

Pleural lavage with distilled water during surgery for esophageal squamous cell carcinoma

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Abstract. This study aimed to investigate cytotoxic effects of hypotonic shock on esophageal squamous cell carcinoma (ESCC) cell lines, and to apply pleural lavage with distilled water to surgery for ESCC. Three human ESCC cell lines, TE5, TE9 and KYSE170 were exposed to distilled water, and morphological changes in ESCC cells were closely observed under a differential interference contrast microscope connected to a high-speed digital video camera. Further, serial cell volume changes after hypotonic shock were measured using a high-resolution flow cytometer. To investigate the cytotoxic effects of hypotonic shock on ESCC cells, re-incubation of ESCC cells was performed after hypotonic shock. Additionally, the effects of 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), a Cl⁻ channel blocker, during hypotonic shock were analyzed. Video recordings by high-speed digital camera demonstrated that hypotonic shock with distilled water induced cell swelling followed by cell rupture. Measurements of cell volume changes using a high-resolution flow cytometer indicated that severe hypotonicity with distilled water increased broken fragments of ESCC cells within 5 min. Re-incubation experiments demonstrated cytotoxic effects of hypotonic shock on ESCC cells. Treatment of cells with NPPB increased cell volumes by the inhibition of regulatory volume decrease, which is observed during hypotonic shock, and enhanced cytotoxic effects. These findings demonstrated the cytotoxic effects of hypotonic shock on ESCC cells, and clearly support the efficacy of pleural lavage with distilled water during surgery for ESCC.

Introduction

The presence of exfoliated cancer cells in the pleural cavity after resection of thoracic esophageal carcinoma has been reported (1-4), and positive pleural lavage cytology before thoracic closure is recognized as a prognostic indicator of recurrence in thoracic esophageal carcinoma (1,3). Therefore, effective pleural lavage is clinically important at the time of initial surgery of thoracic esophageal carcinoma because exfoliated cancer cells may be viable and tumorigenic. Although intraoperative pleural lavage with saline solution has been widely performed, it may be difficult to remove all of the exfoliated cancer cells from the pleural cavity. Accordingly, pleural lavage with distilled water has been performed during thoracic surgery for various cancers, but there is no systematic study elucidating its efficacy during surgery for thoracic esophageal carcinoma. Furthermore, there are no experimental data demonstrating the cytotoxic effects of distilled water on esophageal squamous cell carcinoma (ESCC) cell lines due to hypotonic shock.

In the present study, we analyzed changes in cellular morphology and volume after hypotonic shock in detail, and determined the osmolarity and incubation time necessary to kill ESCC cells using several unique methods, such as a differential interference contrast (DIC) microscope connected to a high-speed digital video camera, a high-resolution flow cytometer and re-incubation experiment. Further, we treated ESCC cells with 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), a Cl⁻ channel blocker, to enhance cytotoxic effects by increasing cell volumes during hypotonic shock via the inhibition of regulatory volume decrease (RVD) (5,6). These findings clearly support the efficacy of pleural lavage with distilled water during surgery for ESCC.

Materials and methods

Cell culture and materials. The human ESCC cell lines, TE5 (poorly differentiated) and TE9 (poorly differentiated) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku

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University, Sendai, Japan) (7). The human ESCC cell line KYSE170 (moderately differentiated) was obtained from Kyoto University (Kyoto, Japan) (8). These cells were grown on plastic culture flasks (Corning Incorporated, NY, USA) in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 100 U/ml of penicillin and 100 μ g/ml of streptomycin, and 10% fetal bovine serum (FBS). The flasks were kept in a humidified incubator at 37°C with 5.0% CO₂ in air. NPPB was purchased from BIOMOL International, L.P (Plymouth Meeting, PA).

Solutions and measurement of osmolality. The 140 mM isotonic NaCl buffer contained 140 mM NaCl, 5.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 5.0 mM glucose and 10 mM HEPES. The osmolality of the solutions was measured using a freezing point osmometer (model 110, Fiske Associates, Norwood, MA) and was determined as 300±4.0 [mean ± standard error of the mean (SEM), n=5] mOsmol/kgH₂O for the isotonic NaCl buffer. In order to analyze cell volume changes after hypotonic shock of various osmolalities, graded hypotonic NaCl buffers were produced by diluting 2-, 4- and 16-fold with distilled water. Accordingly, the osmolality of each hypotonic NaCl buffer was determined as 148±4.4, 90.0±4.6 and 24.3±2.0 (means ± SEM, n=3) mOsmol/kgH₂O, respectively. The osmolality of distilled water was determined as 0 mOsmol/kgH₂O (n=3). All solutions used in the present study were adjusted to pH 7.40.

Observation of morphological changes in ESCC cells after exposure to distilled water. ESCC cells were detached from flasks in a trypsin-EDTA solution and centrifuged. Isotonic (300 mOsmol/kgH₂O) NaCl buffer was added to the pelleted cells and the suspended cells were mounted on a coverslip precoated with neutralized Cell-Tak (Becton-Dickinson Labware, Bedford, MA, USA) for firm attachment of the cells. The coverslip with the cells was set in a perfusion chamber that was mounted on the stage of a DIC microscope (BX50Wi, Olympus, Tokyo, Japan) connected to a video-enhanced contrast (VEC) system (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan), and images were continuously recorded by a video recorder according to the procedure described previously (9). The experiments were performed at 37°C. The volume of the perfusion chamber was approximately 20 μ l and the rate of perfusion was 200 μ l/min. At first, the isotonic NaCl buffer was perfused in the chamber. Then, this was changed to distilled water and serial changes in ESCC cells were observed.

