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| Original Article |

Effect of Conditioned Media Collected from Human Amniotic Fluid-Derived Stem Cells (hAFSCs) on Skin Regeneration and Photo-aging

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Abstract : The purpose of this study is to evaluate the effect of conditioned media (CM) collected from human amniotic fluid-derived stem cells (hAFSCs) on *in vitro* wound healing and on expression change of MMP-1 and procollagen 1A by ultra-violet A (UVA) irradiation. The conditioned media (CM) were collected from hAFSCs cultured with DMEM/F-12 serum free media. AFSC-CM were tested using *in vitro* wound healing model, and the results demonstrated that AFSC-CM facilitated cell proliferation in the dermal fibroblast (132.72 ± 7.48%) and skin epidermal cell (125.95 ± 4.61%), and accelerated cell migration after wound was generated. In order to evaluate the effect of AFSC-CM treatment on gene expressions changed by UVA irradiation, AFSC-CM and CM collected from culture of epidermal cell after UVA irradiation were used in dermal fibroblast culture. qRT-PCR data indicated that up-regulated MMP-1 expression by UVA was down-regulated and down-regulated procollagen 1A expression was recovered by treatment with AFSC-CM. Collectively, the results suggest that hAFSC-CM has a potential to improve skin regeneration which was damaged by photo-aging.

Key words: *amniotic fluid-derived stem cell, conditioned media, skin, MMP-1, procollagen 1A*

1. Introduction

Human skin has two major components of epidermis which play a role of barrier function against infection, and dermis which are distributed with neurons and blood vessels. Dermis are composed of collagen fibril, microfiber and elastin fibril, and thus serve strength and resilience to skin.[1] Production of dermis components decreased by aging, especially decreased collagen fibril, possessing over 90% volume of dermal extracellular matrix (ECM), cause skin to show senescence. Aging is caused by reduction of fibroblast density and ECM production, and increment of collagenase activity enhances aging synergistically. Changes of dermis have impact on epidermis changes. [2, 3]

Skin as the first barrier from outer is aging due to extrinsic pollution and intrinsic factor. Aging by UV, the representative extrinsic pollution, reveals characteristics such as wrinkle, rough, and laxness by complex process.[4-6] UV induces pro-inflammatory cytokines such as TNF-α, IL-1β, -6 and –8 via activation of various signal intermediates.[7-9] MAPK pathways of the activated signal regulate MMP expression.[10, 11] Especially, Up-regulated activity of interstitial collagenases such as MMP-1 causes degradation of nature collagen fibril comprising dermal connective tissue. Because type I collagen has most volume of ECM component comprising dermis, balance between type I collagen synthesis and degradation by collagenase activity considered the touchstone of skin aging.[12-16] Unlike UVB, UVA can directly affect dermis, because UVA can penetrate into the lower dermis. The effect of UVA on dermal fibroblast has been extensively investigated. Especially, these studies had shown that UVA irradiation down-regulates procollagen IA production via ROS induced AP-1 activation.[17-19] These experiments had been performed without considering interrelation between epidermis and dermis.

Mesenchymal stem cells (MSC), extracted from various adult tissues including human amniotic fluid-derived stem cell (hAFSC), were investigated their differentiation capacities to osteocyte, chondrocyte, and adipocytes.[20] It is known that hAFSC has immune-modulatory properties of up-regulating CD274.[21] Recent preclinical studies have shown that

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transplantation of MSC improved diseases in the cases of stroke, renal pathology, and various cancers.[22-24] And transplantation of encapsulated progenitors using various scaffolds facilitates hair follicle regeneration.[25] Moreover only MSC-conditioned media (CM) without cells has capacity to improve disease in stroke, renal pathology, and lung failure model. [26-28]

In the present study, we investigated the effect of hAFSC which can be easily collected by amniocentesis. We examined the effects of hAFSC-CM on proliferation and migration of skin cells, and on gene expression changes by UVA-induced supernatants from HaCaT cell. We used *in vitro* wound healing method for skin regeneration, and analyzed gene expression of MMP-1 and procollagen 1A which were considered as key molecules of photo-aging after UVA irradiation. We found that AFSC-CM facilitated skin regeneration and improve photo-aging modulating gene expression.

