Defective Induction of Stress-activated Protein Kinase Activity in Ataxia-Telangiectasia Cells Exposed to Ionizing Radiation

Timothy D. Shafman, Ahamed Saleem, John Kyriakis, Ralph Weichselbaum, Surender Kharbanda, and Donald W. Kufe

Materials and Methods

Cell Culture. Lymphoblasts from A-T (GM03189, GM03332, and GM01526) and non-A-T (GM02184 and GM01989) individuals were obtained from National Institutes of General Medical Sciences Human Genetic Mutant Cell Repository (Cortell Institute for Medical Research, Camden, NJ). Cells were grown in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Irradiation was performed at room temperature using a Gamma-cell 1000 (Nordion International, Kanata, Ontario, Canada) with a 137Cs source and a fixed dose rate. Cells were also treated with 60 J/m² UVC irradiation using a UV Stratalinker 2400 (Stratagene, Cincinnati, OH) or 50 μg/ml anisomycin (Sigma Chemical Co., St. Louis, MO).

Immune Complex Formation and Kinase Assay. Cells were washed three times in ice-cold PBS and then lysed in 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP40, 1 mM DTT, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin) for 30 min on ice. The insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 4°C. Equal amounts of protein, as determined using a Bio-Rad (Richmond, CA) protein assay, were incubated with rabbit anti-SAP kinase antisera (7) for 1.5 h at 4°C, and then protein A-Sepharose beads were added for an additional 30 min. Lysates from irradiated non-A-T and A-T cells were also incubated with preimmune rabbit serum to ensure the specificity of the anti-SAP kinase immunoprecipitation. Immune complexes were washed twice with the lysis buffer and then once with kinase buffer [20 mM HEPES (pH 7.0), 10 mM NaCl, 10 mM MgCl₂, 0.1 mM sodium vanadate, and 2 mM DTT]. The immune complexes were then resuspended in 30 μl of kinase buffer containing 5 μCi [γ-32P]ATP (3000 Ci/mmol; NEN, Boston, MA) and 5 μg of GST-Jun (amino acids 2 to 100) fusion protein for 15 min at 30°C (11). Reactions were terminated by the addition of concentrated SDS-PAGE sample buffer and boiling. The phosphorylated proteins were resolved by 10% SDS-PAGE, dried, and analyzed by autoradiography. Equal loading of the lanes was determined by Coomassie blue staining of the gel prior to drying.

Results

Lymphoblasts from a normal individual were exposed to 20 Gy IR. Cell lysates prepared at 1 h were subjected to immunoprecipitation with anti-SAP kinase antibody. The immune complexes were then assayed for kinase activity using GST-Jun (amino acids 2 to 100) as the substrate (11). Using this approach, a low level of GST-Jun phosphorylation was detectable in the anti-SAP kinase immunoprecipitates obtained from unirradiated cells (Fig. 1A). More importantly, we found that IR activates SAP kinase to phosphorylate GST-Jun (Fig. 1A) and not GST (data not shown). These findings supported the involvement of SAP kinase in IR-induced signaling. Similar studies were performed with lymphoblasts obtained from an individual with A-T and, in contrast, there was little if any induction of SAP kinase activity when these cells were exposed to IR (Fig. 1B). This result

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2. To whom requests for reprints should be addressed, at Joint Center for Radiation Therapy/SWRL, 50 Binney Street, Boston, MA 02115.
3. The abbreviations used are: A-T, ataxia-telangiectasia; IR, ionizing radiation; MAP, mitogen-activated protein kinase; SAP, stress-activated protein.
suggested that IR-induced activation of SAP kinase may be defective in certain A-T cells.

To further address the activation of SAP kinase by IR, we irradiated lymphoblasts obtained from other non-A-T and A-T individuals. IR exposure of cells from a second non-A-T individual was associated with stimulation of SAP kinase activity. However, there was no detectable induction of Jun phosphorylation when using A-T lymphoblasts from two additional A-T individuals (Fig. 2). Taken together, the results obtained with lymphoblasts from two non-A-T and three A-T individuals support an IR-induced activation of SAP kinase that is defective in A-T cells.

Maximal induction of Jun phosphorylation was obtained at 1 h after IR exposure in non-A-T lymphoblasts (Fig. 3A). Although there was no detectable activation of SAP kinase in the irradiated A-T lymphoblasts at 1 h, we assayed subsequent time points to ask whether there is a delayed response. The finding that SAP kinase is also not activated at 3 and 6 h after IR treatment suggested that the response is defective in the A-T cells. Other studies were performed at IR doses ranging from 2 to 20 Gy. Maximal induction of SAP kinase activity in non-A-T lymphoblasts occurred at a dose of 20 Gy (Fig. 3B). The results also demonstrate that there is an absence of detectable SAP kinase activation in A-T cells following exposure to this dose range (Fig. 3B).

