Therapeutic effect of EGF secreted by human umbilical cord blood-derived mesenchymal stem cells on atopic dermatitis

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Abstract

Human mesenchymal stem cells (MSCs) are emerging as a treatment for atopic dermatitis (AD), which is a common inflammatory skin disorder that affects a large number of people across the world. Treatment of AD using human umbilical cord blood-derived MSCs (hUCB-MSCs) has recently been studied; however, the mechanism underlying the effects of these cells is unclear. This study investigated the effect of epidermal growth factor (EGF) secreted by hUCB-MSCs on AD. hUCB-MSCs secreted a high concentration of EGF compared with other cell types. To elucidate the effect of EGF secreted by hUCB-MSCs, EGF expression was downregulated in hUCB-MSCs using EGF-targeting small interfering RNA, and these cells were co-cultured with keratinocytes, Th2 cells, and mast cells. Depletion of EGF expression disrupted the immunomodulatory effects of hUCB-MSCs on these AD-related inflammatory cells. In a *Dermatophagoides farinae*-induced AD mouse model, subcutaneous injection of hUCB-MSCs ameliorated gross scoring, histopathologic damage, and mast cell infiltration, and significantly reduced the levels of inflammatory cytokines including interleukin (IL)-4, tumor necrosis factor- α (TNF α), thymus and activation-regulated chemokine (TARC), and IL-22, as well as the serum IgE level. These therapeutic effects were significantly attenuated at all evaluation points in mice injected with EGF-depleted hUCB-MSCs. Taken together, these results suggest that EGF secreted by hUCB-MSCs plays an important role in treatment of AD by regulating the inflammatory response in keratinocytes, Th2 cells, and mast cells.

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disorder that usually develops in infancy or early childhood. Patients suffer from itchy, dry, and inflamed skin, and often develop other atopic manifestations such as allergic asthma and allergic rhino-conjunctivitis, and most patients continue to have problems once they reach adulthood [1,2]. AD is most likely caused by interplay between several genetic and environmental factors.

Keratinocytes of AD patients express many chemokines, including CCL27, thymus and activation-regulated chemokine (TARC, also known as CCL17), and CCL22/macrophage-derived chemokine, to induce inflow of dendritic cells (DCs), T cells, and other leukocytes into the skin, which enhance inflammation [3-5]. These chemokines induce Th2 cell migration and infiltration into inflammatory sites via CCR4, leading to a Th2 immune response [6]. Increased production of chemokines (TSLP: thymic stromal lymphopoietin, RANTES: Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted and TARC); increased number of Th2 cells compared with Th1 cells; increased production of cytokines (e.g., interleukin (IL)-1 β , IL-4, and tumor necrosis factor (TNF)- α); and increased production of IgE have been reported as immunological symptoms of AD. Targeting keratinocytes, Th2 cells, B cells, and inflammatory cells involved in the skin immune response might be a novel therapeutic strategy for AD.

Mesenchymal stem cells (MSCs) can rapidly proliferate and differentiate into mesenchymal lineages and regulate immune cells such as T cells, B cells, DCs, and natural killer cells by influencing the innate and

adaptive immune systems [2]. Recent studies reported that MSCs may lead to a breakthrough in the treatment of diverse autoimmune and inflammatory diseases including inflammatory bowel disease, arthritis, and AD [7-9]. In addition, MSCs secrete various immune modulators including transforming growth factor (TGF)- β , hepatocyte growth factor, IL-6, and IL-10 [10]. These secreted cytokines inhibit proliferation of CD4⁺ cells, CD8⁺ T cells, and natural killer cells as well as maturation of DCs, which are highly activated in AD, and have important functions to reduce the allergic response and chronic inflammation in AD.

