Abstract. The effects of Phx-3 on changes in intracellular pH (pHi) in the MKN45 and MKN74 human gastric cancer cell lines were evaluated in order to determine the mechanism for the proapoptotic effects of 2-aminophenoxazine-3-one (Phx-3) on these cells. Phx-3 (100 μM) reduced pHi in MKN45 from 7.45 to 5.8, and in MKN74 from 7.5 to 6.2 within 1 min of engagement with these cells. Such a decrease of pHi was closely correlated with the dose of this phenoxazine and continued for 4 h. The activity of Na+/H+ exchanger isoform 1 (NHE1), which is involved in H+ extrusion from the cells, was dose-dependently suppressed by Phx-3 in these cells, and was greatly suppressed in the presence of 100 μM Phx-3. This result indicates that the decrease of pHi in MKN45 and MKN74 cells is closely associated with the inhibition of NHE1 in these cells. The morphology of these cells at 24 h after treatment with Phx-3 indicated shrinkage of the cells and condensation of the nuclear chromatin structure, which are characteristic of the apoptotic events in these gastric cancer cells. Cytotoxicity of Phx-3 against MKN45 and MKN74 cells was extensive because almost all MKN45 cells lost viability at 24 h in the presence of 20 μM Phx-3, and nearly 50% of the MKN74 cells lost viability in the presence of 50 μM Phx-3. These results suggest that rapid and extensive decrease of pHi in human gastric cancer MKN45 and MKN74 cells caused by Phx-3 might disturb intracellular homeostasis, leading to apoptotic and cytotoxic events in these cells. Phx-3 is a good candidate for therapeutics of gastric cancer that is intractable to conventional chemopreventive therapies.

Introduction

It has been suggested that a decrease of intracellular pH (pHi) precedes apoptotic events in cancer cells (1,2). Therefore, drugs to induce pH reduction have been considered good candidates for treating cancer and causing programmed cell death (3). For example, cyclohexamide, etoposide, and camptothecin decrease pHi in cancer cells, leading to apoptosis of the cells (3-5); however, these drugs often exert significant adverse effects on the body (6,7).

The oxidative phenoxazines [e.g., 2-amino-4,4-dihydro-4a,7-dimethyl-3H-phenoxazine-3-one (Phx-1) and 2-aminophenoxazine-3-one (Phx-3)] exert anticancer effects on various species of cancer cells in vitro (8-10) and in vivo (11), without marked adverse effects (11-13). Recently, Che et al (14) reported that apoptotic events in KB-3-1 cells (human epidermoid carcinoma cell line) and K562 cells (human chronic myeloid leukemia cell line) are preceded by a decrease in pH (7-9) in cells treated with Phx-3, a phenoxazine produced by the reaction of o-aminophenol with bovine hemoglobin. However, the detailed mechanism for pH decrease in these cancer cells with Phx-3 remains unclear.

Regarding the regulation of pH, it has been suggested that among Na+/H+ exchangers, Na+/H+ exchanger isoform 1 (NHE1) is ubiquitously present in the plasma membrane in the cells and plays a pivotal role in regulating pH (15-17). Therefore, it is important to investigate whether a decrease in pHi precedes apoptosis in cancer cells treated with Phx-3, and whether a decrease in Phx-3-inducible pH is associated with the activity of NHE1 that is primarily involved in pH regulation in cancer cells.
Kasuga et al (18) reported that gastric cancer cells such as MKN45 and MKN74 are susceptible to the treatment of Phx-3, i.e., Phx-3 greatly inhibited the growth of these cells and the cell cycle and consequently induced the programmed death of these cells. However, the effects of Phx-3 on changes in pH, of these cells have not been investigated. Thus, we investigated the effect of Phx-3 on these gastric cancer cells in terms of the pH regulation associated with NHE1 and the causal relationship of pH decrease and apoptotic events. The present study is the first report on the inhibitory effects of Phx-3 on NHE1 in human gastric cancer cells, including MKN45 and MKN74 cells, leading to a drastic decrease in pH in these cells, and finally to cellular apoptosis.

