Chloride Ion Modulates Cell Proliferation of Human Androgen-independent Prostatic Cancer Cell

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Key Words

Cell cycle • NKCC • Bumetanide • Furosemide

Abstract

In the present study, we investigated if the intracellular Cl^- affects cell growth and cell cycle progression of androgen-independent prostate cancer PC3 cells. PC3 cells cultured in a medium containing 113 mM Cl^- for 96 h grew up 9-fold in cell number, while PC3 cells cultured in an 8 mM- Cl^- -containing culture medium showed complete arrest of cell growth even after culture for 96 h. Exposure of cells to the 8 mM- Cl^- culture medium diminished phosphorylation levels of Rb and cdc2, which are respectively key accelerators of transition from G_1 to S phase and G_2 to M phase in cell cycle progression. Culturing cells in the 8 mM- Cl^- -containing culture medium upregulated the protein expression level of p21 (a CDK inhibitor) inhibiting transition of G_1 to S phase, and diminished the incorporation of 5-ethynyl-2'-deoxyuridine (EdU; a thymidine analogue) into DNA. These results suggest that cells cultured in the low Cl^- medium prolonged the duration of all phases of the cell cycle (G_1 , S, and G_2 /M), thereby abolishing

overall cell cycle progression. Effects of culturing cells in the low Cl^- culture medium on cell cycle progression would be mediated via a change in the intracellular Cl^- concentration ($[\text{Cl}^-]_i$), since $[\text{Cl}^-]_i$ was decreased under a low Cl^- culture medium. To clarify this possibility, we studied effects of furosemide and bumetanide, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) inhibitors, on proliferation of PC3 cells. Furosemide and bumetanide decreased $[\text{Cl}^-]_i$ and cell growth of PC3 cells. These results suggest that a change in $[\text{Cl}^-]_i$ would play a critical role in this growth mechanism.

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Introduction

In recent years, various studies show that ion channels/transporters play important roles in fundamental cellular functions not only in neuron but also in other tissues [1-20]. Particularly, their physiological roles in cell proliferation have been studied, since ion transport across the cell membrane participates in regulation of cell volume, which would be indispensable in cell cycle progression. For example, there are several reports indicating important roles of Cl^- channels/transporters such as CLC [21-24], CFTR [25], K^+/Cl^- cotransporter (KCC) [13, 26] and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) [27].

	0% Cl ⁻ medium (Cl ⁻ -replaced medium)	50% Cl ⁻ medium	100% Cl ⁻ medium (normal Cl ⁻ medium)
Replacement of the medium Cl ⁻ with NO ₃ ⁻	8.1±0.8	62.3±0.8	113.3±1.1
gluconate	7.3±0.6	59.6±0.5	

Table 1. Cl⁻ concentrations of culture media (mM). 50% Cl⁻ medium was made by an equal volume mixture of 0% Cl⁻ medium with 100% Cl⁻ medium. n=3~6.

K⁺/2Cl⁻ cotransporter (NKCC) [1, 27] on cell proliferation in various carcinoma cells. Further, some studies [15, 28-32] indicate that ion channels would regulate various functions in prostate cancer cells. Namely, a Ca²⁺-conducting channel (TRPM8) is expressed in prostatic cancer cells in a manner dependent on malignancy of cancer [31], and an inhibitor of TRPM8, capsazepine, induces apoptosis and reduces cell viability in an androgen-dependent prostate cancer cell line, LNCaP cell [32]. Abdul et al. [28] have reported that 54 % prostate cancer specimens express a voltage-gated sodium channel (VGSC) at a higher level compared with normal one and an opener of VGSC, veratrine, increases cell growth in several prostate cancer cell lines. In our previous reports, we indicated that the intracellular Cl⁻ could act as signals in various cell functions [1, 11, 33-42]. However, there is no report investigating the relation between cell proliferation of prostate cancer and Cl⁻. In the present study, we studied the role of Cl⁻ on cell proliferation of androgen-independent prostate cancer using a model cell line, PC3 cell.

Materials and Methods

Cell culture

A human androgen-independent prostate cancer cell line, PC3, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in plastic flasks in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS). The flasks were kept in a humidified incubator at 37°C with 5 % CO₂ in air. During each experiment, we cultured cells in RPMI 1640 media containing Cl⁻ at various concentrations (Cl⁻-replaced RPMI 1640 media) supplemented with 5 % charcoal-stripped serum (cFBS), which was used as an androgen-depleted medium for cell culture in this study.