Among the diverse immune modulators and growth factors secreted by MSCs, epidermal growth factor (EGF) is considered a potential therapeutic target to restore the epidermis and suppress immune reactions in AD. The EGF receptor (EGFR) signaling pathway is crucial for skin development and homeostasis. Several studies, including a study that used a genome-wide approach and a histopathologic study, reported that EGFR expression is lower in lesional skin of AD patients than in healthy controls [11]. In addition, there is increasing evidence that the EGFR pathway is an important signal transducer in skin biology and has a major impact on inflammatory/immune reactions of the skin [11-14]. In accordance with the findings regarding EGFR expression, the serum level of EGF is also significantly downregulated in AD patients [15]. EGF plays an essential role in the dermal wound-healing process and stabilization of mast cell degranulation [12]. Additionally, EGF treatment reduces epithelial thickness, cutaneous inflammation, and the serum IgE level in allergen-induced AD skin [16]. Although treatment of AD using human umbilical cord blood-derived MSCs (hUCB-MSCs) has been studied, it is unclear whether growth factors secreted by these cells facilitate skin recovery. Moreover, the mechanism via which EGF secreted by MSCs influences AD symptoms has not been explored in detail. This study investigated the role of EGF in the therapeutic effects of hUCB-MSCs on AD.

Materials And Methods

Cell culture

hUCB-MSCs were provided by Kangstem Biotech GMP Center (South Korea). These cells were maintained in KSB-3 medium (Kangstem Biotech, South Korea) containing 10% fetal bovine serum (FBS) and 100 μ g/mL primocin (Invitrogen, USA). The HaCaT (Addexbio, USA), Human dermal fibroblast (HDF: Cell Applications, USA) and HEK293FT (ATCC, USA) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS and 100 μ g/mL primocin (Invitrogen). Human LAD2 cells were cultured in StemPro-34 medium (Invitrogen) containing 100 ng/mL recombinant human stem cell factor/c-kit ligand (R&D Systems, USA) and 2 mM L-glutamine (Sigma, USA). All cells were maintained at 37°C in a 5% CO₂ incubator. Recombinant human EGF (rhEGF), human interferon (IFN)- γ , and human TNF- α were purchased from PeproTech (NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

All of the cells were cultured in their respective culture media. A total of 2×10^6 cells were suspended in 2 mL culture medium, added to a single well of a 6-well plate, and cultured for 24 h. Thereafter, the culture medium was collected in a 1.5 mL conical tube and centrifuged at 500 g for 5 min. The concentrations of EGF and TARC in the culture medium were measured using a Human EGF Quantikine ELISA Kit and a Human TARC Quantikine ELISA Kit (R&D Systems), according to the manufacturer's instructions.

Scratch assay

Migration of HaCaT and HDF was examined using a scratch wound-healing assay. Cells (5×10^5 cells/well) were grown in complete DMEM containing 10% FBS as a monolayer in 6-well plates until they reached 80–90% confluency. A wound was generated using a sterile P1000 micropipette tip. The cells were washed with phosphate-buffered saline and co-cultured with hUCB-MSCs for 48 h. The wound widths in three fields were examined at 10 $\times$ magnification using a phase-contrast microscope (Ti-U; Nikon, Japan). The wound closure area was calculated using ImageJ 1.41 software (NIH, USA). Cell migration was quantitatively analyzed by determining the average wound areas in three fields.

Quantitative real-time polymerase chain reaction (qRT-PCR)

HaCaT cells (2×10^5 cells/well) were co-cultured with hUCB-MSCs in 6-well plates and stimulated with TNF- α /IFN- γ (each 10 ng/mL) for 24 h. Total RNA was extracted from HaCaT cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 1 μ g purified total RNA was converted into cDNA using a cDNA synthesis kit (Bioneer, South Korea). All cDNA samples were stored at -20°C . cDNA was analyzed by qRT-PCR using PowerUp[™] SYBR Green Master Mix (Applied Biosystems, CA, USA) and appropriate primers. qRT-PCR was performed using an ABI 7700 sequence detection system (Applied Biosystems). mRNA expression was normalized against that of GAPDH. Relative expression was calculated using the comparative CT method ($2^{-\Delta\text{Ct}}$).

