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Long-term Activation of c-Jun N-terminal Kinase through Receptor Interacting Protein is Associated with DNA Damage-induced Cell Death

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Activation of c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase family, is an important cellular response that modulates the outcome of the cells which are exposed to the tumor necrosis factor (TNF) or the genotoxic stress including DNA damaging agents. Although it is known that JNK is activated in response to genotoxic stress, neither the pathways to transduce signals to activate JNK nor the primary sensors of the cells that trigger the stress response have been identified. Here, we report that the receptor interacting protein (RIP), a key adaptor protein of TNF signaling, was required to activate JNK in the cells treated with certain DNA damaging agents such as adriamycin (Adr) and 1-β-D-arabinofuranosylcytosine (Ara-C) that cause slow and sustained activation, but it was not required when treated with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and short wavelength UV, which causes quick and transient activation. Our findings revealed that this sustained JNK activation was not mediated by the TNF (tumor necrosis factor) receptor signaling, but it required a functional ATM (ataxia telangiectasia) activity. In addition, JNK inhibitor SP-600125 significantly blocked the Adr-induced cell death, but it did not affect the cell death induced by MNNG. These findings suggest that the sustained activation of JNK mediated by RIP plays an important role in the DNA damage-induced cell death, and that the duration of JNK activation relays a different stress response to determine the cell fate.

Key Words: DNA damage, Cell death, Receptor interacting protein, c-Jun N-terminal kinase, Poly (ADP-ribose) polymerase

INTRODUCTION

The cellular response to genotoxic stress, such as radiation and DNA damage, includes activation of the transcription factor AP-1 and NF-κB (Canman and Kastan, 1996; Houghton, 1999). An early event in AP-1 induction by genotoxic stress is the activation of c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK) which mediates the phosphorylation of c-jun at serines 63 and 73 located within its transactivation domain (Derijard et al, 1994). Phosphorylation of these serine sites results in not only an increased transactivation potential of c-jun, a major component of AP-1, but also in other targets such as activating transcription factor 2 (ATF-2) and Ets-like transcription factor (ELK) (Raingeaud et al, 1996; Hayakawa et al, 2003). C-jun is a member of a multiprotein family that has been implicated in a number of signal transduction pathways associated with cellular growth, differentiation and cellular stress (Angel and Karin, 1991).

Various forms of cellular stress, including genotoxic agents, inflammatory cytokines and UV irradiation, stimulate the activation of JNK (Kyriakis et al, 1994; Kharbanda et al, 1995; Chen et al, 1996; Liu et al, 1996; Fritz and Kaina, 1999; Tournier et al, 2001). The underlying molecular mechanism of JNK activation by TNF has been well established. Upon TNF-receptor 1 (TNF-R1) trimerization in response to TNF, the TNFR1 is recognized by the adaptor protein TNF receptor-associated death domain (TRADD), which recruits additional adaptor proteins, receptor interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) (Shu et al, 1996; Chen and Goeddel, 2002). RIP and TRAF2 seem to be involved in both NF-κB and JNK activation. It is also believed that several mitogen-activated protein (MAP) kinases cascade in TNF signal transduction. Although MKK7/ JNKK2 has recently been identified as a specific JNK kinase that follows TNF treatment (Tournier et al, 2001), the corresponding MAPKKK remains still unknown. Several upstream kinases involved in this cascade, including

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ABBREVIATIONS: JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; RIP, receptor-interacting protein; Adr, adriamycin; Ara-C, 1-β-D-arabinofuranosylcytosine; MNNG, N-methyl-N-nitro-N-nitrosoguanidine; ATM, ataxia telangiectasia; PARP, poly (ADP-ribose) polymerase; 3-AB, 3-aminobenzamide; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone.

MEKK1 and apoptosis signal-regulating kinase 1 (ASK1), have been suggested to mediate the TNF-induced JNK activation (Liu et al, 1996; Ichijo et al, 1997).

Some efforts have been made to elucidate the underlying molecular mechanism of JNK signaling in response to genotoxic stress. There is some evidence to suggest that c-Abl, ATM or protein kinase C-δ (PKC-δ) functions upstream to JNK in the cellular response to genotoxic stress (Kharbanda et al, 1995; Liu et al, 1996; Wang 2000; Yoshida et al, 2002). However, upstream signaling components induced by genotoxic stress, leading to the activation of JNK or the primary sensors of the cells that trigger the stress response are still largely unidentified. Since we recently found that RIP plays a key role in DNA damage-induced NF-κB activation and p53-independent cell death (Hur et al, 2003; Hur et al, 2006), the present study was undertaken to examine the involvement of RIP in the activation of JNK by DNA damaging agents, such as adriamycin (Adr), 1-β-D-arabinofuranosylcytosine (Ara-c), and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) as well as UV irradiation.

