The association of deamidation of Bcl-xL and translocation of Bax to the mitochondria through activation of JNK in the induction of apoptosis by treatment with GSH-conjugated DXR

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Abstract. We investigated the induction of apoptosis via deamidation of Bcl-xL and translocation of Bax to the mitochondria by treatment with GSH-DXR. GSH-DXR treatment of HepG2 cells, which did not express GST P1-1, exhibited deamidation of Bcl-xL, and the degree of deamidation was related to the activation of caspase-3. Overexpression of GST P1-1 in HepG2 cells decreased both the Bcl-xL deamidation and caspase-3 activation induced by treatment with GSH-DXR. Bcl-xL deamidation and caspase-3 activation were also suppressed by co-treatment with SP600125, a specific inhibitor of JNK activity. Overexpression of wild-type Bcl-xL in HepG2 decreased GSH-DXR-induced apoptosis although deamidation was observed. However, expression of the deamidated mutant of Bcl-xL, in which aspartic acid was substituted for both arginine 52 and 66 (N52,66D-Bcl-xL), exhibited high sensitivity for the induction of apoptosis. Expression of the Bcl-xL mutant, in which alanine was substituted for both arginine 52 and 66 (N52,66A-Bcl-xL), suppressed deamidation and showed resistance to the induction of apoptosis by treatment with GSH-DXR. On the other hand, endogenous Bax and overexpressed Flag-Bax were localized in the cytosolic fraction of HepG2 cells. Treatment of the cells with GSH-DXR caused translocation of Flag-Bax to the mitochondrial fraction following the induction of apoptosis. The induced apoptosis was enhanced by the expression of Flag-Bax. Moreover, Flag-Bax was partly located in the mitochondrial fraction in N52,66D-Bcl-xL-expressed cells without the induction of apoptosis. Therefore, the induction of apoptosis by treatment of HepG2 with GSH-DXR was enhanced, thereby facilitating the release of cytochrome c by both deamidated inactivation of Bcl-xL and functional translocation of Bax to the mitochondria via JNK activation. Deamidation of Bcl-xL might be induced in order to translocate Bax to the mitochondria.

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Introduction

Regulation of apoptosis is essential for normal development and for the maintenance of homeostasis in most metazoans. Various apoptotic signals eventually converge into a common death mechanism, in which members of caspases are activated and cleave various cellular proteins. In mammals, the mitochondria play an essential role in apoptosis by releasing cytochrome c from the intermembrane space into the cytoplasm (1,2). Once in the cytoplasm, cytochrome c binds to Apaf-1, a mammalian homologue of Ced-4, that recruits and activates initiator caspase-9, which subsequently activates effector caspase-3/caspase-7 (3,4).

The Bcl-2 family of proteins includes the best-characterized regulators of apoptosis, comprising anti-apoptotic members, including Bcl-2 and Bcl-xL, and pro-apoptotic members that include multi-domain Bax and Bak and various single-domain BH3-only proteins (1,5,6). Proteins of this family directly regulate the release of mitochondrial cytochrome c. Many of the pro-apoptotic family members, such as Bax, Bid, Bad, Bim, and Bmf, are localized in the cytoplasm, and apoptotic stimulation results in their translocation to the mitochondria and induction of the release of cytochrome c, probably by inactivating anti-apoptotic members of the family and activating multi-domain members like Bax and Bak (7-10). Translocation of the BH3-only proteins appears to involve various post-translational modifications (11-15). Although the mechanism involved is still unknown from Bax has also been shown to undergo translocation and integration into the mitochondrial membrane during apoptosis (16-20), and the translocation process has been suggested to involve a conformational change of the Bax molecule, especially exposure of the C terminus (17,18,21). It has also been reported that translocation of Bax to the mitochondria is enhanced by caspases (18) or by intracellular alkalization (22) and is negatively regulated by Bcl-2 through a still unidentified mechanism (17,19,20). Moreover, it has been reported that phosphorylation of 14-3-3 by c-Jun N-terminal kinase (JNK) caused dissociation of Bax from 14-3-3/Bax complex and allows it undergo translocation to the mitochondria (23).

