Original Paper

Decreased Intracellular pH Induced by Cariporide Differentially Contributes to Human Umbilical Cord-Derived Mesenchymal Stem Cells Differentiation

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Key Words

Na⁺/H⁺ exchanger 1 (NHE1) • Mesenchymal stem cells • Intracellular pH (pH_i) • Osteogenic differentiation • Adipogenic differentiation • β-catenin

Abstract

Background/Aims: Na⁺/H⁺ exchanger 1 (NHE1) is an important regulator of intracellular pH (pH_i). High pH_i is required for cell proliferation and differentiation. Our previous study has proven that the pH_i of mesenchymal stem cells is higher than that of normal differentiated cells and similar to tumor cells. NHE1 is highly expressed in both mesenchymal stem cells and tumor cells. Targeted inhibition of NHE1 could induce differentiation of K562 leukemia cells. In the present paper we explored whether inhibition of NHE1 could induce differentiation of mesenchymal stem cells. **Methods:** MSCs were obtained from human umbilical cord and both the surface phenotype and functional characteristics were analyzed. Selective NHE1 inhibitor cariporide was used to treat human umbilical cord-derived mesenchymal stem cells (hUC-MSCs). The pH_i and the differentiation of hUC-MSCs were compared upon cariporide treatment. The putative signaling pathway involved was also explored. **Results:** The pH_i of hUC-MSCs was decreased upon cariporide treatment. Cariporide up-regulated the osteogenic differentiation of hUC-MSCs while the adipogenic differentiation was not affected. For osteogenic differentiation, β-catenin expression was up-regulated upon cariporide treatment. **Conclusion:** Decreased pH_i induced by cariporide differentially contributes to hUC-MSCs differentiation.

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Introduction

It is well known that the intracellular pH (pH_i) of terminally differentiated cells is lower than the relatively more “stem” cells such as tumor cells and/or stem cells [1, 2]. Higher pH_i is important to keep the stemness of tumor cells and/or stem cells. The pH_i is regulated by the transport system located in the plasma membrane, of which NHE1 is the most important one. NHE1 is a member of the Na^+/H^+ exchanger family and regulates pH_i by catalyzing the extrusion of intracellular proton ions in exchange for extracellular sodium ions [3-6]. Increased pH_i caused by the NHE1 has been proved to provide extremely crucial signal for cell proliferation and differentiation, especially in tumor cells [7]. Our previous study has shown that targeted inhibition of NHE1 induced differentiation of K562 leukemia cells [8].

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal stem cells and mesenchymal stromal cells, are a heterogeneous multipotent population of stromal cells with fibroblast-like morphology and proliferate *in vitro* as plastic-adherent cells. They also possess the properties to form colonies *in vitro* and differentiate into osteoblasts, chondrocytes and adipocytes [9-12]. In addition to the bone marrow stroma, MSCs have been found in other tissues, such as adipose, umbilical cord blood, umbilical cord (UC), amniotic membrane, gingiva, thymus, placenta, synovium and fetal blood and liver [13-21]. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are isolated from discarded extra-embryonic tissue after birth and become a promising source of mesenchymal stem cells.

In this study, we chose hUC-MSCs which have many advantages over other MSCs as the subject. Cariporide, which is a high selective and low cytotoxic NHE1 inhibitor [22], was used to decrease the pH_i . This study was aimed to explore the effect of decreased pH_i on the differentiation of hUC-MSCs and to explore the putative mechanism involved.

Materials and Methods

Preparation of hUC-MSCs

Human UC were collected from healthy full-term pregnant donors with written consent and approval by the institutional review board of the Chinese Academy of Medical Sciences and Peking Union Medical College (Tianjin, China). hUC-MSCs were isolated and expanded as described previously [23]. hUC-MSCs were cultured in complete culture medium containing Dulbecco's minimum essential medium (DMEM)/F12 (1:1) (Gibco; Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin-streptomycin (Sigma; St. Louis, MO, USA), 1% glutamine (Sigma) and 10 ng/mL epidermal growth factor (EGF; Sigma). The hUC-MSCs were incubated in a 5% CO_2 incubator at 37 °C, and cells at passage (P) 4–6 were used for experiments unless otherwise stated. The hUC-MSCs used in each independent comparison assay were derived from the same UC in order to reduce experimental variation.

