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Ionizing Radiation Activates c-Jun NH_2-Terminal Kinase (JNK/SAPK) via a PKC-Dependent Pathway in Human Thyroid Cells

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Thyroid gland is known to be higher sensitive to carcinogenic effects of external ionizing radiation (IR) than other tissues. To clarify the cell-specific response following irradiation, activations of c-Jun NH_2-terminal kinases (JNKs), which is one of mitogen-activated protein kinases (MAPKs) family members, and extracellular signal-regulated kinase (ERK) were examined in primary cultured human thyroid cells in comparison with human diploid fibroblast cells, WI-38. Although UV exposure strikingly induced JNK activity in both cells, the dose-response increase following IR exposure was observed in thyroid cells with the maximal JNK activity (3.5 fold induction) obtained at 10 Gy exposure, but no increase in WI-38 cells. The JNK activity was reached a maximum of 2.2 fold induction at 30 min after 5 Gy exposure and then sustained for at least 12 hr. On the other hand, ERK activity was not stimulated in thyroid cells following irradiation. The effects of 12-*O*-tetradecanoylphorbol *β*-acetate (TPA) mimicked those of radiation on JNK cascade and 1-(5-isoquinolinesulphonyl)-2,5-dimethylpiperazine 2HCl (H7) and pretreatment with TPA blocked JNK activation following irradiation. Our results demonstrate that IR stimulates JNK activity in cultured human thyroid cells but not in fibroblasts indicating distinct activation and regulation mechanisms of JNK cascade. The JNK activation following IR exposure is mediated at least partially through a PKC-dependent pathway.

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Key Words: **ionizing radiation, JNK, ERK, MAPK, thyroid cell, protein kinase C**

Ionizing radiation (IR) is a well-known carcinogenic agent, however, the incidence of radiation induced tumor is various from organ to organ, suggesting the tissue-type specific differences in the sensitivity to carcinogenic effects of radiation. According to in vivo experiments and epidemiological studies including Atomic-bomb survivors and children around Chernobyl, thyroid gland as well as bone marrow seem to be one of the most sensitive organs to the carcinogenic effects of external radiation (1–3). In general, IR triggers several signaling pathways not only through DNA damages, but also by probably direct degradation of cell membrane that leads to hydrolyze membrane phosphatidylinositol (4). Previous studies have revealed that thyroid cell specific responses of p53 downstream genes are induced by DNA damages in human thyroid cells following irradiation (5, 6). However, the knowledge of other intracellular responses induced by IR exposure in human thyroid cells is still uncertain.

The mitogen-activated protein kinases (MAPKs) are key enzymes of highly conserved signaling pathway transmitting extracellular signals into the nucleus (7–10). Three subgroups of MAPKs which were designated ERKs, JNKs also known as SAPKs, and p38 MAPK have been identified. These kinases are structurally related and activated by phosphorylation of threonine and tyrosine residues in a -TXY- motif that is common to most MAPKs. It has been revealed that ERKs, which are primarily activated in response to growth-factor stimulation, are implicated in processes of cellular proliferation (11–14) and differentiation (15–17). In addition, recent studies have identified ERKs to be involved in cellular responses to environmental stresses including IR in certain cell types (18,19). JNKs and p38 MAPK are characterized by their strong response to cellular stresses such as UV light (20), IR (21), osmotic stress (22), DNA-damaging drugs (23) and proinflammatory cytokines (24). JNKs are thought to participate in signaling pathway of apoptosis induced by environmental stresses (21,25). Since MAPKs can determine the cell fates by controlling cellular proliferation, differentiation and apoptosis in response to environmental stresses, they may play a crucial role in intracellular

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responses to lead to be highly sensitive to carcinogenic effects of IR in human thyroid cells. In this study, we therefore investigated whether and how JNKs and ERKs are activated by IR in human thyroid cells.

