Monoamine carboxylate transporters are involved in BI-1-associated cancer metastasis in HT1080 colon fibrosarcoma cells

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Abstract. The overexpression of BI-1 induces an acidic extracellular pH due to alterations of mitochondrial function, leading to cancer metastasis through anaerobic glucose metabolism. In this study, ion transporters such as sodium hydrogen exchangers (NHE) and monoamine carboxylate transporters (MCTs) were studied in BI-1-overexpressing HT1080 cells (BI-1 cells). The extracellular pH became acidic as culture time of BI-1 cells increased, while intracellular pH stayed relatively stable at pH 7.2. The expression of MCTs increased in BI-1 cells as culture time passed. 5-(N-ethyl-N-isopropyl) amiloride or dimethylamiloride, NHE inhibitor, abrogated the elevated MCT expression, indicating that MCT followed NHE activation. An MCT inhibitor, lonidamine, regulated the acidification of extracellular pH, also inhibiting both increased cancer cell migration and infiltration and MMP2/9 activity. The inhibition of either NHE or MCT affected the intracellular pH, leading to a severely acidic intracellular pH and cell death. In BI-1 cells, the activation of ion transporters such as NHE or MCT may offer a survival strategy against metabolism-associated acidic stresses. These findings suggest that BI-1 can lead cancer invasion and metastasis by inducing extracellular environment acidic.

Introduction

Bax inhibitor-1 (BI-1; also known as 'testis-enhanced gene transcript') is an anti-apoptotic protein capable of inhibiting Bax activation and translocation to the mitochondria (1,2). Functionally, BI-1 affects the leakage of Ca^{2+} ions from the ER, as measured with Ca^{2+}-sensitive, ER-targeted fluorescent proteins and Ca^{2+}-sensitive dyes (2). BI-1 has also protective effect against reactive oxygen species through functional inhibition of Bax (3). In the BI-1 overexpression system, increase in heme oxygenase-1 expression has also been proposed as a regulatory mechanism for reactive oxygen species through the activation of Nrf2 transcription factor (4) BI-1 enhances cancer/tumor progression (5,6). We previously demonstrated that the overexpression of BI-1 increases cell mobility and invasiveness, with elevated glucose consumption and cytosolic accumulation of lactate, leading to cancer metastasis (7). In that study, monoamine carboxylate transporter (MCT) as well as sodium hydrogen exchanger (NHE) activities are suggested as homeostatic mechanisms for intracellular pH. Increased intracellular H^+ can affect the pH-homeostasis system, including NHE. Hydrogen ions (H^+) play major roles in many normal cellular processes, including the control of tumorigenic processes such as oncogenesis, oncogene expression and malignant transformation (8,9). The ability to secrete protons is dependent on membrane-localized ion exchangers such as NHEs, Na^+-dependent and independent HCO^{3+}/Cl^- exchangers, and the H^+/lactate cotransporter. High lactic acid production is also a common feature of many solid tumor cells (10-12). The transporters responsible for export of lactic acid have been characterized in detail (13). It was recently reported that MCT isoform 1 (MCT1) and MCT4 have elevated activity in human cancer cells in response to low extra-cellular pH (14), suggesting that the levels and/or affinities of proton export transporters may be increased in cancer cells, and that these transporters function to prevent lethal intracellular pH decreases as tumors grow and become more acidic. Furthermore, the interconnection of NHE and MCT is involved in the regulation of acidic extracellular pH and cancer metastasis (15). We hypothesized that expression of MCT would be associated with the cancer-related characteristics of BI-1 including extracellular acidic pH and the activity of NHE in BI-1-overexpressed HT1080 cells.

Materials and methods

Materials and cell culture. Chemicals, including 5-ethylisopropyl amiloride (EIPA), dimethylamiloride (DMA) and lonidamine were obtained from Sigma Aldrich (St. Louis, MO, USA).

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Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and other tissue culture reagents were supplied by Life Technologies, Inc. (Grand Island, NY, USA). Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Biotechnology (Rockford, IL, USA). All other chemicals of analytical grade were purchased from Sigma Aldrich. Human HT1080 fibrosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mg/ml streptomycin and 100 units/ml penicillin. The cells were stably transfected with pcDNA3 (Neo), pcDNA3-BI-1-HA or C-BI-1-HA (C-terminal-deleted) plasmids using the Superfect transfection reagent (Qiagen, Hilden, Germany) and then cultured for 3 weeks in 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA).

Intracellular pH measurements. Cells were loaded with 10 μM pH-sensitive dye carboxy-seminaphthorhodafluor (SNARF-1)/AM for 30 min at 37˚C, washed, and incubated for 30 min with lactic acid in medium. Internal pH values were determined via the ratio of mean FL-2/FL-3 by flow cytometry analysis. A standard curve was generated by treating cells with 10 μM nigericin in calibration buffers from pH 6.0 to 8.0.

