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Effects of Poly-L-Lactic Acid on Adipogenesis and Collagen Gene Expression in Cultured Adipocytes Irradiated with Ultraviolet B Rays

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Background: Poly-L-lactic acid (PLLA), a synthetic, biocompatible, and biodegradable polymer, has been safely used in several clinical applications. Recently, PLLA has been widely used in the field of dermatology to treat wrinkles in aging skin. Reportedly, PLLA directly acts on dermal fibroblasts causing a significant increase in the expression of type I collagen. However, little is known about the effect of PLLA on adipocytes.
Objective: This study aimed to analyze the effect of PLLA on adipocytes and examine its potential in treating deep wrinkles engendered by the loss of subcutaneous fat because of aging and photoaging.
Methods: To elucidate the effect of PLLA on skin photoaging, cultured 3T3-L1 adipocytes were irradiated with ultraviolet B (UVB) rays. Oil red O staining was used to detect lipid accumulation in the adipocytes. Real-time quantitative polymerase chain reaction and Western blotting were performed to detect types IV and VI collagen mRNA and protein levels, respectively, under different conditions.
Results: The differentiation of 3T3-L1 cells enhanced adipogenesis and the expression of types IV and VI collagens, both of which were inhibited by UVB irradiation. Following this irradiation, PLLA stimulated adipogenesis and the expression of types IV and VI collagens.
Conclusion: PLLA may provide the beneficial effect on adipocytes from the aspect of adipogenesis and collagen expression in the subcutaneous adipose tissues.

Keywords: Adipogenesis, PLLA, UVB

INTRODUCTION

Wrinkles are produced due to the atrophy of the skin's dermal layer and subcutaneous fat, which occurs with age. In particular, the depth and thickness of wrinkles is greater in the skin areas that have been exposed to sunlight for extended periods compared with those in unexposed areas; this phenomenon is known as photoaging. The pathological findings of photoaged skin include not only solar elastosis and basophilic degeneration but also the atrophy of the dermis and subcutaneous layer of the skin, suggesting that although these skin layers are difficult to penetrate for ultraviolet (UV) rays, prolonged sun exposure induces abnormalities in these layers. This phenomenon commonly occurs in areas of thin skin that are constantly exposed, such as that of the face[1,2].

Despite the fact that UV rays cannot directly reach the adipose tissue, it was observed that the subcutaneous adipose tissue of the skin that has been chronically exposed to UV rays contains less fat than that contained in unexposed skin[3]. In a pathological condition where the dermal layer is very thin due to photoaging or skin damage occurs due to blister disease, severe burns, or skin defect by trauma, UV irradiation could directly affects fat cell metabolism. For this reason, we studied the effects of UVB radiation on fat cells and at the same time

tried to found out the substances that inhibit this process.

Therefore, UV rays can be considered a crucial environmental factor that determines the amount of subcutaneous fat in the skin, and ultimately, has a major impact on the occurrence of excessively deep wrinkles that is generally observed in photoaging[4].

Both acute single UV irradiation and repeated chronic exposure have been shown to downregulate the expression of genes associated with adipogenesis and lipogenesis, thereby reducing lipid synthesis in subcutaneous adipose tissue[3]. Therefore, effectively preventing the UV-induced inhibition of adipogenesis will facilitate the development of novel photoaging treatments.

Poly-L-lactic acid (PLLA) is a biodegradable and biocompatible polymer material that has been long used as a raw material for sutures, intratissue implants, and ecofriendly disposable products[5]. After the Food and Drug Administration approved it for cosmetic treatment in 2008, PLLA has been widely used for treating wrinkles and other skin issues due to aging, especially by intradermal injections to increase skin volume. Furthermore, its biodegradable quality ensures stability; thus, the use of PLLA is expected to surge even more in the future[6].