Characteristics of hUC-MSCs

The phenotype of hUC-MSCs were analyzed by flowcytometry according to the criteria by ISCT [10]. The following antibodies were used for fluorescence-activated cell sorting (FACS) analysis: phycoerythrin (PE)-conjugated antibodies against CD11b, CD45, CD73, CD90, CD105 and HLA-DR, while fluorescein isothiocyanate (FITC)-conjugated antibodies against CD19 and CD34. All antibodies were purchased from Becton Dickinson (San Diego, CA, USA).

Cytotoxicity analysis

MTT assay was performed to detect the cytotoxicity of cariporide to hUC-MSCs. Briefly, cells were seeded into 96-well culture plates at a density of 5×10^4 cells/ml. Cariporide was added in medium with a final volume of 200 μ l per well at gradient concentrations. After drug treatment for 72 h, medium was replaced with an equal volume of fresh medium containing 0.5mg/ml MTT. The culture plates were incubated for 4 h at 37 °C. Then, the medium was removed and 100 μ l DMSO was added and incubated for 10min at room temperature. The cytotoxic effects of drugs were determined according to the OD values using a microplate reader at absorption wavelength of 490 nm.

Table 1. The sequence of primers designed for Real-time PCR

Gene	Primer sequence (5'-3')
CEBPD	Forward: GAAGTTGGTGGAGCTGTCCG Reverse: TGAGGTATGGGTCGTTGCTGA
LPL	Forward: TGGATGGAGGAGGAGTTT Reverse: GGATCGAGGCCAGTAATT
BMP	Forward: GACGAGGTCCTGAGCGAGTT Reverse: ACCTGAGTGCCTGCGATACA
Osteocalcin	Forward: CAGGCGCTACCTGTATCAATG Reverse: CTCCTGAAAGCCGATGTGGT
β -Catenin	Forward: GAGCCTGCCATCTGTGCTCT Reverse: ACGCAAAGGTGCATGATTTG
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTTC

Evaluation of pH_i

pH_i of cells was assessed by flowcytometry using the pH-sensitive fluorescent probe BCECF-AM as described previously [24]. Cell suspensions in serum-free medium were washed and labeled with BCECF-AM. BCECF was successively excited at 490 and 440 nm, and the resultant fluorescent signal was monitored at 535 nm. Intensity ratio (490/440) was plotted vs. pH_i . In order to obtain the calibration curve, a linear regression within the pH_i range 6.2–7.4 was obtained. To illustrate the decreased NHE1 activity in cariporide-treated and NHE1 silencing cells, the cells were acidified by an ammonium pulse and the rate of sodium-dependent H^+ extrusion was studied. Following exposure to NH_4Cl , NH_3 enters the cells, binds H^+ to form NH_4^+ and thus leads to a transient cytosolic alkalization. The subsequent removal of NH_4Cl resulted in exit of NH_3 leaving H^+ ions in the cell which causes cytosolic acidification.

Osteogenic and adipogenic differentiation of hUC-MSCs

Specialized cell culture media was purchased from Invitrogen and used according to the manufacturer's instruction to induce osteogenic and adipogenic differentiation of hUC-MSCs respectively *in vitro*. hUC-MSCs were incubated in six-well plates in the presence or absence of 10 μM cariporide for 21 days, and the medium was replaced every three days. The alkaline phosphatase (ALP) staining, Alizarin red (AR) staining and Von Kossa staining were used to detect osteogenic differentiation, and Oil Red O staining was used to detect adipogenic differentiation.

Quantitative RT-PCR

Total RNAs were isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. RNAs were reverse transcribed, and the relative mRNA expression levels of CEBPD, LPL, BMP, Osteocalcin, and β -Catenin were detected by Real-time PCR using ABI Prism 7500 sequence detection system (Applied Biosystems, USA). Meanwhile, GAPDH was used as internal reference gene. All primers are listed in Table 1. The Real-time PCR procedure included 3 min of pre-denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and 60 °C for 40 s. Real-time PCR analysis of each sample was repeated in triplicate.

Quantitative ALP assay

Samples were lysed with 300 μL of ALP lysis buffer and 0.5% Triton X-100 on 10th day during osteogenic differentiation. The hydrogel samples were homogenized with pipetting and sonicated for 10 min. BCA was conducted to assess the total protein to make sure that equal total protein was loaded in each group. The samples were vortexed and incubated with an alkaline buffer solution and a phosphatase substrate solution at 37 °C for 15 min. The ALP activity in nmol/min was calculated by comparing samples' absorbance of p-nitrophenol product at 405 nm with that of p-nitrophenol standards.