Cl⁻-replaced RPMI 1640 medium

To incubate PC3 cells under low Cl⁻ conditions, we used Cl⁻-replaced RPMI 1640 media (Cell science and Technology Institute, Sendai, Japan), which were prepared by replacing Cl⁻ with NO₃⁻. These media were supplemented with 5 % cFBS. Actual Cl⁻ concentrations of culture media were measured

with a Corning Chloride Analyzer 925 (Corning, New York, NY, USA). The Cl⁻ concentration of normal RPMI 1640 medium supplemented with 5 % cFBS (100% Cl⁻ medium) was 113.3 ± 1.1 mM (mean ± SEM; n = 6). The actual Cl⁻ concentration of RPMI 1640 medium with all Cl⁻ replaced by NO₃⁻ supplemented with 5 % cFBS (0% Cl⁻ medium) was 8.1 ± 0.8 mM (n = 3). This Cl⁻ of 8.1 mM was mainly brought by cFBS. For cell proliferation assay, we mixed the 8 mM-Cl⁻ RPMI medium with normal RPMI 1640 medium at various ratios to set Cl⁻ concentrations of media at various levels, and the actual Cl⁻ concentrations in the media were also measured with the Corning Chloride Analyzer 925 (Table 1). We further tried to replace Cl⁻ in culture media with gluconate. The Cl⁻ concentrations of culture media used in the present study are shown in Table 1. We observed no significant difference of Cl⁻ concentrations in the Cl⁻-replaced media with NO₃⁻ and gluconate (Table 1).

Cell proliferation assay in culture media containing various Cl⁻ concentrations

The cells were seeded into 25 cm² flasks at density of 1.0 × 10⁵ cells/flask and incubated for 24 h in the normal RPMI 1640 medium. We defined this time point as time of 0 h. Then, we cultured the cells in the culture media containing various Cl⁻ concentrations. At 48 and 96 h after the change of the culture media, the cells were detached from the flasks in a trypsin-EDTA and then counted on a haemocytometer. The cell numbers at 48 and 96 h were normalized to the cell number at 0 h.

Apoptosis and cell cycle analysis

The cells were seeded into 25 cm² flasks at density of 1.0 × 10⁵ cells/flask and incubated for 24 h in the normal RPMI 1640 medium. Then, we applied the normal or 8 mM-Cl⁻ RPMI medium. At 96 h after culturing cells under each condition, the cells were detached from the flasks by trypsin-EDTA treatment and then centrifuged. A nuclear isolation medium (NIM-DAPI 10; Beckman Coulter Inc., Fullerton, CA, USA) of 0.3 ml was added to cells in the pellets. Ten thousand cells were examined using the Cell Lab Quanta (Beckman Coulter, Beckman Coulter, Fullerton, CA, USA) with an excitation at 365 nm and emission at 450 nm for DAPI. Sub-G₁ hypodiploid (apoptotic) fragments and each cell cycle phase were determined using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western blotting

The cells were seeded into 75 cm² flasks at density of 3.0 × 10⁵ cells/flask and incubated for 24 h in the normal RPMI

1640 medium, and then we applied the normal or 8 mM-Cl⁻ RPMI medium. At 96 h after culturing cells under each condition, the cells were lysed with lysis buffer containing protease inhibitors (aprotinin, leupeptin and PMSF) and phosphatase inhibitors (pyrophosphate, sodium orthovanadate and NaF). The cells were homogenized by sonication and centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant fluid was collected. The samples were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred from gel to nitrocellulose membranes. The blots were incubated with primary antibodies (anti-phospho-Rb (Ser780), anti-Rb, anti-p21, anti-phospho-cdc2 (Thr161), anti-cdc2 and anti-GAPDH) obtained from Cell Signaling Technology (Beverly, MA, USA), which were detected by using chemiluminescence, ECL plus (GE Healthcare, Buckinghamshire, UK). We measured the band densities with an image analyzer, Kodak ID (Kodak, New Haven, CT, USA) after scanned from the film. The content of each protein was normalized with GAPDH.