Measurements of cell volume changes in ESCC cells after hypotonic shock. ESCC cells grown on culture flasks were detached from culture flasks in a trypsin-EDTA solution and centrifuged. Then, 7.5x10⁶ cells in pellets were suspended in 1-ml distilled water or hypotonic NaCl buffer of various osmolalities to induce hypotonic shock. The suspending solution was displaced into a Vi-CELL™ Sample Cup (Beckman Coulter, Fullerton, CA) and the cell volume was measured 1, 5 and 10 min after induction of hypotonic shock using a high-resolution flow cytometer, Cell Lab Quanta (Beckman Coulter). This flow cytometer is designed to measure the electronic volume (EV) by the Coulter Principle

and is more accurate than the volume detected by forward angle light scattering analysis by a conventional flow cytometer. All data on EV were analyzed by Quanta control software. EV of more than 10,000 cells was collected in each measurement. The cell suspension in the isotonic (300 mOsmol/kgH₂O) NaCl buffer was used as a sample without hypotonic shock (0 min). For experiments with NPPB, cells were pre-incubated with NPPB for 30 min at 37°C with 5.0% CO₂ in air.

Re-incubation of ESCC cells after exposure to distilled water. ESCC cells grown on culture flasks were detached from culture flasks in a trypsin-EDTA solution and centrifuged. The cells were suspended in culture medium and 2.5x10⁵ TE5 or TE9 cells, or 1.5x10⁵ KYSE170 cells, respectively, were placed in each centrifuging tube. The cell suspension was centrifuged, and the pelleted cells were re-suspended in distilled water and incubated for 1, 5, 10, 20 or 30 min. Thereafter, the suspension was centrifuged, and the cells in pellets were suspended in culture medium and seeded into Costar® 6-well plates (Corning Incorporated, NY, USA). At 48 h after plating, the cells were detached from the plates in a trypsin-EDTA solution and counted on a hemocytometer. The cell suspension in isotonic (300 mOsmol/kgH₂O) NaCl buffer was used as a sample without hypotonic shock (0 min). For experiments with NPPB, cells were pre-incubated with NPPB for 30 min at 37°C with 5.0% CO₂ in air.

Pleural lavage fluid during surgery. Pleural lavage with distilled water was performed in 5 patients during surgery for ESCC. Before thoracic closure, 1,000 ml of distilled water was poured into pleural cavity and left for 5 min with agitation. Thereafter, lavage fluid was collected and the osmolality was measured using a freezing point osmometer.

Statistical analysis. Results were expressed as means ± SEM. Statistical analyses were carried out using Student's t-test. Differences were considered significant when the P-value was <0.05.

Results

Changes of individual ESCC cells after exposure to distilled water. We observed the morphological changes in ESCC cells after exposure to distilled water using a DIC microscope connected to a VEC system. ESCC cells started swelling as soon as the cells were exposed to distilled water, and then continued swelling followed by rupture. These changes were observed in each of the three ESCC cell lines (Fig. 1). Rupture of ESCC cells occurred within 3 min after perfusion of distilled water.

Cell volume changes of ESCC cells after hypotonic shock. To quantify serial cell volume changes in ESCC cells after hypotonic shock of various osmolalities, we measured the cell volume and cell counts simultaneously after hypotonic shock using Cell Lab Quanta. Fig. 2 shows the results of simultaneous measurements of cell volume and cell counts of TE5 (Fig. 2A), TE9 (Fig. 2B) or KYSE170 cells (Fig. 2C) before and after hypotonic shock of various osmolalities. Once ESCC cells

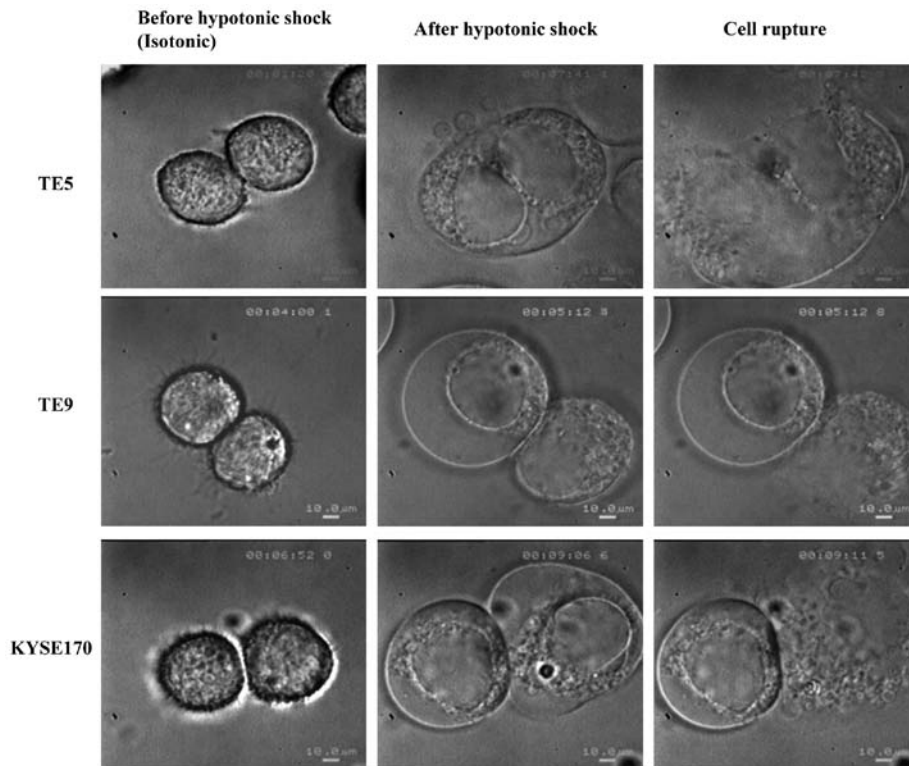


Figure 1. Changes in individual ESCC cells after hypotonic shock with distilled water. Representative images of TE5, TE9 and KYSE170 cells before and after hypotonic shock with distilled water are shown. ESCC cells continued to swell followed by cell rupture after hypotonic shock with distilled water.

