



Original Article

Effects of conditioned medium obtained from human adipose-derived stem cells on skin inflammation



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ABSTRACT

Introduction: Cell therapy using adipose-derived mesenchymal stem cells (ASCs) is a promising avenue of regenerative medicine for the treatment of various diseases. It has been considered that ASCs exert their therapeutic effects through the secretion of multiple factors that are critical for tissue remodeling or the suppression of inflammation. Recently, conditioned medium (CM) from ASCs that contains a complex of secreted factors has received attention as a cost-effective alternative to cell therapy.

Methods: We investigated the effects of CM obtained from ASCs (ASCs-CM) using human dermal fibroblasts (hDFs) and human epidermal keratinocytes with or without interleukin (IL)-1 β and examined mRNA levels of marker genes. We also examined alterations in cell proliferation and morphology of hDFs following treatment with ASCs-CM. We further investigated the effects of ASCs-CM treatment on prevention of skin inflammation using a mouse model.

Results: In hDFs and human epidermal keratinocytes, the ASCs-CM treatment suppressed pro-inflammatory factors and enhanced regenerative and remodeling factors with or without interleukin (IL)-1 β exposure. The ASCs-CM treatment also enhanced cell proliferation of hDFs and prevented morphological changes in response to IL-1 β exposure. Furthermore, in a mouse model of skin inflammation, treatment with ASCs-CM reduced the inflammatory reactions, including redness and thickness.

Conclusions: CM from ASCs may represent a potential alternative to ASC therapy for the treatment of inflammatory skin conditions.

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1. Introduction

Cell therapy using mesenchymal stem cells (MSCs) has been widely investigated [1,2] for the treatment of various degenerative and inflammatory diseases. Formerly, MSCs were known as somatic stem cells, which are capable of self-renewal and retain the potential to differentiate into multiple lineages [3]. Subsequent studies have revealed that MSCs have immunomodulatory effects; furthermore, anti-inflammatory, anti-apoptotic, anti-catabolic,

anti-fibrotic, pro-remodeling, and pro-angiogenic effects are now proposed to be the underlying mechanisms of MSC therapy [4]. MSC therapy is likely to be effective for the treatment of various diseases such as osteoarthritis, cerebral infarction, spinal cord injury, chronic kidney disease, and cirrhosis, in addition to other degenerative and inflammatory diseases [3]. A remarkable number of clinical studies investigating the use of MSCs for the treatment of skin diseases are in progress [5].

Bone marrow and adipose tissue have been widely used as sources of MSCs. Because it is much less invasive to obtain adipose tissue, adipose-derived mesenchymal stem cells (ASCs) are gaining popularity. Nevertheless, there are several limitations for the clinical use of ASCs. For autograft ASCs, it is necessary to culture ASCs for weeks to obtain a sufficient number of cells, and the process is accompanied by large costs as well as the need for staff to maintain cell cultures and a cell processing facility.

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For allograft ASCs, it is difficult to secure a stable supply of adipose tissue from donors. Particularly for the treatment of local skin lesions, a method of ASC administration should be optimized.

Recently, conditioned medium (CM) obtained from ASC culture has received attention as an alternative to ASC therapy [6]. ASCs exert their effects mainly through the release of cytokines, extracellular vesicles, and exosomes [7]. Because CM does not contain cells, it can be applied for allogeneic use. CM can be manufactured, preserved, and transported easily [8]. Moreover, CM can be used as topical medication for local skin lesions. Many studies have reported the efficacy of ASCs-CM for wound healing, the prevention of skin aging, and the reduction of scar formation both *in vitro* and *in vivo* [9].

In the present study, we aimed to clarify the effects of ASC-derived CM on normal and inflamed human dermal fibroblasts (hDFs) *in vitro* and skin inflammation *in vivo*. We applied the ASCs-CM to hDFs and human epidermal keratinocytes with or without interleukin (IL)-1 β and examined mRNA levels of marker genes. We also examined alterations in cell proliferation and morphology of hDFs following treatment with ASCs-CM. We further investigated the effects of ASCs-CM treatment on prevention of skin inflammation using a mouse model.

