Proteasome Inhibitors Sensitize Hepatocellular Carcinoma Cells to TRAIL

Qingfeng Sheng[1]
Yurong Shi[2]
Qiang Li[1]
Jihui Hao[1]
Ruifang Niu[2]
Xiyin Wei[2]
Yi Yang[2]
Lin Zhang[2]

[1] Department of Hepatobiliary Oncology, Tianjin Cancer Institute & Hospital, Tianjin 300060, China.
[2] Oncology Central Laboratory, Tianjin Cancer Institute & Hospital, Tianjin 300060, China.

Correspondence to: Jihui Hao
Tel: 86-22-23340123 ext.5225
E-mail:shengqingfeng@eyou.com

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CJCO http://www.cjco.cn E-mail:cocr@eyou.com
Tel (Fax): 86-22-2352-2919

OBJECTIVE To investigate the effect of proteasome inhibition on the sensitivity of carcinoma cells to TRAIL-inducing apoptosis, and to study the mechanism of the response.

METHODS Human hepatocellular carcinoma cells, pretreated with the proteasome inhibitor, MG132, were cotreated with TRAIL. Western blot assays, immunoprecipitation and RT-PCR were performed to test the expression of the Bcl-2 family proteins and Bax mRNA.

RESULTS We found that (i) proteasome inhibition sensitized the human hepatocellular carcinoma cells to TRAIL; and (ii) resulted in Bax accumulation before release of cytochrome C and induction of apoptosis. These results were associated with the ability of proteasome inhibitors to overcome Bcl-2-mediated antiapoptotic function; (iii) Bax is regulated by an ubiquitin/proteasome-dependent degradation pathway.

CONCLUSION Proteasome inhibition sensitized hepatocellular carcinoma cells to TRAIL by the inhibition of the ubiquitin/proteasome-mediated Bax degradation pathway.

KEYWORDS: proteasome inhibitor, TRAIL, apoptosis, hepatocellulr carcinoma.

Apoptosis, an evolutionarily conserved form of cell suicide, occurs in two physiological stages: commitment and execution.[1] It has been found that several Bcl-2 family proteins are located in the outer mitochondrial membrane, where they control release of some caspase-activating proteins (such as cytochrome C) into the cytosol. Release of cytochrome C can be induced by proapoptotic members of the Bcl-2 family (such as Bax, Bad, and Bid), but inhibited by antiapoptotic Bcl-2 family members (such as Bcl-2 and Bcl-XL). The ratio of proapoptotic to antiapoptotic proteins, therefore, is involved in determination of the cellular fate. In addition, posttranslational modifications of Bcl-2 family proteins also determine their active or inactive conformations.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to kill various tumor cell lines in vitro and in vivo without being toxic to mice and non-human primates. Some conflicting results have been published concerning the sensitivity of primary human hepatocytes.[2] TRAIL induces apoptosis upon binding to TRAIL-R1 and TRAIL-R2, because of receptor crosslinking, which in turn leads to the formation of a death-inducing signaling complex (DISC).[3] Via Fas-associated death domain protein (FADD), caspase-8 and caspase-10 are recruited to and activated at the TRAIL DISC.

Hepatocellular carcinoma (HCC) is one of the most common carcinomas worldwide, and has an especially high frequency in China.

Therapeutic options are very limited because of its chemotherapeutic resistance. A number of studies have shown that cotreatment with chemotherpeutic agents or irradiation resulted in TRAIL sensitization of TRAIL-resistant tumor cell lines.

It has become clear that the ubiquitin/proteasome system plays an important role in the degradation of cellular proteins that are involved in regulating different cellular processes, inducing apoptosis. Proteasomal targets include the nuclear factor-kappa B (NF-κB)/IκB system, p53, and inhibitors of apoptosis proteins (IAPs).[4] Recently it has been suggested that proteasome inhibitors sensitize hepatoma cells for TRAIL-induced apoptosis by NF-κB-independent mechanisms.[5]

MATERIALS AND METHODS

Materials

TRAIL was obtained from Calbiochem (Germany). An Annexin V-FITC Kit was purchased from Beckman Coulter. The proteasome inhibitor, MG132, monoclonal mouse anti-Bax (clone 6A7 and clone YTH 2D2), cytochalasin B, human anti-ubiquitin antibody, and other chemicals were purchased from Sigma.

