# c-Jun N-terminal Kinase Activation is Required for Apoptotic Cell Death Induced by TNF-related Apoptosis-inducing Ligand plus DNA-damaging Agents in Sarcoma Cell Lines

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**Abstract.** *Background: Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in combination with a chemotherapeutic agent, cis-diammine dichloroplatinum (CDDP) or doxorubicin (DXR), has recently been demonstrated to result in enhanced apoptotic cell death in the sarcoma cell lines MG-63 and SaOS-2. DNA-damaging agents, such as CDDP induced sustained activation of c-Jun N-terminal kinase (JNK), probably leading to apoptosis. In the present study, whether JNK activation is involved in apoptotic cell death induced by combined treatment with CDDP/DXR and TRAIL was addressed. Results: MG-63 or SaOS-2 cells overexpressing the dominant-negative (dn) form of JNK (dnJNK1) were established by transfection with dnJNK1 cDNA. Following stimulation with the chemotherapeutic agent CDDP or TRAIL, both MG-63 and SaOS-2 cells demonstrated enhanced cell death compared with stimulation by either agent alone, as assayed for apoptosis using annexin V staining or mitochondrial membrane potential using $DiOC_6$ staining. Interestingly, partial inhibition of the cell death induced by the combined treatment with CDDP/DXR and TRAIL was found in MG-63 or SaOS-2 cells overexpressing dnJNK1, suggesting that JNK activation is required for the combined treatment. Moreover, induction of caspase-8 activation by TRAIL or TRAIL plus CDDP/DXR was substantially prevented by dnJNK. Conclusion: Efficient cell death induced by combined treatment with the chemotherapeutic agents CDDP/DXR and TRAIL is involved in JNK activation in the sarcoma cell lines MG-63 and SaOS-2. These results would be useful for treatment modalities of patients with sarcoma.*

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Adjuvant chemotherapy in combination with surgical treatment has substantially improved the long-term survival rate of patients with diverse tumors, including osteosarcoma, one of the most common malignant tumors in bone (1, 2). However, clinical use is, in some cases, limited by the inherent and/or acquired resistance to these chemotherapeutic agents (3, 4). Several mechanisms of the resistance to chemotherapeutic drugs include defects in normal apoptotic programs, which result from defective apoptotic components, including Bcl-2 family proteins and mutation in p53 (5, 6). The Bcl-2 family members are composed of pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-$x_L$) proteins, and regulate mitochondrial function (7-9). Mitochondrial dysfunction accompanies apoptosis (9) and the induction of apoptosis is regulated by several components, including mitogen-activated protein kinase (MAPK) family proteins, which are comprised of at least 3 members: extracellular signal related kinases (ERKs), p38MAPK and c-Jun N-terminal kinase (JNK) (10, 11). Activation of JNK promotes apoptotic cell death (11-15), whereas ERK activation favors cell survival in some cell lineages after exposure to diverse stimuli.

Mitochondria-mediated signaling pathways have been demonstrated to synergize with death-receptor-mediated pathways to induce apoptotic cell death in some cell types (9, 16). We and others have recently demonstrated that the combined treatment of chemotherapeutic agents with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) synergistically induces apoptosis in a variety of tumors, including sarcoma and lymphoma cell lines (17-21). TRAIL, a member of the TNF family proteins, induces

apoptosis in diverse tumor cell types, but does not affect normal cells (22, 23). Stimulation with TRAIL results in caspase-8 activation, which is amplified through mitochondria in a variety of cell types (23). However, the molecular mechanisms of the combined treatment with chemotherapeutic agents and TRAIL are not completely known.

In the present study, the dominant-negative form of JNK1 (dnJNK1) was employed to assess whether DNA-damaging agents (cis-diammine dichloroplatinum, CDDP, and doxorubicin, DXR) in conjunction with TRAIL utilize JNK activation pathways to induce cell death in the sarcoma cell lines SaOS-2 and MG-63 (24), lacking p53. JNK activation is demonstrated to be involved in the apoptotic cell death induced by the combined treatment of CDDP/TRAIL or DXR/TRAIL.

