Suppression of MG132-mediated cell death by peroxiredoxin 1 through influence on ASK1 activation in human thyroid cancer cells

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Abstract

Proteasome inhibitors represent a novel class of antitumor agents with pre-clinical and clinical evidence of activity against hematologic malignancies and solid tumors. However, emerging evidence indicates that antiapoptotic factors may also accumulate as a consequence of exposure to these drugs, thus it seems plausible that the activation of survival signaling cascades might compromise their antitumoral effects. Peroxiredoxins (PRDXs) are a family of thiol-containing peroxidases identified primarily by their ability to remove cellular hydroperoxides. The function of PRDX1 in particular has been implicated in regulating cell proliferation, differentiation, and apoptosis. Another important finding is that aberrant upregulation of PRDX1 has been discovered in various cancers. Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase (MAPK) kinase kinase that is regulated under conditions of cellular stress. ASK1 phosphorylates c-Jun N-terminal kinase and p38 MAPK, and elicits an apoptotic response. ASK1 activity is regulated at multiple levels, one of which is through interaction with PRDX1. In this study, for the first time we report that upregulation of PRDX1 expression was found in thyroid cancer cells treated with proteasome inhibitors, and PRDX1 knockdown resulted in accelerated proteasome inhibitor-induced cell death. In addition, we demonstrated that ASK1 activity was implicated in the PRDX1-dependent response of thyroid cancer cells to proteasome inhibitor-mediated cell death.

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Introduction

Oxidative stress is widely recognized as a major pathophysiologic mechanism in various injuries that lead to either acute or chronic organ failure. This stress is characterized by the increased production of reactive oxygen species (ROS) such as superoxide radicals, \( \text{H}_2\text{O}_2 \), and hydroxyl radicals, which can result in the oxidation of macromolecules including nucleic acids, lipids, and proteins. Endogenous ROS are usually byproducts of cellular metabolism, and can be induced to high levels. Mammalian cells have developed many antioxidative systems to maintain an appropriate level of ROS and regulate their action. Peroxiredoxins (PRDXs), a family of peroxidases that reduce intracellular peroxydes (one type of ROS) with the thioredoxin system as the electron donor, are highly expressed in various cellular compartments. PRDXs are currently known to possess six mammalian isoforms. Although their individual roles in cellular redox regulation and antioxidant protection are quite distinct, they all catalyze peroxide reduction to balance cellular \( \text{H}_2\text{O}_2 \) levels essential for signaling and metabolism (Shi et al. 2008). Besides their cytoprotective
antioxidant function, PRDXs appear to play a role in cell proliferation, differentiation, immune response, protection of oxidant-sensitive proteins, regulation of cellular H$_2$O$_2$, control of apoptosis, and processes involving redox signaling (Shi et al. 2008).

PRDX1, whose function has been implicated in regulating cell proliferation, differentiation, and apoptosis, is the most abundant and ubiquitously distributed member of the mammalian PRDX family, having been identified in a large variety of organisms. Analysis of cells and mice lacking PRDX1 suggest that it not only regulates H$_2$O$_2$ levels, but also possesses the properties of a tumor suppressor (Neumann et al. 2003), which was further supported by the finding that PRDX1 interacts with c-Myc, thereby selectively inhibiting c-Myc transcripational activity (Egler et al. 2005). However, many studies indicated that aberrant increased expression of PRDX1 is found in various kinds of cancers including thyroid, lung, and breast (Yanagawa et al. 1999, 2000, Chang et al. 2001, Kim et al. 2003, 2007, 2008, Alfonso et al. 2004, Lehtonen et al. 2004, Xin et al. 2005, Park et al. 2006), which obscures its actual role in tumorigenesis. As the hypoxic and unstable oxygenation microenvironment of a tumor is one of the key factors influencing tumor growth and progression, this increase in PRDX1 expression might be an adaptive response. In addition, the expression of PRDX is inducible by various stimuli including H$_2$O$_2$, okadaic acid (OA), 12-O-tetradecanoylphorbol 13-acetate (TPA), and butylated hydroxyanisole, which protects cells from these stimuli (Ishii et al. 2000, Immenschuh et al. 2002, Wijayanti et al. 2008). Furthermore, an increased level of PRDX1 was reported to be involved in the protection of cancer cells against various therapeutic challenges by neutralizing ROS and modulating the related signaling pathways (Butzke et al. 2004, Kim et al. 2006, Song et al. 2009). These findings indicate that PRDX1 functions as an important regulator of resistance against cancer therapy, in which induction might be an adaptive response of cells to various stimuli.