The present study demonstrates that RIP was required for the activation of JNK in the cells when treated with certain DNA damaging agents such as Adr and Ara-C which cause slow and sustained activation, but not required by MNNG and UV-C which cause quick and transient activation. Our results also showed that this response was mediated by ATM-dependent or -independent mechanisms, suggesting that the activation of JNK induced by DNA damage is mediated through two distinct signaling pathways. Moreover, the long term activation of JNK was involved in the activation of poly (ADP-ribose) polymerase (PARP), thereby contributing to the DNA damage-induced cell death.

METHODS

Reagents

Glutathione S-transferase (GST)-c-jun (1-79) was expressed and purified from *Escherichia coli* as described elsewhere (Lin et al, 2000). Anti-JNK-1 and anti-PARP antibody were purchased from BD Pharmingen. Anti-RIP antibody was purchased from Transduction Laboratories. MNNG and Ara-C were purchased from Sigma. PARP inhibitors (3-aminobenzamide, 3,4-dihydro-5-[4-(1-piperidinyl) butoxyl]-1(2H)-isoquinolinone), Adr and SP600125 were purchased from Calbiochem. Anti-TNF-R1 and recombinant mouse TNF-α were purchased from R & D Systems. Protein A sepharose was purchased from Amersham Pharmacia Biotech.

Cell culture and treatment

Wild-type (wt), RIP-/-, ATM-/- and TNFR1-/- mouse embryonic fibroblast cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For UV irradiation of cells, the culture medium was removed and UV-C irradiated (20 J/m^2) where indicated, and the original culture medium was added back to the cells. Chemicals were added in the culture medium from concentrated stock solutions, as described in the figure legends.

Western blot analysis

After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris at pH 7.5, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin). Fifty micrograms of the cell lysates were resolved by SDS-polyacrylamide gel and blotted onto PVDF membrane. After blocking with 5% skim milk in PBST, the membrane was probed with the relevant antibody and visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instruction (Amersham Biosciences).

JNK kinase assay

After various treatments as described in the figure legends, cells were collected and lysed in M2 buffer. JNK1 was immunoprecipitated with anti-JNK1 antibody and protein A-sepharose beads by incubation at 4°C for 4 hrs to overnight. The beads were washed with lysis buffer, and kinase assay was then performed in complete kinase assay buffer (20 mM HEPES at pH 7.5, 20 mM β-glycerol phosphate, 10 mM $MgCl_2$, 1 mM DTT, 10 mM PNPP, 50 μM sodium vanadate, 20 μM ATP) with the addition of ^{32}p-γ-ATP and 1 μg of GST-c-Jun (1-79) as substrates. After 20 min at 30°C, sample buffer was added and proteins were resolved in 4~20% SDS-polyacrylamide gels, and phosphorylated substrates were visualized by autoradiography.

Detection of cell death

Cells were seeded in 6-well plates and treated with indicated concentrations of reagents as described in the figure legends. Cell death, as observed by a microscope, was characterized by rounding up and detaching of the cells from the plates. Cell death was quantified by trypsinization, followed by staining with trypan blue (Bio-Whittaker) and counting with a hemacytometer. The stained cells (blue) were counted as dead cells and were expressed as the percentage of total cells. For each treatment, triplicate experiments were performed three times.

Statistical analysis

Data are expressed as mean±SE from at least three separate experiments, performed in triplicate. The differences between groups were analyzed using a Student's t-test, and p values <0.05 were considered statistically significant. Statistical analyses were carried out using Statistical Package for Social Science software program (version 11.0; SPSS Inc. IL.).

RESULTS

RIP functions in activation of JNK in response to Adr and Ara-C, but not to MNNG and UV-C

Previous studies have demonstrated that genotoxic stresses, including UV and certain classes of DNA-damaging agents such as Adr, Ara-C and MNNG, activate JNK (Osborn and Chambers, 1996; Fritz and Kaina, 1999; Parra et al, 2000; Yoshida et al, 2002). In our present study, these