On the other hand, phosphorylation of Bcl-2 and Bcl-xL occurs in an unstructured loop and involves several sites by various stresses (24-30). Uncertainty still exists whether phosphorylation increases or decreases the anti-apoptotic function of Bcl-2. Most data support the original hypothesis (24) and suggest that phosphorylation inactivates Bcl-2, thus promoting apoptosis, possibly by freeing Bax from Bcl-2/Bax dimers (31).

It has been demonstrated previously (32) that Bcl-xL was expressed in hepatoma cells and that down-regulation of Bcl-xL by antisense oligonucleotide stimulated apoptosis in hepatoma cells in response to cellular stresses, such as serum starvation, p53 activation, and staurosporine treatment. Because down-regulation of endogenously expressed Bcl-xL alone was sufficient for the induction of apoptosis in hepatoma cells by various cellular stresses, Bcl-xL must play an integral role in suppressing apoptosis when hepatoma cells are exposed to pro-apoptotic conditions. It was demonstrated that Bcl-xL is post-translationally modified by deamidation at asparagines 52 and 66 in its loop domain (33). It was also demonstrated that deamidated Bcl-xL is a major form in normal and non-tumor liver tissues, whereas the level of deamidated Bcl-xL is lower than that of unmodified Bcl-xL in the majority of hepatocellular carcinomas tissues (32). Bcl-xL deamidation has been shown to produce a complete loss of the antiapoptotic function of Bcl-xL (34). It was proposed that suppression of Bcl-xL deamidation might be an important mechanism by which malignant tumors acquire resistance to apoptosis (33).

The stress-activated protein kinase, JNK, is predominantly activated by inflammatory cytokines and stress stimuli through phosphorylation of 183T and 185Y, and its active center consisting of an ATP-binding site formed by 55K (35-38). The importance of the JNK pathway has also been shown in the control of cell survival and death pathways, and interference with the JNK pathway suppresses induction of apoptosis by a variety of agents (39). It was important for the induction of cell proliferation, differentiation and apoptosis to activate JNK (40-44). Strong and prolonged activation of JNK has been reported in response to lethal doses of a variety of stresses including UVC, γ radiation and cisplatin, any one of which triggers apoptosis (40-44). Recently, the link between the redox active components of glutathione S-transferase P1-1 (GST P1-1, placental type isozyme of rat GST) and JNK has been redefined as a non-catalytic, ligand binding activity that mediates both stress and apoptotic responses (45-49).

In particular, it has been reported that increased levels of GST P1-1, one of the rat GST isozymes, was often associated with the emergence of resistance to antineoplastic agents in some cancer cells (50-53). The GST P1-1 molecule in the active center consisted of the GSH binding site (G-site) and the substrate-binding site (H-site) formed by 38W and 47C, respectively (54). On the other hand, the positively charged sequence contained residues 194-201 of human GST-pi (especially, 198H and 201R), therefore showing that the C-terminal region of GST P1-1 must bind to the negatively charged C-terminus of JNK (D- and E-rich domain) (46,49). Moreover, Adler *et al* reported that binding of the C-terminal peptide 194-201 of human GST-pi to the JNK molecule and peptide 34-50 of GST-pi containing the active center region suppressed JNK activity without directly binding to JNK (45-49). Additionally, we have reported that the inhibition of GST P1-1 by GSH-DXR activated JNK and induced apoptosis (49).

In the present study, we investigated that the induction of apoptosis via JNK activation by treatment with GSH-DXR was enhanced to facilitate release of cytochrome c by both deamidated inactivation of Bcl-xL and translocation of Bax to mitochondria.

Materials and methods

Materials. DXR was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). GSH, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium tablet (BCIP/NBT) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Dowex 50W x8 and glutaraldehyde were purchased from Nakarai Tesque (Kyoto, Japan). Acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA), DEVD-aldehyde, acetyl-Leu-Glu-His-Asp-aldehyde (LEHD-aldehyde) and 7-amino-4-methyl-coumarin (AMC) were purchased from Peptide Institute Inc. (Osaka, Japan). Ex-Taq DNA polymerase was purchased from Takara (Tokyo, Japan). Murine anti-human GST-pi antibody and murine anti-Flag antibody were purchased from Transduction Laboratories (KY, USA) and Sigma-Aldrich Japan, respectively. Vectors of pcDNA3.1 and pFLAG-CMV4 were obtained from Invitrogen and Sigma-Aldrich Japan, respectively. Rabbit anti-human phospho-c-Jun (Ser63) antibody and rabbit anti-human phospho-JNK antibody were obtained from Cell Signaling Technology Inc. (MA, USA). All other chemicals were of analytical grade.

