Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis

Keiji Shimada, Syuichi Matsuyoshi, Mitsutoshi Nakamura, Eiwa Ishida, Munehiro Kishi and Noboru Konishi

Department of Pathology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan

1To whom correspondence should be addressed Email: nkonishi@naramed-u.ac.jp

FADD has been shown to be phosphorylated at Ser194 at the G2/M transition of the cell cycle. Here we have investigated the contribution of this phosphorylation to apoptosis induced by anticancer drugs in two human prostate cancer cell lines, LNCaP and DU145. Both were arrested at G2/M and FADD was found to be phosphorylated at Ser194 on treatment with paclitaxel. Inhibition of paclitaxel-induced c-jun NH2-terminal kinase (JNK) activation by treatment with a specific inhibitor, SP600125, or overexpression of a dominant-negative mutant form of upstream kinases, MEK kinase 1 (MEKK1) and mitogen-activated protein kinase kinase (MKK) 7, significantly reduced the increase in phosphorylated FADD. It is noteworthy that pretreatment with paclitaxel significantly up-regulated MEKK1 expression, resulting in enhancement of etoposide- or cisplatin-induced MEKK1/MKK7-dependent JNK activation and apoptosis in LNCaP and DU145 cells. Interestingly, MEKK1 up-regulation and the synergistic effects of paclitaxel on anticancer drug-induced apoptosis were abolished by overexpression of mutant FADD (Ser194→Ala). The results clearly show that FADD phosphorylation at Ser194 affects functions both upstream and downstream of the MEKK1/MKK7/JNK1 pathway and is closely associated with chemosensitivity in prostate cancer cells. This is the first report indicating that phosphorylated FADD plays an essential role in the mechanisms of amplifications of chemotherapy-induced apoptosis.

Introduction

The Fas-associated death domain-containing protein (FADD) was originally identified as an adapter molecule for Fas-mediated apoptosis (1,2). Upon Fas stimulation by Fas ligand (3,4) or exposure to agonistic anti-Fas antibody, a death-inducing signaling complex (DISC) is formed by recruitment of at least FADD and procaspase 8. The latter is subsequently activated in DISC and further stimulates downstream caspases such as caspases 3, 6 and 7, which can initiate apoptosis. In addition, a number of investigations have indicated that a FADD-dependent pathway significantly influences the cellular response to cytotoxic drugs; FADD overexpression appears to enhance tumor cell sensitivity, whereas exposure to an anti-sense construct decreases the sensitivity (5).

Scaffidi et al. showed that FADD is exclusively phosphorylated at the C-terminal Ser194, specifically at the G2/M transition, suggesting an essential role of FADD in cell cycle progression (6). Very recently, analysis of mutant mice with FADD unphosphorylated or constitutively phosphorylated at Ser194, the amino acid equivalent to Ser194 in human FADD, showed that FADD phosphorylation is the key to its role in the regulation of cell growth and proliferation (7).

Paclitaxel is an anticancer drug that targets microtubules and arrests cancer cells in mitosis (8,9) and is well known to be an adjuvant drug for enhancement of apoptosis induced by chemotherapy, radiotherapy or death receptor-mediated death signaling in various types of malignancies, including prostate cancer (10,11). G2/M cell cycle arrest is one of the important mechanisms involved in the synergistic effect of paclitaxel on induction of apoptosis because cancer cells arrested at the G2/M transition are known to be most sensitive to chemotherapy and ionizing radiation (12–14). These results raise the possibility that phosphorylation of FADD at Ser194 is closely associated with sensitization to chemotherapy-induced apoptosis.

