The c-j un N-terminal kinases (J NK) are activated by various stimuli, including UV light, interleukin-1, tumor necrosis factor-α (TNF-α), and CD28 costimulation. Induction of J NK by TNF-α, a strong apoptosis inducer, implies a possible role of J NK in the regulation of programmed cell death. Present studies show that lethal doses of γ radiation (GR) induced J NK activities at the early phase of apoptosis in J urkat T-cells. We demonstrate that J NK1 was activated by either the T-cell activation signal, anti-CD28 monoclonal antibody plus phorbol 12-myristate 13-acetate (PMA), or the apoptosis-inducing treatment, GR; however, the induction patterns were different. In contrast to the rapid and transient J NK1 activation caused by CD28 signaling plus PMA, GR induced a delayed and persistent J NK1 activation. This implies a distinct regulatory mechanism and specific function of J NK1 in irradiated cells. The nuclear and cytosolic JNK1 activities were simultaneously increased in the irradiated cells without an evident change in the protein levels. The abilities of GR to induce J NK1 activation and DNA fragmentation were correlated. Peripheral blood lymphocytes were more sensitive to GR than J urkat cells in J NK1 induction. The responsiveness of J NK1 to GR suggests the involvement of J NK1 in the initiation of the apoptosis process.

Apoptosis is the unique morphological pattern of cell death characterized by chromatin condensation, membrane blebbing, and cell fragmentation. The most prominent event in the early stages of apoptosis is internucleosomal DNA cleavage by undefined endonuclease activities. This programmed cell death is widely observed in different cells of various organisms, from nematodes to mammals. It is generally accepted that apoptosis plays important roles in developmental processes, maintenance of homeostasis, and elimination of cells that have suffered serious damage (reviewed in Refs. 1 and 2).

γ radiation (GR) is one of many stimuli that induce cellular damage and apoptosis (2). GR can cause single-stranded and double-stranded breaks in the genomic DNA (3). Hydroxyl radicals generated by radiolytic attack on H₂O in the cellular aqueous environment can cause oxidative damages to macromolecules (4). Although the direct damaging effects of GR have been well studied, the biochemical and genetic mechanisms that initiate the active programmed cell death in radiation-damaged cells remain largely unknown.

The p46/p54 serine/threonine kinases, c-j un N-terminal kinases (J NK1 and J NK2), are emerging members of the MAP kinase-related family (5). Similar to MAP kinase, J NK activation requires phosphorylation at 2 residues, Thr-183 and Tyr-185, by MAP kinase kinase 4 (MEKK4). MAP kinase kinase 4 (MEKK4) kinase is phosphorylated and activated by the upstream kinase MAPK/ERK kinase 1 (MEKK1) (5). The J NK cascade can be induced by various mitogenic factors including growth factors, oncogenic Ras, phorbol esters, and T-cell activation signaling (5, 6). J NK activity is also induced by stimuli such as UV light, protein synthesis inhibitors, osmotic shock, and proinflammatory cytokines (5, 7). This kinase cascade was shown to be the common pathway shared by cell proliferation and stress-response signaling. The exact mechanism of how J NK kinase cascade integrates with other signaling pathways to achieve specific response to different stimuli remains to be elucidated. There are three cellular proteins currently known to be phosphorylated by J NK, which are the transcription factors c-j un (8), ATF-2 (9), and Elk-1 (10). Transcription activities driven by the responsive elements binding these transcription factors are strongly enhanced after J NK activation (5, 9, 10).

Prominent J NK activation was observed in cells treated with TNF-α (7), a potent inducer of apoptosis (2). Moreover, the tumor suppressor p53, which causes apoptosis on some occasions (1), was suggested as an in vivo substrate of J NK1 (11). Based on these findings and the general involvement of J NK in responses to various stresses, we proposed that J NK may be activated by GR. In these studies, we demonstrated that J NK1 was activated in cells exposed to lethal doses of GR. The radiation-induced J NK1 activation showed a unique kinetics in comparison with that induced by other stimuli.

MATERIALS AND METHODS

Cells, Antibodies, and Fusion Proteins—J urkat T-cell culture, peripheral blood lymphocyte preparation, and anti-CD28 mAb stimulation were performed as described (12). Rabbit anti-J NK1 serum (Ab101) was raised against a peptide sequence consisting of the C terminus (amino acids 368–384) of the human J NK1 protein. GST-j un-(1–331) and GST-J NK1-(26–384) were constructed by inserting each into the pGEX-4T-3 vector (Pharmacia Biotech Inc.). Whole cell extracts were prepared according to the procedure of Kyriakis et al. (13). Nuclear and cytosolic fractions were collected as described previously (12).