MATERIALS AND METHODS

Cells and reagents. For primary thyroid cells, human thyroid tissues were obtained during subtotal thyroidectomy in patient with hyperthyroid Graves' disease as described previously (26). WI-38 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were irradiated using a EXS-300 gamma irradiator (200 kV, 15 mA, filter : 0.5 mm aluminum + 0.5 mm copper, 0.47 Gy/min Toshiba, Tokyo, Japan), and UV irradiation was performed using a UVP ultraviolet crosslinker, model: CL-1000 (wavelength: 254 nm, Ulta-Violet products, LTD, Cambridge, UK). 12-*O*-tetradecanoylphorbol β-acetate (TPA) and 1-(5-isoquinoline-sulphonyl)-2,5-dimethylpiperazine 2HCl (H7) were obtained from Sigma Chemical Co. (St. Louis, MO).

JNK and ERK kinase assay. JNK activity was measured using Phospho Plus c-Jun antibody kit (New England Biolabs, Beverly, MA) as followed manufacture's protocol. Briefly, cells were lysed in cell lysis buffer containing 20 mM Tris (pH7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM -Glycerophosphate, 1 mM Na_3VO_4, 1 mg/L Leupeptin, 1 mM PMSF. After separating JNKs from cell lysates using GST-c-Jun (1-89), kinase reaction was performed in the presence of cold ATP. Samples were separated by electrophoresis in 12 % SDS-PAGE, then blotted onto PVDF membrane (Clear Blot Membrane-P^{AE} Atto, Tokyo, Japan). Immunodetection was carried out by incubation the membrane with phospho-specific c-Jun (Ser 63) antibody. ERK activity was determined using PhosphoPlus p44/42 MAPK (Tyr 204) antibody kit (New England Biolabs) following manufacture's protocol. Briefly, cells were lysed in the above cell lysis buffer and ERK activity was immunoprecipitated from cell lysates with phospho-specific p44/42 MAP kinase antibody. The resulting immunoprecipitate was then incubated with a Elk1 fusion protein in the presence of cold ATP. Samples were separated by electrophoresis in 12 % SDS-PAGE, then blotted onto PVDF membrane. Immunodetection was carried out by incubation the membrane with phospho-specific Elk1 (Ser 383) antibody.

RESULTS AND DISCUSSION

An increased activation of JNK was observed in cultured human thyroid cells following IR (Figure 1). Elevated level of JNK activity was detected 30 min after irradiation in a dose-dependent manner. The increase of JNK activity with 10 Gy IR exposure was about 3.5 -fold over control (Figure 1A, top and bottom panels). Western blotting of the same samples with antibody to JNK1 showed that JNK1 protein was constitutively expressed in non-stimulated human thyroid cells and remained at the same levels even after irradiation (Figure 1A, middle panel). In contrast, no increase of JNK activity following IR exposure was observed in human diploid fibroblasts, although a significant increase of JNK activity was induced following UV exposure ranging from 10 to 100 J/m^2 as well as in thyroid cells (Figure 2A). Similarly, failure to detect JNK activation demonstrated in irradiated NIH3T3 cells (26) suggests that the JNK activation is one of cell-type specific cellular responses induced following IR exposure in cultured human thyroid cells. A kinetics of JNK activation in cultured human thyroid cells after 5 Gy irradiation revealed rapid increase and peaked at 30 min after irradiation (about 2.2-fold over control). The increased activity remained 12 hr after irradiation (about 2.0-fold over control) and declined to near-basal levels 24 hr follow-