According to our previous study, PLLA directly affects dermal fibroblasts and influences the gene expression of type I collagen, which occupies a majority of the dermis. It also stimulates the synthesis of proteins, indicating that the response is related to the signaling pathways involving p38, Akt, and Janus kinase (JNK)[7]. The ameliorative effect on deep wrinkles due to increased skin volume observed after PLLA treatment in clinical settings is likely due to the increase in adipogenesis resulting from the influence of PLLA on both the extracellular matrix and adipocytes. However, the softness and naturalness of the skin observed post-PLLA treatment cannot be explained by an increase in the quantity of extracellular matrix alone. This study attempted to investigate the effect of PLLA on the expression of types IV and VI collagens, which demonstrate an increase in quantity during adipogenesis and adipocyte differentiation following UV irradiation.

MATERIALS AND METHODS

This study was reviewed and declared exempt from Keimyung University Dongsan Hospital Institutional Review Board approval (IRB No. 2022-09-013).

Materials

1) Culture and differentiation of adipocytes

The 3T3–L1 preadipocyte (ATCC) were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (WELGENE) containing 10% calf serum (GIBCO-BRL) at 5% CO_2 and 37℃. The cells were subcultured at 60% confluence. After treatment with trypsin/EDTA (WELGENE) to detach the cells, they were removed, centrifuged, dispensed into 60-mm culture dishes at a density of 4×10^5 cells per dish, and then cultured for 2 days until they reached 100% confluence. The cells were treated with 10% fetal bovine serum (FBS; WELGENE), insulin (5 μg/ml; Sigma-Aldrich), dexamethasone (0.5 μM; Sigma-Aldrich), and 3-isobutyl-1-methyxanthine (0.5 mM; Sigma-Aldrich) in DMEM for 48 hours to induce differentiation. Following that, the medium was replaced with DMEM containing 10% FBS and insulin every 48 hours.

2) PLLA

In the culture medium, dry PLLA powder (Sculptra®; Sanofi Aventis) and 5 ml of sterile water was added, and the final concentrations were adjusted to 0.2% to 0.4%.

Methods

1) UVB irradiation and PLLA treatment

To investigate the effect of UVB irradiation on 3T3-L1 preadipocytes, they were treated with phosphate-buffered saline (PBS) every time the DMEM was replaced during the differentiation period, and then irradiated with 50 mJ/cm^2 and 100 mJ/cm^2 of UVB (FS40 sunlamp; Westinghouse). UVB radiation peaked at 310 to 315 nm and the lamp was placed 30 cm above the cells. PLLA was directly added to the DMEM at concentrations of 0.2% and 0.4% after UV irradiation and its effects were compared with the control group.

2) Cell survival rate analysis

Cell viability was detected by Cell Counting Kit-8 (CCK-8) (Dojindo).

The 3T3–L1 preadipocyte cells were first counted, and about 10,000 cells per well were seeded on a 96-well cell culture plate (Thermo Scientific). After incubation at 37℃ in a 5% CO_2 humidifying atmosphere, cells were exposed to UVB doses of 50, 100, 200, 300 mJ/cm^2 and PLLA concentrations of

0.1%, 0.2%, and 0.4% in a 96-well plate for differentiation 7 days. After treatment, 10 μl of the CCK-8 solution was added to each well, the 96-well plate was continuously cultured at 37℃ for 1 hour 30 minutes, and the optical density value of each well was read at a wavelength of 450 nm to determine cell viability in SPARK microplate readers (TECAN). Control cells were considered to be 100% viable.

3) Analysis of adipogenesis in 3T3-L1 adipocytes

To determine adipogenesis in 3T3-L1 cells, they were stained with oil red O, which reacts with triglycerides. On day 7 of differentiation, the medium was removed; cells were washed with PBS, fixed at room temperature for 1 hour using 4% formaldehyde, and washed again with PBS. After staining with oil red O solution (Sigma-Aldrich) for 1 hour, the cells were washed twice with distilled water and the stained cells were observed under a phase-contrast microscope. In addition, 200 μg of fat was transferred into each well of a 96-well plate after extraction using 100% isopropanol, and the absorbance was measured at 510 nm using an ELISA reader (Tecan). The fat content that reacted with the oil red O solution was expressed as a numerical value relative to the average absorbance of the control group.

