Bax Mediates the Apoptosis-Sensitizing Effect of Maspin

Jiayou Liu,1 Shuping Yin,1 Neelima Reddy,1 Craig Spencer,1 and Shijie Sheng1,2

1Department of Pathology and 2Karmanos Cancer Institute Protease Program, Wayne State University School of Medicine, Detroit, Michigan

ABSTRACT

Maspin, a serine protease inhibitor (serpin), can suppress tumor growth and metastasis in vivo and tumor cell motility and invasion in vitro. This may occur through maspin-mediated inhibition of pericellular proteolysis. In a recent report, we provided evidence that maspin may also suppress tumor progression by enhancing cellular sensitivity to apoptotic stimuli. To our knowledge, maspin is the only proapoptotic serpin among all of the serpins implicated thus far in apoptosis regulation. The goal of the present study is to identify the specific target molecule(s), the modification of which by maspin renders tumor cells sensitive to chemotherapeutic agents. Our cellular, molecular, and biochemical studies demonstrate an essential role of Bax in the proapoptotic effect of maspin. First, Bax was up-regulated in maspin-transfected prostate and breast tumor cells, whereas the levels of other Bcl-2 family members including Bcl-2, Bcl-xl, and Bak remained unchanged. Second, on apoptosis induction, a greater amount of Bax was translocated from cytosol to mitochondria in maspin-transfected cells. After treatment with a Bax-silencing small interfering RNA, maspin-transfected cells became significantly more resistant to drug-induced apoptosis. Consistently, the release of cytochrome c and Smac/DIABLO from mitochondria was more responsive to apoptosis stimuli in maspin-transfected cells than in the mock-transfected cells. Third, the apoptosis induction of maspin-transfected cells was associated with increased activation of both caspase-8 and caspase-9. However, a caspase-9-specific inhibitor blocked the sensitization effect of maspin in a dose-dependent and time-dependent manner, demonstrating a rate-limiting role for caspase-9. In line with the central role of the Bax-mediated mitochondrial apoptotic pathway, maspin sensitized the apoptotic response of breast and prostate carcinoma cells to various drugs, ranging from death ligands to endoplasmic reticulum stress. The link between maspin and Bax up-regulation explains the loss of maspin-expressing tumor cells in invasive breast and prostate carcinomas. Our data reveal a novel mechanism for tumor suppressive maspin and suggest that maspin may be used as a modifier for apoptosis-based cancer therapy.

INTRODUCTION

Maspin is a tumor-suppressive serine protease inhibitor (serpin). Accumulated functional evidence demonstrates that maspin blocks tumor metastasis in vivo and tumor cell motility and invasion in vitro (1–4). In line with its sequence homology with many serpines (4), maspin can inhibit cell surface-bound urokinase plasminogen activator (uPA) in prostate tumor cells (1, 5, 6). The maspin effect on pericellular uPA activity may underlie its stimulatory effect at the step of cell adhesion (7, 8) and its inhibitory effect on cell invasion and motility (5, 6). It is well documented that uPA, together with its cell surface receptor, uPAR, are up-regulated in invasive and metastatic carcinomas. Interestingly, plasminogen activator inhibitor type 1, a known uPA inhibitor, is also up-regulated along with uPA in invasive cancers (9). In contrast, we have shown that maspin expression is lost at the critical transition from noninvasive to invasive breast and prostate carcinomas (4, 10, 11). This result has been confirmed recently by a microarray analysis by Chen et al. (10). Interestingly, maspin expression is also inversely correlated with transmembrane-type serine protease hepsin (10), raising the possibility that maspin may cross-inhibit transmembrane serine proteases as well.

