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The effect of extracellular alkalinization on lactate metabolism of breast cancer stem cells: Overview of LDH-A, LDH-B, MCT1 and MCT4 gene expression

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Abstract. Changes in the metabolic status of cancer cells are presumed to be correlated with the adjustment of these cells to extracellular changes. Cell glycolysis increases the production of intracellular lactate catalyzed by the lactate dehydrogenases, both LDH-A and LDH-B. An increase in intracellular lactate can affect extracellular pH balance through monocarboxylate transporters, particularly MCT1 and MCT4. This study aimed to analyze the effects of extracellular alkalinization on the lactate metabolism of human breast cancer stem cells (BCSCs). In this study, human primary BCSCs (CD24-/CD44+ cells) were treated with 100 mM sodium bicarbonate for 0.5, 24, and 48 h in DMEM F12/HEPES. After incubation, extracellular pH was measured and cells were harvested to extract the total RNA and protein. The expression of LDH-A, LDH-B, MCT1, and MCT4 mRNA genes were analyzed using qRT-PCR method. Our study shows that administration of sodium bicarbonate in the BCSC culture medium could increase extracellular pH. To balance the increase of extracellular pH, BCSCs regulated the expression of LDH-A, LDH-B, MCT1, and MCT4 genes. As the extracellular pH increases, the expression of LDH-A that converts pyruvate to lactate increased along with the increase of MCT 4 and MCT 1 expression, which act as lactate transporters. As the incubation time increases, the pH decreases, leading to the suppression of LDH-A and increase of LDH-B expression that converts lactate into pyruvate. Therefore, we suggest that the extracellular alkalinization by sodium bicarbonate in BCSCs affected the genes that regulate lactate metabolism.

1. Introduction

The complexity of carcinogenesis has increased with the discovery of a small population of highly tumorigenic cells and is complemented by stem cell properties in solid tumors, including breast tumors [1,2]. This cell population is presumed to be a tumor initiator, later called as breast cancer stem cells (BCSC). BCSCs are known to play an important role in dormancy, metastasis, recurrence, and tumor resistance to chemotherapy and radiotherapy [3]. The metabolism of BCSCs is not well understood. Vlashi et al. showed the difference between metabolism of BCSCs and differentiated BCSCs. BCSCs show dependence on oxidative phosphorylation, whereas differentiated BCSCs are more dependent on glycolysis [4]. Hanahan’s study demonstrated the unique correlation between CSC metabolism and the

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tumor microenvironment condition [5]. In other words, understanding microenvironment changes has become important to elucidating BCSC metabolism. In general, the microenvironment of BCSCs is similar to other CSC microenvironment, especially in terms of a decreasing pH which leads to an acidic condition outside the cell. Mauro Sola-Penna showed that the acid conditions in CSCs result from the increased lactate production, and the cells continue to maintain these conditions so that immune cell attack is avoided [6,7].

Using nude mice implanted with cancer cell strains MDA-MB-231, Robey et al. demonstrated that oral bicarbonate administration over a period of time may increase the pH of tumor tissues, thereby decreasing the number and size of metastases in the lungs, visceral organs, and lymph nodes [8]. However, the results of this study did not explain the effect of bicarbonate on lactate metabolism of CSCs, especially in BCSCs. Conducting research on the effect of sodium bicarbonate on lactate metabolism in terms of the lactate dehydrogenase (LDH) and monocarboxylate transporters (MCT) genes is therefore necessary. LDH and MCT genes are known to play a role in lactate metabolism. The genes are LDH-A, LDH-B, MCT1, and MCT4. Comparing the expression of LDH-A and LDH-B with that of MCT1 and MCT4 on BCSCs treated with sodium bicarbonate can provide information on the regulation of lactate balance and metabolic status in BCSCs.

Lactate is produced from pyruvate reduction by the LDH and NADH as cofactor. The formation of lactate has effects on CSCs in terms of generating less ATP than through oxidative phosphorylation. To overcome this condition, CSCs increase the speed of pyruvate conversion to lactate, resulting in the increased synthesis of LDH [7]. The lactates continuously produced by the LDH are needed to be removed through a monocarboxylate transporter (MCT) to cause an acidification of BCSCs extracellular with a decrease pH to 6.5 [9]. LDH is a dehydrogenase enzyme that has isozymes [10]. The LDH isozymes that play a role in lactate metabolism are LDH-A and LDH-B. LDH-A converts pyruvate to lactate, and LDH-B converts lactate to pyruvate. LDH-A increases in oxygen deprivation conditions to run the metabolism of glycolysis, while LDH-B increases when enough oxygen is available, and goes into the path of oxidation phosphorylation. The transporters that have been reported to play a role in cancer cells are MCT1 and MCT4. MCT1, which has a high affinity for lactate, takes the lactate from the outside into the cell, whereas MCT4, which has a low affinity for lactate, takes the lactate from the inside into the cell [11]. MCT1 inhibition prevents lactate entry to stromal cells, so it can reduce the glucose taken by cancer cells because of the glucose taken by the stroma. Inhibition of MCT4 may provide immediate potential for dead tumor cells because of the accumulation of lactic acid in cells [12].