## Materials and Methods

*Cell culture.* The human sarcoma cell lines MG-63 and SaOS-2 were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and were maintained in a culture medium (cRPMI-1640) consisting of RPMI-1640 (JRH Biosciences, Lenexa, Australia) with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μg/ml kanamycin, at 37˚C in humidified air with 5% $CO_2$. The MG-63 and SaOS-2 cell lines overexpressing dnJNK1 (MG-63/dnJNK1, SaOS-2/dnJNK1) were generated by transfection using the expression vector dnJNK1/pMKITNeo, followed by selection in the RPMI medium with Geneticin (G418, 400 μg/ml) (Sigma, St. Louis, MO, USA).

*Luciferase reporter assay.* Cells ($1x10^5$) in 1 ml of cRPMI-1640 medium were transfected with 2 μg of the reporter constructs (pAP1-Luc) (BD Biosciences, San Jose, CA, USA) using FuGene 6 (Rosche, Indianapolis, IN, USA), according to the manufacturer's instructions. After an overnight incubation with RPMI-1640 medium alone, the cells were exposed to 3 μg/ml CDDP or DXR for 24 h and luciferase activity from the cell extract was determined with a Luciferase Assay System (Promega, Madison, WI, USA), according to the manufacturer's recommendations. The luciferase activities were monitored using a Lumat LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

*Flow cytometric analysis of apoptosis and mitochondrial membrane potential.* Cells stimulated with chemotherapeutic agents and/or TRAIL were assayed for apoptosis using an Annexin V-Cy5 Apoptosis Detection Kit (BioVision, Inc., Mountain View, CA, USA), which detects redistribution of intracellular phosphatidylserine onto the extracellular surface. Briefly, cells stained with annexin V-Cy5 were subjected to flow cytometric analysis using a Nippon Becton Dickinson FACSCalibur cytometer (Tokyo, Japan). The percentage of apoptotic cells was determined using CELL QUEST software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). For determination of loss of mitochondrial membrane potential ($\Delta\Psi m$), fluorochrome $DiOC_6$ (Molecular Probes, Eugene, OR, USA) was used as previously described (13). Briefly, cells were loaded with 2 nM $DiOC_6$ for 30 min, followed by analysis on a flow cytometer.

*Assay for caspase-8 activation.* The assay for caspase-8 was carried out according to the manufacturer's instructions (MBL, Nagoya, Japan). Briefly, cells were suspended in a lysis buffer, kept on ice for 10 min and then centrifuged. The supernatants were harvested to assess the enzymatic activity. The enzymatic reactions were carried out in a buffer containing supernatant proteins (50 μg/sample) and the caspase-8 substrate IETD-*p*-nitroanilide (*p*NA) at 37˚C for 3 h, followed by colorimetric detection of *p*NA at a wavelength of 405 nm using a microplate reader (BioRad, Hercules, CA, USA).

*Statistical analysis.* Data were expressed as the means±SD for each group. The statistical analysis was carried out with the StatView for Windows software package. To determine the effectiveness of a combination of chemotherapeutic drugs with TRAIL, a two-way factorial ANOVA was performed. Other comparisons were done with the Student's *t*-test. Statistical significance was set at 0.05.

## Results

*Establishment of SaOS-2 and MG-63 cell lines overexpressing dnJNK1.* We have recently found that CDDP or DXR induced sustained activation of JNK in the sarcoma cell lines SaOS-2 and MG-63 (Koyama *et al.,* submitted for publication). To examine the causal effect of JNK activation on the apoptosis induced by chemotherapeutic agents together with TRAIL, cell lines overexpressing dnJNK1 were established. SaOS-2 or MG-63 cells were transfected with dnJNK1 or the control vector alone, followed by selection with G418-containing medium. The resulting transformants were assessed by the expression of exogenous Flag-dnJNK1. Western blot analysis showed that the transformants (MG-63/dnJNK1, #B2 and #H5; SaOS-2/dnJNK1, #B12 and #H3) derived from either MG-63 or SaOS-2 contained exogenous dnJNK1 (47 kDa) in addition to the endogenous (46 kDa) protein (Figure 1A).

