LKB1 is Crucial for TRAIL-mediated Apoptosis Induction in Osteosarcoma

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Abstract. *Background: Despite improvements in chemotherapy and surgery in the treatment of osteosarcoma, satisfactory results are still difficult to achieve. New therapeutic modalities need to be developed for the improvement of these treatments. TRAIL (TNF-related apoptosis inducing ligand) is known as a selective apoptosis inducer in most tumor cells, but not in normal cells. Therefore, TRAIL is a good candidate target for the treatment of tumors. However, sensitivity of osteosarcoma cells to TRAIL-induced apoptosis is lower than that of other types of tumor cells. Recently, DAP3 (death associated protein 3) was demonstrated to play a critical role in TRAIL-mediated apoptosis through activation of pro-caspase-8. Here, we found that LKB1, a serine/threonine kinase, expressed in bone and soft tissue sarcoma cells, associated with DAP3. We also demonstrated that expression of DAP3 induced apoptosis in osteosarcoma cells. Furthermore, expression of LKB1 induced apoptosis and co-expression of LKB1 with DAP3 strongly induced apoptosis in osteosarcoma cells. In addition, expression of LKB1 kinase dead mutant, LKB1 (K78M), inhibited DAP3-induced apoptosis in these cells. These results suggest that LKB1 is critical for TRAIL-induced apoptosis induction, cooperating with DAP3 in osteosarcoma cells. It is predicted that LKB1 and DAP3 could be critical target molecules for the treatment of osteosarcomas.*

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Development of chemotherapy and surgery has resulted in the considerable improvement of survival rates of osteosarcoma patients (1), the current chemotherapeutic agents still have a lot of problems to overcome, such as serious side-effects or resistance to multiple chemotherapeutic agents in 20-30% of the patients (2). New therapeutic modalities for these patients are driven by necessity for the treatment.

TRAIL is a member of TNF (tumor necrosis factor) family proteins (3, 4). TRAIL, which selectively induces apoptosis in most tumor cells, but not in normal cells, is expected to be a new therapeutic agent without significant toxicities to normal tissues (5, 6).

Many experimental studies of the application of TRAIL to osteosarcoma cells have been reported (7-15). However, treatment of osteosarcoma cells with TRAIL did not lead to satisfactory results, since cell sensitivity to TRAIL-induced apoptosis is different in various cells, and the mechanisms for apoptosis induction remain unclear. Therefore, additional agents to sensitize osteosarcoma cells to TRAIL-induced apoptosis have been proposed (7-11, 13-15) but the problem of side-effects still remains.

TRAIL receptors have been identified as TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegrin (OPG). DR4 or DR5 induces apoptosis by TRAIL stimulation (16). In contrast, DcR1, DcR2 and OPG are not able to induce apoptosis, since their cytoplasmic death domains are deficient or incomplete (16, 17, 19-21). Although the expression of DR4 or DR5 is required for TRAIL-mediated apoptosis, the expression of DcR1, DcR2 and OPG does not necessarily relate to cell sensitivity to TRAIL-induced apoptosis (22, 23). Therefore,

the intracellular molecules located downstream of DR4 or DR5 have been proposed to be a critical determinant of cell sensitivity to apoptosis induced by TRAIL.

Pro-apoptotic cytoplasmic DAP3 associates with both the intracellular death domain of TRAIL receptor and the Fas associated death domain (FADD) (23). The complex formation of these proteins by TRAIL receptor-stimulation induces apoptosis through activation of pro-caspase 8 (24). However, details of molecular mechanisms in TRAIL- or DAP3-induced apoptosis are not fully understood.

Therefore, we screened the proteins which bind to DAP3, using a yeast two hybrid system, in order to identify the regulators for TRAIL-mediated apoptosis. As a result, we found that LIP1 (LKB1 interacting protein 1) associated with DAP3. LIP1 was reported to interact with LKB1 (25). Although LIP1 is not a substrate of LKB1, it was shown to anchor LKB1 to the cytoplasm (25). LKB1 was originally described with regard to its gene mutation causing Peutz-Jeghers syndrome (PJS) (25-27), a rare hereditary disease, in which there is predisposition to develop benign and malignant tumors of many organ systems (28, 29). LKB1 was demonstrated to be a tumor suppressor involved in the regulation of cell polarity (30-32). However, the function of LKB1 in TRAIL-induced apoptosis is not clear. Therefore, we studied the functional role of LKB1 in apoptosis induction by TRAIL stimulation. In addition, we examined the function of LKB1 and DAP3 in TRAIL-mediated apoptosis in osteosarcoma cells.