Small interfering RNA (siRNA) transfection

Transient and stable transfection was performed using Lipofectamine3000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, hUCB-MSCs (2×10^5) were plated into 6-well plates in KSB-3 medium containing 10% FBS without antibiotics at 1 day before transfection, such that they were 60–70% confluent at the time of transfection. On the day of transfection, 200 pmol control siRNA (siCTL; Santa Cruz Biotechnology, USA) and human EGF-targeting siRNA (siEGF; Santa Cruz Biotechnology, USA) was mixed with 10 μ L Lipofectamine3000 in 1 mL Opti-MEM medium (Invitrogen). The mixture was incubated for 5 min at room temperature and added to hUCB-MSCs in fresh medium lacking serum. The cell culture medium was exchanged with fresh medium at 24 h after transfection.

Mast cell degranulation

For IgE-mediated mast cell degranulation, hUCB-MSCs were cultured in 24-well plates for 24 h. Thereafter, LAD2 cells were cultured in the presence of hUCB-MSCs, sensitized with 100 ng/mL human IgE (Millipore,

MA, USA) for 24 h, and then challenged with 6 µg/mL anti-IgE for 1 h. Degranulation of LAD2 cells was stopped on ice. Conditioned medium (CM) were harvested and transferred to a 96-well plate in triplicate, and mixed with substrate solution (*p*-nitrophenyl-N-acetyl-β-D-glucosaminide, pH 4.5) at a 1:1 ratio. The mixture was incubated in a shaking incubator at 37°C for 2 h, and then an equal volume of 0.2 M glycine (pH 10.7) was added. Released β-hexosaminidase was quantified by measuring absorbance at 410 nm using a microplate reader.

Th2 cell isolation and polarization

According to the manufacturer's instruction, Naïve CD4⁺ T lymphocytes were isolated from PBMCs using a naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated CD4⁺ cells were treated with 50 ng/ml of interleukin (IL)-2 and anti-CD3/28 beads for proliferation. To polarize Th2 cells, 25 ng/ml of IL-6, 50 µg/ml of anti-IFN-γ, and 25 ng/ml of IL-4 were supplemented in the medium. In the presence of hUCB-MSCs, Th2 cell were further cultured for 5 days. Polarized Th2 cells were analyzed by flow cytometry analysis detecting surface or intracellular markers using. Th2 cells were fixed and incubated with FITC-conjugated anti-CD4 for surface marker staining. For intracellular marker staining, cells were fixed and permeabilized with an intracellular staining buffer set (BD Biosciences, San Jose, USA) and then incubated with PE-conjugated anti-IL-4 antibody. Detection was performed with a FACS calibur flow cytometer and evaluated using Cell Quest software (BD Bioscience).

Animals

Seven-week-old male NC/Nga mice were purchased from Central Laboratory Animal, Inc. (Seoul, Korea). Mice were acclimatized at a temperature of 22°C ± 2°C and humidity of 55% ± 5% in an air-conditioned conventional area with a 12 h light–dark cycle. Three mice were placed in each cage and fed ad libitum. All in vivo experimental procedures were approved by Seoul National University (Approval No. SNU-190925-3-1).

Induction of AD in NC/Nga mice

AD-like symptoms were induced in 8-week-old male NC/Nga mice using *Dermatophagoides farinae* (Df) extract (Biostir, Inc., Hiroshima, Japan). After 1 week of acclimation, mice were divided into four groups (n = 3 in the negative control group and n = 5 in each other group). Df extract was applied to shaved dorsal skin and ears twice per week for 3 weeks. The skin barrier was disrupted by applying 200 µL of 4% sodium dodecyl sulfate to shaved dorsal skin and each ear at 3–4 h before topical administration of 100 mg Df extract. On day 21, mice were subcutaneously injected with 2 × 10⁶ hUCB-MSCs (Fig. 4A). Control vehicle-injected mice were subcutaneously injected with phosphate-buffered saline at the same time point.