To verify that dnJNK1 competes with the endogenous JNK activation cascade, the transcription activity of the JNK downstream element AP-1 was employed. The MG-63/dnJNK1 cells were transfected with an AP-1 reporter plasmid and then stimulated with CDDP for 3 h, followed by assay for reporter activity. After stimulation with CDDP, the MG-63/dnJNK1 cells demonstrated a reduced level of reporter activity, compared with those expressing the vector alone (Figure 1B). Similar dnJNK1 activity was detected in the dnJNK1-expressing SaOS-2 cells (data not shown). These results indicated that dnJNK1 serves as a competitive inhibitor of JNK activation in response to chemotherapeutic agents.

*Mitochondrial membrane potential induced by CDDP or DXR in combination with TRAIL is partially blocked in dnJNK1-overexpressing SaOS-2 and MG-63 cell lines.* Apoptotic cell death induced by DNA-damaging agents is accompanied by loss of $\Delta\Psi m$ in a variety of cell types (7-9, 19). MG-63 cells expressing dnJNK1 or the control vector alone were stimulated with or without CDDP or DXR for 24 h and

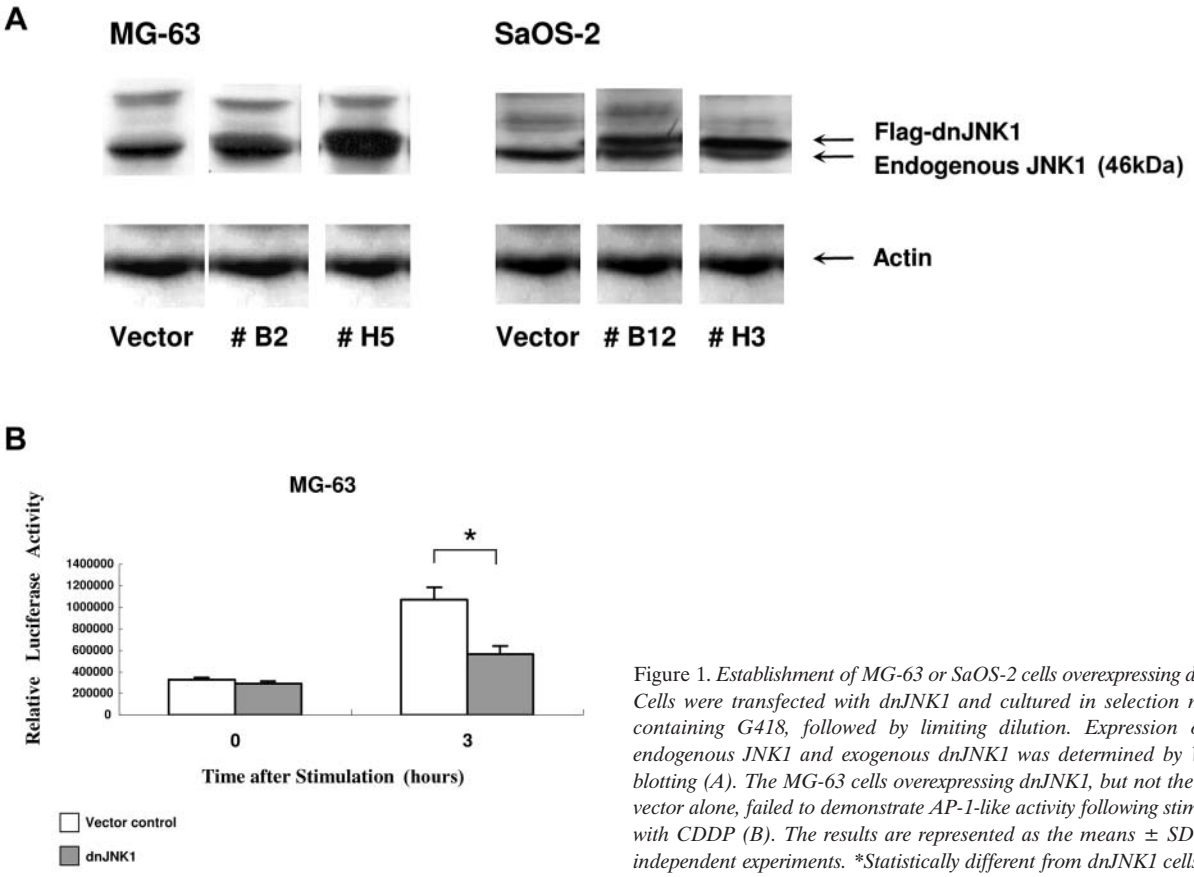


Figure 1. *Establishment of MG-63 or SaOS-2 cells overexpressing dnJNK1. Cells were transfected with dnJNK1 and cultured in selection medium containing G418, followed by limiting dilution. Expression of both endogenous JNK1 and exogenous dnJNK1 was determined by Western blotting (A). The MG-63 cells overexpressing dnJNK1, but not the control vector alone, failed to demonstrate AP-1-like activity following stimulation with CDDP (B). The results are represented as the means ± SD from 3 independent experiments. *Statistically different from dnJNK1 cells.*