were suspended in the hypotonic buffers, the cells initially increased in cell volume. Severe cell swelling was observed depending on low osmolarity. When the cells were exposed to mild hypotonicity (90-148 mOsmol/kgH₂O), the cell volume decreased gradually to their pre-hypotonic shock level, maintaining a monomodal distribution despite the continued presence of extracellular hypotonicity. For example, the temporal changes of mean cell volume (MCV) of TE5, TE9 and KYSE170 cells 0, 1, 3, 5, 10 and 20 min after hypotonic shock of 148 mOsmol/kgH₂O were shown in Fig. 3, suggesting mild hypotonicity could induce RVD in ESCC cells. However, when the cells were exposed to even lower osmolarity (24 mOsmol/kgH₂O), the cell volume showed bimodal distribution. Although one peak of the cell volume was larger than that of their pre-hypotonic shock volume, the other peak was smaller than that of their pre-hypotonic shock volume. Moreover, when the cells were exposed to distilled water (0 mOsmol/kgH₂O), the overall cell volume was smaller than that of the pre-hypotonic shock volume, suggesting that almost all of the cells had broken into fragments (Fig. 4). These findings demonstrated that hypotonic shock with distilled water induced disruption of ESCC cells within 5 min. Additionally, we found differences in the cell volume changes by hypotonic shock among TE5, TE9 and KYSE170 cells. Although the cell volume 1 min after hypotonic shock with distilled water showed bimodal distribution in TE5 and TE9 cells, that in KYSE170 showed monomodal distribution. In TE5 or TE9 cells, the overall cell volume became smaller than the pre-hypotonic one, and the larger one of the two peaks disappeared within 5 min. However, in KYSE170 cells, it took 20 min for the larger peak of the cell volume to

completely disappear after hypotonic shock with distilled water (data not shown), suggesting a difference in resistance to hypotonic shock.

Cytocidal effects of distilled water on ESCC cells. To confirm the cytocidal effects of hypotonic shock with distilled water on ESCC cells, re-incubation of ESCC cells was performed after exposure to distilled water. The decrease in the number of ESCC cells was depended on the duration of exposure to distilled water in each of the three ESCC cell lines (Fig. 5). We also found differences in cytocidal effects of hypotonic shock with distilled water among TE5, TE9 and KYSE170 cells. There were no surviving cells 48 h after 10-min exposure to distilled water in TE5 and TE9 cell lines. However, KYSE170 cells were more resistant to cytocidal effects of hypotonic shock, and 30-min exposure to distilled water was needed for 1.5x10⁵ cells to be killed completely. These findings were consistent with the results of cell volume changes after hypotonic shock, which were more remarkable in TE5 and TE9 cell lines.

Osmolarity of pleural lavage fluid during surgery. It is expected that distilled water poured into pleural cavity may be contaminated by existing intrapleural secretions and many types of cells in the pleural cavity. To demonstrate this, pleural lavage with distilled water was performed in 5 patients during surgery for ESCC. The measured osmolarity of collected fluid in 5 patients is shown in Table I, and determined as 7.6±1.0 (mean ± SEM) mOsmol/kgH₂O. Although the osmolality of collected fluid was not 0 mOsmol/kgH₂O, the elevation was found to be <10 mOsmol/kgH₂O.

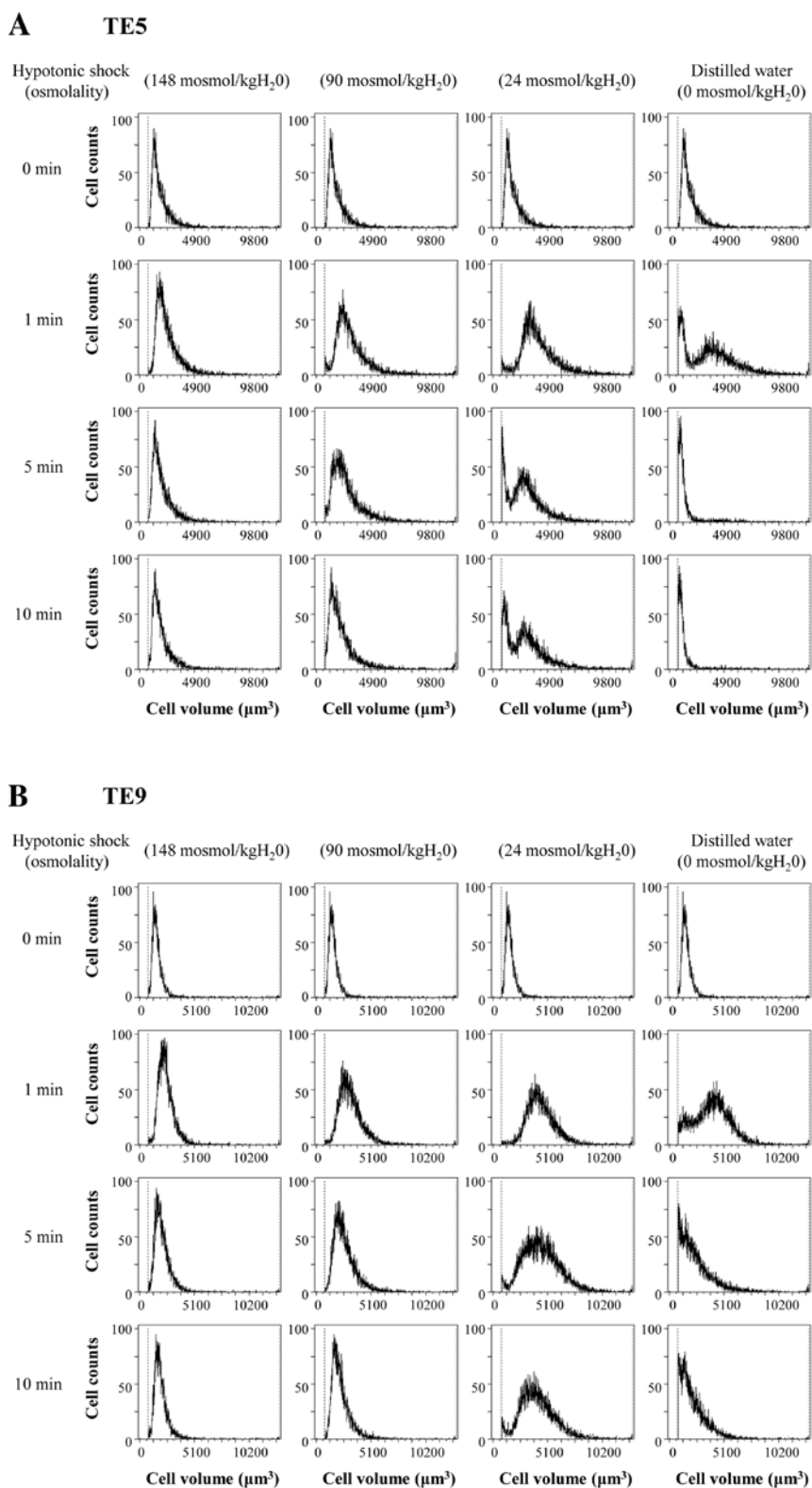


Figure 2A and B. Cell volume changes in TE5 (A) and TE9 (B) ESCC cells after hypotonic shock.