2. Materials and Methods

2.1. Research Sample and Cell Culture

(CD24 -/CD44 +) were obtained from a previous study in the Biochemistry and Molecular Biology laboratory on the basis of a primary culture of human BCSCs sorted with magnetic beads (MACS) conjugated with anti-CD24 and anti-CD44 antibodies to obtain CD24 - /CD44 + and CD24 -/CD44 - cells. The breast CSCs (CD24 -/CD44 +) were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM F-12) medium, which was added with 1% streptomycin-penicillin and 1% amphotericin B. The CD24 -/CD44 + BCSCs were grown in an incubator at 37 °C temperature with 5% CO2 and 19% O2. To change the extracellular environment pH, 100 µm of sodium bicarbonate (NaHCO3) was added to the culture medium, which was then incubated for 0.5, 24 and 48 h.

2.2. Extracellular pH Measurement (pH)

pHe was determined by measuring pH of the culture medium using pH electrode with Micro Bulb for 96-well plate (Hanna®) connected with ph meter (HI 2210®, Hanna). After incubation, 500 µl medium from each alkalinized and non-alkalinized BCSC culture was collected in a test tube for pH

measurement. The pH should be immediately measured upon removing the cell culture plate from incubator.The pH measurements were compared with those of the control pH.

2.2.1. Calculation of the Number of Cells
After the cells were harvested and dissolved in the medium, 10 µl cells were taken, and 10 µl were added with trypan blue. Trypan blue is a colorless dye that does not permeate intact cells, so it cannot dye living cells. Cells are calculated (Luna automated counter cells®) in five fields of view

2.2.2. Calculation of the Population Doubling Time (PDT)
Cells grown in vitro will undergo the following four phases of cell growth: 1) lag phase, 2) logarithmic (log) growth phase, 3) plateau phase, and 4) decline phase. The characteristics of cell growth in the logarithmic phase can be assessed with the population doubling time (PDT). This value indicates the time required by the cell population to grow twice the number of cells during inoculation (seeding). PDT can be used to estimate cell cycle time, which is calculated with the following formula:

***Population doubling time* (PDT)**

$$PDT = 1/r$$

$$r = 3{,}32\ (\log N_H - \log N_I) / (t_2 - t_1)$$

Explanation:
r = multiplication speed
N_I = number of inoculating cells
N_H = number of harvested cells
t_1 = inoculating time
t_2 = harvest time

2.3. Quantitative reverse transcription-PCR
Total RNA was extracted from cell pellets using Tripure® RNA Isolation Kit (Roche, Germany) according manfacture's protocol. Total RNA concentration was quantified using spectrophotomether (Varioskan Flash®, Thermo Scientific, Finland). Samples with an A260/A280 ratio of 1.6-2.0 were considered to be free of DNA and protein. Quantitative RT-PCR was performed using KAPA SYBR Fast® qPCR (Kapa Biosystem, USA) in the ExicycleTM 96 (Bioneer, Korea). The PCR primers for Primary LDH-A Forward: 5' AGC CCG ATT CCG TTA CCT 3', Reverse: 5' CAC CAG CAA CAT TCA TTC CA 3'. Primary LDH-B Forward: 5' CTC CTG GTA GGT TTC GGC TC 3', Reverse: 5' CCA AAC TGA GCG GCA AAG TC 3'. Primary MCT 1 Forward: 5' GCT GCA GTT CGG ATG TCT GT 3', Reverse: 5' AGC GAG GCT GCC TTA TAA CC 3'. Primary MCT 4 Forward: 5' TTT TGC TGC TGG GCA ACT TCT TCT G 3', Reverse: 5' TCA CGT TGT CTC GAA GCA TGG GTT T 3'. All primers have been tested for their efficiency for all genes (>95%). Ct for each gene was determined, and ΔΔCt was normalized to the designated reference sample. Gene expression values were the relatively calculated using the Livak method ($2^{-\Delta\Delta Ct}$).