2. Materials and Methods

2.1 Cell Culture

We used secondary subcultured hAFSC supplied from Joint Institute for Regenerative Medicine in Kyungpook National University Hospital. Amniotic fluid was collected from amniocentesis performed in the second trimester for routine prenatal diagnosis. The samples were collected after obtaining written informed consent from each patient. The experimental protocol was approved by the Kyungpook National University Hospital Institutional Review Board (KNUHBIO_09-1006). The cells were cultured in α-MEM (Gibco, Rockville, MD, USA) supplemented with 15% ES-FBS, 1% glutamine (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin (Gibco, Rockville, MD, USA), 18% Chang B, 2% Chang C (Irvine Scientific, Santa Ana, CA, USA).[29] Media were changed every 2 days, and the cells at passage 5 were used in this study.

Human dermal fibroblast (hDF) and HaCaT cell (Human epidermal cell) were cultured with high-glucose Delbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (100 μl/mL) and changed to fresh media every 2 days.

2.2 Collection of the AFSC-CM

To collect serum free conditioned media, the cells remained with Chang's media were incubated with DMEM/F-12 through incubation with α-MEM (2% FBS) as reported method. [29, 30] hAFSCs were plated at a density of 1×10^6 per a 100 mm culture dish, and the cells were cultured overnight. The cells were washed with phosphate buffered saline (PBS) twice, and were incubated with DMEM/F-12 serum free media for 24 hr. The supernatant was collected by centrifuging at 15000 rpm, and filtered with 0.22 μm membrane filter (BD Biosciences, Bedford, MA, USA) for sterilization, and stored at -80°C until use.

2.3 CCK-8 Assay

To investigate the effects of hAFSC-CM on cell proliferation, HaCaT and hDF were cultured and incubated with hAFSC-CM for 24 hrs. Cell proliferations after treatment with AFSC-CM were determined by using CCK-8 kit according to manufacturer's protocol. Briefly, HaCaT and hDF cells plated in 96 well at a density of 1×10^4 per each well, were cultured with AFSC for 24 hrs. And then, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Rockville, USA) were added to each well, after which cells were incubated for another 4 hrs at 37°C. The absorbance values were read using Microplate Reader (Versa Max, Molecular Devices, CA, USA) at 450 nm.

2.4 *In Vitro* wound Healing

To examine the effect of hAFSC-CM on cell migration, HaCaT and hDF cells were cultured on 6 well plates at density of 3×10^5 cells per each well and these were maintained at 37°C and 5% CO_2. *In vitro* wound healing was performed according to a previous study.[31] Briefly, the confluent monolayers were scraped with a 200 μl yellow pipette tip to generate scratch, and media were immediately removed and washed three times with culture media. After wash, the cells were allowed healing for 24 hrs with DMEM or with AFSC-CM. Images were taken at 0 hr and 24 hrs after scrape. Wound healing rate was determined by evaluating denuded area, un-healed area, using Image J program (NIH image program).

2.5 UVA Irradiation

In order to investigate the effect of hAFSC-CM on expression change of procollagen-1A and MMP-1 related to photo-aging by UVA irradiation, the supernatant secreted from HaCaT cells was analyzed after the cells were exposed to UVA (wavelength 320 nm to 400 nm) as described previously.[32] Sub-confluent HaCaT cells were washed with PBS, irradiated with approximately 1 J/cm^2 of UVA (4W, Vilber Lourmat, France) for 10 min. UVA irradiation was performed under a thin layer of PBS and UVA lamp was located on the uncovered 60 mm culture dish (BD Biosciences, Bedford, MA, USA) during irradiation. After irradiation, the cells were cultured for 24 hrs, and the supernatant was collected and frozen -20°C until use. UVA strength was measured at a distance of 1 cm above using a UV-radiometer (HD 9021, Delta OHM, Italy). To examine the

indirect effect of UVA irradiation on DF, CM from HaCaT irradiated with and without UVA were used as media. Media of DFs were replaced by CM from UVA-irradiated HaCaT and incubated for 24 hrs. After 24 hrs, media were replaced by fresh DMEM/F-12 or hAFSC-CM, and incubated for another 24 hrs. Thereafter, total RNA were collected for qRT-PCR, the cells incubated with supernatant from normal HaCaT cells were used as control.

2.6 qRT-PCR

To evaluate the change of gene expression by UVA and AFSC-CM, total RNA from cultured cells was extracted using RNAspin Mini according to the manufacturer's protocol(GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Equal amount (1 μg) of total RNA was reverse transcribed into cDNA by using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, CA). The transcribed complementary DNAs were used for real-time polymerase chain reaction (RT-PCR). qRT-PCR was operated by using FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) kit and ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA), and relative expression rate of the genes was evaluated with the house-keeping gene GAPDH by using $2^{\Delta\Delta C}{}_{T}$ (delta cycle threshold) which is commonly used method to analyze data from real-time.[33] The result was expressed as increase rate relative to the control group (=1.0). The PCR primers for MMP-1, procollagen 1A, and GAPDH were designed based on published human gene sequences (Table 1).