Measurement of dermatitis severity and ear thickness

The severity of dermatitis was assessed by calculating the sum of individual scores (0, no symptoms; 1, mild; 2, moderate; and 3, severe) of dryness/scarring, erythema/hemorrhage, edema, and erosion three times per week for 4 weeks. Assessment was performed by two investigators who were blinded to the experiment. Ear thickness was measured three times per week using Digimatic Calipers (Mitutoyo Corporation, Kanagawa, Japan). Images of clinical symptoms were acquired using a digital camera (DSC-RX100 III; Sony Inc., Tokyo, Japan) for 4 weeks.

Statistical analysis

All data were analyzed using GraphPad Prism version 5 (GraphPad Software, <http://www.graphpad.com/>) and expressed as the mean $\pm$ standard error of the mean (SEM). In the AD mouse model, the clinical severity and ear thickness were analyzed by two-way analysis of variance (ANOVA) with further post hoc tests. Statistical comparisons were performed using the T-test with GraphPad Prism version 5. $p < 0.05$ was considered statistically significant.

Results

hUCB-MSCs secrete EGF

Recent studies showed that EGFR expression is consistently lower in AD skin than in healthy skin [17], and that the EGFR pathway is an important signal transducer in skin biology and has a major impact on inflammatory/immune reactions of the skin[13,14]. To investigate whether hUCB-MSCs secrete EGF, we measured EGF secretion according to the cell density using an ELISA. The concentration of EGF in cell culture medium was higher than 40 pg/mL when the cell density was 2×10^6 cells/well or greater (Fig. 1A). We further measured EGF secretion according to the cell culture duration. Secretion of EGF was highest at 24 h after cell seeding (Fig. 1B). hUCB-MSCs secreted very high concentrations (>10-fold higher) of EGF compared with other cell lines (Fig. 1C). This demonstrates that hUCB-MSCs secrete a very high level of EGF and have the potential to treat AD.

EGF secreted by hUCB-MSCs accelerate wound healing

Increased keratinocyte migration is related to a high degree of re-epithelialization and wound closure. We examined whether hUCB-MSCs facilitate the wound-healing process. Migration of HaCaT cells and HDFs was evaluated by a scratch wound-healing assay after co-culture with hUCB-MSCs for 48 h. The wound area significantly decreased following co-culture with hUCB-MSCs. The wound was completely closed after co-culture with hUCB-MSCs for 48 h, but this effect was attenuated when EGF was depleted in hUCB-MSCs using siEGF (Fig. 2A and B). The results indicate that hUCB-MSCs enhance migration of keratinocytes and dermal fibroblasts, and that EGF secreted by hUCB-MSCs accelerates wound healing for treatment of AD.

EGF secreted by hUCB-MSCs inhibits expression of proinflammatory cytokines and suppresses degranulation of stimulated mast cells