It is important to note that in several in vivo studies, maspin-expressing tumor cells were inhibited in gross growth (1–4). However, the effect of maspin on tumor growth was not reproduced by in vitro cell culture experiments, suggesting that an inhibitory effect for maspin on tumor cell survival or growth may be further dependent on additional stimuli from the tumor microenvironment. In a recent report, we provided evidence that endogenous maspin expression sensitizes breast carcinoma cells to staurosporine (STS)-induced apoptosis in vitro (12). To our knowledge, this is also the first evidence that a mammalian serpin can act as an apoptosis agonist. The apoptosis-sensitizing effect of maspin appears to be an intracellular rather than an extracellular activity. As compared with the extracellular effect of maspin, which depends on its reactive site loop sequence (2, 13), the intracellular maspin effect on apoptosis depends both on its reactive site loop and its NH2-terminal domain (12). It is intriguing to hypothesize that the intracellular and extracellular maspin may act in concert to help eliminate invasive carcinoma cells in the progression of breast and prostate cancers.

To additionally explore the potential application of maspin in apoptosis-based cancer intervention, it is important to identify the molecular mechanism underlying its proapoptotic effect. Apoptosis, in general, consists of three steps, induction, commitment, and execution. The mechanism of induction can be intrinsic or extrinsic, depending on each specific apoptotic inducer. The commitment of apoptosis is largely a mitochondrial event controlled by proteins in the Bcl-2 family (14–17). The antiapoptotic members of the Bcl-2 family (e.g., Bcl-2 and Bcl-xl) protect against mitochondrial membrane disruption, whereas the proapoptotic members (e.g., Bax and Bak) that undergo cytosol-to-mitochondria translocation in response to death signal cause mitochondrial release of apoptogenic factors such as cytochrome c (18, 19) and Smac/DIABLO (20, 21). Thus, the relative concentrations of anti-versus proapoptotic members of the Bcl-2 family may act as a rheostat for the death program. The execution of apoptosis features a series of common events including activation of effector caspases. In particular, the cytochrome c released into cytosol leads to Apaf-1-mediated apoptosome assembly and a rapid activation of caspase-9 (18, 19), whereas Smac/DIABLO interacts with inhibitors of apoptotic proteases (e.g., XIAP) and, thus, blocks their inhibitory effects on activated caspase-9 and caspase-3 (20, 21). Caspase-3 cleaves many cellular proteins, ultimately leading to nuclear DNA fragmentation and cytoskeleton collapse (14–17).

In the current study, we described cellular, molecular, and biochemical evidence that the apoptosis-sensitizing effect of maspin, which was independent of apoptosis inducers, resulted predominantly from increased Bax expression and activity. These results suggest that maspin may be useful to restore tumor sensitivity toward a broad range of apoptosis-based chemotherapeutics.

MATERIALS AND METHODS

Cell Culture and Apoptosis Induction. Clonal cell lines M3, M7, and M10 were derived from stable transfection of human prostatic carcinoma cells

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Requests for reprints: Shijie Sheng, Department of Pathology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201. Phone: (313) 993–8197; Fax: (313) 993–4112; E-mail: ssheng@med.wayne.edu.
and cytosolic supernatants. The mitochondrial pellets were rinsed twice with KCl, 5 mM EGTA, 5 mM MgCl$_2$, and 1 mM DTT]. The cells were then homogenized with a Dounce homogenizer. Intact cells and nuclei were re-

antibody for Bax (Santa Cruz Biotechnology, San Diego, CA) at 4°C were blocked with 2% BSA for 1 h before being incubated with monoclonal antibody for Bax (Santa Cruz Biotechnology, San Diego, CA); caspase-8, cytochrome c, and Smac/DIABLO (PharMingen, San Diego, CA); caspase-9 (Calbiochem, San Diego, CA); Bcl-2 and Bcl-xl (Santa Cruz Biotechnology, Santa Cruz, CA); and Bak (UPSTATE, Lake Placid, NY). Bax antibody in the Western blotting with fractionated cell lysates (Fig. 8) was a monoclonal antibody purchased from Santa Cruz Biotechnology. The Bax antibody used in all of the other experiments was a polyclonal antibody obtained from UPSTATE. Anti-β-actin (Sigma) was used for loading controls. Immunoprecipitation was performed as described previ-

zyme assay. For analysis, a threshold of arbitrary fluorescence reading was set at 30, at which point the increase in fluorescence exceeded the background noise and entered the exponential phase. The fluorescence readings were taken when the slower reaction in the pairs reached the threshold cycle. The real-time PCR for GAPDH was used as a normalization control.