2.7 Statistic Analysis

Data presented in this study are means ± standard error of the mean. As all data were shown to be not normally distributed, the non-parametric Mann-Whitney U test was used to determine significant differences between samples. $P < 0.05$ was taken as indicating statistical significance.

Table 1. Primers used for qRT-PCR.

Gene	Sequence
GAPDH	5'-GCTTGTCATCAATGGAAATCCC-3
	5'-TCCACACCCATGACGAACATG-3
MMP-1	5-CACAGCTTTCCTCCACTGCTGCTGC-3
	5-GGCATGGTCCACATCTGCTCTTGGC-3
Procollagen-1A	5'CCCCCTCCCCAGCCACAAAG-3'
	5'-TCTTGGTCGGTGGGTGACTCT-3'

Abbreviations : GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), MMP-1 (Metalloproteinase-1)

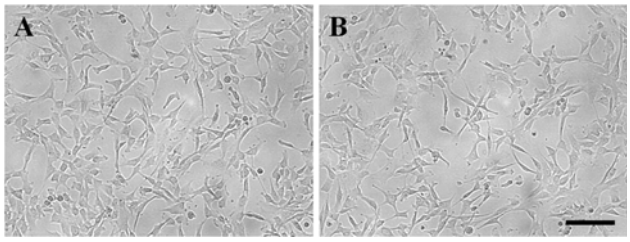

Figure 1. Morphology of the hAFSC cultured in serum free media. Cell morphology of the hAFSC was captured at the time cultured with Chang's media (A) and at 24 hr after change to DMEM/F-12 serum free media (B). The result revealed that the cell morphology did not change by media change (× 200). Scale bar indicates 100 μm. The data shown are representative of at least three independent experiments.

3. Result

3.1 Morphology of hAFSC after Culture with Serum free Media

In order to confirm the effect of serum free media on cell behavior, we observed whether morphological changes can be occurred after media change according to previous reports.[29, 30] The result showed that the morphology of the AFSC did not change after replacement of Chang's media with DMEM/F-12 (Fig 1).

3.2 Effect of the hAFSC-CM on Dermal Fibroblast Proliferations

To investigate the effects of hAFSC-CM on cell proliferation, HaCaT and hDF were cultured and incubated with hAFSC-CM for 24 hrs. Cell proliferation was determined by using CCK-8 kit. HaCaT keratinocytes in hAFSC-CM revealed increased proliferating rate as 125.95 ± 4.61% compared to HaCaT in DMEM/F-12 serum free media. And hAFSC-CM affected increment of DF proliferation as 132.72 ± 7.48% compared to DMEM/F-12 serum free media (Fig 2). The results showed that proliferation of human HaCaT and DF was increased by treatment with hAFSC-CM.

3.3 Effect of the hAFSC-CM on Dermal Fibroblast *In Vitro* wound Healing

In vitro wound healing was assayed to determine whether hAFSC-CM facilitates cell migration or not. After *in vitro* wound generation, the cells allowed wound healing with hAFSC-CM or DMEM/F-12 as control for 24 hrs. After 24 hrs, HaCaT cells hAFSC-CM treated migrated further into the scratched area holding less denude area (70.44 ± 0.88%) than DMEM/F-12 serum free treated (85.64 ± 0.84%). Similarly,

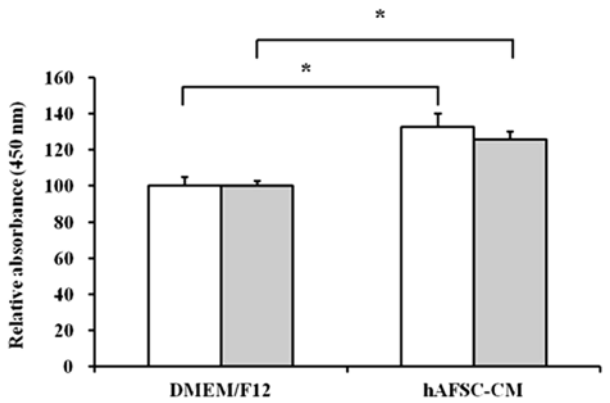

Figure 2. Proliferation assay in HaCaT and hDF treated with or without hAFSC-CM. To determine an effect of hAFSC-CM on skin cell proliferation, proliferating assay was performed using CCK-8 kit. hAFSC-CM increased cell proliferation in hDF (white block) and HaCaT (gray block). Both hDF (125.95 ± 4.61%) and HaCaT (132.72 ± 7.48%) treated with hAFSC-CM showed higher absorbance than treated with DMEM/F-12 in term of proliferation. Each value represents the mean ± SEM of the experiments (n=6). * $P < 0.01$.