Cell lines. The human hepatoma cell line, HepG2, was cultured with RPMI-1640 containing 10% heat inactivated fetal bovine serum (growth medium) under conventional conditions (55-61).

Subcellular fractionation. After treatment of HepG2 cells with 10 μM digitonin in PBS at 37˚C for 15 min, mixture was centrifuged at 10,000 x g for 5 min. The obtained supernatant was used as cytosolic fraction. The other precipitate was suspended with 10 mM sodium phosphate buffer (pH 7.4) and the suspension was centrifuged at 1,000 x g for 5 min. The supernatant was centrifuged at 10,000 x g for 20 min. The obtained precipitate was used as mitochondrial fraction.

Conjugation of DXR with GSH. GSH-DXR was prepared as described previously (56-59). In brief, the combination of 1 mg of each GSH and 0.5 mg of DXR in 0.5 ml of 0.15 M NaCl containing 0.1% glutaraldehyde was incubated at room temperature for 30 min. After incubation, GSH-DXR was separated from GSH and DXR using Dowex 50 W x8 (H^+ form, 5x15 mm). The obtained GSH-DXR was filter-sterilized by a 0.45-μm syringe filter (Corning Coster, Tokyo, Japan). The concentration of DXR was measured by absorbance at 495 nm.

Assay of caspase-3 activity. Caspase-3 activity was measured in 100 μM of DEVD-MCA monitored for AMC liberation at 37˚C for 15 min in a spectrofluorometer at an excitation/