2.4. Statistical Analysis
All data reported as means±SE of at least triplicates. A P-Value of < 0.05 in the independent t-test and one way anova was considered to be statistically.

3. Results and Discussion

3.1 Results
Following of extracellular alkalinization on BCSC showed that the increased pHe compared with non alkalinization (Figure 1). The pHe of extracellular BCSC with alkalinization was increased at 0.5, 24, and 48 h of incubation time compared with BCSC non alkalinization at each incubation time. However, as the incubation time increased, the pHe of the extracellular was decreased. The pHe of the BCSC non alkalinization decreased significantly starting at 0.5 h of incubation time, and lasted until the end of the 48h of incubation time. Thus, the extracellular alkalinization may increase the pHe of BCSC CD24 -/CD44 +. At the end of incubation time, pHe of extracellular BCSC with alkalinisation

was still higher than non alkalinization. The pH changes indicate the presence of cell activity during each incubation time.

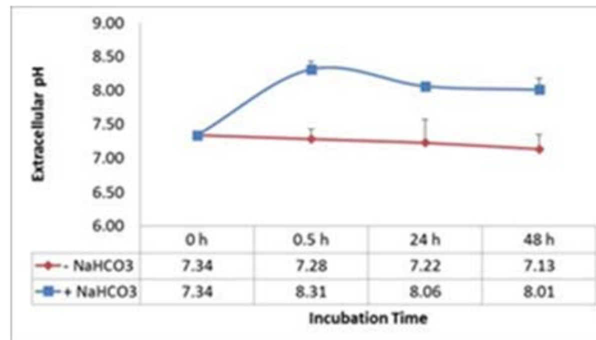

Figure 1. Measurement of the medium pH of BCSC against NaHCO3 treatment. The blue line shows the amount of pHe BCSCs non alkalinization (-NaHCO3), and the red line shows the amount of pHe BCSCs with alkalinization (+NaHCO3). The incubation was started before the alkalinization (0 h), half an hour after the alkalinization (0.5 h), 24 h after the administration of NaHCO 3 (24 h), and 48 h after NaHCO3 release (48 h). The result is the average of the pH measurement with five repetitions. Data are presented as mean ± SD; no significant difference has been found.

BCSC growth were observed by planting 150,000 cells at each well (counted as day 0). Growth was followed during cell incubation periods starting at 0.5, 24, and 48 h. The growth curve of BCSC resulting from the alkalinization from the initial planting to 48 h of incubation is presented in Figure 2.

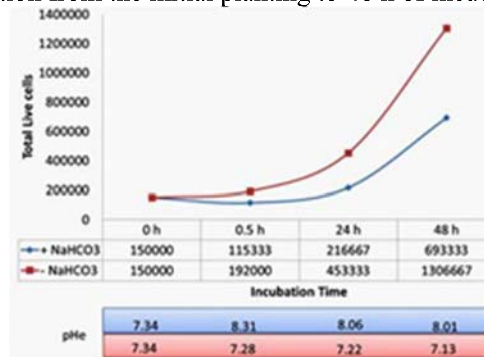

Figure 2. Growth of BCSCs CD24 -/CD44 + with extracellular alkalinization. The blue line shows the number of living BCSCs without extracellular alkalinization(- NaHCO3), and the red line shows the number of living BCSCs with extracellular alkalinization (+NaHCO3). BCSCs are stored below 37oC temperature and at 5% CO2 during incubation time. The number of early incubation living cells with and without extracellular alkalinization (0 h) at each treatment was equal. However, after half an hour (0.5 h), 24 h and 48 h of BCSC with alkalinization have difference in the number of living cells was doubled. The result was an average of living cells obtained from five times of calculation using trypan blue dye and the Luna cell counter®. Data are presented as mean ±SD; no significant difference has been found.

The characteristics of cell growth in the logarithmic phase can be assessed with the PDT. In cells treated with alkalinization, the PDT value was 21,739 h, indicating that the cells required 21,739 h to form a cell population twice that of the initial cell count. For the cells without alkalinization, the PDT value was 14.49 h, indicating that the cells required 14.49 h to form a population twice that of the initial cell count. Thus, the extracellular alkalinization may slow down the growth of BCSCs compared with the BCSCs without alkalinization during 48 h of incubation.