Measurement of DNA synthesis

We measured DNA synthesis by using Click-iT EdU Alexa Fluor Imaging Kit (Invitrogen, Carlsbad, CA, USA). The cells were seeded into 6 well cell culture plates with glass coverslips at density of 4.0×10^4 cells/well and incubated for 24 h in the normal RPMI 1640 medium, and then we applied the normal or 8 mM-Cl⁻ RPMI medium. At 96 h after culturing cells under each condition, the culture medium was replaced with the normal or 8 mM-Cl⁻ RPMI medium containing 10 mM 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen). After 3 h incubation, the cells on glass coverslips were fixed with formaldehyde. After washing 0.5 % Triton X-100 in PBS, the cells were stained with Alexa 594-azide (Invitrogen). The cells were mounted in a mounting medium and imaged with a fluorescence microscopy.

Effects of NKCC inhibitors on cell proliferation

The cells were seeded into 25 cm² flasks at density of 1.0×10^5 cells/flask and incubated for 24 h in the normal RPMI 1640 medium. We defined this time point as 0 h. Then, we cultured the cells in the normal RPMI medium containing bumetanide or furosemide, Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) inhibitors (10, 50, 100 or 200 μM). We used DMSO as a solvent control for NKCC inhibitor. At 96 h after the change of the culture medium containing an NKCC inhibitor (bumetanide or furosemide) or DMSO only, the cells were detached from the flasks with a trypsin-EDTA-containing solution and counted on a haemocytometer. The cell number at 96 h was normalized to that at 0 h.

Measurement of [Cl⁻]_i

The cells were seeded into 75 cm² flasks at density of 3.0×10^5 cells/flask and incubated for 24 h in the normal RPMI 1640 medium, and then we cultured the cells in normal RPMI medium, 8 mM-Cl⁻ (NO₃⁻ or gluconate) RPMI medium, or normal RPMI medium containing NKCC inhibitors (bumetanide or furosemide; 100 or 200 μM). We used DMSO as a solvent control for NKCC inhibitor. At 96 h after the change of the culture medium, we measured [Cl⁻]_i by the same method as we

Fig. 1. Cell proliferation of PC3 cells incubated in the culture media containing various Cl⁻ concentrations (113, 62 and 8 mM Cl⁻) at 48 h and 96 h after application: replacement of Cl⁻ with NO₃⁻. The data shown in here are normalized to the cell number at 0 h (24 h after plating the cells). The proliferation rate in PC3 cells was diminished by the application of Cl⁻-replaced media. When the cells were cultured in the 8 mM-Cl⁻ RPMI medium, cell proliferation was almost completely blocked. Results are presented as means ± SEM (n = 4).

previously reported [10, 11]. Namely, the method is as follows. We incubated the cells with 5 mM *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) (Invitrogen), a halide-sensitive dye, for 1 h before analysis. The methods of MQAE loading and calibration of fluorescence in terms of [Cl⁻]_i were performed by the same method as previously reported [10, 11]. Briefly, we performed calibration of the fluorescence in terms of [Cl⁻]_i by applying 5 μM nigericin, 5 μM valinomycin, and 10 μM tributyltin to cells in high-K⁺ calibration buffers (pH 7.4) containing 0, 20, 40, 60, 80 and 100 mM Cl⁻. MQAE-loaded cells were detached using trypsin-EDTA and centrifuged. We resuspended the cells in pellet in each calibration buffer, and obtained the calibration for the fluorescence in terms of Cl⁻ concentration. To determine the effect of bumetanide or furosemide on [Cl⁻]_i, we resuspended MQAE-loaded cells after culturing cells with or without bumetanide or furosemide in a buffer, a Cl⁻ concentration of which was adjusted to the same Cl⁻ concentration as that of the culture medium. After 10 min incubation at 37°C, we analyzed cells using a high-resolution flow cytometer, the Cell Lab Quanta flow cytometry system (Beckman Coulter Inc.). We measured MQAE fluorescence (FL) with an excitation at 365 nm and emission at 450 nm, and electronic volume (EV) of 10,000 cells. The [Cl⁻]_i was estimated using the Stern-Volmer constant value acquired from calibration.

Data presentation and statistical analysis

Results are expressed as means ± SEM. Statistical analysis was carried out using ANOVA. The differences were considered significant when the p value was less than 0.05.

