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Endothelin-1 augments the therapeutic potency of human mesenchymal stem cells via GATA2 and MZF1 mediated N-cadherin-VEGF axis

엔도셀린-1이 야기하는 GATA2와 MZF1 매개 N-Cadherin-VEGF 경로를 통한 인간 중배엽 줄기세포의 치료 효능 향상

2015년 2월

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분자의학 및 바이오제약학과

김 기 환
Abstract

Endothelin-1 augments the therapeutic potency of human mesenchymal stem cells via GATA2 and MZF1 mediated N-cadherin–VEGF axis

GiHwan Kim
Molecular Medicine and Biopharmaceutical Sciences
WCU Graduate School of Convergence Science and Technology
The Graduate School Seoul National University

In the previous paper, we reported that individual difference of hUCB-MSCs and N-cadherin as a significant marker on therapeutic efficacy of rat myocardial infarction. We performed cDNA microarray analysis with the best and the worst efficacy-representing hUCB-MSCs to identify critical factor which induces augmentation of therapeutic efficacy. In this study, among of several factors, we focused on Endothelin-1 (ET-1). Interestingly, it has been reported that ET-1 is increased in myocardial infarction and high ET-1 levels are related with microvascular dysfunction and lower salvage index. So, there have been many studies that
concentrated to the blocking of ET-1 pathway via endothelin receptor antagonist or neutralization. However, ET-1 was high expressed in the best efficacy-representing hUCB-MSCs and lower in counterpart. To validate the potency of ET-1 as a priming factor, we detected the N-cadherin-VEGF axis after ET-1 treatment. Both N-cadherin and VEGF mRNA and protein expression levels were up-regulated by ET-1 priming event. We also verified that hUCB-MSCs are expressing the endothelin receptor type A not B and ET-1 up-regulates the N-cadherin-VEGF axis via endothelin receptor type A. Then, to screen N-cadherin transcriptional activator depended on ET-1, we searched N-cadherin promoter region and developed GATA2 and MZF1. Finally, to validate therapeutic efficacy of ET-1 priming, we injected ET-1 primed hUCB-MSCs to rat myocardial infarction model and obtained the outcomes that the ET-1 pre-treated hUCB-MSCs improved the therapeutic efficacy in rat myocardial infarction than non-treated hUCB-MSCs. Histological analysis also showed that increased engraftment efficacy and reduced fibrosis area on the ET-1 pre-treated hUCB-MSCs.

Keywords : Endothelin-1, Human umbilical cord blood mesenchymal stem cell, N-cadherin, GATA2, MZF1

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In recent times, human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have received attention as encouraging solution for allogenic cell therapy. Because, not only protocols for isolating, expanding of hUCB-MSCs has been successfully established, but also hUCB-MSCs can be extracted from relatively young and healthy donors. Furthermore, assured unique characteristics that can be differentiated into diverse lineages, hUCB-MSCs have shared the role of cell therapy products with human bone marrow derived MSCs (hBM-MSC). However, hUCB-MSCs isolated from different donors showed different therapeutic efficacies in repairing rat myocardium after myocardial infarction (MI), even though they were isolated and expanded under same standard protocol. That is, hUCB-MSCs have limitation with individual variation.

In the previous paper, we compared therapeutic efficacy of 4hUCB-MSC from different donors (M01, M02, M03, M04) in rat MI model. And we assured that N-cadherin is critical marker for distinguishing whether each hUCB-MSC exhibits
remarkable therapeutic performance or not.\textsuperscript{1} The higher N-cadherin expression hUCB-MSC have, the better effective in cell therapy. Eventually, It is very important to increase N-cadherin expression in hUCB-MSC. Then, It will be the most satisfactory if the priming event for elevating N-cadherin is induced by small molecular protein, not by artificial work as genetic manipulation.

In this study, we verified small molecular protein, ET-1 as priming agent in hUCB-MSC and confirmed their impact on therapeutic efficacy in rat MI model, and the principal mechanisms that ET-1 activates N-cadherin expression.
Materials and methods

Umbilical cord blood mesenchymal stem cell culture.

hUCB−MSCs provided by Medipost Co., Ltd. were isolated from human umbilical cord vein of deliveries with informed consents as previously described. M01 and M02 hUCB−MSCs were grown in −minimum essential medium (−MEM, Gibco BRL), containing 10% FBS (Gibco BRL), 10,000 units/ml penicillin G sodium, 10,000µg/ml streptomycin sulfate, and 25µg/ml Plasmocin™ (Invivogen, San Diego, CA). Plated cells were sustained in a humidified 37°C cell incubator with 5% CO₂. Medium were changed every other day.

