Mitochondrial depolarization and apoptosis associated with sustained activation of c-jun-N-terminal kinase in the human multiple myeloma cell line U266 induced by 2-aminophenoxazine-3-one

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Abstract. We investigated the involvement of c-jun-N-terminal kinase (JNK) in mitochondrial depolarization and apoptosis in a human multiple myeloma cell line, U266, treated with 2-aminophenoxazine (Phx-3). It was found that, with Phx-3 administration to U266 cells, JNK was phosphorylated 2 and 7.5-fold at 6 and 24 h, respectively, compared to the Phx-3-free control. This increasing activation of JNK in U266 cells with Phx-3 correlated with cellular disorders, such as mitochondrial depolarization and cellular apoptosis. When the JNK-specific inhibitor SP6000125 was administered to the U266 cells together with Phx-3, the number of cells exhibiting mitochondrial depolarization and cellular apoptosis was significantly reduced. These results suggest that JNK activation in human multiple myeloma U266 cells may be closely associated with mitochondrial depolarization and apoptosis.

Introduction

It is generally accepted that the loss of mitochondrial membrane integrity causes the release of cytochrome c, caspase-3 activation and cellular apoptosis (1-3). The agents employed to induce these cellular events in cancer cells may be favorable candidates for the treatment of cancer (4,5). We previously found that 2-aminophenoxazine-3-one (Phx-3), produced by the reactions of *o*-aminophenol and bovine hemoglobin solutions (6), induced apoptosis in various cancer cells, including the gastric cancer cell lines MKN45 and KATO III (7), the human glioblastoma cell lines A-172 and U-251 (8), human neuroblastoma NB-1 cells (9), and human multiple myeloma U266 cells (10), regardless of caspase-3-dependent or -independent pathways. We previously reported that Phx-3 induced mitochondrial depolarization and apoptosis in human myeloma U266 cells (10); the caspase-3-dependent apoptosis of the cells was preceded by mitochondrial depolarization, induced by Phx-3. However, the mechanism by which Phx-3 caused mitochondrial depolarization in the cells was not clarified.

It has been demonstrated that c-jun-N-terminal kinase (JNK) plays a crucial role in the activation of the intrinsic apoptotic pathway mediated by mitochondria (11). Tsuruta *et al* (12) demonstrated that JNK promotes Bax translocation to the mitochondria, resulting in cytochrome c release and cellular apoptosis. JNK was activated in multiple myeloma cell lines, including U266 cells, when treated with arsenic trioxide (ATO). Its activation was sustained for a long time and was associated with the ATO-induced apoptosis of U266 cells (13). Therefore, it is conceivable that the Phx-3-induced mitochondrial depolarization and apoptosis in U266 cells that we observed previously (10) is associated with JNK activation. In the present study, we investigated the role of JNK in Phx-3-induced mitochondrial depolarization and cellular apoptosis in human multiple myeloma U266 cells.

Materials and methods

Preparation of 2-aminophenoxazine-3-one. 2-Aminophenoxazine-3-one (Phx-3) was prepared according to the method described by Shimizu *et al* (6). The chemical structure of Phx-3 is shown in Fig. 1. Phx-3 was dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle with a final volume of 20 mM. This was added to culture medium at a final concentration of 10-100 μM during the experiments. The final volume of vehicle in culture medium was equivalent in the Phx-3-free and Phx-3-treated cells in each experiment.

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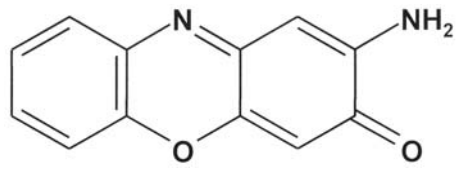

Figure 1. Chemical structure of 2-aminophenoxazine-3-one (Phx-3).

Cell line and culture conditions. The U266 human multiple myeloma cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in RPMI-1640 medium (Wako Pure Chemical, Osaka, Japan) supplemented with 80 mg/l kanamycin sulfate (Wako) and 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) in a humidified incubator containing 5% CO_2 and 95% air at 37˚C.

Cell viability. Cell viability was assessed using a Cell Titer-Blue™ cell assay (Promega, Madison, WI, USA). U266 cells were pre-cultured in a Nucleon 48-well flat-bottomed microtiter plate at $5x10^4$ cells/well for 24 h. After the collection of the cells by centrifugation (8,000 x g for 1 min), the culture medium was discarded. The cells were then treated with fresh medium containing various concentrations of Phx-3 (0-100 μM). After 24, 48 and 72 h of treatment, the cells were collected by centrifugation, and the culture medium was discarded. Fresh medium and 100 μl/well of the Cell Titer-Blue reagent (Resazurin, 7-hydroxy-3H-phenoxazine-3-one-10-oxide) were added, and further incubation continued for 1 h. Resazurin, reduced by living cells, was analyzed using a multi-detection microplate reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) at 560 nm excitation/570 nm emission. Cell viability was determined by referring to the fluorescence of cells treated without Phx-3.

