Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 317 (2004) 192–197
BBRC
www.elsevier.com/locate/ybbrc

Expression and roles of Cl^- channel ClC-5 in cell cycles of myeloid cells

Baohong Jiang, Naoki Hattori, Bing Liu, Yasuhisa Nakayama, Kaori Kitagawa, Kiminobu Sumita, and Chiyoko Inagaki*

Department of Pharmacology, Kansai Medical University, Osaka 570-8506, Japan

Received 3 March 2004

Abstract

ClC-5 is a chloride channel known to be expressed in the kidney. We previously reported that ClC-5 mRNA was also strongly expressed in immature human myeloid cell line (HL-60), but weakly expressed in mature neutrophils. To clarify the underlying mechanisms, we examined the relationship between ClC-5 expression and cell cycle. Dimethyl sulfoxide treatment of HL-60 that causes differentiation with G0/G1 cell cycle arrest decreased the expression of ClC-5 mRNA. Cell sorting and synchronization experiments revealed that ClC-5 mRNA expression was high in S and G2/M phases and low in G0/G1 phase. ClC-5 antisense oligonucleotide suppressed proliferation of HL-60 cells with a decrease in ClC-5 protein expression, probably due to G2 arrest. These results suggest that cell cycle-dependent expression of ClC-5 has a role in cell cycle progression in myeloid cells.

Keywords: Proliferation; Cell cycle; ClC-5; Myeloid cell

The mammalian chloride channel family contains nine members: ClC-1 to ClC-7, ClC-Ka, and ClC-Kb. ClC-5 is known to be expressed in the kidney, playing a role in tubular re-absorption of low molecular proteins [1]. We previously reported that ClC-5 mRNA was also abundantly expressed in immature human myeloid cells (HL-60), while it was scarcely expressed in mature neutrophils [2]. This prompted us to assume the involvement of ClC-5 in myeloid cell maturation or the cell cycle progression. Recently, growing evidence indicates that chloride channels are critical for the cell cycle [3,4], but the data linking a specific Cl^- channel to cell cycle progression are limited [5]. In the present study, we examined the relationship between ClC-5 mRNA expression and the cell cycle progression in myeloid cell line (HL-60).

Materials and methods

Cells and culture. HL-60 (human myeloid cell line) cells from RIKEN Cell Bank (Tsukuba, Japan) were seeded onto culture plates at a density of 5×10^4/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, and cultured in a humidified incubator with a CO_2 concentration of 5% at 37 °C. Differentiation of HL-60 cells toward the granulocytic pathway was induced by 1.25% dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan).

Separation of neutrophils from the blood of normal subjects. Venous blood (10–20 ml) was taken from six normal adult volunteers (20–42 years old) and peripheral blood mononuclear cells (PBMCs) and neutrophils were separated by Ficoll–Paque (Pharmacia Biotech, Uppsala, Sweden) as described previously [6]. Approximately 7×10^6 neutrophils were obtained from 10 ml of blood.

Cell cycle analysis and cell sorting. HL-60 cells were harvested, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at 4 °C for 1 h. Cellular DNA was stained with 20 μg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) containing 500 U/ml RNase (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min and measured using Becton–Dickinson FACScan (Becton–Dickinson, San Jose, CA, USA). The populations of cells in the G0/G1, S, and G2/M phases were estimated using the Modifit cell cycle analysis program (Verity Software House, Topsham, ME, USA). In order to collect the cells in G0/G1 and S phases separately, the harvested HL-60 cells were washed with PBS and treated with RNA later (Ambion, Austin, TX, USA) at 4 °C for 30 min to prevent RNA degradation. Cellular DNA was stained with 20 μg/ml propidium iodide for 30 min. Fluorescence of individual nuclei was monitored with a EPICS ALTRA FACScan flow cytometer (Beckman Coulter, Tokyo, Japan). The cells in G0/G1 and S phases were sorted and RNA was isolated from each fraction using RNeasy Mini Kit (Qiagen, Avenue Stanford, Valencia, CA, USA).