	0% Cl ⁻ medium (Cl ⁻ -replaced medium)	50% Cl ⁻ medium	100% Cl ⁻ medium (normal Cl ⁻ medium)
Replacement of the medium Cl ⁻ with NO ₃ ⁻	8.0±1.1	22.9±0.4	52.1±1.0
gluconate	12.8±0.8	27.8±2.1	

Table 2. Intracellular Cl⁻ concentrations of PC3 cells (mM). 50% Cl⁻ medium was made by an equal volume mixture of 0% Cl⁻ medium with 100% Cl⁻ medium. n=3.

Results

The cell proliferation and the intracellular Cl⁻ concentration ([Cl⁻]_i) of PC3 cultured in media containing various Cl⁻ concentrations

We cultured PC3 cells in media containing various Cl⁻ concentrations. Unless we provide any statements regarding ionic species for replacement of Cl⁻, we reduced the Cl⁻ concentration in the culture medium with NO₃⁻. Figure 1 shows the normalized cell numbers of PC3 cells at 48 and 96 h after culturing cells in media containing various concentrations of Cl⁻ replaced with NO₃⁻. These data indicate that the rate of cell proliferation depends on the extracellular Cl⁻ concentration. The proliferation rate in PC3 cells was decreased according to a decrease in the Cl⁻ concentration of the medium. When the cells were cultured in the 8 mM-Cl⁻ RPMI medium, cell proliferation was almost abolished. Further, the [Cl⁻]_i assay indicated that the [Cl⁻]_i of PC3 cells cultured in media containing various concentrations of Cl⁻ replaced with NO₃⁻ was decreased as the Cl⁻ concentration of the culture media was diminished (NO₃⁻ in Table 2). These results strongly indicate that the Cl⁻ plays a key role in proliferation of PC3 cells. However, we should consider a possibility that the action of decrease in Cl⁻ concentration of culture medium on cell cycle progression is not mediated through a decrease in Cl⁻ concentration of culture medium but an increase in the concentration of NO₃⁻. To rule out this possibility, we studied effects of Cl⁻ replacement with gluconate instead of NO₃⁻ on proliferation of PC3 cells. The numbers of cells cultured for 96 h normalized to the cell number at 0 h were 1.67 ± 0.16 in 7 mM-Cl⁻, 6.90 ± 0.25 in 60 mM-Cl⁻, and 8.98 ± 0.35 in normal Cl⁻ RPMI media replaced with gluconate (n = 4 in each case), suggesting that Cl⁻ replacement with gluconate showed an effect on proliferation of PC3 cells for 96 h similar to that with NO₃⁻. Under this condition, the [Cl⁻]_i of PC3 cells was also decreased as the Cl⁻ concentration of the culture media was diminished (gluconate in Table 2). Therefore, the action of reduction of Cl⁻ concentration in the culture medium on cell proliferation would be really

Fig. 2. The numbers of sub-G₁ hypodiploid (apoptotic) fragments detected by flow cytometry analysis at 96 h after application of the normal (Normal Cl⁻) or the 8 mM-Cl⁻ (Low Cl⁻) RPMI medium. The data shown in here are normalized to the number at 0 h (24 h after plating the cells). The number of sub-G₁ population cultured in the 8 mM-Cl⁻ RPMI medium was much less than that cultured in the normal RPMI medium. * p < 0.01 compared with that in normal RPMI medium. Results are presented as means ± SEM (n = 5).

due to the reduction of Cl⁻ concentration, but not due to an increase in NO₃⁻ or gluconate. We also studied if effects of reduced Cl⁻ on cell growth is reversible. After cell culture for 96 h in 8 mM-Cl⁻ RPMI medium, we cultured cells for 96 h in normal Cl⁻ RPMI medium: the normalized cell number was 2.43 ± 0.13 (n = 4). As a time control, we cultured cells in 8 mM-Cl⁻ RPMI medium for 96 h, and continued cell culture in 8 mM-Cl⁻ RPMI medium for 96 h: the normalized cell number was 0.82 ± 0.03 (n = 4). These experimental results indicate that an effect of reduced Cl⁻ on cell growth is reversible.