To explore the role of LDH-A, LDH-B, MCT1 and MCT4 in first regulating the changes of pHe, we measured it mRNA expression level in BCSC after each NaHCO3 incubation period. LDH-A gene expression level at 0,5 h was significantly high and at 24 h it decreased but not significantly, whereas the LDH-B mRNA did not show a significant expression level (Figure 3A). The MCT4 expression levels were significantly high and began to decrease during the time of incubation. In contrast to that of MCT 1, the relative expression level is the same until 24 h, but it decreased dramatically at 48 h (Figure 3B).

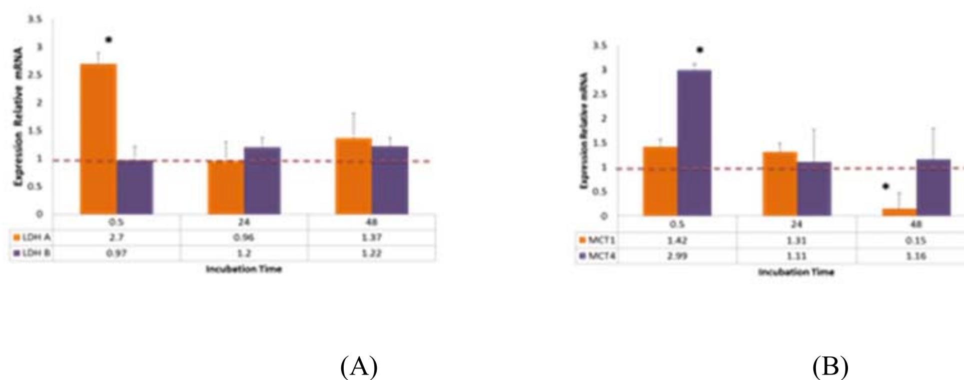

Figure 3. Expression of LDH and MCT mRNA BCSCs CD24 -/CD44+ with alkalinization for 0.5, 24, and 48 h. (A) The expression of LDH-A and LDH-B was analyzed through a comparison of the expression level at each incubation time. At 0.5 h, the expression level of LDH-A was high, but it decreased significantly at 24 h of incubation. (B) The expression level of MCT4 was high at 0.5 h, but that of MCT 1 decreased drastically at 48 h. Comparison analysis of the mRNA expression level based on the expression ratio of cells given NaHCO3 to cells without NaHCO3 (control). Data are presented as a relative expression value ± SD. *: $p < 0.05$

3.2 Discussion

The microenvironmental changes performed in this study were the effects of $NaHCO_3$ supplementation in the BCSC growth medium. As demonstrated in our previous study [13], $NaHCO_3$ could increase the pH of the BCSC medium after 0.5-h incubation. The results obtained in this study also showed an increase of extracellular pH after 0.5-h supplementation with 100 mM of NaHCO3. When NaHCO3 is added, HCO_3^- increases, takes $[H^+]$, and forms H_2CO_3 as a buffer system for the cell [14,15].

In this study, we found that $NaHCO_3$ supplementation could decreases BCSC viability, which have also been reported in our previous study [13] and by Kobayashi *et al.* [16]. Study of Ursula *et al.* [17] has suggested that an increased CO_2/HCO_3^- can suppress cell proliferation. The supplementation of high bicarbonate in the cells increases the production of CO_2 and changes the bicarbonate rate. NaHCO3 administration changed the extracellular environment from acidic (pH 6.5) to alkali (pH 8.2–8.3). The acid–base balance is important in maintaining cell survival. A slight change in the pH affects the cellular system through a balance between CO_2, HCO_3^-, and H^+ as the buffer system.

LDH-A gene expression of breast CSCs (CD24-/CD44+) treated with $NaHCO_3$ increased at 0.5 h of incubation time compared with those of the control (without $NaHCO_3$). The increased expression of LDH-A may be due to an adaptive response of BCSCs in alkaline extracellular pH (8.31) compared with the control pH (7.34) at the beginning of incubation. The study of Robey *et al.* [8] indicated that although the administration of NaHCO3 may alter the pH, but not the intracellular pH. In other words, the increase of LDH-A expression in 0.5-h incubation of $NaHCO_3$ was related to the role of LDH-A in

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converting pyruvate to lactate. Thus, the more that LDH-A is expressed, that more that lactate is increased. Interestingly, after ŧ 24-h of $NaHCO_3$ supplementation, extracellular pH of BCSCs decreased to 8.06, leading to the decrease of LDH-A mRNA expression until it reached the level of control without $NaHCO_3$ supplementation. The decreased pH value after 24-h supplementation was assumed to be the fast response of carbonic anhydrase-9 (CA9) in regulating alkaline pHe, rather than HIF1-α [18]. In breast cancer CSCs (CD24-/CD44+), the decreased LDH-A cells were suspected to slow down cell proliferation [19]. Other previous study reported that inhibition of LDH-A synthesis in hypoxic melanoma cells reduced the cell proliferation [20].