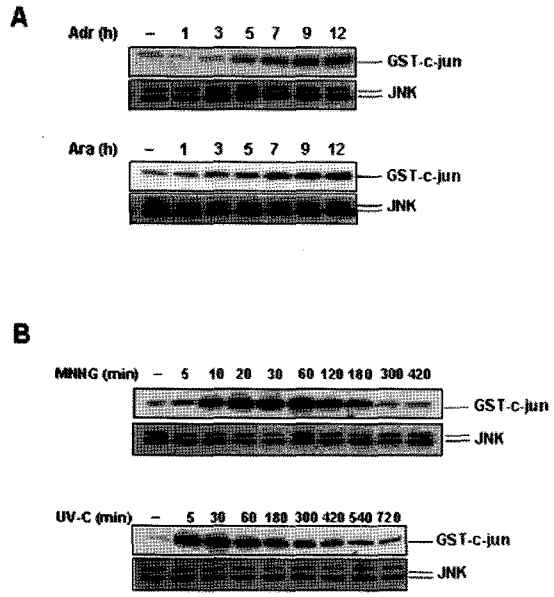

Fig. 1. Different kinetics of DNA damage-induced JNK activation in response to Adr/Ara-C and MNNG/UV-C treatment. Mouse wild-type (WT) fibroblasts were treated with 10 μg/ml of adriamycin (Adr), 50 μM 1-β-D-arabinofuranosylcytosine (Ara-C) (A) or 100 μM *N*-methyl-*N*(-nitro-*N*-nitrosoguanidine (MNNG), or exposed to 20 J/m^2 of UV-C (B) for various times as indicated on the figure. Cell extracts were subjected to immunoprecipitation with anti- JNK1 antibody, and its activity was assayed by immune complex kinase assay with [γ-^{32}P]ATP and GST-c-Jun (1~79) as the substrate. GST-c-Jun phosphorylation was assessed by SDS-PAGE and autoradiography (upper panel). JNK content was analyzed by Western blotting with anti-JNK antibody (lower panel).

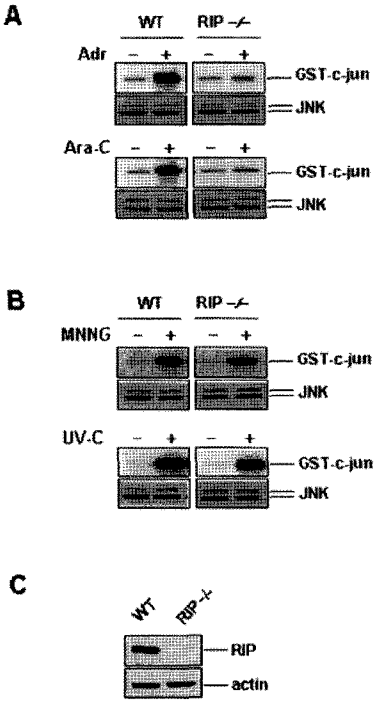

Fig. 2. RIP plays a role in JNK activation by Adr and Ara-C, but not by MNNG and UV-C. Wild-type (WT) and RIP-/- fibroblasts were left untreated (-) or treated with Adr (10 μg/ml) and Ara-C (50 μM) for 12 hrs (A), or MNNG (100 μM) and exposed to UV (20 J/m^2) for 30 min (B). JNK activity and expression were measured as described in the legend of Fig 1. The protein expression levels of RIP and β-actin in WT and RIP-/- fibroblasts. (C) The same amount of cell extracts from each cell line was applied to SDS-PAGE for Western blotting with anti-RIP and anti-β-actin antibodies.

agents were chosen because they represent the widely studied DNA-damaging agents with different mechanisms of action. Adr has a cytotoxicity through its ability to intercalate DNA and generate single or double-strand break; Ara-c is an antimetabolite as an analog of 2-deoxycytidine; MNNG is a monofunctional alkylating agent which reacts with a variety of nucleophilic sites in DNA and protein, generating various types of adducts; and UV induces the formation of thymidine dimmers and DNA strand breaks. To determine whether the activation of JNK is a general response to genotoxic stress, a time course study of UV and DNA-damaging agents, including Adr, Ara-C and MNNG treatment was conducted in wild-type mouse embryonic fibroblasts (WT), and the activity of JNK was measured at different time points after treatment by in vitro kinase assay with GST-c-Jun (1-79) as the substrate. As shown in Fig. 1, Adr and Ara-C induced a slow and sustained activation of JNK activity until after 12 hrs of treatment, whereas MNNG and UV-C induced a quick and transient increase of JNK activity, indicating that diverse DNA-damaging agents can induce JNK activation with different kinetics. Especially, the JNK activation induced by UV-C and MNNG was detected as early as 5 min after treatment. As a control of the amount of immunoprecipitates, the JNK protein level in the same extracts was examined by Western blotting with an anti-JNK antibody, and no difference was observed (Fig. 1A, B, bottom panels).

Because our previous study indicated that RIP plays a key role in DNA damage-induced NF-κB activation (Hur et al, 2003), we investigated whether RIP was involved also in JNK activation by these agents. To address this issue, we treated the cells, which were deficient in RIP (RIP-/-) cells, with DNA damaging agents and UV-C. Interestingly, JNK activation at 12 hrs after treatment with, either Adr or Ara-C was dramatically impaired in RIP-/- cells (Fig. 2A), whereas this was intact upon MNNG or UV-C treatment for 30 min (Fig. 2B). There was no expression of RIP protein in RIP-/- cells, and no change in (-action expression in these cells (Fig. 2C). These results suggest that RIP is required also for the activation of JNK by Adr and Ara-C, but not required for MNNG and UV-C.