Western blot analysis

The preparation of protein lysates and Western blot analysis were performed according to the manufacturer's instruction for each respective antibody. The primary antibodies used were anti- β -catenin (mouse monoclonal, Cell Signaling), and anti-GAPDH (rabbit monoclonal, Cell Signaling).

Fig. 1. Phenotype characteristics of hUC-MSCs. Surface markers of hUC-MSCs were detected by flowcytometric analysis. The experiment shown is representative of three performed.

Statistical analysis

The data was expressed as mean $\pm$ SD over three independent experiments. Statistical significance of differences was analyzed by t-test for comparison between two groups. $P < 0.05$ by two-tailed test was considered as statistically significant.

Results

Characteristics of hUC-MSCs

The surface phenotype was analyzed by flowcytometry according to the criteria defined by ISCT [10]. hUC-MSCs showed positive expression ($>95\%$) of CD73, CD90 and CD105, but negative ($<2\%$) for CD34, CD45, CD11b, CD19 and HLA-DR (Fig. 1). The cells possessed multipotent differentiation potential, as they could be induced to differentiate into osteoblasts and adipocytes under standard *in vitro* differential conditions.

Cytotoxicity of cariporide on hUC-MSCs

To explore the cytotoxicity of cariporide, hUC-MSCs were incubated with different concentrations of cariporide and MTT assay was performed. As Fig. 2a shows, cariporide inhibited hUC-MSCs growth at a concentration higher than $80\ \mu\text{M}$ but exerted little effect on hUC-MSCs at a low concentration. Finally, we chose a concentration of $10\ \mu\text{M}$ at the latter experiment to avoid the cytotoxicity of the inhibitor.

Inhibition of NHE1 by cariporide decreased the pH_i of hUC-MSCs

To investigate the effect of NHE1 inhibitor on pH_i of hUC-MSCs, pH_i was measured in hUC-MSCs with treatment of $10\ \mu\text{M}$ cariporide for 24 h by using the fluorescent dye BCECF-AM. As indicated in Fig. 2b, cariporide resulted in a decrease in pH_i value of hUC-MSCs. Na^+ -dependent alkalinization (Fig. 2c) showed that pH_i recovered soon in the cells without any treatment, while pH_i hardly recovered in cariporide-treated and NHE1 knock-down cells. This further confirmed that the decrease in pH_i was due to the inhibition of NHE1 activity.

NHE1 inhibition promoted the osteogenic differentiation of hUC-MSCs

To explore the effect of NHE1 inhibition on the osteogenic differentiation of hUC-MSCs, hUC-MSCs were incubated in the presence or absence of $10\ \mu\text{M}$ cariporide for 21 days. We performed the alkaline phosphatase (ALP) staining in the 10th day, Alizarin red (AR) staining and Von Kossa staining in the 21th day to detect osteogenic differentiation. As shown in Fig. 3a, the osteogenic differentiation of hUC-MSCs was up-regulated upon

Fig. 2. Effect of cariporide on hUC-MSCs cytotoxicity and pH_i . (a) Cytotoxicity of cariporide. hUC-MSCs were incubated with different concentrations of cariporide for 72 h and MTT was performed to measure the viability. Cariporide has little effect on hUC-MSCs viability at a low concentration. (b) Effect of cariporide on pH_i of hUC-MSCs. hUC-MSCs were cultivated with 10 μ M cariporide for 24 h. pH_i was measured by using the fluorescent dye BCECF-AM ($*P < 0.05$). (c) Na^+ -dependent alkalinization. In the presence of Na^+ , pH_i recovered soon in the cells without any treatment, while the pH_i hardly recovered in the cariporide-treatment cells and NHE1 silencing cells.

Fig. 3. Effect of NHE1 inhibition on hUC-MSCs osteogenic differentiation. (a) hUC-MSCs were incubated in osteogenic differential culture media in the presence or absence of 10 μ M cariporide for 21 days. The alkaline phosphatase (ALP) staining in the 10th day, Alizarin red (AR) staining and Von Kossa staining in the 21th day were used to characterize osteogenic differentiation. (b) hUC-MSCs were incubated in osteogenic differential culture media in the presence or absence of 10 μ M cariporide for 14 days. Total RNA was collected and quantitative RT-PCR was performed to assess the expression of the indicated genes ($*P < 0.05$). (c) The ALP activity was measured by quantitative ALP assay on 10th day during osteogenic differentiation ($*P < 0.05$).

cariporide treatment. Quantitative RT-PCR analysis (Fig. 3b) showed that the expression of main osteoblast markers BMP and Osteocalcin were also up-regulated upon cariporide treatment for 14 days. To further confirm the effect, quantitative ALP assay was performed. As shown in Fig. 3c, the ALP activity increased significantly in cariporide treatment group compared with control group. These results indicated that NHE1 inhibition promoted the osteogenic differentiation of hUC-MSCs.

