MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells

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Proline oxidase (POX), localized on inner mitochondrial membranes, is encoded by a p53-induced gene and metabolically participates in p53-induced apoptosis. Previously, we showed that POX catalyzed the generation of reactive oxygen species (ROS). We and others have demonstrated that overexpression of POX, independent of p53, causes apoptotic cell death in a variety of cancer cells. But a necessary role for ROS remains uncertain. Therefore, we asked whether superoxide dismutases (SOD) and catalase (CAT), important antioxidant enzymes, might interfere with the POX-dependent induction of apoptosis. In this study, we used DLD-1 colorectal cancer cells stably transfected with the POX gene under the control of a tetracycline-inducible promoter. When doxycycline was removed from the culture medium and the expression of POX was induced, apoptotic cell death was initiated. To examine the importance of the ROS-dependent component of the pathway, we infected DLD-1 POX cells with recombinant adenoviruses containing MnSOD, CuZnSOD, CAT or varying combinations of these adenoviruses followed by induced expression of POX. The expression of MnSOD inhibited POX-induced apoptosis, but others did not. Mechanistically, mitochondria-localized MnSOD dramatically reduced the release of cytochrome c to cytosol by POX. Compared with control cells, MnSOD-expressing DLD-1 POX cells generated a higher concentration of H2O2 owing to dismutation of superoxide radicals, which was elevated by POX. Thus, these data further suggest that the generation of superoxide radicals plays a crucial role in POX-induced apoptosis and the process is partially blocked by MnSOD.

Introduction

p53 is the most extensively studied tumor suppressor, and acts in response to diverse forms of cellular stress to mediate antiproliferative processes, either G1 cell-cycle arrest or apoptosis, depending on the cellular context and type of activating agent. In the process of apoptosis, the role of p53 in the transactivation and transrepression of genes is well established. The most studied p53-transactivated genes in apoptosis probably are Bax, Puma and Noxa (1). By serial analysis of gene expression, Polyak et al. (2) showed that p53-dependent apoptosis is preceded by the induction of 14 of 7202 genes, termed PIGs, including proline oxidase (POX), PIG-6. POX, localized on the inner mitochondrial membrane, is an enzyme converting proline to pyrroline-5-carboxylate (P5C). Its activity mediates the proline cycle that shuttles redox equivalents between mitochondria and cytosol (3,4). It is also important to note that PSC is the sole intermediate directly connecting the tricarboxylic acid and urea cycles with amino acid metabolism (4). Recently, we showed that POX catalyzed the generation of reactive oxygen species (ROS) (5). We and others have demonstrated that an overexpression of POX also causes apoptotic cell death in a variety of cancer cell types (6–8). Thus, in p53-induced apoptosis, although the direct line appears to be p53 to PUMA/NOXA to interact with BCL-2 or BCL-XL and free BAX (9,10), the importance of alternative or modulating pathways depends on the metabolic environment. It is possible that POX regulates cell survival in situations of nutritional and energy stress. Therefore, POX may be a good model for matrix/nutrition-dependent modulation of apoptotic phenotype.

ROS are highly reactive oxygen metabolites that include superoxide radical (O2•−), hydroxyl radicals (OH•) and the non-radical hydrogen peroxide (H2O2). In addition to ROS production, PSC generation by POX may also increase production of nitric oxide and its derivatives, reactive nitrogen species (RNS) (11,12). Now, the RNS are also recognized as important radicals. Since different radical species may react with different molecules and play a variety of roles in different cell processes, it is important to determine the specific species that mediates POX-induced apoptosis.