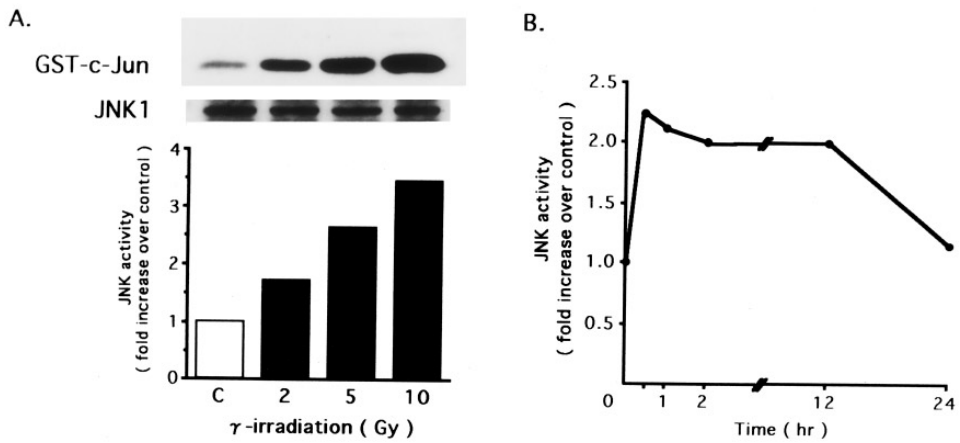

FIG. 1. Dose-response effect of IR on JNK activity and kinetics of IR-induced activation of JNK in cultured human thyroid cells. (A) Cells were irradiated with the indicated dose of IR and harvested 1 h postirradiation. Cell lysates were used for the *in vitro* kinase assay with GST-c-Jun as the substrate. A representative immunoblot obtained with anti-phospho-c-Jun antibody is shown (top panel). JNK1 protein levels were determined by immunoblotting with anti-JNK1 antibody (middle panel). Densitometric analysis of c-Jun N-terminal phosphorylation activity was performed using NIH Image 1.58 (bottom panel). Data were expressed as -fold increase relative to representing the unstimulated levels. Representative results of one of three separate experiments. (B) Cells were irradiated with 5 Gy and harvested at the indicated time. Cell lysates were used for the *in vitro* kinase assay with GST-c-Jun as the substrate. c-Jun N-terminal phosphorylation activities were quantified by densitometry. Data were expressed as -fold increase relative to representing the unstimulated levels. Representative results of one of three separate experiments.

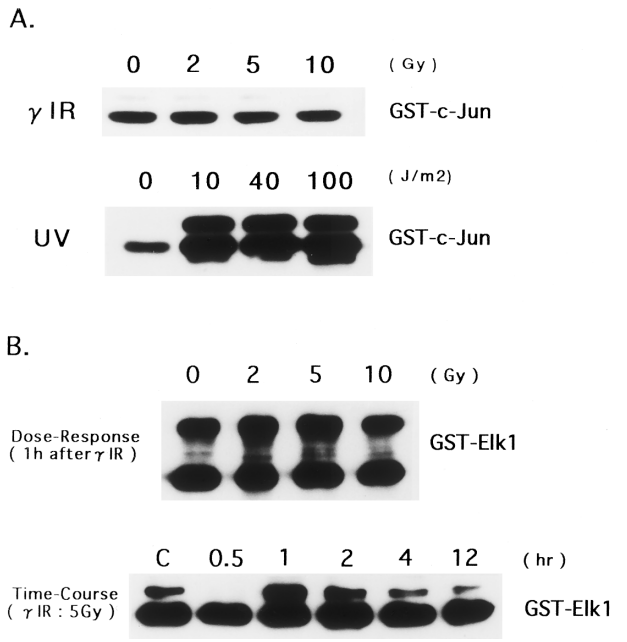

FIG. 2. Dose-response effect of IR on JNK activation in WI-38 cells and dose-response effect of IR on ERK activity and time-course of ERK activity following irradiation in cultured human thyroid cells. (A) WI-38 cells were irradiated with the indicated dose of IR (upper panel) or UV (lower panel) and harvested 1 h postirradiation. Cell lysates were used for the *in vitro* kinase assay with GST-c-Jun as the substrate. A representative immunoblot obtained with anti-phospho-c-Jun antibody is shown. Results are representative example of one of three separate experiments. (B) Cultured human thyroid cells were irradiated with the indicated dose of IR and harvested 1 h postirradiation (upper panel). Cultured human thyroid cells were irradiated with 5 Gy and harvested at the indicated time (lower panel). Cell lysates were used for the *in vitro* kinase assay with GST-Elk as the substrate. A representative immunoblot obtained with anti-phospho-Elk antibody is shown. Results are representative example of one of three separate experiments.

ing irradiation (Figure 1B). No detectable change of JNK1 protein levels was observed after irradiation (data not shown). Although it has been reported that the activation of JNKs by IR was involved in processes of IR-induced apoptosis in lymphocytes (21,25), we did not detect any increase of apoptotic cells number after in vitro exposure of human thyroid cells to IR of up to 5-8 Gy (26), suggesting that the IR-induced JNK activation observed in cultured human thyroid cells is not likely to be involved in process of apoptosis.