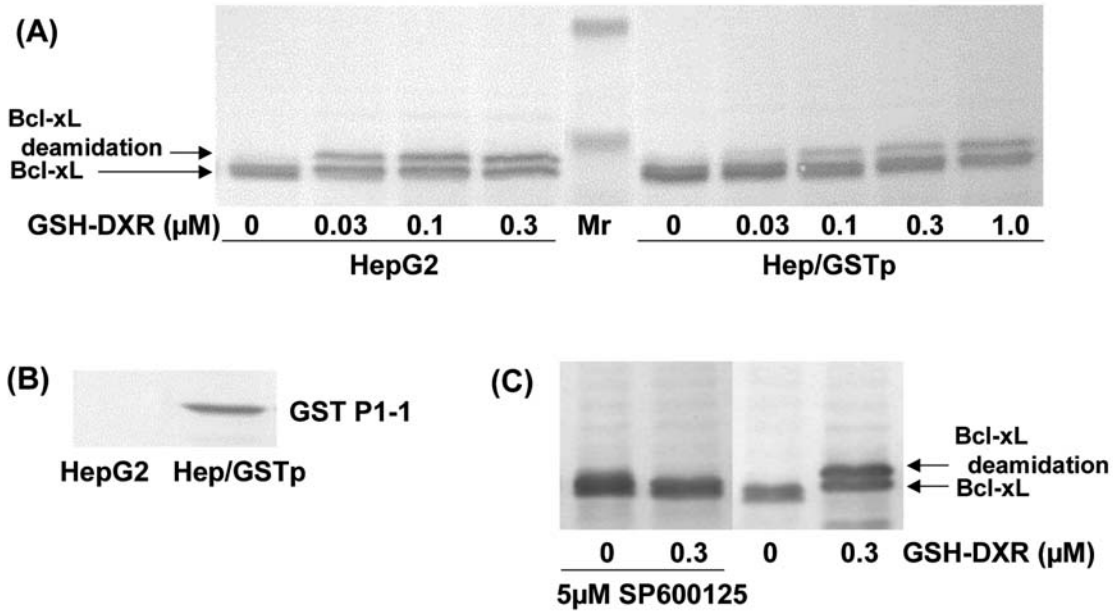

Figure 1. Deamidation of Bcl-xL in HepG2 and HepG2/GSTp cells treated with GSH-DXR. (A) Induction of Bcl-xL deamidation in both cells by treatment with various concentration of GSH-DXR for 24 h. (B) Expression of GST-pi in HepG2/GSTp cells, which was transfected with plasmid of GST-pi/pcDNA3.1 into HepG2 cells, was detected by Western blot analysis using anti-GST-pi antibody. (C) Suppression of GSH-DXR-induced Bcl-xL deamidation in the cells treated with 5 μM SP600125, an inhibitor of JNK. (A and C) Bcl-xL and its deamidation were detected by Western blot analysis using anti-Bcl-x antibody.

emission wavelength of 380/460 nm, and expressed as pmol AMC per min per mg protein (62).

Detection of GST-pi (Western blot analysis). GST-pi protein was analyzed as previously reported (59). After immunoreaction using murine anti-GST-pi antibody (x1/1,000) as the primary antibody and anti-mouse IgG-alkaline phosphatase conjugate as the secondary antibody (x1/20,000), GST-pi band was visualized with BCIP/NBT.

Construction of Bcl-xL/pFLAG, mutated Bcl-xL/pFLAG and Bax/pFLAG plasmids. Bcl-xL cDNA and Bax cDNA, prepared by RT-PCR from total RNA extracted from HepG2 cells, were inserted into pFLAG-CMV4 (mammalian expression vector). Two mutated plasmids of site-directed mutated Bcl-xL cDNA, in which two deamidation sites Asn52 and Asn66 were replaced with Asp [Bcl-xL deamidated mutant (N52,66D-Bcl-xL)] or Ala [Bcl-xL deamidation-resistant mutant (N52,66A-Bcl-xL)], were prepared from the resulting Bcl-xL/pFLAG as the template using the Site-directed Mutagenesis Kit (Stratagene, USA). Each of the plasmids obtained was transfected into HepG2 cells using the FuGENE 6 transfection reagent (Roche). The transfectants were selected by treatment with G418.

Construction of GST-pi/pcDNA3.1 plasmid. GST-pi cDNA, prepared by RT-PCR from total RNA extracted from GST-pi-expressed human epitherial A431 cells, was inserted into pcDNA3.1 vector. The plasmid obtained was transfected into HepG2 cells using the FuGENE 6 transfection reagent (Roche). The transfectant was selected by treatment with G418.

Assay of JNK activity. After treatment of HepG2 cells with GSH-DXR, JNK was extracted with cell lysis buffer (1% Triton X-100, 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 mM Na_3VO_4 and 1 μg/ml leupeptin). JNK activity in the cell extract was measured as follows: the cell extract was incubated with 100 μM ATP and 1 μg c-Jun in kinase buffer (25 mM Tris-HCl (pH 7.5) containing 0.1 mM Na_3VO_4) at 30˚C for 30 min. The phosphorylated c-Jun (JNK activity, Pi-c-Jun) by incubation and the phosphorylated JNK (active form, Pi-JNK) in the cell extract were analyzed by Western blot analysis using rabbit anti-human phospho-c-Jun (Ser63) antibody (x1/1,000) and rabbit anti-human phospho-JNK antibody (x1/1,000), respectively as the primary antibody. After immunoreaction with anti-rabbit IgG-alkaline phosphatase conjugate as the secondary antibody (x1/20,000), Pi-c-Jun and Pi-JNK bands were visualized with BCIP/NBT.

Protein determination. Protein concentration was assayed by a Bio-Rad protein assay kit using BSA as the standard.

