ADIPOCYTE
2022, VOL. 11, NO. 1, 643–652
https://doi.org/10.1080/21623945.2022.2149121

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Plasminogen-derived peptide promotes adipogenic differentiation of preadipocytes *in vitro* and *in vivo*

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ABSTRACT

Soft tissue defects caused by adipose tissue loss can result in various conditions such as lipodystrophy in congenital diseases, trauma secondary to ageing, and mastectomy in breast cancer; fat grafting is commonly performed to restore these defects. Although various enrichment strategies have been studied, novel therapeutics that are cost-effective, safe, technologically easy to manufacture, and minimally invasive are required. In this study, we identified a novel peptide derived from plasminogen, named plasminogen-derived peptide-1 (PLP-1), which showed adipogenic differentiation potential and led to an increase in the expression levels of adiponectin, C1Q and collagen domain containing protein, fatty acid-binding protein 4, and CCAAT/enhancer-binding protein-alpha. *In vivo* experiments confirmed an increase in the rate of adipocyte differentiation and the expression levels of CD31 in the PLP-1-treated mice groups. These results suggested that PLP-1 plays an important role in promoting the differentiation of preadipocytes and may be useful for developing therapeutic approaches to treat adipose tissue defects.

ARTICLE HISTORY
Received 6 March 2022
Revised 25 October 2022
Accepted 15 November 2022

KEYWORDS
Adipose-derived stem cell (ADSC); breast cancer; differentiation; peptide; plasminogen (PLG)

Introduction

Adipose tissue is central to the regulation of whole-body energy homoeostasis, energy storage, and hormone and cytokine secretion, thereby acting as an endocrine organ [1]. Brown adipose tissue is involved in thermoregulation via lipid and glucose oxidation-mediated heat generation [2,3]. In addition to its role as an endocrine organ and thermoregulator, adipose tissue (especially subcutaneous fat present under the skin) fundamentally provides structural support to the whole body. Loss of adipose tissue can result in soft tissue defects, including lipodystrophy in congenital diseases, trauma secondary to ageing, and mastectomy in breast cancer [4]. Correction of these defects remains a challenge. Autologous fat grafting is widely applied to improve soft tissue defects and is more permanent than injectable soft tissue fillers that provide only temporary relief [5,6]. However, the retention rate of grafted fat shows great variability, resulting in unpredictable clinical outcomes [7]. Various approaches, including co-transplantation of stromal vascular fraction (SVF), platelet-rich plasma, and adipose-derived stem cells (ADSCs), have been used to stimulate angiogenesis in grafts and differentiation of preadipocytes [8–10]. Although these procedures can improve the graft retention rate, their application is limited by their exorbitant cost, need for liposuction, specialized ultracentrifugation steps, and long time required to achieve results. In clinical situations, such a delay in achieving results and steep prices are major limitations in the application of these treatment approaches. Therefore, various therapeutics such as peptides that regulate cell differentiation have been studied, as these are promising candidates for molecular medicine and cell therapy [11,12].

Most cellular therapeutic approaches use stem cells. In addition to the therapeutic efficacy of stem cells, the ability of these cells to differentiate into various cells and the paracrine effects of cytokines and growth factors secreted from transplanted stem cells are also being studied, as these are important and effective modes of action. Transplanted stem cells migrate to a damaged area in response to the signalling system activated by the damage (such as inflammation or ischaemia) and secrete growth factors that regulate local and systemic inflammatory

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/21623945.2022.2149121

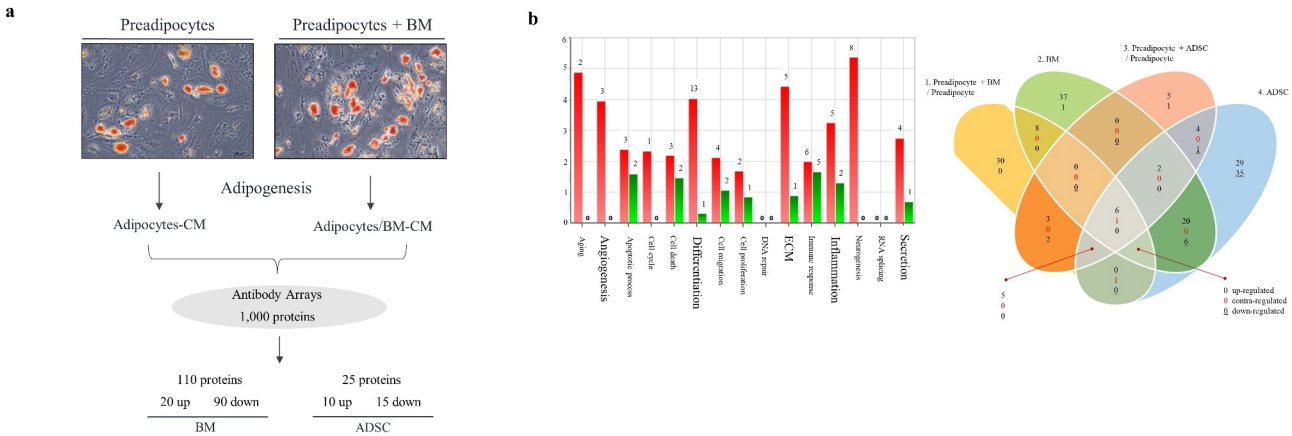
responses and regenerate tissues in the damaged area [13]. Therefore, in this study, we focused on the factors whose levels show an increase during adipocyte differentiation. Peptides that regulate cellular differentiation are promising candidates for molecular medicine and cell therapies. Since plasminogen-derived peptide (PLP) is smaller than 500 Da in size, it is easily absorbed into the skin and is expected to easily penetrate cells because it is a short peptide [11,13–15].