4) Analysis of collagen gene expression by real-time quantitative polymerase chain reaction

RNA was isolated from cultured adipocytes using TRIzol reagent (Invitrogen) and 2 μg of it was prepared using a cDNA synthesis kit (Takara Bio Inc). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix Kit (Roche Diagnostics) on a CFX Connect Real-Time PCR system (Bio-Rad). The following thermal cycle was performed: after running qPCR once at 95℃ for 5 minutes, 40 cycles of 95℃ for 10 seconds and 60℃ for 30 seconds was conducted. The dissolution curve started at 65℃ and ended at 95℃, and the desired fluorescence value was confirmed by increases of 0.5℃. The primers used were as follows: collagen type IV (forward, 5'-CCC TAA CGG TTG GTC CTC AC-3'; reverse, 5'-CGA TGA ATG GGG CGC TTC TA-3'), collagen type VI (forward, 5'-AAA GGC ACC TAC ACC GAC TG-3'; reverse, 5'-GCA TGG TTC CTT GTA GCC CT-3'), and β-actin (forward, 5'-CCT CTA TGC CAA CAC AGT GC-3'; reverse, 5'-GTA CTC CTG CTT GCT GAT CC-3').

5) Analysis of collagen synthesis by Western blot

After washing the cultured cells twice with PBS, RIPA buffer (Thermo Scientific) was added and the cells were centrifuged to extract the proteins. The protein concentration was quantified using a Pierce BCA protein assay kit (Pierce). After performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 20 μg of protein, the isolated proteins were transferred to an immobilon-P transfer membrane (Millipore) and blocked using 5% skim milk solution. The membrane was incubated first with primary anti-collagen type IV (Novus Biologicals), anti-collagen type VI (Novus), and anti-β-actin (Cell Signaling) antibodies, diluted to 1:1,000 in Tbst solution (Biosesang) at 4℃, followed by incubation with secondary antibodies. The protein bands were detected using an ECL detection kit (Amersham Biosciences) on a Fusion Solo 5 (VILBER Lourmat SAS) machine. Quantitative analysis was performed using ImageJ software and illustrated graphically.

6) Statistical analysis

Statistical analysis was performed using one-way analysis of variance. Multiple groups were compared using variance analysis through Bonferroni's multiple comparison test. The results were expressed as mean±standard error of the mean. All the results were considered to have a statistically significant difference when the *p*-value was less than 0.05.

RESULTS

Effect of UVB irradiation and PLLA on the viability of cultured adipocytes

To assess whether UVB and PLLA influence the viability of adipocytes, the CCK-8 assay was performed after treatment with the various doses of UVB and PLLA.

The cell viability decreased from the UVB irradiation dose of 200 mJ/cm^2 and decreased to a significant level from 300 mJ/cm^2. Thus, the UVB doses of 50 mJ/cm^2 and 100 mJ/cm^2 were used in this experiment (Fig. 1A).

None of the tested concentrations of PLLA affected the viability of 3T3-L1 cells (Fig. 1B). Thus, 0.1% PLLA was initially tried as it was shown to stimulate collagen production in dermal fibroblast by other report[7]. However, it was shown to not stimulate enough adipogenesis and collagen synthesis, so 0.2% and 0.4% PLLA were used.

There were no changes in cell viability even in the group

administered with UVB 100 mJ/cm^2 dose and 0.2%, 0.4% PLLA at the same time (Fig. 1C).

Effect of UVB irradiation and PLLA on adipogenesis in cultured adipocytes

After inducing differentiation for 7 days, 3T3-L1 cells were stained with oil red O, and the intracellular accumulation of lipids was measured using ImageJ. In the experimental group that was irradiated with UVB, adipocyte differentiation was statistically significantly reduced compared with the control group. However, adipocytes treated with 0.2% and 0.4% PLLA post-UVB irradiation exhibited increased adipogenesis compared with those that were exposed to UVB alone and left untreated. Although adipogenesis was higher in the group treated with 0.4% PLLA, this increase was not observed to be dose-dependent (Fig. 2).