2. Materials & methods

2.1. Cell culture

Human adult hDFs and human epidermal keratinocytes were purchased from Lonza Walkersville Inc (Walkersville, MD, USA). hDFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). For assays, hDFs at 3 passages were seeded on 12- or 48-well plates (BD, Franklin Lakes, NJ). Human epidermal keratinocytes were cultured in KGM-Gold™ Keratinocyte Growth Medium Single-Quots™ Supplements and Growth Factors (Lonza, Basel, Switzerland). At 2 passages, these keratinocytes were seeded on 12- or 48-well plates (BD) for use in assays. Interleukin (IL)-1 β (1 ng/ml) (AF-200-01B, PeproTech, Rocky Hill, NJ) was added 24 h after passage.

2.2. ASC culture and CM preparation

Primary ASCs were obtained from donors at Avenue Cell Clinic (Tokyo, Japan) [10]. ASCs were maintained in NutriStem medium (NutriStem™, Biological Industries, Kibbutz Beit-Haemek, Israel) containing 1% penicillin-streptomycin and 2% human serum (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in an atmosphere containing 5% CO₂. For CM collection, ASCs at 3–5 passages were seeded with a density of 5×10^5 cells on 15-cm culture dishes and cultured to around 90% confluency. Then, ASCs were washed thoroughly 2 times using 10 ml of PBS and 1 time using 8 ml DMEM, and replenished with 15 ml NutriStem medium without human serum. After 48 h of culture in NutriStem medium without human serum, the supernatant was collected and washed 3 times with 1 ml PBS. Total 3 ml PBS was also collected to the supernatant. ASCs were replenished again with 15 ml NutriStem medium without human serum. These steps were repeated 3 times every 48 h. Then, the supernatant containing PBS was filtered with a 1.2- μ m syringe filter (Sartorius, Göttingen, Germany) and cleaned using Vivaspin® 100,000 MWCO (Sartorius), which is appropriate for efficient collection of exosomes. We obtained 8 ml of the prepared CM from 315 ml of supernatant. The protein concentration of the CM was 30 mg/ml as measured by BCA assay.

2.3. Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using Direct-zol™ micro kit (Zymo Research, Irvine, CA). One microgram of RNA was reverse-transcribed with RevaTraAce qPCR RT Master Mix (TOYOBO, Osaka, Japan) to make single-stranded cDNA. Each PCR reaction contained $1 \times$ THUNDERBIRD SYBR qPCR Mix (TOYOBO), 0.3 mM specific primers, and 20 ng of cDNA. RT-PCR was performed using Thermal Cycler Dice Real Time System III (Takara Bio, Kusatsu, Japan). Copy numbers of target gene mRNA in each total RNA sample were calculated by referencing standard curves and were adjusted to the human standard total RNA (Thermo-Fisher Scientific, Waltham, MA), with human *GAPDH* used as an internal control. The primer sequences of target genes are described in [Supplementary Table 1](#).

2.4. Cell proliferation assay

Cell proliferation and viability were examined using a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Tokyo, Japan). hDFs and human epidermal keratinocytes were seeded into the wells of a 48-well plate at a density of 10,000 cells/well and cultured in DMEM containing 1% penicillin-streptomycin at 37 °C under 5% CO₂. The next day, conditioned medium was added at various concentrations (0, 250, 500, or 1000 μ g/ml). CCK-8 assay was then performed at 48 h after the CM treatment. CCK-8 substrate (10 μ l) was added to each well and incubated for 3 h at 37 °C. A plate reader was used at 450 nm for colorimetric detection (Thermo-Fisher Scientific).

2.5. Phorbol myristate acetate-induced dermatitis

The efficacy of conditioned medium on prevention of skin inflammation was assessed using a mouse model. To induce inflammation, 0.05% phorbol-12-myristate-13-acetate (PMA) (Wako, Osaka, Japan) in dimethyl sulfoxide (DMSO) was applied to the ears of 8-week-old CD-1 male mice (total 6 mice) [11]. First, right and left ears were treated with 20 μ l of PBS and 20 μ l of 30 mg/ml CM (600 μ g), respectively. We placed drops of PBS or CM solution on each ear and waited until the drops were absorbed. Subsequently, both ears were exposed to 20 μ l of PMA solution via the same method. Ear thickness was measured with a caliper at day 1, 2, and 7.

