Mimic of manganese superoxide dismutase to induce apoptosis of human non-Hodgkin lymphoma Raji cells through mitochondrial pathways

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1. Introduction

Lymphoma is a refractory malignant tumor that originates in the lymphatic system. The two main types are Hodgkin’s lymphoma and non-Hodgkin’s lymphoma (NHL) [1]. Human malignant NHL represents a heterogeneous group of tumors, which vary in their biological aggressiveness and clinical course [2]. The standard chemotherapy regimen of NHL is cyclophosphamide, doxorubicin, vincristine, and prednisone, which plays an important role in the treatment of NHL. Unfortunately, a considerable number of patients undergoing cyclophosphamide, doxorubicin, vincristine, and prednisone relapse thereafter or suffer from dose-related side effects and complications. Furthermore, stem cell transplantation, as a treatment of curing NHL [3], is too expensive for patients, so curative treatment can be achieved in only a minority of NHL patients. Thus, novel therapeutic strategies and new anticancer agents are urgently needed to improve palliative treatment, prolong life expectancy, and improve quality of life in patients with lymphoma.

Reactive oxygen species (ROS) play an important role in the regulation of cell redox state [4]. Endogenous ROS generation is counterbalanced by the action of antioxidant enzymes and other redox molecules. Excessive endogenous ROS levels result in oxidative damage that has been implicated as the cause of various pathological states, including cancer. Manganese superoxide dismutase (MnSOD) is one of the primary antioxidant enzymes and is located in mitochondria, which are the main source of ROS production in aerobic organisms. The main function of MnSOD is protection against oxidative stress by conversion of superoxide into H2O2, which is subsequently converted to O2 and H2O by GSH-Px in mitochondria [5]. Multiple studies have shown that increasing MnSOD expression in cancer cells has anti-tumor effects. Cell growth, plating efficiency, cell growth in soft agar assays, and tumor formation in nude mice were all reduced when MnSOD was expressed in melanoma, pancreatic and prostate cancer cell lines [6–8]. Moreover, transgenic over-expression of MnSOD suppressed chemically induced tumor formation in a mouse model of skin tumorigenesis [9]. Similar to the results of previous studies [6,10,11], over-expression of MnSOD altered the phenotype of RWPE-2 cells and resulted in an oxidative shift in cell redox state and growth inhibition.

So far, more than 30 MnSOD have been found, but the intact amino acid sequence only has been completed in less than ten of them. The clinical application of natural MnSOD has been limited as its big molecular quality to induce immunity and allergic reaction when long-term to use. In addition, a short half-life and poor stability caused by hydrolysis of the enzyme also limited the clinical application of natural MnSOD. In order to solve those problems, domestic and foreign scholars mainly applied molecular engineering methods for molecular modification to mimic MnSOD. Those methods have become a useful tool for us to know natural
MnSOD. The synthetics or mimics of MnSOD have changed the clinical application of natural SOD.

In the recent years, many scholars have found that MnSOD mimics have a preferable anti-tumor effect in vivo and in vitro. Deng et al. [12] reported that cells treated with MnSOD mimics [Mn(EDTB)(AC)]{+} obviously inhibited the proliferation of SSMC-7721 in a concentration and a time-dependent manner. In the present study, the mimic compound of MnSOD (C₄₀H₅₇Mn₂N₉O₂₁) was made by a water soluble good flexibility fat amine and a well biocompatibility of adjacent vanillin. In the complexes molecule contained two Mn activities center, which increased the activity of the same site. Fan LL et al. [13] reported that Mimic of MnSOD (C₄₀H₅₇Mn₂N₉O₂₁) obviously inhibited the proliferation of K562 cells and induced apoptotic in vitro. However, anticancer effects of MnSODm in human non-Hodgkin lymphoma cells have not been reported. Since Raji cells (a human B cell Burkitt’s lymphoma cells) provide a useful system for studying cellular and molecular events involved in apoptosis by chemical agents, in the present study, we used Raji cells to investigate the mechanisms of MnSODm-induced apoptosis and the effect of MnSODm against human non-Hodgkin lymphoma.

