JNK inhibition sensitizes tumor cells to radiation-induced premature senescence via Bcl-2/ROS/DDR signaling pathway

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1. Introduction

Premature senescence is considered as a cellular defense mechanism to prevent tumorigenesis. Although recent evidences demonstrate that c-Jun N-terminal kinase (JNK) is involved in the senescence process [1], the target and exact mechanism of JNK signaling in the regulation of cell proliferation has yet to be defined. In this study, we investigated the role of JNK in premature senescence and demonstrated JNK inhibition sensitized tumor cells to radiation-induced premature senescence.

2. Materials and Methods

2.1 Cell culture, antibodies, and constructs

MCF7 and HEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and H460 cells were grown in RPMI 1640 medium. The wild type, phospho-mimetic, and nonphosphorylatable forms of Bcl-2, which were described in *Deng et al*. (2004), were a generous gift from Prof. William S. May (University of Florida, FL, USA).

2.2 Isolation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions were prepared as described in [2].

2.3 Analysis of cellular reactive oxygen species levels and mitochondrial superoxide

Intracellular level of reactive oxygen species (ROS) was determined through 2′, 7′-dichlorofluorescin diacetate (DCF-DA) and MitoSOX staining and FACScan flow cytometry.

2.4 Western blot analysis

Cells were lysed in RIPA buffer. Equal amounts of proteins were separated and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4 °C with one of the specific antibodies. Proteins were visualized using enhanced chemiluminescence with a LAS-3000 image system (Fujifilm Medical systems USA Inc., Stanford, USA).

2.5 Colony formation analysis

The cells were seeded in 60-mm dish and cultured for 10 days. The colonies were fixed and stained with crystal violet.

2.6 Cell cycle analysis

Cells were collected by trypsinization, fixed in 70 % ethanol and resuspended in 1 ml of PBS containing 1 mg/mL RNase and 50 μg/mL propidium iodide. Cell cycle distributions were analyzed using EPICS flow cytometer.

2.7 Comet assay

Alkaline comet assay was performed according to a described procedure of Comet Assay Kit (Cat no. 4250-00-K, Trevigen Inc. Gaithersburg, MD, USA).

2.8 Transfection

Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.9 RNA interference

RNA interferences in JNK and Bcl-2 were performed by using small interfering RNA (siRNA) duplexes. Cells were transfected by using Lipofectamine™ RNAiMAX. Nonspecific siRNA was used as a control for comparison.

2.10 Senescence associated β-galactosidase staining

To determine the senescent phenotype, we performed senescence-associated β-galactosidase assay described in [3] with modifications.

3. Results

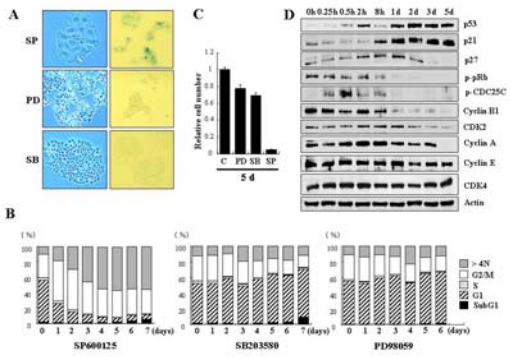

Figure 1. JNK Inhibitor SP600125 induces premature senescence. Effect of SP600125 on Morphological changes (A), cell cycle distribution (B), cell number (C), and cell cycle regulatory genes in MCF7 cells.

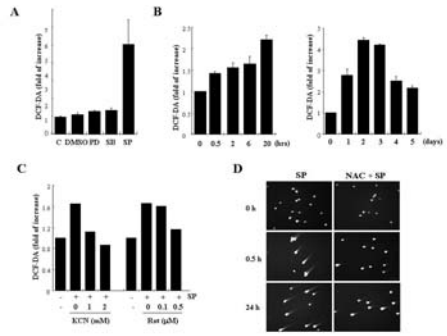

Figure 2. Inhibition of JNK activity increases mitochondrial ROS level (A,B,C), which induces DNA damage through comet assay (D).

