Cytosolic chloride ion is a key factor in lysosomal acidification and function of autophagy in human gastric cancer cell

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Received: September 17, 2013; Accepted: January 22, 2014

Abstract

The purpose of the present study was to clarify roles of cytosolic chloride ion (Cl⁻) in regulation of lysosomal acidification [intra-lysosomal pH (pHlys)] and autophagy function in human gastric cancer cell line (MKN28). The MKN28 cells cultured under a low Cl⁻/C₀ condition elevated pHlys and reduced the intra-lysosomal Cl⁻ concentration ([Cl⁻]lys) via reduction of cytosolic Cl⁻ concentration ([Cl⁻]c), showing abnormal accumulation of LC3II and p62 participating in autophagy function (dysfunction of autophagy) accompanied by inhibition of cell proliferation via G0/G1 arrest without induction of apoptosis. We also studied effects of direct modification of H⁺ transport on lysosomal acidification and autophagy. Application of bafilomycin A1 (an inhibitor of V-type H⁺-ATPase) or ethyl isopropyl amiloride [EIPA; an inhibitor of Na⁺/H⁺ exchanger (NHE)] elevated pHlys and decreased [Cl⁻]lys associated with inhibition of cell proliferation via induction of G0/G1 arrest similar to the culture under a low Cl⁻ condition. However, unlike low Cl⁻ condition, application of the compound, bafilomycin A1 or EIPA, induced apoptosis associated with increases in caspase 3 and 9 without large reduction in [Cl⁻]c compared with low Cl⁻ condition. These observations suggest that the lowered [Cl⁻]c primarily causes dysfunction of autophagy without apoptosis via induction of lysosome induced by disturbance of intra-lysosomal acidification. This is the first study showing that cytosolic Cl⁻ is a key factor of lysosome acidification and autophagy.

Keywords: autophagy • chloride ion • lysosome • pH • apoptosis

Introduction

Autophagy has been found to be a self-nutrient-providing system for cell survival under starvation conditions [1–3], and an important system for degradation of miss-folded or long-lived proteins, and superfluous or damaged organelle such as mitochondria [4–6]. Starvation-caused poverty of nutrients induces autophagy for cell survival via autophagy-mediated recycling of nutrients contained in cells themselves [1]. Cells mainly produce amino acids via autophagy-mediated process by digesting their own proteins [1]. New proteins are synthesized from these amino acids provided by autophagy [1]. As mentioned above, autophagy is, in general, activated by starvation. However, it has been recently suggested that autophagy process functions even under conditions with rich nutrition [7], and that impairment or activation of autophagy closely relates to pathogenesis of diverse diseases including Parkinson disease [6], diabetes mellitus [8], inflammatory disease such as Crohn disease [9] and cancer [10].

As cancer cells survive under hypoxic and hypo-nutrient microenvironments, cancer cells elevate autophagy ability to use recyclable materials [10]. It has been clarified that impairment of...
autophagy system by knocking down Atg5 or Atg7 induces apoptosis of cancer cells, inhibiting cell growth [11–13]. Autophagy is a catabolic process degrading cell components mediated through lysosomal machineries. Lysosome is, therefore, a key organelle in autophagy degrading various compounds [3]. In fact, at the final phase of degradation of proteins in autophagy process, lysosomes fuse to autophagosomes followed by lysosomal enzyme-mediated digestion of proteins.

The digesting activity of lysosomal enzymes depends on intralysosomal acidity, which is primarily generated by V-type H⁺-ATPase (proton pump) co-operating with ClC-7, Cl⁻/H⁺ antiporter, which is assumed to participate in Cl⁻ movement [14]; ClC-7 has 2Cl⁻/1H⁺ exchange stoichiometry [15]. The ClC-7 located on lysosome membrane would primarily behave as a Cl⁻ permeation pathway in lysosomal membrane [14]. Mutation of ClC-7 induces abnormal accumulation of proteins into intra-lysosomal meaning disturbance of lysosomal function [16]. It is also reported that inhibition of ClC-7 by siRNA impairs lysosomal acidification [14] and induces abnormal accumulation of proteins in lysosomes resulting in inhibition of cell proliferation [17]. The observations [14, 16] suggest that Cl⁻ movement/transport would essentially play an important role in lysosomal acidification and cell proliferation via autophagy. However, it has not been confirmed that the functional presence of Cl⁻ transporter, ClC-7, is essentially required for lysosomal acidification and autophagy function. Namely, there are no direct evidence indicating that the presence and movement/transport of Cl⁻ are essentially required for lysosomal acidification and autophagy function. In other words, it is still unclear if the presence of Cl⁻ itself as a target ion transported by ClC-7 plays an essential role in lysosomal acidification and autophagy function.