Cell culture and drug treatment

The human hepatocellular carcinoma cell line, SMMC 7721, was cultured in RPMI-1640 containing 10% fetal bovine serum. For the single treatment of cells, 10^6 cells/ml were treated with 500 ng/ml TRAIL and 2 μmol/L MG132 respectively for up to 24 h. For the cotreatment, cells were pretreated with 2 μmol/L MG132 for 1 h and then treated together with 500 ng/ml TRAIL for up to 24 h.

Measurement of cell survival by the MTT assay

MTT can be degraded by mitochondrial enzymes, to form formazan, a dark blue crystalline product. Less formazan is produced if mitochondrial dehydrogenase activity is decreased, if cell proliferation is inhibited or if cells have died. The cells were treated for 24 h (3 wells for each condition). Enough MTT dye was added to each well to reach the final concentration of 50 μg/ml and the cells allowed to incubate for 4 h at 37 ℃. Media were replaced with 150 μl of dimethyl sulfoxide (DMSO) to solubilize the insoluble formazan complex. Absorbance at 490 nm was determined with an ELISA plate reader.

Apoptosis assay by flow cytometry

Suspensions of 106 Cells were incubated with Annexin V-FITC and propidium iodide (PI) on ice. Then ice-cold binding buffer was added to the preparations after which they were analyzed by 30 min using flow cytometry or fluorescence microscopy. DNA content was measured by flow cytometry. The cells were permeabilized with 80% ethanol and stained with 50 μg/ml PI. PI fluorescence of nuclei was measured by a FACScan flow cytometer and cells with a DNA content less than G0/G1 (hypodiploid) were defined as apoptotic cells.

Western blot analysis and immunoprecipitation

Whole-cell extract, cytosol and mitochondria fractions were prepared and Western blot assays performed as described.[6] The cells were washed with PBS and lysed with Chaps buffer (10 mM HEPES, pH7.4, 150 mM NaCl, 1% Chaps, 1 mM DTT, 0.1 mM PMSF, 3 μg/ml aprotinin, 25 μg/ml leupeptin, and 25 μg/ml pepstatin). Anti-Bax (6A7) monoclonal antibody was preincubated with Dynabeads at 4 ℃ for 3 h. The cell lysates, which were normalized for protein content in Chaps lysis buffer, were then added to the immunoprecipitation tube containing Bax antibody (6A7)-loaded Dynabeads. The tubes were incubated at 4 ℃ overnight, rinsed 4 times with Chaps buffer, and the conformationally changed Bax protein eluted with 25 μl of sample buffer for Western blotting using the monoclonal anti-Bax antibodies clone 2D2 and antibodies to ubiquitin.

Immunocytochemistry and reverse transcriptase-PCR (RT-PCR).

Immunocytochemistry was performed with the rabbit polyclonal Bax antibody and Bcl-2 antibody. To perform RT-PCR, total RNA was isolated from the treated SMMC 7721 cells by QIAGEN. The primer pairs used for amplification of Bax mRNA (538 bp) were : forward, 5'-CAG CTC TGA GCA GAT CAT GAA-GAC A-3' reverse, 5'-GCC CAT CTT CTT CCAGAT GGT GAG C-3'. PCR products were detected by agarose gel analysis.

RESULTS

TRAIL induces apoptosis in human HCC cells after sensitization with the proteasome inhibitor MG132

In our preliminary experiments, we found that SMMC 7721 cells were TRAIL-resistant (data not shown). To test whether the proteasome inhibitor influenced TRAIL sensitivity, we incubated the cells with 500

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ng/ml TRAIL after pretreatment with MG132 (2 μmol/L). The findings clearly indicated sensitization for combined effects on inducing apoptosis (Fig.1A). Measurement of apoptotic cells was performed by fixing the cells, staining with PI, and analyzing by flow cytometry. Apoptotic cells were defined as having a DNA content less than G0/G1. The concentration of MG132, which showed a significant synergistic effect on TRAIL-induced apoptosis, started at 2 μmol/l (data not shown). Therefore, a concentration of 2 μmol/l MG132 was used to study the combined effect on TRAIL. The results indicated that MG132 can sensitize TRAIL-induced apoptosis, and that this sensitization is not a simple additive effect (Fig.1B~D).