* Corresponding author. Fax: +81-6-6993-9427.
E-mail address: inagaki@takii.kmu.ac.jp (C. Inagaki).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.036

Cell synchronization—thymidine double block. The cell cycle of HL-60 was arrested at G1/S boundary by the incubation with an inhibitor of DNA synthesis, thymidine (2.5 mM) for 25 h, and allowed to enter S phase by removing the inhibitor for 8 h. Then the cells were treated by 2.5 mM thymidine again for another 24 h, rendering most cells synchronized at S phase. The cells were harvested, washed with normal medium, plated onto flasks, and incubated in the humidified 5% CO_2 incubator at 37 °C.

RNA isolation and reverse transcription polymerase chain reaction. Total RNA was isolated from human HL-60 cells and normal neutrophils using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco-BRL, Rockville, MD, USA), followed by extraction and precipitation with isopropyl alcohol. The amounts of RNA were quantitated using UV spectroscopy (absorption at 260 nm). Reverse transcription polymerase chain reaction (RT-PCR) was performed with a commercially available RT-PCR system (Promega, Madison, WI, USA) in 50 μl of 10 mM Tris–HCl buffer containing 1 μM each of sense and antisense primers, 0.1 U/μl avian myeloblastosis virus (AMV) reverse transcriptase, 0.1 U/μl *Thermus flavis* (Tfl) DNA polymerase, 0.2 mM dNTP, 1.2 mM $MgSO_4$, and 0.5 μg RNA, which was pretreated with DNase I (Gibco-BRL, Rockville, MD, USA). After producing cDNA by RT at 48 °C for 30 min, several cycles of PCR were carried out in a Gene Amp PCR System 9600 (Perkin–Elmer, Foster City, CA, USA). The primers were 5′-CTGTG CCACTGCTTCAAC-3′ (sense) and 5′-AGTGTTGAAGTGGTTCT C-3′ (antisense) for ClC-5, 5′-ACAGCAGCACCTACCGGATT-3′ (sense) and 5′-CTCTGGGCGACAGCGTTG-3′ (antisense) for ClC-2, 5′-ATTAAATGGATATACCCTTTCTTG-3′ (sense) and 5′-TTGCA ATTGTCAGGTCTCTTCT-3′ (antisense) for ClC-3, 5′-TTGTAACC AACTGGGACGATATGG-3′ (sense) and 5′-CCGCTCATTGCCGA TAGTGATGA-3′ (antisense) for β-actin, and 5′-ACCACAGTCCAT GCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense) for GAPDH. The predicted lengths of the PCR products were 555 bp for ClC-5, 178 bp for ClC-2, 248 bp for ClC-3, 539 bp for β-actin, and 452 bp for GAPDH.

Northern blot analysis. ClC-5, ClC-3, and β-actin cDNA fragments made by the above method were labeled with digoxigenin-11-dUTP by PCR (DIG DNA labeling mixture; Boehringer–Mannheim GmbH, Mannheim, Germany) and used as probes for Northern blotting. Denatured total RNA samples (5 μg/lane) were separated by electrophoresis on a 1% agarose–formaldehyde gel. The gel was washed in 20× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and the RNA was transferred onto positively charged nylon membranes (Boehringer–Mannheim GmbH) by capillary blotting with 20× SSC for 15–17 h. The RNA was fixed by UV cross-linking at 254 nm (0.16 J/cm^2). The membrane was pre-hybridized at 42 °C for 1–2 h in a hybridization solution containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 7.4), 2% blocking reagent (Boehringer–Mannheim GmbH), 50 μg/ml salmon testes DNA, and 0.5% sodium dodecyl sulfate (SDS). Denatured digoxigenin-labeled probe and dextran sulfate were added to the hybridization solution at final concentrations of 12.5 ng/ml and 5%, respectively, and incubation was continued for 16 h. The blots were rinsed briefly and washed twice at 65 °C for 20 min in 1× SSC with 5% SDS. The hybridized probes were detected using alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer–Mannheim GmbH) and a chemiluminescent substrate, 0.26 mM disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.13,7]-decan}-4-yl) phenyl phosphate (CSPD) (Tropix, Bedford, MA, USA), according to the manufacturer's instructions. The membrane sealed in the hybridization bag was exposed to Kodak-OMAT AR film. To normalize the amount of total RNA, the membrane was reprobed with a β-actin cDNA probe after stripping twice with 1× SSC and 5% SDS at 95 °C for 10 min each.