Our previous reports indicated that Cl⁻ plays various important roles in cellular functions; namely, lowering cytosolic Cl⁻ inhibits proliferation of cancer cells [18–26] and elongation of neurite in neuronal cells [27–31], but activates expression of epithelial Na⁺ channel [32–34] and Na⁺-permeant channel [35]. Thus, we tried to clarify the role of Cl⁻ in acidification of lysosome and function of autophagy in the present study by using a model cancer cell line (MKN28) by replacing Cl⁻ with NO₃⁻, which generally has permeability identical to Cl⁻ via itself as a target ion transported.

Materials and methods

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, bafilomycin A1 (an inhibitor of V-type H⁺-ATPase), ethyl isopropyl amiloride (EIPA; an inhibitor of Na⁺/H⁺ exchanger (NHE)), acridine orange (AO) and valinomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Na⁺/Ethoxybenzylmethyl)-6-methoxy quinolinium (MQAE) and carboxy-seminaphthorhodaflo-1 (carboxy-SNARF-1) were obtained from Invitrogen (Carlsbad, CA, USA). Nigericin, tributyltin chloride and monensin were purchased from Wako Pure Chemical Industries (Osaka, Japan). The moderately differentiated human gastric adenocarcinoma cell line, MKN28, was obtained from ATCC (Manassas, VA, USA).

Culture conditions

MKN28 cells were seeded into 25 cm² flasks at a density of 2.5 × 10⁵ cells/flask and incubated for 24 hrs in RPMI1640 medium (Sigma-Aldrich) supplemented with 5% foetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO₂ in air. We defined this time-point as time zero (0 hr) at 24 hrs culture after seeding. Cells were then cultured for 48 hrs under six different conditions: (1) RPMI medium as control, (2) RPMI medium with a low Cl⁻ concentration (low Cl⁻ medium; low Cl⁻ condition), (3) RPMI medium with a low pH (low pH medium; low Cl⁻ condition), (4) RPMI medium containing bafilomycin A1, (5) RPMI medium containing EIPA and (6) RPMI containing dimethyl sulfoxide (DMSO) as solvent control for bafilomycin A1 and EIPA. For RPMI medium with a low Cl⁻ concentration, we used a Cl⁻-replaced RPMI1640 medium (Cell Science and Technology Institute, Sendai, Japan) prepared by substituting Cl⁻ with NO₃⁻, as previously reported [20]. For RPMI with a low pH, we adjusted pH of medium by adding HNO₃ to pH 6.9. The concentrations of bafilomycin A1, EIPA and DMSO (solvent control for bafilomycin A1 and EIPA) were respectively 1 nM, 25 μM and 0.1% as previously reported [18].

Measurement of cytosolic pH (pHc)

Cells were seeded into 35 mm glass bottom dishes at a density of 1.0 × 10³ cells/dish and incubated for 24 hrs in RPMI1640 medium supplemented with 5% FBS in a humidified incubator at 37°C with 5% CO₂ in air followed by culture under six different conditions for 48 hrs in a humidified incubator at 37°C with 5% CO₂ in air. We measured pHc of MKN28 cells under each condition by using carboxy-SNARF-1, a pH-sensitive fluorescent dye, with an inverted confocal laser microscope, LSM510 META (Carl Zeiss, Jena, Germany) at 37°C in air with 5% CO₂. The emission ratio was calibrated and used to estimate the pHc under each condition from the calibration curve. We evaluated the mean value of pHc under different culture dishes. By using the mean values of pHc obtained from different cell culture dishes, we calculated the mean value of pHc shown in the present study. Namely, ‘n’ shown in the present study means the number of cell culture dishes used for measurement of pHc.