Since it had been reported that IR activates ERK in certain cell types and induces cell proliferation (18,28,29),we examined ERK activity following irradiation in cultured human thyroid cells. As shown in Figure 2B, no dose-response effect of IR on ERK activity was observed 1 hr after irradiation (upper panel). In addition, ERK activity did not significantly change during 12 hr after 5 Gy irradiation (lower panel).

The exact mechanism of JNK kinase cascade integration with other signaling pathways to achieve specific response to IR exposure remains to be elucidated. Phosphatidylinositol 3-kinase (PI3-kinase) is one of candidates because JNK and the p85α-subunit of PI3-kinase form a complex in irradiated cells (30). Protein kinase C (PKC) has also been reported to be activated within a few minutes following irradiation and induce expression of some genes including TNF-α (31). To further clarify the signal transduction system to mediate activation of JNK after IR exposure, the interaction of PKC to JNK pathway was examined in human thyroid cells. Similar to IR exposure, an increased JNK activity (about 2.2-fold over control) was found when the cells were treated with TPA (0.1 M) alone for 15 min which stimulates endogenous PKC activity (Figure 3, top and bottom panels). No further increase of JNK activity was, however, observed when the cells were stimulated by IR just after the treatment of TPA (Figure 3, top and bottom panels). To examine the effects of PKC depletion on IR-induced JNK activation, treatment with H7, a PKC inhibitor, or prolonged TPA treatment (5 M, 18 hr) were performed in human thyroid cells with or without irradiation. The IR-induced JNK activation was partially inhibited by the treatment of H7 (50 M, 15 min) or prolonged TPA treatment (Figure 3, top and bottom panels). These results indicate that the

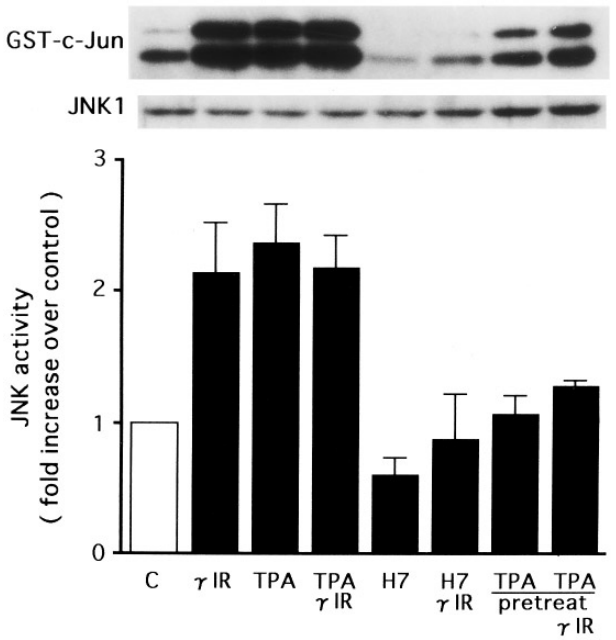

FIG. 3. Effects of TPA and H7 on IR-induced JNK activation in cultured human thyroid cells. Cells were treated with TPA (0.1 μM, 15 min), H7 (50 μM, 15 min) or TPA (5 μM, 18 hr) (indicated as "pretreat") and further incubated with or without irradiation (5 Gy) for 30 min. JNK activity was determined in the cell extracts. Representative immunoblots with anti-phospho-c-Jun antibody (top panel) or anti-JNK1 antibody (middle panel) are shown. c-Jun N-terminal phosphorylation activities were quantified (bottom panel). Data are expressed as -fold increase over control values (mean S.D. n=3).

principal pathway by which IR leads to JNK activation requires at least partially a PKC-dependent pathway. Blumer K.J. et al. reported that mammalian mitogen-activated protein kinase kinase kinase (MEKK) could replace the budding yeast MAP kinase kinase kinase, BCK1, which is located downstream of PKC in the cell wall signal transduction pathway (32), suggesting that PKC is implicated in the activation of MEKK. It is possible that in irradiated human thyroid cells, PKC which is activated shortly after irradiation stimulates MEKK, resulting in the JNK activation.

In conclusion, we demonstrated that IR stimulated JNK activity in cultured human thyroid cells but not in fibroblasts indicating distinct activation and regulation mechanisms of JNK cascade. The JNK activation following IR exposure is mediated at least partially through a PKC-dependent pathway.

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