Materials and Methods

Cell lines and plasmids. HOS, MNNG/HOS, MG63 (human osteosarcoma), HT1080, NIH3T3 (mouse fibrosarcoma) and HFO (primary cultured human fetal osteoblast) cells (15) were kindly donated by the Department of Pathology, Sapporo Medical University, Japan. HEK293T and 143B (human osteosarcoma) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbeccos' modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (SIGMA-ALDRICH, JAPAN), 100 IU/ml penicillin and 100 μg/ml streptomycin (SIGMA) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Jurkat cells were cultured in the medium of RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (SIGMA), 100 IU/ml penicillin and 100 μg/ml streptomycin (SIGMA).

Cell viability assay. Before seeding onto 96-well plates, osteosarcoma or HEK293T cells ($2x10^4$) were mixed with 5 μM troglitazone. After incubation for 24 h, each well of this plate was washed with PBS twice, fixed with 3.7% formaldehyde and then stained with 50 μl/lane 0.05% crystal violet for 1 min. Stained cells were lysed with 100 μl/lane 10% SDS and the absorbance was measured at OD405 nm. In the case of Jurkat cells, cell viability was analysed by the measurement of dead cells detected by trypan blue staining. All cell viability assay experiments were repeated twice. Results of representative experiments are given as the mean+SD.

Yeast two-hybrid screening. Human wild-type DAP3 gene was inserted and fused to the Gal4 DNA-binding domain in the vector pGBKT7 (Trp) (BD Clontech, USA). After transformation into the yeast strain, AH109 with pGBKT7 (Empty vector) or pGBKT7-DAP3, these transformants were selected on SD (Synthetic Dropout) plates, in which the media lacks Trp. These selected yeast cells were transformed with a human fetal brain cDNA library fused with Gal4ad in the vector pActII (Leu) (Clontech), and then selected on SD media lacking Trp and Leu. The positive clones were selected and the confirmation of positive clones was carried out using α-gal or β-gal assay.

Western blotting. Cells were harvested and the protein extracts from these cells were prepared as follows. The protein samples (20 μg/lane) were fractionated by SDS-PAGE on a 4-20% gradient polyacrilamide gel (Daiichi Pure Chemicals, JAPAN). The analysed proteins were transferred to a PMDF filter with a semi-dry blotter. The blotted membrane filter was reacted either with each antibody as shown in the Figure after blocking. This membrane was reacted with HRP-conjugated antibodies and immunodetection of the proteins was performed using the ECL detection system (Amersham, Piscataway, NJ, USA).

Immunoprecipitation. HEK293T cells ($2x10^6$) in 10 cm cell culture plates were transiently transfected with 5 μg of each expression vector plasmid (10 μg as a total DNA amount) together with 30 μl of Fugene 6 transfection reagent (Roche Diagnostic, Mannheim, Germany) and harvested 24 h later. Cells were suspended in 500 μl of lysis buffer containing 0.1% NP-40, 20 mM Tris-HCl at pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl and protease inhibitors Complete Mini (Roche). Alternatively, untransfected HOS cells ($5x10^8$) were harvested and lysed in 1.5 ml of lysis buffer as described above. Cell extracts were reacted either with the indicated antibody in the Figure for 1 h and then washed with the lysis buffer for five times. These samples were fractionated by SDS-PAGE and analyzed by Western blotting assay as described above.

Colony formation assay. HOS cells ($5x10^5$) in 35 mm cell culture plates were transfected with 4 μg of each expression vector plasmid together with 10 μl of Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). After 24 h, these cells were cultured with TRAIL (50 ng/ml) for 1 week. The number of colonies was determined after staining with 0.05% crystal violet solution.