Measurement of lysosomal pH (pHlys)

We cultured cells by using the condition same as measurement of cytosolic pH. We measured pHlys of MKN28 cells cultured under each condition at 37°C with 5% CO₂ incubation, by using LysoSensor™ Yellow/Blue DND-160 (Invtrogen) with an inverted confocal laser microscope, LSM510META (Carl Zeiss). LysoSensor-loaded cells were washed with each conditioning medium, and incubated for 20 min. at 37°C. The intensity of LysoSensor in the chamber on the stage of the microscope (ZILCS) was then measured by LSM510 META system. The sample was excited at 780 nm with a 2-photon excitation laser system (MaTiAir®.
Measurement of cytosolic chloride ion concentration ([Cl\textsuperscript{−}]\textsubscript{c})

We cultured cells by using the condition same as measurement of cytosolic pH. We measured [Cl\textsuperscript{−}]\textsubscript{c} of MKN28 cells cultured under each different condition by using MOAE at 37°C in air with 5% CO\textsubscript{2} with an inverted confocal laser microscope, LSM510 META (Carl Zeiss) as previously reported [18]. The sample was excited at 780 nm for MOAE by using a 2-photon excitation laser system (MaiTai\textsuperscript{®}, Spectra-Physics). The emitted fluorescence was simultaneously collected by a gating, and the separated fluorescence was detected by 24 PMTs. We collected one PMT at 460 nm for MOAE. MOAE is insensitive to pH changes within physiological ranges. The intensity of fluorescence was digitized with a META system. Several ROI were then randomly selected excluding vesicles and nuclear regions. Calibration of the fluorescence based on [Cl\textsuperscript{−}]\textsubscript{c} was accomplished by 5 μM nigericin, 5 μM valinomycin (K\textsuperscript{+} ionophore) and 10 μM tributyltin chloride (Cl\textsuperscript{−}/OH\textsuperscript{−} ionophore) in 140 mM-K\textsuperscript{+} calibration buffers (pH 7.4) with various Cl\textsuperscript{−} concentrations. The ionophores were used to adjust the [Cl\textsuperscript{−}]\textsubscript{c} to a level identical to the extracellular Cl\textsuperscript{−} concentration at constant cytosolic K\textsuperscript{+} and H\textsuperscript{+} concentrations. To evaluate the mean value of [Cl\textsuperscript{−}]\textsubscript{c}, we applied the experimental procedure same as pH\textsubscript{c} measurement. The meaning of ‘n’ is same as that of pH\textsubscript{c}.

Measurement of lysosomal Cl\textsuperscript{−} concentration ([Cl\textsuperscript{−}]\textsubscript{lys})

We cultured cells by using the condition same as measurement of cytosolic pH. Cells cultured in a glass bottom dish with the culture medium were stained with MOAE and a lysosome-detectable dye, AO, accumulating into lysosomes in each culture medium containing 5 mM MOAE and 3 μM AO for 30 min. in a humidified incubator at 37°C with 5% CO\textsubscript{2} in air. We measured [Cl\textsuperscript{−}]\textsubscript{lys} of MKN28 cells under each condition by detecting MOAE fluorescence at 37°C in air with 5% CO\textsubscript{2} by using an inverted confocal laser microscope, LSM510 META (Carl Zeiss). The sample was excited at 780 nm for MOAE by using a 2-photon excitation laser system (MaiTai\textsuperscript{®}, Spectra-Physics). The emitted fluorescence was then simultaneously collected by a gating, and the separated fluorescence was detected by 24 PMTs. We collected one PMT at 460 nm for MOAE. To detect lysosomal areas, the sample was excited at 488 nm by using an Ar laser for measurement of AO fluorescence and the emitted fluorescence was simultaneously collected by a gating, and the separated fluorescence was detected by 24 PMTs. We collected one PMT at 640 nm for AO fluorescence. The intensity of MOAE in lysosome detected by AO fluorescence in the chamber on the stage of the microscope (ZILCS) was measured by the LSM510 META system. The intensity of fluorescence was digitized with a META system. Several ROI in lysosomal areas were then randomly selected. Calibration of the fluorescence based on [Cl\textsuperscript{−}]\textsubscript{lys} was accomplished by 5 μM nigericin, 5 μM valinomycin and 10 μM tributyltin chloride in 140 mM-K\textsuperscript{+} calibration buffers (pH 7.4) with various Cl\textsuperscript{−} concentrations to determine the [Cl\textsuperscript{−}]\textsubscript{lys} under each condition. To evaluate the mean value of [Cl\textsuperscript{−}]\textsubscript{lys}, we applied the experimental procedure same as pH\textsubscript{lys} measurement. The meaning of ‘n’ is same as that of pH\textsubscript{lys}.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by using a multiple comparison test (Tukey-Kramer). The differences were considered significant when the P < 0.05.
Results