2.6. Histological staining

For histological analysis, mouse ears were cut at day 7, fixed in 4% paraformaldehyde for 1 day, embedded in paraffin, and sectioned at 4 μ m thickness. Hematoxylin and eosin (H&E) staining was performed in accordance with standard protocols.

2.7. Ethics statement

We performed all animal experiments in accordance with a protocol approved by the Animal Care and Use Committee of the University of Tokyo. The use of human ASCs was approved by the Institutional Ethics Committee of the University of Tokyo (#622). ASCs from patients who provided written informed consent were used. All methods were performed in accordance with the relevant guidelines and regulations.

2.8. Statistical analysis

Data are expressed as mean \pm standard deviation (SD) and analysis was performed using Graph Pad Prism v.9 (Graph Pad Software, San Diego, CA). For multiple comparisons, statistical significance for differences between groups was determined with a

one-way ANOVA followed by Dunnett's post hoc test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effects of ASCs-CM on hDFs

We first evaluated the effects of ASCs-CM on hDFs. hDFs were treated with CM at concentrations of 0, 250, 500, and 1000 µg/ml for 24 or 48 h, and mRNA levels of *MMP3* and *COX2*, representative pro-inflammatory markers, were quantified by qRT-PCR. mRNA levels of *MMP3* decreased by about half in all ASCs-CM-treated groups at 24 h (Fig. 1A), and this effect was attenuated at 48 h (Fig. 1B). *COX2* mRNA levels were significantly decreased by the ASCs-CM treatment at both time points (Fig. 1A and B). We then examined the effects of ASCs-CM under inflammatory conditions using IL-1β, which is widely employed in inflammatory models [12,13]. At 24 h, IL-1β exposure increased the mRNA levels of *MMP3* and *COX2* about 40- and 80-fold, respectively (Fig. 1A), whereas *MMP3* and *COX2* levels were markedly suppressed by the ASCs-CM treatment (Fig. 1A). The suppression of both genes was also observed at 48 h after ASCs-CM treatment (Fig. 1B). We next examined the expression of regenerative and remodeling factors, such as *FGF2* and *PDGFA* markers. In the normal condition of cells without IL-1β exposure, mRNA levels of *FGF2* and *PDGFA* were

most enhanced by 250 and 500 µg/ml ASCs-CM treatment at 24 and 48 h, respectively (Fig. 1A and B). Treatment with 1000 µg/ml ASCs-CM corresponded with a decrease in *FGF2* at 24 h and a decrease in *PDGFA* at 48 h (Fig. 1A and B). In the inflammatory condition, *FGF2* and *PDGFA* were efficiently increased by treatment with 250 µg/ml ASCs-CM at 24 h (Fig. 1A). These significant increases were maintained in some ASCs-CM groups at 48 h, although the increases were attenuated compared with those observed at 24 h (Fig. 1B).

3.2. Effects of ASCs-CM on cell proliferation and morphology of hDFs

We next examined the effects of ASCs-CM on cell proliferation and morphology of hDFs. Cell proliferation was enhanced by the ASCs-CM treatment at 250 µg/ml; however, it was unchanged by ASCs-CM treatment at 500 or 1000 µg/ml (Fig. 2A). Similarly, crystal violet staining was intense in the 250 µg/ml ASCs-CM group (Fig. 2B). hDFs typically exhibit a spindle-shaped morphology in monolayer culture. Although the cells exhibited a round shape in the presence of IL-1β, the original spindle-shape was maintained with 250 µg/ml ASCs-CM throughout the observation period (Fig. 2C and Supplementary Fig. 1). These results suggest that ASCs-CM treatment enhanced cell proliferation and maintained the characteristic morphology of hDFs.