Protein expression analysis of types IV and VI collagens after treatment of PLLA in cultured adipocytes

After inducing differentiation of 3T3-L1cells for 7 days, Western blot was used to analyze the levels of types IV and VI collagens with β-actin as the reference. The expression of both the collagen proteins increased in the PLLA-treated adipocytes compared with the control group (Fig. 3).

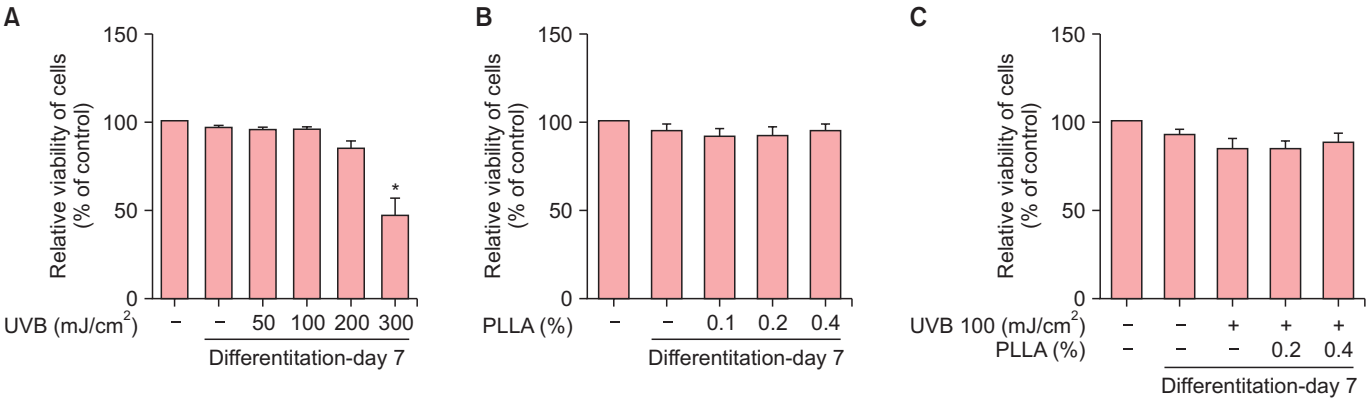

Fig. 1. Effect of UVB and PLLA on the viability of adipocytes. (A~C) The cell viability was determined using Cell Counting Kit-8 assay and OD value was read at a wavelength of 450 nm in SPARK microplate readers. The results shown are mean±SEM. UVB: ultraviolet B, PLLA: poly-L-lactic acid, OD: optical density, SEM: standard error of the mean. *p<0.001 compared with day 7 post-differentiation only.