NHE activity measurement. After a 20 mM NH_4Cl prepulse, cells were washed with Na-free solution (HEPES buffer with NaCl replaced by N-methyl-D-glutamine). NHE activity was calculated from the initial slope of intracellular alkalization upon re-addition of Na. To allow for direct comparison, pH per min was calculated only for intracellular pH values in the range of pH 6.50-6.80. All experiments were performed in pairs with measurement of NHE activity before and after 15 min incubation. Control cells were incubated with only standard HEPES solution.

MCT activity measurement. L-lactate transport was measured by monitoring the change in extracellular pH with a pH- sensitive electrode as described previously (16). Initial rates of transport of lactate were calculated by first-order regression analysis of the time course of pH change and converted into nmol of H^+/min by determining the pH change induced by small additions of standardized NaOH.

Immunoblotting. Cell lysates were prepared and the level of protein expression was measured as described (2). An equal amount of protein extracted from cells with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and protease inhibitor cocktail) was separated on a 10% SDS-PAGE gel. The proteins were transferred to nitro-cellulose membranes. After each membrane was probed with a specific primary antibody (1:1000 for HA, MMP-2 and MMP-9 antibodies), the blot was stripped and re-probed with a polyclonal antibody against β-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) to confirm equal protein loading and transfer. Finally, the blots were developed with enhanced chemiluminescence (ECL).

Invasion assay. The *in vitro* invasion assay was performed in 24-well transwell plates with polycarbonate membranes (Corning Costar, Cambridge, MA, USA) as previously described (7). The bottom of the chamber was coated with type I collagen, and the top was coated with Matrigel (Collaborative Research, Lexington, MA, USA). The lower chamber was filled with serum-free medium containing 0.1% BSA. Cells were placed in the upper chamber, incubated for 17 h, fixed with methanol, and stained with hematoxylin and eosin for 10 min. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the membrane under a microscope. Thirteen fields were counted for each membrane, and each sample was assayed in triplicate.

Wound migration assay. Cells plated in culture dishes at 90% confluency were pretreated with mitomycin C (25 mg/ml) for 30 min before an injury line was made with a tip 2 mm wide. After being rinsed with phosphate-buffered saline (PBS), cells were allowed to migrate in complete medium, and photographs were taken (x40).

Gelatin zymography. Cells were cultured in serum-free DMEM, and the medium was collected and centrifuged at 1,200 x g for 10 min to remove cell debris. The protein concentration was measured using bicinchoninic acid (BCA) protein assay reagents (Pierce). Equal amounts of proteins from conditioned media were mixed with 2X Laemmli non-reducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 three times for 30 min, and then rinsed for 15 min with 50 mM Tris-HCl buffer (pH 7.6).

Lactate production analysis. Lactate production rates were determined in cells grown to confluence in 24-well plates (Falcon, model 3047; Becton-Dickinson, Franklin Lakes, NJ, USA). Cells were inoculated at a density of $1x10^4$ cells per well and allowed to grow for 3 to 4 days until confluent. Lactate levels were measured by commercial kit (BioVison, Mountainview, CA, USA) by manufacturer's protocol.

Statistical analysis. Results are presented as mean ± SEM of n cells, and paired and unpaired Student's t-tests were applied where appropriate. Origin software (Microcal, Northampton, MA, USA) was used for statistical calculations. P-values were determined by Student's t-tests. Statistical significance was set at $P<0.05$.

Results

BI-1 overexpression induces acidic intracellular pH. Recently, it was shown that the overexpression of BI-1 induces an acidic extracellular pH because of the alteration of mitochondrial function, which affects glucose metabolism (7). Considering the enhancement of glucose metabolism in BI-1 cells, we expected that the intracellular pH would decrease or at least activate greatly the H^+ transporter systems to extrude the accumulating intracellular H^+ outside the cells. We examined intracellular pH through a flow cytometer analysis (FACS) system, and confirmed that the intracellular pH in BI-1 cells was significantly lower than in Neo cells (Fig. 1A). Treatment with NH_4CL also caused a relatively acidic intracellular pH in

