Novel Smad Proteins Localize to IR-induced Double-strand Breaks: Interplay between TGFβ and ATM Pathways

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Abstract
Irradiating radiation (IR) induced DSBs can cause cell death or mutation, and initiate genetic instability contributing to carcinogenesis in humans. High LET radiation is more biologically effective compared to low LET radiation for many endpoints due to greater clustered DNA damage and oxidative stress. Cellular/tissue damage from IR is partly due to reactive oxygen species (ROS), which activate cytokine signaling and DNA damage response pathways, including the ATM/Chk2 pathway. IR can activate TGFβ, which in turn regulates the Smad pathway. Using classic DSB markers, we studied the role of Smad proteins in DNA damage response and the crosstalk between TGFβ and ATM pathways. Human fibroblasts and epithelial cells were irradiated with γ-rays and high LET particles. We observed co-localization of phospho-Smad2 (but not Smad3) and Smad7 with DSB repair proteins following IR. The decay of both foci was similar to that of γH2AX foci. High LET particles induced pSmad2 and Smad7 foci tracks characteristic of the particle trajectory through cells, with a slower decay kinetics compared to γ-rays. pSmad2 foci were observed mainly in G1 phase while Smad7 foci have no cell-cycle dependence. ATM inhibitor abolished Smad2 foci formation, and pSmad2 foci were not detected in ATM-deficient cells. Smad7 foci were not ATM dependent. In contrast, a TGFβRI inhibitor completely abrogated Smad7, but not pSmad2 foci at DSBs. In summary, we suggest that Smad2 and Smad7 contribute to IR-induced DSB signaling in an ATM or TGFβ receptor signaling dependent manner, respectively.

Materials and Methods

Cell lines and chemicals
Human hTERT-immortalized adult skin fibroblast cells (182-6) were grown in DMEM supplemented with 10% FBS and antibiotics-antimycotic. Human fibroblast cells (IMR90) were maintained in MEM supplemented with 10% FBS and Pen-Strep. hTERT-immortalized human esophageal epithelial cells (EPC) were cultured in keratinocyte-SFM medium supplemented with 50 ng/mL bovine pituitary extract, 1.0 ng/mL TGF and Pen-Strep. AT mutant cells (GM03025) were maintained in MEM supplemented with 10% FBS, L-glutamine and Pen-Strep. 2mg/mL recombinant human TGFβ, 1mg/mL ATM kinase inhibitor KU55933 and 1mg/mL TGFβ receptor 1 (TGFβRI) kinase inhibitor SD208 were added to medium one hour prior to radiation.

Irradiation
Experiments using particle irradiation were performed at the NASA Space Radiation Laboratory (NSRL) in Brookhaven National Laboratory (BNL, NY). Oxygen (O2) particles with an energy of 17 MeV/n, and a LET of 58 keV/μm, and iron (Fe) particles with an energy of 600 MeV/n, and a LET of 180 keV/μm were delivered. A 20x20 cm beam with a dose uniformity of ±2%, and a dose-rate of 72 Gy/min was used. 10Cs gamma radiation (γ-rays) experiments were completed at NASA Lyndon B. Johnson Space Center (Houston, TX) with a dose-rate of 0.3 Gy/min. 725 flasks containing exponentially growing cells were exposed vertically with the cell surface perpendicular to the beam. 8-well chamber slides were exposed either horizontally or vertically as indicated.

Western blotting and immunofluorescence
To detect nuclear specific proteins nuclear extracts were prepared using NE-PER Nuclear Protein Extraction Kit from Thermo Scientific, supplemented with Halt protease inhibitor cocktail. The membranes were probed using the enhanced chemiluminescence Plus kit as recommended by the manufacturer. The signal was detected using a Storm 840 scanner.

For immunofluorescence, cells were grown on LabTek eight-well chamber slides and fixed with 4% paraformaldehyde for 15 min. After permeabilization with 0.3% Triton X-100 in PBS for 3 min, cells were blocked with 10% normal goat serum in PBS and incubated with the indicated primary antibodies, at 4°C overnight. Detection was accomplished using Alexa Fluor 488 or 594 conjugated secondary antibodies (Invitrogen) and nuclei were counterstained with DAPI. Immunofluorescence was evaluated with a fluorescence microscope Axiosplan2 (Zeiss, Sweden). Rabbit anti-phospho-Smad2 (S465/467) and rabbit anti-phospho-p53 were from Cell Signaling Technology Inc.; mouse anti-Smad2/1/3/7 antibody was from Santa Cruz Biotechnology; rabbit anti-phospho-Smad3 (S423/425) was from Abcam; mouse anti-Smad7 was from R&D; mouse anti-ATP1 and mouse anti-γH2AX (S139) antibodies were from Millipore; rabbit anti-phospho-AKT (S473) and rabbit anti-p53 were from Cell Signaling Technology Inc.; rabbit anti-β-ACT was from Sigma; mouse anti-Rb2p80 and rabbit anti-Rb2p86 antibodies were from Cell Signaling Technology Inc.; rabbit anti-Cyclin A and mouse anti-S3B1 antibodies were from BD Transduction Laboratories; and rabbit anti-RAD51 antibody was from Calbiochem.

Conclusion
1. Our novel finding supports roles for Smad2 and Smad7, the key players of TGFβ/Smad signaling pathway, in DNA damage response.
2. Smad2 and Smad7 contribute to radiation-induced DSB signaling in an ATM or TGFβ receptor signaling dependent manner, respectively.

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