then exposed to TRAIL for a further 24 h, followed by assay for ΔΨm. The loss of ΔΨm triggered by the combined treatment was partially abrogated in the MG-63/dnJNK1 cells, compared with control cells (Figure 2A). Similarly, dnJNK1 reduced the loss of ΔΨm in SaOS-2 cells after the combined stimulation (Figure 2B). These results suggest that JNK activation is required for loss of ΔΨm following stimulation with one of the chemotherapeutic drugs (CDDP or DXR) plus TRAIL.

*Apoptotic cell death induced by CDDP or DXR in combination with TRAIL is partially blocked in dnJNK1-overexpressing SaOS-2 and MG-63 cell lines.* We recently demonstrated that the chemotherapeutic agents CDDP and DXR sensitized sarcoma cells to TRAIL-induced apoptosis (19, 25). Diverse apoptotic stimuli induce prolonged JNK activation, leading to apoptosis (13, 14). To examine if JNK activation is involved in apoptotic cell death induced by the combined treatment with CDDP/DXR and TRAIL, cell lines overexpressing dnJNK1 were utilized. The annexin V staining method was employed to detect the early phase of apoptosis (26). Either chemotherapeutic agent (CDDP or DXR) or TRAIL induced marginal levels of apoptosis in MG-63 cells (Figure 3A). When MG-63 cells were pretreated with CDDP or DXR, TRAIL induced enhanced cell death (Figure 3A, B). The apoptotic cell death induced by either the combined treatment or a single reagent alone was partially prevented in the MG-63/dnJNK1 cells, compared with control cells (Figure 3B). A similar dnJNK1-mediated blockade of apoptotic cell death was also found in SaOS-2 cells (Figure 3C). These results suggest that JNK activation mediates apoptotic cell death triggered by CDDP/DXR and TRAIL in sarcoma cells.