Results

Sensitivity of osteosarcoma cells to TRAIL-induced apoptosis. We first examined the effects of TRAIL stimulation for the induction of apoptosis in osteosarcoma cells. Human osteosarcoma cell lines, HOS, MG63 and 143B were cultured in the presence of TRAIL for 24 h. As shown in Figure 1, cell viability of every cell line was reduced in a dose-dependent manner. However, loss of cell viability was only about 60% even in the most sensitive 143B cells at a high concentration of TRAIL (500 ng/ml) for 24 h. Generally, cell sensitivity to

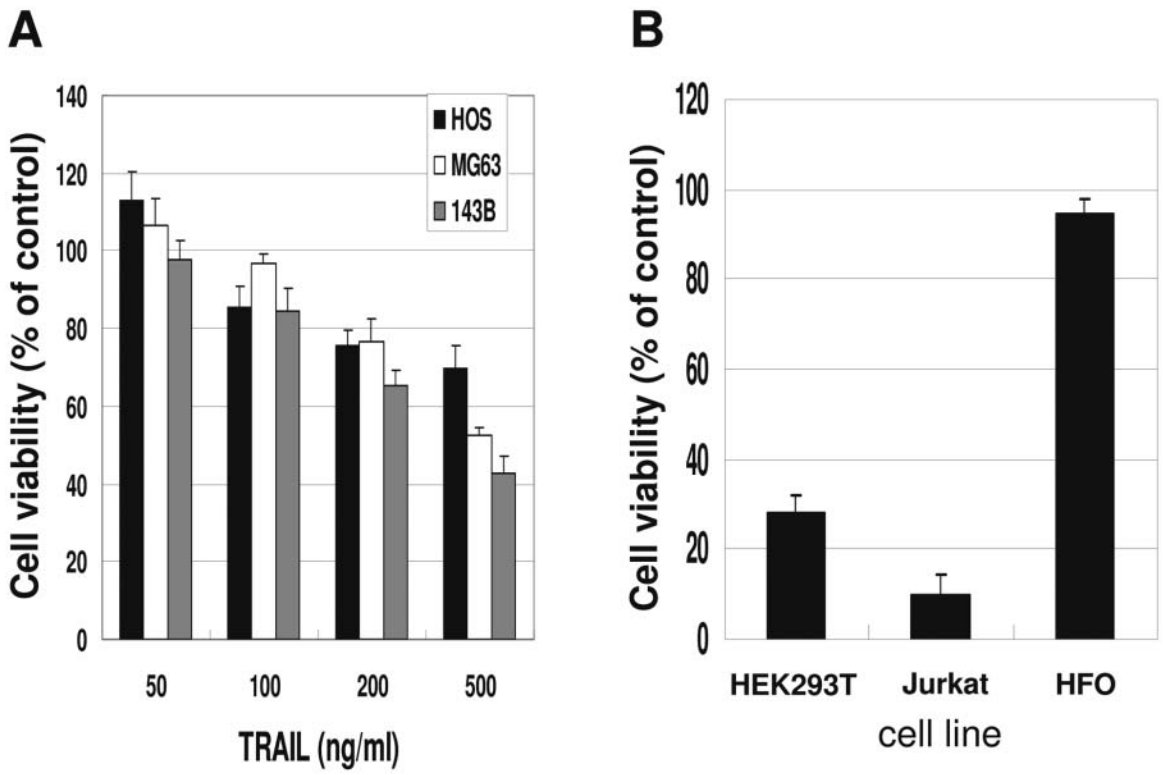

Figure 1. *Sensitivity of osteosarcoma cells to TRAIL-induced apoptosis. (A) Cell viability of osteosarcoma cells after treatment with TRAIL. HOS, MG63 and 143B osteosarcoma cells were cultured in the presence of TRAIL at various concentrations. After 24 h, cell viability as a percentage of the control was determined by crystal violet assay (n=3, mean+SD). (B) Cell viability of HEK293T, Jurkat and HFO cells after treatment with TRAIL. HEK293T, Jurkat, and HFO (human normal osteoblastic cells) were cultured with TRAIL (200 ng/ml). After 24 h, the percentage for cell viability was determined (n=3, mean+SD).*

TRAIL-induced apoptosis in osteosarcoma cells is lower than that of other tumor cells, for example, loss of viability is 80% in HCT116 (human colon cancer) cells (data not shown) or 90% in Jurkat (human T cell line) cells in the presence of TRAIL for 24 h (Figure 1B).

It is strongly suggested that there is the necessity for an enhancement of the sensitivity to TRAIL-induced apoptosis in osteosarcoma cells to improve their treatment *via* this method.