ET−1 priming and ETRA blocking.

Treated human recombinant ET−1 (Sigma, St Louis, Missouri) concentration was 0.025ug/ml (10nM) and incubation time was 24h. Human recombinant ET−1 was diluted in phosphate buffered saline (PBS). Before ET−1 treatment on M01 hUCB−MSCs, medium was changed to fresh culture medium.
ETRA blocker, BQ123 (Sigma, St Louis, Missouri) was treated for 1h with 5uM concentration. Cell culture medium was replaced to fresh culture medium before BQ123 treatment.

Real-time PCR analysis.

QIAshredder and RNeasy mini kit (Qiagen, Inc.) were used to prepare Total RNA according to manufacturer’s instruction. 1µg of RNA was changed into cDNA with the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Tokyo, JAPAN).

PCR was performed using the Power SYBR Green PCR master mix (Applied Biosystems, INC., Foster city, CA). Real-time samples were run on an ABI PRISM-7500 sequence detection system (Applied Biosystems, INC., Foster city, CA). GAPDH used as an internal control and for normalization. Real-time PCR primer sequence followed as

**Endothelin-1**
- forward: 5’- gctcgctccctgtggataaa-3’
- reverse: 5’- ttctgtctggcaaaaaattc-3’

**N-cadherin**
- forward: 5’- gacaatgccctcaagtt-3’
- reverse: 5’- ccattaacgccgatggtggt-3’

**VEGF**
- forward: 5’- ggcagaatcatacgaagt-3’
- reverse: 5’- tggatgttgactctca-3’

**GAPDH**
- forward: 5’- tggaggagggagattca-3’
- reverse: 5’- caacgaattttgctacagca-3’
Western blot analysis and ELISA assay.

Protein lysate buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% NP 40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate and Protease Inhibitor Cocktail (Roche, Indianapolis, IN)) were used to prepare all cell protein lysates. Total protein lysates (25–30 µg) were fractionated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with anti-EDNRA antibody (Santa Cruz biotechnology, Santa Cruz, CA) with 1:200 dilution and anti-N-cadherin antibody (Santa Cruz biotechnology, Santa Cruz, CA) with 1:1000. Internal control utilized primary antibody against α-tubulin (Sigma, St Louis, Missouri) with 1:5000. ELISA for VEGF was performed by the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The absorbance (450 m) was measured on the ELISA reader (VERSAmax; AccuScan Instruments, Columbus, OH).

Site-directed mutagenesis and Luciferase assay.

Among the 7 different pGL3 basic vectors that containing 7 different proximal N-cadherin promoter regions, respectively, the pGL3 basic vector including promoter fragment from −462bp to −18bp was used as key vector for site-directed mutation.
A commercial QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) were utilized for point mutation of specific DNA sequence for GATA2 and MZF1 binding of N-cadherin promoter region. Following mutation sequences were used.

GATA2 binding site mutation sequence:
5′-gctctttggggccgcgccgctccacttccacc-3′

MZF1 binding site mutation sequence:
5′-tccacggccaaggtcagcgccgctccactccac-3′

The Constructs and mutants were checked by sequencing process.

The made site-directed mutation vector were transfected using Metafectene-Pro (Biontex, Planegg, Bavaria, Germany) with -MEM medium without FBS. After 7 hours later, Non-FBS containing medium was changed into 10% FBS containing culture medium.

After transfection, luciferase assays were performed using the Luciferase Assay System kit (Promega, Fitchburg, Wisconsin) and luminometer (Turner Design).

The relative luciferase activity was normalized to relative light units / renilla luciferase activity.

**Immunofluorescence staining.**

Sections of rat infarcted myocardium were de-paraffinized, subjected to antigen retrieval with citrate buffer (DAKO,
Glostrup, Denmark). Blocking was conducted with 0.1% bovine serum albumin and 0.01% triton-X-100 in PBS. Then, sections were incubated overnight at 4°C with Lectin from Bandeiraea simplicifolia TRICC conjugated antibody, 1:100 (Sigam-Aldrich, St. Louis, Missouri). After 3 times washing, tissue sections were mounted by fluorescent mounting medium with DAPI (4-6-diamidino-2-phenylindole, IHC world, Woodstock, New York).