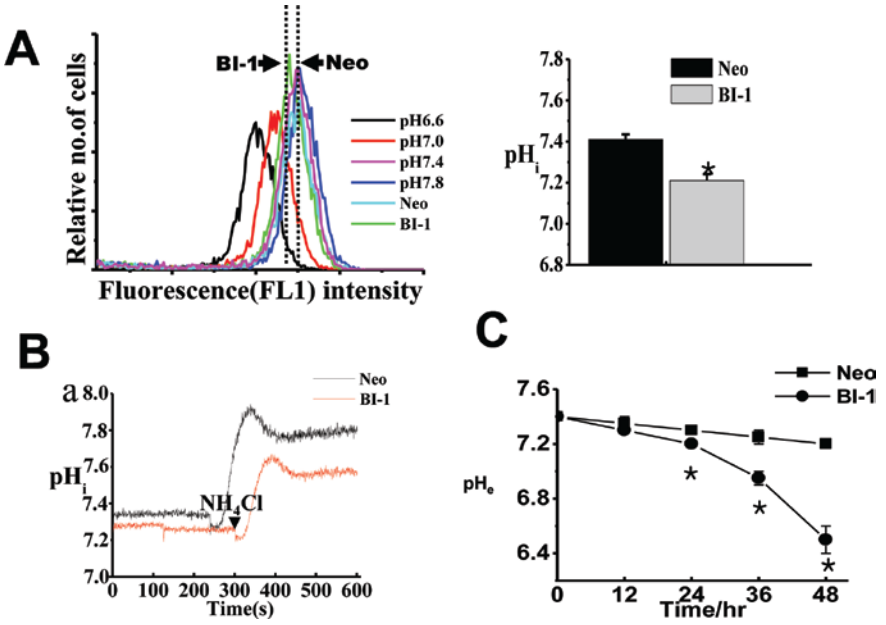

Figure 1. Intracellular pH is acidic in BI-1-overexpressing cells. (A) Neo and BI-1 cells were incubated with SNARF1 dye in 150 mM KOH/nigericin buffer after calibrating intracellular pH as described in Materials and methods. The intracellular pH was quantified (right). *P<0.05, significantly different from Neo cells (B) Neo and BI-1 cells were incubated with SNARF1 dye in 150 mM KOH/nigericin buffer and exposed to NH_4Cl, and the fluorescence was measured in a spectrofluorometer. (C) Neo and BI-1 cells were incubated with 20 mM glucose media. Extracellular pH was determined with phenol red after the indicated periods. *P<0.05; significantly different from the indicated time-cultured Neo cells. Neo, neomycin-resistant vector-transfected cells; BI-1, BI-1-transfected cells.

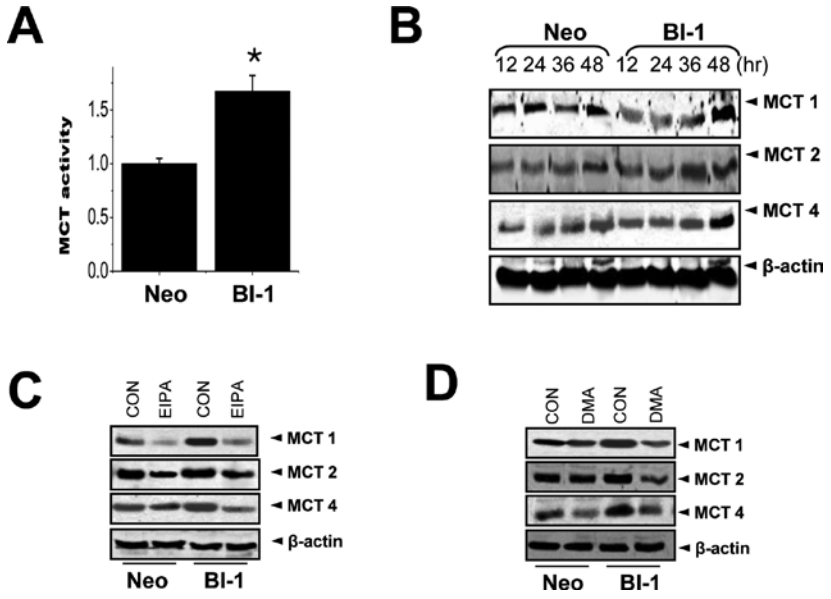

Figure 2. MCT activity is enhanced in BI-1-overexpressing cells. (A) MCT activity were measured in Neo and BI-1 cells. *P<0.05, significantly different from Neo cells. (B) Neo and BI-1 cells were harvested at the indicated culture time periods and immunoblotting was performed with anti-MCT1, 2 or 4 antibody. Neo and BI-1 cells were exposed to either 20 μM EIPA (C) or 100 μM DMA (D) for 24 h, followed by immunoblotting with anti-MCT1, 2 or 4 antibody.

BI-1 cells, compared with Neo cells (Fig. 1B). The extracellular pH of BI-1 cells sharply decreased with culture time, ultimately dropping to approximately pH 6.5 (Fig. 1C). At the same time, the intra-cellular pH of the BI-1 cells was maintained at about pH 7.2, at the various culture time points, including at 48 h (data not shown). These finding suggest that intracellular pH homeostasis was probably maintained through ion transporter activation.