To evaluate the anti-inflammatory effects of hUCB-MSCs on human keratinocytes, we measured production of proinflammatory cytokines in HaCaT cells upon TNF- α /IFN- γ stimulation. We first investigated changes in mRNA expression of the proinflammatory factors IL-1 β , TNF- α , and TARC. HaCaT cells were co-cultured with hUCB-MSCs and stimulated with TNF- α /IFN- γ for 24 h. qRT-PCR analysis showed that TNF- α /IFN- γ treatment significantly increased mRNA expression levels of these proinflammatory factors, and siEGF showed a poor effect on proinflammation (Fig. 3A). Secretion of proinflammatory factors was next investigated in HaCaT cells co-cultured with hUCB-MSCs using an ELISA. TNF- α /IFN- γ treatment increased secretion of TARC by HaCaT cells. Secretion of TARC by HaCaT cells was significantly decreased upon co-culture with hUCB-MSCs, but this effect was attenuated when EGF was depleted in hUCB-MSCs using siEGF (Fig. 3B). We further investigated the effects of EGF on TARC secretion upon TNF- α /IFN- γ treatment. TARC secretion was increased in TNF- α /IFN- γ -treated HaCaT cells but was significantly decreased by EGF in a concentration-dependent manner (Supplementary Fig. 2). Furthermore, we investigated whether EGF secreted by hUCB-MSCs regulates degranulation of stimulated mast cells. We measured secretion of β -hexosaminidase by LAD2 cells to investigate IgE-mediated mast cell degranulation. Secretion of β -hexosaminidase was increased in stimulated LAD2 cells and was suppressed by co-culture with hUCB-MSCs. However, this effect was attenuated when EGF was depleted in hUCB-MSCs using siEGF (Fig. 3C). These results suggest that hUCB-MSCs inhibit expression of proinflammatory factors including TARC in keratinocytes in inflammatory environments and suppress degranulation of stimulated mast cells, and that secreted EGF is crucial for these effects.

EGF secreted by hUCB-MSCs inhibits maturation of Th2 cells

TARC is produced by keratinocytes in AD patients, suggesting that it plays an important role in maturation of Th2 cells [18,19]. It suggests that EGF can regulate the maturation of Th2 cells by inhibiting TARC expression. Furthermore, we investigated the direct role of EGF secreted by hUCB-MSCs on maturation of Th2 cells. We isolated immature Th2 cells from human peripheral blood mononuclear cells and co-cultured them with hUCB-MSCs transfected with control siRNA (siCTL) or siEGF under maturation conditions. Co-culture with hUCB-MSCs inhibited maturation of CD4/IL4⁺ Th2 cells; however, these effects were attenuated by depletion of EGF in hUCB-MSCs (Fig. 3D). These results suggest that EGF secreted by hUCB-MSCs suppresses maturation of Th2 cells directly.

EGF secreted by hUCB-MSCs ameliorates the symptoms of AD

Our in vitro study demonstrated that EGF secreted by hUCB-MSCs is involved in the regulation of diverse inflammatory cells associated with the pathology of AD. To confirm the therapeutic effect of EGF secreted by hUCB-MSCs in vivo, we evaluated the effects of EGF-depleted hUCB-MSCs in mice with Df-induced AD. To induce AD-like lesions, Df was applied to the shaved dorsal skin, including the surfaces of ears, of mice for 3 weeks. Mice were injected with vehicle, siCTL-transfected hUCB-MSCs, and siEGF-transfected hUCB-MSCs on day 21. After injection of siEGF-transfected hUCB-MSCs, a human anti-EGF antibody was further injected (Fig. 4A). The clinical scoring index, ear thickness, and spleen weight were significantly lower in mice administered siCTL-transfected hUCB-MSCs than in those administered the

vehicle control. However, the therapeutic effects of siEGF-transfected hUCB-MSCs were inferior to those of siCTL-transfected hUCB-MSCs (Fig. 4B–D). Histological analysis demonstrated that the epidermal thickness of skin lesions and ear thickness was reduced in mice administered siCTL-transfected hUCB-MSCs; however, depletion of EGF in hUCB-MSCs attenuated these effects (Fig. 5A–B). Toluidine blue staining was also performed to determine the degree of infiltration of inflammatory leukocytes and mast cells in skin tissues. The disappearance of cells from the basal layer of the epidermis was remarkable in mice administered the vehicle and siEGF-transfected hUCB-MSCs, and the number of infiltrated inflammatory cells was significantly higher in these mice than in mice administered siCTL-transfected hUCB-MSCs (Fig. 5C).