Adr and Ara-C-induced JNK activation is independent of TNF signaling, but requires functional ATM

Previous studies have suggested that UV and DNA damage can induce the synthesis and release of cytokines, and that JNK activation by genotoxic stress is mediated through cell surface receptors such as TNF receptor 1 (TNFR1) and G protein-coupled receptors (Coso et al, 1996; Rosette and Karin, 1996; Sheikh et al, 1998; Tobin et al, 1998). Since our present data suggested that RIP, the adaptor protein of TNF signaling, was required for the activation of JNK in response to Adr or Ara-C, we examined the JNK

activation in wild-type and TNFR1-/- MEF cells in response to Adr and Ara treatment to rule out the involvement of TNF signaling in Adr- or Ara-C-induced JNK activation. The result showed that all these treatments (Adr/Ara-C as well as MNNG/UV-C) resulted in a similar level of JNK activation in the TNFR1-/- cells, compared to wild-type cells (Fig. 3, top panel), suggesting that Adr or Ara-C stimulates the JNK activation by TNF-signaling independent. The expression of TNF-R1 in these two types of cells is shown in the middle panel of Fig. 3. To be sure that TNF signaling

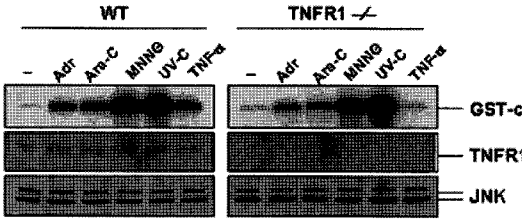

Fig. 3. Genotoxic stress-induced JNK activation is independent of TNF receptor 1 signaling. Wild-type (WT) and TNFR1-/- fibroblasts were left untreated (-) or treated with Adr (10 μg/ml) and Ara-C (50 μM) for 12 hrs , MNNG (100 μM) for 30 min or TNF-α (15 ng/ml) for 15 min and exposed to UV (20 J/m^2) for 30 min. JNK activity (top) was measured as described in the legend of Fig 1. The same cell lysates were also analyzed for the expression of TNFR1 (middle) and JNK (bottom) by Western blotting with corresponding anti-TNFR1 and anti-JNK antibodies.

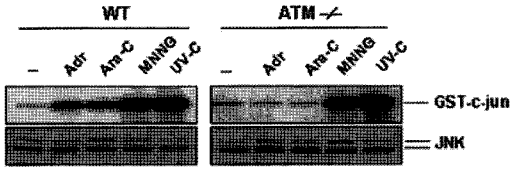

Fig. 4. ATM is required for JNK activation by Adr and Ara-C, but not by MNNG and UV-C. Wild-type (WT) and ATM-/- fibroblasts were left untreated (-) or treated with Adr (10 μg/ml) and Ara-C (50 μM) for 12 hrs or MNNG (100 μM) for 30 min, and exposed to UV (20 J/m^2) for 30 min. JNK activity and expression were measured as described in the legend of Fig. 1.

