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鳥胺酸去羧化酶抗細胞凋亡之研究

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行政院國家科學委員會補助專題研究計畫成果報告

（計畫名稱）

鳥胺酸去羧化酶抗細胞凋亡之研究

The Role of anti-Apoptotic Mechanism on Ornithine Decarboxylase

計畫類別：■個別型計畫

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Part 1.

Abstract

Ornithine decarboxylase (ODC) plays an essential role in various biological functions, including cell proliferation, differentiation and cell death. However, how it prevents the cell apoptotic mechanism is still unclear. Previous studies have demonstrated that decreasing the activity of ODC by difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, causes the accumulation of intracellular reactive oxygen species (ROS) and cell arrest, thus inducing cell death. These findings might indicate how ODC exerts anti-oxidative and anti-apoptotic effects. In our study, tumor necrosis factor alpha (TNF-α) induced apoptosis in HL-60 and Jurkat T cells. The kinetic studies revealed that the TNF-α-induced apoptotic process included intracellular ROS generation (as early as 1 h after treatment), the activation of caspase 8 (3 h), the cleavage of Bid (3 h) and the disruption of mitochondrial membrane potential ($\Delta\psi_m$) (6 h). Furthermore, ROS scavengers, such as glutathione (GSH) and catalase, maintained $\Delta\psi_m$ and prevented apoptosis upon treatment. Putrescine and overexpression of ODC had similar effects as ROS scavengers in decreasing intracellular ROS and preventing the disruption of $\Delta\psi_m$ and apoptosis. Inhibition of ODC by DFMO in HL-60 cells only could increase ROS generation, but did not disrupt $\Delta\psi_m$ or induce apoptosis. However, DFMO enhanced the accumulation of ROS, disruption of $\Delta\psi_m$ and apoptosis when cells were treated with TNF-α. ODC overexpression avoided the decline of Bcl-2, prevented cytochrome c release from mitochondria and inhibited the activation of caspase 8, 9 and 3. Overexpression of Bcl-2 maintained $\Delta\psi_m$ and prevented apoptosis, but could not reduce ROS until four hours after TNF-α treatment. According to these data, we suggest that TNF-α induces apoptosis mainly by a ROS-dependent, mitochondria-mediated pathway. Furthermore, ODC prevents TNF-α-induced apoptosis by decreasing intracellular ROS to avoid Bcl-2 decline, maintain $\Delta\psi_m$, prevent cytochrome c release and deactivate the caspase cascade pathway.

Keywords: TNF-α; apoptosis; ODC; ROS; Bcl-2.

Abbreviations: ODC, Ornithine decarboxylase; TNF-α, tumor necrosis factor alpha; DFMO, difluoromethylornithine; ROS, reactive oxygen species; $\Delta\psi_m$, mitochondrial membrane potential; DCFH-DA, 2', 7'-dichlorofluorescin diacetate; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone.

Results

TNF-α induces increase of intracellular ROS, caspase-dependent apoptosis and decrease of $\Delta\psi_m$

Generation of ROS and disruption of $\Delta\psi_m$ were both found to be obligatory in TNF-α-mediated apoptotic signaling by previous studies. In our experiments, after HL-60 and Jurkat T cells were treated with TNF-α at different concentrations and a variety of times, we found dose-dependent (Fig. 1A) and time-dependent (data not shown) manner of decreasing cell viability. To characterize the pattern of TNF-α-induced cell death, we analyzed morphologic changes and DNA fragmentation upon treatment. The morphologic changes, observed by light microscope after 20 ng/ml TNF-α treatment for 24 h, were chromatin condensation, membrane blebbing and shrinkage, as well as apoptotic body formation. In the experiments with DNA gel electrophoresis, we found dose-dependent and time-dependent manner of DNA fragmentation after TNF-α treatment (Fig.1B). All these morphologic and molecular changes are typical features of apoptosis.[33] Additionally, a general caspase inhibitor, z-VAD-fmk, dramatically increased cell viability up to 90% for 24 h after TNF-α (20ng/ml) treatment (data not shown), prevent the formation of apoptotic bodies and DNA fragmentation.

To clarify the early events of TNF-α-induced apoptosis, we performed kinetic studies to evaluate intracellular ROS, $\Delta\psi_m$, the activation of caspase 8 and the cleavage of Bid (substrate of caspase 8). HL-60 cells were treated with different concentrations (0, 20 and 40 ng/ml) of TNF-α or the fixed concentration for 1, 3, 6, 12 and 24h. Intracellular ROS and $\Delta\psi_m$ were quantified by flow cytometry with measuring DCF and rhodamine 123. The activation of caspase 8 and tBid were analyzed by fluorometric analysis and immunoblotting, respectively. The results of flow cytometric analysis showed dose-dependent increase of intracellular ROS generation and decrease of $\Delta\psi_m$ in HL-60 (Fig. 1C) and Jurkat T cells (data not shown) after TNF-α treatment. The kinetic studies revealed the significant increase of intracellular ROS (as early as 1 h), the activation of caspase 8 (3 h), the cleavage of Bid (3 h), and the disruption of $\Delta\psi_m$ (6 h) (Fig. 1D). All these findings indicate that TNF-α-induced apoptosis as well as the changes of intracellular ROS and $\Delta\psi_m$.