LKB1 was associated with DAP3. A yeast two-hybrid screening for DAP3 as a bait using a human embryonic brain cDNA library was performed to identify proteins binding to DAP3. As a result, 41 positive clones in both α- and β-galactosidase assays were obtained (data not shown). One of them coded the sequence of LIP1. LIP1 was first identified to interact with a serine-threonine kinase, LKB1 by the yeast two-hybrid method using a mouse embryonic cDNA library and LKB1 gene as a bait (25). It was shown that LIP1 binds to LKB1 and anchors LKB1 to cytoplasm (25). LKB1 was demonstrated to be a tumor suppressor involved in the regulation of the mitochondrial apoptotic pathway (27-30).

We confirmed the interaction of DAP3 with LIP1 in mammalian cells by co-immunoprecipitation as described in Materials and Methods (Figure 2A). It was shown that LIP1 was associated with DAP3 in mammalian cells.

We found that endogenous DAP3 could interact with LKB1 in osteosarcoma cells (Figure 2B). However, DAP3 could not be immunoprecipitated by control antibody. These results suggested that LKB1 was associated with DAP3 through LIP1.

Expression of DR4, DR5, DAP3 and LKB1 in bone and soft tissue cells. Expression of LKB1 and also DR4, DR5 and DAP3 in HOS, MNNG/HOS, MG63 (osteosarcoma), HFO (primary cultured normal human fetal osteoblastic cells), HT1080 and NIH3T3 (fibrosarcoma) cells was determined by western blotting analysis. As shown in Figure 3, the expression level of DR4 is slightly lower in HT1080 and NIH3T3 cells with compared to other cells. In addition, expression of DR5 and LKB1 was slightly lower in normal osteoblastic cells (HFO), with compared to other tumor cells. These results suggest that the expression level of these molecules is not

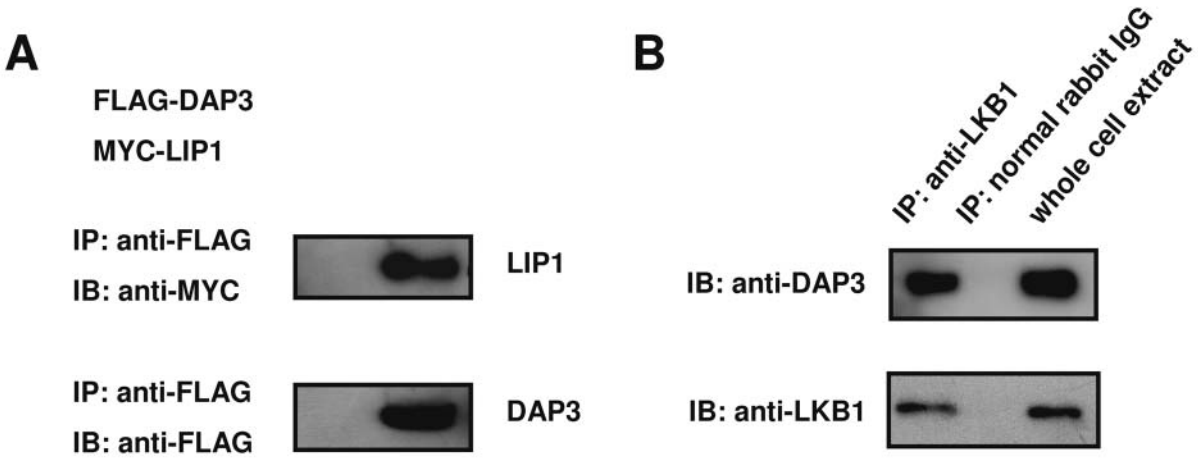

Figure 2. *Association of LKB1 and LIP1 with DAP3. (A) LIP1, which is a LKB1 binding protein, associates with DAP3. Expression vector for MYC-tagged LIP1 was co-transfected with empty vector plasmid or expression vector encoding FLAG-tagged DAP3 into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG M2 antibody, analysed by SDA-PAGE and blotted with anti-MYC or anti-FLAG M2 antibody. (B) Endogenous LKB1 associates with the endogenous DAP3 in osteosarcoma cells. Cell lysates from osteosarcoma HOS cells ($5x10^8$) were immunoprecipitated with normal rabbit IgG or anti-LKB1 antibody, analysed by SDA-PAGE and blotted with anti-DAP3 or anti-LKB1 antibody.*

correlated with apoptosis induction by TRAIL stimulation in normal osteoblastic cells and osteosarcoma cells.

