene Co. Plasmid DNA was purified by isopropanol precipitation by centrifugation using Plasmid Midi Kit (Qiagen). U937 cells were cultured in fresh medium for one day, prior to suspension in Electroporation Medium (10 μl BioBrene Plus per 100 ml and 0.2% (w/v) Glucose in PBS). DNA, suspended in 0.3 ml of Electroporation Medium at room temperature, was mixed with 0.5 ml of the cell suspension (final volume was 0.8 ml) in an electroporation cuvette. The mixture was electroporated at 960 μF, 300 V, left in the cuvettes at room temperature for 5 min, and then transferred to 1 ml of medium in a 15 ml tube and incubated for 5 to 10 min at room temperature. Cells were then cultured in the medium, which was replaced every two days.

2.6. Cytoplasmic extract preparation and western blot analysis

Cells were lysed in cell extraction buffer (Invitrogen), supplemented with 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma-Aldrich). Nuclei were separated by centrifugation and sonicated cytoplasmic extracts were obtained. The protein concentration in each sample was determined using the method of Bradford Protein Assay. Immunoblotting was performed with various antibodies p21, phospho-ATM (S1981), ATM, phospho-IKK-γ (Ser376), DNA-PK, ATR, and actin from Cell Signaling Technology Corp. (Danvers, MA). Blots were washed with TBS (1×)-Tween (1%) and incubated with anti-mouse IgG, or anti-rabbit IgG HRP-linked antibody. Immunoreactivity of the blots was detected by ECL western blot detection reagent (Amersham Biosciences).

2.7. Assay for quantitative determination of GSH and GSSG levels

Intracellular GSH (reduced glutathione) and GSSG (glutathione disulfide) levels were measured using the GSH reductase recycling assay as previously described [30]. Concentrations of GSH and GSSG were expressed as nmol/mg of protein and the ratios of GSSG/(GSH + GSSG) were calculated.

2.8. Transient siRNA transfection of U937 cells

These experiments used HP validated (by quantitative RT-PCR) ATM siRNA from Qiagen, AllStars Negative (nonsilencing) Control siRNA (5 nmol), HiPerfect transfection reagent and RNAse free water, and were performed in an RNAse-free environment with 20 μM siRNA solutions. Transfection was performed per manufacturer's instructions.

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