Putrescine maintains $\Delta\psi_m$ and prevents TNF-α-induced apoptosis by decreasing intracellular ROS, functioning as ROS scavengers

Although ROS was assumed to affect $\Delta\psi_m$ on the basis of findings that the stimulation of ROS damaged mitochondria and that antioxidants maintained mitochondrial permeability, the mechanism by which these cellular events are integrated in TNF-α signaling is presently unclear. To study the mechanism of TNF-α-induced apoptosis and the role of putrescine, we pretreated putrescine (0, 0.1, 0.5 and 1 mM) and ROS scavengers, such as GSH (10 mM) and catalase (100 U) for 3 h before 20 ng/ml TNF-α treatment. Compared with cells treated by TNF-α only, we found putrescine, GSH and catalase significantly decreased the ratio of apoptosis upon the treatment and these ROS scavengers could rescue about 70% of treated cells. In DNA fragmentation assay, not only GSH and putrescine prevented DNA fragmentation, but also putrescine displayed a dose-dependent manner (data not shown). Furthermore, in flow cytometric assay, when compared with TNF-α only, there were 40.2% and 36% decreases of cell death after GSH and putrescine treatments, respectively (Fig. 2).

Intracellular ROS and $\Delta\psi_m$ were determined by measuring DCF and rhodamine 123 with flow

cytometry, respectively. Putrescine and ROS scavengers, such as GSH and catalase decreased intracellular ROS (Fig. 3A) and maintained $\Delta\psi_m$ (Fig. 3B) after TNF-α treatment. Taken together, TNF-α-induced apoptosis and $\Delta\psi_m$ disruption occurred mainly through increasing intracellular ROS. Putrescine could reduce intracellular ROS and maintain $\Delta\psi_m$ (Fig. 3A, B) and prevent cell apoptosis (Fig. 2), functioning as ROS scavengers.

Overexpression of ODC decreases intracellular ROS, maintains $\Delta\psi_m$ and prevents cytochrome c release from mitochondria and apoptosis after TNF-α treatment

Since putrescine is a product of ODC, we further examined the role of ODC during TNF-α-induced apoptosis. Therefore, we constructed ODC cDNA into a mammalian expression plasmid, pCMV-Tag and generated cell lines harboring an overexpressed *odc* gene in HL-60 and Jurkat T cells. The overexpression of ODC (WT-ODC) cells and the frame-shift mutant of ODC (m-ODC) cells were detected by the intracellular transcriptional and translational levels. The quantities of mRNA expressions of WT-ODC and m-ODC cells were similar, but higher than the expression of parental HL-60 cells. However there were about two folds greater protein expression in WT-ODC cells than in m-ODC cells (Fig. 4A). According to the original design of this experiment, the transcripts of m-ODC cells could not translate ODC protein in cell system, because only one nucleotide mutation of this cDNA forms a stop codon by frame-shift.[34] After stimulating WT-ODC, m-ODC and DN-ODC cells by 10% FBS, WT-ODC cells expressed about two folds higher ODC enzyme activity than HL-60 and m-ODC cells (Fig. 4B). In WT-ODC cells, ODC enzyme activity was dramatically inhibited by DFMO. The level of ODC enzyme activity in DN-ODC cells was lower than in WT-ODC cells, but higher than in m-ODC cells. In addition, intracellular ROS was decreased in WT-ODC cells after 10% FBS stimulation from 0.5 to 4 h with untreated TNF-α (Fig. 4C). After TNF-α treatment, WT-ODC cells were more resistant to TNF-α-induced apoptosis than HL-60, m-ODC and DN-ODC cells in the experiments of phase light microscopy, DNA fragmentation assay (data not shown), cell viability and flow cytometry (Fig. 5A and B). The anti-apoptotic effects of ODC in WT-ODC cells were reduced about 26.9% by DFMO treatment (Fig. 5B). Overexpression of ODC in Jurkat T cells had the similar results in HL-60 cell system. In the flow cytometric assay of Jurkat T cell system, WT-ODC cells showed decreased cell death by about 38% and 35.6% when compared with m-ODC cells and parental Jurkat T cells after TNF-α treatment, respectively (Fig. 5C). These results showed that ODC inhibited apoptosis when it was overexpressed in human promyelocytic leukemia HL-60 and Jurkat T cells. Furthermore, intracellular ROS accumulated in m-ODC cells higher than in WT-ODC cells by about 2.8 and 2.5 folds after TNF-α treatment in HL-60 (Fig. 6A) and Jurkat T cells (data not shown), respectively. Not only did WT-ODC cells significantly decrease the loss of $\Delta\psi_m$ (Fig. 6B), but also WT-ODC cells notably prevented cytochrome c release from mitochondria to cytosol (Fig. 6C). Our results indicate that overexpression of ODC enforces the ability of antioxidant system to maintain $\Delta\psi_m$, prevent cytochrome c release and apoptosis.