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed serine–threonine protein kinase that functions as a mitogen-activated protein kinase (MAPK) kinase kinase to activate the c-Jun N-terminal kinase (JNK) and p38 MAPK signaling cascades (Nishitoh et al. 1998, Saitoh et al. 1998, Tobiume et al. 2001). ASK1 is activated in response to various stresses and plays a critical role in the regulation of signaling in response to oxidative stress, which is a major contributor to proteotoxic damage and cell death (Nishitoh et al. 1998, Saitoh et al. 1998, Tobiume et al. 2001, Goldman et al. 2004). Recently, PRDX1 has been reported to function as a negative regulator of ASK1 activity (Kim et al. 2008), further suggesting a role for PRDX1 in protecting cancer cells from harmful stimuli.

Proteasome inhibitors represent a novel class of antitumor agents with pre-clinical and clinical evidence of activity against hematologic malignancies and solid tumors. However, it has to be noted that proteasome inhibitors paradoxically also trigger accumulation or even de novo synthesis of antiapoptotic factors and can compromise the cytotoxic effect of the drug (Yang et al. 2006, Zaarur et al. 2006, Podar et al. 2008, Wang et al. 2007, 2008). Suppression of these unwanted proteins in combination with an effective conventional cancer chemotherapeutic agent is therefore a rational approach to improve cancer therapy. In this study, we demonstrated for the first time that PRDX1 is another molecule which is induced by proteasome inhibitors and compromised the anticancer effects of proteasome inhibition. In addition, we showed that the specific, potent proteasome inhibitor MG132 activated ASK1 as well as its downstream effectors p38 and JNK, and induction of PRDX1 suppressed MG132-mediated thyroid cancer cell death by its influence on ASK1 activity.

**Materials and methods**

**Culture of multiple cancer cell lines**

FRO82-1 (simply FRO) cell lines were initially obtained from Dr James A Fagin (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and were provided to us by Dr Shunichi Yamashita (Nagasaki University Graduate School of Biomedical Sciences, Japan). KTC1 and KTC3 cell lines were generously provided by Dr Junichi Kurebayashi (Kawasaki Medical School, Japan). 8305C and 8505C cells were obtained from the European Collection of Animal Cell Cultures. All cell lines were maintained in DMEM (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Sigma–Aldrich).

**Chemicals**

MG132, epoxomycin, Z-Ile-Glu(OtBu)-Ala-Leu-H (PSI), and lactacystin were purchased from Calbiochem (La Jolla, CA, USA). Actinomycin D and tumor necrosis factor α (TNFα) were obtained from Sigma and PeproTech (London, UK) respectively. As a vehicle control, 0.02% dimethyl sulfoxide (DMSO) was used.
Detection of cell death

For cell death assays, cells were washed twice in PBS, and were then stained with Annexin V-FITC (Biovision, Mountainview, CA, USA) and propidium iodide (PI, Sigma–Aldrich) according to the manufacturer’s instructions. After staining with annexin V-FITC and PI, samples were analyzed by fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

RNA isolation and real-time reverse transcription-PCR

RNA isolation and real-time reverse transcription (RT)-PCR were performed as previously reported (Wang et al. 2007). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and were the following:

For PRDX1:
- Forward: 5'-CACAGCTGTTATGCAGATG-3'
- Reverse: 5'-ACTGAAAGCAATGATCTCCG-3'

For PRDX6:
- Forward: 5'-AACAGCTGTGATCGATGGAG-3'
- Reverse: 5'-TCA-TAGTCGTGCCAAAGC-3'