c-Jun NH2-terminal kinase (JNK), a member of the mitogen-activated protein (MAP) kinase family, acts as an important factor for apoptotic transduction initiated by toxic stimuli (15). It has been shown that overexpression of constitutively active mutant forms of upstream kinases such as MAP kinase kinase (MKK) or MEK kinase (MEKK) can lead to further activation of caspases and induction of apoptosis (16). In contrast, overexpression of dominant-negative mutant forms of these molecules effectively inhibits apoptosis induced by anticancer drugs (17–19). Recently we have demonstrated that activation of JNK and its dependent Fas-mediated death signaling significantly affects the sensitivity to etoposide-induced apoptosis in hormone-insensitive prostate cancer cells (19). Thus, JNK strongly contributes to chemosensitization through a variety of pathways, including death receptor-related ones. In the present study we have investigated the role of paclitaxel-mediated FADD phosphorylation in synergistic effects on MAP kinase activation and apoptosis induced by etoposide or cisplatin, in an attempt to clarify the molecular mechanisms of FADD-dependent chemosensitization in prostate cancer cells.

Materials and methods

Cell culture, plasmids and chemicals

The human prostate cancer cell lines LNCaP and DU145 were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI supplemented with 10% fetal bovine serum. FLAG or Myc-tagged human FADD and MEKK1 or M KK7 cDNA were prepared by conventional RT–PCR.
and mutant FADD, S194→A, and kinase-inactive mutants of MEKK1, K1253→M and MKK7, K165→L, were generated using a Quick-change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) as described earlier (17,19,20). To establish stable cell lines, the pTK-Hyg vector (Clonetech Laboratories Japan Ltd, Tokyo, Japan), harboring the hygromycin-resistant gene, was co-transfected with the expression vector encoding dominant-negative Myc-tagged MKK7, FLAG-tagged MEKK1 or FLAG-tagged S194A FADD as previously described (19). Etoposide and cisplatin were purchased from Calbiochem (San Diego, CA), anti-phospho-FADD (194S) was from Cell Signaling (Beverly, MA), anti-FLAG was from Sigma-Aldrich Japan Ltd (Tokyo, Japan), anti-poly(ADP)ribose polymerase (PARP) polyclonal antibody and anti-FADD antibody were from Transduction Laboratories (Lexington, KY), anti-c-Myc antibody was from Clontech Laboratories Japan Ltd, anti-JNK1 and MEKK1 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-actin monoclonal antibody was from Oncogene Research Products (Darmstadt, Germany).

Preparation of cell lysates 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, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM sodium orthovanadate) with a protease inhibitor mixture (1 μg/ml aprotinin, leupeptin and pepstatin). Cell lysates were resolved in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore Ltd, Bedford, MA). The membranes were blocked in TBST buffer (20 mM Tris containing 150 mM NaCl and 0.1% Tween 20) with 5% skimmed milk at room temperature for 1 h and then incubated with anti-FADD, anti-phospho-FADD (S194), anti-MEKK1, anti-JNK1, anti-c-Myc or anti-FLAG 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.

Flow cytometry and apoptosis analysis

Cells were transfected with paclitaxel, etoposide, cisplatin or combinations for the indicated times, harvested, centrifuged and fixed in 80% ethanol. The cells were co-stained with annexin V FITC alone or for annexin V-FITC and propidium iodide. Flow cytometry was performed using a FACSort flow cytometer (Becton Dickinson, CA) and the percentage of apoptotic cells was calculated based on forward and sideward scatter analysis.

In vitro kinase assay

Lysates were immunoprecipitated with 2 μg anti-JNK1 antibody or 10 μg anti-MEKK1 antibody, followed by incubation with 1 μg N-terminal peptide of c-Jun (amino acids 1–79; Santa Cruz Biotechnology) or MKK7 β1 (inactive, amino acids 2–419) (Upstate Biotechnology, Waltham, MA) in 25 μl of kinase reaction buffer (40 mM HEPES, pH 7.5, with 20 mM MgCl2, 20 mM β-glycerophosphate and 0.1 mM vanadate) containing 25 μM ATP and 2.5 μCi [γ-32P]ATP for 30 min at 30°C. Endogenous JNK1 or MEKK1 kinase activity was determined using Kodak 1D Image Analysis Software (Tokyo, Japan) as described previously (19,22).