Although evidence points to a role for oxidant species in carcinogenesis, a parallel series of observations suggest that ROS may also be important in limiting cell or tumor growth. Multiple lines of evidence suggest that ROS actively participate in the process of apoptosis, in either early or late stage, through mitochondrial or death receptor pathway (13,14). To protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase. Whether cells die from ROS-induced apoptosis depends on the balance between the generation of oxidant species and the intracellular antioxidant system. While superoxide is rapidly removed by conversion to H2O2 in a reaction catalyzed by superoxide dismutases, the H2O2 generated from either side of

Abbreviations: Ad, adenovirus; DCFH, 2',7'-dichlorofluorescein diacetate; DOX, doxycycline; CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; MOL, multiplicity of infection; NAC, N-acetyl cysteine; PSC, pyrroline-5-carboxylate; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PIG, p53-induced gene; POX, proline oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species.
the mitochondrial membrane by MnSOD or CuZnSOD is converted by CAT or glutathione peroxidase to water. Indeed, MnSOD is widely recognized as an antiapoptotic agent (15). On the basis of these findings, we proposed that antioxidant enzymes might have an effect on POX-induced apoptosis. In this study, we employed the DLD-1 Tet-Off POX cell line, where DLD-1 colorectal cancer cells were stably transfected with the POX gene under the control of a tetracycline-inducible promoter (5). We found that POX-induced apoptosis in DLD-1 colorectal cancer cells was partially blocked by MnSOD. In contrast, CuZnSOD, CAT had no effect. However, CAT blocked the antiapoptotic effect of MnSOD, suggesting a role for H2O2 in the process. Mechanistic studies demonstrated that MnSOD inhibited apoptosis by reducing the release of cytochrome c into the cytosol. In addition, we demonstrated the increased generation of superoxide by POX induction. MnSOD overexpression lowered superoxide radical levels but increased H2O2 levels. Thus, we propose that it is superoxide radicals rather than other forms of ROS that directly mediate the apoptosis induced by POX.

Materials and methods

Reagents and cell culture

The antioxidant reagent N-acetylcysteine (NAC) was purchased from Calbiochem. The fluorescent dyes hydroethidine and DCFH were obtained from Molecular Probes (Eugene, OR). Doxycycline (DOX) was from Sigma (St Louis, MO). The generation of the DLD-1 Tet-Off POX cell lines has been previously described (5). The cells were maintained in McCoy’s 5A medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) in the presence of 0.4 mg/ml G418, 0.25 mg/ml hygromycin B and 20 ng/ml DOX.

Cell growth assays

The CellTiter 96TM aqueous non-radioactive cell proliferation assay (MTS assay; Promega, Madison, WI), performed according to the manufacturer’s protocol, was used to measure cell growth. Approximately 25,000 cells were seeded in wells of a 24-well plate and doxycycline or vehicle was added to block or induce the expression of POX by DLD-1 Tet-Off POX cell lines. Solution 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (20:1 v/v) was added to the cells for 2 h at 37°C and absorption at 490 nm was determined. Each data point was performed in triplicate, and the results were reported as mean absorbance ± standard error.

Adenovirus infection

Recombinant adenoviruses (Ads), Ad-GFP, Ad-Empty, Ad-MnSOD, Ad-CuZnSOD and Ad-CAT were purchased from Gene Therapy Vector Core Facility of the University of Iowa. Ad-GFP was used to determine the infection efficiency. The multiplicity of infection (MOI) of different recombinant viruses was used to reach 100% infection but did not cause dramatic cell death by virus alone.

Western blot analysis

The cell lysates were prepared in a buffer containing 50 mM Tris–HCl (pH 6.8), 20% SDS, 10% glycerol and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). For preparation of mitochondrial and cytosolic fractions, a Mitochondria Isolation Kit (Pierce Biotechnology, Rockford, IL) was employed according to the manufacturer’s protocols. Equal amounts of total cellular protein extract were electrophoresed on an acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The primary antibodies used were anti-CuZnSOD (Calbiochem, San Diego, CA), anti-MnSOD (Upstate USA, Inc., Chicago, IL), anti-CAT (Sigma), anti-cytochrome c (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PARP (Cell Signaling Technology, Inc., Beverly, MA), anti-actin (Sigma) and anti-POX. Blots were developed using the chemiluminescence (ECL) procedure (Amersham, Piscataway, NJ).