For b-actin:
- Forward: 5'-GAGACCTTCAACACTCAGACAGGC-3'
- Reverse: 5'-GGATCTTCATGAGGTAGTCAG-3'

Results

Induction of PRDX1 expression by proteasome inhibitors in thyroid cancers

We first performed real-time RT-PCR to study the regulation of PRDX mRNA expression by MG132 in a panel of thyroid cancer cell lines. Incubation with MG132 significantly increased PRDX1 mRNA levels in the panel of thyroid cancer cells (Fig. 1A). Although to a much lesser extent, MG132 also enhanced PRDX6 mRNA levels (Fig. 1A). MG132 had no obvious effect on the mRNA expression levels of PRDX2–5 (Fig. 1A). Western blot analysis also demonstrated a marked increase in PRDX1 protein expression by MG132 treatment (Fig. 1B). Paradoxically, MG132 exposure had no stable effects on PRDX6 in the panel of thyroid cancer cells (Fig. 1B). The PRDX6 protein levels were slightly increased in 8305C and 8505C cells, no clear alteration was observed in KTC1 and KTC3 cells, and MG132 caused a decrease in FRO cells (Fig. 1B). To examine whether the increase in PRDX1 mRNA on MG132 treatment was due to an increase in PRDX1 mRNA stability or a transcriptional induction, we pretreated FRO cells with 10 μg/ml actinomycin D for 1 h, and then treated with vehicle or MG132 for an additional 8 h. Pretreatment with the transcription inhibitor actinomycin D completely blocked the upregulation of PRDX1 by MG132 (Fig. 1C), indicating that MG132 induced increased PRDX1 expression at the transcriptional level. To further assess whether upregulation of PRDX1 was a general response to proteasome inhibition, we investigated PRDX1 expression in thyroid cancer cells.
treated with different proteasome inhibitors. Similar to MG132, four other proteasome inhibitors bortezomib, PSI, lactacystin, and epoxomycin also elevated PRDX1 mRNA (Fig. 1D) and protein (Fig. 1E) expression levels in FRO cells. In addition, these different proteasome inhibitors also increased PRDX1 expression in 8305C and 8505C cells (Fig. 1F), indicating that induction of PRDX1 is a general effect of proteasome inhibition.

Promotion of MG132-mediated thyroid cancer cell death by suppression of PRDX1 induction

We next examined the functional roles of PRDX1 in MG132-mediated apoptosis in thyroid cancer cells. FRO cells were transfected with PRDX1 siRNA (siPRDX1), the level of knockdown was then determined by real-time RT-PCR (Fig. 2A) and western blot analysis (Fig. 2B). siPRDX1 efficiently suppressed MG132-mediated upregulation of PRDX1, ~30–40% of the PRDX1 protein detected in the control cells (scramble) was detected in siPRDX1-transfected cells (Fig. 2B). To determine the effect of PRDX1 knockdown on proteasome inhibitor-mediated cell death, siPRDX1-transfected cells were cultured for 24 h in the presence of MG132, labeled with annexin V-FITC/PI, and subjected to flow cytometry. Cells transfected with siPRDX1 displayed an abrupt increase in cell death with MG132 treatment (Fig. 2C). siPRDX1 also increased the sensitivity of FRO cells to other proteasome inhibitors including lactacystin and epoxomycin (Fig. 2D). In addition, siPRDX1 also increased the sensitivity of two other thyroid cancer cell lines, 8305C and 8505C cells, to MG132-induced cell toxicity (Fig. 2E). To further confirm whether the exogenously expressed PRDX1 could compromise the sensitizing effect of siPRDX1 on MG132, we investigated the effect of PRDX1 overexpression (Fig. 2F). Overexpression of PRDX1 per se had no
A significant effect on MG132-mediated apoptosis, but it recovered the responsiveness of siPRDX1 to MG132-induced cell death (Fig. 2G).