RESULTS

Maspin Sensitizes Apoptotic Response of Prostate Carcinoma Cells to Distinct Stimuli. Maspin has been shown to sensitize breast carcinoma cells MDA-MB-435 to STS-induced apoptosis (12). To explore further the potential of maspin to be an agonistic modifier of cancer chemotherapeutic agents, it is critical to investigate: (a) whether maspin sensitize apoptotic response in other types of cancer cells; and (b) whether the maspin effect depends on specific apoptosis inducers. In the present study, we extended our investigation to prostate carcinoma cells, DU145-derived stable maspin-transfected clones (5). We reported previously that expression of maspin in these transfec tant cells resulted in reduced cell motility and invasion without altering cell growth kinetics in culture (5), a finding similar to that found with MDA-MB-435-derived maspin-transfected cells (12). When treated with STS, the maspin-expressing clone M7 exhibited concentration- and time-dependent phenotypic changes typical of apoptosis, including cell shrinkage and detachment (data not shown). Six h after treatment, M7 had undergone significant nuclear DNA condensation and fragmentation as revealed by a Hoechst fluorescent staining method (Fig. 1A). In contrast, the mock-transfected control Neo cells were markedly more resistant to STS-induced apoptosis. In fact, after the same treatment, some Neo cells remained mitotic. As summarized in Fig. 1B, STS treatment produced approximately three times more apoptotic cells in M7 cells than in Neo cells. Notably, the numbers of apoptotic M7 and Neo cells were similarly insignificant when untreated.

STS is a synthetic chemical known to induce apoptosis via an intrinsic pathway, presumably by changing the mitochondria membrane permeability (24). To test whether maspin regulates tumor cell response to pathophysiologic apoptosis inducers such as extrinsic TRAIL or TNF-α, M7 and Neo cells were treated with TRAIL, an exclusive apoptotic cytokine (25). Ninety min into treatment, >70% of M7 cells showed nuclear DNA fragmentation to various extents. In contrast, only 13% of TRAIL-treated Neo cells underwent significant nuclear DNA fragmentation (Fig. 1B). Similar differential responses were observed when M7 and Neo cells were treated with TNF-α for 3 h in the presence of cycloheximide. As shown in Fig. 1C, untreated maspin-transfected (M3, M7, and M10) and mock-transfected (Neo)
cells expressed PARP at a similar level. Furthermore, in the absence of apoptosis stimuli, maspin did not lead to spontaneous cleavage of 116 kDa PARP to its 85-kDa product. However, when treated with TNF-α/cycloheximide, all three of the maspin-transfected clones were significantly more sensitive than Neo cells to apoptosis. The extent of PARP cleavage in these clonal cell lines semiquantitatively correlated with their nuclear morphologic changes (data not shown) and inversely correlated with the levels of maspin expression (Fig. 1D). Clone M7 exhibited the highest sensitivity to TNF-α/cycloheximide and TRAIL, and was chosen for most of the subsequent experiments.

Apoptosis may also result from endoplasmic reticulum (ER) stress (26). Brefeldin A, for example, inhibits ER-Golgi transport and induces ER stress and apoptosis. To test the effect of maspin on ER stress-induced apoptosis, M7 and Neo cells were treated with brefeldin A. As compared with Neo cells, M7 cells underwent more rapid cell shrinkage and detachment (data not shown). Furthermore, among the remaining adherent cells, approximately one third of M7 cells showed nuclear DNA fragmentation, whereas only 14% of Neo cells were positive for nuclear DNA fragmentation (Fig. 1B). In parallel, thapsigargin, another cytotoxic ER-targeting drug, induced similar differential responses in M7 and Neo cells (data not shown). Taken together, these results demonstrate that the maspin effect on apoptosis