Also, to calculate fibrosis area of infarcted region, sections were stained with hematoxylin-eosin (H&E staining) and Masson’s trichrome (MT staining). SABIA software program (Metaosoft, Seoul, Korea) was used for quantitative analysis of fibrosis area on MT staining. Engraftment percentage of DiI-stained injected cells and fluorescent intensity of capillary density were calculated by Image J software program.

**Rat myocardial infarction model.**

All animal experiments procedures were performed under approved protocol ‘Guide for the Care and Use of Laboratory Animals’ announced by the National Institutes of Health, the United States, and by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital, Seoul, Korea.
Male 8 to 10 weeks old–Sprague–Dawley rats (weighting 240 to 290g) were used to experiment. SD rats were anesthetized with 50mg/kg Zoletil (Virbac, Carros, France) and Rompun (Bayer, Leverkusen, Germany) by intraperitoneal injection and then fixed in the supine position. A left thoracotomy was conducted in the fourth intercostal space and the pericardium was opened. Initiation of MI was performed by making an incision about 3~4cm along the left side of the sternum. Ligation of the main trunk of the left coronary arteries was performed with 6–0 silk suture. After ligation of proximal LAD, an irreversible pale area was demarcated on the surface of the middle and apical portion of left ventricle, and then the chest was closed in layers.

To confirm rat myocardial infarction state, Echocardiographic study (Vivid I, GE Healthcare) using 11.5 MHz transducer was performed at two days before and eight weeks after hUCB–MSC transplantation. Left ventricular (LV) fractional shortening (FS) was calculated as

\[ FS = \frac{(LVEDD - LVESD)}{LVEDD}, \]

where LVEDD is left ventricular end-diastolic diameter and LVESD is left ventricular end-systolic diameter.

For histological analysis, injected hUCB–MSCs were stained with Cell Tracker CM–DiI (Invitrogen, Carlsbad, CA). Before 1 day ago for injection, hUCB–MSCs were cultured in growth medium containing CellTracker CM–DiI with dilution range 1:200 for 24h.
Statistical analysis.

GraphPad Prism 5 (GraphPad Software, La Jolla, Calif) were used to perform statistical analysis. Results are expressed as means±SEM. Comparisons between two groups were conducted using the unpaired t-test analysis of variance, according to post hoc testing with Bonferroni analysis. P-value <0.05 were regarded statistically significant.
Result

Potency of ET−1 for augmentation in cell therapy via N−cadherin−VEGF axis

Previously, we reported individual difference induced the difference in therapeutic efficacy and which is marked by N−cadherin−VEGF axis. To select the augmentable factor in therapeutic efficacy, we performed cDNA microarray analysis with the best and the worst efficacy−representing hUCB−MSCs. Interestingly, high level of ET−1 which is known as a negative biomarker in myocardial infarction was distinguished (supple Fig.1A.). ET−1 mRNA and protein were high expressed in the best efficacy−representing hUCB−MSCs when compared with counterpart (supple Fig.1B,C). We used almost 10, 100 times (0.025, 0.25ug/ml) ET−1 concentration (2.64 pg/ml) than which of plasma levels in control subjects14 for 24 hours. According to 0.025 μg/ml ET−1 treatment, both N−cadherin and VEGF mRNA and protein level were up−regulated. whereas, 0.25 μg/ml ET−1 treatment represented neglectful effect (Fig. 1A). To optimize treatment time, we performed the ET−1 treatment for 1h, 6h, 24h and 72h and evaluated the expression levels of N−cadherin−VEGF. The expression of N−cاهرین mRNA
was higher at 6hr treated group (Fig. 1B) but final functional product, secretion VEGF was higher at 24hr and 72hr treatment groups with increasing mRNA depend on time (Fig. 1C,D). However, for safety on clinical application, critical points of priming factor are short time-expose in vitro and treatment with low concentration as possible as. Exposure for long time and in high concentration could induce the unexpected toxicity and transformation on cell therapeutics. We selected 0.025 ug/ml ET-1 concentration for 24hr that is suitable condition to augment the therapeutic efficacy in hUCB-MSCs via N-cadherin-VEGF axis.