To determine which inflammatory cytokines are affected by EGF, we measured mRNA expression of the main inflammatory cytokines in the ears of mice (Fig. 6A–D). IL-4 is a representative proinflammatory cytokine secreted by Th2 cells and induces differentiation of mast cells and eosinophils. TARC is a biomarker of AD and causes infiltration of CD4⁺ T cells at lesion sites. TNF- α and IL-22 are inflammatory cytokines that produce inflammatory mediators and thus exacerbate AD. Administration of siCTL-transfected hUCB-MSCs significantly reduced expression of IL-4, TNF- α , TARC, and IL-22 in the ears of mice, but these effects were attenuated by silencing of EGF in hUCB-MSCs. Similarly, the serum IgE level was reduced in mice administered siCTL-transfected hUCB-MSCs, but this effect was significantly attenuated by depletion of EGF in hUCB-MSCs (Fig. 6E).

Discussion

MSCs elicit therapeutic effects on various immune-related diseases, including AD, due to their immunomodulatory ability. On the other hand, EGF helps to restore the skin barrier in AD. However, the effect of EGF secreted by MSCs on immune-related diseases including AD has not been clearly determined.

This study investigated the therapeutic effects of EGF secreted by hUCB-MSCs on AD for the first time. To confirm that hUCB-MSCs secrete EGF, we first measured the amount of EGF secreted by these cells in the absence of supplemental growth factors. Secretion of EGF by hUCB-MSCs increased in a time- and cell density-dependent manner. In addition, hUCB-MSCs secreted significantly more EGF than HaCaT cells, HDFs, and HEK293FT cells. EGFR signaling in MSCs reportedly enhances cell migration, proliferation, and angiogenic effects [20]. Autocrine and EGFR signaling promotes secretion of EGF [21]. This study confirmed that hUCB-MSCs secreted EGF even in the absence of a specific stimulating signal, suggesting that EGF secretion is promoted via autocrine effects.

We previously reported that conditioned medium of hUCB-MSCs contains various growth factors and promotes migration of HDFs and synthesis of extracellular matrix proteins [22,23]. Although the roles of EGF in proliferation, regeneration, and wound healing of skin cells are well known, it has not been determined whether EGF secreted by MSCs elicits regenerative effects [24–27]. The present study found

that EGF at concentrations secreted by hUCB-MSCs in the absence of any additional stimulatory factor is functional and is a key factor in the wound-healing effect of hUCB-MSCs.

To investigate the role of EGF secreted by hUCB-MSCs, hUCB-MSCs in which EGF had been depleted using siEGF were co-cultured with responder cells, and immunomodulatory effects were investigated. These analyses revealed that EGF secreted by MSCs is important for effectively controlling inflammatory cells and associated cells that trigger an allergic reaction in AD, including Th2 cells, mast cells, and keratinocytes.

Kim et al. reported that topical administration of EGF suppresses immune responses and protects the skin barrier in NC/Nga mice with dinitrochlorobenzene-induced AD [28]. They suggested that EGF improves AD symptoms, consistent with the findings of the present study. Moreover, several reports suggest that EGF regulates innate immunity by affecting TLR expression, especially in keratinocytes [14,29]. Shibata et al. reported that EGF regulates TNF- α -induced TARC in canine keratinocytes [30]. The present study revealed for the first time that EGF secreted by hUCB-MSCs directly regulates differentiation of Th2 cells, degranulation of mast cells (LAD2 cells), and TARC expression in human keratinocytes (HaCaT cells) via in vitro co-culture experiments.

An imbalance between Th1 and Th2 cells is the major pathogenic mechanism in AD, and biologics targeting the Th2 cell-derived cytokines IL-4 and IL-13 effectively improve AD symptoms [31-33]. We previously confirmed that administration of hUCB-MSCs significantly downregulates the cytokine IL-4 in AD model mice. However, this effect is abrogated when secretion of TGF- β by hUCB-MSCs is downregulated. Kim et al. reported that various inflammatory cytokines whose levels are increased in AD, including IL-4 and IL-13, are downregulated upon topical treatment of EGF in DNCB-induced AD model mice. However, they did not elucidate whether these effects are induced indirectly by recovery of the skin barrier or directly by modulation of specific immune cells. Interestingly, the present study demonstrated that hUCB-MSCs suppressed differentiation and maturation of Th2 cells, and these effects were significantly attenuated by depletion of EGF in hUCB-MSCs. This suggests that hUCB-MSCs ameliorate AD symptoms by regulating Th2 cells, and that EGF is a key factor in Th2 cell regulation.

