Tumor necrosis factor-related apoptosis-inducing ligand induces apoptotic cell death through c-Jun NH_2-terminal kinase activation in squamous cell carcinoma cells

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Abstract. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptosis in MIT6 cells derived from primary oral squamous cell carcinoma (OSCC), whereas it does not induce apoptosis in MIL6 cells derived from metastases. The present studies were performed to examine whether activation of c-Jun NH_2-terminal kinase (JNK) is implicated in the differential sensitivity to TRAIL-induced apoptosis. The TRAIL-induced JNK activation in MIT6 cells was stronger than in MIL6 cells, as assessed by Western blotting using antibodies specific for phospho-JNK. To evaluate the role of JNK1 in TRAIL-induced cell death, one clone expressing the dominant-negative form of JNK1 (dnJNK1) was established. The dnJNK1-expressing cells and MIL6 cells expressed TRAIL protein at levels similar to or even greater than MIT6 cells did. When cell death was assessed by annexin V staining and mitochondrial membrane potential, kinetic studies demonstrated that the dnJNK1-expressing cells were substantially more resistant to 100 ng/ml TRAIL, comparable to MIL6 cells, at 36 and 48 h after stimulation. Collectively, the primary OSCC cell line, MIT6, is sensitive to TRAIL but its metastatic line MIL6 is resistant to TRAIL exposure. Thus, the underlying molecular mechanism of TRAIL-induced cell death involves JNK activation. These results suggest that the acquisition of TRAIL resistance provides some metastatic capacity to primary tumors.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, and cases have recently increased in number (1,2). Although conventional treatments, including surgery, adjuvant chemotherapy and radiotherapy have some efficacy, survival rates have not improved significantly, partly because of local recurrence and/or distant metastases (3,4). Although the molecular mechanisms of tumor development and metastases are not completely understood, induction of apoptosis has been demonstrated to be involved in tumor metastases in some cell types (5,6). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family of proteins, induces apoptosis of transformed cells, including SCC cells (7) but not normal cells, *in vitro* and *in vivo* (7-10). Interaction of TRAIL with the receptors TRAIL-receptors (TRAIL-Rs) TRAIL-R1 and -R2 results in caspase-8 activation, by forming a death-inducing signaling complex (DISC) (11-13). Moreover, we and others have demonstrated that TRAIL has a synergistic action on tumor growth in combination with conventional DNA-damaging agents (7,14-16). The DNA-damaging agents activate an intrinsic apoptotic pathway, mainly involving mitochondria, while TRAIL induces activation of an extrinsic apoptotic pathway through caspase-8 activation (12,17). Furthermore, TRAIL on natural killer (NK) cells was shown to play a crucial role in tumor immune surveillance because it prevented liver metastasis in a mouse model (17,18). However, whether TRAIL plays a role in multistep tumorgenesis remains controversial (19).

We previously established several OSCC lines derived from primary lesions and their metastatic counterparts (20). The OSCC line MIT6 derived from primary OSCC was substantially more sensitive to chemotherapeutic agents than its metastatic counterpart MIL6. In the present study, we examined the sensitivity to TRAIL of both cell lines and found that MIT6 is more sensitive to TRAIL than MIL6. These findings suggest that TRAIL serves as a suppressor of metastasis in humans.

Materials and methods

Cell culture. Human SCC lines (MIT6 and MIL6) originally established in our laboratory were maintained in cRPMI-1640 consisting of RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml kanamycin. At each passage, cells were harvested as single cell suspensions using trypsin/EDTA. Several stable cell lines expressing the dominant-negative form of JNK1 (dnJNK1) were established by a previously described procedure (21). Briefly, MIT6 cells were transfected with an expression vector containing dnJNK1

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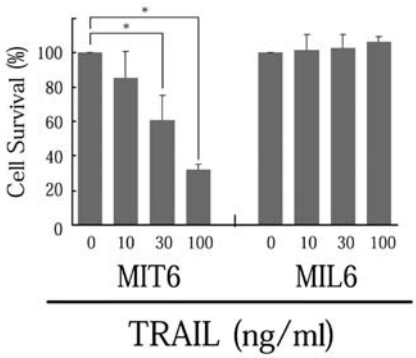

Figure 1. MIT6 is sensitive to TRAIL exposure, while MIL6 is totally resistant *in vitro*. Both MIT6 and MIL6 cells were cultured with indicated concentrations of TRAIL for 48 h, followed by assay for cell survival using a WST-8 assay kit. The results are shown as mean ±SD from three independent experiments. *Significantly different from medium alone.