Function of LKB1 to induce TRAIL-mediated apoptosis co-operatively with DAP3. To investigate the function of LKB1 in TRAIL- or DAP3-induced cell death, HEK293T cells were transfected with the expression vector plasmid for LKB1, DAP3, dominant negative DAP3 (DAP3ΔC) or dominant negative LKB1 (LKB1 (K78M)) as shown in Figure 4A. These transfected cells were cultured with TRAIL, and after 24 h, the percentage of dead cells was determined. Expression of DAP3 enhanced TRAIL-mediated cell death and expression of LKB1 also induced cell death (Figure 4A). Interestingly, expression of both LKB1 and DAP3 strongly enhanced TRAIL-mediated cell death. In addition, the induction of cell death by the expression of LKB1 together with DAP3 mutant, DAP3ΔC was low compared with the expression of LKB1 only. In addition, induction of cell death through DAP3 was inhibited by the expression of LKB1 mutant, LKB1 (K78M) (Figure 4A).

Furthermore, we assessed the function of DAP3 and LKB1 in TRAIL-induced cell death in osteosarcoma cells using a colony formation assay as described in Materials and Methods. As shown in Figure 4B, the number of surviving colonies was reduced by the expression of LKB1 as well as DAP3 in the presence of TRAIL in HOS cells. In addition, it was severely reduced by co-expression of LKB1 with DAP3. However, LKB1-induced inhibition of colony formation was recovered by the expression of DAP3 mutant, DAP3ΔC. DAP3- induced inhibition of colony formation was strongly recovered by the expression of LKB1 mutant, LKB1 (K78M).

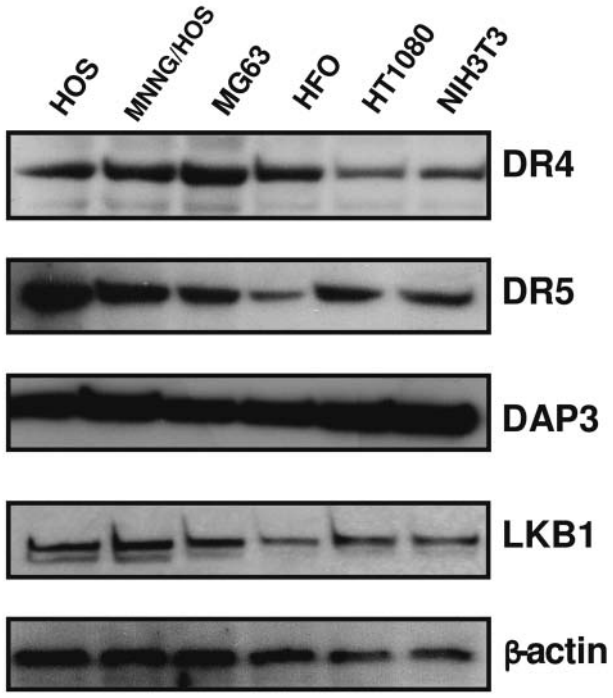

Figure 3. *Expression of DR4, DR5, DAP3 and LKB1 proteins in osteosarcoma cells. Cell lysates from HOS, MNNG/HOS, MG63, HFO (primary cultured human normal fetal osteoblastic cells), HT1080 and NIH3T3 cells were analysed by SDS-PAGE and blotted with anti-DR4, anti-DR5, anti-DAP3 or anti-β-actin antibody.*

These results suggest that LKB1 is critical for cell death induction and inhibition of colony formation by TRAIL stimulation cooperatively with DAP3 in osteosarcoma cells.