Inhibition of mast cell degranulation by EGF in AD has not been studied well; however, EGF reportedly stabilizes mast cell degranulation and histamine secretion in a gastric damage model and protects the gastric mucosa[12]. We confirmed that EGF secreted by hUCB-MSCs also regulates degranulation and infiltration of mast cells in allergic skin lesions. Our previous studies confirmed that TGF- β and prostaglandin E2 secreted by MSCs are involved in differentiation, maturation, degranulation, and activation of mast cells. The present study demonstrated that EGF is another key molecule in regulation of mast cell activity by hUCB-MSCs.

The immunomodulatory function of EGF secreted by hUCB-MSCs observed in vitro was confirmed in a Df-induced AD mouse model. The improvements in gross lesions, spleen weight, histologic damage, the serum IgE level, and levels of inflammatory cytokines, observed upon injection of hUCB-MSCs, were significantly attenuated when EGF expression was downregulated in these cells. This study clearly

demonstrates that EGF secreted by hUCB-MSCs is important for improving AD. Moreover, EGF secreted by hUCB-MSCs effectively controlled differentiation/maturation of Th2 cells and activities of mast cells and keratinocytes, as well as regeneration of the skin barrier in AD model mice. EGF has been reported to improve the symptoms of AD in several studies; however, this is the first study to confirm that EGF secreted by MSCs play a key role in ameliorating AD symptoms. The serum IgE level, IL-22 level, and spleen weight were significantly improved in AD model mice injected with hUCB-MSCs; however, these effects were attenuated when EGF was depleted in hUCB-MSCs. These results suggest that EGF secreted by hUCB-MSCs affects immune cells such as Th2 cells and B cells. Additional studies are needed to further elucidate these effects and the underlying mechanism.

Taken together, our findings reveal that EGF secreted by hUCB-MSCs play a key role not only in promoting skin regeneration, but also in effectively controlling Th2 cells, mast cell degranulation, and keratinocyte-derived cytokines, which induce AD symptoms. It is important to clarify the mechanisms underlying the actions of stem cells, although this is often difficult. Elucidation of these mechanisms provides a scientific basis for cell therapy products and is important for quality control during commercial manufacturing. The novel findings presented in this study highlight the potential of hUCB-MSCs for atopic treatment of AD and can be utilized for quality control or to predict efficacy. Moreover, this study indicates that the method of enhancing EGF secretion by MSCs can be considered as one of the way to increase the therapeutic efficacy in AD.

SUMMARY

In conclusion, this study demonstrates that hUCB-MSCs ameliorate AD symptoms by secreting EGF. EGF is involved in regeneration of the epidermis and regulation of cellular immune responses, including TARC secretion by keratinocytes, degranulation of mast cells, IgE secretion by B cells and polarization of Th2 cells, (Fig. 7). To develop stem cell therapeutics, the mechanisms underlying the effects must be clarified, and efficacy markers must be developed. This study suggests the scientific rationale for the therapeutic use of hUCB-MSCs in allergic diseases including AD.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

- Not applicable

Author's contributions

Conception and design, Supervising all experimental testing, Data analysis and interpretation, Manuscript writing: SL, KSK

Conception and design, Data analysis and interpretation, Manuscript writing: NJ

Collection and assembly of data, Data analysis and interpretation, Manuscript writing: THK

Conception and design, Data analysis and interpretation: YY

Collection and assembly of data: HP, EL, SMY, SYB

All authors read and approved the final manuscript.