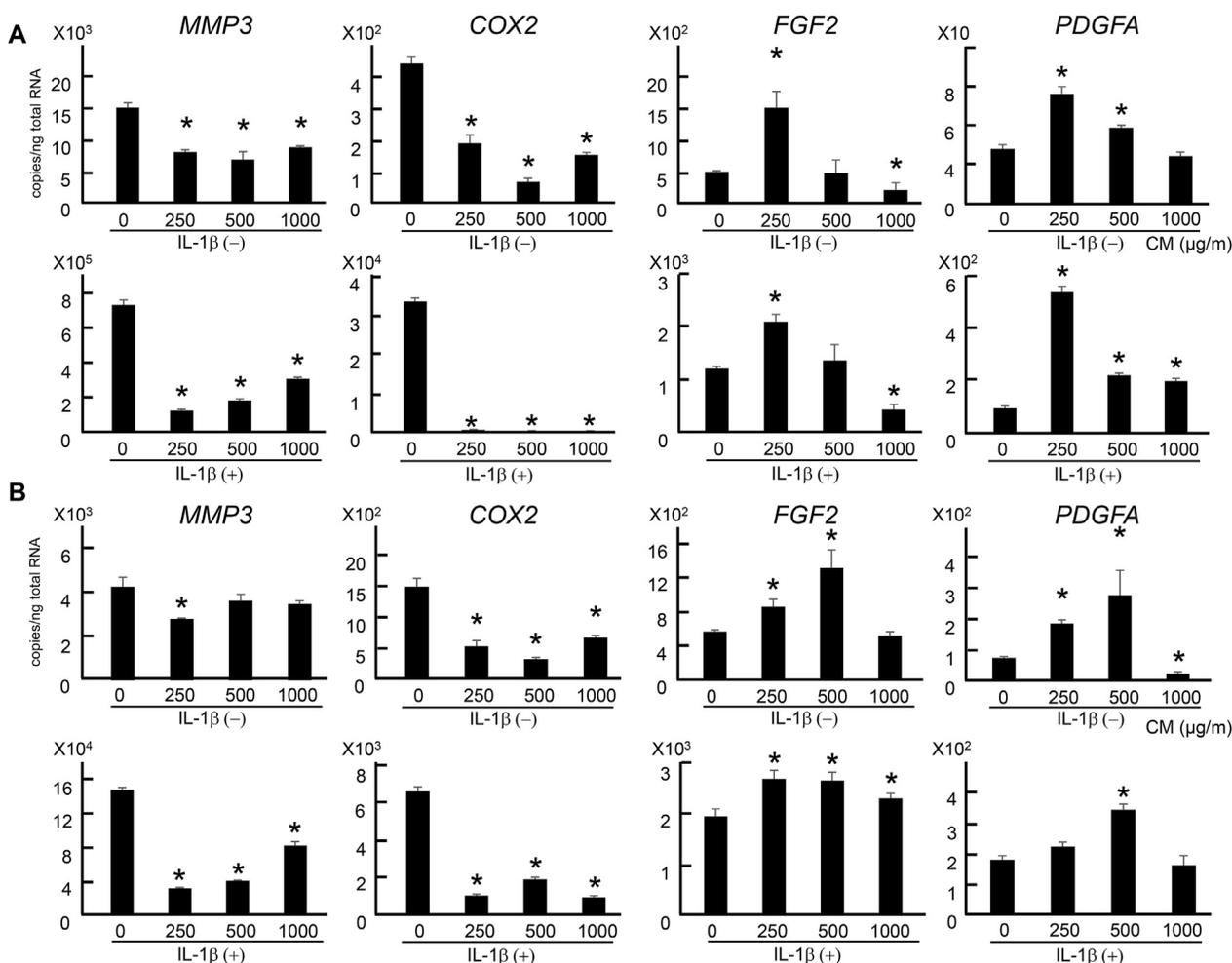


Fig. 1. Effects of conditioned medium (CM) obtained from adipose-derived stem cells (ASCs) on human dermal fibroblasts (hDFs). mRNA levels of marker genes in hDFs treated with ASCs-CM at concentrations of 0, 250, 500, and 1000 µg/ml for 24 h (A) and 48 h (B) in the presence of interleukin (IL)-1β (1 ng/mL) or vehicle. All data are expressed as the mean ± SD (n = 3). *P < 0.05 vs. 0 µg/ml, determined by ANOVA followed by Dunnett's post hoc test.