Difference in osmolality between the solutions with and without suspended cells. It was expected that the osmolality of each hypotonic solution with suspended cells would be higher than that without suspended cells as a result of the increase of osmolytes arising from the rupture of ESCC cells. To demonstrate this, the osmolality of each solution with or

without suspended TE5 cells was measured, and there was not a marked difference between two groups (Table II). Although the osmolality of distilled water with suspended cells was around 10 mOsmol/kgH₂O higher than that without suspended cells, this level was higher than the actual osmolality of pleural lavage fluid during surgery.

C KYSE170

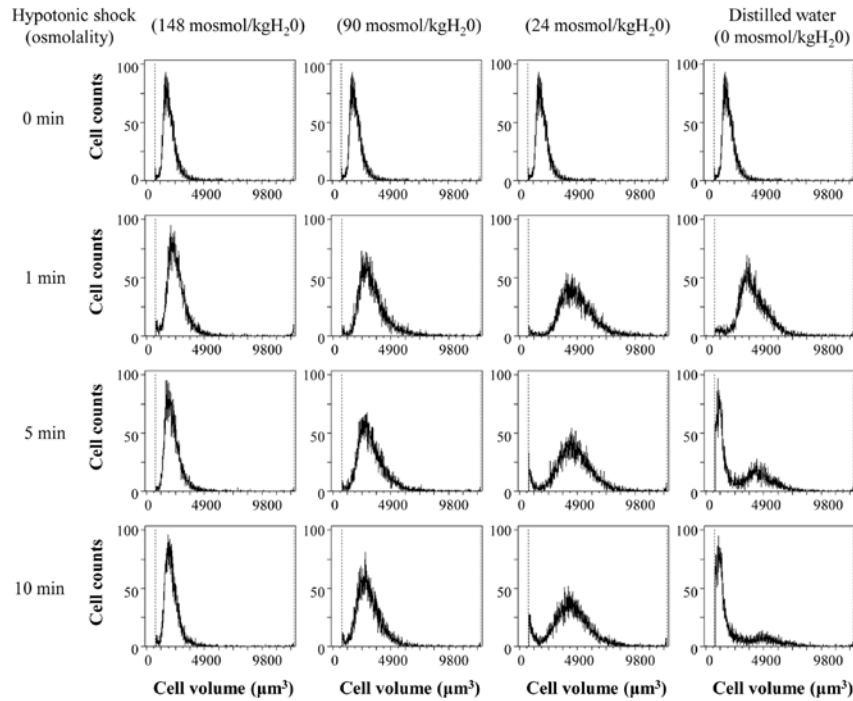


Figure 2C. Cell volume changes in ESCC cells after hypotonic shock. The cell volume and the cell counts of TE5 (A), TE9 (B) and KYSE170 cells (C) were simultaneously measured at 1, 5 and 10 min after hypotonic shock of various osmolarities using a high-resolution flow cytometer, Cell Lab Quanta. The isotonic (300 mOsmol/kgH₂O) NaCl buffer was diluted 2-, 4- and 16-fold with distilled water, and the osmolarity of each hypotonic NaCl buffer is described in the figure. The cell suspension in the isotonic (300 mOsmol/kgH₂O) NaCl buffer was used as a sample without hypotonic shock (0 min). When the cells were exposed to mild hypotonicity (90-148 mOsmol/kgH₂O), the cell volume initially increased and then decreased gradually to the pre-hypotonic shock level, maintaining a monomodal distribution despite the continued presence of the extracellular hypotonicity. When the cells were exposed to even lower osmolarity (24 mOsmol/kgH₂O), the cell volume showed a bimodal distribution. When the cells were exposed to distilled water (0 mOsmol/kgH₂O), the overall cell volume was smaller than the pre-hypotonic shock volume, suggesting that almost all of the cells had broken into fragments.

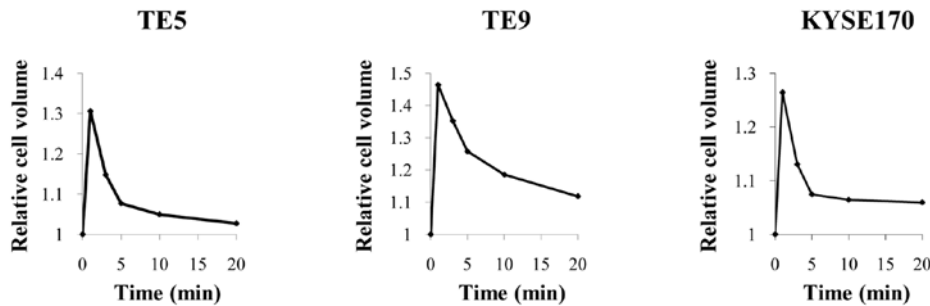


Figure 3. Cell volume changes in ESCC cells after mild hypotonic shock. Mean cell volumes (MCV) of TE5, TE9 and KYSE170 cells 1, 3, 5, 10 and 20 min after hypotonic shock of 148 mOsmol/kgH₂O were presented as values relative to those at 0 min. The cell suspension in isotonic (300 mOsmol/kgH₂O) NaCl buffer was used as a sample without hypotonic shock (0 min). Mild hypotonicity caused a biphasic change in cell volume; i.e., initial cell swelling followed by regulatory volume decrease (RVD) returning the cell volume toward the original level.