Results

Deamidation of Bcl-xL by treatment with GSH-DXR following JNK activation. After treatment of HepG2 cells with GSH-DXR, deamidation of Bcl-xL was observed in a concentration-dependent manner (Fig. 1A). The deamidation of Bcl-xL in GST-pi-overexpressed transfectant Hep/GSTp cells treated with GSH-DXR was more decreased than in HepG2 cells (Fig. 1A). Expression of GST-pi was observed in Hep/GSTp cells, but not in HepG2 cells (Fig. 1B). On the other hand, deamidation was suppressed by co-treatment with 5 μM SP600125, an inhibitor of JNK (Fig. 1C). Recombinant wild-type Bcl-xL (WT-Bcl-xL), a deamidated mutant (N52,66D-Bcl-xL) in which Asp was substituted for Arg 52 and 66, and a deamidation-resistant mutant (N52,66A-Bcl-xL) in which Ala was substituted for Arg 52 and 66, were prepared, and the electrophoretic mobility of

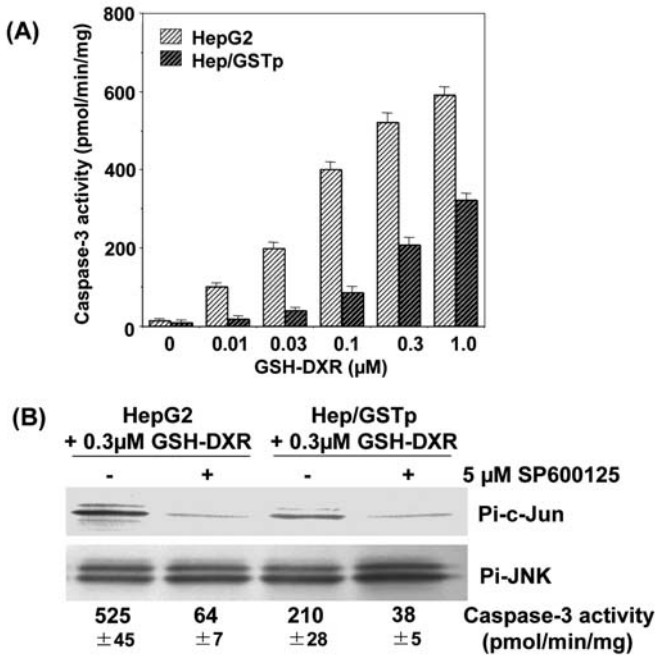

Figure 2. Change in caspase-3 and JNK activities in HepG2 and Hep/GSTp cells treated with GSH-DXR. (A) Concentration-dependent increase in caspase-3 activity in both cells treated with GSH-DXR. Hep/GSTp cells were resistant against GSH-DXR. Results are mean ± SD (three independent experiments). (B) Suppression of JNK activity in HepG2 and Hep/GSTp cells treated with GSH-DXR by co-treatment with 5 μM SP600125. Both cells were co-treated with 0.3 μM GSH-DXR and 5 μM SP600125 for 24 h. Pi-JNK and Pi-c-Jun show active form of JNK and phosphorylation activity of c-Jun by active JNK, respectively. Results in caspase-3 activity are mean ± SD (three independent experiments).

these proteins confirmed. N52,66A-Bcl-xL showed a slightly large electrophoretic mobility compared with WT-Bcl-xL on SDS-PAGE, but the mobility of N52,66D-Bcl-xL was apparently smaller than that of the WT-Bcl-xL (data not shown).

Treatment of HepG2 cells with GSH-DXR caused potent activation of caspase-3 and JNK (Fig. 2). However, co-treatment with SP600125 inhibited caspase-3 activation. On the other hand, these activations in Hep/GSTp cells treated with GSH-DXR were partly suppressed; moreover, inhibition of JNK activity by co-treatment with SP600125 also inhibited caspase-3 activation, although JNK was processed to phosphorylated JNK as active form (Fig. 2B).

Overexpression of Flag-Bcl-xL deamidation mutant. Flag-WT-Bcl-xL, Flag-N52,66D-Bcl-xL and Flag-N52,66A-Bcl-xL overexpressed in HepG2 and Hep/GSTp cells, and were detected by Western blot analysis using anti-Flag antibody. Overexpressed Flag-Bcl-xL mutant, which fused Flag-tagged peptide, were expressed in several times more than endogenous Bcl-xL.