Plasminogen is an inactive precursor that is converted into its active form, plasmin, by the plasminogen activator (PA). The PA system plays a major role in extracellular proteolytic activities such as fibrinolysis. It is known to contribute to extracellular matrix (ECM) degradation and tissue remodelling. In addition, studies have shown that the regulation of urokinase plasminogen activator receptor (uPAR) activity affects adipogenesis [16]. In this study, we wanted to confirm that plasminogen induces adipocyte differentiation. We also identified a novel peptide derived from plasminogen. The overall aim of conducting the present study was to develop potential therapeutic strategies for the treatment of adipose tissue defects.

Results

Selection of proteins for inducing adipocyte differentiation

Proteins secreted into the medium during cell culture were analysed. Proteins secreted into the medium during the differentiation of preadipocytes into adipocytes were analysed using a protein array (Label-free quantification (LFQ) method; Figure 1). Among them, the protein increased by 1.5 times or more in the bone marrow-derived stem cell (BM) + preadipocyte co-culture compared to the preadipocyte single-culture was selected. In total, 31 proteins were identified, and the main categories of pathways in which these proteins were involved were ECM organization, angiogenesis, negative regulation of smooth muscle cell–matrix adhesion, protein folding, and axon regeneration in the peripheral nervous system. Notably, the involvement of these proteins in ECM organization is a significant result, as it plays a major role in tissue engraftment following filler injection into a patient. Finally, 10 candidate proteins (plasminogen (PLG), fibronectin, serpin A1, haptoglobin, activin RIIA, activin A, ghrelin, galectin-3, differential screening-selected gene aberrative in neuroblastoma (DAN) and decorin were selected based on their



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	Proteins
Extracellular Matrix	TGFb1 , FN1, NTN4, SERPINA1, DCN, LGALS3, MMP11
Immune response	CHGA, IL34, IRF6, TNFRSF13C, CD40LG, TNFRSF6B, CCL26, LGALS3, CXCL6,IL19CXCL2
Inflammation	FN1, IL34, TNFRSF6B, CXCL6, CXCL2
ECM&Immune& Inflammation	SERPINA1, ICAM1, KNG1
Secretion	FN1, GHRL, CHGA, HP, PLG, INHBA, LGALS3
Angiogenesis	TGFB1, FN1, ANG-1
Differentiation	DGFB1, FN1, GHRL, IRF6, TFF1, INHBA, ADIPOQ, ANGPT1, CHRDL1, DKK1, FGF20, FST, LGALS3

Figure 1. Identification of proteins secretedinto the medium during the differentiation of preadipocytes into adipocytesusing protein array analysis. (a) Confirmation of changes in expression of 1000proteins through protein array for proteins secreted into the medium duringculture. A doubling increases of 100 proteins in BM co-culture and 25 proteinsin ADSC co-culture compared to monoculture. (b, c) Functional disease analysisbased on GO (GO; Gene ontology) of proteins that increase more than twice whenco-cultured with BM compared to single culture.

predicted involvement in the adipose-related categories and a review of previously published reports.

Subsequently, the preadipocytes were cultured in the differentiation medium, the selected proteins were added to the medium to examine the degree of adipocyte differentiation. Analysis using the Oil Red O staining revealed that the maximum increase in extent of adipogenesis occurred when the cells were treated with 50 ng/mL of plasminogen

Cytotoxicity assay and increased gene expression levels of adipogenic markers

We performed a cell counting kit-8 (CCK-8) assay to confirm that the selected proteins were not cytotoxic. Cytotoxicity was evaluated by treating the cells with different dilutions of the plasminogen (50, 100, and 200 ng/mL). We found no significant differences in cell viability on days 3, 6, and 9 (Figure 2(a)). When the cells were treated with 50 ng/mL plasminogen, the gene expression levels of the adipogenic markers Peroxisome Proliferator-Activated Receptor gamma (PPARγ), adiponectin, C1Q, and collagen domain containing (ADIPOQ), fatty acid-binding protein 4 (FABP4) and CCAAT/enhancer-binding protein-alpha (CEBPα) significantly increased, as observed by the quantitative polymerase chain reaction (qPCR) experiment (**P < 0.01, ***P < 0.001, Figure 2(c)). To check the expression, change of adipogenic markers (*FABP4, PPARγ and CEBPα*) at the protein level, Preadipocytes were treated with plasminogen at a concentration of 50 ng for 14 days. As a result of protein level confirmation, it was confirmed that *FABP4* significantly increased compared to the plasminogen untreated group (***P < 0.001, Figure 2(d)).