Overexpression of Bcl-2 maintains $\Delta\psi_m$ and prevents apoptosis after TNF-α treatment, but cannot reduce intracellular ROS during the initial onset

Our experimental data have explained that ODC and putrescine maintain $\Delta\psi_m$ and prevent apoptosis by decreasing the accumulation of intracellular ROS. In addition, cytochrome c released after TNF-α treatment in parental HL-60 and m-ODC cells, but not in WT-ODC cells. It is known that Bcl-2 can prevent cytochrome c release, maintain $\Delta\psi_m$, prevent apoptosis and decrease ROS.[35] To resolve this contradiction, we transiently transfected *bcl-2*, *odc* gene or control plasmid into parental HL-60 cells and these cells were treated with DFMO, TNF-α or both. DFMO alone could not cause disruption of $\Delta\psi_m$ and apoptosis in HL-60 cells, but it enhanced these effects after TNF-α treatment. Furthermore, cells that overexpressed Bcl-2 decreased the $\Delta\psi_m$ collapse and the percentage of apoptosis after treatment with TNF-α only, or both DFMO plus TNF-α (Fig. 7A). Cells treated with DFMO increased intracellular ROS both with and without TNF-α treatment, although there were no significant differences between Bcl-2 cells and parental HL-60 cells. In the time-course experiments of measuring DCF in Bcl-2, HL-60 and WT-ODC cells by flow cytometry, WT-ODC cells reduced the accumulation of intracellular ROS as compared with HL-60 cells, but Bcl-2 cells could not do so until 4 h later (Fig. 7B). This indicated that ODC decreased intracellular ROS, maintained $\Delta\psi_m$ and protected apoptotic cell death from early onset, however Bcl-2 did not exhibit the antioxidant effect until a later period after TNF-α treatment.

Overexpression of ODC reduces the decline of Bcl-2 and caspase activation after TNF-α treatment

According to the above results, TNF-α-induced apoptosis seems to occur mainly through a mitochondria-dependent pathway and the anti-apoptotic effect of ODC is also mediated by this process. To clarify the ODC-protected TNF-α-induced apoptotic pathway, we used 20 ng/ml TNF-α for 24 h to treat parental HL-60, m-ODC and WT-ODC cells for immunoblotting with anti-Bcl-2 antibody. In addition, caspase 8, 9, 3, Bid, and PARP (the substrate of caspase 3) were observed by immunoblotting at different times, respectively. In WT-ODC cells, overexpression of ODC could increase the expression of Bcl-2 and reduce the decline of it after TNF-α treatment for 24 h (Fig. 8A). The cleavage of caspase 8, Bid, 9, 3 and PARP after TNF-α treatment was inhibited when WT-ODC cells had a higher ODC enzyme activity in the initial 3~6 h after serum induction (Fig. 8B). Based on these results, the anti-apoptotic mechanism of ODC seems to act through maintaining $\Delta\psi_m$ by Bcl-2 to prevent

cytochrome c release from mitochondria. In addition, ODC overexpression in parental cells attenuates the caspase activation of apoptosome and closely coincides with the inhibition of apoptosis, as shown in our previous results.

Discussion

TNF-α causes cell death through necrosis or apoptosis, depending on the cell types.[36] In HL-60 and Jurkat T cells, we have analyzed cytological alterations, DNA fragmentation and apoptotic bodies (the group of sub-G1) upon TNF-α treatment. There were typical morphologic and molecular changes, consistent with apoptosis, including induced cell death. Recently, there have been advanced studies to explore the mechanism of TNF-α-induced apoptosis. In this apoptotic mechanism, the binding of TNF-α to TNFR1 first causes rapid recruitment of TRADD, RIP1 and TRAF2. The next step is that TRADD and RIP1 associate with FADD to form death-inducing signaling complex (DISC). DISC activates caspase 8 to execute the following death signals.[37] In the module of TNF-α-induced apoptosis in HeLa cells, there were at least two apoptotic signaling cascades, one was an early mitochondria-dependent ROS production and the other was ROS-independent.[23] These two apoptotic signaling cascades were blocked by z-VAD, a general caspase inhibitor. Caspase 8, a z-VAD sensitive caspase, is thought to play a role in the primary phase of the TNF-α-induced apoptosis.[38] In addition, there is a "non-classical" caspase-independent pathway of TNF-α-induced apoptosis.[39, 40] In our study, increasing accumulation of intracellular ROS and disruption of $\Delta\psi_m$ in TNF-α-induced apoptosis were found. TNF-α-induced apoptosis was blocked by z-VAD-fmk. Therefore, TNF-α-induced apoptosis in HL-60 and Jurkat cell lines is mainly through caspase-dependent pathway. Moreover, ROS scavengers, such as GSH and catalase reduced intracellular ROS, as well as preventing the loss of $\Delta\psi_m$ and apoptosis. The ROS scavengers rescued more than 70% cells from apoptosis after TNF-α treatment. These results suggest that TNF-α-mediated apoptosis is mainly through a ROS-dependent pathway and that ROS can trigger $\Delta\psi_m$ collapse. Many previous reports have suggested that overexpression of Bcl-2 could maintain $\Delta\psi_m$ and prevent apoptosis, as well as showing that TNF-α triggered the mitochondria-mediated apoptotic pathway. However, it has been shown that Bcl-2 overexpression can prevent apoptosis mediated by decreasing ROS,[35] although in our study, Bcl-2 overexpression, as shown in Fig. 7B, could not reduce ROS in the initial four hours after TNF-α treatment. In the study of TNF-α-induced apoptosis of HeLa cells,[23] the generation of ROS was separated into two stages. One was the induction phase, which is mitochondria-dependent and is reduced by ROS scavengers; and the destruction phase, which is mitochondria-independent and cannot be reduced by ROS scavengers. We also found there are two different stages of ROS during the course of TNF-α-induced apoptosis. In the first onset of the initial four hours after TNF-α stimulation, ROS is reduced by ODC overexpression, but not by Bcl-2 overexpression in HL-60 cells. After to four hours later, ROS could be reduced by either ODC or Bcl-2 overexpression after TNF-α stimulation in the second period. ROS generated at the first onset may disrupt the $\Delta\psi_m$ and induce more ROS release after cytochrome c escapes from the mitochondria. Subsequently, at the second stage, the serious insults of ROS generation join to damage cells through mitochondria-mediated death signaling again. ODC could reduce the generation of ROS during both stages on the course of TNF-α-induced apoptosis, however Bcl-2 did not prevent the initial generation of ROS, which could cause a transient burst of mitochondrial ROS production. Of course, in mitochondria the major function of the electron transport chain is the production of ATP and ATP levels rapidly falling in apoptotic cells following caspase activation.[41] Therefore, the major function of Bcl-2 might be maintaining the $\Delta\psi_m$ and the generation of ATP, as well as preventing ROS generation from electric transport chain in mitochondria, not in cytosol after different cell insults.[35, 42]