Superoxide dismutase assay

SOD activities were determined using Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI). It utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

In brief, the cells were collected and sonicated in a cold buffer containing 20 mM HEPES, (pH 7.2), 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. The supernatant was collected after centrifuging at 1500×g for 5 min at 4°C. To separate the two enzymes, the 1500×g supernatant was centrifuged at 10 000×g for 15 min at 4°C. The resulting 10 000×g supernatant contained cytosolic SOD (CuZnSOD) and pellet contained mitochondrial SOD (MnSOD). The samples were analyzed using a plate reader with a 540 nm filter. Each data point was performed in triplicate, and the results were reported as mean absorbance ± standard error.

Catalase (CAT) assay

CAT activity was measured using Catalase Assay Kit (Cayman Chemical). It utilizes the peroxidatic function of CAT for determination of enzyme activity. In brief, the cells were collected and sonicated in a cold buffer containing 50 mM potassium phosphate (pH 7.0) and 1 mM EDTA. The supernatant was collected after centrifuging at 10 000×g for 15 min at 4°C. The samples were analyzed using a plate reader with a 540 nm filter. Each data point was performed in triplicate, and the results were reported as mean absorbance ± standard error.

Hydroethidine assay and DCFH assay

These assays are designed to measure hydroethidine and DCFH fluorescence, which reflect the levels of superoxide and H2O2, respectively (16,17). Cells were grown in 6-well plates. The cultures were washed twice with PBS and then stained with either 10 μM DCFH for the detection of H2O2 or 10 μM hydroethidine for the detection of superoxide, for 30 min at 37°C. The cells were washed twice with PBS and fluorescence was measured using a Cytofluor II Fluorescent Multi-Plate Reader (Applied Biosystems, Foster City, CA). The wavelengths used were 485/535 nm for DCF and 485/585 nm for hydroethidine. Each data point was performed in triplicate, and the results were reported as mean absorbance ± standard error.

Flow cytometry

DLD-1 Tet-Off POX cells were cultured in the presence or absence of DOX to block or induce the expression of POX. On days 0, 2, 4, 6 and 8, one million cells were harvested and washed twice with cold PBS. The pellets were fixed in 70% ice-cold methanol for 30 min. Before running on the flow cytometer, the cells were washed twice again with cold PBS and incubated with 5 μl RNase (200 U/ml, DNase-free) (Sigma) for 15 min. The cells were stained with 10 μg/ml propidium iodide for at least 1 h in the dark. Stained cells were analyzed with an EPICS-XL-MCL flow cytometer (Beckman Coulter, Inc., Miami, FL). Each data point was performed in triplicate, and the results were reported as the mean ± standard error.

Results

We and others have demonstrated that POX induced apoptosis in several types of cancer cells (6–8). We then showed that POX caused cytochrome c release from mitochondria and the activation of caspases 9 and 3, indicating POX-induced apoptosis was through the mitochondrial or intrinsic apoptotic pathway (6). Previously, we isolated DLD-1 colorectal cancer cell clones that expressed POX under the control of an inducible promoter, the Tet-Off promoter (5). In the present study, we utilized DLD-1 Tet-Off POX cells to determine the effect of POX on apoptosis. First, the expression of POX protein was determined using western blotting (Figure 1A). As can be seen, no POX was expressed in the presence of DOX, while time-dependent increase of POX expression was observed when the cells were cultured in the absence of DOX. Control vector clone did not express POX in the presence or absence of DOX.

To confirm the previous results, we showed that POX expression inhibited DLD-1 cell growth by inducing apoptosis. DLD-1 Tet-Off POX and DLD-1 Tet-Off vector cells were cultured in medium with or without DOX. The expression of POX after the removal of DOX inhibited cell growth and caused cell death. Cells transfected with vector without the POX gene grew well in the presence or in the absence of DOX (Figure 1B).