Western blotting. U266 cells were exposed to 50 μM Phx-3 for 24 h, and the levels of total and phospho-JNK were determined by Western blotting using antibodies specific for phospho-JNK and total JNK, respectively, as described by Takata *et al* (14). Briefly, samples were resolved on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Blots were incubated with anti-phospho-JNK (Thr183/Tyr185; 1:1,000; Cell Signaling Technology, Beverly, MA) polyclonal antibody (Abs), total anti-JNK1 Ab (1:100), or rabbit anti-actin antiserum (1:100; Sigma). After several washes, the blots were developed with horseradish peroxidase-labeled goat anti-rabbit IgG (1:2,000; MP Biomedicals, Aurora, OH), followed by enhanced chemiluminescence according to the manufacturer's recommendations (GE Healthcare, Buckinghamshire, UK). The density of each band was measured using NIH Image (National Institute of Health, Bethesda, MD).

c-Jun N-terminal kinase inhibitor treatment. In the present study, SP600125 (Wako Pure Chemicals Co., Ltd., Tokyo) was used as a JNK-specific inhibitor. SP600125 was dissolved in DMSO to make a 20-mM solution. This was added to culture medium for a final concentration of 20 μM in the experiments. SP600125-free cells were treated with culture medium containing the equivalent volume of DMSO.

Detection of apoptosis and necrosis. The detection of apoptosis and necrosis in the U266 cells was performed by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories, Nagoya, Japan). U266 cells ($1x10^6$/10 ml) were treated with or without SP600125 for 1 h in 25 cm^2 cell culture flasks, and were incubated for 24 h with or without 20 μM Phx-3. After treatment, the cells were collected by centrifugation (8,000 x g for 1 min), washed once with PBS (pH 7.4) and resuspended in 500 μl binding buffer to which 5 μl of fluorescein isothiocyanate (FITC)-labeled annexin V (Annexin V-FITC) and propidium iodide (PI) were added. These samples were incubated at room temperature for 5 min in the dark and analyzed using a flow cytometer. Annexin V-FITC binding (a marker of cells experiencing apoptosis) and PI staining (a marker of cells experiencing necrosis) were monitored using an FITC signal detector (FL1, 520 nm) and a phycoerythrin emission signal detector (FL3, 590-650 nm), respectively.

Mitochondrial membrane depolarization analysis. Mitochondrial membrane depolarization analysis was performed by flow cytometry (Partec PAS; Partec, Münster, Germany) using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenz-imidazolyl-carbocyanine iodide (JC-1) (Wako). U266 cells ($1x10^6$/10 ml) were treated with or without SP600125 for 1 h in 25 cm^2 cell culture flasks, and incubated with or without 20 μM Phx-3 for 12 h. Cell suspension was subjected to centrifugation at 8,000 x g for 1 min. The cells were resuspended in 1 ml of fresh culture medium containing 10 μg/ml of JC-1, incubated at 37˚C for 10 min in the dark and washed twice with phosphate-buffered saline (PBS) (pH 7.4). Mitochondrial depolarization in the cells was analyzed using a flow cytometer. JC-1 monomer in cytosol (a marker of cells containing depolarized mitochondria) and J-aggregates in mitochondria (a marker of cells containing normal mitochondria) were monitored using the FITC (FL1, 520 nm) and phycoerythrin emission (FL3, 590-650 nm) signal detectors, respectively.

Results

Fig. 2 shows U266 cell proliferation in the absence or presence of different concentrations of Phx-3 for 72 h. Cell proliferation was almost completely suppressed at concentrations >60 μM at 72 h. The 50% inhibition concentration (IC_{50}) was 20 μM at 72 h and ~50 μM at 24 h, indicating that, *in vitro,* Phx-3 inhibits U266 cell proliferation in a time- and dose-dependent manner.

Changes in the JNK phosphorylation levels of U266 cells treated with 50 μM Phx-3 for 24 h (Fig. 3) were examined. JNK phosphorylation was intensified 2- and 7.5-fold compared to the control 6 and 24 h, respectively, after the administration of 50 μM Phx-3 (Fig. 3A), while total JNK levels remained unchanged (Fig. 3B). These results support the hypothesis that JNK is activated in U266 cells treated with Phx-3.