Western blot for ClC-5. HL-60 cells were washed in PBS and lysed by 100 mM Tris (pH 7.5) containing 300 mM NaCl, 2 mM EDTA, 2% Nonidet P40 (NP40), 1% sodium deoxycholate (NaDOC), 0.2% SDS, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions at 4 °C for 1 h. The cell lysates were centrifuged at 10,000 rpm for 10 min. The aliquots (50 μg protein) of the supernatant were electrophoresed on a SDS–polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA) and blotted with ClC-5 antibody (Alomone Labs, Jerusalem, Israel) at a 1:200 dilution. The bands of ClC-5 were visualized by the chemiluminescence reagent (Amersham–Pharmacia Biotech, Little Chalfont Buckinghamshire, England) and the density of the band was quantitated by Fluorochem IS-8000 (Alpha Innotech, CA, USA). To normalize the amount of protein, the membrane was reprobed with β-actin antibody.

Transfection of antisense oligonucleotides for ClC-5. The phosphorothioated antisense and sense oligonucleotides for human ClC-5 mRNA were synthesized and purified by high performance liquid chromatography. The sequence of antisense and sense was 5′-TTCAG AGGTTTCTGTGGTGTA-3′ and 5′-TACACCACAGAAACCTCT GAA-3′, respectively. HL-60 cells were cultured at a density of 10^5/ml in 10 ml flasks. The sense or antisense oligonucleotide (3 μM each) was added twice to the culture medium immediately after the washout of the first and second incubation with thymidine for cell cycle synchronization. The cell number was counted from the day of the first thymidine application (day 0) to day 6. Cell cycle profiles were analyzed by FACS and ClC-5 protein by Western blot on day 3.

Statistical analysis. All values are presented as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to test for statistical significance ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Results

Expression of ClC-5 in undifferentiated human myeloid cells (HL-60) and mature neutrophils

Fig. 1A shows the mRNA expressions of ClC-5, ClC-2, and ClC-3 in HL-60 cells and neutrophils from six normal subjects. ClC-5 mRNA was strongly expressed in HL-60, while the expression levels were very low in neutrophils from normal subjects (40 cycles). In contrast, ClC-2 and ClC-3 were equally expressed in the cells of both groups (32 cycles). FACS analysis revealed that 42% of HL-60 cells were in G0/G1 phase, 48% in S phase, and 10% in G2/M phase, while almost all neutrophils from normal subjects were in G0/G1 phase (Fig. 1B). These results suggest that ClC-5 expression is related with cell cycle distribution or cell differentiation.

G0/G1 arrest and differentiation of HL-60 cells by DMSO treatment

We examined whether ClC-5 mRNA expression decreased in HL-60 cells treated with 1.25% DMSO which is known to arrest cell cycle in G0/G1 phase and to differentiate them into neutrophils [7]. HL-60 cells treated in this way acquired morphological changes (segmented nuclei) characteristic to mature neutrophils (data not shown), and eventually underwent growth arrest in G0/G1 phase (Fig. 2A). ClC-5 mRNA expression decreased

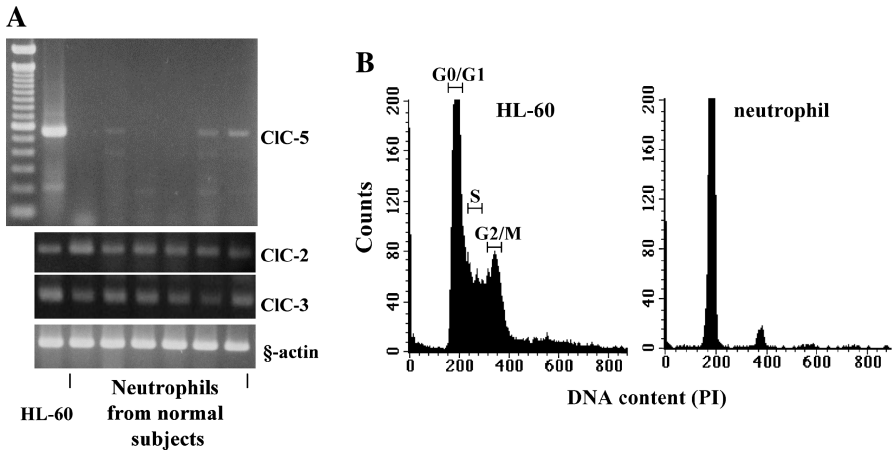

Fig. 1. Expressions of ClC-5, ClC-2, and ClC-3 mRNA in myeloid leukemic cell (HL-60) and neutrophils from six normal subjects determined by RT-PCR (A). The representative cell cycle distribution profiles in HL-60 and neutrophils from normal subjects are shown (B).