**ET-1 in hUCB-MSCs: up-regulates the signature axis of therapeutic efficacy via EDNRA**

ET-1 acts through endothelin receptor A (ETRA) and/or B(ETRB). The ET-1-receptor (endothelin receptor A (ETRA) and ETRB) axis triggers the activation of diverse network of other signaling pathway. There are two different types of ET-1 receptors, Endothelin receptor type A (ETRA) and Endothelin receptor type B (ETRB). Several papers reported that ETRA and ETRB create different ripple effects through dissimilar downstream signaling pathways. And its aspect also appeared in a different way in diverse tissues.\textsuperscript{15-16}

To identify underlying mechanism that closely correlated
with ET-1, first of all, we investigated what type of ET-1 receptor was expressed in general in hUCB-MSCs. Both of M01 hUCB-MSC and M02 hUCB-MSC indicated high levels of ETRA mRNA and protein expression than that of ETRB. (Figure 2A, 2B)

Next, to investigate whether ETRA is related with priming event by ET-1, we treated ETRA specific blocker, BQ123 in M01 hUCB-MSC.

When compared to ET-1 only treatment group, N-cadherin expression of BQ123 treatment group was suppressed both mRNA and protein level. (Figure 2C, 2D) Following diminishment of N-cadherin, quantitative analysis of VEGF secretion was also drastically declined more than 2 times in ETRA blocking group. (Figure 2E)

These results suggested that disruption of ETRA function were closely associated with N-cadherin and VEGF activation. Consequently, this means that ET-1 priming event in M01 hUCB-MSC was induced through ETRA mediated downstream axis.

**GATA2 and MZF1 activated by ET-1, promote N-cadherin expression.**

To confirm what transcription factors were related with N-cadherin activation by ET-1-EDNRA axis, we carried out luciferase reporter assay on N-cadherin promoter region.
We used a pGL3 basic vector that was composed of human N-cadherin putative promoter and firefly luciferase expression region. (a kind gift from P.J. Marie, Lariboisiere Hospital, France) Additionally, we utilized 4 pGL3 basic vectors that each vector was made up of 4 different positions of N-cadherin promoter region, respectively. (-462bp, -335bp, -214bp, -74bp)

In a concrete way, all 4 vectors basically included promoter position where 18 base pairs upstream out of N-cadherin start codon ATG. (-18bp) Then, fragment gradationally deleted 5’ part of the N-cadherin promoter region, from maximum -462 base pair to minimum -74 base pair, was inserted into each vector, individually.

Resulting constructs were transfected into M01 hUCB-MSC with renilla luciferase containing normalization vector. And then, we separated them into two groups depending on ET-1 treatment or not. Interestingly, luciferase activity was drastically dropped when deletion from -462bp to -335bp was occurred. (Figure 3A)

Because the decline in the luciferase activity meant this promoter region was essential for N-cadherin expression by ET-1 priming, we thoroughly screened transcription factors that had possibility to bind this position. Finally, we nominated two candidates, GATA2 and MZF1.

To validate whether GATA2 and MZF1 regulate N-cadherin
increase directly, we manufactured 4 point-mutated vectors. These vectors included mutated binding sequence for GATA2 or MZF1 at N-cadherin promoter sequence from -462bp to -335bp. (Figure 3B)

When mutated construct was transfected into M01 hUCB-MSC, as shown in Figure 3C, luciferase activity measurement dramatically decreased in spite of ET-1 treatment. That is, inhibition of GATA2 or MZF1 approach to specific binding position on N-cadherin promoter region showed reduced luciferase activity.

Overall, GATA2 and MZF1 elevated by ET-1 were fundamental transcription factors that interacting directly in N-cadherin promoter region, especially from -462bp to -335bp sequence position.

**ET-1 priming induces enhancement of therapeutic efficacy on hUCB-MSCs in rat infarced myocardium model.**

To verify how ET-1 priming on hUCB-MSCs affect therapeutic efficacy on post-infarction left ventricle (LV) remodeling in a rat model, we prepared 3 experiment groups. 3 groups were composed of PBS treatment negative group, M01 hUCB-MSC naive group, ET-1 primed M01 hUCB-MSC group. According to minimum effective cell dose for infarcted myocardium repair that was found in previous study, we
injected $1 \times 10^5$ M01 hUCB-MSC cells per head. And injected cells could be chased by DiI-labeling.

At post MI 3days, there were no significant differences between 3groups in terms of MI related indexes. So we proceeded as planned in vivo experiment scheme.