In the kinetic studies, we found the ROS generation is earlier than the activation of caspase 8 and the loss of $\Delta\psi_m$. ROS, generated as early as 1 h after TNF-α treatment, is not the consequence of caspase 8 activation or the loss of $\Delta\psi_m$. However, we did not investigate whether increased ROS could activate caspase 8 or not. But it has been showed that phagocytosis-induced ROS promote cleavage and activation of caspase 8 in neutrophils.[43]

Putrescine and overexpressed ODC could be functioning as ROS scavengers that reduce intracellular ROS, and then maintain $\Delta\psi_m$ and prevent apoptosis after TNF-α treatment. Although DFMO induced cell apoptosis in previous studies,[13, 44] the different cell types and the diverse concentrations of DFMO determine the effects of apoptotic induction. Our findings have demonstrated that the inhibition of ODC by DFMO increased intracellular ROS in HL-60 cells, but could not disrupt $\Delta\psi_m$ and cause apoptosis with untreated TNF-α. On the contrary DFMO further enhanced ROS accumulation, disruption of $\Delta\psi_m$ and apoptosis after TNF-α treatment. ODC presents an important signaling role during TNF-α-induced apoptosis as well since it seems to play a function similar to an oncogenic action to prevent cell death. Through the mitochondria-mediated apoptotic pathway, ODC prevented cytochrome c release from mitochondria and inhibited the activation of downstream caspases, such as caspase 9 and 3, and the cleavage of the substrate of caspase 3, PARP. Additionally, the activation of caspase 8, an upstream caspase in TNF-α-induced apoptotic signaling cascades, was inhibited in WT-ODC cells. In previous studies, it has been reported that TNF-α regulated the activation of ODC.[45, 46] Expression of FLICE inhibitory protein (FLIP), an inhibitor of apoptosis induced by cytokines of TNF family, was regulated by ROS in cardiac myocytes.[47] Alternatively, TNF-induced nonapoptotic cell death was also observed due to ROS accumulation.[48] This evidence indicates that, ROS

accumulation plays a critical role, and that ODC exerts a protective function during TNF-mediated cell death.

WT-ODC cells exhibit smaller amounts of intracellular ROS than m-ODC cells after 10% FBS stimulation and they express more Bcl-2 protein than parental HL-60 or m-ODC cells upon untreated TNF-α. Furthermore, they have the ability to maintain the expression of Bcl-2 compared with parental HL-60 and m-ODC cells after TNF-α treatment. However, ODC affects the expression of Bcl-2 is currently uncertain. In a previous report, the antioxidant Mn(III) tetrakis (5, 10, 20-benzoic acid) porphyrin (MnTBAP) and overexpression of catalase increased the expression of Bcl-2.[49] In a culture of active T cells, MnTBAP reversed the decline in Bcl-2. It is thus possible that overexpression of ODC, maintaining the expression of Bcl-2, is mediated by ROS.

Furthermore, how can putrescine and ODC reduce ROS *in vivo*? Considering this in terms of the physiological function of ODC, there are several possible explanations suggested by this study. First, overexpression of ODC or treatment with putrescine in HL-60 cells increases the concentration of polyamines. A polyamine such as spermine can function directly as a free radical scavenger.[50] Second, inhibition of ODC by DFMO could increase intracellular ROS and lead to an imbalance in polyamine pools. Polyamine catabolism by polyamine oxidase (PAO) could continue and result in the production of ROS.[51] Thus, overexpression of ODC may have reduced the ROS generation during polyamine catabolism. Third, polyamines modulate ligand-receptor interactions, such as N-methyl-D-aspartate receptors[52] and estradiol binding to estrogen receptors.[53] Although the inactivation of caspase 8 in WT-ODC cells was found in this study, further investigation is needed to determine whether polyamines and ODC could directly affect more upstream components, such as TNFR1 or DISC in the TNFR1-dependent apoptotic pathway.

Conclusion

TNF-α induces the apoptosis of HL-60 cells and Jurkat T cells mainly by a ROS-dependent, mitochondria-mediated pathway. Overexpression of ODC can maintain $\Delta\psi_m$ and prevent apoptosis after TNF-α treatment through decreasing intracellular ROS. In addition, ODC overexpression also could decrease ROS generation, avoid Bcl-2 decline, prevent cytochrome c release from the mitochondria, and inactivate caspase 8, 9 and 3. These findings indicate a novel anti-apoptotic mechanism of ODC during TNF-α-induced cancer cell death. Simultaneously, ODC regulates cell survival not only to provoke cell proliferation, as shown by previous studies, but also to defend apoptotic cell death, as shown by our present results, and it provides a potential illumination why tumor cell still live on the TNF-α existence.

