CONTRIBUTION OF ERK & NRF-2-ARE PATHWAY TO
CONSTITUTIVE AND INDUCIBLE RADIARESISTANCE OF
TUMOR CELLS VIS A VIS NORMAL CELLS

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Introduction

Radiation therapy is one of the main line treatment modalities for different types of solid tumors. Tumor cells possess inherent and/or exhibit acquired resistance to radiation induced cytotoxicity (1). Ionizing radiation (IR) mediates damage to cells by both direct and indirect processes. Reactive oxygen species (ROS) generated as a result of indirect effect cause damage to biomolecules resulting in cell death. Generation of ROS disturbs redox balance within the cells (2). Effective scavenging of ROS depends on how efficiently they are neutralized by antioxidants present inside cells so that ionizing radiation induced damage is not permanently fixed.

We have shown that intrinsic radioresistance of lymphoma cells vis-à-vis normal lymphocytes may be due to lower basal and inducible ROS levels. Further, lymphoma cells had higher GSH levels and antioxidant enzyme activities as compared to normal lymphocytes (3). The levels of intracellular antioxidants and antioxidant enzymes are regulated by redox sensitive transcription factor nuclear factor erythroid-2 related factor-2 (Nrf-2), that induces transcription of a battery of antioxidant enzymes viz. catalase, Mn-superoxide dismutase, glutathione peroxidase, glutathione-s-transferase, hemeoxygenase I etc.

It is important to identify newer targets to sensitize tumor cells to radiation without affecting normal tissues. Recent reports indicate that ionizing radiation activates Nrf-2 pathway through oxidative stress and targeting this pathway may improve outcome of radiation therapy. Based on these reports, we hypothesized that Nrf-2-ARE pathway may contribute to the constitutive as well as inducible radioresistance in tumor cells. We compared murine splenic lymphocytes from C57/BL6 mice with syngenic murine T cell lymphoma cells (EL-4). The aim of this study is to determine the contribution of ERK/Nrf-2-ARE pathway in tumor radioresistance.

Results

EL-4 cells showed higher resistance to ionizing radiation induced cell death than normal murine splenic lymphocytes

Fig.1 shows IR induced apoptosis in mouse T lymphoma cell line EL-4 and mouse splenic lymphocytes (Fig. 1A). Ionizing radiation induced apoptosis in about 60% of murine splenic lymphocytes over control. However, EL-4 lymphoma cells showed significantly lower radiation induced apoptosis (~10%) as compared to murine lymphocytes. Basal levels of cellular ROS were significantly lower in tumor cells as compared to their normal counterpart as measured by different redox-sensitive fluorescent probes.
Murine T cell lymphoma cells showed higher GSH to GSSG ratio

There was a significant decrease in GSH/GSSG ratio in tumor cells at 2h post-irradiation but stabilized at later time points (Fig. 2). However, in normal lymphocytes the change in ratio of GSH to GSSG was not significant post-irradiation.

ERK or Nrf-2 inhibitor significantly enhanced radiation induced cell death in tumor cells

Inhibitors of ERK, Nrf-2, HO-1 and thioredoxin reductase significantly enhanced radiation induced cell death in EL-4 cells suggesting their involvement in cellular radioresistance (Fig 3).

EL-4 cells showed upregulation of Nrf-2 and its dependent gene expression at different time points after radiation exposure

Murine splenic lymphocytes showed complete down regulation of Nrf-2 at 24hrs but, EL-4 cells showed upregulation in Nrf-2 mRNA levels at all the time points studied (Fig. 4a). There was also a significant increase in mRNA copy number of Nrf-2 dependent genes viz. Mn-SOD, catalase, thioredoxin reductase, GCLC and HO-1 in EL-4 cells (Fig. 4b).

Exposure of EL-4 cells to radiation increased nuclear levels of Nrf-2

Nuclear translocation of Nrf-2 was assessed by electrophoretic mobility shift assay. EL-4 cells exposed
to radiation showed increased levels of Nrf-2 in nuclear extracts at 6h but decreased at later time points (Fig. 5).

**ERK or Nrf-2 knockdown EL-4 cells showed significantly higher radiosensitivity than wild type cells**

Cells were transfected with scrambled shRNA plasmids or shRNA against ERK/Nrf-2 and then exposed to radiation. Cell death was measured by propidium iodide staining followed by flow cytometry (Fig. 6). ERK or Nrf-2 knockdown cells showed higher radiation induced apoptosis as compared to wild type cells. However, double knockdown ERK and Nrf-2 showed increased level of basal apoptosis.

**Discussion**

Our earlier report showed that decreased generation of radiation induced ROS in mouse lymphoma cells was associated with reduced extent of radiation induced apoptosis as compared to that in normal lymphocytes (2,4). The present studies also confirmed these observations and further probed the role of cellular redox signaling in murine lymphocytes and EL-4 lymphoma cells after exposure to ionizing radiation.

The ratio of GSH to GSSG and activity of thioredoxin in EL-4 cells (data not shown) was significantly higher in
EL-4 lymphoma cells as compared to normal lymphocytes under basal conditions (Fig 2). Cellular redox state can influence the pro-survival/pro-apoptotic signaling targets and thereby decide the fate of a cell [2]. Pharmacological inhibitors of MAPK, Nrf-2, HO-1 and thioredoxin reductase significantly enhanced radiosensitivity of EL-4 cells. Interestingly it was observed that, incubation of EL-4 cells with ERK or Nrf-2 inhibitor decreased their cell survival (Fig. 3) highlighting their importance in survival of these lymphoma cells.

To further confirm these findings, we examined the radiosensitivity of EL-4 cells after knocking down ERK and Nrf-2. It was observed that knockdown of ERK or Nrf-2 enhanced radiation induced apoptosis (Fig 5). Nrf-2 was found to be constitutively active in EL-4 cells which may help to express genes involved in scavenging cellular ROS (Fig. 4). Our results show that constitutive and inducible activation of Nrf-2 and its upstream kinase ERK plays an important role in determining tumor radio-responsiveness.

References