At 7days later after MI operation, we carried M01 hUCB-MSCs transplantation out to peri-infarction regions of each group. Then, we compared degree of therapeutic efficacy between 3 groups at post MI 8 weeks. (Figure 4A) Interestingly, ET-1 primed M01 hUCB-MSCs injection groups showed remarkable performance in left ventricle systolic function compared with non ET-1 treatment group. (Figure 4B) In addition, ET-1 priming on M01 hUCB-MSC also improved left ventricle fractional shortening than those of naïve group. (Figure 4C)

Next, we measured fibrosis area of infarcted heart to investigate pathological changes. As a result, ET-1 primed M01 hUCB-MSCs showed much smaller infarction size compared to hUCB-MSCs group without ET-1 priming. (Fibrosis area %, Control group 10.4975% ±0.8433% vs. Control+ET-1 treatment group 5.81% ±0.4924 % p < 0.05; Figure 5A)

Furthermore, when we traced DiI-positive hUCB-MSCs to detect maintenance and survival of injected cells, ET-1 priming on hUCB-MSCs group showed approximately 1.5 folds more DiI-labeled cells in the peri-infarct area than
those of not primed group. (Engraftment %, Control group 16.4404% ± 2.3707% vs. Control+ET-1 treatment group 28.8110% ± 3.5566 % p < 0.05; Figure 5B)

We also observed that blood vessel capillary formation was more stimulated in ET-1 primed hUCB-MSCs group compared to naïve group.

(Fluorescence intensity per 0.1804㎟, Control group 31526.33±4420.95 vs. Control+ET-1 treatment group 130136.8±32666.94 p < 0.05;Figure 5C)

Based on these results, we established that ET-1 priming could augment survival possibility and engraftment rate to peri-infarct area of M01 hUCB-MSCs. Then, ET-1 primed cells made even stronger paracrine effects by secreting much more VEGF than non ET-1 primed cells. These harmonious events induced improved therapeutic efficacy of ET-1 priming on hUCB-MSCs.
Endothelin-1 (ET-1) is a small molecular protein that well known as constricting vessels and raising blood pressure. Up to the present time, the studies of ET-1 have focused on effects of ET-1 to receptor axis in various types of cancer cell lines. Furthermore, Most of the studies have reported that ET-1 is closely related with serious problems that induce cancer cells more deterioration. Synthetically, appraisal of ET-1 has oriented to negative direction, so far. ET-1 arouses pleiotropic effects extensively in various kinds of tumor cells. It promotes tumor cell proliferation and survival.\textsuperscript{17-18} In addition, not only ET-1 induces migration, invasion, epithelial–mesenchymal transition (EMT) of tumor cells\textsuperscript{19–21,25} but also it gets involved in angiogenesis and lymphangiogenesis.\textsuperscript{22–23} According to one study, ET-1 palpated cancer cell angiogenesis. It promoted VEGF secretion through ETAR related downstream pathway and this event made tumor cells more aggressive by generating many micro vessels and stimulating extravasation.\textsuperscript{23–24} In another study revealed that ET-1 caused epithelial–mesenchymal transition (EMT) by up-regulating Snail as EMT related marker, at the same time, down-regulating epithelial marker, E-cadherin.\textsuperscript{20, 25}
Therefore, based on these researches, preclinical studies either ongoing or scheduled progress, also take notice of developing antagonists that work specific on two types of ET-1 receptor, ETRA and ETRB.

**ET-1 can act positive effect as a priming agent on hUCB-MSC**

We assumed that hUCB-MSCs which was ET-1 treated could have an effect on recovering rat infarcted myocardium more powerfully by increasing their engraftment to infarction area and augmenting their survival possibility, compared with ET-1 non-treatment hUCB-MSCs. Moreover, infarction area of ET-1 treated hUCB-MSCs transplanted group showed significant reduction than control hUCB-MSCs. We also validated that ET-1 caused these superior performances through N-cadherin mediated VEGF release. Overall, ET-1 is not a molecule that has to be suppressed. In hUCB-MSCs, ET-1 can increase survival and engraftment of transplanted hUCB-MSCs. In addition, it is able to work ultimately positive factor to improve therapeutic efficacy.
GATA2, MZF1 is a key transcription factor for ET-1 mediated N-Cadherin signaling.