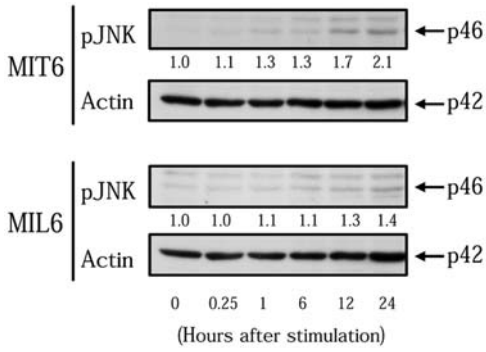

Figure 2. JNK activation is found in MIT6 cells, while it is reduced in MIL6 cells. Both MIT6 and MIL6 cells were cultured with 30 ng/ml TRAIL for the indicated time periods and assayed for JNK activation by Western blotting using phospho-specific antibodies. Levels of phospho-JNKs relative to those of actin are expressed as multiples of phospho-JNKs from unstimulated cells. The results are representative of three independent experiments.

or a control vector alone, and cultured in the presence of G418. One representative clone, DN12, was employed in this study. Similar results were obtained from other cell lines.

Cell survival assay. Cells were cultured with or without the indicated concentrations of TRAIL for various times, and cell survival was estimated using a WST-8 assay kit (Dojndo Laboratories, Kumamoto, Japan), as previously described (20).

Western blot analysis. Western blotting was carried out as previously described (20). The antibodies (Abs) used here included anti-phospho-specific JNKs, total anti-JNK1, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The density of each band was measured using the NIH image program.

Flow cytometric analysis of TRAIL expression, mitochondrial membrane potential, and apoptosis. To demonstrate TRAIL expression on the cell surface, direct staining methods were performed according to the manufacturer's recommendations. Briefly, cells were stained with phycoerythrin (PE)-labeled anti-TRAIL-R1 and -R2, and PE-labeled irrelevant control Abs (R&D Systems, Minneapolis, MN, USA). Samples were analyzed on a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company, Tokyo, Japan) using Cell Quest software (Becton Dickinson Immunocytometry System, San Jose, CA, USA). For determination of mitochondrial membrane potential ($\Delta\Psi m$) and apoptosis, the cells stimulated with or without TRAIL were loaded with 20 nM $DiOC_6$ or stained with annexin V-Cy5 using an Annexin V-Cy5 Apoptosis Detection kit (BioVision, Mountain View, CA, USA), followed by analysis on a flow cytometer.

Establishment of stable clones. Cells were transfected with expression vectors encoding dnJNK1 or control vector alone (21), then cloned by limiting dilution to obtain clone(s). Expression of dnJNK1 was confirmed by Western blotting.

Statistical analysis. Data are expressed as mean ±SD for each group. Statistical significance was determined by Student's t-test and was set at $p \leq 0.05$.

Results

Primary SCC cells are sensitive to TRAIL-induced cytotoxicity, whereas those from its metastatic counterpart are resistant. To explore the role of TRAIL in metastasis of SCC cells, primary SCC (MIT6) cells and those derived from its metastatic counterpart (MIL6) were exposed to various concentrations of TRAIL for 48 h, followed by a WST-8 assay for cell survival. The MIT6 cells showed sensitivity to TRAIL in a dose-dependent manner, while the MIL6 cells were completely resistant up to 100 ng/ml TRAIL (Fig. 1). These results suggest that metastatic SCC cells have acquired TRAIL resistance.

TRAIL induces higher JNK activation in MIT6 cells than in MIL6 cells. We recently demonstrated that TRAIL-induced JNK activation is implicated in the induction of apoptosis in sarcoma cells following treatment with chemotherapeutic agents (22). To examine whether TRAIL induces JNK activation in the SCC cell lines, cells were cultured with 100 ng/ml TRAIL for the indicated periods and JNK phosphorylation was assessed by using antibodies specific for phospho-JNK. MIT6 cells showed substantial levels of the p46 kDa form of phospho-JNKs for 12-24 h after stimulation with TRAIL, while the levels of phospho-JNK were considerably reduced in MIL6 cells (Fig. 2). These findings suggest that the JNK signaling pathway is involved in the TRAIL-induced cytotoxicity in SCC cells.