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Part 1. has be accepted in Apoptosis: **G.-Y. Liu***, Y.-C. Hung, P.-C. Hsu, Y.-F. Liao, W.-H. Chang, G.J. Tsay and H.-C. Hung. Ornithine decarboxylase prevents tumor necrosis factor alpha-induced apoptosis by decreasing intracellular reactive oxygen species. ***Apoptosis*** 10(3):569-581, 2005. (SCI: 4.54; Biochemistry and Molecular Biology 19.5%) (First and corresponding author)

Part 2.

Methotrexate (MTX) is developed for the therapy of malignancies, rheumatoid arthritis (RA) and other chronic inflammatory disorders. One of its mechanisms of anti-inflammatory and immunosuppressive effects may be the production of reactive oxygen species (ROS). Ornithine decarboxylase (ODC) played an important role in diverse biological functions, including cell development, differentiation, transformation, growth and apoptosis. In our previous studies, ODC overexpression could prevent TNFα-induced apoptosis via reducing ROS. There, we additionally investigated the mechanism of MTX-induced apoptosis and anti-apoptotic effects of ODC to MTX. We found MTX could induce caspase-dependent apoptosis, ROS generation and disruption of mitochondrial membrane potential ($\Delta\psi_m$) in HL-60 and Jurkat T cells. Putrescine and ROS scavengers could reduce MTX-induced apoptosis, loss of $\Delta\psi_m$ by reducing intracellular ROS. Overexpression of ODC in parental cells had the same effects of putrescine and ROS scavengers. Moreover, ODC overexpression prevented the decline of Bcl-2 which maintained $\Delta\psi_m$, the cytochrome c release and activations of caspase 9 and 3 after MTX treatment. The results demonstrate that MTX-induced apoptosis is through ROS-dependent and mitochondria-mediated pathway. ODC overexpression prevents MTX-induced apoptosis by reducing intracellular ROS.

Keywords: Apoptosis; methotrexate; ODC; ROS; caspase.

Abbreviations: MTX, methotrexate; ODC, ornithine decarboxylase; ROS, reactive oxygen species; $\Delta\psi_m$, mitochondrial membrane potential; DFMO, difluoromethylornithine; DCFH-DA, 2', 7'-dichlorofluorescin diacetate; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

Results

MTX induced caspase-dependent apoptosis, ROS generation and loss of mitochondrial membrane potential ($\Delta\psi_m$).

We first investigated the cell death induced by MTX. HL-60 and Jurkat T cells were treated with 0, 0.1, 0.5, 1 and 10 μM of MTX with or without zVAD-fmk, a total caspase inhibitor, for 24 h. Cells were observed by fluorescence-microscope and counted for the percentage of apoptosis. We found MTX could induce apoptosis in a dose-dependent manner (Fig. 1A). The morphologic changes were chromatin condensation, membrane blebbing and shrinkage, and apoptotic body formation. Then, cells were harvested for DNA gel electrophoresis and flow cytometry with propidium iodide (PI) staining. Again, there were time course as indicated and dose-dependent increasing in DNA fragmentation after MTX treatment, and zVAD-fmk (500 nM, pretreated one hour before MTX) could block this phenomenon (Fig. 1B). In the experiment of flow cytometry with PI staining, sub-G1 peak ratio were 3.1 %, 41.8 % and 11.2 % in cells treated with vehicle, MTX (1 μM) and MTX combined with zVAD-fmk (500 nM, pretreated 1 h before MTX), respectively. zVAD-fmk could decrease the sub-G1 peak ratio after

MTX treatment up to 82 %. All these morphologic and molecular changes are typical features of apoptosis [34]. Compatible with previous study [15], MTX-induced apoptosis could be blocked by z-VAD-fmk. The result indicated that MTX could induce caspase-dependent apoptosis.

For measuring the intracellular ROS, HL-60 cells were treated with 0, 0.1, 0.5 and 1 μM MTX for 1 h, and then incubated with 5 μM DCFH-DA after MTX treatment at 37°C. Intracellular ROS was quantified by flow cytometry, using a permeable cell fluorescent probe DCFH-DA. DCFH-DA is hydrolyzed by intracellular esterases to nonfluorescent 2', 7'-dichlorofluorescin (DCFH), which is then rapidly oxidized by ROS to generate the fluorescent 2',7'-dichlorofluorescein (DCF). DCF is very useful in quantifying overall oxidative stress in cells [35,36]. To assess $\Delta\psi_m$, rhodamine 123 [31], a green fluorescent mitochondrial dye, was measured by flow cytometry after MTX treatment for 24 h. The results of flow cytometric analysis showed there were dose-dependent increase of intracellular ROS generation and dose-dependent decrease of $\Delta\psi_m$ in HL-60 cells after MTX treatment (Fig. 2).

Putrescine and ROS scavengers could reduce MTX-induced apoptosis, loss of $\Delta\psi_m$ by reducing intracellular ROS.