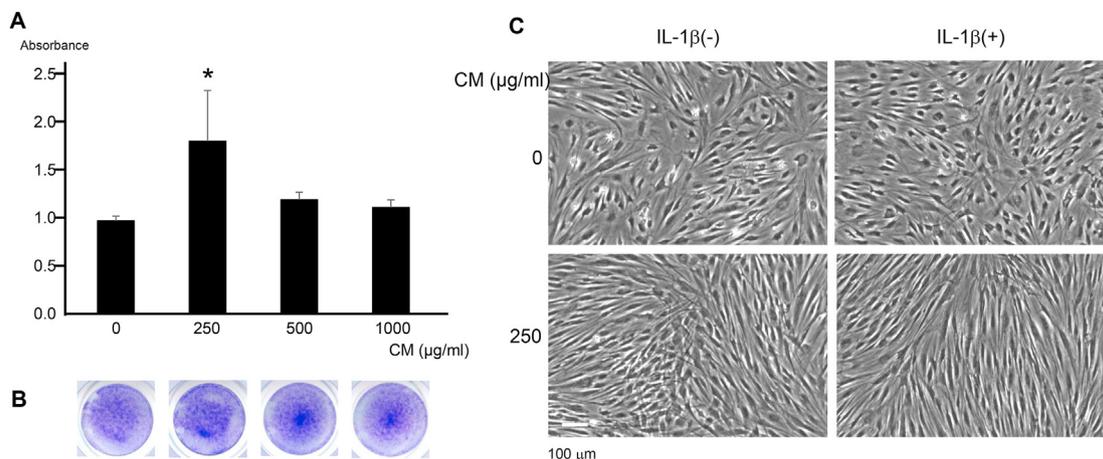


Fig. 2. Effects of ASCs-CM on cell proliferation and morphological changes of hDFs. (A) CCK-8 assay of hDFs treated with ASCs-CM at concentrations of 0, 250, 500, and 1000 µg/ml for 48 h. All data are expressed as the mean ± SD (n = 4). *P < 0.001 vs. 0 µg/ml determined by the Kruskal–Wallis test and Dunn’s post hoc test for unequal variance. (B) Crystal violet staining after the CCK-8 assay. (C) Morphology of hDFs treated with 250 µg/ml ASCs-CM for 7 days with or without 1 ng/ml IL-1β. Scale bars, 100 µm.

3.3. Effects of ASCs-CM on human epidermal keratinocytes

We further examined the effects of ASCs-CM treatment on human epidermal keratinocytes, which compose the outermost layer of skin. mRNA levels of *MMP3* and *COX2* in keratinocytes were significantly suppressed by the ASCs-CM treatment (Fig. 3A). *FGF2* levels were increased by 250 µg/ml ASCs-CM treatment, whereas *PDGFA* levels were not changed (Fig. 3A). Cell proliferation was enhanced by the ASCs-CM treatment in a dose-dependent manner (Fig. 3B). Similarly, crystal violet staining was intense in all ASCs-CM groups (Fig. 3C).

3.4. Effect of ASCs-CM on in vivo model of inflammatory conditions

Finally, we examined the efficacy of the ASCs-CM treatment on prevention of skin inflammation induced by PMA using a mouse model; topical application of PMA induces an acute inflammatory reaction in which IL-1β is involved [14]. PMA exposure caused redness and thickening of the ears in vehicle-treated groups (Fig. 4A and B and Supplementary Fig. 2). Notably, these reactions were inhibited by the ASCs-CM treatment (Fig. 4A and B and Supplementary Fig. 2). In the vehicle group, both epidermal and dermal layers were hypertrophic and thickened, and the boundary