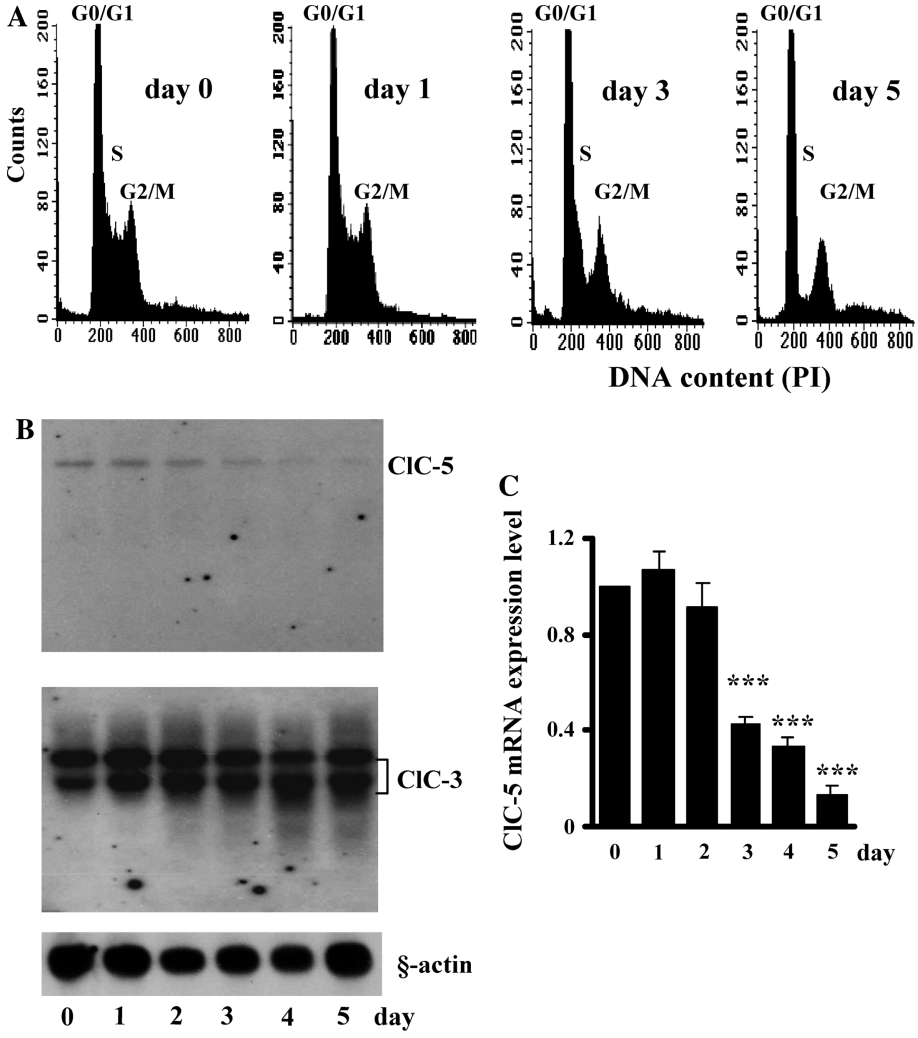

Fig. 2. Effects of DMSO treatment on cell cycle of HL-60 (A) and the expression of ClC-5 and ClC-3 mRNA determined by Northern blotting (B). The density of the band was analyzed by densitometry and the results on ClC-5 are summarized in (C) ($n = 3$, $^{***}p < 0.001$).

significantly during 5 days in parallel with the decline of cells in S and G2/M phases, while ClC-3 mRNA expression did not change (Figs. 2B and C).