BI-1 overexpression induces MCT activation. To examine the role of ion transporter in pH regulation of BI-1, MCT activity was measured and compared between Neo and BI-1 cells. MCT activity was much greater in the BI-1 cells (Fig. 2A), and MCT expression also increased in BI-1 cells depending on culture time (Fig. 2B), demonstrating the correlation of MCT with extracellular pH (Fig. 1C). Sodium-hydrogen exchanger has been studied regarding the extracellular pH in

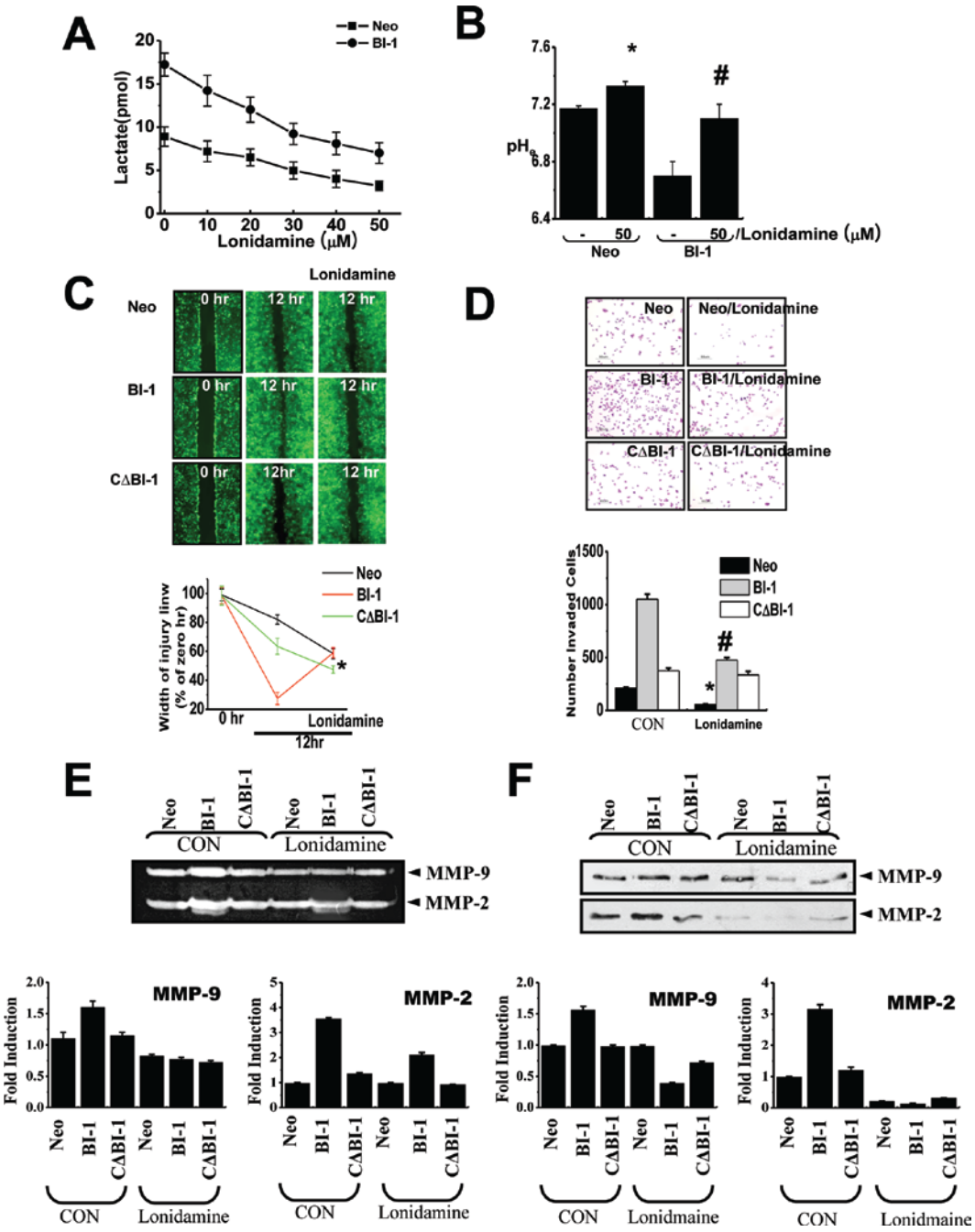

Figure 3. MCT activity is related to BI-1-associated cancer metastasis. (A) Neo and BI-1 cells were cultured with the indicated concentrations of lonidamine for 24 h and the MCT activity was measured as described in Materials and methods. (B) Neo and BI-1 cells were cultured with the indicated concentrations of lonidamine for 24 h and the extracellular pH was measured. (C) In Neo and BI-1 cells, an injury line was made on a confluent monolayer of cells. Cell motility was examined with a light microscope (x40) after 12 h, and the wounding width was quantified (lower). (D) In Neo, BI-1 or CΔBI-1 cells, the expression of BI-1 was confirmed (upper) and the cells were seeded onto a Matrigel invasion chamber. Samples were then incubated for 72 h in serum-free medium. The cells that invaded through the Matrigel-coated membrane were stained with 0.1% crystal violet and then examined under a light microscope (x200) (middle). The number of invading cells was determined by counting the stained cells in the lower well (lower). After Neo, BI-1 and CΔBI-1 cells were treated with 30 μM lonidamine for 24 h, MMP2/9 activity (E) was measured and expression analysis (F) was performed using zymography and immunoblotting. In the lower panel, quantification was performed.