NPPB, a Cl⁻ channel blocker, enhances swelling of ESCC cells by hypotonic shock. To investigate whether NPPB, a Cl⁻ channel blocker, effects on the cell volume changes of ESCC cells after hypotonic shock, the cell volume was measured at 1, 5, 10, 20 and 30 min after exposure to the hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 200 µM NPPB or 0.2% DMSO by using Cell Lab Quanta. The serial changes in MCV of TE5, TE9 and KYSE170 cells after exposure to hypotonic solutions with NPPB or DMSO are shown in Fig. 6A. NPPB enhanced swelling of ESCC cells during

hypotonic shock. Furthermore, NPPB drastically slowed down the decrease in cell volume following cell swelling by hypo-tonic shock, suggesting that these effects were induced by the inhibition of RVD (5,6). Therefore, ESCC cells treated with NPPB were forced to maintain an extremely large cell volume for a longer time than those treated with DMSO. The cell volume measured 10 min after exposure to the hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 100 µM NPPB, 200 µM NPPB or 0.1% DMSO is represented as a value relative to that at 0 min in Fig. 6B. We found that NPPB

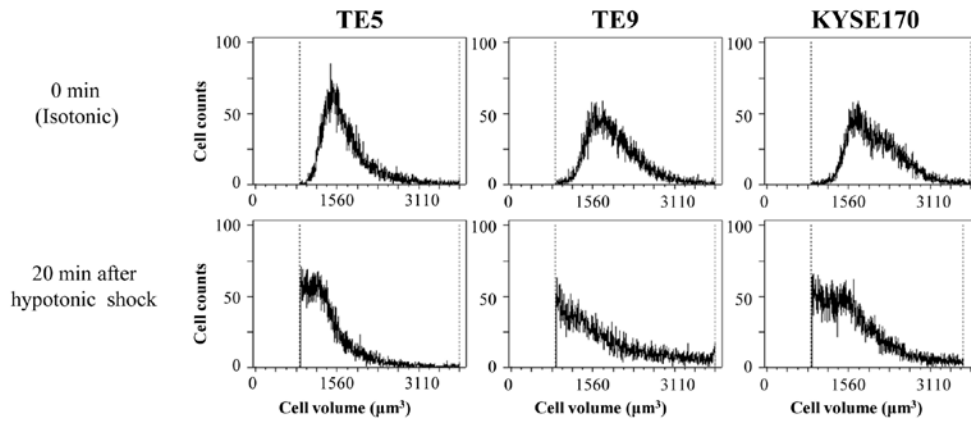


Figure 4. Cell volume of ESCC cells 20 min after hypotonic shock with distilled water. The cell volume and cell counts of TE5, TE9 or KYSE170 cells 20 min after hypotonic shock with distilled water were simultaneously measured by Cell Lab Quanta. The cell suspension in isotonic (300 mOsmol/kgH₂O) NaCl buffer was used as a sample without hypotonic shock (0 min). In the figure, the small area of the cell volume was emphasized. The overall cell volume 20 min after hypotonic shock was smaller than that obtained at 0 min, suggesting that the cells were broken into fragments by hypotonic shock.