Fig. 4 shows the flow-cytometric analysis of apoptotic/necrotic cells in the U266 cell line with or without Phx-3 and with or without the JNK-specific inhibitor SP600125. Viable

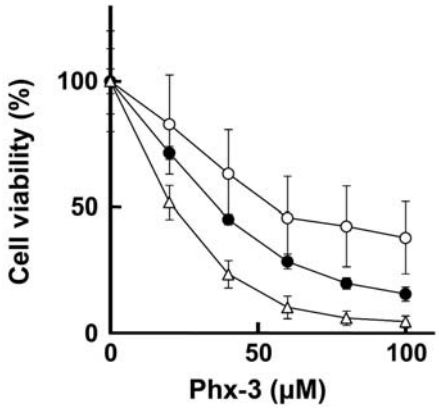

Figure 2. Viability of the human multiple myeloma U266 cells in the presence or absence of different concentrations of Phx-3. U266 cells were treated with fresh medium containing various concentrations of Phx-3 (0-100 μM) and were incubated for 72 h. (○) 24 h; (●) 48 h; (Δ) 72 h.

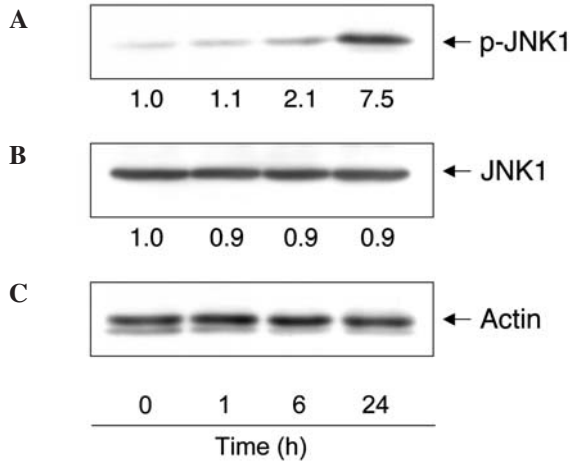

Figure 3. Time-dependent JNK phosphorylation in U266 cells treated with 50 μM Phx-3 as detected by Western blotting. Phospho-JNK or total JNK1 expression was assayed as described in Materials and methods. The numbers indicate phosphorylation levels in U266 cells with Phx-3, as compared to those in the control without Phx-3. (A) Time course of JNK phosphorylation in U266 cells with Phx-3 for 24 h. (B) Time course of total JNK1 in U266 cells with Phx-3 for 24 h. (C) Actin levels were determined as a control.

cells (bottom-left quadrant) were abundant (~75-77%), while late-phase apoptotic/necrotic cells (top-right quadrant) were few (~4-5%) in the Phx-3-free cells (Fig. 4A). The addition of SP600125 did not affect the cells (Fig. 4B).

The viable cell population decreased markedly to 57% and the late-phase apoptotic/necrotic cells clearly increased up to 16% in Phx-3-treated cells without SP600125 (Fig. 4C). In this case, the number of early-phase apoptotic cells (bottom-right quadrant) increased from normal levels, indicating that Phx-3 induces apoptosis in U266 cells. However, the increased numbers of early-phase apoptotic and late-phase apoptotic/necrotic cells observed with Phx-3 treatment were returned to almost normal levels by treatment with SP600125 (Fig. 4D). The present results strongly suggest that JNK activation is involved in apoptosis in U266 cells treated with Phx-3.

We therefore examined whether mitochondrial depolarization in U266 cells, which was shown to be induced by Phx-3 (10), is prevented by SP600125. Fig. 5 shows the flow-cytometric analysis of cells with normal mitochondria (top-left quadrant) and depolarized mitochondria (bottom-right quadrant) in U266 cells without Phx-3 (Fig. 5A), with SP600125 alone (Fig. 5B), with 50 μM Phx-3 alone (Fig. 5C), and with both 50 μM Phx-3 and 20 μM SP600125 (Fig. 5D).

Cells with normal and depolarized mitochondria were observed to be dominant (~84-86%) and slight (~6%), respectively, in Phx-3-free cells. The addition of SP600125 did not affect the cells with normal and depolarized mitochondria. However, the number of cells with normal mitochondria decreased from 85.7 to 40.8% and that of the cells with depolarized mitochondria increased from 5.6 to 33.4%, indicating that Phx-3 strongly induced mitochondrial depolarization in U266 cells. When SP600125 was administered to U266 cells with Phx-3, the number of cells with normal mitochondria increased from 40.8 to 66%, while cells with depolarized mitochondria decreased from 33.4 to 13.9% (Fig. 5D). These results suggest that the mitochondrial depolarization caused by Phx-3 is closely related to JNK phosphorylation.