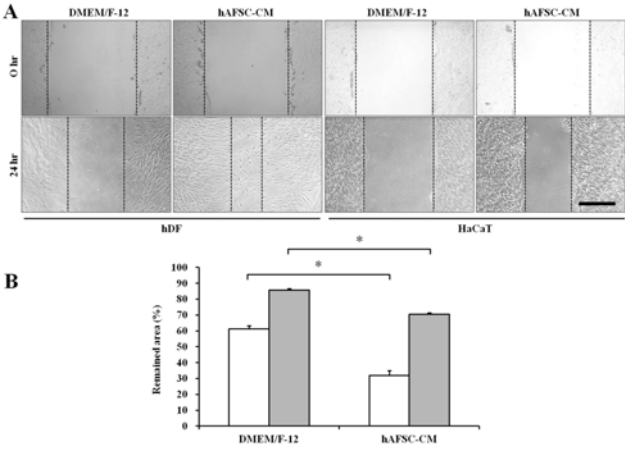

Figure 3. AFSC supernatant accelerated cell migration *in vitro* wound healing. We investigated hAFSC-CM effects on cell migration during *in vitro* wound healing, followed by wound generation using 200 μl yellow tip. The healing rate was evaluated by remained area at 24 hrs compared to at 0 hr (A). HaCaT treated with hAFSC-CM revealed significantly facilitated wound healing with less denuded area (70.44 ± 0.88%) than DMEM/F-12 serum free treated (85.64 ± 0.84%). In the hDF, wound healing was facilitated in hAFSC-CM treated (31.88 ± 1.9%) compared to DMEM/F-12 treated (61.29 ± 3.06%) (B). Dermal fibroblast showed that higher number of invaded cells found within the indicated area than epidermal cells when both types of the treatment were used. Original magnification is × 40. Scale bar indicates 500 μm. Each value represents the mean ± SEM of the experiments (n=12). * $P < 0.01$.

when denuded area of hDF wound healing assay was 61.29 ± 3.06% in DMEM/F-12 serum free at 24 hrs, it was 31.88 ± 1.9 % in AFSC-CM. When HaCaT keratinocyte and hDF *in vitro* wound healing assay were performed in hAFSC-CM which generated under serum free conditions. Significant enhancement of wound closure was found for both of the cell types (Fig 3).

3.4 Effect of the AFSC-CM on Expression of MMP-1 and type I Procollagen in Dermal Fibroblast after UVA Irradiation

MMP-1 is interstitial collagenase breaking cross-links of collagen triple helix. Previous reports have shown that UVA irradiation caused photo-aging due to induction of MMP-1.[34] Conditioned media from UVA-irradiated HaCaT lead to up-regulate MMP-1 expression as score of 1.216 ± 0.076 compared to DMEM/F-12 treated, however hAFSC-CM lead to down-regulate its level as score of 1.038 ± 0.017 which is similar to DMEM/F-12 treated. In contrast, procollagen 1A expression level in hDF was significantly decreased (0.658 ± 0.211) by treatment with conditioned media from UVA-irradiated HaCaT. The down-regulated level was recovered by treatment with AFSC-CM (1.021 ± 0.035). These results demonstrated that hAFSC-CM can inhibit MMP-1 expression and increase procollagen 1A expression changed by UVA irradiation (Fig 4).