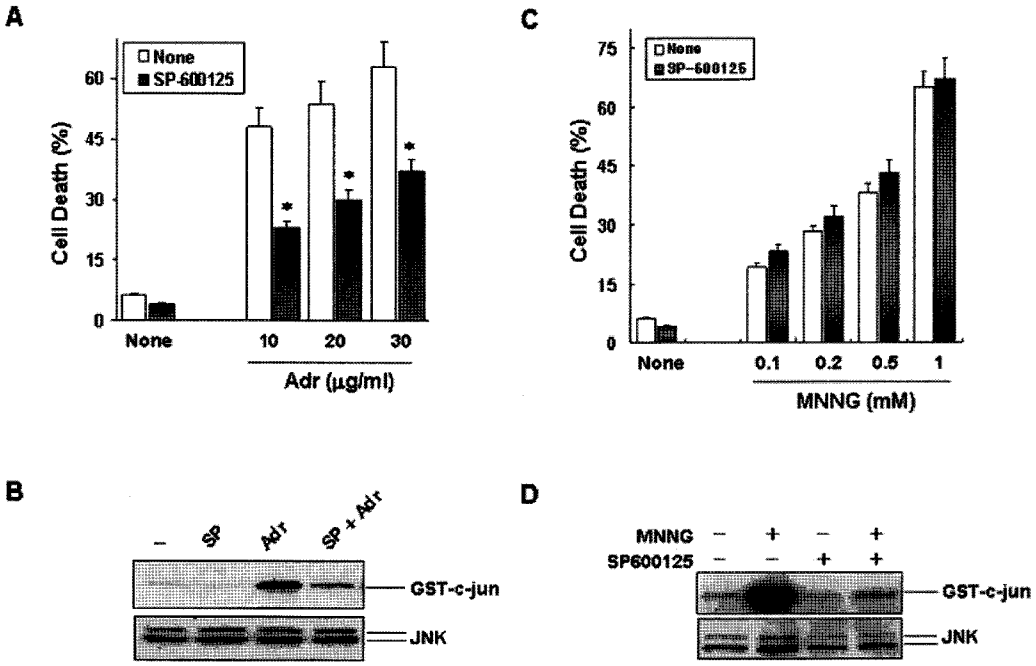

Fig. 5. Duration of JNK activation has an important role in DNA damage-induced cell death. (A) Long term activation of JNK is associated in the Adr-induced cell death. Wild-type (WT) fibriblasts were pretreated with SP-600125 (20 μM) for 30 min and then treated with various doses of Adr as indicated on the figure. At 24 hrs after treatment, the percentage of cell death was determined by trypan blue exclusion assay, as described under "Materials and Methods". Each bar shows mean±SE of at least three independent experiments. *$p<0.05$, when compared with Adr-treated group. (B) SP-600125 abrogates the Adr-induced JNK activation. WT fibriblasts were pretreated with SP-600125 (20 μM) for 30 min and treated with Adr (10 μg/ml) for 12 hrs. JNK activity and expression were measured as described in the legend of Fig. 1. (C) Rapid and short term activation of JNK is not associated with the MNNG- induced cell death. (WT) fibriblasts were pretreated with SP- 600125 (20 μM) for 30 min and treated with various doses of MNNG as indicated on the figure. At 24 hrs after treatment, the percentage of cell death was determined as described in (A). (D) WT fibriblasts were pretreated with SP-600125 (20 μM) for 30 min and then treated with MNNG (100 μM) for 30 min, and JNK activity was determined as described in (B).

is defective in TNFR1-/- cells, the TNF-induced JNK activation was examined. As shown in the top panel of Fig. 3, no JNK activation was detected in TNFR1/- cells after TNF treatment, as compared with wild-type cells.

The serine/threonine protein kinase ATM (ataxia telangiectasia mutated) has previously been suggested to be activated in response to genotoxic stresses such as IR and DNA damaging agents, and that it is required for DNA double breaks-induced JNK activation (Zhang et al, 2002; Bakkenist and Kastan, 2003). Furthermore, our present study indicated above that Adr (or Ara-C) and MNNG (or UV-C) may induce the JNK activation through a different upstream signaling component (Fig. 2), therefore we further examined whether ATM also has a role in the JNK activation as an upstream event after treatment with UV or these agents. As shown in Fig. 4, the JNK activation could not be detected in ATM-/- fibroblasts after treatment with either Adr or Ara-C for 12 hrs. On the other hand, treatment of ATM-/- cells with MNNG or UV-C for 30 min resulted in the JNK activation, almost comparable with that in the wild-type cells, suggesting that, unlike MNNG or UV-C, the Adr- or Ara-C induced JNK activation required ATM.

Duration of JNK activation has an important role in DNA damage-induced cell death

The signaling pathway in response to TNF and genotoxic stress is thought to modulate cell death, although JNK alone may not be sufficient to induce cell death (Karin and Lin, 2002; Tang et al, 2002). Since our data suggested that Adr and MNNG have different kinetics of JNK activation (Fig. 1), we next investigated whether the duration of JNK activation may contribute to determination of proliferation or death of cell. Thus, we first tested whether the specific JNK inhibitor SP600125 can inhibit the Adr- or MNNG-induced cell death in wild-type cells. As shown in Fig. 5A, pretreatment of the wild-type cells with SP600125 led to a significant suppression of Adr-induced cell death. The results of an in vitro kinase assay showed that SP600125 efficiently inhibited the Adr-induced JNK activation (Fig. 5B), and that this JNK inhibition was correlated with the inhibition of cell death. However, SP600125 failed to protect the MNNG-induced cell death (Fig. 5C), although it could completely block the JNK activation induced by MNNG treatment (Fig. 5D). These data suggest that the sustained activation of JNK in response to Adr, but not in response to MNNG, may contribute to directing the outcome to cell death.

Recent reports indicated that the activation of PARP and the depletion of ATP also may play a role in stress-induced nonapoptotic cell death, including DNA damaging agents or oxidative stress (Byun et al, 2006; Lewis and Low, 2007; Heeres and Hergenrother, 2007; Zhang et al, 2007). Since sustained JNK activation contributed to DNA damage-induced cell death (Fig. 5A), we evaluated the relationships between each of these signaling components. Pretreatment with either PARP inhibitors, 3-aminobenzamide (3-AB) or 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ), suppressed the DNA damage-induced cell death to an extent comparable to that by the specific JNK inhibitor SP600125 (Fig. 6A), indicating that the activity of PARP may participate in the DNA damage-induced cell death. Furthermore, immunoblotting with anti-PAR antibody, specific for poly (ADP-ribose) polymers, showed that poly(ADP-ribosyl)ation was increased significantly by Adr, and pretreatment of JNK inhibitor SP600125 markedly suppressed PARP activation (Fig. 6B), suggesting that the sustained JNK activation contributes to the activation of PARP. Because RIP was required for sustained activation of JNK upon treatment with DNA damaging agent (Fig. 2), we next compared the extent of poly(ADP-ribosyl)ation of RIP-/- and wild type MEF cells after Adr treatment. As expected, when wild-type cells were treated with Adr, the amount of poly(ADP-ribosyl)ation increased gradually in a time-dependent manner (Fig. 6C, left panel). In RIP-/- cells, however, the PARP activity did not increase (Fig. 6C, right panel). These data provide additional evidence that the activation of PARP mediated by RIP contributes to DNA damage-induced cell death.