In contrast to LDH-A, LDH-B expression did not increase at 0.5-h incubation of $NaHCO_3$. It has been widely reported that cancer cells have increased LDH-A, but not for LDH-B expression [20]. At 24-h alkalinization, a slightly increase in LDH-B expression was observed compared with that of the control. LDH-B began to increase its expression possibly because of lactate production, which started to increase in the cells so that lactate is converted into pyruvate for use in oxidative phosphorylation. Schulte *et al*. [21] found an increase in LDH-B expression, indicating the stress response experienced by southern fish. Therefore, an increased LDH-B expression in breast cancer cells (CD24-/CD44+) becomes an adaptive response for stress cells to survive.

The MCT1 mRNA expression levels in $NaHCO_3$-treated cells at 0.5-h to 24-h incubation were slightly higher than that of the control (without NaHCO3). This result is presumed to be an adaptive response of CD24-/CD44+ cells. According to Hong *et al*. [22], an increase in MCT1 expression is needed to promote the glycolysis in breast cancer cells. MCT1 transports lactate from the extracellular into the cell. In addition, the increase of MCT1 has been associated with the increase of cell proliferation and growth [22]. Koukourakis *et al*. [23] found that a high MCT1 is also associated with fibroblast cell expression. Interestingly, at 48-h alkalinization, a lower and significant decrease of LDH-B expression was observed in breast CSCs (CD24-/CD44+). This might be due to the increased intracellular lactate level after 24-h alkalinization [13]. In addition, cancer cells adapt to the loss of MCT1 by increasing the oxidative phosphorylation of glucose metabolism [24].

A higher MCT4 mRNA expression was found in cells treated with NaHCO3 at 0.5-h of incubation compared to that without NaHCO3. The increased MCT4 expression was possibly an adaptive response of breast CSCs (CD24-/CD44+) to remove the accumulation of intracellular lactate into extracellular [13]. According to Ullah *et al.* [25], MCT4 mediated lactate removal from the cells depending on the glycolysis which produce ATP. After 24-h incubation, the MCT4 expression significantly decreased compared with that at the previous incubation. A decreased MCT4 expression was presumed to be a consequence of the increase of intracellular lactate production at 24-h alkalinization, which resulted from the ongoing glycolysis in breast CSCs [19]. According to Izumi *et al*. [26], high level of intracellular lactate was found simultaneously with low extracellular lactate level. We suggest that the MCT4 expression is closely related to the intracellular lactate level. A similar pattern between MCT4 and LDH-A occurred in this study, suggesting that LDH-A expression may also correlated with MCT4 expression. Both of these genes have a HRE promoter which activated through hypoxic conditions [26]. Our findings indicate that MCT4 expression is influenced by LDH-A expression and intracellular lactate production. Najjar [20] explained that gene expression regulated by HIF1α transcription factors is able to undergo aerobic metabolism during hypoxia in the presence of lactate [20]. Pinheiro *et al.* [27] suggested that CD44 is associated with an increased expression of MCT1, but it decreases MCT4 expression in plasma membranes of lung cancer cells.

The increased expression of LDH-A compared with that of LDH-B and the increased expression of MCT4 compared with that of MCT4 in cells treated with $NaHCO_3$ allow cells to undergo glycolysis. In cancer cells, glycolysis is a suitable metabolic process due to hypoxic condition of cancer cells. To balance the increased pH value after extracellular alkalinization, BCSCs decreased the pH after 24 hours mimicking the hypoxic condition. In our previous study, we have demonstrated that the expression of HIF1α increased after 24-h alkalinization. One of the target gene expression that induced by the HIF1α transcription factor is LDH-A. According to Ullah *et al*. [26], MCT4 expression is also

regulated by HIF1α. Therefore, our present study suggests that HIF1α, like CA9, plays a role in the regulation of LDH-A and MCT-4 expression.

4. Conclusion

The extracellular alkalinization through NaHCO3 treatment affects the regulation of lactate metabolism on the basis of LDH-A, LDH-B, MCT-1, and MCT-4 gene expressions. The expression of LDH-A increases along with MCT-4. As LDH-A expression begins to decrease, LDH-B increases. Human BCSCs (CD24-/CD44+) regulate the expression of these lactate metabolism genes to maintain lactate balance.

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