Cytosolic proton concentration (pHc)

Cell culture in low Cl⁻ medium significantly decreased pHc compared with that in normal medium (Fig. 1). As low Cl⁻ condition led pHc to a lowered level, we tried to mimic the effect of low Cl⁻ condition on pHc by culturing cells in low pH medium expected to decrease pHc. As expected, pHc of cells cultured in low pH medium was almost identical to that in low Cl⁻ medium, which was much lower than that in normal medium. Bafilomycin A1 slightly decreased pHc, but its effect on pHc was much smaller than that in low Cl⁻ or pH medium (Fig. 1). Ethyl isopropyl amiloride had no significant effect on pHc (Fig. 1). Application of DMSO alone (solvent control for bafilomycin A1 and EIPA) had no significant effects on pHc compared with normal condition (Fig. 1).

![Fig. 1 Measurement of pHc in MKN28 cells exposed to low Cl⁻ condition, low pH condition, bafilomycin A1 (BA), ethyl isopropyl amiloride (EIPA) or DMSO alone as solvent control for bafilomycin A1 or EIPA. Low Cl⁻ condition, low pH condition and bafilomycin A1 caused significant decreases of pHc in MKN28 cells; n = 4, *P < 0.05.](image1)

Lysosomal pH (pHlys)

As shown in Figure 2, cells cultured in normal medium showed pHlys a little bit lower than 4.0, and application of DMSO alone had no significant effect on pHlys. pHlys in low Cl⁻ medium was much higher than that in normal medium (Fig. 2). However, although low pH condition decreased pHlys similar to low Cl⁻ condition, pHlys in low pH medium was lower than that in normal medium unlike low Cl⁻ condition (Fig. 2). Application of bafilomycin A1 or EIPA increased pHlys to a level identical to that in low Cl⁻ medium (Fig. 2).

![Fig. 2 Measurement of pHlys in MKN28 cells exposed to low Cl⁻ condition, low pH condition, bafilomycin A1 (BA), ethyl isopropyl amiloride (EIPA) or DMSO alone. Low Cl⁻ condition, bafilomycin A1 and EIPA caused significant increases of pHlys in MKN28 cells; n = 4, *P < 0.05.](image2)

Cytosolic chloride ion concentration ([Cl⁻]c)

As shown in Figure 3, [Cl⁻]c of cells cultured in low Cl⁻ medium was much lower than that in normal medium; application of DMSO alone (solvent control for bafilomycin A1 and EIPA) had no significant effects on [Cl⁻]c compared with normal condition (Fig. 1). Low Cl⁻ condition and EIPA significantly decreased [Cl⁻]c in MKN28 cells. Low pH, bafilomycin A1 (BA) or DMSO caused no significant change in [Cl⁻]c in MKN28 cells; n = 4, *P < 0.05.

![Fig. 3 Measurement of [Cl⁻]c in MKN 28 cells exposed to low Cl⁻ condition, low pH condition, bafilomycin A1 (BA), ethyl isopropyl amiloride (EIPA) or DMSO alone. Low Cl⁻ condition and EIPA significantly decreased [Cl⁻]c in MKN28 cells. Low pH, bafilomycin A1 (BA) or DMSO caused no significant change in [Cl⁻]c in MKN28 cells; n = 4, *P < 0.05.](image3)

Lysosomal chloride ion concentration ([Cl⁻]lys)

As shown in Figure 4, application of DMSO alone (solvent control for bafilomycin A1 and EIPA) had no significant effects on [Cl⁻]lys compared with normal condition (Fig. 1). Low Cl⁻ condition, bafilomycin A1 (BA) and EIPA caused significant decreases of [Cl⁻]lys in MKN28 cells; n = 3, *P < 0.05.