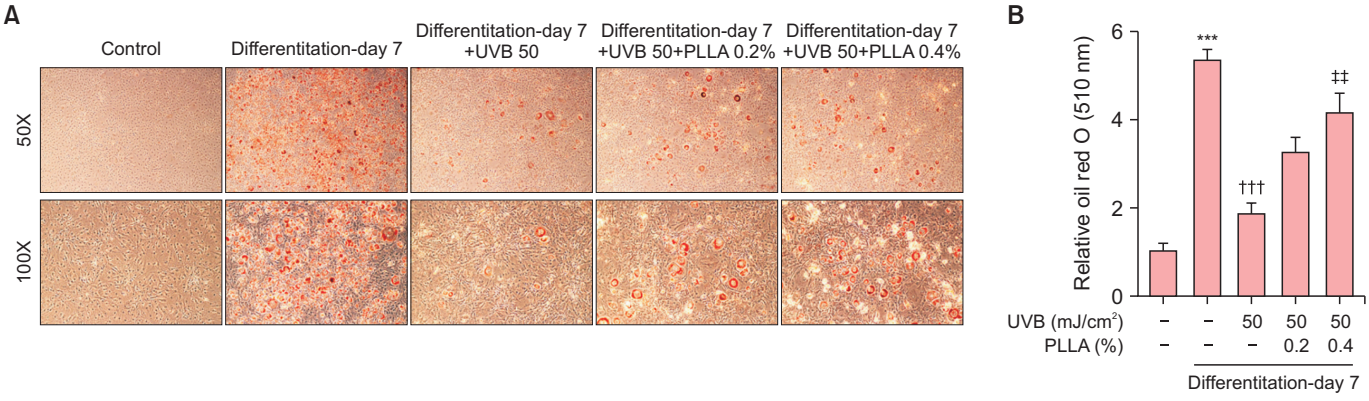

Fig. 2. Effect of UVB and PLLA on adipocyte differentiation. (A) Photomicrography of 3T3-L1 cells (Oil red O stain, ×50, ×100). (B) The 3T3-L1 cells were irradiated with UVB, followed by treatment with 0.2% or 0.4% PLLA for 7 days. The lipid content of the adipocytes was determined using oil red O staining and ImageJ. The results shown are mean±SEM. UVB: ultraviolet B, PLLA: poly-L-lactic acid, SEM: standard error of the mean. ***p<0.001 compared with control, $^{\dagger\dagger\dagger}p$<0.001 compared with day 7 post-differentiation only, $^{\ddagger\ddagger}p$<0.01 compared with day 7 post-differentiation post-UVB treatment.

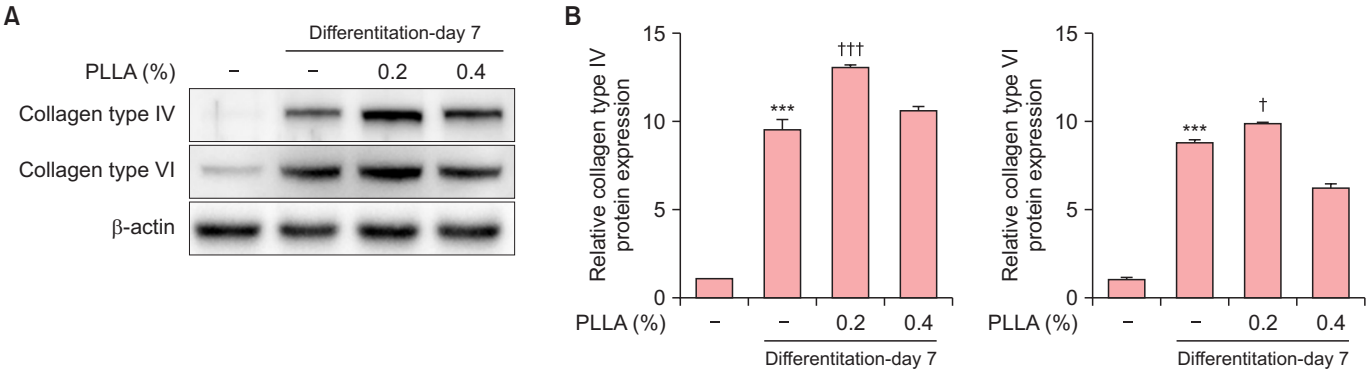

Fig. 3. Effect of PLLA on types IV and VI collagen expressions. (A) The levels of types IV and VI collagen proteins were determined using Western blot. (B) The relative levels of types IV and VI collagen proteins were normalized against β-actin levels from the same protein preparation. Results shown are mean±SEM. PLLA: poly-L-lactic acid, SEM: standard error of the mean. ***$p<0.001$ compared with control, $^{\dagger}p<0.001$ compared with day 7 post-differentiation only, $^{\dagger\dagger\dagger}p<0.001$ compared with day 7 post-differentiation only.