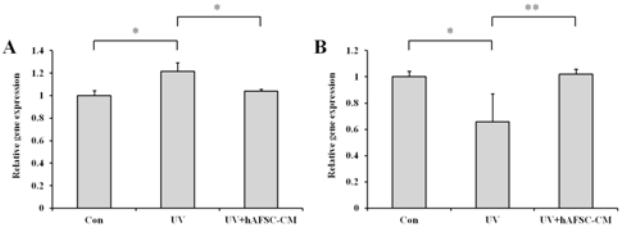

Figure 4. Effects of hAFSC-CM on DF treated with UVA stimulated HaCaT-CM. To determine gene expression changes of MMP-1 and procollagen 1A in DF by UVA and hAFSC-CM, DF was cultured with HaCaT-conditioned media (CM) exposed to UVA, and then further cultured with or without AFSC-CM for 24 hrs. qRT-PCR was performed and analyzed and relative expression rate of the genes was evaluated with the house-keeping gene GAPDH. The mRNA expression of MMP-1 was induced in the culture with HaCaT-CM collected from UVA treated, but it was suppressed by treatment with the AFSC-CM similar to DMEM/F-12 treated control (A). However, procollagen 1A expressions decreased in the cells treated with UVA-induced HaCaT-CM, but its expression level was rescued by treatment with hAFSC-CM (B). Each value represents the mean ± SEM of the experiment (n=4). * $P < 0.05$, ** $P < 0.01$.

4. Discussion

Chang's media, a well established media for growing AFSC, is composed of α-MEM, 15~30% FBS and Chang's supplements.[24, 25] In order to rule out the effect of FBS on cells, serum free culture was performed with DMEM/F-12 serum free media through incubation with α-MEM containing 2% FBS for the present study. Such serum free conditioned media of ASFC were collected after confirmation of cell morphology (Fig 1).[26]

Analysis of AFSC-CM revealed that various growth factors such as bFGF, EGF, and TGF-α were produced by AFSC (data not shown). Previous study also showed that it contains various soluble proteins including growth factors, cytokines, and matrix metalloproteinase.[30]

Proliferation and migration of skin cells are most important processes in wound healing and skin regeneration. Conditioned media from MSC facilitates wound closure of keratinocyte and human dermal fibroblast *in vitro,*[31] furthermore, AFSC-CM facilitates migration via TGF-β-Smad2 pathway and increases cell proliferation of DF *in vitro* and *in vivo.*[30, 31] In this study, we examined cell proliferation and migration assays of skin cells such as HaCaT keratinocyte and DF to determine the effect of AFSC-CM on skin regeneration. Similar to previous data, we observed that AFSC-CM elevated cell proliferation and facilitated cell migration in both of HaCaT and DF (Fig 2 and 3).

Skin is outmost barrier of the body, and consistently exposed to UV which resulted in photo-aging.[28] Only UVA and B can reach ground because of absorption by the ozone layer, and they affect skin diseases including photo-aging.[35-37] In particular, UVA exposure results in the decrease of type I collagen synthesis which is the main component of the dermal ECM.[34, 38] Photo-aging by UVA is well known. UVA damages DF directly by penetrating into the dermis, and it causes decrease in the amount of collagen.[34] UVA can also affect DF indirectly via TNF-alpha secretion from keratinocyte to dermal ECM. Secreted TNF-alpha induces expression of MMP-1 in DF.[38] MMP-1, interstitial collagenase, causes breakdown of fibril collagen. Decrease of collagen synthesis and degradation by UVA lead to wrinkling.[34] Preliminary data showed that 1 J/cm^2 of UVA changed MMP-1 and procollagen IA gene expression in DF. However, 3 J/cm^2 of UVA demonstrated numerous cell deaths in DF when cultured for 48 hrs similar to other research.[18] In the present study, we focused on the therapeutic effect of AFSC-CM on dermal changes by intermediates secreted from epidermis after UVA irradiation. According to previous reports, AFSC-CM includes several growth factors and cytokine, especially leptin.[30, 31] They suggested that leptin showed anti-oxidative effects in fibroblast and intestine.[39, 40] Our finding also supported AFSC-CM involved in cellular antioxidant pathways in the DF.

Results of our study demonstrated that CM from UVA irradiated HaCaT cells induced MMP-1 expression, but reduced procollagen 1A synthesis. However, these changes were restored by AFSC-CM treatment. Data suggest that AFSC-CM has a potential function to improve photo-aging effect including wrinkling.

Various UVA strengths were employed in the previous reports and we used 1 J/cm^2 of UVA irradiation for this study.[18,32,41] This variation occurred due to presence of different reflectors of the irradiation such as media and plastic wares. Therefore, further studies are required to establish UVA irradiation method, which would determine cell viability and gene expression changes. Recently, a few researches had been reported on the analysis of the supernatant from AFSC for their presence of growth factors and cytokines by using protein based array.[30, 42] Thus, further investigations are required on the functional analysis of the growth factors, cytokines and its signal intermediates.

Taken together, our findings suggest that AFSC-CM has a great potential in the skin regeneration as increasing cell proliferation and facilitating wound healing. Additionally, AFSC-CM appeared to have the therapeutic potential to improve UVA induced damages.

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