Overexpression of Flag-WT-Bcl-xL in HepG2 suppressed GSH-DXR-induced caspase-3 activation (525 pmol/min/mg in Fig. 2B) to 380 pmol/min/mg, although deamidation was observed (Fig. 3). However, expression of the deamidated mutant, Flag-N52,66D-Bcl-xL, showed high sensitivity for apoptosis and potently activated caspase-3 in an GSH-DXR-independent manner (Fig. 3). Basal activity of caspase-3 in Flag-N52,66D-Bcl-xL-expressed cells (180 pmol/min/mg) also showed high level compared with that in Flag-WT-Bcl-xL-expressed cells (25 pmol/min/mg). Expression of the deamidation-resistant mutant, Flag-N52,66A-Bcl-xL, showed resistance to apoptosis (a little activation of caspase-3, 240 pmol/min/mg) caused by treatment with GSH-DXR (Fig. 3). On the other hand, GSH-DXR-induced caspase-3 activation in Hep/GSTp, which was overexpressed in each mutant of Flag-Bcl-xL, was partly suppressed compared with the level in HepG2 cells.

Translocation of Bax by treatment of HepG2 cells with GSH-DXR. Localization of overexpressed Flag-Bax was detected in subcellular fractions of Hep/Bax cells by Western blot analysis using the anti-Flag antibody. Flag-Bax was localized largely in the cytosolic fraction of Hep/Bax cells and slightly in the mitochondrial fraction (Fig. 4A). After treatment of Hep/Bax cells with 0.3 μM GSH-DXR, Flag-Bax

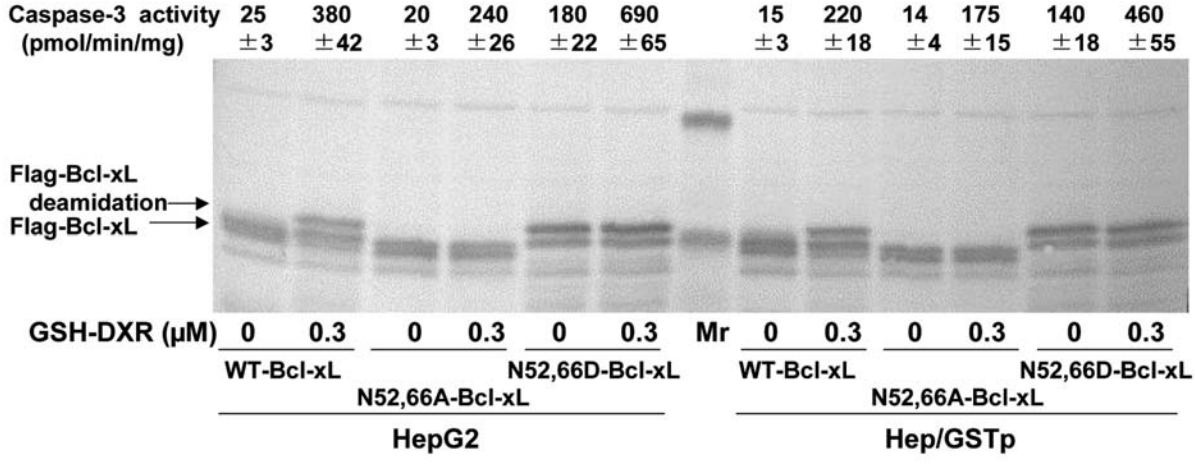

Figure 3. Change in deamidation of Bcl-xL in Bcl-xL mutant-expressed cells treated with GSH-DXR. Plasmids of WT-Bcl-xL/pFLAG, N52,66A-Bcl-xL/pFLAG and N52,66D-Bcl-xL/pFLAG were transfected into HepG2 and Hep/GSTp cells. Deamidation of exogenously overexpressed Flag-Bcl-xL mutant in HepG2 and Hep/GSTp cells treated with 0.3 μM GSH-DXR for 24 h. Flag-Bcl-xL and its deamidation were detected by Western blot analysis using anti-Flag antibody. Results in caspase-3 activity are mean ± SD (three independent experiments).