2. Materials and methods

2.1. Chemicals and reagents

Mimic of manganese superoxide dismutase (MnSODm, C₄₀H₅₇Mn₂N₉O₂₁) was kindly provided by Professor Wei-Sheng Liu (College of Chemistry and Chemical Engineering, Lanzhou University, China); dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture medium RPMI-1640 and the other chemicals and solvents were of the highest analytical grade. The antibodies against poly (ADP-ribose) polymerase (PARP), Bcl-2 and Bax were purchased from Cell Signaling Technology (Beverly, Mass, USA). Goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, CA, USA) were used as secondary antibody. All the other chemicals and solvents were of the highest analytical grade.

2.2. Cell culture

The human non-Hodgkin lymphoma Raji cells, acute myelocytic leukemia HL-60 cells, and human histiocytic lymphoma U937 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells of Raji, HL-60 and U937 were all cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. The cells in all of the experiments were gathered when they were in the exponential growth phase.

2.3. Cell proliferation assay

The inhibition effects of MnSODm on cells proliferation were measured by MTT assay. The cells were incubated with different concentrations of MnSODm (0.5, 1, 2.5, 5 and 10 μg/mL) in a 96 well-plates (1 x 10⁴/well) for 24, 48, and 72 h. After incubation, MTT was dissolved in PBS at 5 mg/mL and added to culture media at a final concentration of 0.5 mg/mL. After incubation at 37 °C for 4 h, the media were removed, 100 μL DMSO was added to each well to dissolve purple crystals of formazan. The plate was shaken for 10 min to allow complete solubilization. The absorbance was read at 570 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, USA). Based on these results, the IC 50 values (concentration of the compound that caused 50% inhibition of cancer cell growth) of the compound were tested. Moreover, the morphological changes of Raji cells were examined in cell smears using the light microscopy of cytospin preparation stained with May–Gruenwald–Giemsa solution (Merck, Darmstadt, Germany) [14].

2.4. Electron microscopy

For electron microscopy [15], the cells were treated with MnSODm (2.5 and 10 mg/L) at 37 °C for 24 h and then were collected. The pellet was fixed in 5% glutaraldehyde for 30 min and then placed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate (pH 7.4) for 1 h. The cells were desiccated in graded series of acetone and embedded with EPON-812. Ultra-thin sections were prepared and observed under a Hitachi transmission electron microscope with 8000 magnifications after double staining with uranium and plumbum. At the same time, the untreated cells were used as the control.

2.5. Assay for cell apoptosis

Raji cells were seeded in 12-well plates and cultured with physiological saline or various concentrations of MnSODm (0.5, 1, 2.5, 5 and 10 mg/L).
10 mg/L) for 48 h. Then, the cells (1 × 10^5 cells) were collected, washed, and resuspended in PBS. Apoptotic cell death was identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated Annexin V and PI, using the Annexin V-FITC apoptosis detection kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Flow cytometric analysis was performed immediately after supravital staining. The fraction of cell population in different quadrants was analyzed using quadrant statistics. Cells in the lower right quadrant represented early apoptosis and in the upper right quadrant represented late apoptotic cells.

2.6. Analysis of the production level of ROS

Cells from exponentially growing cultures were seeded in 12-well culture plates and treated with MnSODm at 1, 5 and 10 mg/L for 24 h. The cells were harvested, washed, and resuspended in PBS. The resuspended cells were stained at 37 °C with a 10 μM fluorescent dye DCFH-DA solution, rewashed 3 times by PBS after 1 h and then subjected to flow cytometric analyses.