GATA2 and MZF1 are transcription factors that have zinc finger motif based DNA binding domain in common. Wang Y. and coworkers have reported that GATA2 was potential metastasis-driving gene in prostate cancer. And GATA2 also has performed an important function in regulatory network related with erythroid lineage differentiation.

With Sp1/Sp3, MZF1 have regulated human N-cadherin promoter in osteoblast. In this study, we established that GATA2 and MZF1 played a significant role in N-cadherin expression activated by ET-1.

Although there might be many binding sequences in N-cadherin promoter region with which GATA2 and MZF1 could interact. However, in ET-1 primed hUCB-MSCs, binding sequence from -462bp to -335bp of N-cadherin promoter region especially carried out the key role. Consequently, Binding of GATA2 and MZF1 to this promoter sequence is in close association with therapeutic efficacy of ET-1 primed hUCB-MSCs.
ET-1 can increase therapeutic efficacy directly without genetic manipulation in hUCB-MSC.

As mentioned earlier, ET-1 can improve therapeutic efficacy as priming agent in hUCB-MSCs. Of course, there are many possible ways to prime hUCB-MSCs except ET-1 treatment. For example, according to N-cadherin mediated VEGF axis, either N-cadherin or VEGF over-expression by virus infection is able to be a probable alternative method. In addition, we can place more emphasis on other growth factors (ex, HGF, bFGF) as final constructs to enhance therapeutic efficacy rather than VEGF. Then, it is also possible alternatives to up-regulate or down-regulate various signaling molecules related with other growth factors. However, to execute alternatives method, it is essential to use genetic manipulation technologies such as virus infection or vector transduction to increase or decrease specific genes. And these genetic manipulation ways certainly have to concern side effects that might be going to occur during clinical cell therapy trials using primed hUCB-MSCs. Whereas, hUCB-MSC priming event by ET-1 has a creative benefit that it was realized without any genetic manipulation. Furthermore, this simple priming performance can be achieved by small molecular protein, ET-1 with low concentration and short time treatment.
Figure 1A.

**N-cadherin**

![N-cadherin bar chart]

**VEGF**

![VEGF bar chart]
Figure 1B.

Figure 1C.
Figure 1D.
Figure 1. N-cadherin mediated VEGF signaling pathway of ET-1 priming.

(A) Determination of ET-1 concentration that can cause optimal priming efficacy on M01 hUCB-MSCs. Transcription level of N-cadherin and VEGF mRNA measured by real-time PCR was augmented noticeably when 0.025ug/ml (10nM) ET-1 concentration was treated compared to 0.25ug/ml (100nM) ET-1 concentration. (n=3 per group) *P < 0.05 vs. control group.

(B, C, D) Confirmation of the best ET-1 treatment time that can induce activation and secretion of signaling related genes. N-cadherin mRNA was the highest expression at ET-1 treatment group for 6h as shown in (B). Measurement of VEGF mRNA expression level (C) and secreted VEGF (D). VEGF mRNA level which was increased the most at ET-1 treatment group for 6h influenced rapid growth of VEGF secretion at the time from 6h to 24h after ET-1 treatment (D). (n=3 per group)

Taken these results together, optimal condition for ET-1 priming on M01 hUCB-MSCs are 0.025ug/ml concentration and 24h treatment time.
Figure 2A.

hUCB-MSC

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hUCB-MSC

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Figure 2B.
Figure 2C.

Figure 2D
Figure 2E

Secreted VEGF

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0.5 1 1.5 2 2.5 3

-28-
Figure 2. ET-1 priming event on hUCB-MSCs via ETRA.

(A, B) The existence of ET-1 receptor was checked at the mRNA level and at the protein level (A) in M01 and M02 hUCB-MSCs. Both mRNA and protein results revealed that ETRA was expressed more dominantly than ETRB in both M01 and M02 hUCB-MSCs. Immunofluorescence staining also showed that ETRA was expressing intensively in M01 hUCB-MSCs (B). (n=3 per group)

(C, D) ETRA blocker, BQ123 reduced both N-cadherin mRNA level (C) and protein level (D) under the ET-1 treatment condition.

(E) VEGF secretion was measured by ELISA exposed that BQ123 also aroused decrease of secreted VEGF quantification.

Overall, ETRA plays an important role in ET-1 inducing N-cadherin-VEGF axis. (n=3 per group)

ETRA: Endothelin receptor type A.
ETRB: Endothelin receptor type B.
Figure 3C
Figuer3. Screening of N-cadherin transcriptional factors which were activated by ET-1 priming.