Apoptosis and cell cycle analysis

Abolishment of cell proliferation by culturing cells in the 8 mM-Cl⁻ RPMI medium led us to two possibilities: 1) culturing cells in the 8 mM-Cl⁻ RPMI medium induces

Fig. 3. A) The percentage of PC3 cells staying in each phase of the cell cycle 96 h after culturing cells in the normal (Normal Cl⁻) or the 8 mM-Cl⁻ (Low Cl⁻) RPMI medium. Culturing cells in the 8 mM-Cl⁻ RPMI medium increased the population of cells staying in the G₂/M phase, and decreased the population of cells staying in the G₀/G₁ phase. B) The number of cells staying in each cell cycle phase (a, G₁ phase; b, S phase; c, G₂/M phase) at 0 and 96 h after culturing cells in the normal or 8 mM-Cl⁻ RPMI medium. Culturing cells in the normal RPMI medium significantly increased the number of cells staying in all cell cycle phases, whereas the number of cells staying in any cell cycle phase was not changed by culturing cells in the 8 mM-Cl⁻ RPMI medium. * $p < 0.01$ compared with that in normal RPMI medium. Results are presented as means $\pm$ SEM ($n = 5$).

apoptosis, resulting in abolishment of an increase in cell number, and 2) the cell cycle completely arrests at any phases before entering into M phase. To clarify these possibilities, we analyzed the apoptosis and cell cycle. To determine whether culturing cells in the 8 mM-Cl⁻ RPMI medium induces more apoptosis, we measure the number of sub-G₁ hypodiploid (apoptotic) fragments by

Fig. 4. A) Expression and phosphorylation levels of regulatory proteins for G₁/S (a, pRb; b, p21) and G₂/M (c, cdc2) phases in PC3 cells cultured in the normal (N) or 8 mM-Cl⁻ (L) RPMI medium. The western blots were performed using anti-pRb (total and phospho-Ser 780), anti-p21, and anti-cdc2 (total and phospho-Thr161) antibodies. B) The normalized level of expression and phosphorylation of each protein in PC3 cells cultured in the 8 mM-Cl⁻ RPMI medium to that in the normal RPMI medium for 96 h. * $p < 0.01$ compared with that in the normal RPMI medium. Results are presented as means $\pm$ SEM ($n = 4$).

flow cytometry analysis. As shown in Fig. 2, culturing cells in the 8 mM-Cl⁻ RPMI medium did not increase the number of sub-G₁ apoptotic fragments, but rather decreased the number of sub-G₁ apoptotic fragments to about one-fifth of that in the normal Cl⁻ medium. This observation indicates that abolishment of an increase in cell number by culturing cells in the 8 mM-Cl⁻ RPMI

Fig. 5. The percentage of PC3 cells incorporating EdU in the normal (Normal Cl⁻) or the 8 mM-Cl⁻ (Low Cl⁻) RPMI medium. Culturing cells in the 8 mM-Cl⁻ RPMI medium decreased EdU incorporation into PC3 cells. * $p < 0.01$ compared with that in the normal RPMI medium. Results are presented as means $\pm$ SEM (n = 4).

Fig. 6. Proliferation of PC3 cells exposed to NKCC inhibitor, bumetanide or furosemide for 96 h. The data shown in here are normalized to the cell number at 0 h (24 h after plating the cells). The proliferation rate was diminished in a manner dependent on the concentration of NKCC inhibitor. * $p < 0.05$ compared with that in the DMSO containing media. Results are presented as means $\pm$ SEM (n = 4).

medium is not due to an increase in apoptosis. To clarify the second possibility (complete arrest), we analyzed the cell cycle. Flow cytometer measurements show the percentage population of PC3 cells staying at each phase of the cell cycle (Fig. 3A). Culturing cells in the 8 mM-Cl⁻ RPMI medium increased the percent population of PC3 cells staying in G₂/M phase and decreased the percent population of cells staying in G₀/G₁ and S phases (Fig. 3A). The doubling time of PC3 cells was 30.3 ± 0.6 h (n = 4) in the normal RPMI medium. Therefore, if only stalling the G₂/M cell cycle progression caused this growth inhibition in the 8 mM-Cl⁻ RPMI medium, all PC3 cells cultured in the 8 mM-Cl⁻ RPMI medium for 96 h should pass through G₀/G₁ and S phases, and stay in G₂/M cell cycle phase. However, more than half (about 56%) of the PC3 cells cultured in the 8 mM-Cl⁻ RPMI medium for 96 h stayed in G₁ or S phase (Fig. 3A). Therefore, it is impossible to explain the complete cell growth arrest (Fig. 1) only due to the cell cycle arrest at the G₂/M checkpoint of PC3 cells cultured in the 8 mM-Cl⁻ RPMI medium. Furthermore, we calculated cell number staying in each cell cycle phase at 0 and 96 h after exposure to the normal or 8 mM-Cl⁻ RPMI medium (Fig 3B-a, b, and c) by using results of the cell proliferation assay and the cell cycle analysis. Culturing cells in the normal Cl⁻ medium significantly increased cell number staying in each cell cycle phase (Fig. 3B-a, b, and c).