BI-1-overexpressing cells (7). Because it was reported that NHE is directly or indirectly related to the transport of mono amine carboxylates including lactate (13), the intercorrelation of NHE and MCT was examined. The expression level of MCT was checked after treatment of NHE inhibitors, EIPA or DMA (17). The expression of MCT decreased in the presence of the NHE inhibitors (Fig. 2C, D), but an MCT inhibitor did not affect NHE activity (data not shown), indicating that NHE activation was followed by MCT activation. As previously reported (7), C-terminal sequence of BI-1 plays a key role for NHE activation. Therefore, the C-terminal motif, EKDKKKEKK was examined on the function of MCT in this study. Expectedly, deletion of the motif in BI-1-transfected cells regulated the highly increased MCT activation in BI-1 cells

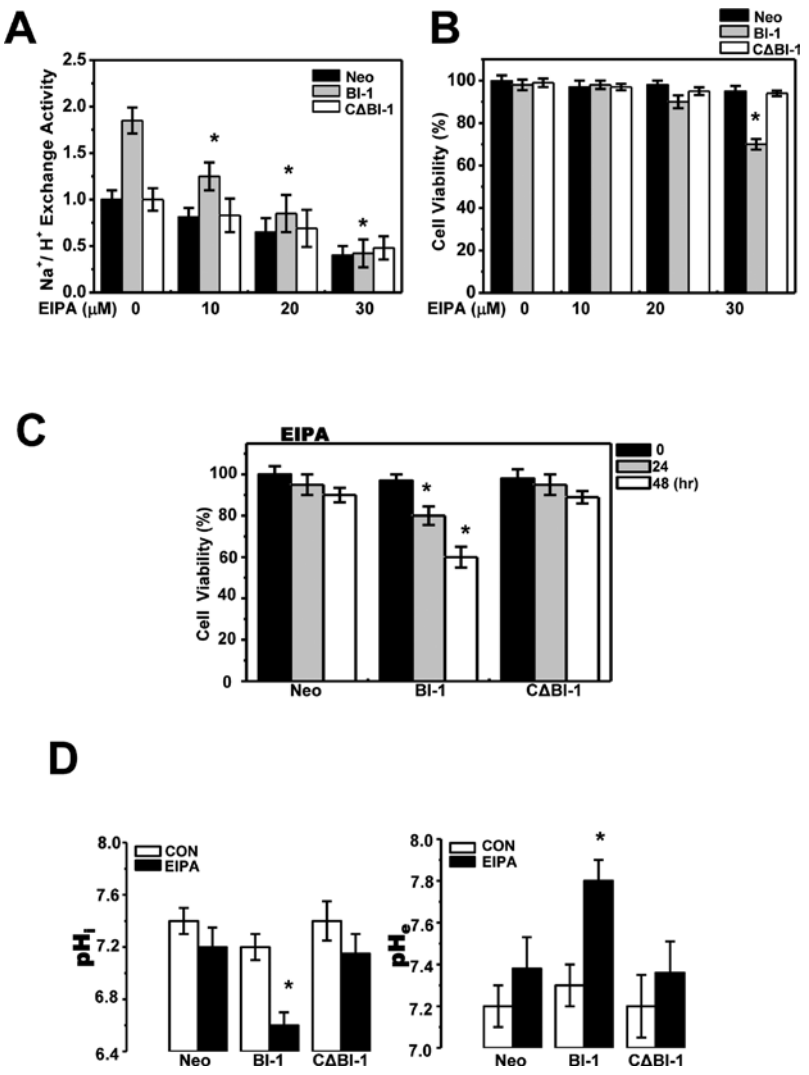

Figure 4. NHE activity is required for cell survival through maintenance of intracellular pH homeostasis. NHE activity (A) and cell viability (B) were measured when Neo, BI-1 and CΔBI-1 cells were exposed to the indicated concentrations of EIPA for 24 h. (C) After Neo, BI-1 and CΔBI-1 cells were exposed to 30 μM EIPA for the indicated time periods, cell viability was evaluated, along with (D) intracellular and extracellular pH (right). *P<0.05, significantly different from non-treated BI-1 cells.