Results

Contribution of the MEKK1/MKK7/JNK1 pathway to phosphorylation of FADD at Ser194

Paclitaxel induced apoptosis of the human prostate cancer cell lines LNCaP and DU145 in a concentration-dependent manner, as confirmed by the results for PARP cleavage (Figure 1A). Consistent with a previous report, FADD was phosphorylated on treatment with paclitaxel in both cell lines, as determined by western blots using a specific antibody to FADD phosphorylated at Ser194 (Figure 1B). Although passage through the G2/M transition of the cell cycle continued to be observed, the amount of phosphorylated FADD was greater in cells treated with a higher concentration of paclitaxel (Figure 1B). It has been shown that cell cycle arrest in the G2/M phase can be affected with paclitaxel even at low concentrations, whereas JNK activation and apoptosis can only be induced by a high concentration (9). As shown in Figure 2A, JNK was activated and the activity was sustained for up to 24 h by stimulation of LNCaP and DU145 cells with 25 nM paclitaxel, but not with 10 nM paclitaxel (data not shown). However, in cells stably expressing dominant-negative mutant forms of the upstream kinases MKK7 [MKK7 (K/L)] and MEKK1 [MEKK1 (K/M)], 25 nM paclitaxel failed to activate JNK, with phosphorylated FADD being reduced to almost the same level as after stimulation by 10 nM paclitaxel. Similar results were obtained when cells were treated with the JNK inhibitor SP600125 (23) (Figure 2B and C). These data suggest that JNK activation through MEKK1/MKK7 is closely associated with phosphorylation of FADD (6). Recently, Screaton et al. demonstrated that FADD is mainly located in the nucleus in many types of adherent cancer cells, such as Caco-2 colorectal carcinoma cells (24). However, we determined the localization of FADD to be mainly in the cytoplasm of the prostate cancer cells used in the current study (data not shown).

Phosphorylated FADD contributes to chemosensitization by paclitaxel through regulating JNK activation

To identify the role of FADD phosphorylation in sensitization to anticancer drug-induced apoptosis, we constructed cell lines stably expressing mutant FADD in which Ser194 was replaced by alanine (S194A FADD). As shown in Figure 3A, overexpression of S194A FADD significantly inhibited phosphorylation of FADD at Ser194 induced by 25 nM paclitaxel in LNCaP and DU145 cells. When cells were treated with 25 nM paclitaxel for 12 h then stimulated by 5 μM etoposide or cisplatin for 36 h, neither of which was active alone, paclitaxel-induced apoptosis and JNK activation were significantly enhanced, i.e. apoptosis and JNK activation by sub-lethal concentrations of etoposide and cisplatin could be induced by 12 h treatment with paclitaxel (Figure 3B and C). The synergistic effects were strongly reduced in cells expressing S194A FADD, indicating that phosphorylation of FADD is closely linked to paclitaxel-induced chemosensitization in prostate cancer cells. We have observed that in both LNCaP and DU145 cells expressing S194A FADD, JNK can be activated by other reagents, such as anisomycin and concentrated sodium hydrochloride (data not shown). Interestingly, overexpression of S194A mutant FADD significantly inhibited the delayed phase of JNK activation by paclitaxel and apoptosis, as well as the synergistic effects (Figure 3B and D). As to JNK activation, the same results were also observed in DU145 cells (data not shown). The data demonstrate that phosphorylation of FADD is required for sustained activation of JNK and plays an important role in sensitivity to apoptosis induced by paclitaxel. Overexpression of a dominant-negative mutant form of upstream kinase, MEKK1, significantly canceled the enhancement of JNK activation and apoptosis by paclitaxel treatment (data not shown), suggesting that the MEKK1/JNK1 pathway is involved in paclitaxel-mediated chemosensitization through phosphorylation at Ser194.
FADD phosphorylation at Ser194. The slightly greater activation of JNK in LNCaP than in DU145 cells may reflect differences in sensitivity to etoposide or cisplatin induction of apoptosis between the two lines (25).