POX catalyzes the generation of ROS, which is considered an important mediator of apoptosis. Thus, it is reasonable
to think that antioxidant chemicals or enzymes may inhibit POX-induced apoptosis. To test this, we first used NAC, a widely used antioxidant agent. NAC dramatically reduced POX-induced apoptosis as demonstrated by flow cytometry (Figure 2). Then, to identify the induction of specific oxidant species, we employed recombinant adenoviruses to introduce MnSOD, CuZnSOD or CAT into the cells. The expression and activity of these enzymes after infection were determined using western blotting and their respective activity assays (Figure 3A-C). To confirm the specific localization of enforced MnSOD and CuZnSOD, the mitochondrial and cytosolic fractions were isolated. Western blottings showed that overexpressed MnSOD was principally localized in the mitochondria, while overexpressed CuZnSOD was principally in the cytosol (Figure 3D). On removal of DOX to induce POX, the cells underwent apoptosis. Importantly, the growth assay also clearly showed that MnSOD dramatically inhibited POX-induced apoptosis while CuZnSOD and CAT did not (Figure 4A). This was not surprising for CuZnSOD/CAT combination since neither enzyme had an effect on POX-induced apoptosis. However, CAT appeared to abrogate the affect of MnSOD. This suggested that H2O2 generated from MnSOD inhibited apoptosis. The inhibition by MnSOD was also confirmed by flow cytometry and the blockade of PARP cleavage (Figure 4B and C).

ROS are a set of different oxidizing equivalents. Owing to differences in their nature, their specific regulatory effects on cellular functions, including apoptosis, can be distinct. The above data, that MnSOD inhibits POX-induced apoptosis, but not CuZnSOD or CAT, alone or in combination, also indicate this difference and suggest that it is superoxide rather than other forms of ROS mediating POX-induced apoptosis. To test this directly, we performed hydroethidine assay to determine the level of superoxide generated by POX. The DLD-1 Tet-Off POX cells were cultured in the specified conditions with or without DOX. The time-dependent increase of superoxide with POX induction after removal of DOX was observed, whereas in cells exposed to DOX it remained unchanged (Figure 5).

The presence of MnSOD inhibited POX-induced apoptosis presumably because MnSOD decomposed superoxide radicals to H2O2. To verify experimentally that this was the case, we determined the effect of MnSOD on POX-mediated accumulation of superoxide and its conversion to H2O2. When DOX was removed and POX induced, the elevated levels of superoxide was dramatically reduced by the expression of MnSOD (Figure 6A). This decrease in superoxide coincided with decreased apoptosis with MnSOD (Figure 4B). Accompanying the decrease in superoxide, the level of H2O2 by MnSOD was markedly increased (Figure 4B).

MnSOD blocked POX-induced apoptosis although other antioxidant enzymes did not. This prompted us to examine the mechanisms that mediated POX-induced apoptosis inhibited by MnSOD. Since MnSOD was localized mainly to the mitochondria, and POX is bound to inner mitochondrial membrane, it was likely that SOD interacted directly with superoxide anions in the mitochondria. Therefore, we examined mitochondrial pathways that directly lead to apoptotic cell...
death. Our attempts to define the early POX-mediated event, e.g. loss of membrane potential, yielded inconclusive results, but the critical release of cytochrome $c$ by mitochondria was clearly defined. In the studies monitoring the release of cytochrome $c$ in POX-induced cells, the expression of MnSOD markedly reduced the release of cytochrome $c$ from mitochondria into cytosol (Figure 7). Thus, superoxide generated by POX expression plays a critical role in the loss of mitochondrial membrane integrity during POX-induced apoptosis.

