Phosphorylation of Fas-associated Death Domain Contributes to Enhancement of Etoposide-induced Apoptosis in Prostate Cancer Cells

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Fas-associated death domain (FADD) plays an important role as an adapter molecule in Fas (CD95/APO-1)-mediated apoptosis and contributes to anticancer drug-induced cytotoxicity. We treated three human prostate cancer cell lines with etoposide, a topoisomerase II inhibitor with activity against various tumors including prostate cancer. We found that the overexpression of FADD sensitizes etoposide-induced apoptosis through a rapid activation of c-Jun NH2-terminal kinase (JNK) and, subsequently, of caspase 3. In addition, phosphorylation of FADD at serine 194 coincided with this sensitization. Treatment with the caspase 3 inhibitor, N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), or overexpression of either mitogen-activated protein kinase kinase (MKK) 7 or Bcl-xL canceled FADD-mediated sensitization to etoposide-induced apoptosis. Moreover, treatment with the caspase 8 inhibitor, benzoylxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-IETD-fmk), or overexpression of viral FLICE/caspase-8-inhibitory protein (FLIP) from equine herpesvirus type 2 E8 also had an inhibitory effect, supporting a major involvement of a caspase 8-dependent mitochondrial pathway. Interestingly, FADD was phosphorylated, and etoposide-induced JNK/caspase activation and apoptosis were enhanced in the cells arrested at G2/M transition, but not in those overexpressing mutant FADD, in which 194 serine was replaced by alanine. Our results demonstrate that phosphorylated FADD-dependent activation of the JNK/caspase pathway plays a pivotal role in sensitization to etoposide-induced apoptosis in prostate cancer cells.

Key words: FADD — Phosphorylation — Etoposide — Prostate cancer

Fas-associated death domain (FADD) was originally identified as an adapter molecule for Fas-mediated apoptosis. Upon Fas stimulation by Fas ligand1–3 or exposure to agonistic anti-Fas antibody,4 death-inducing signaling complex (DISC) is formed by recruitment of at least FADD and procaspase 8. Procaspase 8 is subsequently activated in DISC and activated caspase 8 further stimulates downstream caspases such as caspases 3, 6 and 7, which can initiate apoptosis. In addition, other reports indicate that a FADD-dependent pathway significantly influences the cellular response to cytotoxic drugs; FADD overexpression seems to enhance tumor cell sensitivity to the drugs, whereas exposure to an antisense construct decreased sensitivity.5

c-Jun NH2-terminal kinase (JNK) is a mitogen-activated protein kinase that regulates a number of physiological and pathological processes. JNK is strongly activated by and always linked to cell death under stressful stimuli such as ultraviolet (UV), γ irradiation and cytotoxic drugs.6 The entire role of JNK in cell death under a variety of stressful stimuli remains controversial. Tournier et al.,7 however, have recently demonstrated that, in JNK1−/− and JNK2−/− mouse embryonic fibroblasts, activation of JNK and the release of cytochrome c into the cytoplasm by JNK-mediated mitochondrial cell death signaling are required for UV radiation-induced apoptosis. In addition, JNK is required for the apoptotic response to the genotoxic methyl-methanesulfonate and the drug anisomycin. Chen and Lai8 also showed that persistent JNK activation in Jurkat T cells induces apoptosis, which is dependent on FADD and caspase 8.

Prostate cancer is a common diagnosis in both American and Japanese men. Improved procedures for surgical intervention and radiation have significantly reduced the number of fatalities. However, this type of cancer is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced disease, especially in hormone-independent cases.9–14 Recent reports have demonstrated novel mechanisms pertaining to chemosensitivity in prostate cancer cells by targeting FADD or JNK.15,16 There is thus growing evidence suggesting that a FADD- or JNK-dependent cell death mechanism plays an important role in chemotherapeutic sensitivity in various tumors, including prostate cancer, but the interaction between these proteins remains elusive. Scaffidi et al.17 showed that FADD is phosphorylated at serine 194, which correlates with cell cycling, but is not apparently essential for...
Fas-mediated apoptosis. However, they did not exclude the possibility that phosphorylation of FADD is important for other types of cell death.