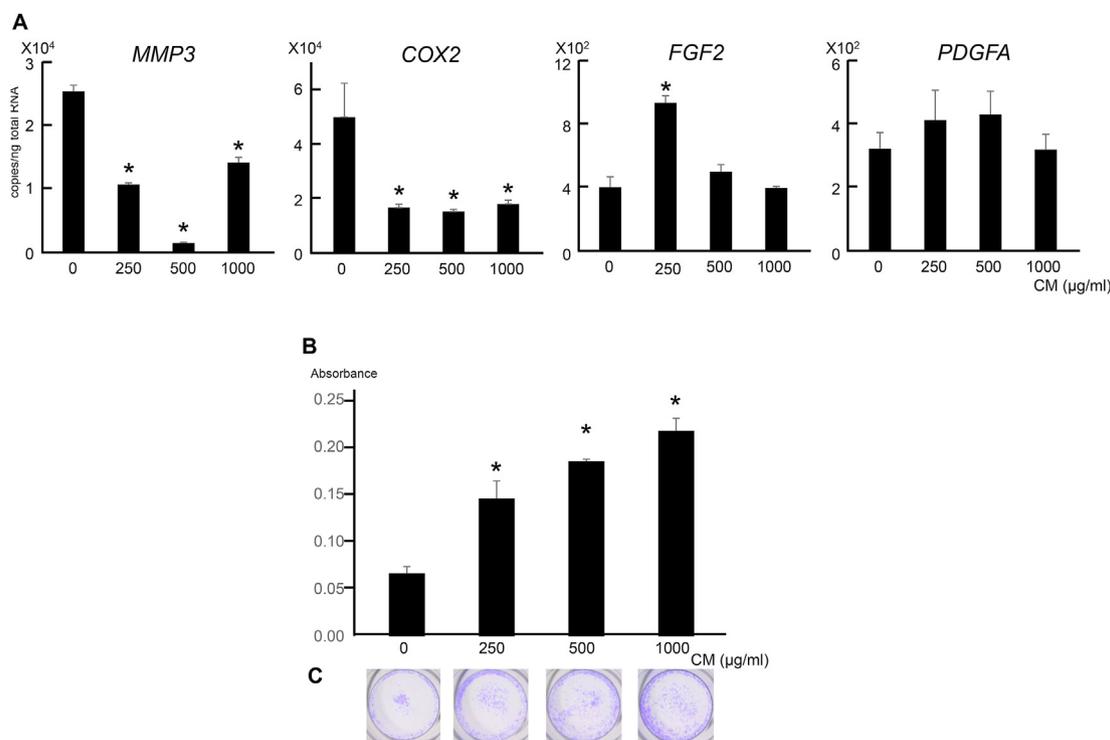


Fig. 3. Effects of ASCs-CM on human epidermal keratinocytes. (A) mRNA levels of marker genes in human epidermal keratinocytes treated with ASCs-CM at concentrations of 0, 250, 500, and 1000 µg/ml for 48 h. All data are expressed as the mean ± SD (n = 3). *P < 0.05 vs. 0 µg/ml, determined by ANOVA followed by Dunnett’s post hoc test. (B) CCK-8 assay of human epidermal keratinocytes treated with ASCs-CM at concentrations of 0, 250, 500, and 1000 µg/ml for 48 h. All data are expressed as the mean ± SD (n = 4). *P < 0.001 vs. 0 µg/ml determined by the Kruskal–Wallis test and Dunn’s post hoc test for unequal variance. (C) Crystal violet staining after the CCK-8 assay.