Implication of ASK1 activation in MG132-mediated thyroid cancer cell death

ASK1 is activated in response to various stresses including H$_2$O$_2$, serum withdrawal, endoplasmic reticulum stress, and TNF$\alpha$ (Ichijo et al. 1997, Tobiume et al. 1997, Chang et al. 1998, Morita et al. 2001). Therefore, we investigated whether ASK1 was also involved in MG132-mediated thyroid cancer cell death. We first examined the effect of MG132 on the catalytic activity of ASK1 and its downstream effectors p38 and JNK by using their respective anti-phosphorylated antibodies. Similar to TNF$\alpha$, the well-known activator of ASK1, treatment of FRO cells with MG132 activated endogenous ASK1, as well as its downstream effectors p38 and JNK (Fig. 3A), indicating that MG132 activates the ASK1–p38 and the ASK1–JNK pathways. We next examined whether ASK1 is required for the activation of p38 and JNK, as well as whether ASK1 is required for the induction of cell death mediated by proteasome inhibition, by using siRNA raised against ASK1 (siASK1) to knock down endogenous ASK1. siASK1 successfully reduced the expression of total and phosphorylated ASK1 (Fig. 3B), siASK1 also dramatically decreased the activation of p38 and JNK mediated by MG132, as well as by lactacystin (Fig. 3B). In addition, the apoptotic FRO cell death induced by MG132 or lactacystin was clearly suppressed by siASK1 (Fig. 3C), indicating that ASK1 is an essential component of p38 and JNK activation, as well as FRO cell death induced by proteasome inhibition. To investigate the general involvement of ASK1 activation in proteasome inhibition-mediated thyroid cancer cell death, we checked the effect of siASK1 in thyroid cancer KTC1 and 8505C cells, in which cell death could be induced by proteasome inhibitors (Wang et al. 2007). siASK1 treatment significantly protected both KTC1 and 8505C cells from MG132-induced cell death when compared with scrambled siRNAs (Fig. 3D).

Suppression of MG132-mediated ASK1 activation by PRDX1

It has been reported that PRDX1 negatively regulates ASK1 activity (Kim et al. 2008), we hypothesized that induction of PRDX1 may affect MG132-induced apoptosis via its influence on the activation of ASK1. To explore this question, we tested whether PRDX1

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**Figure 2** Promotion of MG132-mediated apoptosis by knockdown of PRDX1 by siRNA. (A) FRO cells were transfected with scrambled siRNA or siRNA against PRDX1 (siPRDX1) for 48 h, and were then treated with vehicle control or MG132 for additional 24 h, PRDX1 mRNA was then measured using real-time RT-PCR. (B) FRO cells were treated as in (A), and western blot analysis was performed. (C) FRO cells were treated as in (A), and apoptotic cells were analyzed using flow cytometry. (D) FRO cells were transfected with scramble or siPRDX1 for 48 h, and were then treated with vehicle control, lactacystin or epoxomycin for an additional 24 h, and apoptotic cells were analyzed. (E) 8305C and 8505C cells were transfected with scramble or siPRDX1 for 48 h, and were then treated with vehicle control or MG132 for an additional 24 h, and apoptotic cells were analyzed using flow cytometry. (F) FRO cells transfected with mock (1), siPRDX1 (2), PRDX1 expression vector (3) or both siPRDX1 and PRDX1 expression vector (4), were then treated with vehicle control or MG132 for 24 h, and western blot was performed. (G) FRO cells were treated as in (E), and apoptotic cells were analyzed. *P<0.01 and NS, not significant.
knockdown can affect the activation of ASK1-mediated signaling by MG132. FRO cells were transiently transfected with scramble or siPRDX1 and stimulated with MG132 for various time periods, and then the level of phosphorylation of ASK1, p38, and JNK was evaluated. Upon the stimulation of MG132, phosphorylated ASK1 was first detected after 2 h, the levels gradually increased in a time-dependent manner and peaked at 8–12 h (Fig. 4A). Similarly, phosphorylation of p38 and JNK increased in a time-dependent manner and peaked at 8–12 h (Fig. 4A). Interestingly, the levels of phosphorylated ASK1 quickly and strongly appeared at 1 h, peaked at 4–8 h, and were then continuously maintained in PRDX1 knockdown cells (Fig. 4A). Similar patterns of p38 and JNK phosphorylation could be detected in PRDX1 knockdown cells (Fig. 4A). To further confirm the role of ASK1 in the PRDX1-dependent response of thyroid cancer cells to MG132-mediated apoptosis, we knocked down ASK1 in FRO cells transfected with siPRDX1. Suppression of ASK1 clearly decreased siPRDX1-mediated promotion of apoptotic cell death induced by MG132 (Fig. 4B), these results strongly suggest that the induction of PRDX1 as a negative regulator plays a key role for ASK1-mediated cell death in response to MG132 in undifferentiated thyroid cancer cells.