To clarify the role of ROS in the mechanism of MTX-induced apoptosis and the effects of putrescine, we pretreated putrescine (1 mM) and NAC (10 mM) for 3 h, and other ROS scavengers, such as catalase (100 U) and vitamin C (1 mM) were pretreated for 1 h before MTX (1 μM) treatment. After MTX treatment for 24 h, cells were observed by fluorescence-microscope. We found MTX could induce apoptotic morphologic changes, including chromatin condensation, membrane blebbing and shrinkage, and apoptotic body formation. These apoptotic morphologic changes occurred less if cells were pretreated with ROS scavengers or putrescine (data not shown). Then cells were harvested for flow cytometry with PI fluorescence. We found that putrescine, vitamin C, NAC and catalase decreased the sub-G1 peak ratio after MTX treatment (Fig. 3). Among these ROS scavengers, catalase had the best effect of decreasing the sub-G1 ratio up to 55 % and putrescine decreased about 50 % as compared with vehicle, respectively.

Intracellular ROS and $\Delta\psi_m$ were determined by measuring DCF (1 h after treatment) and rhodamine 123 (24 h after treatment) with flow cytometry, respectively. Putrescine (1 mM) and NAC (10 mM) could decrease intracellular ROS and maintain $\Delta\psi_m$ after MTX treatment significantly (Fig. 4). Putrescine and ROS scavengers could prevent more than half of cells from apoptosis. Taking together, it is indicated MTX-induced apoptosis is mainly through ROS-dependent pathway. Putrescine exerts a protective effect in MTX-induced apoptosis via decreasing intracellular ROS.

ODC overexpression could reduce intracellular ROS and prevent MTX-induced loss of $\Delta\psi_m$ and apoptosis.

We further examined the role of ODC in MTX-induced apoptosis. We constructed ODC cDNA into a mammalian expression plasmid, pCMV-Tag and generated cell lines overexpressing ODC in parental HL-60 and Jurkat T cells, named by WT-ODC and JK-WT-ODC cells, respectively. Parental HL-60 and Jurkat T cells were transfected by its frame-shift mutant vector as control which were named by m-ODC and JK-m-ODC cells, respectively. At the same time, we also used site-directed mutagenesis to constructed dominant-negative ODC and built DN-ODC cells [37]. ODC was overexpressed in WT-ODC cells in the transcriptional and translational level (data not shown). Four hours after stimulated by 10 % FBS in parental HL-60, m-ODC, WT-ODC and DN-ODC cells with or without DFMO (1mM), WT-ODC cell expressed about two folds higher ODC enzyme activity than parental HL-60 and m-ODC cells and overexpressed ODC activity could be inhibited by DFMO (Fig. 5). In addition, intracellular ROS was decreased more in WT ODC cells than in m-ODC significantly after 10 % FBS stimulation for 4 h (Fig. 6A). One hour after MTX (1 μM) treatment in m-ODC and WT-ODC, intracellular ROS was accumulated more in m-ODC cells than in WT-ODC cells significantly (Fig. 6B), that is, ODC overexpression reduced intracellular ROS after induced by MTX. Twenty-four hours after treatment, WT-ODC was more resistant to MTX-induced apoptosis than parental HL-60, m-ODC and DN-ODC cells in experiments of phase light microscopy (data not shown) and flow cytometry with PI staining. The percentage of apoptosis in WT-ODC cells was approximately decreased 20 % and 34 % as compared with parental HL-60 by 1 μM MTX treatment for 24 h and parental Jurkat T cells by 10 μM MTX treatment for 48 h, respectively (Fig. 6C and 6D). The protective effect of ODC in WT-ODC cells was reversed by DMFO (Fig. 6E). In the experiment of flow cytometry with rhodamine 123, WT-ODC cells had less loss of $\Delta\psi_m$ than parental HL-60, m-ODC and DN-DOC cells significantly. In addition, DFMO, inhibiting the activity of ODC, blocked the effect of maintaining $\Delta\psi_m$ in WT-ODC cells (Fig. 7A). MTX also induced disruption of $\Delta\psi_m$ and apoptosis in JK-m-ODC cells, but less in JK-WT-ODC cells (Fig. 7B), same as in HL-60 cell system. Furthermore, WT-ODC cells prevented cytochrome c release from mitochondria after MTX treatment (Fig. 7C). Based on these data, MTX-induced apoptosis seemed though mitochondria-mediated pathway. ODC overexpression reduced ROS accumulation, disruption of $\Delta\psi_m$ and cytochrome c release after induced by MTX. Simultaneously ODC overexpression reduced at least a part of apoptosis after MTX treatment in HL-60 and Jurkat T cell systems.

ODC overexpression prevented the decline of Bcl-2 which maintained $\Delta\psi_m$ and reduced MTX-induced apoptosis.

After stimulated by 10 % FBS, m-ODC, WT-ODC and DN-ODC cells were treated with or without MTX. ODC overexpression could efficiently prevent the decline of Bcl-2 when MTX untreatment (1.4 folds) and treatment (3 folds), respectively (Fig. 8A). Mitochondria-mediated pathway maybe play an important role in MTX-induced apoptosis. Bcl-2 can maintain $\Delta\psi_m$, avoid cytochrome c release from mitochondria and prevent apoptosis [38]. In previous studies, Bcl-2 overexpression can prevent MTX-induced apoptosis [39,40]. To confirm the importance of mitochondria-mediated pathway in this apoptosis after MTX treatment, we transfected *bcl-2* gene (Bcl-2 cells) or its vector only (vector cells) into parental HL-60 cells [26]. Bcl-2 cells and vector cells were treated with or without MTX, and then harvested for flow cytometry with rhodamine 123 and PI staining. We found Bcl-2 cells had less loss of $\Delta\psi_m$ and sub-G1 peak ratio than its vector cells, respectively (Fig. 8B and C). Indeed, ODC overexpression prevented the decline of Bcl-2 and Bcl-2 overexpression in parental HL-60 cells decreased disruption of $\Delta\psi_m$ and apoptosis induced by MTX. Therefore, maintaining $\Delta\psi_m$ by ROS scavengers, putrescine, ODC overexpression and Bcl-2 in parental cells is a way to prevent MTX-induced apoptosis.