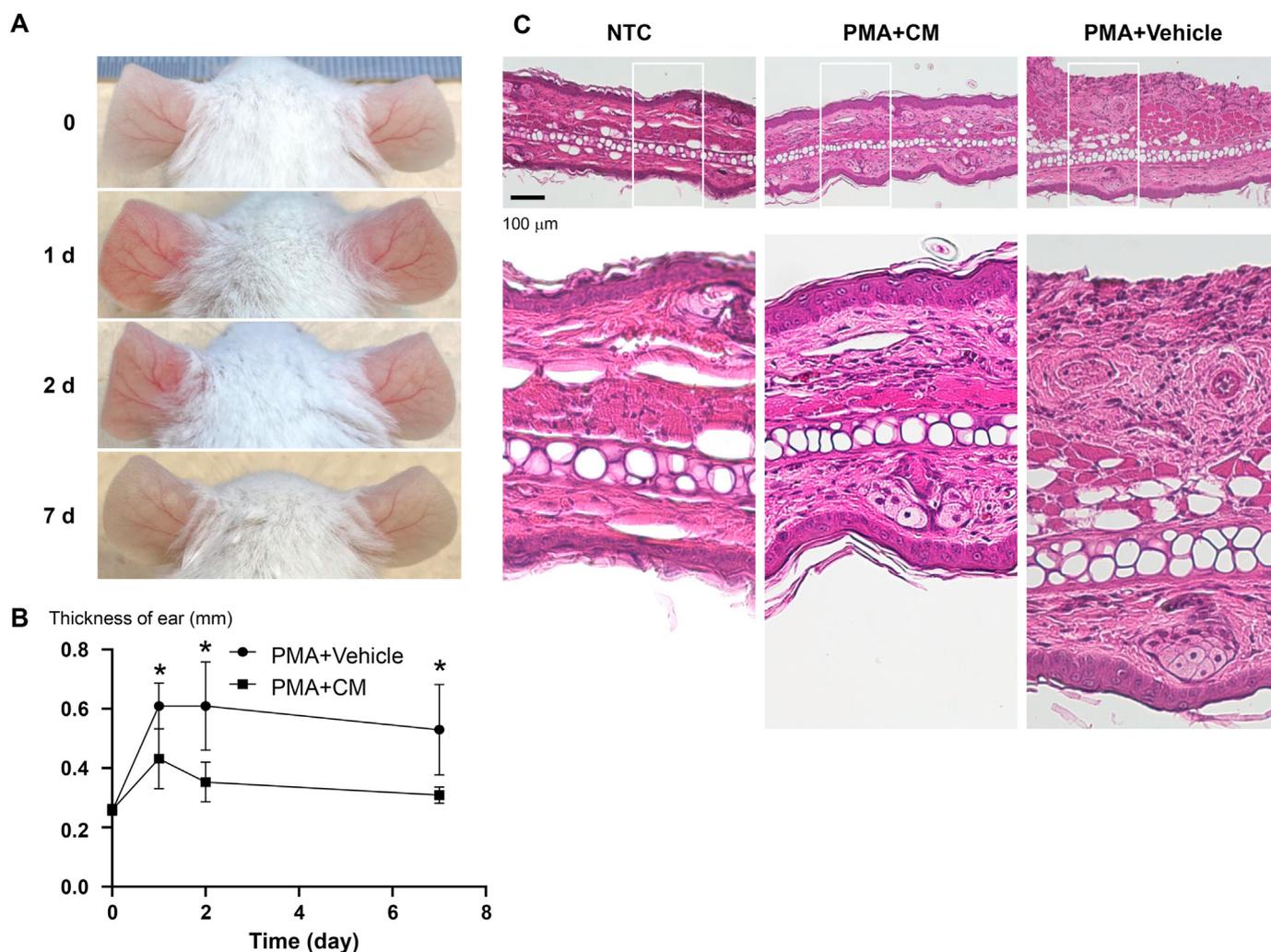


Fig. 4. Effects of ASCs-CM on prevention of skin inflammation induced by phorbol myristate acetate (PMA) using a mouse model. (A) Gross appearance of ears at day 0 (before induction), 1, 2, and 7. Right and left ears were treated with 20 μ l of PBS (vehicle) and 20 μ l of 30 mg/ml CM (600 μ g), respectively. Both ears were then exposed to 20 μ l of PMA solution. (B) Thickness of the ears at day 0, 1, 2, and 7. * $P < 0.05$ vs. PBS at each time point, determined by two-way ANOVA for unequal variance. (C) Hematoxylin and eosin (H&E) staining of the ears at day 0 and 7. Inset boxes in H&E staining indicate enlarged images below. NTC, no treatment control. Scale bar, 100 μ m.

became unclear (Fig. 4C). Parakeratosis, which is characterized by retention of nuclei in the keratinocytes of the stratum corneum, and the infiltration of many inflammatory cells were prominent in the vehicle group, whereas the ASCs-CM group displayed only minimal changes (Fig. 4C).

4. Discussion

The present study showed that ASCs-CM contributes to the reduction of inflammatory responses in both hDFs and a mouse skin model. In hDFs and human epidermal keratinocytes, the ASCs-CM treatment suppressed pro-inflammatory factors and enhanced regenerative and remodeling factors with or without IL-1 β exposure. The ASCs-CM treatment also enhanced cell proliferation of hDFs and maintained their characteristic spindle-shape even in the presence of IL-1 β . In a mouse model of skin inflammation, the ASCs-CM treatment reduced the inflammatory reactions, including redness and thickness. These data illustrate the anti-inflammatory effects of ASCs-CM.