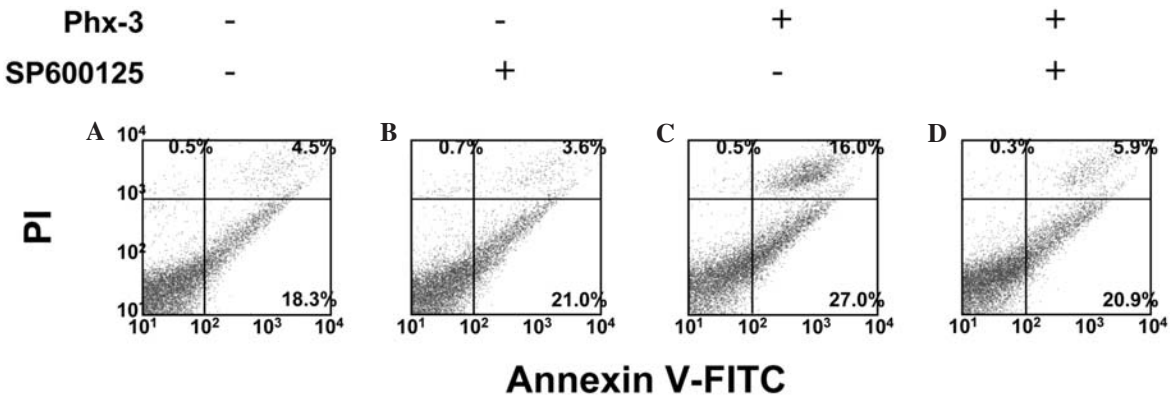

Figure 4. Inhibitory effect of SP600125 on Phx-3-induced apoptosis in U266 cells. U266 cells were treated with (+) or without (-) 20 μM SP600125 for 1 h prior to the experiment, and then with (+) or without (-) 100 μM Phx-3 and with (+) or without (-) 20 μM SP600125 for 24 h. Annexin V- and PI-negative (bottom-left quadrant), annexin V-positive and PI-negative (bottom-right quadrant), and annexin V- and PI-positive (top-right quadrant) cells were considered viable, early-phase apoptotic, and late-phase apoptotic/necrotic (dead), respectively. The percentages (%) of each population are indicated in each quadrant.

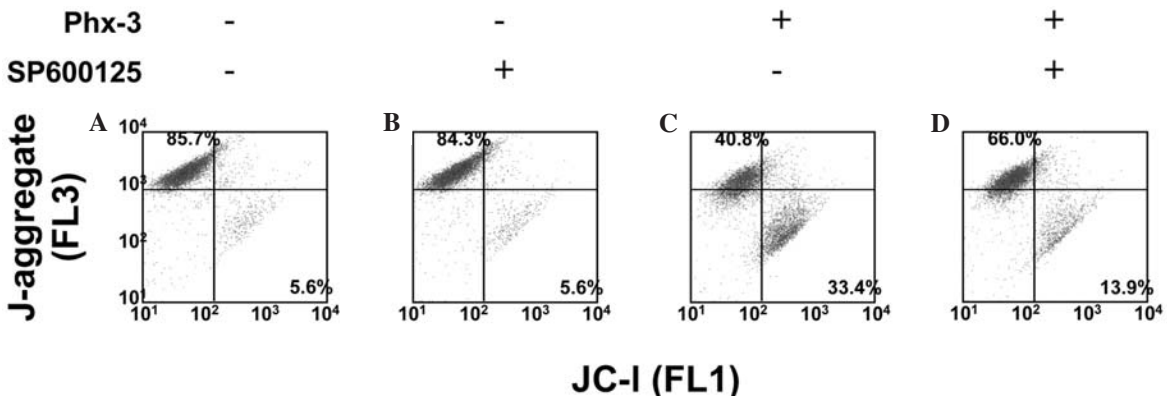

Figure 5. Inhibitory effect of SP600125 on Phx-3-induced mitochondrial membrane depolarization in U266 cells. U266 cells were treated with (+) or without (-) 20 μM SP600125 for 1 h prior to the experiment, and then with (+) or without (-) 100 μM Phx-3 and with (+) or without (-) 20 μM SP600125 for 12 h. J-aggregate-positive (top-left quadrant) and JC-1-positive (bottom-right quadrant) cells were considered to be those exhibiting normal and depolarized mitochondria, respectively. The percentages (%) of each population are indicated in each quadrant.