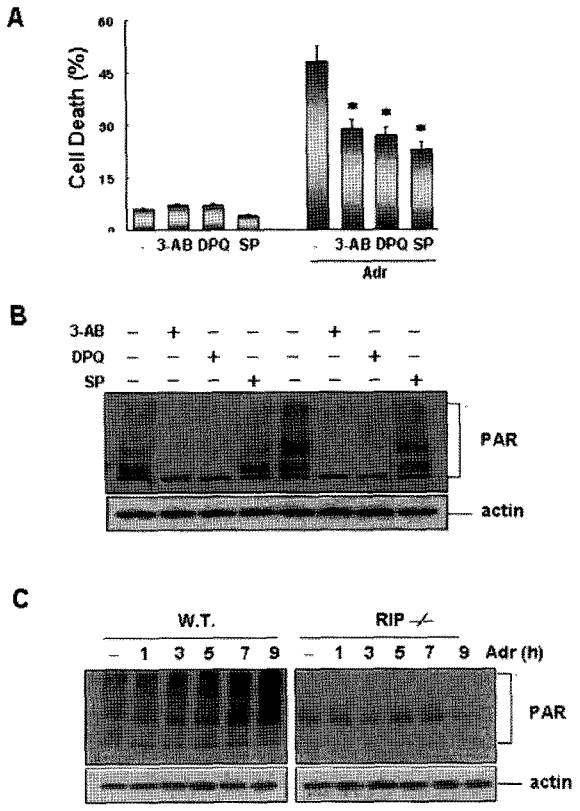

Fig. 6. JNK plays as an upstream effector of DNA damage-induced PARP activation. (A) Wild-type (WT) fibriblasts were pretreated with either SP-600125 (20 μM) or PARP inhibitors (1 mM 3-AB or 30 μM DPQ). They were then treated with Adr (10 μg/ml) for 24 hrs, and cell death was then quantified by trypan blue exclusion assay, as described in the legend of Fig 5A. Each bar shows mean ±SE of at least three independent experiments. *$p<0.05$, when compared with Adr-treated group. (B) Wild-type (WT) fibriblasts were treated with Adr (10 μg/ml) for 12 hrs in the absence or presence of SP-600125 or PARP inhibitors (1 mM 3-AB or 30 μM DPQ), as indicated. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR and β-actin. (C) Wild-type and RIP-/- cells were treated with Adr (10 μg/ml) for various times, as indicated. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR and β-actin.

DISCUSSION

The cellular response to genotoxic stress such as UV, IR and DNA-damaging agents has been analyzed extensively, and it is known to activate different MAPKs (ERK, JNK, and p38) as well as NF-κB depending on the cell systems (Kyriakis et al, 1994; Kharbanda et al, 1995; Canman and Kastan, 1996; Chen et al, 1996; Bulavin et al, 1999; Fritz and Kaina, 1999; Houghton, 1999; Tang et al, 2002). Although some efforts have been made to elucidate the underlying molecular mechanism of JNK signaling in response to genotoxic stress, the pathway to transduce signals to JNK remained unsolved. We have recently demonstrated that RIP plays a key role in DNA damage-induced NF-κB activation (Hur et al, 2003). On the basis of this observation, the present study was undertaken to elucidate whether RIP, an important effector of TNF signaling, is involved in JNK activation in response to various genotoxic stresses, including different types of DNA-damaging agents and UV-C. In particular, to address the question of primary cellular targets that induce the JNK activation by this genotoxic stress, we investigated physiological significance of JNK activation in the DNA damage-induced cell death.