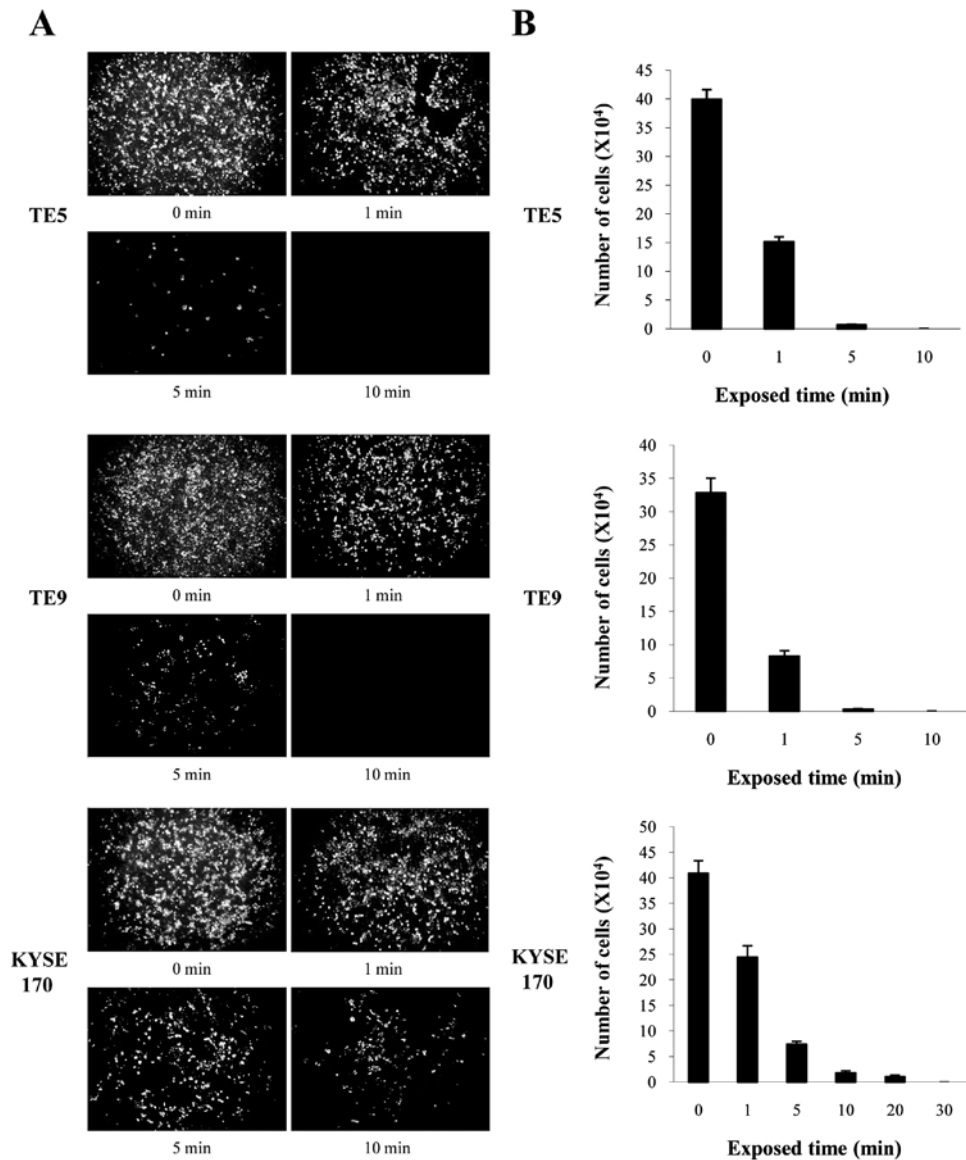


Figure 5. Re-incubation of ESCC cells after exposure to distilled water. (A) Representative pictures of cultured cells 48 h after 1-, 5- and 10-min exposure to distilled water. The cells exposed to isotonic (300 mOsmol/kgH₂O) NaCl buffer were used as a sample without hypotonic shock (0 min). (B) Cell numbers were counted 48 h after exposure to distilled water. The decrease in the number of surviving ESCC cells was dependent on the duration of exposure to distilled water in all three ESCC cell lines. Cytocidal effects induced by hypotonic shock with distilled water differed among TE5, TE9 and KYSE170 cells. The results are presented as the mean ± SEM (n=8).

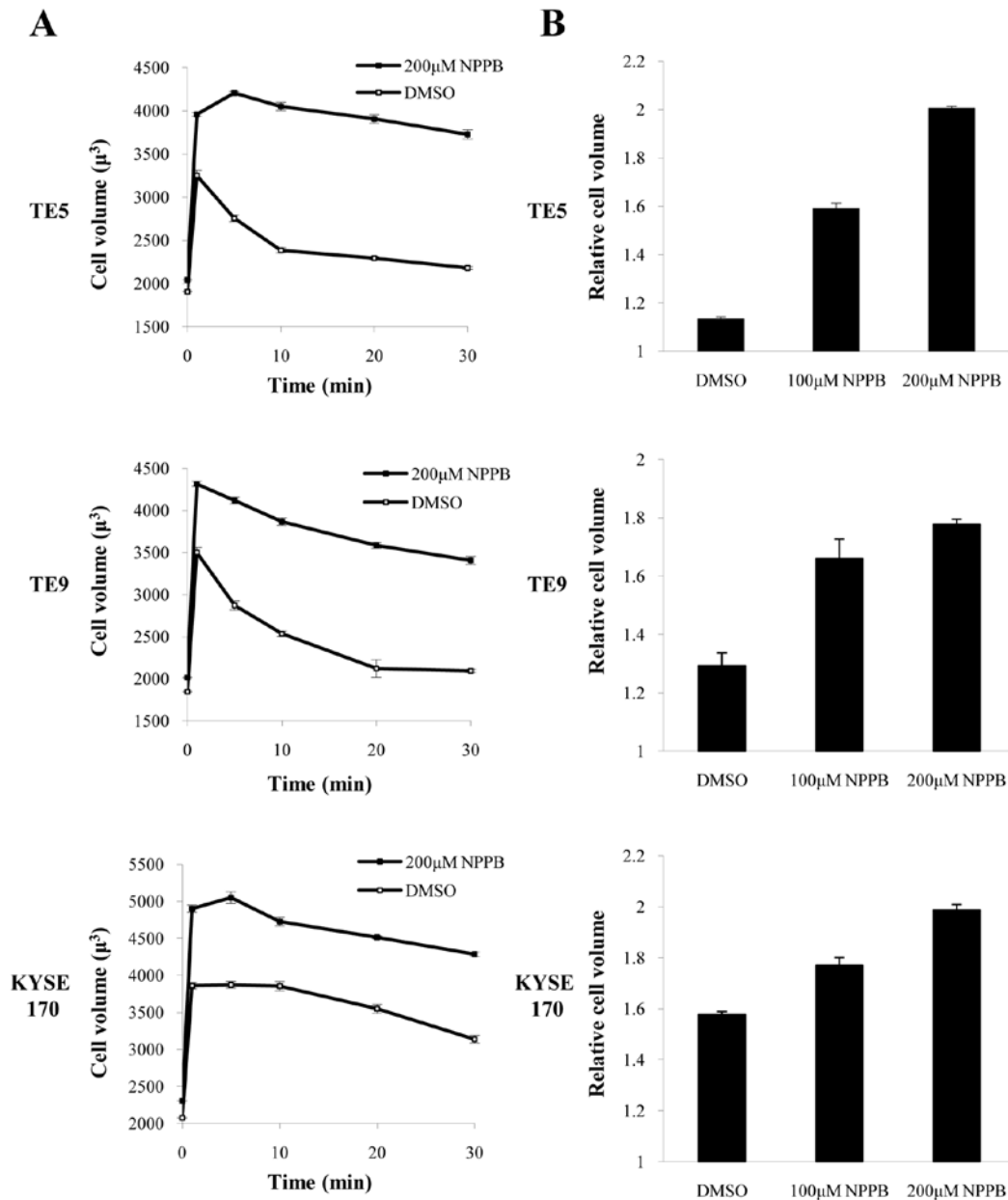


Figure 6. Effects of NPPB on hypotonicity-induced cell volume changes in ESCC cells. (A) The cell volume was measured 1, 5, 10, 20 and 30 min after exposure to hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 200 µM NPPB or 0.2% DMSO. The cell suspension in isotonic (300 mOsmol/kgH₂O) NaCl buffer containing NPPB or DMSO was used as a sample without hypotonic shock (0 min). NPPB enhanced swelling of ESCC cells by hypotonic shock and drastically slowed down the decrease in cell volume after swelling. Each point shows the mean ± SEM (n=4). (B) The cell volume measured 10 min after exposure to hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 100 µM NPPB, 200 µM NPPB or 0.1% DMSO is represented as a value relative to that at 0 min. NPPB enhanced swelling of ESCC cells by hypotonic shock in a dose-dependent manner. The results are presented as the mean ± SEM (n=4).

Table I. Osmolarity of pleural lavage fluid during surgery for ESCC.

	Osmolarity (mOsmol/kgH ₂ O)
Case 1	9
Case 2	8
Case 3	4
Case 4	7
Case 5	10
Mean ± SEM (n=5)	7.6±1.0

enhanced swelling of ESCC cells during hypotonic shock in a dose-dependent manner. The effect of NPPB on cell volume was more remarkable in TE5 and TE9 cells than in KYSE170 cells.