In the present study, we focused on the effects of FADD, especially the phosphorylation of FADD at serine 194, and JNK activation on etoposide-induced apoptosis in three human prostate cancer cell lines, PC3, DU145 and LNCaP.\(^{19}\) Our results clearly show that JNK activation, mediated by phosphorylated FADD, sensitizes prostate cancer cells equally to etoposide-induced apoptosis through caspase 3 activation. This phosphorylated FADD-dependent enhancement of JNK activation and its subsequent execution of the caspase cascade may be critical for sensitivity to drug-induced apoptosis in prostate cancer cells.

**MATERIALS AND METHODS**

**Cell culture, plasmids and chemicals** We purchased the human prostate cancer cell lines, LNCaP, DU145 and PC3, from American Type Culture Collection (Manassas, VA) and cultured the lines in RPMI supplemented with 10% fetal bovine serum. Plasmid Myc-tagged dominant-negative MKK7\(^{19}\) was a gift from Dr. Nishida (Kyoto University, Kyoto). The expression vectors GFP (pTRE-d2GFP), β-gal (pβgal-control), and hygromycin-resistance gene (pTK-Hyg) were obtained from Clontech Laboratories Japan, Ltd. (Tokyo). Etoposide was purchased from Oncogene Research Products (Boston, MA); N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-IETD-fmk) were from MBL, Inc. (Nagoya); anti-FLAG antibody was from Sigma-Aldrich Japan, Ltd.; anti-194S-p-FADD was from Transduction Laboratories (Lexington, KY); anti-Bcl-xL was from Clontech Laboratories Japan, Ltd.; anti-β-gal for 1 h at 4℃ and precipitated with protein A-Sepharose (Amersham Pharmacia Biotech). After further washing with TBST, peroxidase activity was detected on X-ray films using an enhanced chemiluminescence detection system.

**Transfection of expression vectors** For transient transfection, cells were seeded at 5×10^5^ cells per well in 6-well plates. The cells were cultured in fresh medium for 24 h and then transfected with various vectors (0.2 μg/each vector) by using LipofectAmine (Invitrogen Japan, K.K., Tokyo) in accordance with the manufacturer’s protocol. To establish stable cell lines, the pTK-Hyg vector, harboring the hygromycin-resistance gene, was co-transfected with an expression vector encoding dominant-negative Myc-tagged MKK7. The transfectants were then grown and selected in the presence of hygromycin.

**Preparation of cell lysates, immunoprecipitation, and immunoblotting analysis** Cells were washed once with phosphate-buffered saline (PBS) and suspended in lysis buffer (40 mM Hepes (pH 7.4) with 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 20 mM β-glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM vanadate) with protease inhibitor mixtures (1 μg/ml apro tinin, leupeptin, and pepstatin). Cell lysates were cleared by centrifugation at 15 000 rpm for 30 min. For immunoprecipitation, cell lysates were incubated with 2 μg of anti-HA polyclonal antibody for 1 h at 4℃ and precipitated with protein A-Sepharose (Amersham Pharmacia Biotech, Japan, Ltd., Tokyo). Cell lysates and immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Ltd., Bedford, MA). The membranes were blocked in Tris-buffered saline-Tween 20 (TBST) buffer (20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20) with 5% skim milk at room temperature for 1 h. The membrane was then incubated with anti-FLAG, anti-HA, anti-FADD, anti-c-Myc, or anti-Bcl-xL for 1 h, washed with TBST, and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech). After further washing with TBST, peroxidase activity was detected on X-ray films using an enhanced chemiluminescence detection system.

**Fluorescence microscopy** A total of 5×10^5^ non-treated and 5×10^5^ hydroxyurea- or nocodazole-treated DU145 cells were harvested for flow cytometric and protein analyses as described below.