*Caspase-8 activation induced by CDDP or DXR combined with TRAIL is partially blocked in dnJNK1-overexpressing SaOS-2 and MG-63 cell lines.* TRAIL induces caspase-8 activation through interaction with its receptor, TRAIL-Rs. We assessed if TRAIL-induced caspase-8 activation is enhanced by pretreatment with chemotherapeutic agents and, if so, whether JNK activation is necessary for the enhanced caspase-8 activation. Pretreatment with CDDP or DXR enhanced TRAIL-induced caspase-8 activation, which was substantially reduced in the MG-63/dnJNK1 cells

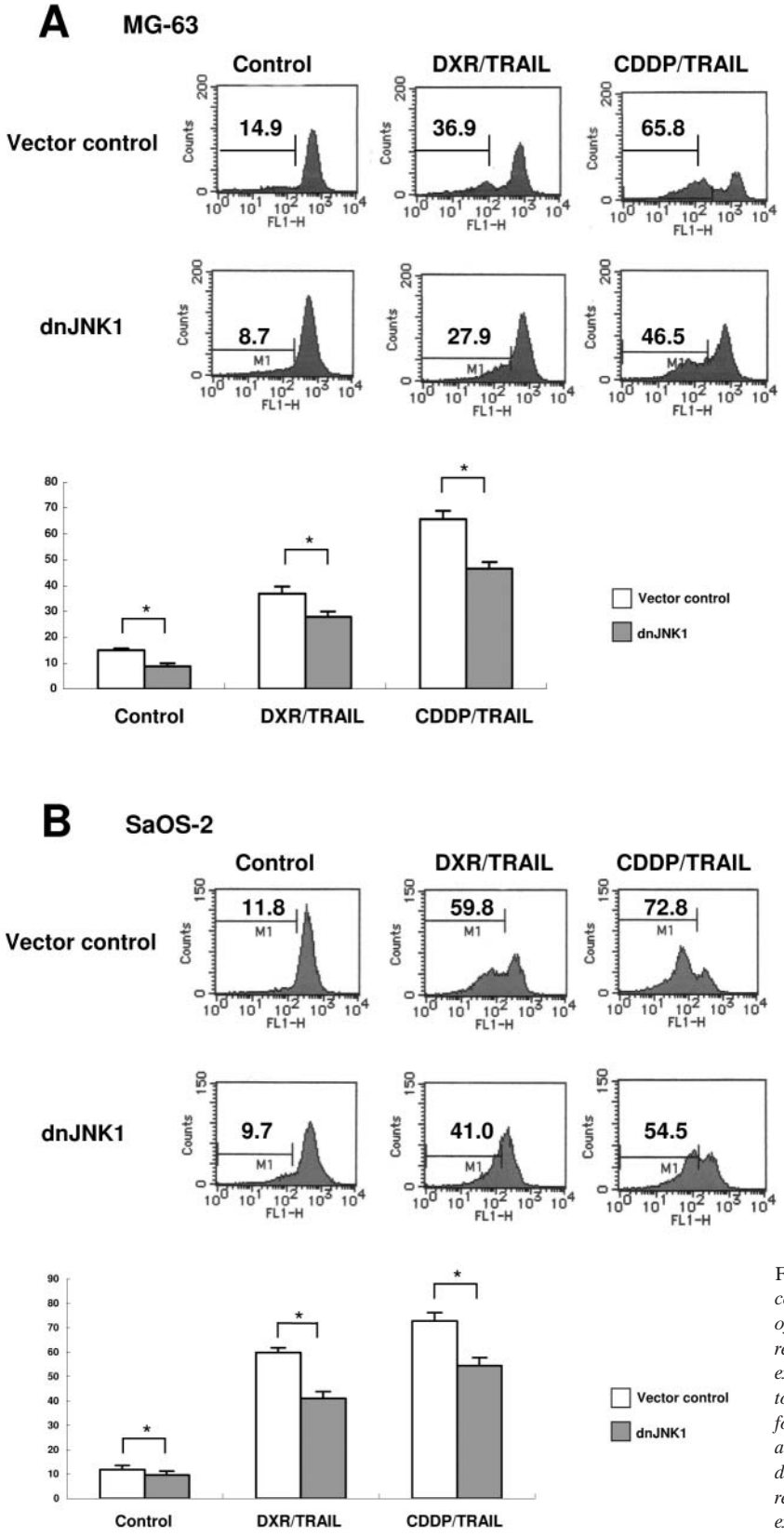


Figure 2. *Treatment with CDDP or DXR in combination with TRAIL resulted in an enhanced loss of mitochondrial membrane potential, which was reduced by dnJNK1. MG-63 (A) or SaOS-2 (B) cells expressing dnJNK1 or the control vector were exposed to DXR (3 μg/ml) or CDDP (3 μg/ml) for 24 h, followed by stimulation with TRAIL (20 ng/ml) for an additional 24 h. The cells were assayed for ΔΨm, as described in Materials and Methods. The results are represented as the means±SD from 3 independent experiments. *Statistically different from dnJNK1 cells.*