NPPB enhances cytotoxic effects of hypotonic shock on ESCC cells. To investigate whether NPPB enhances cytotoxic effects of hypotonic shock on ESCC cells, re-incubation of ESCC cells was performed after exposure to the hypotonic solution containing NPPB. Fig. 7 showed the number of surviving cells counted 48 h after 10-min exposure to the hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing

Table II. Osmolarity of the solutions with or without suspended TE5 cells.

Dilution ^a	Before cell suspension (mosmol/kgH ₂ O)	After cell suspension (mosmol/kgH ₂ O)
X1 (Isotonic NaCl buffer)	300±4.0	299±6.0
X2	148±4.4	152±2.9
X4	90.0±4.6	78±2.2
X16	24.3±2.0	17±0.3
X0 (Distilled water)	0	10±6.5

The results are presented as the means ± SEM. The isotonic NaCl buffer before cell suspension, n=5; the other solutions, n=3. ^aThe isotonic NaCl buffer was diluted 2-, 4- and 16-fold with distilled water.

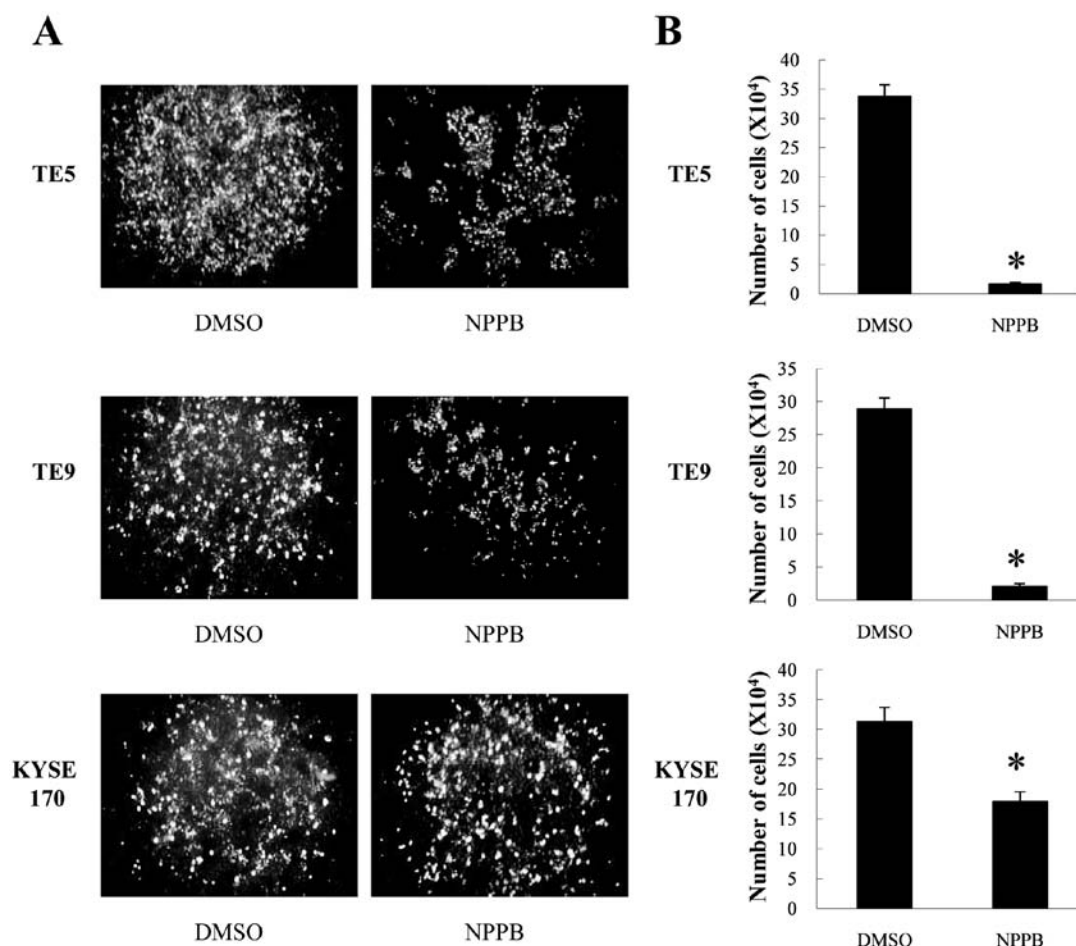


Figure 7. Re-incubation of ESCC cells after exposure to hypotonic solution containing NPPB. (A) Representative pictures of cultured cells 48 h after 10-min exposure to hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 200 μM NPPB or 0.2% DMSO. (B) Cell numbers were counted 48 h after 10-min exposure to hypotonic (90 mOsmol/kgH₂O) NaCl buffer containing 200 μM NPPB or 0.2% DMSO. NPPB enhanced cytotoxic effects of hypotonic shock on ESCC cells. However, KYSE170 cells were more resistant to hypotonicity-induced cytotoxic effects enhanced by NPPB than TE5 and TE9 cells. The results are presented as the mean ± SEM (n=12). *p<0.05 (DMSO vs. NPPB).

200 μM NPPB or 0.2% DMSO. We found that NPPB enhanced the cytotoxic effects of hypotonic shock on all three ESCC cell lines. In this experiment, we also found differences in enhanced cytotoxic effects of hypotonic shock with NPPB among TE5, TE9 and KYSE170 cells. These findings were consistent with the results showing cell swelling induced by NPPB, suggesting that cell volume regulation was the main mechanism underlying its cytotoxic effects.