Expression of ClC-5 mRNA in G0/G1 and S phases separated by cell sorting

To estimate whether the changes in ClC-5 mRNA expression were depending on cell cycle, we used sorting technique to isolate the cells in G0/G1 phase and those in S phase directly (Fig. 3A). ClC-5 mRNA expression was higher in S phase than that in G0/G1 phase, while ClC-2 and ClC-3 mRNA expressions did not change (Figs. 3B and C). The amounts of cells in G2/M phase were too small to be analyzed for the mRNA.

Expression of ClC-5 mRNA in cell cycle-synchronized HL-60 cells

To determine the expression level of ClC-5 mRNA in G2/M phase during the cell cycle, HL-60 cells were

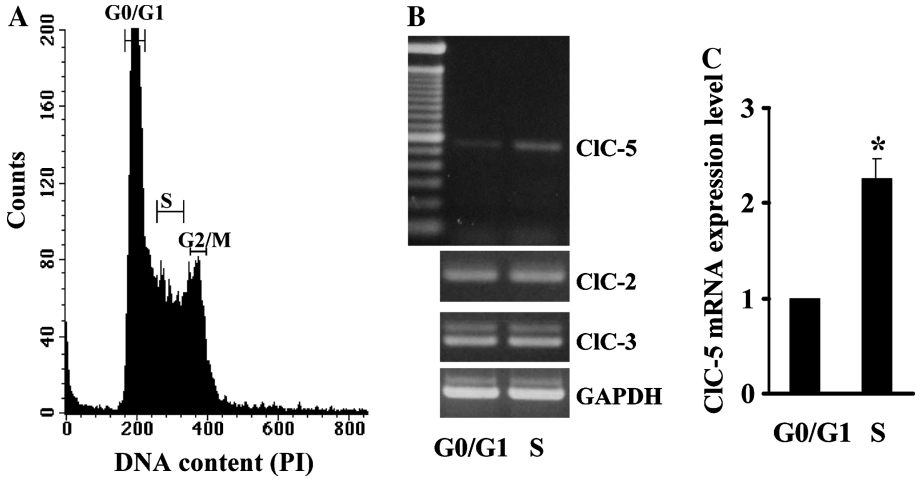

Fig. 3. Expression of ClC-5 mRNA in HL-60 cells in G0/G1 or S phase isolated by cell sorting. The cells in G0/G1 and S phases were separately collected by ALTRA FACScan flow cytometer (A). ClC-5 mRNA expression was higher in S phase than in G0/G1 phase, whereas ClC-2 and ClC-3 mRNA expression did not change (B). The density of the band was analyzed by densitometry and the results on ClC-5 are summarized in (C) ($n = 4$, $^{*}p < 0.05$).