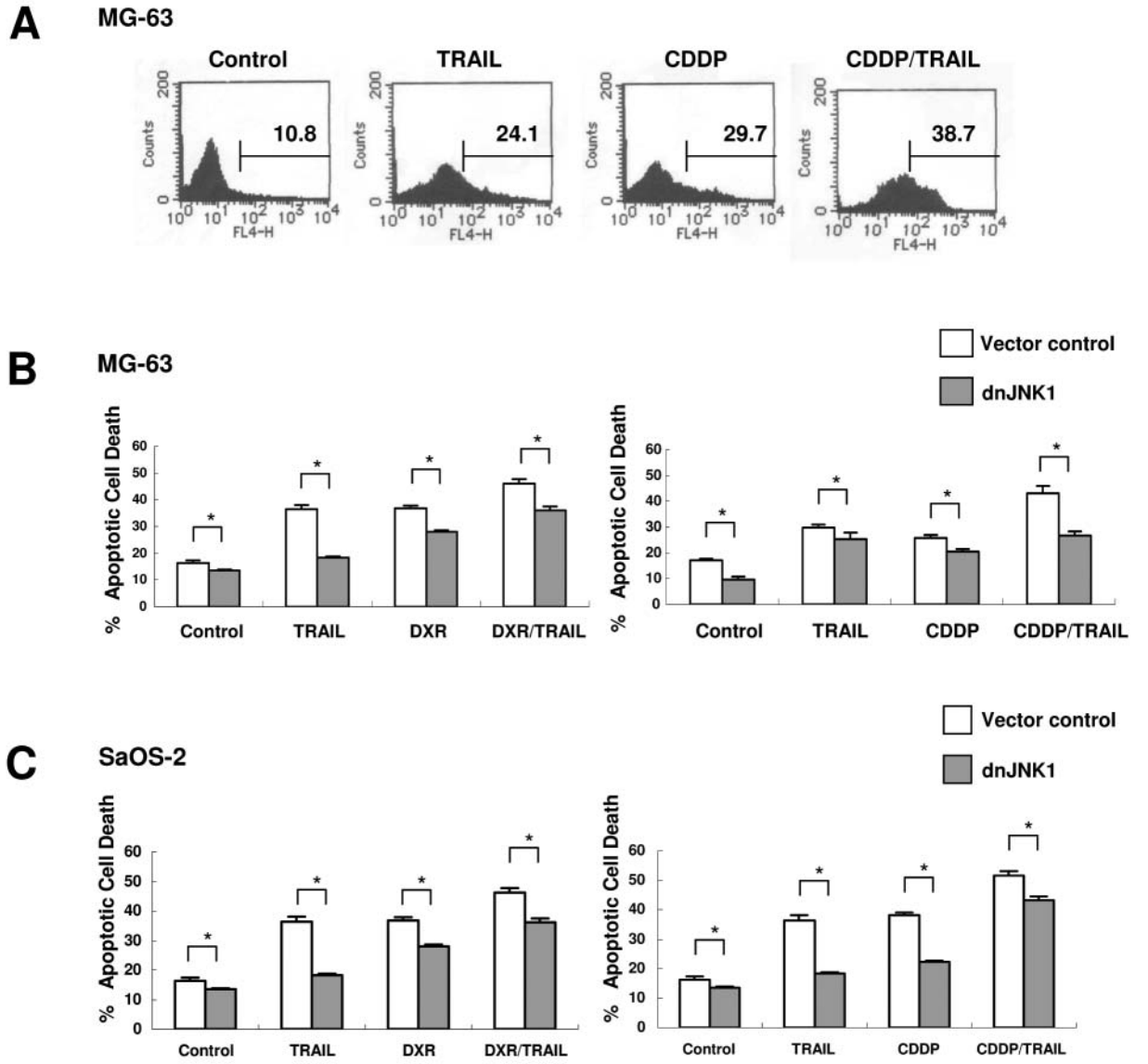


Figure 3. *Treatment with CDDP or DXR plus TRAIL resulted in enhanced apoptotic cell death, which was partially prevented by dnJNK1. MG-63 (A and B) or SaOS-2 (C) cells expressing dnJNK1 or the control vector were stimulated, as described in Figure 2. The cells were then assessed by the annexin V-staining method, as described in Materials and Methods. The profile of annexin V-staining in MG-63 cells is shown (A). Each point was carried out in triplicate and the results are represented as the means±SD (B and C). *Significantly different from dnJNK1 cells. Essentially similar results were obtained from 2 other experiments.*

(Figure 4). JNK activation is required for the caspase-8 activation due to the combined treatment with CDDP or DXR and TRAIL, resulting in apoptosis.

## Discussion

Chemotherapeutic agents, such as CDDP and DXR, have been used in a variety of tumors, including sarcoma, with some beneficial effects (1, 2, 27, 28). However, inherent and/or acquired resistance to these agents limits their clinical use (3, 5, 6). We and others have recently