2.7. Western blotting analysis

Cells were incubated for 48 h in the presence of indicated concentrations of MnSODm. After the incubation, the cells were harvested, and washed with PBS solution. Mitochondrial protein and cytosolic protein were isolated using Mitochondrial Fractionation Kit (Active Motif, Carlsbad, Calif, USA) according to the manufacturer’s instructions [16]. Protein concentrations were quantified by the method of BCA (the kit of BCA protein assay was obtained from Applygen Technologies Inc., Beijing, China). Samples were diluted to a concentration of 2 mg/ml in SDS-loading buffer and boiled for 5 min. For Western blotting analysis, 30 μg of protein from each sample was separated by 10–12% SDS-PAGE and transferred to nitrocellulose membranes. Gels were also loaded with colored molecular weight markers to assess electrophoretic transfer and biotinylated protein ladder marker to estimate the molecular weights of bands of interest. The membranes were blocked with 0.5% BSA in TBST (pH 8.0) for 1.5 h and then incubated overnight at 4 °C with suitably diluted primary antibodies against PARP, cleaved caspase-3, cleaved caspase-9, and cytochrome c, COX-IV, Bcl-2, or Bax. The expression of β-actin was used to show equal protein loading. After extensive washing with TBST (3 × 10 min), the membranes were incubated in horseradish peroxidase (HRP)-linked anti-biotin and the appropriate secondary antibody in TBST with 0.5% BSA for 1 h at room temperature then rewashed (TBST, 3 × 10 min). The blots were detected using the enhanced chemiluminescence (ECL) reaction. Quantification of protein bands was achieved by densitometric analysis using Image-Pro Plus® software (Media Cybernetics, Inc., USA). All Western blot analyses were carried out at least three times.

Fig. 2. Effects of MnSODm on morphology of Raji cells demonstrated by light microscopy. Morphological changes of Raji cells treated with MnSODm. Cells were treated without (A) or with (B) 0.5 mg/L, (C) 1 mg/L, (D) 2.5 mg/L, (E) 5 mg/L and (F) 10 mg/L MnSODm for 48 h. Cell smears were stained with May–Grunwald–Giemsa solution. The results are representative of 3 independent experiments.

Fig. 3. Effects of MnSODm on ultrastructure of Raji cells demonstrated by transmission electron microscope of ultra-thin section (24 h×8000). (A) Cells from control sample with intact cell wall; (B and C) cells were incubated with MnSODm (2.5 and 10 mg/L) at 37 °C for 24 h. All images are shown at the same magnification.
2.8. Statistical analysis

Results were expressed as mean±SD of three independent experiments and each experiment included triplicate sets. Data were statistically evaluated by one-way ANOVA followed by Dunnett’s test between control group and multiple dose groups, with the level of significant chosen as \( P < 0.05 \).

3. Results

3.1. MnSODm concentration-dependently and time-dependently inhibited proliferation of Raji cells

To examine the effects of MnSODm on different kinds of tumor cells, exponentially growing cells were treated with various concentrations of MnSODm (0.5, 1, 2.5, 5 and 10 mg/L) for 48 h. As shown in Fig. 1A, MnSODm significantly reduced the proliferation of U937, Raji and HL-60 cells in a dose-dependent manner. The results also demonstrated that the Raji cells were most sensitive to MnSODm, treatment with MnSODm (0.5, 1, 2.5, 5 and 10 mg/L) resulted in a dose-dependent manner and time-dependent inhibition of Raji cells proliferation. This effect was more pronounced at 24, 48, and 72 h post-treatment compared with that pre-treatment (Fig. 1B). The IC\(_{50}\) values of MnSODm for the Raji cells at 24, 48, and 72 h were 4.88, 2.96, and 1.97 mg/L, respectively.

3.2. MnSODm induced apoptosis in Raji cells

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis [17]. Therefore, induction of apoptotic cell death is a promising emerging strategy for prevention and treatment of cancer. Treatment of MnSODm for 48 h induced morphological changes typical of apoptosis, such as nuclear shrinkage, chromatin condensation, and nuclear fragmentation in Raji cells (Fig. 2A–F), and there were no change in control group cells. Morphological ultrastructural appearance was also evaluated by transmission electron microscopy (TEM). The Raji cell images under TEM after incubation with MnSODm for 24 h are shown in Fig. 3. Apoptotic cells observed in response to MnSODm were characterized by nuclear fragmentation and strong...
condensation of chromatin (Fig. 3A–C). Annexin-V positive cells were considered as early and late apoptosis populations. As shown in Fig. 4A (a–f) and B, treatment of MnSODm induced apoptosis of Raji cells in a dose-dependent manner.