![Fig. 4 Measurement of [Cl⁻]lys in MKN28 cells exposed to low Cl⁻ condition, low pH condition, bafilomycin A1 (BA), ethyl isopropyl amiloride (EIPA) or DMSO alone. Low Cl⁻ condition, bafilomycin A1 (BA) and EIPA, but not low pH condition caused significant decreases of [Cl⁻]lys in MKN28 cells; n = 3, *P < 0.05.](image4)
had no significant effects on $[\text{Cl}^-]_c$ compared with normal condition (Fig. 3). Furthermore, $[\text{Cl}^-]_c$ of cells treated with EIPA was significantly lower than that in normal or DMSO-containing medium, however the EIPA-induced decrease in $[\text{Cl}^-]_c$ was much smaller than that induced by culture in a low Cl$^-$/medium. On the other hand, low pH or bafilomycin A1 had no significant effects on $[\text{Cl}^-]_c$ compared with that in normal or DMSO-containing medium (Fig. 3).

**Lysosomal chloride ion concentration ([Cl$^-]$$_{ly}$)**

$[\text{Cl}^-]_{ly}$ significantly decreased in low Cl$^-$/medium compared with that in normal or DMSO-containing medium (Fig. 4). DMSO alone had no significant effect on $[\text{Cl}^-]_{ly}$ (Fig. 4). Bafilomycin A1 or EIPA decreased $[\text{Cl}^-]_{ly}$ to a level identical to that in low Cl$^-$/medium (Fig. 4). Low pH medium had no significant effect on $[\text{Cl}^-]_{ly}$ (Fig. 4).

**Cell proliferation**

Cell proliferation of MKN28 cells cultured in low Cl$^-$/low pH, bafilomycin A1 or EIPA condition was significantly diminished (Fig. 5).

**Cell cycle analysis**

As mentioned above, low Cl$^-$/low pH, bafilomycin A1 or EIPA condition diminished cell proliferation. Therefore, we analysed cell cycle under these conditions. Cell cycle analysis indicates that application of low Cl$^-$/medium, bafilomycin A1 or EIPA significantly increased the G0/G1 fraction associated with reduction of the S-phase fraction (Fig. 6). However, although low pH condition diminished cell proliferation, low pH condition has no significant effect on any cell cycle phases (Fig. 6). These observations suggest that application of low Cl$^-$/bafilomycin A1 or EIPA induces G0/G1 arrest, but that low pH condition induces no specific arrest of cell cycle (Fig. 6).

**Autophagy-related proteins**

Markers of autophagy degrading function, LC3 I, LC3 II and p62 [37, 38], were detected under each different condition. Application of low Cl$^-$/bafilomycin A1 or EIPA significantly elevated amounts of those proteins compared with those under normal or DMSO-containing medium (Fig. 7).
medium, but low pH condition did not significantly affect amounts of those proteins (Fig. 7).

**Discussion**

Our present study focused on clarifying roles of cytosolic Cl\(^{-}\) in lysosomal acidification required for autophagy. Our observations shown in the present study indicate that cell culture in low Cl\(^{-}\) medium significantly increased compared with that in normal medium, but not under other conditions (Fig. 8A and C). These observations suggest that apoptosis would be prevented by lowering [Cl\(^{-}\)], via an increase in phosphorylated Akt, and that low Cl\(^{-}\) condition diminishes cell proliferation without induction of apoptosis.

Phosphorylated Akt, active form of Akt, is well known to prevent apoptosis via several pathways [39]. Phosphorylated Akt (Ser473) in low Cl\(^{-}\) medium significantly increased compared with that in normal medium, but not by low Cl\(^{-}\) or pH condition. Taken together, these observations shown in Figures 7 and 8 suggest that impairment of autophagy would not be functionally correlated with apoptosis.