ODC overexpression prevented the activation of caspase 9, 3 and the formation of apoptosome after MTX treatment.

According to the above results, cytochrome c released after MTX treatment in parental HL-60, m-ODC and DN-ODC cells, but not in WT-ODC cells. In addition, MTX-induced apoptosis in HL-60 cells seemed mainly through mitochondria-mediated pathway. To clarify the downstream of ODC-involved mitochondria-mediated pathway after MTX-induced apoptosis, m-ODC, WT-ODC and DN-ODC cells were treated with or without MTX. Total proteins from diverse cells were detected by immunoblotting with anti-Apaf-1, caspase 9, 3 and PARP (the substrate of caspase 3). In WT-ODC cells, we found that Apaf-1 was decreased, the activation of caspase 9 and 3 were inhibited, and the cleavage of PARP was protected, significantly (Fig. 9). The results indicated that ODC overexpression prevented the formation of apoptosome (the complex of Apaf-1, cytochrome c and caspase 9) and inhibited the activation of caspase 3 cascade during apoptotic progression.

Discussion

When cells are treated by MTX, extracellular MTX is transported into cells by at least two different energy-dependent mechanisms. One is a relatively low-affinity reduced folate transmembrane carrier in the micromolar range and the other is a membrane-associated folate-binding protein with nanomolar affinity. Intracellular MTX is converted to polyglutamate forms by binding of two to five polyglutamate groups, resulting in increasing intracellular half-life. MTX and its polyglutamate derivatives inhibit dihydrofolate reductase (DHFR), the major MTX target, and several folate-dependent enzymes such as thymidylate synthase (TS) and 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR) transformaylase [41].

The main pharmacological action of MTX is attributed to its inhibition of these enzymes during thymidine and purine synthesis [42-45]. Purine and pyrimidine nucleotides play critical roles in DNA and RNA synthesis as well as in membrane lipid biosynthesis and protein glycosylation. They are necessary for the development and survival of mature T lymphocytes. Pyrimidines control progression from early to intermediate S phase in the cell cycle. Inhibition of pyrimidine synthesis by MTX is a way to induce apoptosis of activated T lymphocytes [10,46]. There are several reports demonstrating that apoptosis caused by anticancer drugs, including MTX, may be mediated via the CD95 system [6]. MTX-induced apoptosis could proceed via expression of CD95-L and ligation of the death receptor CD95 in leukemia T cell lines and hepatoma, gastric cancer, colon cancer, and breast cancer cell lines [6,8,47]. However, in activated human peripheral blood T-lymphocytes (PBL), MTX triggered apoptosis by a CD95-independent pathway[10]. Here, we demonstrate another novel mechanism of MTX-induced apoptosis. MTX induced caspase-dependent apoptosis in HL-60 and Jurkat T cells in a time- and dose-dependent manner. After MTX treatment, intracellular ROS accumulates in the initial one hour and $\Delta\psi_m$ loss is detected 24 h later. ROS scavenges, such as NAC, vitamin C and catalase, could reduce the accumulation of intracellular ROS, prevent the loss of $\Delta\psi_m$, and rescue a part of cells from apoptosis. These data provide the evidence that MTX causes $\Delta\psi_m$ disruption and apoptosis in HL-60 and Jurkat T cells via ROS generation. Overexpression of Bcl-2 also prevents MTX-induced disruption of $\Delta\psi_m$ and apoptosis. Furthermore, apoptosome formation, activation of caspase 3 and cleavage of PARP (substrate of caspase 3) are found after MTX treatment. It is indicated mitochondria-mediated pathway participates in MTX-induced apoptosis.

How can MTX induce the generation of ROS? One possible mechanism is that MTX inhibits the activity of enzymes involved in enzyme defense mechanisms against ROS, including glucose-6-phosphate dehydrogenase, glucose-6-phosphogluconate dehydrogenase, glutathione reductase and gamma-glutamylcysteine synthetase. High dose of MTX decreases the cellular levels of glutathione and leads to a reduction of effectiveness of the antioxidant enzyme defense system [48]. It has been demonstrated that glutathione depletion induces ROS generation, $\Delta\psi_m$ disruption, resulting in mitochondrial cytochrome c release, caspase 3 activation, DNA fragmentation and then apoptosis [49]. In another study, MTX increases the amount of hydrogen peroxide released by stimulated polymorphonuclear neutrophils (PMNs) in a

dose-dependent manner [50]. Low dose of MTX also induced a time- and dose-dependent increase in cytosolic peroxide in U937 monocytes and Jurkat T cells. ROS generation by MTX is important for cytostasis in monocytes and cytotoxicity T cells [12]. Our results confirm previous studies and show one of the mechanisms of MTX-induced apoptosis is through ROS-dependent, mitochondria-mediated pathway.