It has been reported that the activation of JNK after IR or DNA-damaging agent treatment appears to be mediated by ATM or c-Abl, but is not entirely dependent on it (Liu et al, 1996; Pandey et al, 1996), therefore, suggesting that ATM or c-Abl might be an upstream effector of the JNK pathway in response to certain types of genotoxic stress. In the present study, we demonstrated that diverse types of genotoxic stress can induce JNK activation through different signaling pathways: Adr- and Ara-C-induced JNK activation was greatly decreased in RIP-/- cells, however, MNNG and UV-C elicited the same extents of JNK activation in RIP-/- cells as compared to wild-type fibroblasts (Fig. 2). These findings provided a novel evidence for the involvement of RIP as an upstream effector in the Adr and Ara-C-induced JNK activation. On the other hand, MNNG and UV-C induced the activation of JNK by a RIP-independent mechanism. Since the present data suggested that this key TNF signaling molecule had an important role in the Adr- and Ara-C-induced JNK activation, we further investigated whether TNFR1 signaling was involved. Consistent with our recent study that the DNA damage-induced NF-κB activation is TNFR1-independent (Hur et al, 2003), Adr- and Ara-C-induced JNK activation was not affected in TNFR1-/- cells, suggesting that the JNK activation induced by these DNA damaging agents was not achieved through TNF signaling, although RIP was required (Fig. 3). Therefore, the mechanism of how RIP mediates the JNK activation after DNA damage remains unclear. One possibility is that DNA damage may induce a complex between RIP and other adaptor proteins such as TRAF2, leading to the JNK activation, as previously shown for TNF signaling. Unfortunately, we failed to detect the complex of RIP and TRAF2 by an immunoprecipitation (data not shown). Therefore, further studies are necessary to elucidate the role of RIP and other adaptor proteins in the DNA damage-induced JNK activation.

One of the interesting findings in this study was that the induction kinetics of JNK activation in response to these DNA-damaging agents were quite different. The Adr- and Ara-C- induced JNK activation peaked at 12 hrs and showed a sustained activation, whereas the MNNG-induced JNK activation occurred much earlier and was as transient as that of UV-C exposure, showing a maximal activation at 10 min to 1 hr after the treatment (Fig. 1): Indeed, the MNNG-induced JNK activation could be detected as early as 5 min after the treatment. Previous studies have provided clear evidence that UV-C can affect targets at the cell membrane, suggesting that UV responses are independent of nuclear event (Devary et al, 1993; Rosette and Karin, 1996; Sheikh et al, 1998). On the basis of these observations, we hypothesized that the primary cellular targets of Adr (or Ara-C) and MNNG might be different, even though it is generally accepted that nuclear DNA is the critical target of these agents. Interestingly, we found that ATM, which is crucial for initiation of signaling pathway in the nucleus following DNA damage, was required for the induction of JNK activation by Adr (or Ara-C), but was not required by MNNG (Fig. 4). These finding indicate that Adr and MNNG clearly affect different primary targets to trigger the initiation of signaling pathway which involves JNK. However, the signaling event that was initially triggered by MNNG, but leading to JNK activation, remains to be identified. Since it has been reported that UV induces a ligand-independent clustering of TNFR1 to recruitment of TRAF2 (Tobin et al, 1998), it is possible that MNNG may activate the cell surface receptor such as TNFR1, which is sufficient for JNK activation in this case. We found in the present study that the TNFR1-/- cells did not show a significant difference from wild-type cells in response to the MNNG-induced JNK activation, therefore it is unlikely that TNFR1 is responsible for the induction of JNK activation in response to MNNG.

Several studies have demonstrated that JNK is involved both in cell growth and cell death pathways (Karin and Lin, 2002; Tang et al, 2002). However, the factors which determine the various outcomes of JNK signaling are still unknown. Recently, Tang et al. (2002) have reported that the duration of activation appears to be critical for determining whether JNK affects the susceptibility of cells to TNF killing. Consistent with this finding, we also found that difference in the duration of the activation of JNK is correlated with DNA damage-induced cell death. Inhibition of the sustained JNK activation by specific JNK inhibitor SP600125 significantly reduced Adr-induced cell death, whereas inhibition of the early MNNG-induced JNK activation had no effect (Fig. 5). Although we cannot rule out the possibility that SP600125 can block Adr-induced cell death by inhibiting targets other than JNK, it is likely that the sustained JNK activation is involved in cell death induced by Adr. This possibility is further supported by our recent observation (Hur et al, 2006) that the Adr-induced cell death was more resistant in JNK1-/- as well as RIP-/- cells.

Overall, our findings show that the signals generated by distinct forms of DNA damage are transduced by different signaling pathways. We demonstrated that RIP has a crucial function in Adr- or Ara-C-induced JNK activation through ATM. On the other hand, MNNG induces JNK activation through RIP-independent mechanism and a hitherto unknown sensor. These findings led us to conclude that the activation of JNK by different signaling pathways may relay different stress responses to determine the cell fates.