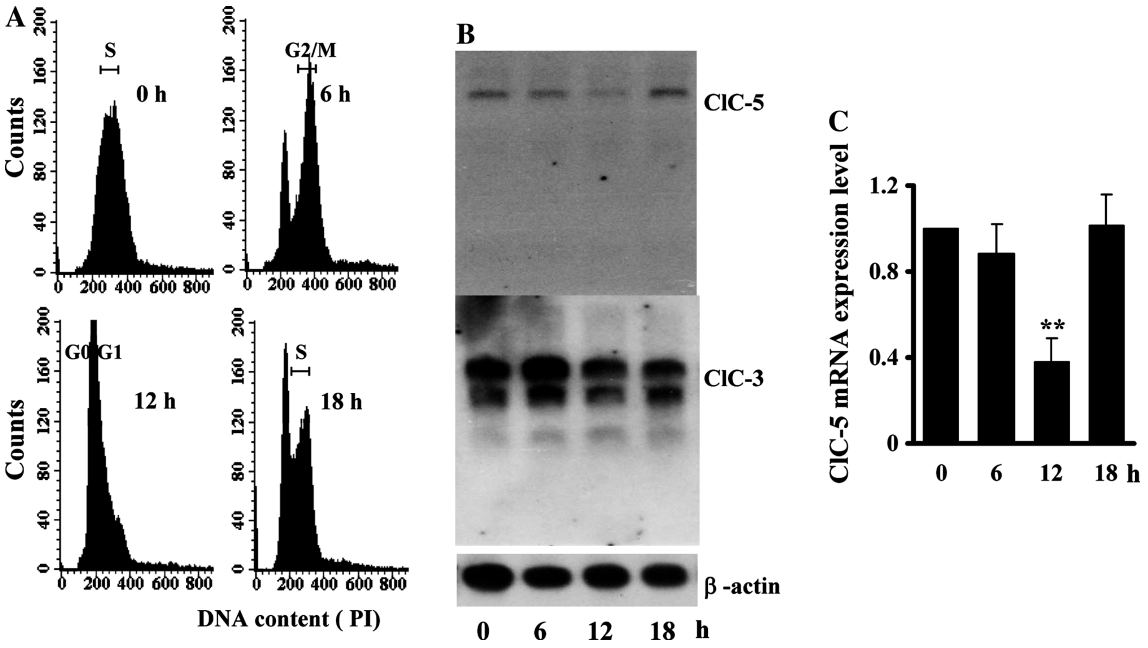

Fig. 4. Expression of ClC-5 mRNA in HL-60 cells synchronized by thymidine double block method. S phase at 0 h, G2/M plus G0/G1 phases at 6 h, G0/G1 phase at 12 h, and G0/G1 plus S phases at 18 h (A). The mRNA expression of ClC-5 and ClC-3 was assessed by Northern blotting (B). The density of the band was analyzed by densitometry and the results on ClC-5 are summarized in (C) ($n = 3$, $^{**}p < 0.01$).

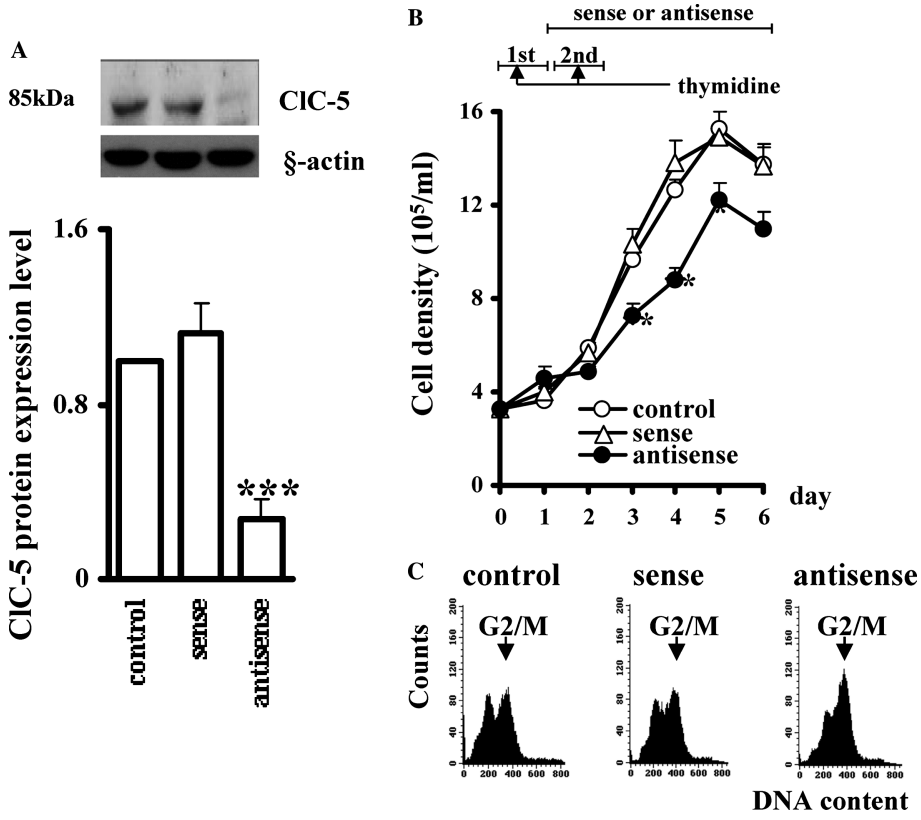

Fig. 5. The effect of ClC-5 antisense oligonucleotide on the proliferation of HL-60. The effect of ClC-5 antisense oligonucleotide on ClC-5 protein expression assessed by Western blotting. The density of the band was analyzed by densitometry and the results are summarized (A) ($n = 4$, $^{***}p < 0.001$). The effects of ClC-5 antisense oligonucleotide on the growth of HL-60 cells (B) and the cell cycle distribution analyzed by FACS (C). HL-60 cells were synchronized by thymidine double block. The sense or antisense oligonucleotide was added twice to the culture medium immediately after the washout of the first and second incubation with thymidine.