Application of EIPA had an action on pH\(_{\text{lys}}\) and [Cl\(^{-}\)]\(_{\text{lys}}\) similar to

**Apoptosis-related proteins**

Expression of cleaved caspase 3 and 9, apoptosis-related proteins, drastically increased in cells treated with bafilomycin A1 or EIPA compared with those in normal or DMSO-containing medium (Fig. 8A and B). In low Cl\(^{-}\) or pH medium, we detected no significant change in expression of cleaved caspase 3 compared that in normal or DMSO-containing medium (Fig. 8A and B). We also observed that expression of caspase 9 was slightly increased by low Cl\(^{-}\) condition but decreased by low pH, however the effect of low Cl\(^{-}\) or pH on expression of cleaved caspase 3 and 9 was much less than that of bafilomycin A1 or EIPA (Fig. 8A and B). These observations suggest that apoptosis would be induced by application of bafilomycin A1 or EIPA, but not by low Cl\(^{-}\) or pH condition. Taken together, these observations shown in Figures 7 and 8 suggest that impairment of autophagy would not be functionally correlated with apoptosis.

Phosphorylated Akt, active form of Akt, is well known to prevent apoptosis via several pathways [39]. Phosphorylated Akt (Ser473) in low Cl\(^{-}\) medium significantly increased compared with that in normal medium, but not by other conditions (Fig. 8A and C). These observations suggest that apoptosis would be prevented by lowering [Cl\(^{-}\)], via an increase in phosphorylated Akt, and that low Cl\(^{-}\) condition diminishes cell proliferation without induction of apoptosis.

Our present study focused on clarifying roles of cytosolic Cl\(^{-}\) in lysosomal acidification required for autophagy. Our observations shown in the present study indicate that cell culture in low Cl\(^{-}\) medium decreased [Cl\(^{-}\)]\(_{\text{c}}\) with lowered pH\(_{\text{c}}\) and also increased pH\(_{\text{lys}}\) with decreased [Cl\(^{-}\)]\(_{\text{lys}}\), leading cells to dysfunction of autophagy via impairment of lysosomal digestion and G0/G1 arrest-caused inhibition of cell growth without apoptosis. It is suggested that the presence of Cl\(^{-}\) itself but not CIC-7 itself might be essentially required for lysosomal acidification and autophagy function.

The mechanism of low pH\(_{\text{c}}\) induced by culture in low Cl\(^{-}\) medium would be because of lowered activity of NHE and/or Na\(^{+}\)-driven Cl\(^{-}\)/bicarbonate (HCO\(_3^{-}\)) exchanger (NDCBE) participating in HCO\(_3^{-}\) uptake into cytosolic space caused by lowered [Cl\(^{-}\)]\(_{\text{c}}\), as NHE activity is diminished by lowering [Cl\(^{-}\)]\(_{\text{c}}\) [40], and NDCBE activity also depends on [Cl\(^{-}\)]\(_{\text{c}}\) [41]. The mechanism increasing pH\(_{\text{lys}}\) observed in low Cl\(^{-}\) medium would be because of an insufficient amount of counter anion, Cl\(^{-}\), in the lysosomal space (lowered [Cl\(^{-}\)]\(_{\text{lys}}\)) co-transported into the extra-luminal space at proton moving into intra-lysosomal space disturbing proton movement into intra-lysosomal space. Thus, an insufficient amount of cytosolic Cl\(^{-}\) would cause elevation of pH\(_{\text{lys}}\) associated with low [Cl\(^{-}\)]\(_{\text{lys}}\), and NO\(_3^{-}\) would not play an identical role to Cl\(^{-}\) in lysosomal acidity or autophagy function (see the conclusion and Fig. 9 in detail).