Fig. 1. Maspin sensitizes apoptotic response of prostate carcinoma cells to distinct stimuli. A, apoptotic nuclear morphology in staurosporine (STS)-induced M7 and Neo cells. Nuclear DNA was stained with Hoechst (blue). White arrows indicate DNA condensation/fragmentation (original magnification ×400). B, apoptosis induced by different stimuli in M7 and Neo cells. Cells were treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) at 50 ng/ml for 1.5 h, tumor necrosis factor (TNF)-α (50 ng/ml)/cycloheximide (CHX; 2 μg/ml) for 3 h, STS at 1 μM for 6 h, and brefeldin A at 30 μg/ml for 30 h. Apoptotic cells with fragmented nuclei as visualized by Hoechst staining were presented as a percentage of the total number of cells counted in the same field. Data shown are means of triplicate experiments; bars, ±SD. C, top, poly(ADP-ribose) polymerase (PARP) in untreated DU145-derived transfected clones Neo, M10, M3, and M7. Bottom, PARP cleavage in DU145-derived transfected clones Neo, M10, M3, and M7. Cells were treated with TNF-α (50 ng/ml)/CHX (2 μg/ml) for 3 h. Whole-cell extracts were loaded (50 μg/lane) for Western blotting of PARP (116 kDa) and PARP-derived fragment (85 kDa). D, Western blotting of maspin in untreated DU145-derived transfected clones Neo, M3, M10, and M7. The same membrane was stripped and probed for β-actin to monitor the protein loading.
is independent of the types of death stimuli and suggest that maspin may regulate a step downstream of the converging point for extrinsic and intrinsic pathways.

**Maspin Expression Correlates with Increased Activation of Caspase-9, Caspase-8, and Caspase-3.** To identify the specific apoptotic step(s) regulated by maspin, we first examined the effect of maspin on caspase-3 by a fluorogenic DEVDase assay. On TRAIL treatment, M7 cells exhibited a rapid increase in caspase-3 activation during the first 30 min (Fig. 2A). The caspase-3 activation in Neo cells, however, started after a 30-min attenuation. Moreover, the maximum caspase-3 activity in Neo cells was approximately three fifths of that in M7 cells. Then, caspase-3 activity began to decrease in both cell lines, reflecting the loss of apoptotic cells to complete cell death. In parallel, TNF-α/cycloheximide-treated M7 cells exhibited a rapid increase in caspase-3 activity beginning from the second hour of treatment (Fig. 2B). The caspase-3 activity in Neo cells, conversely, began to increase after a 2-h attenuation period and reached the maximum level 5 h after induction. The maximum caspase-3 activity in Neo cells was approximately two thirds of that in M7. It was noted that although maspin sensitized indiscriminately the cellular apoptotic response to TRAIL and TNF-α, the effect of maspin did not diminish the difference between these two death ligands in their cytotoxicities. These results further demonstrate the maspin independence of apoptotic stimuli.

We showed previously that maspin does not directly inhibit or activate caspase-3 (12). In this study, M7 cells and Neo cells expressed a comparable amount of caspase-3 protein as judged by Western blotting analyses (data not shown). Furthermore, as shown in Fig. 3A, the endogenous caspase-inhibitor XIAP was slightly increased by maspin transfection (~1.5-fold). Thus, the differential kinetics of caspase-3 activation in M7 cells and Neo cells may result from differential activities of caspase-3 activating enzyme(s) in these two cell populations. Caspase-9 is a known caspase-3-activating enzyme in both intrinsic and extrinsic apoptotic pathways. Real-time PCR detection of caspase-9 showed that the expression of caspase-9 at the RNA level was not different in all clonal cell lines tested (data not shown). Western blotting, however, showed significantly less procaspase-9 in untreated M3 and M7 cells than in untreated Neo cells (Fig. 3B). The reduction of procaspase-9 might be due to autoproteolytic activation of the enzyme (27). Consistent with this notion, on TRAIL treatment (50 ng/ml/50 min), the remaining procaspase-9 was reduced further in M3 and M7, whereas it remained largely unchanged in Neo cells. The 50-min treatment was chosen because the difference of caspase-3 activity between M7 and Neo cells was maximal at this time point (Fig. 2A).