Meanwhile, the cell number staying in each cell cycle phase was not changed by the application of 8 mM-Cl⁻ RPMI medium for 96 h (Fig. 3B-a, b, and c). Therefore, we indicate that the doubling time of PC3 cells cultured in the 8 mM-Cl⁻ RPMI medium is more than 96 h, although we could not obtain accurate information on the doubling time of PC3 cells in the 8 mM-Cl⁻ RPMI medium. These results lead us to a hypothesis that culturing cells in the 8 mM-Cl⁻ RPMI medium would induce the cell cycle arrest in all cell cycle stages.

Western blotting

The observations shown above suggest that culturing cells in the 8 mM-Cl⁻ RPMI medium would induce the cell cycle arrest in all stages of cell cycle. To clarify this point, we analyzed the expression of cell-cycle-associated proteins by Western blot analysis to determine the mechanisms how the decrease of Cl⁻ concentration in the culture medium inhibited the proliferation of PC3 cells. Phosphorylation of the retinoblastoma protein, pRb, by the cyclin-dependent kinase (CDK) is essential for G₁-S phase progression [43]. Therefore, we investigated the effect of culturing cells in the 8 mM-Cl⁻ RPMI medium on the phosphorylation level of pRb and on the expression level of CDK inhibitor, p21. Culturing cells in the 8 mM-Cl⁻ RPMI medium for 96 h significantly decreased phosphorylation of pRb on

Fig. 7. Intracellular Cl^- concentration ($[\text{Cl}^-]_i$) of PC3 cells 96 h after exposure to NKCC inhibitor, bumetanide or furosemide. * $p < 0.01$ compared with DMSO as a solvent control. Results are presented as means $\pm$ SEM ($n = 4$).

serine-780 and total pRb protein (Fig. 4A-a). Expression of p21 protein, which exists in the upstream of pRb in a signal pathway [43], was significantly increased by culturing cells in the 8 mM- Cl^- RPMI medium compared with that in the normal medium (Fig. 4A-b). These results suggest that the decrease of Cl^- concentration of the culture medium diminishes the transition rate from G_1 phase to S phase of the cell cycle progression in PC3 cells by increasing the expression of p21 protein.

Next, we analyzed the effect of culturing in the 8 mM- Cl^- RPMI medium on the phosphorylation level of cdc2, which is a key regulator of the transition from G_2 to M phase of the cell cycle progression [43]. Phosphorylation of cdc2 on threonine-161 is necessary to progress from G_2 to M phase of the cell cycle. Culturing PC3 cells in 8 mM- Cl^- RPMI medium significantly decreased phosphorylation of cdc2 on threonine-161 at 96 h after the culture (Fig. 4A-c). Furthermore, it was also revealed that the total expression of cdc2 protein was significantly decreased by culturing cells in the 8 mM- Cl^- RPMI medium (Fig. 4A-c).

Figure 4B shows the relative amount of each protein expressed in PC3 cells cultured for 96 h in the 8 mM- Cl^- RPMI medium to that in the normal Cl^- RPMI medium. These results suggest that a decrease in the Cl^- concentration of culture medium delays the transition from both G_1 to S phase and G_2 to M phase of the cell cycle progression in PC3 cells.

Measurement of DNA synthesis

We analyzed the effect of culturing cells in 8 mM- Cl^- medium on S phase of the cell cycle in PC3 cells by measuring DNA synthesis directly. DNA synthesis was measured by using a nucleotide analogue of thymidine, 5-ethynyl-2'-deoxyuridine (EdU), which is incorporated into DNA during DNA synthesis during S phase in the cell cycle. We cultured PC3 cells in the normal or 8 mM- Cl^- RPMI culture medium and measured the percentage of the cells incorporating EdU under each condition by imaging with a fluorescence microscopy. In the cells cultured in the normal RPMI medium, cells of 44.4 % incorporated EdU (Fig. 5). On the other hand, in the cells cultured in the 8 mM- Cl^- RPMI medium, the percentage of cells incorporating EdU was only 8.6 % (Fig. 5). These results suggest that culturing cells in the 8 mM- Cl^- RPMI medium prolonged the duration of S phase of the cell cycle. Consequently, in PC3 cells, culturing cells in the 8 mM- Cl^- RPMI medium prolonged the duration of all cell cycle phases (G_1 , S and G_2/M).