Materials and methods

Phx-3 and other reagents. For this study, Phx-3 was obtained by the reaction of o-aminophenol (Wako Pure Chemicals, Osaka, Japan) with bovine erythrocytes and was purified using column chromatography, as described by Nakachi et al (19). The chemical structure of Phx-3 is presented in Fig. 1. Phx-3 was dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle to make 20 mM solution.

This solution was diluted appropriately with an isotonic saline and was used for the experiments with MKN45 and MKN74 cells.

Cell line and culture condition. Human gastric cancer cell lines MKN45 and MKN74 were obtained from the Health Science Research Resources Bank (Osaka, Japan). MKN45 is a poorly differentiated adenocarcinoma, and MKN74 is a moderately differentiated tubular adenocarcinoma. These cells were cultured in Eagle's minimum essential medium (MEM) (Kohjin Bio, Saitama, Japan) supplement with 10% heat inactivated fetal calf serum (Equitech-Bio, Kerrville, TX) at 37°C in a 5% CO₂ humidified atmosphere.

Measurement of pH in MKN45 and MKN74 cells treated with Phx-3. The pH of MKN45 and MKN74 cells was determined according to the method described by Litman et al (20). Briefly, these gastric cancer cells (4x10⁶/mкл) were incubated with a pH-sensitive fluorescent probe (2,7)-bis(carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) (3 μM) (Dojin Chemical, Kumamoto, Japan) in HEPES buffer (pH 7.4), which caused a slight increase in fluorescence as neutral NH₃ entered the cells. This increase was followed by a slow decline that was due to the external and internal pH, which enabled determining the unknown pH. A linear calibration curve for pH was obtained (data not shown). Therefore, it was possible to estimate the pH of MKN45 or MKN74 cells loaded with BCECF-AM.

Measurement of NHE activity in MKN45 and MKN74 cells, and the inhibitory effects of Phx-3 on NHE. NHE activity was determined by ammonium chloride (NH₄Cl)-prepulse technique (21), monitoring the changes in fluorescence of BCECF (an excitation wavelength of 500 nm and an emission wavelength of 530 nm) in MKN45 and MKN74 cells, as described above. Briefly, cells were washed twice after BCECF-AM loading, and were poured in a 2 ml quartz cell. NH₄Cl from 2 M stock solution (approximately pH 7.3) was added to cell suspensions at a final concentration of 50 mM. The pH, which was monitored by the changes in fluorescence at 530 nm, rapidly increased as neutral NH₃ entered the cells. This increase was followed by a slow decline that was due to an influx of NH₄⁺, accompanied by its partial dissociation. After 3 min, the cells were centrifuged at 400 x g for 30 sec and resuspended in HEPES buffer (pH 7.4), which caused a rapid acidification as the NH₄⁺ ions trapped inside the cells crossed the membrane in the form of neutral NH₃, leaving protons behind. Total elapsed time to centrifuge and resuspend was ~2 min. The sample was poured in a 2 ml quartz cell, again. Final concentration of 0, 20, 50 or 100 μM Phx-3 was quickly added to the sample, and was mixed well. The fluorescence spectra were taken to obtain a record of pH, using FP-6200 spectrofluorometer as described above.

Morphological assessment of culture cells. For morphological assessment, the cell suspension was taken out at the indicated time and was sedimented in a Shandon Cytospin II (Shandon, Pittsburgh, PA). The preparations were then stained with May-Grünwald-Giemsa solution. Morphological changes were assessed by digital microscopy using BZ-8000 (Keyence Co., Osaka, Japan).

Estimating the viability of gastric cancer cells treated with various concentrations of Phx-3. MKN45 and MKN74 cells (3,000/well) in 100 μl of culture medium were incubated with or without various concentrations of Phx-3 for 24, 48 and 72 h in 96-well plates. The number of viable cells was assessed using a cell viability assay kit, Cell Titer Blue (Promega Co. Ltd., Madison, WI), with fluorescence measurements at 570 nm for excitation and 590 nm for emission using Multidetection microplate reader, Powerscan HT (Dainippon Pharmaceutical, Osaka, Japan).