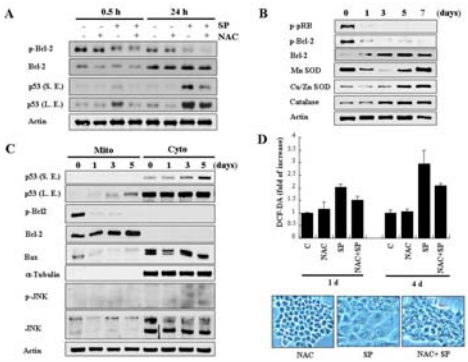

Figure 3. Inhibition of JNK activity results in loss of phosphorylated Bcl-2. (A) Status of p53 and Bcl-2 in SP600125-treated cells in the presence or absence of NAC. (B) Western blot analysis of whole cell lysates. (C) Western blot analysis of mitochondrial (Mito) and cytosolic (Cyto) fractions.

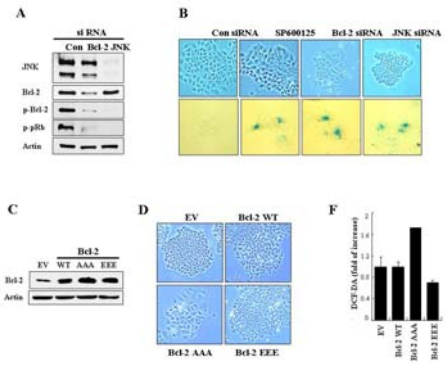

Figure 4. Loss of JNK and Bcl-2 induces premature senescence. **(A)** Effects of siRNA of JNK, Bcl-2 (A, B, C) or mutants of Bcl-2 (D, E, F) on premature senescence.

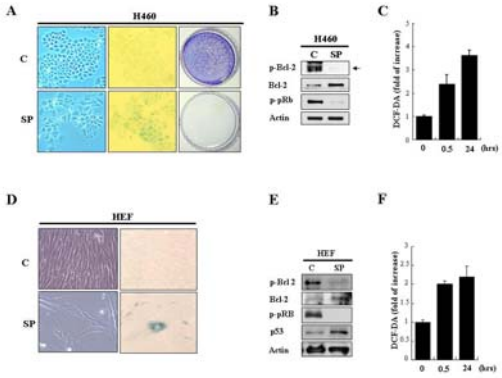

Figure 5. Loss of Bcl-2 phosphorylation triggers premature senescence in the H460 lung carcinoma cells (A, B, C) and HEF primary human embryonic fibroblast cells (D, E, F).

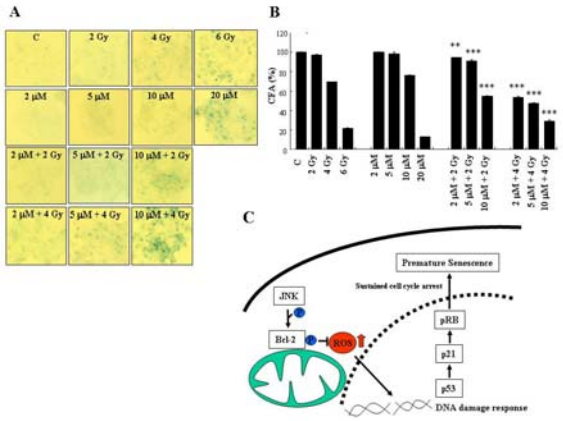

Figure 6. JNK inhibition sensitizes gamma radiation-induced premature senescence

3. Conclusion

Treatment of cells with the JNK specific inhibitor caused senescence and triggered a rapid increase in mitochondrial reactive oxygen species (ROS) production and DNA damage response (DDR). ROS generation was attributed to the suppression of Bcl-2 phosphorylation, and resulted in DNA damage and p53 activation. The essential roles of JNK and phosphorylated Bcl-2 in preventing premature senescence was confirmed using RNA interference and ectopic expression of mutants of Bcl-2, including phosphomimetic and nonphosphorylatable forms. Altogether, our results demonstrated that loss of JNK activity triggers a Bcl-2/ROS/DDR signaling cascade that ultimately leads to premature senescence, and that JNK inhibition sensitizes tumor cells to radiation-induced premature senescence.

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