Discussion

The proteasome represents the major degradation pathway for proteins involved in the regulation of cell survival, proliferation, apoptosis, and other critical cellular functions (Adams 2004). The proteasome also has a critical role in the selective removal of mutant, damaged, and misfolded proteins. Based on these unique properties of the ubiquitin-proteasome system, the proteasome pathway has recently emerged as an attractive target for the development of anticancer agents. Paradoxically, proteasome inhibitors also activate survival signaling cascades, thus compromising their anticancer effects (Mimnaugh et al. 2004, Nencioni et al. 2005). Therefore, the identification of the antiapoptotic activities of proteasome inhibitors could lead to the design of novel, rational combination regimens with enhanced antitumoral efficacy.

The PRDX family includes six known members (PRDX1–6). In the current study, we found that MG132 specifically upregulated expression of PRDX1 and PRDX6 at the transcriptional level. At the protein level, consistent with mRNA expression, MG132
in most cancers, although the physiological mechanism by various stimuli. PRDX1 expression is also elevated in 2008), indicating the expression of PRDX1 is inducible inhibitors, H2O2, OA, TPA, and butylated hydroxyanisole could also induce the expression of PRDX1 (Ishii et al. 2000, Immenschnuh et al. 2002, Wijayanti et al. 2008), indicating the expression of PRDX1 is inducible by various stimuli. PRDX1 expression is also elevated in most cancers, although the physiological mechanism is still unclear (Yanagawa et al. 1999, 2000, Chang et al. 2001, Kim et al. 2003, 2007, 2008, Alfonso et al. 2004, Lehtonen et al. 2004, Xin et al. 2005, Park et al. 2006). Previous studies have reported that an increased level of PRDX1 might be involved in the protection of cancer cells against various therapeutic challenges (Butzke et al. 2004, Kim et al. 2006, Song et al. 2009). Consistent with these reports, this study demonstrated that the induction of PRDX1 by proteasome inhibition compromised any antitumoral cytotoxicity. Based on these results, it is tempting to propose that increased PRDX1 in cancer cells may play a critical role in providing protection against harmful stimuli.

In mammalian cells, ASK1 participates in the JNK and p38 MAPK signaling cascades by phosphorylating MKK4/MKK7 and MKK3/MKK6 respectively (Nishitoh et al. 1998). ASK1 is activated in response to various extracellular and intracellular stimuli, such as lipopolysaccharide, ROS, endoplasmic reticulum (ER) stress, and signaling through Fas and TNFα (Matsukawa et al. 2004). ASK1 is well known as a proapoptotic, stress-activated signaling molecule, and it is under tight regulation at multiple levels, one of which is negatively regulated by PRDX1 (Kim et al. 2008). In this study, we demonstrated that ASK1 played an essential role in MG132-mediated thyroid cancer cell death, as ASK1 knockdown by siRNA markedly suppressed the cytotoxic effects of MG132. Consistent with previous reports, we detected that induction of PRDX1 reduced MG132-mediated cell death via suppression of ASK1 activation.

In conclusion, we have described that PRDX1 is another ‘unwanted molecule’ in cancer therapy with proteasome inhibitors, which compromises the antitumoral cytotoxic responses of thyroid cancer cells treated with MG132 via the negative modulation of ASK1 activity and inhibited ASK1-mediated apoptosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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