Caspase-8 is another major caspase-3-activating enzyme. Both TRAIL and TNF-α are known to bind to their cell surface receptors, leading to caspase-8 activation. Activated caspase-8, in turn, may directly activate caspase-3, and it indirectly activates caspase-9 via a mitochondrial-dependent mechanism (28). The expression of TRAIL receptors (DR4 and DR5) and TNF-α receptor (TNR2) was not different between maspin-transfected cells and the mock control cells, as judged by Western blots (data not shown). Western blotting was performed using an antibody that recognizes both pro- and activated caspase-8. As shown in Fig. 3C, untreated M3, M7, and Neo cells

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**Fig. 2.** Maspin expression leads to increased caspase-3 activation. A and B, M7 (▲) and Neo (□) cells were treated with (A) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or (B) tumor necrosis factor (TNF)-α/cycloheximide (CHX) at the same dosage as described in Fig. 1. A total of 30 μg of cytosolic extracts prepared at the indicated time points were assayed for caspase-3 activity using fluorogenic Ac-DEVD-CMA as a substrate. The mean fluorescence productions obtained from triplicate repeats are used to represent caspase-3 activities, bars, ±SEs.

**Fig. 3.** Maspin expression leads to increased cleavage of procaspase-8 and procaspase-9. A, Western blotting of XIAP using whole-cell extracts from untreated M7 and Neo cells. * indicates that the total density of XIAP Western blot is normalized against the total density of the corresponding β-actin and is presented as a percentage of XIAP detected in untreated Neo cells. B, Western blotting of procaspase-9 using the whole-cell extracts from M3, M7, and Neo cells that were either untreated or treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, 50 ng/ml/50 min). C, Western blotting of pro- and cleaved caspase-8 (p43/36 and p23) using the whole-cell extracts from M3, M7, and Neo cells that were either untreated or treated with TRAIL (50 ng/ml/50 min). The Western blots in A–C were rebotted with β-actin antibody to normalize protein loading.
expressed procaspase-8 protein at a comparable level. The same 50-min TRAIL treatment led to a greater reduction of procaspase-8 in M3 and M7 cells than in Neo cells. Conversely, a greater amount of activated caspase-8 (i.e., p43/36 and p23) was found in M3 and M7 cells than in Neo cells. The extent of caspase-8 activation in the two maspin-transfected clones correlated with the levels of maspin expression (Fig. 1D). These data indicate that the death receptor-mediated proteolytic activation of caspase-8 is enhanced by maspin expression.

**Mitochondrial Pathway Is Essential for the Proapoptotic Effect of Maspin.** Using a caspase-9-specific fluorogenic substrate, we found that the caspase-9 activity increased >3-fold in TRAIL-treated M7 cells but remained at the basal level in TRAIL-treated Neo cells (Fig. 4A). In similar biochemical assays using a caspase-8-specific fluorogenic substrate, caspase-8 activity was barely detectable in all of the clonal cell lines tested (data not shown). The discrepancy between this result and the Western blotting of caspase-8 (Fig. 3C) may be due to different sensitivities of each assay. Alternatively, it is possible that caspase-9 was enzymatically more important for the proapoptotic effect of maspin. To assess further the relative significance of caspase-8 and caspase-9 in caspase-3 activation, caspase-3 activity was determined after apoptosis was induced in the presence of z-LEHD-fmk. Western blotting of PARP further confirmed that z-LEHD-fmk. Western blotting did not detect Bcl-2 in maspin-transfected and mock-transfected DU145 cells (data not shown). In addition, as shown in Fig. 6A, Bcl-xl, an anti-apoptotic Bcl-2 homologue, and Bak, a proapoptotic Bcl-2 homologue, were each expressed at comparable levels in M7 and Neo cells.