As shown in Figure 3D, apoptosis could be induced by lethal doses of etoposide or cisplatin alone even in clones expressing S194A FADD, suggesting that phosphorylation status of FADD is closely related to chemosensitization but not to resistance to apoptosis induced by etoposide or cisplatin.

The role of the MEKK1/MKK7/JNK1 pathway in anticancer drug-induced apoptosis in prostate cancer cells

Etoposide and cisplatin induced apoptosis in both LNCaP and DU145 cells in a dose-dependent manner, LNCaP being the more sensitive line (Figure 4A). JNK was similarly activated by the drugs, and this was canceled out by overexpression of a dominant-negative mutant form of MKK7 or MEKK1 or treatment with the JNK inhibitor SP600125, together with etoposide- or cisplatin-induced apoptosis (Figure 4B). Thus, activation of the MEKK1/MKK7/JNK1 pathway plays a critical role in execution of apoptosis due to anticancer drugs in prostate cancer cells, with involvement of FADD phosphorylation at Ser194 in paclitaxel-mediated chemosensitization.

MEKK1 is up-regulated by paclitaxel through phosphorylated FADD

To identify the underlying molecular mechanisms, we investigated the effect of paclitaxel on MEKK1 regulation. MEKK1 has been shown to be cleaved by activated caspase, therefore, we selected 10 nM paclitaxel, which can induce FADD phosphorylation but not apoptosis. As shown in Figure 5, MEKK1 was up-regulated by 10 nM paclitaxel in the LNCaP and DU145 parental lines, but this was completely canceled out in the cells expressing S194A mutant FADD (Figure 5A). Consistent with an increase in MEKK1 expression, MEKK1 activation by etoposide or cisplatin was significantly enhanced by pretreatment with 10 nM paclitaxel in the parental cell lines, but not in the clones transfected with S194A FADD (Figure 5B). As expected, overexpression of wild-type MEKK1 can successfully lead to induction of apoptosis in
both control clones and the clones expressing S194A mutant FADD by sub-lethal concentrations of etoposide and cisplatin (Figure 5C). The results clearly show that up-regulation and activation of MEKK1 in a FADD phosphorylation-dependent manner are key mechanisms in paclitaxel-mediated enhancement of JNK activation, rendering prostate cancer cells highly sensitized to apoptosis induced by anticancer drugs.

