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Inter- and intra-cellular mechanism of NF-kB-dependent survival advantage and clonal expansion of radio-resistant cancer cells

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ABSTRACT

Understanding the underlying mechanism by which cancer cells acquire resistance to radiation and favorably selected for its clonal expansion will provide molecular insight into tumor recurrence at the treatment site. In the present study, we investigated the molecular mechanisms prompted in MCF-7 breast cancer cells in response to clinical radiation and the associated coordination of intra- and inter-cellular signaling that orchestrate radio-resistance and tumor relapse/recurrence. Our findings showed that 2 or 10 Gy of ^{137}Cs γ-rays at a dose rate of 1.03 Gy/min trigger the activation of nuclear factor kappa B (NF-κB), its DNA-binding activity and recycles its own transcription. NF-κB DNA-binding kinetic analysis demonstrated both sustained and dual phase NF-κB activation with radiation. Gene manipulation approach revealed that radiation triggered NF-κB-mediated TNF-α transcriptional activity. TNF-α blocking approach confirmed that the de novo synthesis and secretion of TNF-α serves as a pre-requisite for the second phase of NF-κB activation and sustained maintenance. Radiation-associated NF-κB-dependent secretion of TNF-α from irradiated cells, in parallel, activates NF-κB in the non-targeted un-irradiated bystander cells. Together, these findings demonstrated that radiation-triggered NF-κB-dependent TNFα secretion is critical for self-sustenance of NF-κB (through autocrine positive feedback signaling) and for coordinating bystander response (through inter-cellular paracrine mechanism) after radiation exposure. Further, the data suggest that this self-sustained NF-κB in the irradiated cells determines radio-resistance, survival advantage and clonal expansion of the tumor cells at the treatment site. Parallel maintenance of NF-KB-TNF-α-NF-κB feedback-cycle in the un-irradiated non-targeted bystander cells initiates supportive mechanism for the promotion and progression of surviving tumor cells. Intervening this molecular pathway would help us to achieve disease-free cancer survivors.

1. Introduction

Combined with breast-conserving surgery (or mastectomy), radiation therapy, chemotherapy along with radiation treatment, and hormonal intervention result in immense advances in breast cancer diagnosis and therapy. These advancements yielded an advantage of increased longevity of cancer patients. Despite, increased survival revealed an associated increase in tumor recurrence and high-risk metastasis after treatment of the primary tumor [1–3]. Local tumor recurrence at the treatment site or near the mastectomy scar demonstrated a tight association between radiotherapy and tumor relapse at or near the treatment site. It is speculated that radiation, while alleviating the cancer burden, can itself be involved in redevelopment of the disease at the treatment site. Surviving tumor cells at the treatment site may elicit signaling molecules that may be responsible for clonal selection, tumor cell proliferation/tumor growth, and metastasis. Hence, it is imperative to understand the relationship between tumor re-growth and those altered responses after radiation exposure. If this were to occur, a subgroup of tumor cells should develop resistance and maintain functional integrity to elicit communication with both irradiated and non-targeted bystander neighboring cells. The cross talk between these cells might allow the cancer cells that survived the radiation exposure to develop a clone (clonal selection), re-grow (tumor cell proliferation and growth), and cause tumor relapse at the treatment site. However, the molecular mechanism responsible for possible radiation-induced tumor recurrence is not well understood.

The effects of radiation on the activation of transcription factors have been reported earlier [4]. Of several transcription factors evaluated in different mammalian cells, NF-κB is profoundly activated in irradiated cell populations [5,6]. Our group [7–9] and others [10] have demonstrated that radiation at low doses ranging from 0.1 to 2 Gy could activate

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transcription factor NF-κB. Similarly, results from our and other groups have shown that NF-κB was activated in MCF-7 cells after exposure to a single high dose of ionizing radiation [11,12]. Pro-inflammatory cytokine, TNF-α is an another factor that is widely known to be triggered by radiation in many cell types. TNF-α mRNA and protein production increased after cellular exposure to ionizing radiation [13]. Both in vivo and in vitro studies have demonstrated that pretreatment with TNF-α resulted in increased protection from cell killing by radiation injury or insult [13–15]. Depending upon cell type/strain, TNF-α exerts its function through two different pathways with opposite effects [16] i.e. promotion or attenuation of apoptosis in target cells. Attenuation of the apoptotic pathway may largely be attributed to the capacity of TNF-α to initiate preferred NF-κB-dependent signaling, which has been demonstrated in vitro [17] and in vivo [18].

Molecular cloning analysis has revealed the presence of one or more NF-κB binding sites in the promoter/enhancer region of TNF-α [16,19,20]. Thus, TNF-α synthesis and secretion and NF-κB activation could form a positive feedback loop. Several inducers have been found to induce a positive feedback loop between NF-κB and TNF-α [21,22]. However, it is unknown whether radiation exposure can trigger this positive feedback loop in breast cancer cells, and whether this loop is involved in tumor recurrence. In the present study, for the first time, we investigated and established the significance of the interplay of two decisive signaling mediators (TNF-α and NF-κB), and how those signaling mediators orchestrate a series of events that could be responsible for tumor reappearance at the treatment site, posing a high risk for invasion and metastasis.