Results

Changes in pH, in MKN45 and MKN74 cells treated with Phx-3. Human gastric cancer cell lines MKN45 and MKN74
were treated with different concentrations of Phx-3 in order to examine changes in pHi. At the resting state, pHi in MKN45 was 7.45, and that in MKN74 was 7.5 (Fig. 2), which was higher than the medium pH (7.4) and extensively higher than pHi in normal cells [e.g., pHi is 7.0 in normal hematopoietic cells (17) and 7.2 in human erythrocytes (22)]. When MKN45 and MKN74 cells were treated with 100 μM Phx-3, pHi was decreased to 5.8 in MKN45 and 6.2 in MKN74 within 1 min (Fig. 2). The decreased level of pHi was closely dependent on the concentrations of Phx-3 added to these cells. Such intracellular acidification continued for 4 h in both MKN45 and MKN74 cells, dependent on the dose of Phx-3 (Fig. 3).

**Effect of Phx-3 on the activity of Na⁺/H⁺ exchanger isoform 1 in MKN45 and MKN74 cells.** Among Na⁺/H⁺ exchangers, the ubiquitous NHE1 is the membrane transporter primarily involved in regulating pHi (15-17). We studied the effects of Phx-3 on the activity of NHE1 in MKN45 and MKN74 cells according to the method described by Coates et al (21). Fig. 4 depicts changes in pHi that were caused by NH₄Cl pulse loading, washing out NH₄Cl, and administration of different concentrations of Phx-3 in MKN45 and MKN74 cells. When MKN45 cells were treated with NH₄Cl alone, pHi increased instantly from 7.38 to 7.65, and then decreased gradually for 3 min (Fig. 4A). When NH₄Cl was washed out from the cells, pHi rapidly decreased from 7.4 to 6.3 for 2 min, and then began to increase (0 μM Phx-3). This recovery process is assumed to be due to proton extrusion by NHE1 (21,23).

When 20 μM Phx-3 was administered to MKN45 cells, 2 min after washing-out NH₄Cl, the recovery of pHi was extensively suppressed. Such inhibition of the recovery of pHi after washing out NH₄Cl was complete when 50 or 100 μM Phx-3 was administered to the cells (Fig. 4A). These results indicate that Phx-3 inhibited the activity of NHE1, which is involved in proton extrusion from the cells (21,23). Similar results were obtained for MKN74 cells treated with different concentrations of Phx-3 (Fig. 4B). When the cells were treated with 100 μM Phx-3, the recovery of pHi in MKN74 cells was completely suppressed, indicating that NHE1 activity was suppressed by Phx-3 in MKN74 cells.

**Effect of Phx-3 on apoptosis and viability in MKN45 and MKN74 cells.** Kasuga et al (18) previously demonstrated that...
apoptosis occurs in MKN45 and MKN74 cells in response to Phx-3 engagement. Thus, we examined whether the morphological changes that are characteristic of apoptotic events may occur in these cells when treated with 10 or 50 μM Phx-3 (Fig. 5). Results indicated that MKN45 treated with 10 μM Phx-3 and MKN74 treated with 50 μM Phx-3 for 24 h exhibited features of apoptosis, including cell shrinkage and condensation of nuclear chromatin.

Since Cell Titer Blue is reduced by mitochondria in viable cells, we evaluated the viability of MKN45 cells and MKN74 cells after treatment with various concentrations of Phx-3, using Cell Titer Blue. Fig. 6 illustrates changes in the viability of MKN45 and MKN74 cells during 72 h of incubation with different concentrations of Phx-3. Almost all the MKN45 cells lost viability 24 h after treatment with >20 μM Phx-3 (Fig. 6A). However, at lower concentrations of Phx-3 (0.5 to 5 μM), resulting in slight decrease of pH_i (Fig. 2A), the number of viable MKN45 cells significantly increased, though the detailed mechanism is unclear. Relatively fewer cytotoxic effects of Phx-3 were observed in MKN74 cells than in MKN45 cells during 72 h, but loss of viability of MKN74 occurred at different concentrations (Fig. 6B). In particular, at 50 μM Phx-3, 85% of the MKN74 cells lost viability at 72 h.