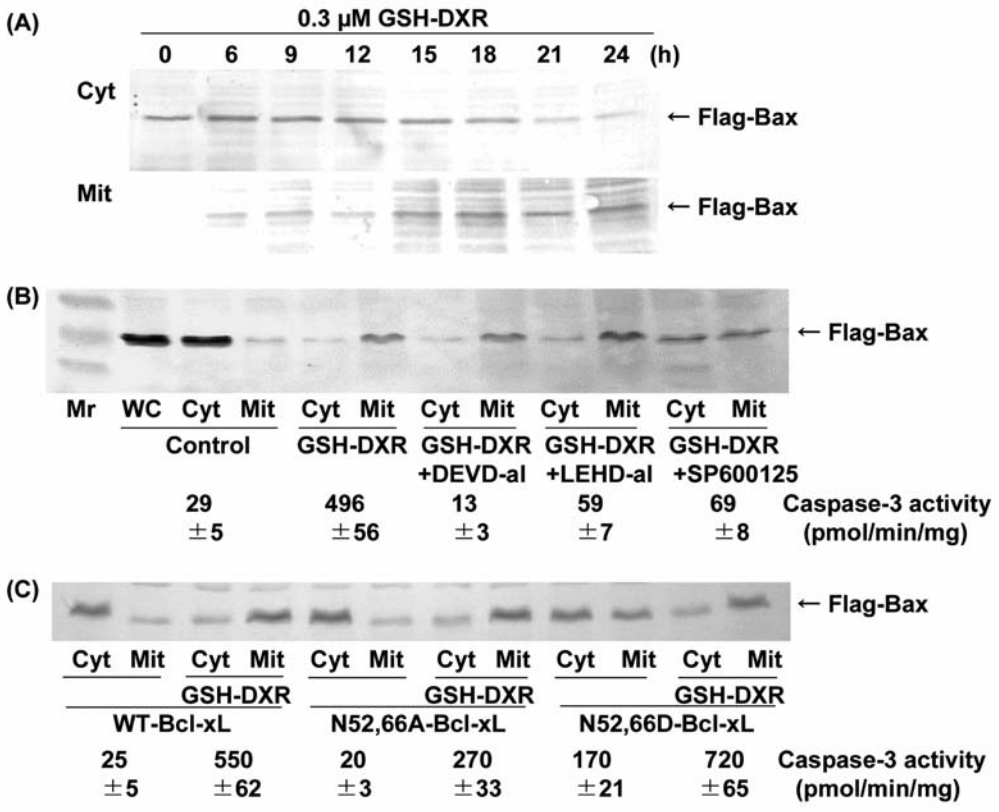

Figure 4. Translocation of Flag-Bax in Hep/Bax by treatment with 0.3μM GSH-DXR at 37˚C for various periods of time (A), or in the presence or absence of caspase inhibitor and JNK inhibitor (B). DEVD-al, DEVD-aldehyde, caspase-3 inhibitor; LEHD-al, LEHD-aldehyde, caspase-9 inhibitor; SP600125, JNK inhibitor. Each inhibitor was co-treated with GSH-DXR at 37˚C for 24 h. (C) Translocation of Flag-Bax in Hep/Bax transfected additionally Flag-Bcl-xL mutant plasmid, WT-Bcl-xL/pFLAG, N52,66A-Bcl-xL/pFLAG and N52,66D-Bcl-xL/pFLAG. After treatment of each transfectant with 0.3μM GSH-DXR for 24 h, subcellular fraction was prepared in accordance with Subcellular fractionation. Flag-Bax was detected by Western blot analysis using anti-Bax antibody. WC, whole cell extract; Cyt, cytosol; Mit, mitochondria. Results in caspase-3 activity are mean ± SD (three independent experiments).

was detected in the mitochondrial fraction at 6 h after treatment. The effects of caspase inhibitor and JNK inhibitor on GSH-DXR-induced translocation of Flag-Bax were examined. Translocation of Flag-Bax was partly suppressed by co-treatment with SP600125, although the translocation was not inhibited by co-treatment with DEVD-aldehyde, an inhibitor of caspase-3 and LEHD-aldehyde, an inhibitor of caspase-9 (Fig. 4B).