**Discussion**

ROS include free radicals, such as the superoxide anion ($O_2^-$), hydroxyl radicals (OH$^-$) and the non-radical hydrogen peroxide ($H_2O_2$). ROS are constantly generated under normal conditions as a consequence of aerobic metabolism, but they are frequently associated with cytotoxicity, often being described as damaging, harmful or toxic. Their role in apoptosis has been established. ROS are not a single species. Our data strongly support the notion that it is superoxide rather than other forms of ROS that mediates POX-induced apoptosis.

The mitochondria are believed to be a major site of ROS production. Complex I, NADH-ubiquinone oxidoreductase and Complex III, ubiquinol-cytochrome $c$ oxidoreductase of the respiratory chain, are the two major sites where superoxide is produced. A crucial role for superoxide anions for the induction of apoptosis in colon cancer cells has been suggested (18–20). The increase of superoxide production during apoptosis has been shown to require mitochondrial respiratory chain activity and may also be a direct consequence of mitochondrial membrane permeabilization and the loss of cytochrome $c$ (21–24). Not only the site of ROS production but also the mitochondria are the target of ROS. More recently, important insights in the role of mitochondria during triggering of apoptosis have allowed a better understanding of how oxygen species participate in the apoptotic cascade. When cytochrome $c$ is released from mitochondria into the cytosol to activate (together with apaf-1) the effector caspase 3, a switch from the normal 4-electron to a 1-electron reduction of oxygen leads to the production of superoxide anion in excess (22).

Superoxide, in turn, amplifies the death cascade by the damaged mitochondria and favors the oxidative opening
Fig. 3. Adenovirus-mediated expression and activity of CuZnSOD, MnSOD and CAT and their specific localization. DLD-1 Tet-Off POX cells were infected with 1000 and 3000 MOI of Ad-CuZnSOD, Ad-MnSOD or Ad-CAT. Ad-Empty infection was used as control. After 2 days the cell lysates were harvested. Western blottings were used to demonstrate the expression of these proteins with actin as a loading control. SOD activity and CAT activity were determined using Superoxide Dismutase Assay Kit and Catalase Assay Kit (Cayman) according to the manufacturer’s direction. (A) The expression and activity of CuZnSOD. (B) The expression and activity of MnSOD. (C) The expression and activity of CAT. (D) The cells were infected with 500 MOI of Ad-CuZnSOD, Ad-MnSOD or Ad-Empty as control. After 2 days the cells were harvested and the mitochondrial and cytosolic fractions were isolated. The localization of enforced MnSOD and CuZnSOD was demonstrated using western blottings.
of the permeability transition pores and the further release of cytochrome c and apoptosis-inducing factor (AIF). An amplification cycle is thus established, which accelerates apoptosis. That POX-induced release of cytochrome c was mediated by this cycle involving ROS was clearly demonstrated by the marked decrease in cytochrome c release in cells expressing MnSOD. Owing to its special localization (inner mitochondrial membrane), POX may directly act on the mitochondrial respiratory chain and cause overproduction of superoxide anions, which then amplify the apoptotic signal resulting in enlargement of pores in mitochondrial membranes (25).

Superoxide can also be generated by various enzymes (xanthine oxidase, lipoxygenases, NADPH oxidases, etc.). One oxidase gaining prominence in the studies of apoptosis is NADPH oxidase, found in the plasma membrane, because activation of this enzyme and subsequent superoxide production has been found necessary for apoptosis in a variety of systems (26,27). Thus, both mitochondrial and cytosolic superoxide productions are possibly important for apoptosis. In the case of POX, since cytosolic CuZnSOD did not inhibit

Fig. 4. MnSOD inhibition of POX-induced apoptosis. (A) DLD-1 Tet-Off POX cells were seeded in 24-well plates in the presence of DOX, then were infected with 3000 MOI of Ad-MnSOD, Ad-CuZnSOD, Ad-CAT or varying combinations. DOX was removed from the medium 2 days later and the cell growth was determined as described in Materials and methods. (B) DLD-1 Tet-Off POX cells were infected with 3000 MOI of Ad-MnSOD. After 2 days, the DOX was removed from the medium to induce the expression of POX. Cells were harvested on days 0, 2, 4, 6 and 8, then stained with propidium iodide for cell-cycle analysis using flow cytometry. (C) DLD-1 Tet-Off POX cells were infected with 3000 MOI of Ad-MnSOD. After 2 days, the DOX was removed from the medium to induce the expression of POX. Cell lysates were prepared and western blotting to PARP was performed. Western blotting for actin was used as loading control (*P < 0.05; **P < 0.01).