**Flow cytometry** A total of 5×10^5^ non-treated and hydroxyurea- or nocodazole-treated DU145 cells were incubated with propidium iodide, then DNA contents were measured by flow cytometric analysis.

**Assay of etoposide-induced apoptosis by β-gal staining in the transfected cells** Cells were transfected for 24 h with either 0.5 μg of various expression vectors or with 0.5 μg of β-gal expression vector (pβgal-control). Transfection was followed by stimulation with various concentrations of etoposide for 48 h; the cells were then fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS.
for 5 min at 4°C. After washing with PBS, the cells were overlaid with X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM MgCl₂, 0.02% NP-40, and 1 mg/ml X-gal in PBS) for 1 h at 37°C in the dark. The numbers of blue β-gal-positive cells with characteristic viable (flat, adherent) and apoptotic morphology (shrunken, round or blebbing formation) were characteristic viable (flat, adherent) and apoptotic morphology (shrunken, round or blebbing formation) were calculated. At least 500 β-gal-positive cells were scored for each transfection in triplicate, and the mean percent-ages±their standard deviations of viable blue and apoptosis before and after treatment with etoposide were calculated.

**Quantitative assay of etoposide-induced apoptosis in transfected cells** Cells were transiently transfected with an expression vector encoding green fluorescence protein (GFP) (pTRE-2EGFP) as previously described.23, 24) After an expression vector encoding green fluorescence protein (GFP) (pTRE-2EGFP) as previously described.23, 24) After

**Cell viability assay** DU145 cells were treated with hydroxyurea or nocodazole for 24 h on 96-well plates, then stimulated with 25 μg/ml of etoposide for 48 h, after which 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonfonyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, Tokyo) was added. After a 3 h incubation period, optical absorbance at 490 nm was measured using a microplate reader. Cell viability was expressed as mean percentage±the standard deviation of absorbance before and after treatment with various reagents. All experiments were performed in triplicate.

**DNA fragmentation assay** DU145 cells were untreated or treated with hydroxyurea or nocodazole for 24 h, then cells (10³) were prepared and stimulated with 25 μg/ml of etoposide for 48 h. The stimulated cells were placed in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0) and 0.5% Triton X-100 for 20 min at 4°C. The lysate was centrifuged at 15,000 rpm for 20 min and the resulting supernatant was incubated with 4 μl of RNase A (10 mg/ml) for 1 h at 37°C, followed by incubation with 4 μl of proteinase K (10 mg/ml) again for 1 h at 37°C. DNA was then extracted using 20 μl of 5 M NaCl and 120 μl of isopropanol for 10 min at room temperature followed by centrifugation at 20,000 rpm for 20 min. The DNA pellet was washed with 70% ethanol, then resuspended and loaded onto a 2% agarose gel and photographed under UV light.

**In vivo analysis of caspase 3 activation** Cells were transfected with various expression vectors, using pME18S-FLAG-tagged mammalian STE20-like kinase 1 (MST1)-KR as a substrate for caspase 3. Following 24 h cultivation, they were stimulated with 25 μg/ml etoposide for 48 h, lysed with lysis buffer and separated on 12% SDS-polyacrylamide gels. After immunoblotting with anti-FLAG antibody, caspase 3 activation was assessed by the detection of intact and cleaved MST1-KR.23, 24)

**In vitro JNK kinase assay** A standard aliquot of cells (5×10⁵ cells) was transfected with various expression vectors incorporating the HA-tagged JNK1 gene. After 24 h of transfection, the cells were stimulated with 25 μg/ml of etoposide for the indicated times. Alternatively, 10³ non-treated or hydroxyurea- or nocodazole-treated DU145 cells were stimulated with 25 μg/ml of etoposide for 48 h. Then, the cells were washed with PBS, lysed with lysis buffer, and immunoprecipitated with 2 μg of anti-JNK1 antibody. Immunoprecipitates were further incubated with 2 μg of the N-terminal peptide of c-Jun (amino acids 1–79; Santa Cruz Biotechnology, Inc.) in 25 μl of kinase reaction buffer (40 mM Hepes (pH 7.5) with 20 mM MgCl₂, 20 mM β-glycerol phosphate, and 0.1 mM vanadate) containing 25 μM adenine triphosphate (ATP) and 2.5 μCi of [γ-³²P]ATP for 30 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli’s sample buffer and boiling for 5 min. A portion of the sample (20 μl) was separated on a 12% SDS-polyacrylamide gels and autoradiographed.