(data not shown) implying the correlation between BI-1 and MCT via NHE.

The MCT inhibitor, lonidamine reverses the acidic extracellular pH and infiltration and migration. Lonidamine has previously been described as an MCT inhibitor (18). Neo and BI-1 cells were treated with lonidamine and extracellular lactate was measured. The amount of lactate was reduced in a lonidamine-dose-dependent manner in both Neo and BI-1 cells (Fig. 3A). Extracellular pH was also more significantly reversed in lonidamine-treated BI-1 cells, compared with Neo cells (Fig. 3B).

Acidic pH is related to cancer invasion or enhanced motility (19). We used wound healing and gel infiltration assays to represent the endogenous characteristics of cancer metastasis. In wound healing and gel infiltration analyses, the MCT inhibitor regulated the activation of invasion and motility, especially in BI-1 cells (Fig. 3C and D). In cancer metastasis, the expression and activity of matrix metalloproteinases (MMPs) are highly increased. MMP2/9 activities were highly activated in BI-1 cells where the MCT inhibitor regulated the activation (Fig. 3E) and elevated expression of MMP2/9 was also regulated by the MCT inhibitor (Fig. 3F). These data consistently show the involvement of MCT in BI-1 characteristics, acidic pH and the associated functions.

Prolonged incubation with either NHE or MCT inhibitors induces cancer cell death through disruption of pH homeostasis. BI-1-associated acidic extracellular pH was related to cancer metastasis, based on our *in vitro* assays (Fig. 3). Although the intracellular pH was also slightly decreased in BI-1 cells, the pH was maintained during the entire incubation period, even up to the 48 h. Our hypothesis was that the ion and related transporter systems are highly activated in this context to maintain intracellular pH for cell survival especially in BI-1 cells. In order to document the hypothesis, we inhibited NHE activity with EIPA, an NHE inhibitor. NHE activity was regulated by pre-treatment by EIPA in a dose-dependent manner (Fig. 4A). Cell viability of BI-1 cells was reduced when cells were exposed to high concentrations of EIPA (Fig. 4B). EIPA

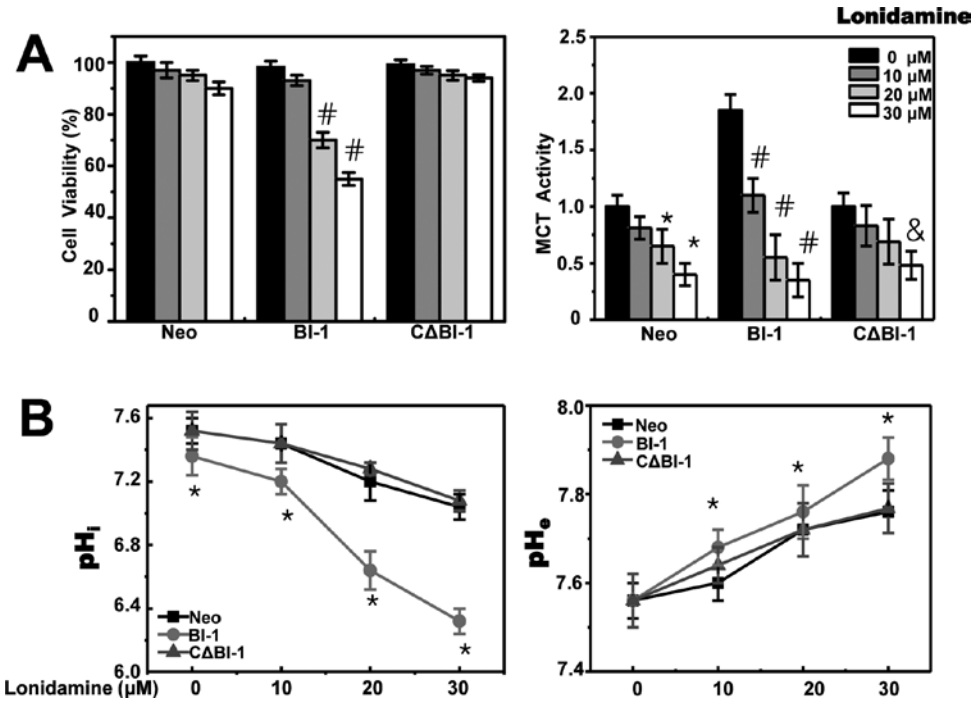

Figure 5. MCT is also required for cell survival through maintenance of intracellular pH homeostasis, an approach with a chemical inhibitor. MCT activity and cell viability (right) (A) and intra and extracellular pH (B) were measured when Neo, BI-1 and CΔBI-1 cells were exposed to the indicated concentrations of lonidamine for 24 h. (A) *P<0.05; significantly different from non-treated Neo cells, #P<0.05, significantly different from non-treated BI-1 cells; &P<0.05, significantly different from non-treated CΔBI-1 cells, (B) *P<0.05, significantly different from the indicated concentration of lonidamide-treated Neo cells.