DNA Fragmentation and Western Blot Analysis—10⁶ irradiated cells were lysed in 50 μL of NTE buffer (100 mM NaCl, 40 mM Tris-Cl, pH 7.4, 20 mM EDTA) containing 0.5% SDS. The lysate was heated at 65 °C for 10 min to inactivate nucleases and digested by a 2-h incubation with 0.5 μg of a polyclonal antibody. Gy, gray; Ab, antibody; Mops, 4-morpholinepropane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis.
GR and T-cell Activation Signals Induce Different Kinetics of JNK1 Activation—To determine if the GST-J un-(1–79) phosphorylation detected by solid-phase kinase assay is due to JNK or other JNK-related kinases, JNK1-specific antiserum (Ab101) was used for the detection of JNK1 activity. As shown in Fig. 2, this antiserum recognizes the 46-kDa JNK1 protein in J urkat cell extract (Fig. 2A, lane 1). The anti-J NK1 Ab also recognized the affinity-purified GST-J NK1 protein (Fig. 2A, lanes 2 and 3). To further determine the specificity of the anti-J NK1 Ab, we incubated Ab101 and protein A-conjugated agarose beads with cell lysate prepared from J urkat cells exposed to various J NK stimuli. The precipitated kinase activities were resolved in SDS-PAGE copolymerized with GST-J un-(1–331) protein, and the in-gel kinase reaction was performed after denaturing and renaturing of the protein gel. As shown in Fig. 2B, single kinase activity around 46-kDa, which phosphorylated GST-J un, was precipitated by anti-J NK1 from UV light, anisomycin, and GR-treated cell lysates (lanes 2–4, respectively). These results clearly showed that Ab101 specifically recognized only one kinase activity, J NK1, which can phosphorylate the c-j un protein.

Because the kinetics of c-j un N-terminal phosphorylation activities induced by GR is very different in comparison with those induced by other stimuli, it is possible that GR may induce several kinases, in addition to JNK, that will phosphorylate the N terminus of c-j un. The kinetics pattern detected by solid-phase kinase assay could be the additive effect of different kinase activities. To determine the JNK1 activation kinetics, we used the anti-J NK1 Ab for immunocomplex kinase assay. As shown in Fig. 3A, the precipitated J NK1 activity from irradiated cells has the same kinetics as that determined by solid-phase kinase assay. This shows that GR induced a delayed and sustained J NK1 activation. To exclude the possibility that this unique kinetics is the specific property of certain J urkat cell clones, we used anti-CD28 mAb plus PMA, which are the potent stimuli for T-cell activation (12), to induce J NK activation. As shown in Fig. 3B, after addition of anti-CD28 plus PMA, J NK1 activity rapidly increased within 15 min and reached the peak at the 30-min time point. However, the J NK1 activity induced by anti-CD28 mAb plus PMA diminished sig-
tion are integrated.

The abilities of irradiation to induce apoptosis and JNK activation in Jurkat cells, induced a significant DNA fragmentation (data not shown), also failed to induce JNK1 activity. JNK1 induction increased as the dosage of irradiation increased and reached the plateau at 60 Gy. However, in normal peripheral lymphocytes, 5 Gy of GR induced a significant JNK1 activity (Fig. 5B). This dosage of radiation, which did not cause detectable DNA fragmentation in Jurkat cells, induced a significant DNA fragmentation in normal lymphocytes (data not shown). These results show that the abilities of irradiation to induce apoptosis and JNK activation are integrated.

DISCUSSION

Because (i) overexpression of MAPK/ERK kinase (MEKK), the JNK-activating kinase, has a lethal effect on fibroblasts (16) and (ii) TNF-α strongly induces JNK activity (7), we suspected that the JNK kinase cascade may be involved in the induction of cell death. In these studies we show that JNK1 activity is strongly activated during the early phase of apoptosis caused by GR. Because of the correlation between JNK1 activity and apoptosis induced by GR, we propose that JNK1 activation may be involved in the initiation of programmed cell death in response to radiation damages.

Immediate and transient kinetics of activation is universal in JNK responses to various stimuli (14). However, the JNK1 induction by GR in Jurkat cells was delayed and persistent. Since the T-cell activation signals, anti-CD28 mAb plus PMA, induced a rapid and transient JNK1 activation in the same cell line, the unique pattern of JNK1 induction should be a specific cellular response to GR rather than a unique property of Jurkat cells. The induction of JNK1 in both T-cell activation and apoptosis indicates that JNK1 is the common kinase shared by these two distinct phenomena. However, the opposite outcomes imply that the presence or absence of other co-activators at different times may be important. In addition, prolonged JNK1 induction in irradiated cells may cause the persistent activation of some cellular factors (e.g. c-Jun or p53) and results in detrimental effects to the cells. Therefore, the different timing and/or duration of JNK1 induction may lead to opposite outcomes, T-cell proliferation or apoptosis.

JNK (JNK1/J NK2) and p38-Mpk protein kinases are coordinately regulated, although to a different extent, by proinflammatory cytokines, UV light, and other environmental stresses (7, 14). Since the substrate specificities of these kinases are different (14), they may have distinct functions in response to the stimuli. It will be important to determine whether GR induces all of these stress-responsive kinases. It is possible that different combinations of the kinase members, with various kinetics, may mediate diverse cellular signaling and dictate...
final outcomes, proliferation or cell death.

The delayed kinetics of JNK1 induction in irradiated cells also implies the existence of a distinct activation or regulation mechanism. In the known JNK activation mechanism, a nuclear translocation of JNK after activation at the proximity of the plasma membrane is required for nuclear function of the kinase (5). However, we show that nuclear JNK1 is constitutively present and can be activated without evident, nuclear translocation in irradiated cells. The delay of JNK1 activation may be due to the time needed for accumulation of cellular damage to certain threshold levels. Furthermore, the existence of unrepairable damage could be the reason for persistent JNK1 activation, probably through the continued activation of upstream kinases. The other possible explanation for the prolonged JNK1 induction after ionizing radiation is the absence of dual specificity phosphatase activities, which were shown to dephosphorylate ERK and JNK causing down-regulation of the kinase activities (17, 18). The imbalance between kinase and phosphatase activities may have detrimental effects and lead to cell death. Finally, identification of JNK1 as a potential signaling molecule in mediating apoptosis will open a new avenue for unraveling the signal transduction mechanisms of apoptosis.

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