In the cell culture experiments, marked suppression of MMP3 and COX2 was observed in IL-1 β -exposed hDFs at both 24 and 48 h, and in human epidermal keratinocytes at 48 h (Figs. 1 and 2). In the mouse skin inflammation model, ear thickness was

significantly suppressed in the ASCs-CM group at all time points (Fig. 4B). The ASCs-CM treatment efficiently suppressed hypertrophy and inflammation of the epidermal and dermal layers induced by PMA exposure and these observations were made after 7 days of ASCs-CM treatment and PMA exposure (Fig. 4C). Internal and external stimuli induce epidermal and dermal cells to produce inflammatory cytokines, which cause subsequent reactions from immune cells. Additionally, PMA, which was employed in this study, is a potential activator of protein kinase C (PKC). PKC regulates conventional effector T cell function in the immunological synapse [15], and activation of PKC by PMA induces granulocytes and proinflammatory cytokines including TNF, IL-1 β , and IL-6 [16]. Considering the anti-inflammatory and immunomodulatory effects of ASCs [17], it is reasonable to assume that the ASCs-CM has similar effects. Notably, ASCs-CM treatment promoted an anti-inflammatory phenotype of macrophages in *ex vivo* experiments that used human peripheral blood mononuclear cells [18]. Although we could not investigate how immune cells were modulated in the present study, the suppression of skin inflammation by the ASCs-CM treatment is partially because of its effects on immune cells in the late phase, as well as direct effects on epidermal and dermal cells in the early phase.

Generally, the skin barrier is an essential issue to be addressed for the delivery of topically applied compounds or drugs. The cuticle, which is the outermost layer of the epidermis, prevents the penetration of various cosmetics and macromolecular drugs into the dermis [9]. Therefore, lasers and microneedles are used *in vivo* studies and the clinic to create microchannels to increase drug absorption [9]. Although we did not use these adjuvant methods, dermatitis was efficiently suppressed by ASCs-CM that was topically applied to the ear. This may be because ASCs-CM could partially protect the skin barrier that was disrupted by PMA exposure. It would be necessary to optimize delivery of ASCs-CM for application on healthy skin for anti-aging purposes or for the prevention of ultraviolet ray-induced damage. Ultimately, ASCs-CM is much more useful for these objectives than ASCs themselves, because it is difficult to apply ASCs topically on skin lesions other than wounds or ulcers.

ASCs-CM and exosomes have promising applications in wound healing as well as skin inflammatory conditions. Previous studies have shown that ASCs-CM promotes proliferation and migration of fibroblasts and contributes to healing processes [9]. Various cytokines, such as epidermal growth factor, FGF, PDGF, and vascular endothelial growth factor, are considered to be essential in this process [9]. FGF2 is the cytokine that is most significantly involved in cell proliferation and has been used clinically for the treatment of ulcers and chronic wounds [19,20]. PDGF contributes to self-renewal and proliferation of ASCs, and its expression in ASCs has been found to decrease with aging [21]. PDGF also stimulates Nestin-expressing MSCs to differentiate toward profibrotic cell types [22]. Interestingly, proliferation of hDFs was enhanced only by 250 µg/ml ASCs-CM, and *FGF2* and *PDGFA* mRNA levels were most significantly induced by treatment with 250 µg/ml ASCs-CM at 24 h (Figs. 1 and 3A). Considering the efficient suppression of *MMP3* and *COX2* in all ASCs-CM groups exposed to IL-1β (Fig. 1), optimal doses of ASCs-CM for anti-inflammation and pro-proliferation may be different.

5. Conclusions

We demonstrated that the ASCs-CM treatment suppressed skin inflammation *in vitro* and *in vivo*. ASCs-CM enhanced cell proliferation of hDFs. This study may contribute to the wide application of ASCs-CM for the treatment of various diseases and conditions of the skin.

Author contributions

Study conception and design: F.Y., R.C., S.Ta, and T.S. Data collection: F.Y., T.Ta., T.K. Data Analysis: F.Y., T.Ta, T.K., T.Ts. Data interpretation: F.Y., K.I., S.Ts., T.S. Drafting of the paper: F.Y., T.S.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2022.03.009>.

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