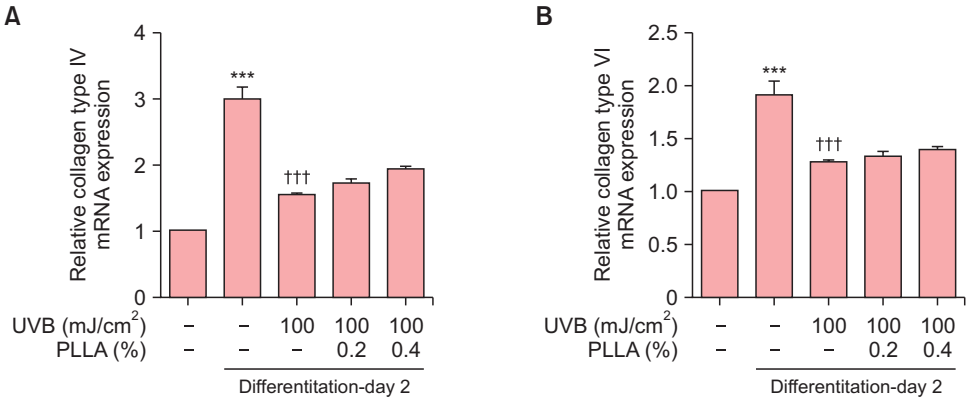

Fig. 4. Effect of UVB and PLLA on types IV and VI collagen mRNA expressions. (A) Type IV collagen expressions were determined using real-time quantitative polymerase chain reaction (PCR). (B) Type VI collagen expressions were determined using real-time quantitative PCR. The relative levels of mRNA were normalized against β-actin levels from the same cDNA preparation. Results shown are mean±SEM. UVB: ultraviolet B, PLLA: poly-L-lactic acid, SEM: standard error of the mean. ***$p<0.001$ compared with control, $^{\dagger\dagger\dagger}p<0.001$ compared with day 2 post-differentiation only.

Effect of PLLA on collagen synthesis in cultured adipocytes following UVB irradiation

1) Analysis of the mRNA expressions of types IV and VI collagens following UVB irradiation and PLLA treatment

To investigate the changes in the expression of extracellular matrix protein accompanying the differentiation of 3T3-L1 cells, the levels of types IV and VI collagen mRNA were analyzed using qPCR after 2 days following differentiation induction. The expressions of types IV and VI collagens significantly increased on the second day after differentiation was induced, while following UVB irradiation, the expression of both the collagen transcripts statistically significantly decreased compared with the control group.

In the adipocytes treated with 0.2% and 0.4% PLLA after UVB irradiation, the expression of both collagen transcriptions dose-dependently increased; however, the effect was not statistically significant (Fig. 4).

2) Types IV and VI collagen protein expression analysis following UVB irradiation and PLLA treatment

The 3T3–L1 preadipocyte cells were differentiated for 1 day following UVB irradiation and PLLA treatment. Then, the protein levels of types IV and VI collagens were analyzed based on β-actin as a reference. The expression of both the collagen proteins statistically significantly increased 7 days after differentiation while it statistically significantly decreased following UVB irradiation.