Discussion

We have shown here that MEKK1/MKK7/JNK1 activation contributes to phosphorylation of FADD at Ser194 in prostate cancer cells. Consistent with a previous report (6), FADD was quantitatively phosphorylated at the G2/M transition by paclitaxel in prostate cancer cells. Inhibition of JNK inhibited phosphorylation of FADD and FADD and JNK1 activity were determined by western blotting and in vitro kinase assay, respectively (raw data for LNCaP only). (C) Phosphorylated FADD levels were detected using anti-phosphorylated FADD antibody and the blots were subjected to densitometric analysis. The data are presented as the ratio of phosphorylated to unphosphorylated FADD (means ± SE of three independent experiments, n = 3). Clones 1, 2, 7 and 9 are those of DU145 which express MEKK1 K/M and MKK7 K/L, respectively.
Fig. 3. Phosphorylation of FADD at Ser194 and sensitization to anticancer drug-induced apoptosis. (A) LNCaP and DU145 cells were transfected with the hygromycin B resistance gene, with an empty vector or with a FLAG-tagged vector encoding S194A mutant FADD. After incubation in the presence of hygromycin B, one control clone (Hyg B) and two clones for S194A mutant FADD transfectants were selected. Expression of S194A mutant FADD was determined by western blotting with anti-FLAG antibody. These clones were stimulated with 25 nM paclitaxel for 12 h, then phosphorylation of FADD was determined using anti-phosphorylated FADD at Ser194 antibody. (B) LNCaP and DU145 cells and clones (HygB and S194A mutant FADD) were treated with or without 25 nM paclitaxel for 12 h, then stimulated with dimethyl sulfoxide (DMSO), etoposide (5 μM) or cisplatin (5 μM) alone for 36 h. After stimulation, apoptosis was assessed by flow cytometric analysis. (C) Similarly, cells were stimulated with anticancer drugs for the indicated times with or without paclitaxel pretreatment, then the kinase activity of JNK1 was determined by in vitro kinase assay (data from LNCaP cell only). (D) LNCaP and DU145 cells and clones were stimulated with etoposide (25 μM), cisplatin (25 μM) or paclitaxel (25 nM) alone for 36 h, then apoptosis was assessed by flow cytometric analysis. The data are average values ± SE (flow cytometric analysis, n = 4).
FADD phosphorylation induced by paclitaxel but this was only partial. The effects of paclitaxel were not significantly canceled out by treatment with the JNK inhibitor SP600125 (21). Moreover, JNK could not directly phosphorylate FADD at Ser194 as assessed in an in vitro kinase assay in the present study (data not shown). From these results we suggest that induction of the MEKK1/MKK7/JNK pathway by paclitaxel is not essential but might stimulate a yet to be identified cell cycle regulating kinase involved in FADD phosphorylation (6) and the G2/M transition, resulting in successful enhancement of FADD phosphorylation (Figure 6). There are a number of conflicting reports as to the role of MEKK1 in activation of JNK by paclitaxel. Gibson et al. (16) demonstrated that taxol dissociates the activation of MEKK1 from the JNK pathway in HEK293 cells, while other investigators (17,26,27), in contrast, indicated that microtubule-interfering agents activate JNK through both the Ras/Rac/MEKK1 and apoptosis signal-regulating kinase (ASK)-1 pathways. At present, we have no critical evidence, but cell type specificity is a likely explanation for this discrepancy and MEKK1 closely regulates JNK activity in response to paclitaxel at least in prostate cancer cells.

The present study demonstrated one more important finding. FADD phosphorylation at Ser194 is closely associated with paclitaxel-induced up-regulation of MEKK1 expression and enhancement of the downstream JNK pathway by which prostate cancer cells are highly sensitized to apoptosis by etoposide or cisplatin. It has been demonstrated that both MEKK1 expression and activity are increased at G2/M by nocodazole or taxol and that this is cancelled in cells blocked at G1/S by hydroxyurea (26). This result and the findings shown in Figure 5 clearly indicate that up-regulation of MEKK1 and activation of the downstream pathway are mediated through FADD phosphorylation at Ser194. Interestingly, G2/M phase arrest induced by paclitaxel or paclitaxel plus etoposide/cisplatin was inhibited in prostate cancer cells expressing S194A FADD, but the inhibitory effect was not complete (data not shown), even though both FADD phosphorylation and sensitization to apoptosis were almost thoroughly canceled out in the clones. The results indicate that other mechanisms than cell cycle arrest appear to be involved in chemosensitization through FADD phosphorylation in prostate cancer cells. In addition, we suggest that FADD phosphorylation might be more essential for regulation of MEKK1 expression by paclitaxel than for the G2/M transition. A number of reports have shown that DNA-damaging agents such as etoposide, cisplatin and UV radiation can activate MEKK1/JNK and induce the proteolytic cleavage of MEKK1, whereas the kinase mutant form blocks apoptosis in response to these reagents (16,17,26). We also demonstrated in Figure 4 that

![Figure 4](image_url)