The absence of SAP kinase activation raised the possibility that either this kinase cannot be activated in A-T cells or that it is not present in a concentration sufficient for the activity to be detected. To address these issues, we treated cells with anisomycin, a protein synthesis inhibitor shown previously to activate SAP kinase (7). Activation of SAP kinase by anisomycin in non-A-T cells was comparable to its activation in these cells by IR (Fig. 4A). Treatment of A-T lymphoblasts with anisomycin resulted in activation of SAP kinase, while IR had no detectable effect (Fig. 4A). These findings indicated that SAP kinase is functionally expressed in A-T cells. UV light has also been identified as a potent inducer of SAP kinase (8), and it is known that A-T cells are not hypersensitive to UV irradiation (2). Indeed, we found that exposure of both A-T and non-A-T cells to UV light induces SAP kinase (Fig. 4B). Taken together, these results indicate that A-T cells respond to certain stress-inducing agents with the activation of SAP kinase and that this response is defective following IR exposure.

Discussion

A-T cells fail to exhibit a delay at the G1-S phase transition following IR exposure (12, 13). This inability to inhibit DNA synthesis has been proposed as a mechanism responsible for increased IR sensitivity of A-T cells (14). The failure of these cells to undergo G1-S phase delay following irradiation has been attributed to a defective pathway leading to the absence of IR-induced increases in p53 protein levels (15). In contrast, other studies with A-T cells have found an attenuation or delay in the p53 response (13, 16). To date, there have been no reports of defective signal transduction pathways in A-T cells in response to IR. The present findings demonstrate that A-T lymphoblasts exhibit defective IR-induced activation of SAP kinase. The results indicate that activation of SAP kinase is absent in γ-irradiated A-T cells. Moreover, an extended time course demonstrates that IR-induced SAP kinase activity is defective rather than delayed in A-T cells.

SAP kinase has been shown to be activated by both the DNA-damaging agent UV light and anisomycin, a protein synthesis inhibitor. Our results demonstrate that exposure of A-T cells to anisomycin is associated with the induction of SAP kinase activity. This finding indicates that functional SAP kinase is expressed in A-T cells. Furthermore, we demonstrate that UV light induces SAP kinase activity in A-T cells, indicating that these cells have an intact signaling pathway responsive to UV light. This suggests that we have identified a defective signaling pathway in A-T cells that is specific for IR-induced DNA damage. It is unclear, however, if activation of SAP kinase is in direct response to DNA damage or if this event is a necessary step leading to DNA repair. In either case, a defective SAP kinase response may be involved in the hypersensitivity of A-T cells to IR.

Given the presence of functional SAP kinase in A-T cells, the results also indicate that the defect in IR-induced signaling resides upstream of SAP kinase. Recent work has demonstrated that SEK1 is a potent activator of SAP kinase (9). SEK1 is structurally related to the MAP kinase kinases (MEK) and is activated by MEK kinase (10). Activation of MEK/MAP kinase by mitogens and SEK1/SAP kinase by stress signals associated with growth inhibition has raised the possibility of interplay between different pathways. Thus, defects in the response of A-T cells to IR could involve signals upstream to SAP kinase that regulate MEK kinase or SEK1 activation. In this context, the demonstration that SAP kinase activation is undetectable in A-T cells provides an opportunity to define the upstream signals that are defective in the IR response.

The role of SAP kinase in the stress response is unclear, and the finding that its response is defective in A-T cells provides evidence...
DEFECTIVE ACTIVATION OF SAP KINASE IN A-T

Fig. 3. Activity of SAP kinase following exposure to IR of varying times and doses. A. lymphoblasts were exposed to 20 Gy IR and harvested after 1, 3, and 6 h. B. lymphoblasts were exposed to 2, 5, 10, or 20 Gy and harvested after 1 h. SAP kinase activity was analyzed as described.

Furthermore, in addition to the phosphorylation of the c-Jun activation domain, there may be multiple unknown substrates regulated by the activation of this kinase. Therefore, an alternative role for SAP kinase in the response to IR may be possible, and this may have implications for the identification of the defect(s) responsible for the hypersensitivity of A-T cells to IR.

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