Adipocytes treated with 0.2% or 0.4% PLLA following UVB

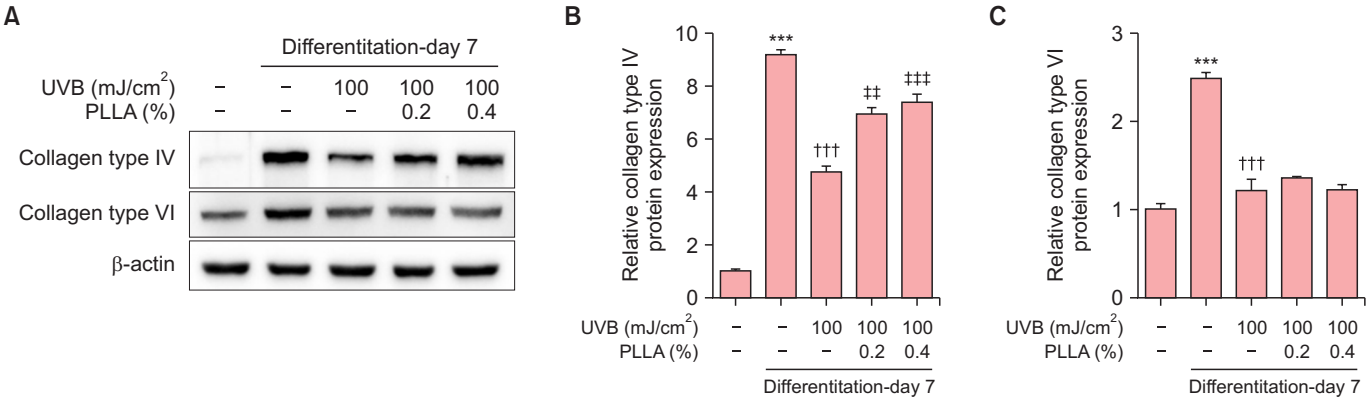

Fig. 5. Effect of UVB and PLLA on type IV, VI collagen expression. (A) The levels of types IV and VI collagen proteins were determined using Western blot. (B) The relative levels of type IV collagen proteins were normalized against β-actin levels from the same protein preparation. (C) The relative levels of types VI collagen proteins were normalized against β-actin levels from the same protein preparation. Results shown are mean±SEM. UVB: ultraviolet B, PLLA: poly-L-lactic acid, SEM: standard error of the mean. ***$p<0.001$ compared with control, $^{\dagger\dagger\dagger}p<0.001$ compared with day 7 post-differentiation only, $^{\ddagger\ddagger}p<0.01$,$^{\ddagger\ddagger\ddagger}p<0.001$ compared with day 7 post-differentiation post-UVB treatment.

irradiation showed a dose-dependent statistically significant increase in the expression of type IV collagen. The expression of type VI collagen also showed an increase; however, the level was lower in the 0.4% PLLA-treated cells than in the 0.2% PLLA-treated cells and not statistically significant (Fig. 5).

DISCUSSION

Premature skin aging or photoaging occurs most severely in perpetually UV-exposed areas, such as facial skin. UV ray induced skin aging is characterized by pigmented moles, decreased collagen, vasodilation, and decreased subcutaneous fat[8]. Compared with endogenous aging that occurs over time, the effects of aging induced by UV rays include wrinkles that are deep and rough and an overall thinning and sagging of the skin due to a decrease in skin volume, thereby making the skin look older than it actually is[1].

In physiological environments, the UV can penetrate the epidermis and into the mid-dermis, but not into the subcutaneous fat tissue. By the way, Kim et al.[3] reported that subcutaneous fat tissue in chronically sun-damaged skin contains less fat than naturally aged skin, and even a single UV exposure of human skin reduced lipid synthesis in the underlying subcutaneous fat tissue through transcriptional regulation of the lipogenic enzymes and adipogenic transcription factors. They reported an indirect reaction of adipocytes to UV by conducting an experiment for skin tissue and skin constituent cells, so we wanted to know the direct reaction of adipocytes by UV radiation.

We hypothesized that UV rays cloud have direct effect on subcutaneous fat cells in special environments such as burn, skin defect, or very thin skin condition (bullous disease, photoaging) and conducted this experiment. And we thought it would be meaningful to study ways to alleviate the negative effect of UV in the lipogenesis.

Little has been revealed regarding the role and direct mechanism of UV rays in decreasing the volume of subcutaneous fat. To determine the biological response to UV rays, we first cultivated adipocytes, exposed them to UV rays to determine its effects on adipogenesis and the expression of extracellular matrix proteins, and investigated the effects of PLLA, which is a compound that has lately been widely used for treating wrinkles.