demonstrated that chemotherapeutic agents in combination with TRAIL caused cell death more efficiently than either agent alone (17-21). A chemotherapeutic agent, such as CDDP or DXR, alone (29, 30), or TRAIL alone (31) has been shown to induce JNK activation in some cell types. However, the molecular mechanisms by which the chemotherapeutic agent plus TRAIL induce efficient cell death remain largely unresolved.

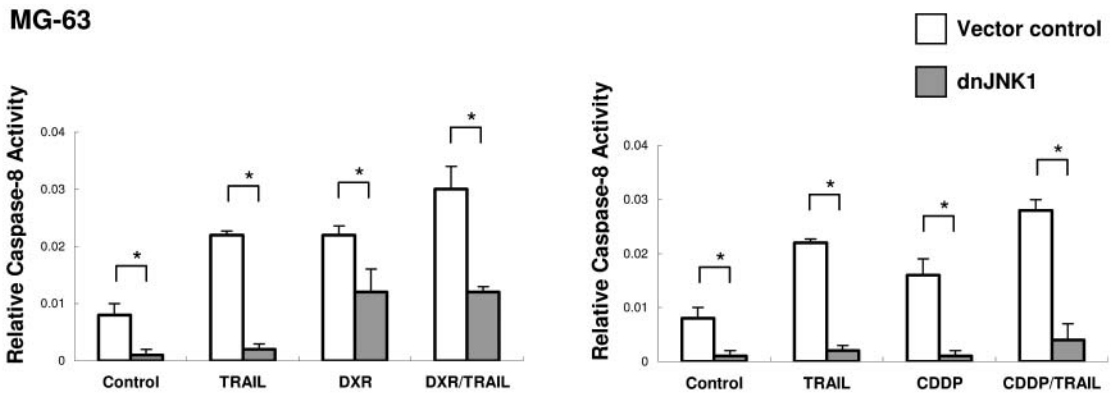


Figure 4. *dnJNK1-expressing MG-63 cells showed decreased caspase-8 activity following stimulation with CDDP/DXR plus TRAIL. MG-63 cells expressing dnJNK1 or control vector were exposed to CDDP or DXR and then stimulated, as described in Figure 2. The cells were then assayed for caspase-8 activity. Each point was carried out in triplicate and the results are represented as the means ± SD. *Significantly different from dnJNK1 cells. Essentially similar results were obtained from 2 other experiments.*

In the present study, it was examined whether JNK contributes to efficient cell death following combined treatment with chemotherapeutic agents and TRAIL.