Accumulation of the Bax protein and subsequent induction of cytochrome C-dependent apoptosis by MG132 in SMMC 7721 cells over-expressing the Bcl-2 protein

Treatment of the SMMC 7721 cells over-expressing Bcl-2 with MG132 for 24 h increased the level of cytosolic cytochrome C, accompanied by a decrease in the level of the mitochondrial cytochrome C (Fig.2A). The increased cytosolic cytochrome C was not the result of contamination from the mitochondria preparation because expression of cytochrome oxidase, an enzyme that is localized in mitochondria, was detected only in the membrane-bound, but not in the cytosolic fraction. These data suggest that apoptosis induced by MG132 was associated with cytochrome C release.

To test the hypothesis that Bax is a direct target protein of the ubiquitin/proteasome pathway, Bax protein levels were measured in the same experiment by a Western blot assay. The level of the Bax protein (Bax/p21) was increased after 6 h or longer of MG132 treatment (Fig.2B). In contrast, the Bax mRNA level remained unchanged during proteasome inhibition (Fig.2C). Only slight changes were observed in levels of the over-expressed Bcl-2 protein in the cells (Fig. 2D). Therefore, MG132 treatment of cells over-expressing Bcl-2 increased the Bax protein level and the Bax/Bcl-2 ratio, which is associated with the ability of this proteasome inhibitor to overcome apoptosis protection mediated by Bcl-2.

Bax degradation depends on the ubiquitin/proteasome pathway

If Bax is a direct target of the ubiquitin/proteasome pathway, inhibition of the proteasome activity should accumulate ubiquitinated forms of the Bax protein. To investigate this possibility, protein extracts of SMMC 7721 cells cotreated with MG132 and TRAIL were immunoprecipitated with a Bax mAb, followed by a Western blot assay using a polyclonal ubiquitin antibody. Several polypeptide bands including p55 and p47 were detected in the untreated cell lysate. The cotreatment group significantly increased both p55 and p47 levels, suggesting that they are probably polyubiquitinated forms of Bax (Fig.2E).

DISCUSSION

In the current study, we reported that (i) a proteasome inhibitor can sensitize human hepatocellular carcinoma cells to TRAIL; (ii) proteasome inhibition results in Bax accumulation before release of cytochrome C and induction of apoptosis, which is associated with the ability of proteasome inhibition to overcome Bcl-2-mediated antiapoptotic function; (iii) Bax is regulated by an ubiquitin/proteasome-dependent degradation pathway.

Human hepatocellular carcinoma cell lines are relevantly resistant to TRAIL-induced apoptosis because of low caspase-8 activity, induction of Bcl-2, and degradation of the Bax protein.[7] Proteasome inhibitors are a new class of chemotherapeutic agents with significant clinical potential for the treatment of different tumors.[8] It has been reported that the activation status of NF-κB is not sufficient to determine the fate of HCC cells with respect to TRAIL-induced apoptosis. We investigated the molecular basis for the ability of a proteasome inhibitor to overcome Bcl-2 antiapoptotic function and have demonstrated that Bax, an inhibitor of Bcl-2, is a direct target of the proteasome. First, Bax protein levels were increased before cytochrome C was released from mitochondria into the cytosol. Second, Bax primarily accumulated in the cytoplasm during proteasome inhibition; the observation that the increased Bax signals clustered around nuclei suggests accumulation in mitochondria. Third, proteasome inhibition resulted in accumulation of the Bax protein which was able to interact with Bcl-2. Fourth, Bcl-2 protein levels remained relatively unchanged during proteasome inhibition. It has been found that dephosphorylated Bad and cleaved Bid [9] are able to interact with Bcl-XL or Bcl-2 in mitochondria and overcome their antiapoptotic function. Whether proteasome inhibitors also induce dephosphorylation of Bad and cleavage of Bid remains to be investigated.

In vitro studies have demonstrated that Bax is degraded via an ATP- and ubiquitin/proteasome dependent pathway. [10] Treatment of cells with MG132