Interestingly, a significantly higher amount of proapoptotic Bax prologue, were each expressed at comparable levels in M7 and Neo cells. This result and the Western blotting of caspase-8 (Fig. 3C) may be due to different sensitivities of each assay. Alternatively, it is possible that caspase-9 was enzymatically more important for the proapoptotic effect of maspin. To assess further the relative significance of caspase-8 and caspase-9 in caspase-3 activation, caspase-3 activity was determined after apoptosis was induced in the presence of z-LEHD-fmk. Western blotting of PARP further confirmed that z-LEHD-fmk. Western blotting did not detect Bcl-2 in maspin-transfected and mock-transfected DU145 cells (data not shown). In addition, as shown in Fig. 6A, Bcl-xl, an anti-apoptotic Bcl-2 homologue, and Bak, a proapoptotic Bcl-2 homologue, were each expressed at comparable levels in M7 and Neo cells. Interestingly, a significantly higher amount of proapoptotic Bax protein was found in maspin-transfected cells (M3, M7, and M10) as compared to different sensitivities of each assay. Alternatively, it is possible that caspase-9 was enzymatically more important for the proapoptotic effect of maspin. To assess further the relative significance of caspase-8 and caspase-9 in caspase-3 activation, caspase-3 activity was determined after apoptosis was induced in the presence of z-LEHD-fmk. Western blotting of PARP further confirmed that z-LEHD-fmk. Western blotting did not detect Bcl-2 in maspin-transfected and mock-transfected DU145 cells (data not shown). In addition, as shown in Fig. 6A, Bcl-xl, an anti-apoptotic Bcl-2 homologue, and Bak, a proapoptotic Bcl-2 homologue, were each expressed at comparable levels in M7 and Neo cells. Interestingly, a significantly higher amount of proapoptotic Bax protein was found in maspin-transfected cells (M3, M7, and M10) as compared to Neo cells.
To investigate further the differential regulation of Bax expression in maspin-transfected versus mock control tumor cells, semiquantitative real-time PCR was performed with total RNAs extracted from untreated cells. As shown in Fig. 7A, the RNA from M7 and Tn 16 (maspin-transfected MDA-MB-435 cells) gave rise to a more rapid amplification of Bax cDNA than the corresponding mock control RNA (Neo and Nn12, respectively), whereas GAPDH, a housekeeping gene commonly used as RNA loading control, was amplified equally in the same set of RNA samples. To confirm the difference in Bax expression, real-time PCR was performed multiple times using two different real-time thermal cyclers and independently prepared RNA samples. Reproducibly, the Bax amplification was significantly more rapid with the RNA of M7 cells than with that of Neo cells (Fig. 7B). These data suggest that the up-regulation of Bax in maspin-transfected cells may occur at the transcriptional level.

To investigate whether the higher amount of Bax protein present in maspin-transfected cells was functionally relevant, we examined Bax localization. M7 and Neo cells were treated with TRAIL. The resulting cell lysates were subjected to fractional centrifugation to separate membrane-bound and cytosolic fractions. As shown in Fig. 8A, cytosolic Bax protein that was detected in untreated M7 cells largely disappeared after TRAIL treatment. Concomitantly, the Bax level significantly increased in the membrane fraction of TRAIL-treated M7 cells as compared with that of untreated M7 cells. In Neo cells, Bax protein, albeit at a much lower level, also appeared to undergo translocation from cytosol to mitochondria. Immunofluorescent staining showed that, on TRAIL treatment (Fig. 8B), most of the cytosolic Bax in M7 cells was translocated to perinuclear space with a punctate staining pattern, typical for mitochondria localization. In Neo cells, Bax protein translocation was hardly detected by this method. To test further the significance of the Bax up-regulation in the proapoptotic effect of maspin, maspin-transfected cells were treated with a Bax-specific siRNA. As shown in Fig. 8C, this Bax siRNA greatly compared with that found in Neo cells (Fig. 6B). Furthermore, Bax expression appeared to correlate with the level of maspin expression in these transfected cell lines (Fig. 1D). Neither TRAIL, nor STS further affected the differential expression pattern of Bax between M7 cells and Neo cells (Fig. 6C). Additionally, MDA-MB-435-derived maspin-transfectant cells, which are more sensitive to STS-induced apoptosis (12), expressed Bax at a significantly higher level than the corresponding mock control cells, whereas Bcl-2 and Bcl-xL expression remained the same in all of those clonal cell lines tested (data not shown).