**RESULTS**

**Effect of phosphorylation of FADD at serine 194 on etoposide-induced apoptosis** We analyzed whether FADD with or without phosphorylation at serine 194 plays a role in sensitization to etoposide-induced apoptosis in prostate cancer cells by using various FADD mutants. Expression of endogenous FADD and various FADD mutants in three prostate cancer cell lines was determined by immunoblotting of a detergent-soluble fraction (Fig. 1A). Since the upper band of FADD was detected in DU145 overexpressing wild type but not in S194A mutant by immunoblotting using anti-phosphorylated FADD at 194S antibody, we confirmed that the upper band is derived from phosphorylation at 194S. It is very difficult to strictly evaluate endogenous levels of FADD phosphorylation by this commercially anti-phosphorylated FADD...
antibody (data not shown). To investigate the effect of phosphorylated FADD on etoposide-induced cytotoxicity, we used two approaches—morphological quantification of apoptosis in a blinded fashion and flow cytometric quantification of cell viability. As shown in Fig. 1, B and C, the results obtained from these two assays correlate well. Transient transfection of FADD alone induced apoptosis in all prostate cancer cell lines, as did the various FADD mutants, including FADD S194A, in which serine 194 is replaced by alanine, which cannot be phosphorylated. The figures indicate the percentage of apoptotic cells (Fig. 1B) and the numbers of viable cells expressing each FADD mutant and a GFP expression vector. After cultivation for the time indicated, cells were stimulated with 25 μg/ml of etoposide or left unstimulated for 48 h. Viable transfected cells expressing various amounts of GFP were then quantified by flow cytometry. Numbers of GFP-positive (log GFP>1.0) cells were calculated, and the percentage (±standard error) of those stimulated with etoposide against those left unstimulated was calculated as indicated in the lower panel (PC3, DU145, LNCaP). W.B.: western blotting.

Fig. 1. Effects of transfected FADD and FADD mutants on etoposide-induced apoptosis in prostate cancer cell lines. (A) Expression of endogenous or transfected FADD and its mutants S194A and S194D in three prostate cancer cell lines, PC3, DU145 and LNCaP, was analyzed by immunoblotting with anti-FADD, anti-phosphorylated FADD (anti-p-FADD) at 194S or anti-FLAG antibody. (B) Cells were transfected with control empty vector, FLAG-tagged FADD, FADD S194A or FADD S194D together with a β-gal expression vector. After cultivation for the times indicated, cells were stimulated with various concentrations of etoposide for 48 h. The mean percentages (and standard error) of the number of apoptotic blue cells before and after treatment with etoposide were calculated in a blinded fashion as described in “Materials and Methods” (empty vector, FADD, S194D FADD, S194A FADD). (C) Cells were co-transfected with control empty vector or various FADD mutants and a GFP expression vector. After cultivation for the time indicated, cells were stimulated with 25 μg/ml of etoposide or left unstimulated for 48 h. Viable transfected cells expressing various amounts of GFP were then quantified by flow cytometry. Numbers of GFP-positive (log GFP>1.0) cells were calculated, and the percentage (±standard error) of those stimulated with etoposide against those left unstimulated was calculated as indicated in the lower panel (PC3, DU145, LNCaP). W.B.: western blotting.
FADD, in which glutamic acid was substituted for serine 194, gave the same results as FADD S194D (data not shown).