Fig. 4. The MEKK1/MKK7/JNK pathway is essential for apoptosis induced by etoposide or cisplatin in prostate cancer cells. (A) LNCaP and DU145 cells were stimulated with the indicated concentrations of etoposide or cisplatin for 36 h, then cells were harvested for assessment of the percentage of apoptotic cells by flow cytometric analysis. (B) LNCaP and DU145 cells or the clones described in Figure 2 were pretreated with or without 20 μM SP600125 (SP) for 30 min, then stimulated with 25 μM etoposide or cisplatin for 36 h. After stimulation, the percentage of apoptotic cells was determined by flow cytometric analysis and cleavage of PARP by western blotting (left). The data for (A) and (B) are average values ± SE (flow cytometric analysis, n = 4). After stimulation with etoposide or cisplatin (5 or 25 μM) for 6 h the kinase activity of JNK1 was determined by in vitro kinase assay (right).
the MEKK1/MKK7/JNK pathway plays a critical role in etoposide- and cisplatin-induced apoptosis in prostate cancer cells, suggesting that the enhancement of this pathway through FADD phosphorylation can lead to chemosensitization. The present results show the synergistic effect of paclitaxel on etoposide- and cisplatin-induced apoptosis to be slightly greater in LNCaP than DU145 cells. Previous studies have demonstrated that expression of constitutively active

Fig. 5. The regulation of MEKK1 is closely dependent on FADD phosphorylation. (A) Human prostate cancer cell lines LNCaP and DU145, transfected with the hygromycin B resistance gene, with an empty vector (HygB) or with a FLAG-tagged vector encoding S194A mutant FADD (clones 1 and 4 for LNCaP and 2 and 11 for DU145), were stimulated with 10 nM paclitaxel for the indicated times. Then, cells were lysed and expression of MEKK1 was determined by western blotting. (B) LNCaP and DU145 cells, transfected with HygB or S194A mutant FADD (clone 1 for LNCaP and clone 11 for DU145), were pretreated with or without 10 nM paclitaxel, then stimulated with 5 μM etoposide or cisplatin for the indicated times. After stimulation, the kinase activity of MEKK1 was determined by in vitro kinase assay. Phosphorylation of kinase-inactive MKK7 was indicated as a fold increase over control. Error bars represent the SE of three separate experiments (n = 3). (C) Clones were transfected for 24 h with β-gal expression vector plus wild-type MEKK1 or empty vector. Transfection was followed by stimulation with 5 μM etoposide or cisplatin. After treatment for 48 h, the numbers of blue β-gal-positive cells with characteristic viable (flat, adherent) and apoptotic morphology (shrunken, round or with bleb formation) were calculated.
MEKK1 induces apoptosis in androgen receptor-positive (LNCaP) but not in androgen receptor-negative (DU145) prostate cancer cells (28,29). Androgen receptor-mediated signaling is mainly involved in MEKK1-induced apoptosis in prostate cancer cells and the synergistic effect of paclitaxel on etoposide- and cisplatin-induced apoptosis, through regulating MEKK1 activity, seems to be greater in LNCaP than DU145 cells. We also examined JNK activation and apoptosis induced by an agonistic anti-Fas monoclonal antibody in control and S194A mutant FADD clones, and the results showed that JNK activation and apoptosis were more prominent in mutant FADD clones, probably due to overexpression of FADD. Therefore, we conclude that phosphorylation and dephosphorylation of FADD at Ser194 affect MAP kinase and apoptotic signaling only in the case of specific stimulation, including by anticancer drugs.

In summary, phosphorylation of FADD at Ser194 plays an important role in sensitization to DNA-damaging agents by enhancing the MEKK1/MKK7/JNK pathway. It is of interest that amplification of chemotherapy-induced apoptosis through FADD phosphorylation at Ser194 and MEKK1-mediated signaling can be effected by a sub-lethal concentration of paclitaxel. A similar mechanism may also operate with Bcl-2, whose phosphorylation is mediated through the ASK-1/JNK pathway and results in activation of the latter (10,27). The present study provides useful clues to understanding the influence of the cell cycle on chemosensitivity and for the development of chemoadjunctive therapy in prostate cancer.

Acknowledgement

This research was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (14770103).

References


Received July 30, 2003; revised February 3, 2004; accepted February 20, 2004.