synchronized by thymidine double block method. Almost all cells were synchronized in S phase immediately after the second thymidine treatment (0 h); G2/M plus G0/G1 phases 6 h later, G0/G1 phase at 12 h, and G0/G1 plus S phases at 18 h (Fig. 4A). The expression of ClC-5 mRNA in S and G2/M phases was significantly higher than that in G0/G1 phase, but the expression of ClC-3 did not change (Figs. 4B and C).

Suppression of cell proliferation by ClC-5 antisense due to G2 arrest

Since specific blockers for ClC-5 are not available, we used antisense oligonucleotide against ClC-5 to inhibit endogenous ClC-5 protein expression in cell cycle-synchronized HL-60 cells. Fig. 5A illustrates that ClC-5 protein expression decreased by 73% in antisense treated cells ($p < 0.01$, $n = 4$). Antisense oligonucleotide of ClC-5 significantly inhibited the proliferation of HL-60. No significant inhibition was observed with sense oligonucleotide (Fig. 5B). Fig. 5C shows the cell cycle distributions of sense or antisense oligonucleotide-treated HL-60. The population of cells in G2/M phase increased in ClC-5 antisense-treated HL-60, suggesting that the blockade of ClC-5 expression caused G2 cell cycle arrest.

Discussion

Characteristic expression pattern of ClC-5 mRNA in mature neutrophils and myeloid cells was confirmed, in contrast with the comparable mRNA expression of ClC-2 and ClC-3 between the two groups. Since DMSO-treatment, which is known to differentiate immature myeloid cells to mature neutrophils with the cell cycle arrest at G0/G1 phase [8] lowered ClC-5 expression (Fig. 2), ClC-5 was presumed to be highly expressed in specific phases of cell cycle. Sorting study in untreated HL-60 cells showed that ClC-5 mRNA expression was much higher in S phase than in G0/G1 phase. Further, cell cycle synchronization study clearly demonstrated that the cells in G2/M phase also expressed ClC-5 mRNA at a level similar to that in S phase cells. These findings suggest that ClC-5 mRNA is up-regulated in S and G2/M phases and down-regulated in G0/G1 phase regardless of cell maturation.

Cell cycle dependent changes in chloride channel activities have been reported in glioma and fibroblast cells. Ullrich and Sontheimer [9] reported that a glioma-specific chloride current increased significantly in early G1 and M phases, speculating the link to the cytoskeletal rearrangements associated with cell division. Zheng et al. [10] showed that chloride permeability increased at G0-to-G1 transition and the G1/S checkpoint in fibroblast cells, assuming that such an increase in Cl^- permeability plays a role in reentry of quiescent cells into proliferating phases. Further, there is a report showing the changes in protein level of chloride channel during a cell cycle. Zheng et al. [11] showed that ClC-2 channel protein in NRK-49F cells was expressed predominantly in M phase without changes in ClC-2 mRNA levels. Since this channel was ubiquitinated at M phase via phosphorylation by M phase specific cyclin B, they assumed its physiological role in the cell cycle. These previous reports together with our findings suggest that chloride channels dynamically change their activities with changes in protein and/or mRNA expression levels during a cell cycle.