2. Materials and methods

2.1. Cell culture

Estrogen receptor-positive human adenocarcinoma (MCF-7) breast cancer cells (obtained from American Type Culture Collection, Bethesda, MD) were maintained as monolayer cultures by serial passage in 100-mm tissue culture plates in Dulbecco's Modified Eagle medium (DMEM, Life Tech., Grand Island, NY) supplemented with 2 mM L-glutamine, 10.2 I.U/ml penicillin, 10.2 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum (JRH Tech., Lenexa, KS) in a 95% air/5% CO_2 humidified incubator. For all experiments, the cells were serum-starved by incubating in 2% serum containing complete growth medium for at least 16 h, unless otherwise specified. Cell viability was determined by the trypan blue dye exclusion method.

The murine L929 fibroblast cell line (ATCC) was used as the target cell line for the TNF-α cytotoxicity bioassay [23]. The cells were grown in complete DMEM as specified for MCF-cells in a 95% air/5% CO_2 humidified incubator. Since not all L929 fibroblasts are TNF-α sensitive, and those that are vary in their degree of sensitivity [24], cellular susceptibility to TNF-α was evaluated using serial dilution of recombinant human TNF-α (rhTNF-α, R&D system, Minneapolis, MN) by WST-1 cell proliferation and cytotoxicity assay (Roche Diagnostics, Indianapolis, IN, USA).

2.2. Exposure to ionizing radiation

For the low LET (linear energy transfer) radiation exposure, the cells were removed from a 37 °C incubator and exposed to a total dose of 2 or 10 Gy of ^{137}Cs γ-rays at a dose rate of 1.03 Gy/min (Atomic Energy of Canada Ltd. GammaCell-40 Irradiator) at room temperature (~22 °C). Immediately after exposure, the cultures were returned to the 37 °C incubator and harvested at selected time points specified for each experiment. Mock-irradiated control cells (0 Gy) were treated identically, except the cells were kept outside the γ-ray exposure chamber.

2.3. Actinomycin D solution

A stock solution of 0.5 mg/ml actinomycin D–mannitol was prepared in sterile water, aliquoted into small volumes, and stored at −20 °C. Just before use, the stock solution was diluted to a final concentration of 8 μg/ml in DMEM medium containing 2% serum, and was kept on ice until used.

2.4. Cell viability by trypan blue dye exclusion assay

To determine the cell viability, trypsinized cells were stained with trypan blue (nuclear stain) as described previously [25]. The chromophore is negatively charged; therefore, it only interacts with cells that have damaged membranes. The single-cell suspension in complete growth medium was diluted 1:1 (v/v) with 0.4% trypan blue solution (Sigma). After mixing, 10 μl of the sample was applied to a Neubauer chamber, and the cell number per mL was obtained in duplicate for each sample. The trypan blue-negative cells were considered viable. Viability was expressed as the percentage of the trypan-blue negative cells in untreated control sample.

2.5. Electrophoretic mobility shift analysis (EMSA)

Nuclear protein extraction and electrophoretic mobility shift assay were performed as described in our earlier studies [26–28]. For the competition assay, the nuclear extract was pre-incubated with unlabeled homologous NFκB oligonucleotide followed by addition of [γ-^{32}P]-ATP labeled NFκB probe.

2.6. TNF-α bioassay

The activity of TNF-α was determined by a cell-based cytotoxic bioassay [29]. The murine L929 fibroblast cells (4×10^4 cells/well) were seeded into flat bottom 96-well plates in 100 μl of complete medium and incubated overnight at 37 °C in a 95% air/5% CO_2 incubator. Plating density was optimized and care was taken to keep the cell density equal for all the wells seeded. The cells were then replaced with fresh growth medium containing 2% serum and were equilibrated for at least 2.5 h. The medium was replaced with 50 μl of test samples (i.e., conditioned medium from irradiated and mock-irradiated MCF-7 cells) and 50 μl of actinomycin D at a final concentration of 8 μg/ml. The micro-titer plates were further incubated for 16 h. Then, 10 μl of WST-1 cell proliferation and cytotoxicity assay reagent, WST-1 (Roche Diagnostics, Indianapolis, IN) was added to each well. Plates were incubated for an additional 2 h. The levels of TNF-α activation were determined against the background control as blank at 460 nm using a Microplate reader (MR-5000, DYNEX, Chantilly, VA). The mean ± SD of the percentage of L929 cytotoxicity was calculated with data obtained from quadruplicate wells.

2.7. Transient transfection and NF-κB-dependent reporter assays

The pTAL-Luc and pNF-κB-Luc plasmids (Mercury Pathway Profiling Luciferase System, Clontech Laboratories, Palo Alto, CA) were amplified using the *E. coli* strain HB101 and were purified using Qiagen Maxiprep protocol (Qiagen, Valencia, CA). These constructs, diluted at a concentration of 1 μg/μl, were used for transfection. MCF-7 cells, seeded in 60-mm culture dishes at a density of 1×10^5 cells/plate, were allowed to grow up to 70% confluence. Then, medium was replaced with fresh growth medium containing 2% serum. Transfection was performed using Effectene™ transfection reagent (Qiagen, Valencia, CA), and incubation continued for 8 h at 37 °C. When the transfection was complete, the medium containing the transfection mixture was replaced with fresh growth medium containing 2% FBS and was equilibrated at 37 °C in the 95% air/5% CO_2 incubator for 2.5 h. At that time, the cells were exposed to the selected dose of radiation or sham-exposed to radiation.