Fig. 5. Superoxide radical generation by POX expression. DLD-1 Tet-Off POX cells were cultured in the presence and absence of DOX. At different time points, hydroethidine assay was performed to measure the generation of superoxide radicals (*P < 0.05; **P < 0.01).
apoptosis, it was unlikely that cytosolic superoxide, which was not permeable, played a major role.

Finally, we could not exclude the involvement of the death receptor or extrinsic pathway in POX-induced apoptosis. TNF-induced, FasL-induced and TRAIL-induced apoptosis may all act through this pathway (28–31), and in all these cases, ROS are involved. Actually, our preliminary data showed the activation of this pathway by POX expression (Y.Liu and J.Phang, unpublished data).

The identification of specific forms of activated oxygen is only one of the complexities in understanding their role in cancer. They may have many other effects in cell regulation. Their roles in mitogenesis, tumor promotion and angiogenesis have been investigated. However, ROS clearly play an important role in apoptosis. Even the widely-studied antiapoptotic BCL-2 and BCL-XL produce their effects, at least in part, through antioxidant mechanisms (14,32–34). Various antioxidant enzymes, including MnSOD, have been used to block apoptosis induced by different agents (15,35–37).

MnSOD inhibits apoptosis induced by a wide range of agents, including p53 (15), and p53-mediated cell killing is, in part, mediated by ROS (2,38). Even though our observations were in DLD-1 cells which lack functional p53 (5,39), they are the source of implications applicable to cells with intact p53 in which others have suggested that p53 may downregulate MnSOD to maximize oxidative effects on mitochondria for apoptosis (15). They are also consistent with the finding that POX is a p53 target gene and POX induces apoptosis through oxidative stress; other p53 target genes may also act through the generation of ROS.

CAT did not inhibit POX-induced apoptosis, suggesting that H2O2 was not a trigger of this apoptosis. Since the MnSOD/CAT combination did not block this apoptosis either, it raised a possibility that H2O2 was acting as an antiapoptotic agent, a role suggested for H2O2 in MnSOD-induced resistance to irradiation (40). At low concentrations, H2O2 has frequently been shown to stimulate cell proliferation in different systems, especially in cancer cells (41), although at high concentrations it is known to cause cell injury and death. Indeed, ROS are known to activate antiapoptotic pathways, such as NF-κB and the heat-shock proteins Hsp70 and Hsp27 (42,43). The role of H2O2 in POX-induced apoptosis needs to be further elucidated.

In summary, POX induces apoptosis in colorectal cancer cells, which is mediated by superoxide production in mitochondria. MnSOD, but not CuZnSOD or CAT inhibits this apoptosis. Furthermore, CAT reverses the protection afforded by MnSOD, suggesting an anti-apoptotic role for H2O2. MnSOD inhibited the release of cytochrome c from mitochondria, probably suppressing superoxide radical-caused damage/opening of permeability transition pores. ROS/superoxide signaling can be divided into two general mechanisms of action. One is the alteration of intracellular redox state. Another is the oxidative modification of protein. Here we show the change of redox status by POX and superoxide. POX-induced changes in signaling pathways, such as its effects on kinases and proteins of the BCL-2 family, which participate in the formation of mitochondrial channels, will be the future emphasis of our studies.

Thus, as a nutrition factor, POX may modulate apoptosis induced by other agents and enhance apoptosis in stress situations. Thus, it is possible to use POX as an adjunct target to inhibit cancer cell growth.

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