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inhibited Bax expression. The transfection efficiency with Bax siRNA was estimated to be approximately 60–80% as judged by a parallel experiment using a FITC-labeled nonspecific siRNA 6-Sam (data not shown). TRAIL-induced PARP cleavage was also reduced by Bax siRNA, but less substantially. Because the Bax effect is cascaded before PARP cleavage, even trace amounts of Bax may still lead to a substantial PARP cleavage. Together, these data demonstrated a dominant role of Bax in maspin-mediated apoptosis sensitization.

DISCUSSION

To our knowledge, maspin is the only serpin that sensitizes apoptosis, whereas all of the other serpins thus far implicated in apoptosis regulation appear to be antiapoptotic (17, 32, 33). Thus, the existing literature offers little insight into the possible molecular mode of maspin action. The goal of the present study was to identify the specific target molecule(s), the modification of which by maspin sensitizes prostate and breast tumor cells toward potential cancer chemotherapeutic agents. By using multiple maspin-transfected cell lines in vitro, we obtained cellular, molecular, and biochemical evidence that supports a key role for Bax in maspin-mediated apoptosis sensitization. Although we may not have exhausted our search because of the ever-growing number of apoptosis regulators, our data seem sufficient to support our new hypothesis that the specific up-regulation of Bax, without changing Bcl-2, Bcl-xL, and Bak expression, may tip the balance of pro-versus anti-apoptotic regulators and potentiate mitochondrial membrane for apoptosis. In particular, the following considerations further support this hypothesis.

Specific Bax-silencing in maspin-transfected cells conferred an increased cellular resistance to TRAIL-induced apoptosis, thus demonstrating an essential role for Bax in the proapoptotic effect of maspin. Consequently, Bax overexpression would predict a predominant role for mitochondrial pathway in the maspin effect on apoptosis (34). Indeed, we found that the level of Bax expression and translocation correlated positively with mitochondrial cytochrome c and Smac/DIABLO release, activation of caspase cascade from apical caspases to effector caspases, and nuclear phenotypes (PARP cleavage and DNA fragmentation). Bax overexpression may further predict an elevated cellular apoptotic response to a broad spectrum of apoptosis stimuli. We showed that maspin-expressing clones were sensitized to a broad range of apoptosis inducers, including TRAIL, TNF-α, STS, brefeldin A, and thapsigargin. Although the early signaling pathways elicited by these inducers differ, they may all converge at the step of mitochondria membrane potentiation (14, 15, 31).

For example, STS is known to cause Bax translocation to mitochondria and disruption of mitochondria membrane pores in many different cells, leading to the release of mitochondrial factors (35, 36). Brefeldin A and thapsigargin, conversely, are commonly used to deplete the ER Ca²⁺ pool, causing extensive ER stress. The Ca²⁺ released from ER is rapidly accumulated in mitochondria (37, 38), leading to a decrease in mitochondrial transmembrane potential and the release of mitochondrial factors (37–41). Consistently, antiapoptotic Bcl-2 has been shown to inhibit death stimuli-induced increase of Ca²⁺ concentration in mitochondria (42).

The mode of death ligand action (e.g., TRAIL, TNF-α) is complex. The death ligands may inflict apoptosis by activating caspase-8 through the death-inducing signaling complex (43, 44). The amplification of down-stream death signal, however, may involve Bcl-2 family protein-mediated mitochondrial pathways (15). Thus, it was not surprising that TRAIL and TNF-α induced activation of both caspase-8 and caspase-9, as was observed in this study. To date, there is no evidence for a direct regulatory role for maspin in death receptor-mediated death-inducing signaling complex activity. Conversely, emerging evidence suggests that activation of caspase-8 in TRAIL (or TNF-α)-induced epithelial apoptosis may also be mediated by a mitochondria-dependent mechanism (45). Considering the up-regulated Bax expression in maspin-transfected cells and the increase of caspase-8 activation in MDA-MB-435-derived maspin-transfected cells after treatment with STS, a non-death-ligand-type apoptotic inducer (12), we speculate that the increased caspase-8 activation in
these cells was a result of increased mitochondrial apoptotic activities. It is important to note that despite the increased caspase-8 activation in maspin-transfected cells, a caspase-9-specific inhibitor completely abolished the enhanced caspase-3 activation and PARP cleavage in these cells (Fig. 4). Although we cannot exclude the possibility that caspase-8 may cleave other substrates, such as Bid (46, 47) and the cytoskeletal protein plectin (48), and facilitate further the apoptosis to completion, our data thus far support that caspase-9 was the predominant apoptosis-executing enzyme.