To maintain acidic conditions of intra-lysosomal space is very important for function of lysosomal digestion mediated by more than 70 kinds of enzymes such as proteinase, lipase, nuclease etc. In autophagy, proteinases including cathepsin B, D and L [42–44] are potent enzymes to degrade proteins in lysosomes. The pH maintaining optimal activity of these cathepsins ranges from 3.0 to 4.0 [45–48]. This means that increased pH\(_{\text{lys}}\) higher than 4.0 might diminish activity of lysosomal digestion of proteins, leading to disturbance of autophagy. Application of EIPA had an action on pH\(_{\text{lys}}\) and [Cl\(^{-}\)]\(_{\text{lys}}\) similar to
bafilomycin A1; i.e. each compound significantly increased \( \text{pH}_{\text{lys}} \) to a level higher than 4.0 associated with a decrease in \( [\text{Cl}^-]/[\text{C}^0] \), causing dysfunction of autophagy. However, the mechanism of \( \text{pH}_{\text{lys}} \) increased by EIPA is different from that by bafilomycin A1, which primarily inhibits proton uptake into the intra-lysosomal space. As we have previously reported, EIPA primarily causes a decrease in \( [\text{Cl}^-]/[\text{C}^0] \) by inhibiting NHE [18] unlike primary inhibition of \( \text{H}^+ \) movement by bafilomycin A1. This decreased \( [\text{Cl}^-]/[\text{C}^0] \) would diminish \( [\text{Cl}^-]/[\text{C}^0] \) associated with increased \( \text{pH}_{\text{lys}} \) via a mechanism similar to that under the low \( [\text{Cl}^-]/[\text{C}^0] \) condition, although the effect of EIPA was smaller than that of low \( [\text{Cl}^-]/[\text{C}^0] \) condition. Based on these observations shown in the present study, it is clear that lowered \( [\text{Cl}^-]/[\text{C}^0] \) has potential to increase \( \text{pH}_{\text{lys}} \), disturbing autophagy because of elevated \( \text{pH}_{\text{lys}} \)-induced inhibition of enzymatic digestion in lysosomes.

In the present study, we indicate that the proliferation of MKN28 cells was diminished under each tested condition except DMSO alone. In cell cycle analysis, low \( [\text{Cl}^-]/[\text{C}^0] \) condition, bafilomycin A1 or EIPA induced G0/G1 arrest, whereas low pH condition inhibited proliferation without any specific cell cycle arrest. We have already reported that lowered \( [\text{Cl}^-]/[\text{C}^0] \) causes G0/G1 arrest in MKN28 cells via activation of MAP kinase [18, 36]. It has been demonstrated that decreased \( \text{pH}_{\text{c}} \) elevates the expression of checkpoint proteins for cell cycle (e.g. cyclinB1 and cdc2) or signal transduction-related proteins (e.g. Wee1 kinase) [49]. However, the present study shows that low \( \text{pH}_{\text{c}} \) condition did not induce any specific cell cycle arrest, but would cause slow transition rates at all phases of cell cycle or cell stemness similar to an observation that low \( \text{pH}_{\text{c}} \) condition induces dormancy in cancer cells [50].

Low \( \text{Cl}^- \) condition caused increases in LC3 II and p62 (Fig. 7). Inhibition of autophagy leads to an increase in p62 [51], while the amount of LC3 II is correlated with the number of autophagosomes [52]. The observations on low \( \text{Cl}^- \)-caused increases in LC3 II and p62 look contradictory. However, it is also reported that LC3 II itself is degraded by autophagy [53]; i.e. impairment of autophagy elevates the amount of LC3 II by inhibiting the degradation of LC3 II [53]. The increase in both LC3 II and p62 observed under low \( \text{Cl}^- \) condition would be because of impairment of autophagy, suggesting that low \( \text{Cl}^- \) condition would impair autophagy.

It is clarified that knock down of Atg5 or Atg7 causes impairment of autophagy system associated with apoptosis of cancer cells and
inhibition of cell growth [11–13]. However, the relationship between induction of apoptosis and impairment of autophagy still is unclear. Amaravadi et al. [11] and Guo et al. [12] report that impairment of autophagy induces caspase-dependent apoptosis. On the other hand, Racoma et al. [54] report that a lysosome acidification inhibitor, weak base, induces caspase-independent apoptosis associated with dysfunction of autophagy via release of cathepsin B from lysosome. Thus, the present study indicates that each application of low Cl\(^-\) condition, bafilomycin A1 or EIPA caused dysfunction of autophagy. On the one hand, increased expression of caspase3 and caspase9 related to apoptosis was observed in cases of bafilomycin A1 and EIPA, but not under low Cl\(^-\) condition. Furthermore, bafilomycin A1 inhibited phosphorylation of Akt, which could accelerate apoptosis. On the other hand, low Cl\(^-\) condition could prevent apoptosis by enhancing phosphorylation of Akt. These observations suggest that: (i) both low Cl\(^-\) condition and application of bafilomycin A1 inhibited cell growth associated with dysfunction of autophagy; (ii) the inhibition of cell growth caused by low Cl\(^-\) condition would not induce apoptosis via enhancement of Akt phosphorylation, but bafilomycin A1-caused inhibition of cell growth would induce apoptosis via diminution of Akt phosphorylation. Akt is a key regulator of a variety of proteins involved in cell proliferation, survival and apoptosis. The PI3K/Akt pathway is important in cancer cells to survive, proliferate and prevent apoptosis. Phosphorylated Akt, the active form of Akt, is well known to prevent apoptosis via several pathways [39, 55]. Phosphorylated Akt in low Cl\(^-\) medium significantly increased compared with that in normal medium, but not under other conditions. These observations suggest that apoptosis would be prevented by lowering [Cl\(^-\)], via an increase in phosphorylated Akt, and that low Cl\(^-\) condition diminishes cell proliferation without induction of apoptosis. However, it is still unclear whether the low Cl\(^-\)-induced impairment of autophagy performs cross-talk to phosphorylation of Akt or occurs in parallel with phosphorylation of Akt. Further studies are required to clarify the relationship between autophagy dysfunction and phosphorylation of Akt, although low Cl\(^-\) condition could prevent apoptosis by enhancing phosphorylation of Akt.