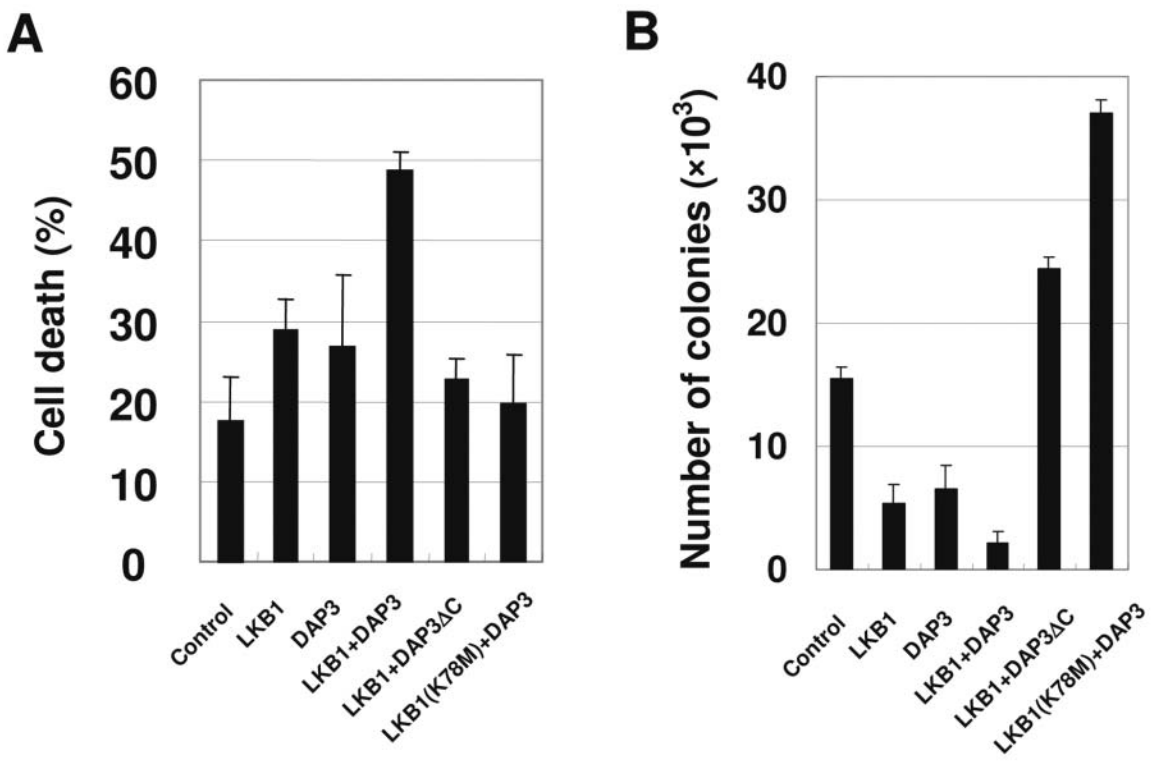

Figure 4. *TRAIL-mediated cell death induced by LKB1 and DAP3 in osteosarcomas. (A) Expression of DAP3 and LKB1 enhances TRAIL-mediated apoptosis. HEK293T cells were transfected with empty plasmid or plasmid vector encoding LKB1, DAP3, LKB1, DAP3ΔC or LKB1 (K78M) with an indicated combinations. These transfected cells were cultured with 10 ng/ml TRAIL for 24 h. The percentage of dead cells was determined by trypan blue dye exclusion (n=3, mean+SD). (B) Expression of DAP3 and LKB1 inhibited colony formation with TRAIL in osteosarcoma cells. HOS cells were transfected with plasmid vectors same as above. After 24 h, these cells were stimulated by TRAIL (50 ng/ml) for 1 week and the number of colonies was determined by crystal violet staining (n=3, mean+SD).*

Activation of caspases by LKB1 after TRAIL stimulation. To investigate the molecular mechanism for apoptosis induction by LKB1 after TRAIL stimulation, we examined whether LKB1 regulated activation of caspase-8, caspase-9 or caspase-3.

Cleavage of caspase-8, caspase-9 or caspase-3 was detected at 8 h in the cells transfected with control vector plasmids. However, cleavage of these caspases was detected at 3 h in the cells expressing LKB1. On the other hand, cleavage was detected at 8 h in the cells expressing LKB1 (K78M) as in the control vector (Figure 5).

These results suggest that LKB1 positively regulates the cleavage of caspases under TRAIL stimulation, and kinase activity of LKB1 is important for this function.