Fig. 4. NHE1 shRNA knock-down experiment. (a) Effect of NHE1 shRNA knock-down on pH_i of hUC-MSCs (* $P < 0.05$). (b) The alkaline phosphatase (ALP) staining in the 10th day was used to detect osteogenic differentiation. (c) The ALP activity was measured by quantitative ALP assay on 10th day during osteogenic differentiation. The normal group is a negative control, measuring the ALP activity of undifferentiated hUC-MSCs cultured in complete culture medium (* $P < 0.05$).

Fig. 5. Effect of NHE1 inhibition on hUC-MSCs adipogenic differentiation. (a) hUC-MSCs were incubated in adipogenic differential culture media in the presence or absence of 10 μ M cariporide for 21 days. Oil Red O staining was used to characterize adipogenic differentiation. (b) hUC-MSCs were incubated in adipogenic differential culture media in the presence or absence of 10 μ M cariporide for 21 days. Total RNA was collected and quantitative RT-PCR was performed to assess the expression of the indicated genes.

NHE1 knock-down experiment

To further confirm the effect of NHE1 inhibition on osteogenic differentiation, we performed the NHE1 knock-down experiment. As shown in Fig. 4, NHE1 knock-down induced a decrease in pH_i and promoted the osteogenic differentiation of hUC-MSCs.

Effect of NHE1 inhibition on adipogenic differentiation of hUC-MSCs

To evaluate the effect of NHE1 inhibition on adipogenic differentiation of hUC-MSCs, hUC-MSCs were incubated in the presence or absence of 10 μ M cariporide for 21 days, Oil Red

Fig. 6. Effect of NHE1 inhibition on expression of β -catenin in osteogenic differentiation of hUC-MSCs. (a) hUC-MSCs were incubated in osteogenic differential culture media in the presence or absence of 10 μ M cariporide for 3 days. Total RNA was collected and quantitative RT-PCR was performed to assess the expression of β -catenin in mRNA level (* P <0.05). (b) hUC-MSCs were incubated in

osteogenic differential culture media in the presence or absence of 10 μ M cariporide for 3 days. Western blot was performed to assess the expression of β -catenin in protein level.

O staining was used to detect adipogenic differentiation. As shown in Fig. 5a, the adipogenic differentiation of hUC-MSCs was not significantly affected upon cariporide treatment. Quantitative RT-PCR analysis (Fig. 5b) showed that the expression of main adipogenic markers CEBPD and LPL were also not significantly affected upon cariporide treatment. These results indicated that NHE1 inhibition had little effect on adipogenic differentiation of hUC-MSCs.

NHE1 inhibition up-regulated the expression of β -catenin in osteogenic differentiation of hUC-MSCs

We then investigated the potential mechanisms through which NHE1 inhibition promoted the osteogenic differentiation of hUC-MSCs. Wnt/ β -catenin signaling pathway has been proven to play an important role in osteoblastogenesis. Quantitative RT-PCR (Fig. 6a) and Western blot (Fig. 6b) analysis showed that the expression of β -catenin was up-regulated upon cariporide treatment for 3 days.

Discussion

It is well known that pH_i is important for cell metabolism since many cellular mechanisms are sensitive to changes in pH_i [25-28]. In normal differentiated adult cells, pH_i is generally ~ 7.2 . The cancer cells and the stem cells, however, possess a higher pH_i [1]. The pH_i is regulated by transport systems including proton extruders like NHE, the H^+ pump, and/or bicarbonate transporters which mediate the influx or extrusion of bicarbonate [29]. One of the most important transporters is NHE1, which is a ubiquitously expressed member of the Na^+/H^+ exchanger family. To confirm the effect of NHE1 on pH_i , we performed the Na^+ -dependent alkalization assay. The slight recovery of pH_i in cariporide-treated and NHE1 knock-down cells indicated that other pH_i regulatory factors may be involved in this process. For instance, monocarboxylate transporters (MCTs) [30, 31] and the plasma membrane proton pump vacuolar ATPase (V-ATPase) are also responsible for the regulation of pH_i in Na^+ -independent manner [32-34]. Our previous study has shown that targeted inhibition of NHE1 induced differentiation of K562 leukemia cells and NHE1 was highly expressed in mesenchymal stem cell. So, we presume that whether decreased pH_i could also induce differentiation of mesenchymal stem cells.