It is known that overexpression of ODC can prevent cells from apoptosis in many insults, including H_2O_2, radiation and some chemotherapeutic drugs including cisplatin, doxorubicin, paclitaxel, 5-flourouracil [23]. We provide the evidence that putrescine or overexpression of ODC can prevent MTX-induced apoptosis. Furthermore, treatment with putrescine or overexpression of ODC in HL-60 and Jurkat T cells can decrease intracellular ROS without MTX treatment and reduce the accumulation of ROS after adding MTX. Putrescine and overexpression of ODC also maintain $\Delta\psi_m$ and prevent apoptosis after MTX treatment as ROS scavengers, such as NAC, vitamin C, and catalase. These protective effects of ODC are not seen in DN-ODC cells and blocked by DFMO. Through the mitochondria-mediated apoptotic pathway, WT-ODC prevented cytochrome c release from mitochondria to cytosol and the formation of apoptosome. As well, it inhibited the activation of caspase 3 and the cleavage of PARP (the substrate of caspase 3). A previous study suggested that the inherent ROS level might be determinative in tumor cells for their apoptotic susceptibility to As_2O_3 [51]. Overexpression of ODC seems to enforce the ability of anti-oxidant defense system to decrease the inherent ROS level and enhance the resistance of tumor cells to anti-cancer drugs.

Furthermore, how can putrescine and ODC reduce ROS *in vivo*? Overexpression of ODC or treatment with putrescine in cells increases the concentration of polyamines. Spermine, one of polyamines, can function directly as a free radical scavenger [52]. Inhibition of ODC by DFMO could increase intracellular ROS, and might lead to an imbalance in polyamine pools. Polyamine catabolism by polyamine oxidase (PAO) could continue and result in the production of ROS [53]. MTX also inhibits the synthesis of spermidine and spermine in stimulated lymphocytes from patients of rheumatoid arthritis through inhibition of the SAM-dependent pathway [54]. Thus, overexpression of ODC possibly reduces the ROS generation during polyamine catabolism, and overcomes the inhibitory effects of MTX on polyamine biosynthetic pathway.

In conclusion, low dose of MTX induces apoptosis of HL-60 and Jurkat T cells in a time- and dose-dependent manner. MTX-induced apoptosis is via ROS-dependent and mitochondria-mediated pathway at least in a part of cells. Putrescine and overexpression of ODC can maintain $\Delta\psi_m$, avoid cytochrome c release and the formation of apoptosome, inhibit the activation of caspase 3 and prevent apoptosis, functioning as ROS scavengers. Our results explain why ODC activity affects the chemosensitivity of anti-cancer drugs.

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Part 3.

Prolactin has more than 300 separate functions including affecting mammary growth, differentiation, secretion and anti-apoptosis. In the previous studies, prolactin induced Bcl-2 expression to prevent apoptosis and also provoked the activity of ornithine decarboxylase (ODC). Our previous data showed that ODC overexpression upregulates Bcl-2 and prevents tumor necrosis factor alpha (TNF-α)- and methotrexate (MTX)-induced apoptosis. Here, we further investigate whether prolactin prevents MTX-induced apoptosis through inducing ODC activity and the relationship between ODC and Bcl-2 upon prolactin stimulation. Prolactin prevented MTX-induced apoptosis in a dose-dependent manner in HL-60 cells. Following prolactin stimulation, ODC enzyme activity also shows an increase in a dose-dependent manner, expressing its maximum level at 3 h, and rapidly declining thereafter. Prolactin-induced ODC activity is completely blocked by a protein kinase C delta (PKCδ) inhibitor, rottlerin. However, there are no changes in the expressions of ODC mRNA and protein level after prolactin stimulus. It indicates that prolactin may induce ODC activity through the PCKδ pathway. Besides, Bcl-2 expresses within 1 h of prolactin treatment and this initiating effect of prolactin is not inhibited by alpha-difluoromethylornithine (DFMO). However, Bcl-2 is further enhanced following prolactin stimulation for 4 h and this enhancement is blocked by DFMO. Bcl-2 has no effect on ODC activity and protein levels, but ODC upregulates Bcl-2, which is inhibited by DFMO. Overall, there are two different forms of prolactin effect, it induces Bcl-2 primarily, and following this it stimulates ODC activity. Consequently induced ODC activity further enhances the expression of Bcl-2. The anti-apoptotic effect of prolactin is diminished by DFMO and recovered by putrescine. Obviously, ODC activity is one basis for the anti-apoptotic mechanisms of prolactin. A Bcl-2 inhibitor, HA14-1, together with DFMO, completely blocks the anti-apoptotic effects of prolactin. These results suggest that increasing ODC activity is another way of prolactin preventing MTX-induced apoptosis and that this induction of ODC activity enhances the expression of Bcl-2 strongly enough to bring about the anti-apoptotic function.

Keywords: Prolactin; methotrexate; apoptosis; crosstalk; ODC; Bcl-2.

Abbreviations: ODC, ornithine decarboxylase; TNF-α, tumor necrosis factor alpha; MTX, methotrexate; PKCδ, protein kinase C delta; DFMO, alpha-difluoromethylornithine; ROS, reactive oxygen species; FBS, fetal bovine serum; RNase A, ribonuclease A; PI, propidium iodide; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; WT-ODC, overexpressing ODC; m-ODC, frame-shift mutant ODC; CKII, casein kinase II; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; HGF, hepatocyte growth factor.

Reference

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