**Phosphorylated FADD enhances etoposide-induced caspase 3 activation** To investigate the role of serine 194 phosphorylation of FADD on etoposide-induced caspase 3 activation in prostate cancer cell lines, we transiently co-transfected the substituted FADD mutants with FLAG-tagged MST1-KR as a substrate for caspase 3 and analyzed the cleavage of MST1-KR after stimulation with etoposide. We first verified whether MST1-KR affects etoposide-induced apoptosis due to its dominant-negative effect. Overexpression of MST1-KR had no effect on etoposide-induced apoptosis in any of the prostate cancer cell lines examined (data not shown).

In both control cells and in cells transiently transfected with the F25Y FADD variant S194A, MST1-KR cleavage began 36 h after stimulation with etoposide (Fig. 2B). However, MST1-KR began to be cleaved by etoposide about 9 h earlier in cells transiently transfected with variant S194D, while in FADD-transfected cells, initial cleavage of MST1-KR was observed at 18 h after stimulation and was nearly complete at 36 h (Fig. 2B).

**Inhibition of sensitization to etoposide-induced apoptosis by Bcl-xL and viral FLIP E8** As shown in Fig. 3, A and C, FADD-dependent enhancement of etoposide-induced apoptosis or MST1-KR cleavage was almost completely nullified by treatment with DEVD-CHO, a specific inhibitor of caspase 3. This suggests that caspase 3 activation is required for FADD-mediated sensitization to etoposide-induced apoptosis in prostate cancer cells. We then analyzed whether caspase 3 is activated via a so-called mitochondrial pathway or is dependent on caspase 8 activation.

As illustrated in Fig. 3, B and C, transient transfection with Bcl-xL significantly inhibited any enhancement by phosphorylated FADD of etoposide-induced apoptosis and caspase 3 activation in all prostate cancer cell lines. Transient transfection of FLIP E8 also suppressed FADD sensitization, as did treatment with the caspase 8 inhibitor, z-IETD-fmk (Fig. 3, A, B, and C). Expression of Bcl-xL or viral FLIP E8 in each cell line was confirmed by immunoblotting (Fig. 3D).

**Activation of JNK contributes to phosphorylated FADD enhancement of etoposide-induced apoptosis** In all cell lines transfected with either empty vector or FADD S194A, initial activation of JNK occurred 12 h after stimulation with etoposide and treatment with a pan-inhibitor of caspses, z-VAD-fmk, had no effect on such FADD-dependent JNK activation (data not shown). In contrast, JNK activation was observed at 6 h after stimulation in the cells expressing FADD S194D and at 9 h after in FADD-transfected cells (Fig. 4A). Interestingly, in all prostate cancer cell lines stably expressing the dominant-negative mutant of mitogen-activated protein kinase kinase (MKK) 7, phosphorylated FADD-dependent enhancement of eto- poside-induced JNK activation, MST1-KR cleavage, and apoptosis were completely negated (Fig. 4, B and C). In addition, inhibition of etoposide-induced JNK activation by MKK7-KL had no significant effect on the phosphorylation status of co-transfected or endogenous FADD (data not shown), suggesting no major involvement of JNK activation in phosphorylation of FADD at 194S.