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal stem cells and mesenchymal stromal cells, are a heterogeneous multipotent population of stromal cells with fibroblast-like morphology and proliferate *in vitro* as plastic-adherent cells. They also possess the properties to form colonies *in vitro* and differentiate into osteoblasts, chondrocytes and adipocytes. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs), have incomparable advantages: (1) Take full advantage of discarded materials from the new born baby, which are easily accessible and less troublesome in ethical controversy. (2) hUC-MSCs are readily long-time preparation and can be scaled up in large numbers

because of short doubling time. (3) hUC-MSCs contain both human embryonic stem cells and human mesenchymal stem cells markers. (4) hUC-MSCs maintain "stemness" for several serial passages. hUC-MSCs offer tremendous promise in cellular therapeutics [35].

In this experiment, the surface phenotype and functional characteristics of hUC-MSCs which are obtained from human umbilical cord were analyzed. To explore the effect of NHE1 inhibition on differentiation of MSCs, selective NHE1 inhibitor cariporide was used to treat hUC-MSCs in different differential culture media. To avoid the cytotoxicity, we selected a low concentration of 10 μ M. In this concentration, the growth of hUC-MSCs was not affected but the pH_i was decreased significantly.

The differentiation of hUC-MSCs was confirmed by both cytochemical staining and differential marker expression. The alkaline phosphatase staining in the 10th day, Alizarin red staining and Von Kossa staining in the 21th day showed that osteogenic differentiation of hUC-MSCs was up-regulated upon cariporide treatment. The up-regulation of the main osteoblast markers BMP and Osteocalcin expression and quantitative ALP assay confirmed the effect. However, the Oil Red O staining and main adipogenic markers CEBPD and LPL expression showed that the adipogenic differentiation was not affected upon cariporide treatment. NHE1 inhibition differentially contributes to hUC-MSCs differentiation. The differential effect may have potential clinical value, especially osteoblast associated disease, such as tissue engineering, bone fracture healing and bone disease in patients with multiple myeloma (MM) [36, 37]. Our recent results have shown the similar effect on BM-MSCs derived from patients with multiple myeloma. Why adipogenic differentiation was not affected upon cariporide treatment? In our opinions, the hUC-MSCs are prone to differentiated into adipocyte so easily in *ex vivo* culture media that the effect of cariporide is negligible. However, the effect *in vivo* needs further investigations.

Lots of evidence indicated that Wnt/ β -catenin signaling pathway plays an important role in osteoblastogenesis [38-40]. Our results showed that cariporide induced MSCs differentiation towards osteoblast, so we explored whether Wnt/ β -catenin signaling is involved in this process. Indeed, cariporide treatment up-regulated β -catenin expression in both mRNA level and protein level suggesting that NHE1 inhibition up-regulated the osteogenic differentiation of hUC-MSCs at least in part through the up-regulation of β -catenin. NHE1 inhibition may up-regulate the expression of β -catenin through MAPK signaling pathway. Previous study has shown that NHE1 plays a central role in the regulation of MAPK activity. Takewaki et al. found that the specific NHE1 inhibitor cariporide partially inhibited stretch-induced activation of ERK as well as of NF- κ B in cultured cardiomyocytes [41]. Mukhin et al. showed that Ras and MEK1/2 activation by Ang II or ET-1 were also attenuated by NHE1 inhibition, indicating that the effect of NHE1 was at or above the Ras level [42]. Chen et al. demonstrated that HOE642 prevented glucose-induced ERK1/2 activation and hypertrophy in a high glucose model of cardiomyocyte hypertrophy, further supporting the notion that NHE1-ERK1/2 pathway plays an important role in hypertrophy development [43]. MAPK is involved in β -catenin expression [44, 45]. But how NHE1 inhibition up-regulated the expression of β -catenin and which other signaling pathway is also involved needs further study.

In summary, our study demonstrates for the first time that decreased intracellular pH induced by cariporide differentially contributed to human umbilical cord-derived mesenchymal stem cells differentiation. These findings may open a new way for better use of MSCs in clinical trials.

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