Proliferation of HL-60 cells treated with ClC-5 antisense oligonucleotide was suppressed in accordance with a decrease in ClC-5 protein expression. FACS analysis revealed that G2 arrest might occur resulting in the suppression of cell cycle progression and cell proliferation. There are several reports demonstrating that chloride channel blockers suppress cell proliferation via G0/G1 arrest [12], and we also recently reported that a chloride channel blocker, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), caused suppression of leukemic cell proliferation via G0/G1 arrest [13]. To our knowledge, there has been only one report showing the involvement of chloride channels in G2 arrest, in which blockade of nuclear chloride ion channel NCC27 suppressed proliferation of Chinese hamster ovary cells via a G2 arrest [14]. The present study clearly showed that blockade of ClC-5 also caused G2 arrest in myeloid cells. ClC-5 is involved in acidification of intracellular compartments together with V-ATPase in the kidney cells [15]. Blockade of ClC-5 may change the ionic mileau, which in turn may prevent cell division or other downstream events related to V-ATPase function such as induction of IL-2 [16]. Future studies are necessary to clarify the role of ClC-5 in the cell cycle progression.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, Japan Private School Promotion Foundation, and Salt Science Research Foundation. We thank Kansai Medical University Laboratory Center for providing experimental instruments.

References

[1] Y. Sasaki, J. Nagai, Y. Kitahara, N. Takai, T. Murakami, M. Takano, Expression of chloride channel, ClC-5, and its role in receptor-mediated endocytosis of albumin in OK cells, Biochem. Biophys. Res. Commun. 282 (2001) 212–218.

[2] B.H. Jiang, N. Hattori, B. Liu, K. Kitagawa, C. Inagaki, Expression of swelling- and/or pH-regulated chloride channels (ClC-2, 3, 4 and 5) in human leukemic and normal immune cells, Life Sci. 70 (2002) 1383–1394.

[3] S.P. Yu, D.W. Choi, Ions, cell volume, and apoptosis, Proc. Natl. Acad. Sci. USA 97 (2000) 9360–9362.

[4] B. Nilius, Chloride channels go cell cycling, J. Physiol. 532 (2001) 581.

[5] G.L. Wang, X.R. Wang, M.J. Lin, H. He, X.J. Lan, Y.Y. Guan, Deficiency in ClC-3 chloride channels prevents rat aortic smooth muscle cell proliferation, Circ. Res. 91 (2002) E28–E32.

[6] N. Hattori, T. Saito, T. Yagyu, B.H. Jiang, K. Kitagawa, C. Inagaki, GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils, J. Clin. Endocrinol. Metab. 86 (2001) 4284–4291.

[7] S.J. Collins, F.W. Ruscetti, R.E. Gallagher, R.C. Gallo, Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds, Proc. Natl. Acad. Sci. USA 75 (1978) 2458–2462.

[8] A. Yen, Control of HL-60 myeloid differentiation, Exp. cell Res. 156 (1985) 198–212.

[9] N. Ullrich, H. Sontheimer, Cell cycle-dependent expression of a glioma-specific chloride current: proposed link to cytoskeletal changes, Am. J. Physiol. 273 (1997) C1290–C1297.

[10] Y.J. Zheng, T. Furukawa, K. Tajimi, N. Inagaki, Cl^- channel blockers inhibit transition of quiescent (G0) fibroblasts into the cell cycle, J. Cell Physiol. 194 (2003) 376–383.

[11] Y.J. Zheng, T. Furukawa, T. Ogura, K. Tajimi, N. Inagaki, M phase-specific expression and phosphorylation-dependent ubiquitination of the ClC-2 channel, J. Biol. Chem. 277 (2002) 32268–32273.

[12] M.R. Shen, G. Droogmans, J. Eggermont, T. Voets, J.C. Ellory, B. Nilius, Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells, J. Physiol. 529 (2000) 385–394.

[13] B.H. Jiang, N. Hattori, B. Liu, Y. Nakayama, K. Kitagawa, C. Inagaki, Suppression of cell proliferation with induction of p21 by chloride channel blockers in human leukemic cells, Eur. J. Pharmacol. (in press).

[14] S.M. Valenzuela, M. Mazzanti, R. Tonini, M.R. Qiu, K. Warton, E.A. Musgrove, T.J. Campbell, S.N. Breit, The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle, J. Physiol. 529 (2000) 541–552.

[15] W. Gunther, A. Luchow, F. Cluzeaud, A. Vandewalle, T.J. Jentsch, ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells, Proc. Natl. Acad. Sci. USA 95 (1998) 8075–8080.

[16] A. Rao, C. Luo, P.G. Hogan, Transcription factors of the NFAT family: regulation and function, Annu. Rev. Immunol. 15 (1997) 707–747.