Discussion

The upstream component of the apoptotic pathway is regulated by several factors, including mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases, p38-MAPKs and JNKs (11,15). JNK, once activated, is involved in the promotion of inflammation and cell death upon diverse stress stimuli in several cell lines (16,17). However, in some cell lines, JNK activation promotes cell survival (18). Therefore, JNK activation appears to have different results in terms of cellular survival or death depending on cell type. In cancer cells, cellular apoptosis is triggered by JNK activation due to chemotherapeutic agents (13,16,17).

In the present study, we demonstrated that when U266, a human multiple myeloma cell line, was treated with Phx-3, JNK phosphorylation was significantly and increasingly enhanced 6 and 24 h after the exposure of cells to the agent (Fig. 3). We also showed that this event is consistent with the prevention of cell proliferation (Fig. 2), increased numbers of apoptotic and necrotic cells (Fig. 4) and increased numbers of cells with depolarized mitochondria (Fig. 5) in the U266 cell line. In addition, it was found that the enhanced apoptosis and mitochondrial depolarization of U266 cells treated with Phx-3 was significantly suppressed by the addition of the JNK-specific inhibitor SP600125 (Figs. 4 and 5). These results strongly suggest that JNK activation, which was induced by the exposure of the cells to Phx-3, is critical to cellular apoptosis and mitochondrial depolarization in U266 cells. These results are in agreement with reports that apoptosis is associated with JNK activation in U266 cells treated with ATO (13) and in the MG63 sarcoma cell line treated with cisplatin (19).

We previously demonstrated that apoptosis in U266 cells treated with Phx-3 was dependent on the activation of caspase-3, and preceded by mitochondrial depolarization (10). Mitochondrial membrane integrity is regulated by B cell lymphoma/leukemia (Bcl)-2 family proteins (20), composed of antiapoptotic members such as Bcl-2 and Bcl-x long (xL), proapoptotic members such as Bcl-2 associated x (Bax) and Bcl-2 associated death promoter (Bad), and proapoptotic Bcl-2 homology domain (BH)3-only proteins, such as BH3-interacting domain death agonist (Bid) (21,22). Bax and Bad are located in cytosol in their complexed forms with 14-3-3 proteins (23). However, when JNK is activated, Bax and Bad dissociate from the 14-3-3 proteins and translocate to the mitochondria (23), with Bax forming a channel on the outer mitochondrial membrane and Bad binding to Bcl-2, thereby suppressing the antiapoptotic function of Bcl-2 and resulting in mitochondrial membrane depolarization. Taking the above-mentioned reports and the present results concerning JNK into account, the sequential pathways for apoptosis in U266 cells may be proposed: JNK activation promotes the translocation of Bax to mitochondria and the binding of Bad to Bcl-2, resulting in mitochondrial depolarization and the release of cytochrome c, caspase-3 activation and apoptosis in U266 cells treated with Phx-3.

The mechanism for the activation of the upstream enzymes, such as MAPK, associated with JNK in U266 cells with Phx-3 remains to be clarified. It is possible that the perturbation of the cellular membrane caused by Phx-3 is associated with the activation of the upstream components of JNK. Hendrich *et al* (24) reported that phenoxazine molecules are located close to the polar/apolar interface of lipid bilayers, with which they weakly interact, resulting in the alteration of the lipid phase properties of the cell membrane and the activation of the molecular mechanism underlying the biological activity of these compounds by changing the intramembrane conditions. However, a detailed mechanism of signal transduction from cell membrane perturbation to JNK activation in U266 cells with Phx-3 is in need of elucidation.

Though the Fig. 2 results indicating that U266 cell proliferation was significantly inhibited by Phx-3 at 24 h are consistent with JNK activation (Fig. 3) with regards to time, it is currently unclear whether the inhibition of cellular proliferation is linked to JNK activation or not. A plausible explanation for the Phx-3-induced inhibition of cellular proliferation is that Phx-3 intercalates to DNA, inhibiting its replication. Phx-3 has been shown to intercalate to DNA (25), though more weakly than actinomycin D, a strong intercalater (26). We previously demonstrated that thymidine incorporation into DNA was significantly suppressed when U266 cells were treated with Phx-3 (10), indicating that the replication of DNA was inhibited by Phx-3, possibly leading to the suppression of cellular proliferation.

It is generally accepted that apoptosis is an important part of the action mechanism of conventional chemotherapeutics and novel anticancer agents (4,5). Therefore, Phx-3 is a promising candidate for the treatment of multiple myeloma, a disseminated malignancy of monoclonal plasma cells in the bone marrow that is refractory to chemotherapy.

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