(A) Promoter assay of N-cadherin promoter region from -462bp to -18bp to screen ET-1 activating transcription factors. When luciferase activity was measured using 4 pGL3 basic vectors that each vector was including gradually deleted N-cadherin promoter site, remarkable decline was observed when promoter position from -462bp to -335bp was removed, even though under the condition of ET-1 treatment. (n=3)

(B, C) Site-directed mutagenesis showed that point mutation at either GATA2 or MZF1 where position between -462bp and -335bp of N-cadherin promoter induced prominent decrease of luciferase activity, although ET-1 was treated. This performance was also observed when both GATA2 and MZF1 binding sequence simultaneously point mutated.

Consequently, with respect to ET-1 priming on hUCB-MSCs, GATA2 and MZF1 binding to N-cadherin promoter region from -462bp to -335bp act as a fundamental role to up-regulate N-cadherin. (n=3)
Figure 4A
Figure 4. Transplantation of ET-1 primed hUCB-MSCs improved therapeutic efficacy after rat myocardial infarction model.

(A) Scheme for transplantation of ET-1 primed hUCB-MSCs and non-primed hUCB-MSCs to appraise therapeutic effectiveness. Injected hUCB-MSCs were M01 hUCB-MSCs that had showed the worst therapeutic potential in prior study.

(B, C) Improvement in systolic function related indexes as LVEF (B) and LVFS (C) after ET-1 priming on hUCB-MSCs transplantation compared to PBS group or naïve hUCB-MSCs group. Each group sample was harvested at post-MI 8 weeks.

LVEF: left ventricle ejection fraction, LVFS: left ventricle fractional shortening, MI: myocardium infarction. *P=0.0148 PBS vs. naive group, **P=0.0019 naïve group vs. ET-1 primed group, ***P=0.0007 PBS vs. ET-1 primed group in LVEF, *P=0.0129 PBS vs. naive group, **P=0.0005 naïve group vs. ET-1 primed group, ***P=0.0001 PBS vs. ET-1 primed group in LVFS. (n=21; n=7 in each group)
Figure 5A

Naive hUCB-MSCs

ET-1 treated hUCB-MSCs

1 mm

[Fibrosis area(%)]

P value = 0.0201

ET-1 - +
Figure 5B

![Image: Naive hUCB-MSCs vs ET-1 treated hUCB-MSCs](image)

**Engraftment (%)**

![Graph showing engraftment with ET-1 treatment](graph)

P value = 0.0030
Figure 5C

[Capillary density by BS-1-lectin]

Fluorescence Intensity (per 0.1804mm²)

ET-1 -  +

P = 0.0319
Figure 5. ET-1 priming effects related with engraftment and vasculogenesis potential.

(A) Masson’s trichrome staining of infarcted rat heart and quantitative analysis of Fibrosis area. Percentage of fibrosis areas (blue color) were more reduced in ET-1 primed hUCB-MSCs injected group than hUCB-MSCs without ET-1 priming injected group. (Fibrosis area %, Control group 10.4975% ±0.8433% vs. Control+ET-1 treatment group 5.81% ±0.4924 % p=0.0201 n=7 in each group)

(B) ET-1 priming enhanced maintenance and engraftment of injected hUCB-MSCs to peri-infarct region. Calculated engraftment percentage showed that Dil-labeling cells were about 1.5 times more monitored in the same observed area in ET-1 primed hUCB-MSCs when observed 7 days later after MI. (Engraftment %, Control group 16.4404% ±2.3707% vs. Control+ET-1 treatment group 28.8110% ±3.5566 % p=0.0030 n=5 in each group)

(C) Immunofluorescence staining to verify potential of vasculogenesis. The data showed that ET-1 primed hUCB-MSCs indicated a tendency to develop much faster and more number of capillary formations than not primed group. More developed blood vessels might be stimulated by VEGF, angiogenesis factor, secreted by ET-1-primed
hUCB-MSCs. (Fluorescence intensity per 0.1804㎟. Control group 31526.33±4420.95 vs. Control+ET-1 treatment group 130136.8±32666.94 p=0.0319 n=7 in each group)
Supplementary Figure 1A
Supplementary Figure 1B

Supplementary Figure 1C

ELISA of ET-1 (pg/ml)
Supplementary Figure 1.