Discussion

Esophageal carcinoma is one of the main causes of cancer-related death in the world (10). In Asian countries, the predominant histological type of esophageal carcinoma is squamous cell carcinoma, and tumors are frequently located in the thoracic esophagus. As in lung cancer (11-14), the presence of exfoliated cancer cells in the pleural cavity after

resection of thoracic ESCC has been reported, and positive pleural lavage cytology has been recognized as a prognostic indicator of recurrence (1,3). However, esophageal carcinomas are less likely to spontaneously exfoliate into the pleural cavity because most of the thoracic esophagus is not covered directly by the pleura but by adventitia. Therefore, it is suggested that surgical manipulation only occasionally exfoliates and spreads cancer cells in the surgical field. Some previous studies have indicated cytotoxic effects of hypotonic shock on cancer cells (15-17). A previous report demonstrated the cytotoxic activity of distilled water on colorectal cancer cell lines in culture, and also discussed the optimal method of peritoneal lavage with distilled water during colorectal cancer surgery (15). However, there is no underlying data demonstrating the cytotoxic efficacy on ESCC cell lines and hence consensus on the optimal method of pleural lavage during surgery for ESCC is lacking.

As a basic point, we first confirmed that hypotonic shock with distilled water actually has cytotoxic effects on individual ESCC cells using a DIC microscope connected to a high-speed digital video camera. Hypotonic shock with distilled water caused cell swelling followed by rupture of ESCC cells. Although the rupture of ESCC cells was observed within 3 min after perfusion of distilled water in the present experiments, it occurred where the extracellular osmolality might remain at nearly 0 mOsmol/kgH₂O by continuous perfusion of distilled water in the chamber, and hence the changes in ESCC cells in a closed space such as in the pleural cavity were unclear. In addition, this study investigated the effects on only a small number of cells. Therefore, we subsequently investigated the changes in a large number of ESCC cells induced by hypotonic shock in a closed space.

It is very important to understand cell volume changes in ESCC cells after hypotonic shock of various osmolarities and elucidate how much hypotonic shock is needed for ESCC cells to be killed when we consider the mechanism involved in cytotoxic effects by hypotonic shock. We quantified serial cell volume changes of ESCC cells after hypotonic shock of various osmolarities using a high-resolution flow cytometer, Cell Lab Quanta. When the cells were exposed to mild hypotonicity, the cell volume initially increased and subsequently decreased gradually to the pre-hypotonic shock level maintaining a monomodal distribution despite the continued presence of extracellular hypotonicity. Thus, mild hypotonicity could not break ESCC cells into fragments. With respect to these changes, many previous studies reported that hypotonicity caused a biphasic change in the cell volume; i.e., initial cell swelling followed by RVD returning the cell volume toward the original level. Although RVD was reported in many types of cells (5,6,18-22), the present study is the first to report RVD in ESCC cell lines. We found that extremely severe hypotonicity was needed for ESCC cells to be broken into fragments, and demonstrated that distilled water was the most effective.

A previous report found that water used for peritoneal lavage was contaminated by secretions in the peritoneal cavity, but the degree of contamination was reduced after sequential lavage (15). In the present study, distilled water poured into the pleural cavity during surgery became contaminated and the osmolality of collected fluid after lavage

was not 0 mOsmol/kgH₂O, but the elevation was only 7.6±1.0 (mean ± SEM) mOsmol/kgH₂O. However, we also found that the elevation in the osmolality was 10±6.5 (mean ± SEM) mOsmol/kgH₂O as the result of rupture of 7.5×10⁶ TE5 cells exposed to 1-ml distilled water. Therefore, the osmolality of the solution before lavage has a more important influence on cytotoxic effects than that after lavage.

We confirmed that hypotonic shock with distilled water actually has cytotoxic effects on ESCC cells by re-incubation of ESCC cells after exposure to distilled water. Although 1-min exposure to distilled water reduced the surviving cell numbers to a certain extent, it took 10 min for 2.5×10⁵ TE5 or TE9 cells, or 30 min for 1.5×10⁵ KYSE170 cells to be killed completely. However, given the actual situation of surgery for thoracic esophageal carcinoma, it is unrealistic to perform pleural lavage for 30 min before thoracic closure. A previous report found that abdominal lavage with 0.9% saline more significantly reduced the numbers of cells during the first to third lavage cycle (23). Sequential lavage with distilled water for 10 min may be an appropriate method as long as significant proportions of ESCC cells are removed and killed because our study examined a much greater number of ESCC cells than the practical number of exfoliated cells likely to exist in the pleural cavity. However, it can not be denied that the elevation of the osmolality deteriorated the cytotoxic effects on ESCC cells by distilled water. In the sense, sequential lavage may also be effective in that the osmolality of lavage fluid would be closer to 0 mOsmol/kgH₂O with each lavage.

Considering these problems, we added NPPB, a Cl⁻ channel blocker, to the hypotonic (90 mOsmol/kgH₂O) solution to enhance the cytotoxic effects on ESCC cells. The efficiency of RVD depends on many factors. The involvement of K⁺ and Cl⁻ channels (5,6,19,21) as well as stretch-activated channels (SACs) (18,22) in RVD is well documented. Previous reports demonstrated that NPPB inhibits RVD as much as in other cell types (5,6). In our study, we inhibited Cl⁻ channels by application of NPPB and found that NPPB enhanced swelling of ESCC cells during hypotonic shock. Furthermore, NPPB drastically slowed the decrease in cell volume following cell swelling during hypotonic shock. As a result, ESCC cells treated with NPPB were forced to maintain an extremely large cell volume for a long time, and most ESCC cells finally died. Our findings clearly demonstrated that inhibition of Cl⁻ channels during hypotonic shock enhances the cell swelling and cytotoxic effects on ESCC cells.

Throughout these experiments, we found differences in the cell volume changes and the cytotoxic effects induced by hypotonic shock among the three ESCC cell lines examined. As possible mechanisms, the strength of the cytoskeleton or the membrane, or the expression of the Cl⁻ channels may differ among these ESCC cell lines. Further, overexpression of several types of aquaporins (AQP), transmembrane water channel proteins which play an important role in transcellular water movement, has been reported in different types of human cancer (24-27). cAMP has been reported to up-regulate AQP5 expression in lung epithelial cells, hence increasing water permeability (28). Therefore, cell swelling and disruption induced by hypotonic shock may be caused by the expression levels of certain types of ion channels or transporters in cancer cells.

In conclusion, we demonstrated the cytotoxic effects of hypotonic shock induced by distilled water on ESCC cells, and clearly support the efficacy of pleural lavage with distilled water during surgery for ESCC.

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