Effects of NKCC inhibitors on the proliferation of PC3 cell

Based on the results shown above, we hypothesized that effects of culturing cells in the 8 mM- Cl^- RPMI medium on cell cycle progression are mediated via a change in $[\text{Cl}^-]_i$, and the intracellular Cl^- is one of the most important regulators of the proliferation in PC3 cells. Therefore, we studied if the cell proliferation is influenced by inhibition of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC), which is one of important transporters controlling $[\text{Cl}^-]_i$ via uptake of Cl^- into the intracellular space. Thus, we investigated effects of NKCC inhibitors (bumetanide and furosemide) on the proliferation of PC3 cells. Figure 6 shows the normalized cell numbers at 96 h after addition of bumetanide or furosemide at various concentrations (10, 50, 100, and 200 μ M). The proliferation rate of PC3 cells decreased in a manner dependent on the concentration of NKCC inhibitors.

Effects of NKCC inhibitors on $[\text{Cl}^-]_i$ of PC3 cells

We further investigated effects of NKCC inhibitors on $[\text{Cl}^-]_i$ in PC3 cells at 96 h after addition of bumetanide, furosemide or DMSO (a solvent control for bumetanide and furosemide) (Fig. 7). Bumetanide or furosemide of 200 μ M significantly diminished the $[\text{Cl}^-]_i$ in the normal RPMI medium compared with that with addition of DMSO only (control) (Fig. 7). These results suggest that NKCC inhibitors diminish cell proliferation by decreasing $[\text{Cl}^-]_i$ in PC3 cells.

Discussion

In the present study, we showed that culturing cells in the 8 mM-Cl⁻ RPMI medium induced complete arrest of cell growth of PC3 cells. Reduction of [Cl⁻]_i upregulated the expression of p21, CDK inhibitor, and downregulated the phosphorylation and expression of pRb, which is a key accelerator for cell cycle progression from G₁ into S phase [43]. We also showed reduction of [Cl⁻]_i diminished the expression of cdc2, which is an important enhancer for cell cycle progression from G₂ into M phase [43]. By measuring DNA synthesis, we showed that culturing cells in the 8 mM-Cl⁻ RPMI medium diminished the percentage of the cells incorporating EdU. This suggests that reduction of [Cl⁻]_i also prolonged the duration of S phase of the cell cycle. Accordingly, these results demonstrate that the reduction of [Cl⁻]_i induces delay of the overall cell cycle progression. In our previous study [11], we showed that reduction of [Cl⁻]_i also suppressed the cell proliferation in gastric cancer cell line, MKN28 cells. In MKN28 cells, diminution of [Cl⁻]_i delayed the cell-cycle transition from G₁ to S phase through a pathway dependent on p21 [11], a CDK inhibitor, whereas there were no substantial effects on the regulatory mechanism of G₂/M checkpoint [11] unlike PC3 cells shown in the present study. Furthermore, we should indicate that reduction of [Cl⁻]_i in MKN28 cells showed much weaker effects on cell proliferation than that in PC3 cells. Namely, the proliferation of PC3 cells was completely inhibited by culturing cells in the low Cl⁻ medium, but the proliferation of MKN28 cells under the low Cl⁻ condition reduced only to 50% of control [11]. We also studied the effect of culturing cells in the low Cl⁻ medium on proliferation of an androgen-dependent prostate cancer cell line (LNCaP cell), indicating that the proliferation of LNCaP cells was diminished to 50% of control (our unpublished data). This suggests that this inhibitory action of low Cl⁻ in androgen-dependent prostate cancer LNCaP cell is much weaker than that in PC3 (complete inhibition). Consequently, these observations strongly suggest that the proliferation of androgen-independent prostatic cancer PC3 cells is strongly affected by the intracellular Cl⁻, which regulates the overall cell cycle progression.