Establishment of MIT6 cell lines overexpressing dominant-negative form of JNK1. To explore the requirement for JNK1 activation in the TRAIL-induced cytotoxicity in SCC cells, a line of TRAIL-sensitive MIT6 cells overexpressing dnJNK1 was established using the procedure as previously described (20,21). One (DN12) of several clones was demonstrated to express exogenous dnJNK1 (p47) as well as a major endogenous form of JNK1 (p46) (Fig. 3A). As expected, the p47 band for exogenous dnJNK1 was not detected in the control clone expressing Neo alone (Neo-1), indicating the specificity of the anti-JNK1 Abs used in this study. The levels of TRAIL-R1/2 were evaluated by flow cytometry. TRAIL expression levels in MIL6 cells were comparable to or ever higher than in MIT6 cells (Fig. 3B). The levels of dnJNK1-

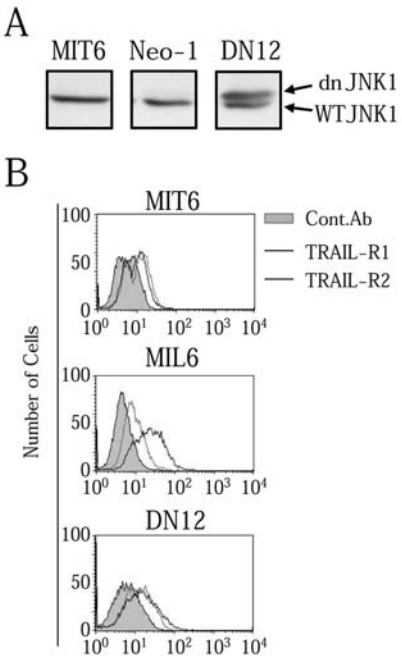

Figure 3. Establishment of MIL6 cells overexpressing dominant-negative form of JNK1. MIT6 cells were transfected with expression vector containing dnJNK1 or the control Neo alone and cultured with G418 for ~10 days. The resultant transformants were subjected to limiting dilution to obtain clones. The clone DN12 was confirmed by Western blotting to express both dnJNK1 and endogenous JNK1 (A). Cells were also examined for expression of TRAIL-Rs (B). Similar results were obtained from two other experiments.

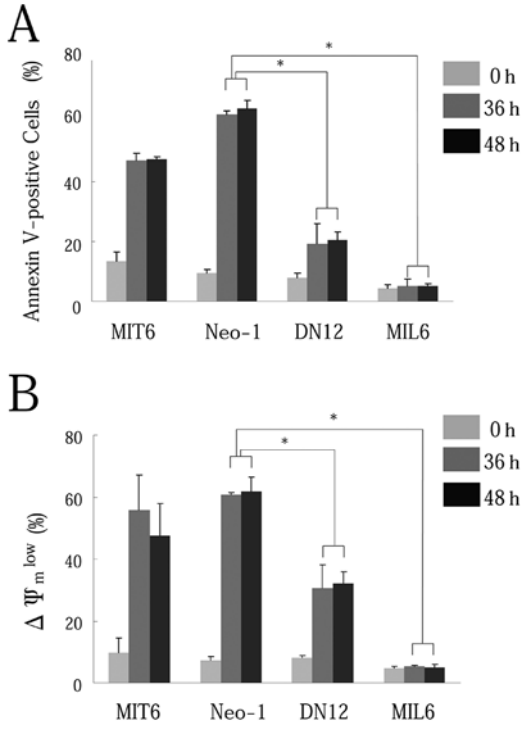

Figure 4. The dnJNK1-overexpressing clone DN12 is substantially resistant to TRAIL-induced loss of mitochondrial membrane potential and apoptosis *in vitro*. Cells were cultured with 100 ng/ml TRAIL for 36 or 48 h, and apoptosis (A) and mitochondrial membrane potential (B) were assessed. The results are shown as mean ±SD from three independent experiments. *Significantly different from control Neo-1 alone.

expressing cells were almost identical to the parental cell line MIT6. These results suggest that expression of TRAIL-Rs does not account for the unresponsiveness to TRAIL.