**Cell cycle-dependent phosphorylation of FADD is well correlated with the sensitization to etoposide-induced apoptosis** Scaffidi et al. showed that phosphorylation of FADD at 194 serine was subject to the G2/M transition by 70 kDa cell cycle-regulating kinase. Here, we investigated
whether etoposide-induced apoptosis in prostate cancer cells is affected by endogenous phosphorylation or unphosphorylation of FADD at the G2/M or G1/S. DU145 cells were arrested at the G1/S or G2/M boundary by treatment with hydroxyurea or nocodazole for 24 h. Cell cycle arrest was confirmed by flow cytometric analysis (Fig.
Fig. 4. Effect of JNK activation on the sensitization to etoposide-induced apoptosis by phosphorylated FADD. (A) Human prostate cancer cell line DU145 was left untreated or treated with hydroxyurea or nocodazole for 24 h. Then, DNA contents were measured by flow cytometric analysis as described in “Materials and Methods.” (B) Cultures of cells stably expressing Myc-tagged dominant-negative MKK7 were established and the expression of dominant-negative MKK7 was analyzed by immunoblotting with anti-Myc antibody. 5×10⁵ cells were then co-transfected with 0.2 µg each of FADD and HA-tagged JNK. After 24 h cultivation, cells were stimulated with 25 µg/ml of etoposide for the times indicated and immunoprecipitated with anti-HA antibody. An in vitro kinase assay of immunoprecipitated JNK was performed and phosphorylated GST-c-Jun (p-c-Jun) was quantified as described in “Materials and Methods.” (C) Cultures of cells stably expressing Myc-tagged dominant-negative MKK7 were stably expressing HA-JNK. After 24 h cultivation, cells were stimulated with 25 µg/ml of etoposide for the times indicated and immunoprecipitated with anti-HA antibody. An in vitro kinase assay of immunoprecipitated JNK was performed and phosphorylated GST-c-Jun (p-c-Jun) was quantified as described in “Materials and Methods.” (D) Cultures of cells stably expressing Myc-tagged dominant-negative MKK7 were co-transfected with 0.2 µg each of FADD and FLAG-tagged MST1-KR. After 24 h cultivation, cells were stimulated with 25 µg/ml of etoposide for 24 h. Then, DNA contents were measured by flow cytometric analysis. Alternatively, immunoblotting using anti-caspase 8 or anti-caspase 3 antibody was performed. (D) After non-treatment or treatment with hydroxyurea or nocodazole for 24 h, cells were stimulated with 25 µg/ml etoposide for 48 h. Then, cell viability was examined by MTS assay, and DNA fragmentation was analyzed. As shown in Fig. 5B, cells arrested at G1/S predominantly contained unphosphorylated FADD, whereas FADD was quantitatively phosphorylated in cells arrested at G2/M. These results were consistent with the previous report by Scaffidi et al. 17 Interestingly, in cells arrested at G2/M, etoposide-induced activation of the JNK/caspase 8/3 pathway and apoptosis were enhanced, while such an enhancement was not found in those arrested at G1/S (Fig. 5C).
Overexpression of non-phosphorylable FADD blocked the sensitization at G2/M transition to etoposide-induced apoptosis. To clarify whether cell cycle-dependent phosphorylation of FADD at serine 194 is essential for the sensitization to etoposide-induced apoptosis in the cells arrested at G2/M transition, we examined the effect of non-phosphorylable FADD (S194A FADD) overexpression on such sensitization using DU145. As shown in Fig. 6, A and B, in the cells overexpressing control vector, G2/M arrest induced by nocodazole resulted in enhancement of apoptosis and reduction of cell viability as measured by morphological and flow cytometric analysis, respectively. In contrast, in those overexpressing S194A FADD, such a G2/M arrest-related sensitization to etoposide-induced apoptosis was not seen. In addition, enhancement of etoposide-induced JNK activation at G2/M arrest was observed in control cells but not in S194A FADD-overexpressing cells (Fig. 6C).

**DISCUSSION**

Our work here demonstrates for the first time that serine 194 phosphorylation of FADD is correlated to enhanced sensitization to etoposide-induced apoptosis. However, a major problem had to be overcome to enable us to assess the physiological role of FADD in this particular system. We discovered that the FADD S194A variant, which cannot be phosphorylated at all, might not be the proper vehicle to evaluate the role of phosphorylation due to the presence of endogenous phosphorylated FADD within the cell. To compensate, we analyzed the amount of expression of both phosphorylated and unphosphorylated FADD by immunoblotting with an anti-phospho-serine 194 antibody. We found that, fortunately, all the cell lines used in the present experiment have relatively little phosphorylated FADD as compared to the unphosphorylated protein (data not shown). In addition, we compared the amount of cell death, MST1-KR cleavage, or JNK activation in the cells transfected with the FADD mutants with those transfected with empty vector. All of these approaches gave us a clearer picture of the role of phosphorylated FADD in etoposide-induced apoptosis.