Discussion

Sensitivity of osteosarcoma cells to TRAIL-induced apoptosis was found to be lower than that of other tumor cell types (Figure 1). Therefore, it is very important to enhance TRAIL-induced apoptosis signal in osteosarcoma cells for the improvement of the treatments for osteosarcomas. The determinants for cell sensitivity to TRAIL-induced apoptosis in osteosarcomas need to be clarified at the molecular level. The intracellular signaling molecules located downstream of the TRAIL receptor (DR4 or DR5) have been proposed as essential determinants for this sensitivity. DAP3 protein was shown to play a critical role in TRAIL-mediated apoptosis through activation of death-inducing signaling complex (DISC) including pro-caspase-8 (24). However, details of the molecular mechanisms in TRAIL- or DAP3-induced apoptosis in osteosarcoma cells are not well known.

Therefore, we screened the molecules which bind to DAP3, by yeast two-hybrid method using cDNA libraries, in order to identify critical molecules for TRAIL-mediated apoptosis. We found LIP1 was associated with DAP3 (Figure 2A). LIP1 has been reported to interact with LKB1 and anchors it to the cytoplasm (26). In addition, LKB1 is responsible for Peutz-Jeghers syndrome (PJS) (27), a rare hereditary disease in which there is a predisposition for benign and malignant tumors of many organ systems (28,

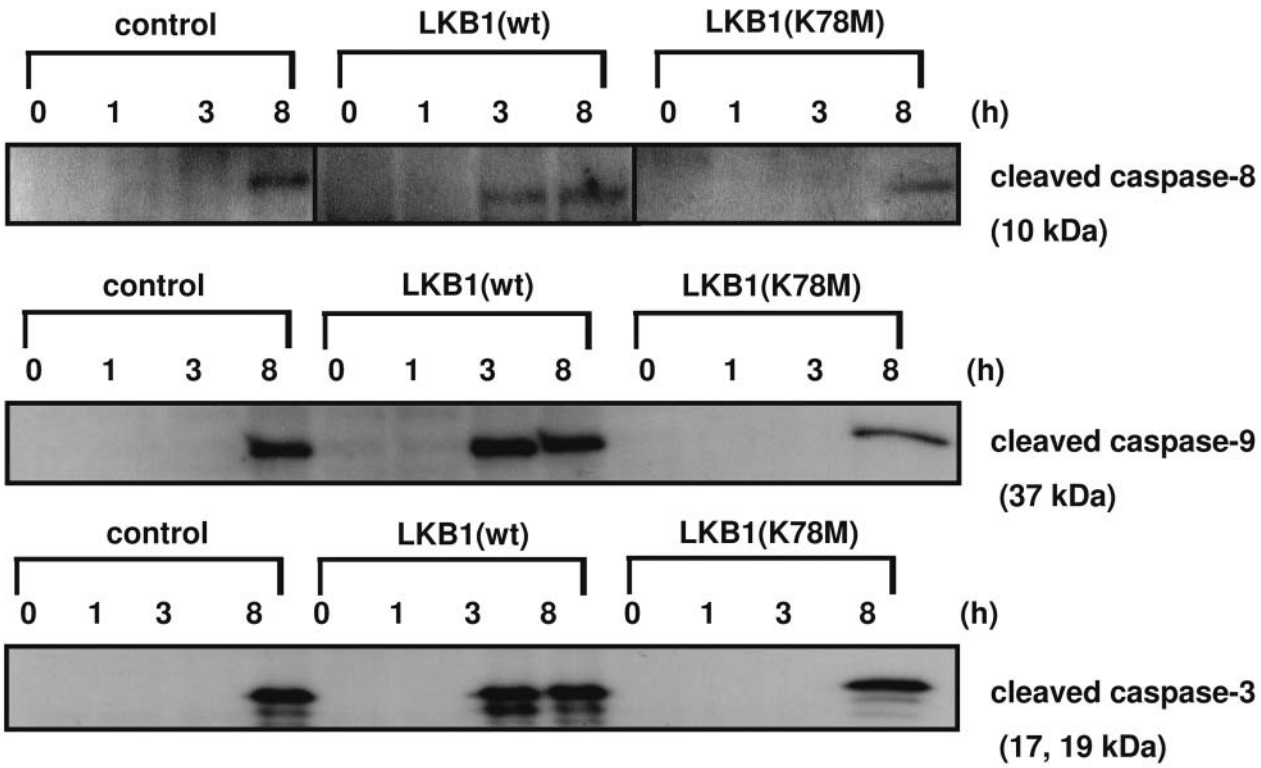

Figure 5. *Activation of caspases by LKB1 after TRAIL stimulation. HEK293T cells were transfected with empty plasmid or plasmid vector encoding LKB1 or LKB1 (K78M). After 18 h, these transfected cells were cultured with 10 ng/ml TRAIL, and then harvested at 0, 1, 3 or 8 h later. These cell lysates were analyzed by SDS-PAGE and blotted with anti-cleaved caspase-8, -9 or -3 antibody.*