High levels of Bax expression may be sufficient to confer apoptosis (49). Interestingly, the up-regulated Bax expression did not lead to spontaneous apoptosis of maspin-transfected cells. To this end, evidence exists that a rapid execution of apoptosis occurs when activation of caspase-9 and inactivation of caspase inhibitor XIAP are provoked simultaneously. It has been shown that cytochrome c and Smac/DIABLO release are, respectively, prerequisites for caspase-9 activation and XIAP inactivation (18). Such an imbalance of release is assumed to lead to a higher level of active caspase-9 without causing apoptosis because of the overwhelming inhibitory effect of XIAP. Indeed, in the absence of apoptosis stimuli, the procaspase-9 cleavage was detected in maspin-transfected cells than in mock control cells (Fig. 3). However, XIAP expression was abolished the enhanced caspase-3 activation and PARP cleavage in maspin-transfected cells but not in control cells (Fig. 4).

The overexpression of Bax in maspin-transfected cells appeared to be regulated at a step before protein translation. Current evidence suggests the following two mechanisms by which maspin may affect Bax expression at the transcriptional level. A recent study by Odero-Marah et al. (50) showed that maspin underwent epidermal growth factor receptor-mediated tyrosine phosphorylation in vitro. In addition, a fraction of maspin protein seems to localize to cell nuclei (51). It is not yet known whether intracellular maspin regulates directly the signal transduction, leading to increased Bax expression. Alternatively, it has been shown that mitogen-activated protein kinase plays a central role in cell survival partly by down-regulating the expression of proapoptotic genes such as Bax (52, 53). Among the increasing number of proteins that regulate mitogen-activated protein kinase activation, the uPA/uPAR complex has been shown to activate p38 through its interaction with integrins, subsequently leading to activation of mitogen-activated protein kinase (54, 55). Because endogenous maspin expression in tumor cells not only inhibits pericellular uPA activity but also dramatically reduces cell surface-associated uPA and uPAR proteins via rapid lipoprotein receptor related protein-mediated internalization (5), it is possible that constitutive expression of maspin positively regulates Bax transcription by eliminating the signal transduction from uPA/uPAR to mitogen-activated protein kinase.

In summary, our biochemical and molecular analyses identified Bax as a key effector of maspin in the regulation of cellular apoptotic sensitivities. This finding helps to explain the general sensitizing effect of maspin on cellular response to multiple apoptotic inducers that act by distinct mechanisms. Currently, a barrier for designing apoptosis-based therapies is that many types of tumors manifest a substantial drug resistance because of an unfavorable Bcl-2:Bax ratio (56). Results of this study suggest that re-expression of maspin in tumor cells may reverse or reduce drug resistance. It is important to note that maspin is expressed in, and, therefore, tolerated by, benign epithelial cells in breast and prostate glands that still have supportive and relatively inactive stroma. Maspin expression is shown to be lost in invasive breast and prostate carcinomas (4, 11). In view of our evidence that maspin did not provoke but rather sensitized tumor cell apoptotic responses and the clinical observation that invasive cancers are often associated with highly immunoreactive stroma marked with increased secretion of cytokines such as TNF-α (57–60), it is likely that maspin-expressing tumor cells, but not the maspin expression in tumor cells, are selected in tumor progression. Consistent with this notion, oxidative stress-induced apoptosis in breast cancer cells, after manganese-containing superoxide dismutase overexpression, was associated with an elevated level of maspin expression (61). Together, our data suggest a novel mechanism for the tumor suppressive activity of maspin. Moreover, the reactive stroma in invasive prostate and breast carcinomas may offer a unique advantage in maspin-based therapies for achieving a greater tumor specific toxicity.

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# Bax Mediates the Apoptosis-Sensitizing Effect of Maspin

Jiayou Liu, Shuping Yin, Neelima Reddy, et al.


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