dnJNK1-overexpressing clones are substantially resistant to TRAIL-induced apoptosis and loss of mitochondrial membrane potential. DN12 cells were cultured with 100 ng/ml TRAIL for 36 to 48 h and assayed for ΔΨm and annexin V. DN12 cells were substantially resistant to TRAIL-induced loss of ΔΨm and apoptosis, compared with controls (Fig. 4A and B). MIL6 cells were completely resistant to the TRAIL-induced loss of ΔΨm and apoptosis. Because TRAIL receptors (TRAIL-R1 and -R2) were expressed at comparable levels in all cell lines tested, signaling components, including JNK1, downstream of TRAIL-Rs may be defective in MIL6 cells.

Discussion

Conventional therapy, including surgery, adjuvant chemotherapy and radiation, has improved the quality of life for OSCC patients (23,24), but recurrent and/or distant metastases are a major obstacle to survival (3,4). The molecular mechanisms of metastasis remain incompletely understood. TNF superfamily proteins, including TRAIL, have been proposed to be involved in the metastases of some tumors (17,18,25,26). Our present study demonstrates that MIL6 cells derived from a metastatic lesion were markedly resistant to TRAIL exposure, while MIT6 cells from the primary tissues were considerably sensitive (Fig. 1). Moreover, TRAIL-induced JNK activation was impaired in MIL6 cells, and the dnJNK1-expressing MIT6 cells became substantially resistant to TRAIL exposure, suggesting that JNK activation is involved in TRAIL-induced apoptosis in OSCC cells.

Recent findings demonstrate that apoptosis is a crucial process for regulating metastasis (5,6). During metastasis, tumor cells have to overcome multiple stresses, including loss of adhesion and hemodynamic shearing (5). The initial steps of metastasis include detachment of epithelial cells from the extracellular matrix, resulting in cell death (anoikis) (6,27). When MIT6 cells were incubated in a non-coated bacteria culture dish, they immediately underwent apoptosis, whereas MIL6 cells became round-shaped and remained viable for certain periods of time (anoikis-resistant) (Itoh *et al* unpublished data). The anoikis resistance of MIL6 cells appears to be associated with the acquisition of metastasis, although a causal relationship between anoikis-resistance and metastasis is not yet established in murine models.

Tumor immunosurveillance is considered to provide a mechanistic basis for detecting and eliminating cancer cells, and also preventing tumor metastasis (28). Despite immunosurveillance, some metastases arise, probably through suppression of immune system. In other circumstances, tumor cells might acquire resistance to immune-mediated attacks including TRAIL and CD95-L. Indeed, Vigneswaran *et al* demonstrated that primary oral cancer cells are sensitive to TRAIL, whereas their metastatic counterparts are substantially resistant (29). We confirmed their findings and further determined that TRAIL-induced apoptosis involves JNK activation in OSCC cells as well as other cell types (22,30).

It is proposed that sustained activation of JNK initiates cell death, while transient activation of JNK favors cell survival

(21,31-33). Sustained JNK activation was found in TRAIL-sensitive MIT6 cells, whereas JNK activation was decreased in TRAIL-resistant MIL6 cells (Fig. 2). TRAIL-sensitive MIT6 cells overexpressing dnJNK1 showed some resistance to TRAIL exposure compared with control cells, suggesting that sustained JNK activation is involved in TRAIL-induced apoptosis in SCC cells. Because TRAIL-R1 and -R2 were expressed almost equally in MIT6 and MIL6 cells, these findings suggest that intracellular component(s) connecting TRAIL-Rs to JNK activation are defective in MIL-6 cells.

The binding of TRAIL to its receptors recruits DISC, resulting in activation of caspase-8 (11,13). Sufficient activation of caspase-8 induces cleavage of downstream effector caspases, leading to cell death (extrinsic pathway) (12). This extrinsic pathway often cross-talks with the intrinsic pathway, which integrates a variety of apoptotic stimuli leading to a release of small toxic molecules from mitochondria into the cytosol, especially when initial activation of caspases are not sufficient to induce cell death. However, how activation of the extrinsic pathway connects to JNK activation remains unresolved in the present study.

Collectively, we propose that resistance to apoptosis in response to TRAIL involving insufficient activation of JNK contributes to metastasis.

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