Ethics Approval

This study was approved by the Institutional Review Board (IRB) of the Public Institutional Bioethics Committee designated by the MOHW (Approval No. P01-201605-BS-02). All in vivo experimental procedures were approved by Seoul National University (Approval No. SNU-190925-3-1)

Consent to Participate

- Not applicable

Consent for publication

- Not applicable

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Figures

Figure 1

Quantitative analysis of EGF secreted from hUCB-MSCs. EGF secretion was analyzed using an ELISA. (A) Measurement of EGF secretion according to the density of hUCB-MSCs. (B) Measurement of EGF secretion according to the culture duration of hUCB-MSCs. (C) HaCaT cells, HDFs and HEK293FT cells secrete low levels of EGF, whereas hUCB-MSCs secrete a high level of EGF.

Figure 2

Effect of hUCB-MSCs on migration of HaCaT and HDF. (A) HaCaT cells and (B) HDFs were cultured until they reached 90% confluency and then scratched with a sterile pipette tip. Representative microscopic images of scratched cells after co-culture with hUCB-MSCs for 48 h. The length of the wound was expressed as mean $\pm$ SEM of three independent experiments. * $p < 0.05$

Figure 3

Effects of hUCB-MSCs in an immune-stimulated environment. (A) HaCaT cells were co-cultured with hUCB-MSCs transfected with siCTL or siEGF under inflammation conditions. Co-culture with hUCB-MSCs inhibited mRNA level of inflammation cytokines. (B) Inhibitory effects of hUCB-MSCs on TARC secretion in TNF- α /IFN- γ -stimulated HaCaT cells. (C) hUCB-MSCs transfected with 200 nM siCTL or siEGF were co-cultured with LAD2 cells in a 24-transwell plate for 48 h. LAD2 cells were treated with IgE and an anti-IgE antibody. The level of β -hexosaminidase in the culture medium was determined using PNAG solution. (D) Th2 cells were co-cultured with hUCB-MSCs transfected with siCTL or siEGF under maturation conditions. Co-culture with hUCB-MSCs inhibited maturation of CD4/IL4+ Th2 cells. *p < 0.05

Figure 4

EGF secreted by hUCB-MSCs suppresses Df-induced AD in NC/Nga mice. (A) Schedule of the sensitization of AD and administration of hUCB-MSCs. (B) Dermatitis severity score. Five symptoms (dryness, itching, erythema, edema, and excoriation) were each scored on a scale between 0 and 3 (0, none; 1, mild; 2, moderate; and 3, severe). (C) Ear thickness. (D) Spleen weight. Results are expressed as mean $\pm$ SEM for five mice per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 5

Histopathological analysis of Df-induced AD mice following administration of hUCB-MSCs. Mice with Df-induced AD were administered vehicle, siCTL-transfected hUCB-MSCs, or siEGF-transfected hUCB-MSCs and sacrificed. Hematoxylin-and-eosin staining was performed to determine (A) epidermal thickness and (B) ear thickness. (C) Skin lesions on the back were stained with toluidine blue. The number of mast cells in toluidine blue-stained sections was counted. Results are expressed as mean $\pm$ SEM for five mice per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 6

mRNA expression of IL-4, TNF- α , TARC, and IL-22 in skin lesions and the serum IgE level of mice with Df-induced AD following administration of hUCB-MSCs. Mice with Df-induced AD were administered vehicle, siCTL-transfected hUCB-MSCs, or siEGF-transfected hUCB-MSCs and sacrificed. mRNA levels of (A) IL-4, (B) TNF- α , (C) TARC, and (D) IL-22 were determined by qRT-PCR. (E) The serum IgE level was determined by an ELISA. Experiments used five mice per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 7

Schematic diagram showing the broad immunomodulatory properties of hUCB-MSCs in AD. EGF secreted by hUCB-MSCs inhibits the functions of Th2 cells, B cells, and mast cells, and promotes keratinocyte regeneration with TARC suppression.

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