3.3. Effect of MnSODm on reactive oxygen species (ROS)

Most apoptosis-inducing agents release reactive oxygen species, which are considered to be key mediators of apoptosis signaling [18]. Since increased ROS production in leukemic cells may lead to the activation of mitochondrial pathways and cell death, we decided to investigate whether ROS are involved in MnSODm-induced apoptosis. As shown in Fig. 5, cells exposed to increasing concentrations of MnSODm (1, 5 and 10 mg/L) exhibited a significant enhanced accumulation of intracellular ROS. When compared to untreated cells, a 40.9–72.5% increase of the fluorescence emitted by the ROS-induced oxidation of DCFH-DA was detected in cells treated with 1–10 mg/L of MnSODm after 24 h (Fig. 5A–D).

3.4. MnSODm induced cytochrome c release from mitochondria to cytosol

To confirm if MnSODm induces apoptosis through mitochondrial pathway, we incubated Raji cells with MnSODm for 48 h. Then, we collected cells and isolated mitochondria protein. 30 μg of each protein was subjected to Western blotting. Equal protein loading was confirmed by immunodetection of COX-IV for mitochondria protein. As shown in Fig. 6, MnSODm greatly increased the cytosol cytochrome c and decreased the mitochondria cytochrome c as compared with the control group.

3.5. MnSODm activated caspase-3 and its related proteins to promote apoptosis

The PARP protein, a substrate of caspase-3, is an early hallmark of apoptosis. To further address the apoptotic effect of MnSODm on Raji cells, we analyzed PARP cleavage by Western blotting using a PARP antibody that recognizes native and fragment PARP. Incubation of Raji cells with MnSODm (0.5–10 mg/L for 48 h) induced a cleavage pattern of PARP characterized by the appearance of an 85 kD fragment. As shown in Fig. 7, the amounts of the cleaved PARP were enhanced with the doses of MnSODm increased. The amounts of cleaved caspase-3 and caspase-9 were also increased significantly in the cells treated with MnSODm in a dose-dependent manner.

3.6. Expression of Bcl-2 and Bax

To investigate the molecular events involved in MnSODm-induced apoptosis, the expressions of the Bcl-2 and Bax were also assessed by Western blot. The results showed that expression of anti-apoptotic Bcl-2 was dose-dependently decreased in MnSODm groups, whereas the expression of pro-apoptotic Bax protein was increased (Fig. 8).

4. Discussion

Cancer is a highly prevalent disease that poses an increasing public health threat worldwide. However, the low selectivity and high
toxicity of most currently used anti-cancer drugs compromise the beneficial treatment effects of these agents [19,20]. Therefore, it is of great significance to develop new chemotherapeutic agents with relatively high selectivity and low toxicity. A mimic of manganese superoxide dismutase (MnSODm) is synthesized by chemical method, which has been shown that obviously inhibited the proliferation of K562 and K562/ADM cells and induced apoptosis [13,21]. However, antiproliferative studies of the MnSODm in human non-Hodgkin lymphoma Raji cells have not yet been assessed. In the present study, we examined the effect of MnSODm on some kinds of cancer cell lines. Though the inhibitions of proliferation rate by MnSODm were observed to different degrees against different kinds of cancer cells, a concentration-dependent action was shown in all three kinds of cell lines used. This finding suggests that MnSODm may have a relatively wide spectrum of antitumor growth effects. In Raji cells, the mimic also showed a time-dependent inhibition against the cell proliferation. As the human non-Hodgkin lymphoma Raji cells showed the most significant inhibitory rate, we chose it to do the following studies.

The normal cell function and tissue homeostasis are maintained by a balance between proliferation and apoptosis. Cancer is a typical disorder in which clones of malignant cells escape such balance and proliferate inappropriately without compensatory apoptosis [22]. Generally, the growth rate of preneoplastic or malignant cells outpaces that of normal cells because of malfunctioning or deregulation of their cell growth and cell death machineries [23]. The success of cancer therapies, therefore, greatly relies on the extent to which they preferentially induce tumor cell death while allowing survival of normal tissue. Blockade of proliferation or induction of apoptosis has been recognized as a rational approach to eliminate genetically damaged or preneoplastic cells before any malignancy manifests [24–27]. Our results showed that MnSODm inhibit against the cell proliferation of Raji cells in a time and does-dependent manner.