(A) Scatter plot figure based on cDNA microarray result. M02 hUCB–MSC showed higher ET–1 expression than M01 hUCB–MSC.

(B, C) According to result of real-time PCR and ELISA, transcriptional and translational ET–1 expression measured more eminently in M02 hUCB–MSC compared with M01 hUCB–MSC.
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국문초록

엔도셀린-1이 야기하는 GATA2와 MZF1 매개 N-Cadherin-VEGF 경로를 통한 인간 중배엽 줄기세포의 치료 효능 향상

김 기 환

이전에 발표한 논문에서 우리는 공여자가 다른 인간 재대혈 중배엽 줄기세포(hUCB-MSC)에서의 N-cadherin 발현 정도 차이가, 쥐의 심근경색 모델에서 치료효과의 차이를 나타내주는 지표라는 사실을 입증하였다. 이와 관련하여 우리는 어떤 유전자의 발현 차이가, 치료 효과가 가장 좋았던 중배엽 줄기세포와 가장 좋지 않았던 중배엽 줄기세포 사이에서의 차이를 야기하는지 (즉, 어떤 요소가 N-cadherin의 발현 정도차이를 유발하는 인자로 작용하고 있는지) 알아보기 위해 상보적 DNA 마이크로어레이 분석을 실시하였다. 그리고 그 결과로써, 엔도셀린-1(ET-1)을 최종 요소로 선정하였다. 치료효과가 좋았던 인간 중배엽 줄기세포는 엔도셀린-1의 발현이 높았던 반면, 치료효과가 좋지 않았던 중배엽 줄기세포는 엔도셀린-1의 발현이 매우 낮았기 때문이다. 그 동안 엔도셀린-1은 급성 심근경색 모델에서 현저히 증가되어 미세혈관의 기능장애 및 여러 심장기능 지표를 악화시키는 요인으로 지목되어온 물질이다. 따라서 엔도셀린 수용체 저해제를 처리하여 엔도셀린이 야기하는 신호 전
달 경로를 막기 위한 연구가 중심이 되어왔다. 그러나 이번 연구에서는 엔도셀린-1이 부정적인 요소로 작용하는 것이 아니라, 인간 중배혈 줄기세포의 치료 효능을 향상시키는 기폭제로서의 긍정적 역할을 입증하고자 한다. 먼저, 엔도셀린-1이 기폭제로서 작용하는지의 여부를 증명하기 위해, 엔도셀린-1을 처리한 후 N-cadherin-VEGF 경로의 변화 양상을 추적하였다. 그 결과, 엔도셀린-1 처리로 인해 N-cadherin 전령 RNA와 단백질의 발현양이 증가하였고, VEGF의 전령 RNA와 단백질의 발현양 또한 증가하는 것을 확인하였다. 그리고 이러한 효과는 엔도셀린 수용체 A를 통해 일어나는 현상이었으며, 엔도셀린 수용체 B는 엔도셀린-1에 의한 기폭효과에 관여하지 않았다. 다음으로, 엔도셀린-1 처리 시, N-cadherin의 발현을 증가시키는 매개체로 작용하는 전사요소가 무엇인지 밝혀내기 위해, N-cadherin의 프로모터 영역에 결합할 수 있는 인자들을 탐색하였고, GATA2와 MZF1을 찾아내었다. 마지막으로, 엔도셀린-1이 인간 중배혈 줄기세포의 기폭제로 작용하여 치료효과를 향상시키는지의 여부를 동물실험을 통해 증명하였다. 쥐의 급성 심근경색 모델에 적용해 보았을 때, 엔도셀린-1에 의해 자극을 받은 중배혈 줄기세포는 그렇지 않은 중배혈 줄기세포에 비해 더 향상된 치료 효과를 나타내었다. 조직학적 분석을 통한 결과에서도, 심근경색이 일어난 부위의 중배혈 줄기세포가 이동하여 생장하는 비율이 증가하였으며, 섬유화된 부분이 감소하는 것을 확인할 수 있었다.

이번 연구를 통해 우리는 엔도셀린-1이 인간 재대혈 중배혈 줄기세포의 치료 효과를 향상시키는 기폭제로 작용한다는 사실을 입증하였다.
주요어: 엔도셀린-1, 인간 재대혈 중배엽 줄기세포, N-cadherin, GATA2, MZF1

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