To assess the role of JNK in the induction of apoptosis induced by DNA-damaging agents (CDDP and DXR) together with TRAIL, dnJNK was employed. We have recently shown that dnJNK1 effectively competed with endogenous JNK, resulting in diminished JNK activation following treatment with type I interferon in both human Daudi and murine CH31 B lymphoma cell lines (12, 32). In MG-63 cells, CDDP induced sustained JNK activation leading to AP-1 transcriptional activity, which was also partially prevented by dnJNK1 (Figure 1B). The JNK inhibitor SP600125 was confirmed to reduce CDDP-induced apoptosis in MG-63 as well as SaOS-2 cells (Toyota and Koyama, unpublished observation). Although JNK inhibitors, such as CEP-1347 and SP600125, have been employed in *in vitro* and *in vivo* experiments, these reagents are not specific for JNKs (33, 34). Thus, the conclusions drawn from their use in *in vitro* cell culture can be misleading.

The chemotherapeutic agents CDDP or DXR combined with TRAIL more efficiently resulted in loss of $\Delta\Psi m$, compared to either agent alone (Figures 2A and 2B). This efficient loss in $\Delta\Psi m$ due to CDDP/DXR plus TRAIL was reduced in SaOS-2 and MG-63 cells overexpressing dnJNK1, compared with cells expressing the control vector alone (Figures 2A and B). The apoptosis induced by CDDP or DXR plus TRAIL was also attenuated in the dnJNK1-overexpressing cells, suggesting that JNK activation is involved in the apoptosis caused by chemotherapeutic agents plus TRAIL (Figures 3A, B and C). In agreement with our findings, either chemotherapeutic agent (29, 30, 35, 36) or TRAIL (31) induces JNK activation and the JNK activation contributes to apoptosis induced by the combined treatment in some cell types (21, 37).

The interaction of TRAIL with its receptor TRAIL-Rs results in caspase-8 activation through formation of a death-inducing signaling complex (DISC), comprising adaptor protein Fas-associated death domain (FADD) and procaspase-8 (38, 39). Efficient caspase-8 activation induced by TRAIL plus DNA-damaging agents was also reduced in dnJNK1-overexpressing cells compared with the controls (Figure 4), suggesting that JNK activation somehow contributes to the apical caspase-8 activation induced by TRAIL or TRAIL plus DNA-damaging agents. Although the precise molecular mechanisms underlying the CDDP- or DXR-mediated caspase-8 activation remain unresolved in the present study, a recent report has demonstrated that methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate (CDDP-Me) up-regulated DR5 expression through JNK activation (37). In addition, other chemotherapeutic agents have shown that JNK activation increased the down-regulation of the anti-apoptotic protein c-FLIP, resulting in caspase-8 activation (19, 40). These findings suggest that modulation of apoptotic or anti-apoptotic proteins might depend on cell lineages.

JNK signaling augments apoptosis through phosphorylation of tumor suppressor protein p53 (41), and the gene encoding p53 is mutated most frequently in human cancer (42). The CDDP/DXR-mediated apoptosis in combination with TRAIL appears to be p53-independent, because the 2 cell lines MG-63 and SaOS-2 lack wild-type p53. In conclusion, combined treatment with TRAIL and either of the DNA-damaging agents CDDP or DXR resulted in efficient apoptotic cell death through JNK activation. Our results are valuable for determining treatment modalities of patients with osteosarcoma.

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