Discussion

It has been recognized that pH_i is strictly regulated in normal cells and even in cancer cells, in order to maintain their cellular homeostasis (1,2,15-17). In normal cells, pH_i is usually kept lower than the pH of the extracellular medium (17,22). However, pH_i in cancer cells is maintained higher than extracellular pH (pHe) (3-5,17). Such higher pH_i in cancer cells seems to be suitable for proliferation, and oncogene transformation of the cells (24,25) and increases tumorigenesis (16). Rotin et al (26) indicated that Na^+H^+ exchange...
exchanger is indispensable for keeping pH_i higher in cancer cells. On the other hand, suppression of cellular proliferation and induction of apoptotic cell death have been known to be promoted by intracellular acidification (3-5). In particular, endonuclease II, which is responsible for digestion of genomic DNA, inducing programmed cell death, and is activated at acidic pH lower than pH 6.8 (27). This enzyme operates when cancer cells are treated with drugs to cause cellular acidification. Drugs that cause intracellular acidification may thus induce apoptosis of cancer cells (1,2,17), and are considered potential anticancer agents (3-5).

In this study, we found that pH_i in human gastric cancer cell lines MKN45 and MKN74 is higher than pHe (pH_i of 7.48 for MKN45 and 7.5 for MKN74 cells, versus medium pH of 7.4) at a resting state. When these cells were treated with Phx-3, pH_i decreased rapidly, dependent on the dose of Phx-3 (Fig. 2), and such intracellular acidification was sustained for at least 4 h (Fig. 3). In particular, pH_i decreased by 1.6 pH units in MKN45 cells and 1.2 pH units in MKN74 cells immediately after administration of 100 μM Phx-3 (Fig. 2). Such rapid and drastic changes in pH_i may be explained by the inhibition of NHE1 caused by administering Phx-3, as described later.

NHE1 is most likely to be involved in the mechanism for pH_i decrease in MKN45 and MKN74 because it has been demonstrated that among Na+/H+ exchangers, NHE1 plays the most important role in regulating pH_i in various cells (15-17). NHE1 exists ubiquitously in the plasma membranes of normal and cancer cells (15,16), and its major role is to extrude intracellular proton to the extracellular medium, thereby regulating pH_i. Drugs administered to inhibit the activity of NHE1 (e.g., amiloride and methylamilorides) decrease pH_i by inhibiting the extrusion of intracellular proton (17,23). Thus, we studied the effects of Phx-3 on the activity of NHE1 in MKN45 and MKN74 cells (Fig. 4) and found that this phenoxyazine compound exerted inhibitory effects on NHE1 in these cells, dependent on concentrations of Phx-3, consistent with the decrease of pH_i (Fig. 2). Thus, the decrease of pH_i in MKN45 and MKN74 cells caused by Phx-3 may be associated with inhibition of the activity of NHE1 by this phenoxyazine compound. The mechanism for inhibiting NHE1 by Phx-3 is still unclear but may be clarified by the findings of Hendrich et al (28) that phenoxyazine molecules are located close to the polar/apolar interface of lipid bilayers and weakly interact with lipid bilayers, altering the lipid phase properties of the cellular membranes. It is possible that rapid changes in the conditions of the plasma membranes induced by Phx-3 extensively affect the activity of NHE1 existing in the cellular membranes.

Gastric cancer is a common malignancy in adults but is often refractory to chemotherapy alone (29,30). Thus, it is crucial to develop drugs exerting anticancer effects but with low adverse reactions. In the present study, we demonstrated that Phx-3 causes apoptosis of human gastric cancer cells in vitro. Phx-3 has proapoptotic effects against various cancer cells in vitro (8-10) and anticancer effects in vivo, with few adverse effects on mice (11-13). Moreover, Kohno et al (31) reported that oral administration of 500-1,500 mg/kg Phx-3 to ddY mice did not cause gastrointestinal injury and that repeated oral administration of 10 mg/kg Phx-3 to mice for four weeks caused no diarrhea. Therefore, Phx-3 holds promise as an agent to treat human gastric cancer in the future.

**Figure 6. Effects of various concentrations of Phx-3 on the viability of MKN45 cells or MKN74 cells during 72 h. MKN45 or MKN74 cells were treated with various concentrations of Phx-3 (0.5, 1, 2, 5, 10, 20, and 50 μM Phx-3), and the viability of these cells was evaluated by an Cell Titer Blue method, which estimates the capacity of mitochondria in the cells, to reduce Cell Titer Blue.**
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