PLLA is a biodegradable and biocompatible polymer used to treat wounds and implants, and in dermatological treatments and medical devices[6,9]. PLLA is superior to conventional fillers in terms of stability and durability of the effect of clinical tissue restoration, and is currently being used worldwide for not only the face but also as a supplement to fill tissue defects in any part of the body, and hence, its demand is anticipated to further increase in the future. The main mechanism of action of PLLA has mainly been attributed to its paracrine effect due to the various cytokines secreted by macrophages, mast cells, and lymphocytes whose quantities increase in response to the infiltration of foreign bodies[10].

However, according to our previous study, PLLA was confirmed to directly affect fibroblasts and greatly increase gene expression and protein synthesis of type I collagen, which is most abundant in the extracellular matrix. This effect was found to be associated with cell signaling through the p38, Akt, and JNK pathways[7,11]. We investigated the effect of PLLA on the subcutaneous adipose layer and the influence of UV rays on damaged adipocytes as a follow-up study. When 3T3L-1 cells were treated with PLLA, an increase in adipogenesis was observed, indicating that PLLA injected intradermally influences not only fibroblasts but also adipocytes, and increases adipogenesis. Following UVB irradiation, adipogenesis was reduced in adipocytes, and the expressions of types IV and VI collagen transcripts and proteins also significantly decreased, indicating that the function of adipocytes was impaired by UV radiation. When adipocytes damaged by UVB irradiation were treated with PLLA, adipogenesis and the expressions of types IV and VI collagen transcripts and proteins increased, suggesting that PLLA may provide the beneficial effect on adipocytes in terms of adipogenesis and collagen expression in subcutaneous adipose tissues.

When the preadipocytes differentiate into mature adipocytes, the alteration in the expression of the extracellular matrix plays an essential role[12,13]. A portion of collagen that comprises the extracellular matrix exhibits changes in synthesis in addition to adipocyte differentiation, and a specific type of collagen shows increased expression in this stage of adipogenesis[14,15].

According to Liu et al.[16], types I~VI collagens play a role in the differentiation and metabolism of adipocytes, of which types IV and VI collagens are very crucial. The substantial expression of type IV collagen in adipocyte membranes suggests that it is deeply involved in the stability of adipocytes. Type VI collagen stimulates the expression of SREBP-1c, C/EBPα, C/EBPβ, and PPARγ, all of which regulate adipogenesis and promote adipocyte differentiation. The promotion of type VI collagen synthesis by PLLA might be partially responsible for inducing adipogenesis[16].

In the present study, we determined that both adipocyte differentiation and adipogenesis were enhanced by PLLA, and the expressions of types IV and VI collagens were also increased at the transcript and protein levels. Based on the results, we confirmed that PLLA alleviates UV-induced damage on adipocytes, and the increased expressions of types IV and VI collagens are involved in this mechanism.

A limitation of this study is that it only provides in vitro results regarding the effect of PLLA on adipogenesis and collagen synthesis in adipocytes. Since these biological processes are complicated and multifactorial, several questions remain to be resolved through in vitro and, more importantly, in vivo experiments. Moreover, in this study, UVB was employed as a skin aging factor to assess the impact of PLLA on adipocytes, as we believed that UVB's high energy could effectively reveal changes in adipocytes. However, considering that UVB is unable to affect subcutaneous fat due to its limited penetration depth, the utilization of UVB in this study has certain limitations. Nevertheless, this study provides a proof-of-concept regarding PLLA's direct biological effects on adipocytes. In addition, it is thought that long-term studies investigating the influence of PLLA on adipocyte differentiation and adipose synthesis and maintenance will lay the groundwork for developing novel photoaging treatments in the future.

When adipocytes differentiate, adipose is produced and accumulates within the cell. The present study confirms that UVB irradiation reduces the adipogenesis as well as the expressions of types IV and VI collagens, which increases with differentiation. Injection of PLLA into adipocytes recovered the lost adipogenesis, indicating that PLLA prevents the inhibition of adipose and collagen syntheses triggered by UV radiation. Therefore, we suggest that PLLA may expert the beneficial effect on adipocytes from the aspect of adipogenesis and collagen expression in the subcutaneous adipose tissues.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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