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REFERENCES

Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1072: 129−157, 1991

Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421: 499−506, 2003

Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, Fornace AJ Jr. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J* 18: 6845−6854, 1999

Byun HS, Park KA, Won M, Yang KJ, Shin S, Piao L, Kwak JY, Lee ZW, Park J, Seok JH, Liu ZG, Hur GM. Phorbol 12-myristate 13-acetate protects against tumor necrosis factor (TNF)-induced necrotic cell death by modulating the recruitment of TNF receptor 1-associated death domain and receptor-interacting protein into the TNF receptor 1 signaling complex: Implication for the regulatory role of protein kinase C. *Mol Pharmacol* 70: 1099−1108, 2006

Canman CE, Kastan MB. Signal transduction. Three paths to stress relief. *Nature* 384: 213−214, 1996

Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* 271: 31929−31936, 1996

Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science* 296: 1634−1635, 2002.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 81: 1137−1146, 1995

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76: 1025−1037, 1994

Devary Y, Rosette C, DiDonato JA, Karin M. NF-kappa B activation by ultraviolet light not dependent on a nuclear signal. *Science* 261: 1442−1445, 1993

Fritz G, Kaina B. Activation of c-Jun N-terminal kinase 1 by UV irradiation is inhibited by wortmannin without affecting c-iun expression. *Mol Cell Biol* 19: 1768−1774, 1999

Hayakawa J, Depatie C, Ohmichi M, Mercola D. The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *J Biol Chem* 278: 20582−20592, 2003

Heeres JT, Hergenrother PJ. Poly (ADP-ribose) makes a date with death. *Curr Opin Chem Biol* 11: 644−653, 2007

Houghton JA. Apoptosis and drug response. *Curr Opin Oncol* 11: 475−481, 1999

Hur GM, Lewis J, Yang Q, Lin Y, Nakano H, Nedospasov S, Liu ZG. The death domain kinase RIP has an essential role in DNA damage-induced NF-kappa B activation. *Genes Dev* 17: 873−882, 2003

Hur GM, Kim YS, Won M, Choksi S, Liu ZG. The death domain kinase RIP has an important role in DNA damage-induced, p53-independent cell death. *J Biol Chem* 281: 25011−25017, 2006

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275: 90−94, 1997

Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 3: 221−227, 2002

Kharbanda S, Ren R, Pandey P, Shafman TD, Feller SM, Weichselbaum RR, Kufe DW. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature* 376: 785−788, 1995

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156−160, 1994

Lewis C, Low JA. Clinical poly (ADP-ribose) polymerase inhibitors for the treatment of cancer. *Curr Opin Investig Drugs* 8: 1051−1056, 2007

Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S, Liu ZG. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. *Mol Cell Biol* 20: 6638−6645, 2000

Liu ZG, Baskaran R, Lea-Chou ET, Wood LD, Chen Y, Karin M, Wang JY. Three distinct signalling responses by murine fibroblasts to genotoxic stress. *Nature* 384: 273−276, 1996

Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. *J Biol Chem* 271: 30950−30955, 1996

Pandey P, Raingeaud J, Kaneki M, Weichselbaum R, Davis RJ, Kufe D, Kharbanda S. Activation of p38 mitogen-activated protein kinase by c-Abl-dependent and independent mechanisms. *J Biol Chem* 271: 23775−23779, 1996

Parra M, Lluis F, Miralles F, Caelles C, Munoz-Canoves P. The cJun N-terminal kinase (JNK) signaling pathway mediates induction of urokinase-type plasminogen activator (uPA) by the alkylating agent MNNG. *Blood* 96: 1415−1424, 2000

Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* 16: 1247−1255, 1996

Rosette C, Karin M. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* 274: 1194−1197, 1996

Sheikh MS, Antinore MJ, Huang Y, Fornace AJ. Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1. *Oncogene* 17: 2555−2563, 1998

Shu HB, Takeuchi M, Goeddel DV. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc Natl Acad Sci* 93: 13973−13978, 1996

Tang F, Tang G, Xiang J, Dai Q, Rosner MR, Lin A. The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. *Mol Cell Biol* 22: 8571−8579, 2002

Tobin D, van Hogerlinden M, Toftgard R. UVB-induced association of tumor necrosis factor (TNF) receptor 1/TNF receptor-associated factor-2 mediates activation of Rel proteins. *Proc Natl Acad Sci* 95: 565−569, 1998

Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev* 15: 141914−141926, 2001

Wang JY. Regulation of cell death by the Abl tyrosine kinase. *Oncogene* 19: 5643−5650, 2000

Yoshida K, Miki Y, Kufe D. Activation of SAPK/JNK signaling by protein kinase Cdelta in response to DNA damage. *J Biol Chem* 277: 48372−48378, 2002

Zhang Y, Ma WY, Kaji A, Bode AM, Dong Z. Requirement of ATM in UVA-induced signaling and apoptosis. *J Biol Chem* 277: 3124−3131, 2002

Zhang S, Lin Y, Kim YS, Hande MP, Liu ZG, Shen HM. c-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly (ADP-ribose) polymerase-1 activation. *Cell Death Differ* 14: 1001−1010, 2007