29). LKB1 has also been proposed to be a tumor suppressor involved in cell polarity (30-32). Furthermore, LKB1 associates with p53 by the stimulation of microtubule damaging agents (*e.g.* paclitaxel or vincristine) and promotes the activation of pro-caspase-9 through the p53-dependent mitochondrial apoptotic pathway (31). The overexpression of LKB1 in G361 melanoma cells induced the expression of p21 (WAF1/CIP1), resulting in a G1 cell cycle arrest (33). These results demonstrate that LKB1 is critical for apoptosis induction and cell cycle regulation in tumor cells.

We demonstrated that endogenous DAP3 associated with LKB1 (Figure 2B) and these proteins were expressed in bone and soft tissue cells (Figure 3). These results demonstrate the possibility that LKB1, in addition to DAP3 (23) was involved in TRAIL-mediated apoptosis induction in osteosarcomas.

Expression of LKB1 or DAP3 enhanced TRAIL-induced cell death. Co-expression of LKB1 with DAP3 strongly induced cell death under TRAIL stimulation (Figure 4A). Furthermore, induction of cell death by LKB1 is slightly reduced by co-expression of DAP3 mutant, DAP3ΔC (Figure 4A). In addition, induction of cell death by DAP3 was inhibited by co-expression of LKB1 mutant, LKB1 (K78M) (Figure 4A). These results suggested that DAP3 and LKB1 could cooperatively induce TRAIL-mediated cell death.

As shown in Figure 4B, in osteosarcoma cells, expression of LKB1 or DAP3 induced a reduction of the number of surviving colonies compared with the expression of the control vector. The combined expression of DAP3 and LKB1 cooperatively enhanced TRAIL-mediated cell death (Figure 4A) and the number of surviving colonies of osteosarcomas was severely reduced (Figure 4B). However, the number of surviving colonies with LKB1 expression was increased by co-expression of the DAP3 dominant negative mutant, DAP3ΔC (Figure 4B). In addition, DAP3-induced inhibition of colony formation was strongly recovered by co-expression of LKB1 mutant, LKB1 (K78M). These results suggest that LKB1 and DAP3 cooperatively regulate cell death induction by TRAIL stimulation in osteosarcomas.

To investigate whether LKB1 induced the activation of caspases by TRAIL stimulation, we analyzed the cleavage of caspases by LKB1 expression. As shown in Figure 5, LKB1 expression induced the cleavage of caspase-8, caspase-9 and caspase-3 at 3 h after TRAIL stimulation.

However, expression of the control vector or LKB1 mutant, LKB1 (K78M) could not induce the cleavage of caspases at 3 h after TRAIL stimulation (Figure 5). LKB1 was shown to be critical for the acceleration of the caspase activation after TRAIL stimulation. These results suggested that LKB1 was involved in the caspase activation and apoptosis induction by TRAIL stimulation. It was proposed that LKB1 might be a potential sensitizer of TRAIL treatment in osteosarcoma.

In addition, expression of the dominant negative form of LKB1, LKB1 (K78M), lacking the kinase activity (31), did not affect the caspase activation induced by TRAIL stimulation (Figure 5). This result suggested that the kinase activity of LKB1 was important for the activation of caspases. Furthermore, expression of LKB1 (K78M) inhibited TRAIL-induced apoptosis (Figure 4A) and increased the number of surviving colonies of osteosarcoma cells (Figure 4B). Therefore, it was demonstrated that LKB1 kinase activity was critical for TRAIL-mediated apoptosis induction in osteosarcomas. The function of DAP3 to induce apoptosis is regulated by another type of serine/threonine kinase, Akt (34). The functional relationship among these molecules in osteosarcomas should be clarified for their effective treatment in the near future.

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