With the exception of FADD S194A, overexpression of both endogenous and substituted mutant FADDs enhances etoposide-induced activation of JNK via MKK7 and MST1-KR cleavage. The kinase MST1 is directly activated by caspase 3 during apoptosis both in vivo and in vitro. When co-transfected with MST1-KR, a mutant form of MST1, the cleavage of MST1-KR is similarly indicative of caspase 3 activation in vivo. Watabe et al. showed that a kinase mutant of MST1 can suppress a certain type of apoptosis due to its dominant-negative effect. However, in our prostate cancer cell lines, we found that MST1-KR had no significant effect on apoptosis. This suggests that caspase 3 activation via MKK7 plays a major role in the etoposide sensitization cascade initiated by phosphorylated FADD.

JNK activation by etoposide was completely suppressed by overexpression of dominant-negative MKK7 (Fig. 4, B and C), but not by caspase inhibitors (data not shown), supporting the theory that JNK activation acts upstream of the caspase cascade. Recent reports have demonstrated that JNK can be activated via Fas in both caspase-depen-
dent and -independent pathways. Since cytotoxic drugs such as etoposide, doxorubicin, and cisplatin are thought to induce Fas activation and subsequent interaction with FADD, it is possible that etoposide-induced Fas-FADD activation might, in turn, lead to acceleration of JNK activation through the caspase-independent manner, for which phosphorylation of FADD at serine 194 may be essential.

Bcl-xL, a member of the anti-apoptotic Bcl-2 gene family, clearly prevents apoptosis by blocking cytochrome c release from mitochondria followed by caspase 9 and caspase 3 activation. Bcl-xL is found in and profoundly associated with chemosensitivity in prostate cancer cell lines. Viral FLIP E8 inhibits Fas-induced apoptosis without stimulating apoptosis itself, even though it consists of two repeated death-effector domains without a pro tease-like domain. We found that both Bcl-xL and viral FLIP E8 suppress FADD-mediated enhancement of etoposide-induced caspase 3 activation and apoptosis (Fig. 3B). A similar result was demonstrated upon treatment with relative specific inhibitors for caspase 8 or 3 (Fig. 3A). This result suggests a pathway following JNK activation involving caspase 3 activation via caspase 8 and mitochondrial cytochrome c release. Tournier et al. similarly proposed that stress-induced apoptosis follows a pre-apoptotic pathway initiated by JNK activation and progressing to cleavage of Bid, cytochrome c release from mitochondria, and caspase 3 activation of an apoptotic cascade7; Bid is cleaved and activated by caspase 8 and induces cytochrome c release. As mentioned earlier, Chen and Lai showed that persistent JNK activation, mediated by overexpression of MKK7, induces apoptosis in Jurkat T cells and that this process is dependent on FADD and caspase 8. Given these results, we propose that a rapid activation of JNK through phosphorylated FADD might activate caspase 8, thus triggering the execution of apoptosis via cytochrome c release and caspase 3 activation. In the present study, inhibition of caspase 8 suppressed enhancement of etoposide-induced caspase 3 activation and did so almost as effectively as overexpression of Bcl-xL, indicating that caspase 3 is mainly activated through a caspase 8-dependent mitochondrial pathway.
sition. Interestingly, such G2/M arrest-related enhancement of etoposide-induced JNK activation or apoptosis was canceled by overexpression of non-phosphorylatable FADD, probably through a dominant-negative effect. These results suggest that cell cycle-dependent phosphorylation of FADD at serine 194 may contribute to the susceptibility to etoposide-induced apoptosis, and raise the possibility that the phosphorylation of FADD/JNK/caspase pathway plays a critical role in the sensitization of prostate cancer cells to anticancer drug-induced apoptosis.

Our study indicates that phosphorylation of FADD at serine 194 accelerates the etoposide-induced cytotoxicity mechanism, in which JNK and caspases are sequentially activated dependently on the mitochondrial pathway (Fig. 7), and that regulation of FADD phosphorylation, or the kinase responsible for this phosphorylation, may provide a novel strategy for treatment of prostate cancer.

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