**Fig. 9** Action of low Cl\(^-\) on lysosomal function. (1) lowered Cl\(^-\) concentration of extracellular space ([Cl\(^-\)]\(_e\)) decreases [Cl\(^-\)]\(_i\) ([Cl\(^-\)]\(_i\) ↓ → [Cl\(^-\)]\(_i\)↓); (2) lowered [Cl\(^-\)]\(_i\); diminishes activity of CIC-7 ([Cl\(^-\)]\(_i\)↓ → CIC-7↓); (3) lowered activity of CIC-7 decreases [Cl\(^-\)]\(_lys\) ([CIC-7↓ → [Cl\(^-\)]\(_lys\)↓]); (4) both lowered CIC-7 activity and [Cl\(^-\)]\(_lys\) elevate the amount of positive charges remaining in intra-lysosomal space (CIC-7↓/ [Cl\(^-\)]\(_lys\)↓ → positive charges in lysosome↑); (5) elevation of amount of positive charges remaining in intra-lysosomal space diminishes transporting activity V-ATPase (V-type H\(^+\)-ATPase) via an increase in electrical barrier for V-ATPase transporting a positive charge against the electrical barrier across the lysosomal membrane (positive charges in lysosome↑ → V-ATPase↓); (6) lowered activity of V-ATPase induces an increase in pH\(_lys\) (a decrease in H\(^+\) concentration intra-lysosomal space; V-ATPase↓ → pH\(_lys\)↑) and (7) lowered pH\(_lys\) disturbs autophagy function (V-ATPase↓ → autophagy↓).

**Conclusions**

Observations shown in the present study suggest the following points (Fig. 9): (i) lowered Cl\(^-\) concentration of extracellular space ([Cl\(^-\)]\(_e\)) decreases [Cl\(^-\)]\(_i\) ([Cl\(^-\)]\(_i\)↓ → [Cl\(^-\)]\(_i\)↓); (ii) lowered [Cl\(^-\)]\(_i\) diminishes activity of CIC-7; (iii) lowered activity of CIC-7 with exchange of 2Cl\(^-)/1H\(^+\) diminishes [Cl\(^-\)]\(_lys\)↓; (iv) both lowered activity of CIC-7 and [Cl\(^-\)]\(_lys\)↓ elevate the amount of positive charges remaining in intra-lysosomal space; (v) elevation of amount of positive charges remaining in intra-lysosomal space diminishes activity V-type H\(^+\)-ATPase via an increase in electrical barrier for V-type H\(^+\)-ATPase transporting a positive charge against the electrical barrier across the lysosomal membrane; (vi) lowered activity of V-type H\(^+\)-ATPase induces an increase in pH\(_lys\) (a decrease in H\(^+\) concentration intra-lysosomal space) and (vii) lowered pH\(_lys\) disturbs autophagy function.

**Acknowledgement**

This work was supported by Grants-in-Aid from Japan Society of the Promotion of Science (24790220, 25670111), Salt Science Foundation (1235) and Cell Research Conference.

**Conflicts of interest**

The authors confirm that there are no conflicts of interest.
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