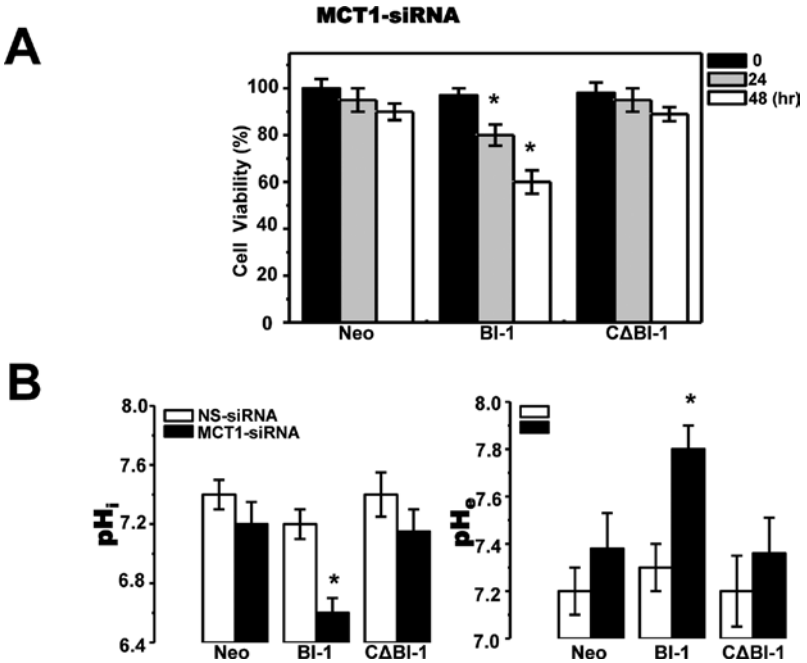

Figure 6. MCT is also required for cell survival through maintenance of intracellular pH homeostasis, gene knock-down approach. (A) Neo, BI-1 and CΔBI-1 cells were transfected with non-specific or MCT-1 siRNA, and cell viability was measured after the indicated periods, along with (B) intracellular and extracellular pH (right). *P<0.05, significantly different from non-specific siRNA-transfected BI-1 cells; NS, non-specific.

induced cell death at high concentrations when incubated with the cells for long periods, such as 24 or 48 h (Fig. 4C).

Next, we attempted to correlate intracellular pH with NHE inhibition. Treatment with the NHE inhibitor markedly and significantly reduced the intracellular pH (previously stable at about 7.2) to lower than pH 6.8, demonstrating the disruption of intracellular pH homeostasis by the inhibition of NHE (Fig. 4D) in BI-1 cells. As expected, the inhibition of NHE also regulated the acidic extracellular pH, leading to an alkaline extracellular pH in BI-1 cells (Fig. 4D, left). Similarly,

the effect of MCT was confirmed in the BI-1 cells. The MCT inhibitor, lonidamine also induced cell death, especially at high concentrations, with the almost complete abrogation of MCT activity (Fig. 5A).

To understand the impact of MCT on intracellular pH homeostasis, we measured intracellular pH in the presence of lonidamine. Treatment with lonidamine reduced intracellular pH more significantly, showing the effect on extracellular pH (Fig. 5B). These data explain the mechanism of cell death, following disruption of intracellular pH homeostasis. We further used a knock-down approach with MCT siRNA to confirm the impact of MCT on cell viability. When the cells were incubated with a high concentration of glucose (20 mM) to increase metabolism, the siRNA induced cell death during incubations longer than 48 h (Fig. 6A). The intracellular pH of BI-1 cells dropped dramatically due to the disturbance of intracellular pH homeostasis. Extracellular pH increased for the same reason (Fig. 6B). In this setting, the CΔBI-1 cell reacted similarly to the Neo cells. These data suggest that the maintenance of pH homeostasis is essential for cell survival in BI-1 cells.

Discussion

We observed that the expression of BI-1 increased NHE and MCT activities, leading to the extracellular accumulation of lactate and acidic extracellular hydrogen. Through these transporters, BI-1-overexpression induces cancer metastasis and maintains the homeostasis of intracellular pH. Furthermore, the relationship between MCT and NHE was also studied in cells overexpressing BI-1 (BI-1 cells).