Apoptosis is an active process of cell death that takes place under a variety of conditions and is important to induce tumor destruction. It is characterized by distinct morphologic changes and regulated by a series of biochemical events that lead to cell death. As apoptosis is often caused by chemotherapeutic drugs [28], we used annexin V-PI staining to study the apoptosis rate of cells treated with MnSODm. The result indicated that MnSODm could dose-dependently induce the apoptosis of Raji cells.

Chemotherapeutic agents have been described to induce apoptosis in two major routes: the extrinsic or death receptor-associated route and the intrinsic or mitochondrial route [29]. In the intrinsic route, the execution phase is initiated by release of cytochrome c and other polypeptides from the mitochondrial intermembrane space [30]. This release is accompanied by a dissipation of mitochondrial inner transmembrane potential [31]. To further access the anti-tumor mechanism of MnSODm, we observed that MnSODm treatment greatly induced the cytochrome c to release from mitochondria to cytosol in the cells, indicating involvement of the intrinsic apoptosis route via mitochondria.

Reactive oxygen species (ROS) have been shown to induce various biological processes, including apoptosis [32]. This implies that the redox state of a cell is a crucial factor in deciding its susceptibility to apoptotic stimuli. ROS at low concentration plays the role of an
intracellular messenger in many molecular events, including cell proliferation and apoptosis, while the production of large amounts of ROS contributes to apoptosis [33]. In the present study, a dramatic ROS burst was observed in various concentrations of MnSODm-treated cells, suggesting that ROS generation is involved in the MnSODm-induced apoptosis in Raji cells. Generated ROS can directly activate the mitochondrial permeability transition.

Apoptosis is a tightly controlled process that regulates tissue homeostasis, normal development, and the removal of damaged cells. The Bcl-2 family can be broadly divided into those members that inhibit (Bcl-2) or promote cell death (Bax). Bcl-2 family members primarily function at the mitochondria where they control the permeability of the outer mitochondrial membrane and result in cytochrome c release and caspase activation. Many cancers have demonstrated defects in apoptotic pathways. Caspases play an important role in the execution phase of apoptosis. “Initiator” caspases, which have long prodomains, such as caspase-8 and caspase-9, either directly or indirectly activate “effector” caspases, such as caspase-3 and caspase-7. Once released into the cytoplasm, cytochrome c usually interacts with Apaf-1 and procaspase-9 to form a complex known as the apoptosome [34]. In the apoptosome, caspase-9 is activated which in turn activates effector caspases, like caspase-3 and -7 [35–37]. The effector caspases may cleave a number of structural and regulatory cellular proteins including PARP and lamin protein and are responsible for the typical morphologic and biochemical features of an apoptotic cell [38–40]. Our results showed that the effects of MnSODm on these events occurred in the apoptosis, where MnSODm markedly caused increases in the cleaved form of PARP, caspase-3, and caspase-9. We also have found an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 in Raji cells. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome and the cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and PARP [41]. This result further confirmed that MnSODm promotes apoptosis through caspase-3 via mitochondria.

The present study showed that MnSODm obviously inhibited the proliferation of Raji cells and induced apoptosis. It showed that MnSODm could down-regulate the expression of Bcl-2 while up-regulate the expression of Bax, change the morphological structures and stability of mitochondria, increase the release of Cyt c from mitochondria to cytoplasm, enhance the production level of ROS and activity of the caspase-3, thus, MnSODm induced apoptosis of Raji cells through the mitochondrial pathway. Similar results had been reported by Fan et al. [13] and An et al. [21]. Overall, our study has proved an antitumor effect of MnSODm. Its mechanism underlying the effect mainly involves the induction of apoptosis through activating caspase-3 via mitochondrial pathway. To our knowledge, this is the first study to examine the functions of MnSODm in human non-Hodgkin lymphoma Raji cells. The findings obtained here may be helpful for understanding the properties of MnSODm as a candidate of antitumor drugs.

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