In the present study, we indicate that reduction of Cl⁻ concentration in culture medium diminished phosphorylation level of Rb and cdc2 proteins and induced upregulation of p21 expression level. A report [44] indicates that sensory rhodopsin has a binding site for Cl⁻, which regulates rhodopsin activity. Further, it has been reported that Cl⁻ regulates mitochondrial ADP/ATP carrier

by inhibiting the binding of adenine nucleotides to the carrier [45], and that ENaC activity in the kidney is regulated intracellular Cl⁻ [33]. These reports and our observations in the present study suggest that Cl⁻ plays important roles in cell functions.

We suggest a possibility that reduction of extracellular Cl⁻ inhibits cell growth via a decrease in intracellular Cl⁻ concentration. However, extracellular Cl⁻ has been reported to affect ENaC activity [37, 46]. Therefore, we should suggest a possibility that extracellular Cl⁻ controls cell growth indirectly by regulating some ion channels and/or transporters such as ENaC. Another possibility is that reduction of extracellular Cl⁻ would inhibit cell growth via a decrease in cell volume by inhibiting Cl⁻ entry (mainly via NKCC) [47, 48]. This cell shrinkage would activate a signaling pathway inhibiting cell growth. The cell shrinkage, recently termed apoptotic volume decrease (AVD), is considered a hallmark of the apoptotic process [49, 50]. Activation of K⁺ and Cl⁻ channels causes AVD, which is followed by release of intracellular-apoptotic inducers such as caspases [49, 50]. Blockade of K⁺ and/or Cl⁻ channels prevents apoptotic cell death [49]. The mechanism causing AVD is almost identical to that causing regulatory volume decrease (RVD), which is observed in response to cell swelling. In our previous study, we demonstrated that [Cl⁻]_i was drastically decreased during RVD [10]. Hence, these results raise a possibility that the decline of [Cl⁻]_i is one of the intracellular signals in the initiation of apoptosis. If so, the reduction of [Cl⁻]_i is a possible target as the therapy for cancer. In this study, we revealed that reduction of [Cl⁻]_i in PC3 cells caused the delay of the overall cell cycle progression and the drastic inhibition of cell proliferation. There were several reports on the expression of Cl⁻ channels/transporters in cancer cells. For instance, volume-regulated anion channels have been found in human prostate and lung cancer cells, and several members of Ca²⁺-activated Cl⁻ channel family are related to breast and colorectal cancers [14, 51-53]. In addition, we found that mRNA and the functional expression levels of NKCC were higher in poorly differentiated type gastric adenocarcinoma cell than in moderately differentiated one [1]. Therefore, it is predictable that inhibition of Cl⁻ channels and/or transporters causes greater changes in [Cl⁻]_i, providing a larger inhibition in growth of poorly differentiated cancer cells than moderately differentiated ones. The normal prostate cells express NKCC as an ion transporter involved in the regulation of [Cl⁻]_i [27]. Consequently, we examined whether NKCC plays important roles in the cellular uptake of Cl⁻ and the

proliferation of PC3 cells. First, we confirmed the mRNA expression of NKCC in PC3 cells by using reverse transcription-polymerase chain reaction (data not shown). Furthermore, we investigated whether NKCC was involved in the regulation of the $[Cl^-]_i$ and cell proliferation of PC3 cells. As shown in the present study, NKCC inhibitors (bumetanide and furosemide) dose-dependently inhibited cell proliferation (Fig. 6) and decreased the $[Cl^-]_i$ (Fig. 7) in PC3 cells. These observations indicate that the intracellular Cl^- would play a key role in cell proliferation of PC3 cells. Although NKCC inhibitors showed inhibitory effects on cell proliferation of PC3 cells, the effects were weaker than replacement of Cl^- . Even if NKCC is completely inhibited, the change in $[Cl^-]_i$ was small (Fig. 7), since NKCC contributes to elevation of $[Cl^-]_i$ from the $[Cl^-]_o$ equilibrating to the membrane potential. To induce much more reduction of $[Cl^-]_i$ to the level caused by Cl^- replacement shown in the present study, we should apply compounds actively stimulating Cl^- release from the intracellular space to PC3 cells such

as an activator of K^+/Cl^- cotransporter, N-ethylmaleimide (NEM) [54].

In conclusion, the present study shows reduction of $[Cl^-]_i$ inhibits cell proliferation in androgen-independent prostate cancer, PC3 cells, by inhibiting overall cell cycle progression, although extracellular Cl^- would play a role in cell growth not via regulation of $[Cl^-]_i$.

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