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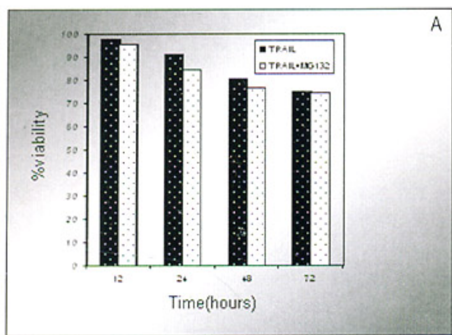
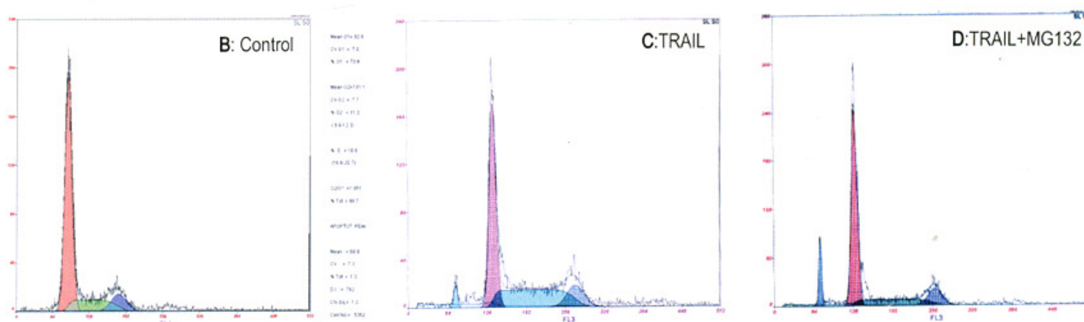
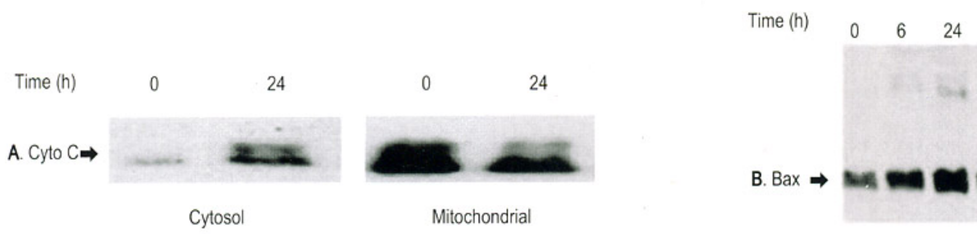


Fig.1. A:Cell survival was analyzed by the MTT assay. Data are shown as percent viability of 2 treated groups (3 wells for each condition). **B~D**: Flow cytometry assay for apoptosis at 24h. Histogram of DNA content was obtained from FL3-area versus cell numbers.





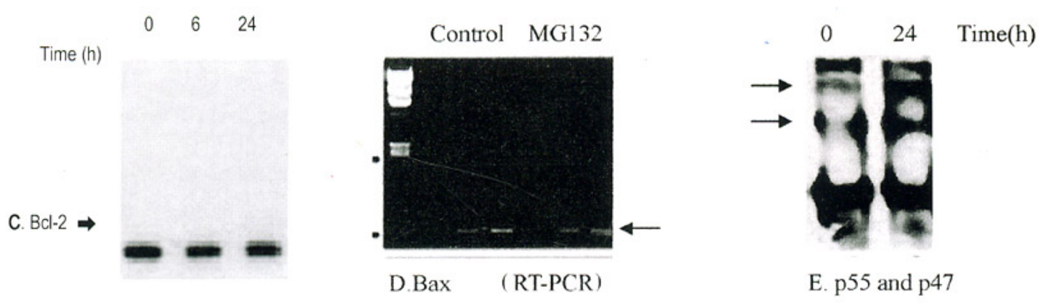


Fig.2. A: The level of cytochrome C increased in cytosol and decreased in mitochondria. **B~D**: The level of Bax protein (21 kDa) increased after treatment with MG132, but the level of Bcl-2 protein (26 kDa) and Bax mRNA (538 bp) remained unchanged. E. Putative ubiquitinated Bax proteins (p55 and p47) were expressed after treatment with MG132.

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caused the accumulation of Bax protein (but not Bax mRNA) and the ubiquitinated forms of Bax. The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin system. In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein.[11] p55 and p47, detected in the treated cells, can be recognized by antibodies to be both Bax and ubiquitin proteins, suggesting that they were probably polyubiquitinated forms of Bax. This hypothesis needs to be confirmed by further study. In the process of ubiquitin-mediated protein degradation, usually there is a single E1, but there are many species of E2s and multiple families of E3s or E3 multiprotein complexes.[12] The special E3s in the ubiquitin-mediated Bax degradation are still unknown.

Taken together, our findings showed that the proteasome inhibitor, MG132, effectively sensitized HCC to TRAIL. Ubiquitin-mediated Bax degradation is an important regulatory mechanism for controlling Bax protein levels. Proteasome inhibitors have significant potential as antitumor therapies that enhance apoptosis by blocking this or other pathways.

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