On the other hand, localization of Flag-Bax in over-expressed Flag-Bcl-xL mutants of HepG2 cells was examined (Fig. 4C). Flag-Bax was largely localized in the cytosolic fraction of both Flag-WT-Bcl-xL-expressed cells and Flag-N52,66A-Bcl-xL-expressed cells. However, Flag-Bax was partly located to the mitochondrial fraction in Flag-N52,66D-Bcl-xL-expressed cells (Fig. 4C). Activity of caspase-3 in each of these cells was 25, 20 and 170 pmol/min/mg, respectively. By treatment with GSH-DXR, the activity was increased to 550, 270 and 720 pmol/min/mg, respectively.

Discussion

We have determined that GSH-DXR-induced apoptosis caused translocation to the mitochondrial pathway via JNK activation in rat ascites hepatoma AH66 cells (49). Treatment with GSH-DXR induced the release of cytochrome c, activation of caspases-9 and -3, and DNA fragmentation (49). Moreover, JNK activity was suppressed by the binding of GST P1-1 to the JNK molecule, and overexpression of GST P1-1 decreased GSH-DXR-induced apoptosis in AH66 cells (49). In HepG2 cells, GSH-DXR-induced apoptosis was caused to the mitochondrial pathway via JNK activation as same as in AH66 cells.

We demonstrated here that treatment with GSH-DXR caused both deamidation and hence inactivation of Bcl-xL and also activation of Bax through its translocation to the mitochondria via JNK activation, leading to the induction of potent apoptosis.

The Bcl-2 family of proteins includes the best-characterized regulators of apoptosis, comprising anti-apoptotic members that include Bcl-2 and Bcl-xL, and pro-apoptotic members that include multi-domain Bax and Bak and various single-domain BH3-only proteins (1,5,6). Proteins of this family directly regulate the release of mitochondrial apoptogenic factors. Anti-apoptotic family members, such as Bcl-2 and Bcl-xL, are localized in the mitochondrial membrane and suppress the release of cytochrome c from mitochondria. Following the induction of apoptosis, phosphorylation of Bcl-2 occurs in an unstructured loop and several sites. However, it remains unknown whether phosphorylation increases or decreases the anti-apoptotic function of Bcl-2 (24-26). We demonstrated here that the deamidation of Bcl-xL plays an important role in the induction of apoptosis. On the other hand, many of the pro-apoptotic family members, such as Bax, Bid, Bad, Bim, and Bmf, are localized in the cytoplasm,

and apoptotic stimulation results in their translocation to the mitochondria and induction of the release of cytochrome c, probably by inactivating the anti-apoptotic members of the family and activating multi-domain members like Bax and Bak (8-10). We demonstrated here that treatment with GSH-DXR inactivated the Bcl-xL function through its deamidation and activated the Bax function by translocation to the mitochondria. Moreover, the association of both Bcl-xL deamidation and Bax translocation exhibited potent induction of apoptosis. Co-treatment with JNK inhibitor SP600125 and GSH-DXR suppressed Bcl-xL deamidation and Bax translocation following caspase activation. Although co-treatment with the caspase-3 and -9 inhibitors, DEVD-aldehyde and LEHD-aldehyde, respectively, inhibited DNA fragmentation, the induction of Bcl-xL deamidation and Bax translocation were not suppressed. It was confirmed that GSH-DXR treatment of Bcl-xL deamidation and Bax translocation to mitochondria facilitated apoptosis (activation of both caspase-3 and -9). Overexpression of the deamidated form of N52,66D-Bcl-xL localized in the mitochondria induced Bax-translocation to the mitochondria, and basal caspase-3 activity was increased. Therefore, deamidation of Bcl-xL might induce the translocation of Bax to the mitochondria.

It was suggested that deamidation of Bcl-xL was caused through JNK activation since an inhibitor of JNK activity, SP600125, and also overexpression of GST-pi suppressed deamidation. However, the mechanism of deamidation of Bcl-xL via JNK activation remained unknown. Some enzymes causing deamidation of protein may be activated by phosphorylation with JNK. On the other hand, it has been reported that Bax was localized in the cytoplasm by binding of cytosolic protein such as 14-3-3, and phosphorylation of cytosolic protein by apoptotic stimulation caused liberation of Bax and translocation to the mitochondria (23), which was consistent with our results.

Further investigation will attempt to identify a specific deamidation enzyme activated by JNK that plays a role in GSH-DXR-induced apoptosis.

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