Our findings suggest that MCT transporters can be critical for maintaining intracellular pH in the BI-1-induced acidic environment and associated cancer metastasis in HT1080 cells. In this study, expression of MCT1, 2 and 4, among the 14 MCT family proteins (13), was examined, showing highly increased expressions of MCT proteins in BI-1 cells (Fig. 2B). Through magnetic resonance spectroscopy, lonidamine was proposed to be an inhibitor of lactic acid efflux that causes intracellular acidification (20). The lonidamine inhibited lactate production with higher rate in BI-1 cells than in Neo cells (Fig. 3A), suggesting a role of MCT in BI-1-associated lactate production which explains a novel mechanism of BI-1-induced acidic surroundings and resulting cancer metastasis.

In addition, NHE1 and MCT are also interconnected in the mechanisms regulating the acidic surroundings in BI-1 cells. We also found that NHE activation was followed by MCT activation (Fig. 2C and D). Actually, members of the MCT family of transporters are responsible for the export of lactate, pyruvate, and a variety of other monocarboxylates from cells (21). Consistently with our findings, the activation of MCT was reported to require sodium-hydrogen exchanger activation, leading to intracellular pH homeostasis; in highly activated conditions, the activation results in the extracellular accumulation of lactate and acidification of extracellular surroundings (22). The involvement of NHEs in MCT activity is further supported by the decrease in uptake rates of l-lactic acid as extracellular sodium levels decrease in normal cell physiology (23,24). In the cancer model described here, MCT is suggested to affect the extrusion of lactate, but not lactate uptake, which influences lactate accumulation in the extracellular cancer environment similarly to previous studies (14,22). Similarly, several studies have also suggested that acidic pH_i and oncogenic transformation activate NHE1 and subsequently MCT, thereby creating an acidic extracellular microenvironment (15,25).

The role of hydrogen/lactate transporters is diverse in various kinds of cells, showing different physiological and pathological phenomena. Usually, NHE and Na^+/HCO^{3-} symporter and MCT are involved in acid-equivalence physiological systems in normal cells (23,26). Recently, various inhibitors of the exchanger or transporter proteins have displayed protective effects against pathological stress, especially in the cardiac system. Recently, a novel NHE-1 selective inhibitor, KR-33028, was shown to have potently cardioprotective effects in perfused rat hearts subjected to global ischemia and reperfusion, suggesting that the inhibition of NHE may prevent hypoxic cell death (27). However, cancer cell physiology is very different from that of other normal cells, in terms of oxygenation states, metabolites, ions and the signaling transduction systems (28). This study showed that a high concentration of EIPA increased cell death, and showed that silencing of the MCT1 gene with siRNA or inhibition of MCT activity with lonidamine in BI-1 cells resulted in a precipitous decline in cell survival, probably through the effect on intracellular pH (Figs. 5A and 6A).

The characteristics of BI-1 have been introduced as an oncogene (29). In non-small cell lung carcinoma patient samples, the expression of BI-1 is significantly higher in lymph node metastasis cases (not published). Enhancement of the glycolytic metabolism of BI-1 is also related to cancer physiology through the Warburg effect. In other cancer cells, including cholangiocarcinoma cells, selective inhibition of ion transport mechanisms that regulate intracellular pH, including NHE, induces apoptosis (30). Intracellular acidification caused by the inhibition of Na^+/H^+ exchanger and H^+-ATPase also triggers apoptosis (31). In addition, the inhibition of the regulation of intracellular pH: potential of 5-(N,N-hexamethylene) amiloride has been suggested as a tumor-selective therapy (32). Tumor metabolism consists of complex interactions between oxygenation states, metabolites, ions and the vascular network cascades. Accumulation of lactate within tumors is correlated with poor clinical outcomes (33). Carcinomas produce high amounts of lactic acid and up-regulate the H^+-linked MCT isoform 1 (MCT1/SLC16A1). MCT1 mRNA levels in fresh carcinoma biopsy samples are positively correlated with the risk of fatal disease (18).

In conclusion, BI-1 increases NHE and MCT activities for intracellular pH homeostasis. Inhibition or knock-down of the transporters inhibited BI-1-induced extracellular acidity and cancer metastasis, ultimately leading to cell death. Until now, the cancer-related function or endogenous expression of BI-1 had not been determined. The next step is to evaluate the relationship between the expression of BI-1 and clinical symptoms or diagnoses. If the expression of BI-1 is significantly higher in cancer tissues, and is correlated with clinical symptoms, ion exchangers or transporters offer potential as cancer therapeutic candidates.

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