Plasminogen-derived peptides (PLPs) promote adipocyte differentiation

Analysis using a panel of 14–20 amino acid-long peptides covering all the domains of plasminogen revealed that amino acid positions 20–97 and 581–810 were the activation peptide (helix; 46–55) and the plasmin light chain B (helix; 796–809) sites, respectively.

We determined the complete plasminogen sequences and selected candidate peptides with respect to their active sites; four peptides (PLP-1, PLP-2, PLP-3, and PLP-4) were selected as candidates (Table 1). Oil red O staining revealed a significant increase (**P < 0.01,

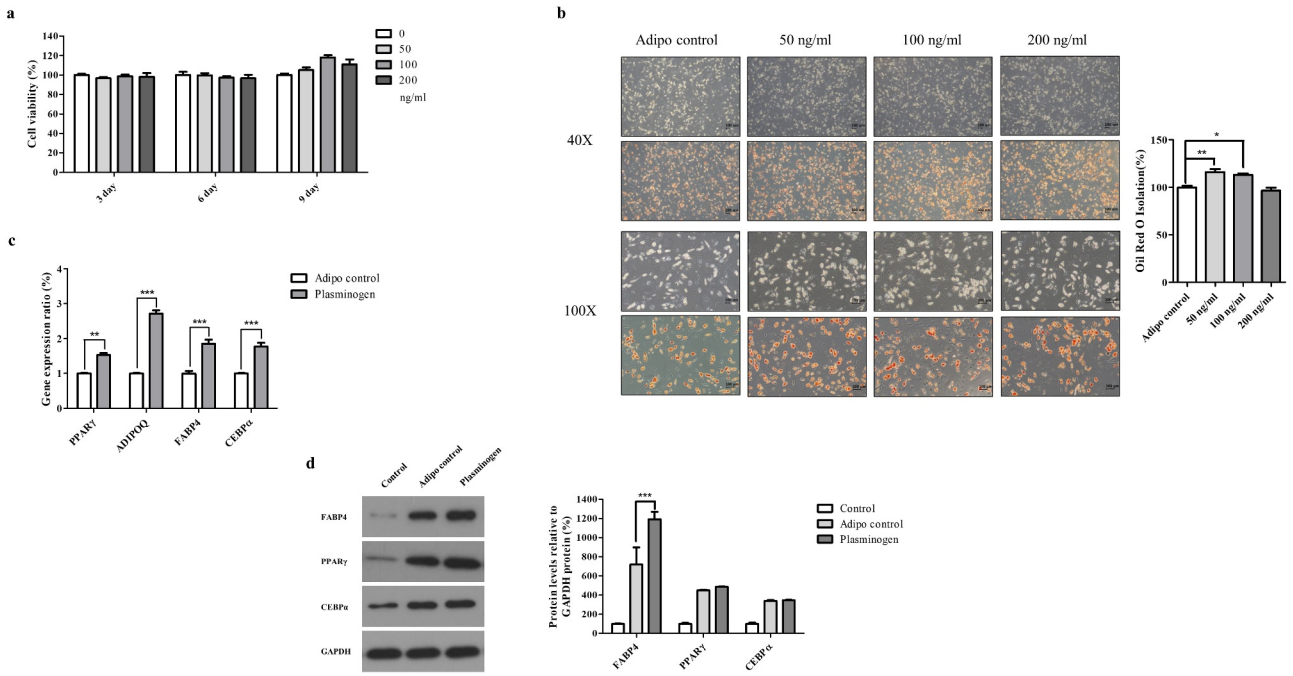

Figure 2. Confirmation of toxicity and adipocytedifferentiation ability after treatment with plasmonogen protein inpredipocytes. (a) Confirmation of cytotoxicity in preadipocytes 3, 6 and 9 daysafter plasminogen protein treatment (n=3for experiment, CCK8). (b) The degree of adipocytedifferentiation confirmed after treatment of preadipocytes with plasminogenprotein for 21 days (n=4 for experiment, Oil RedO). (c) Confirmation of adipogenesis-specific gene expression in cells treatedwith plasminogen at a concentration of 50 ng/ml for 14 days (n=3for experiment, Real time PCR). (d) Confirmation ofadipogenesis-specific protein expression in cells treated with plasminogen at aconcentration of 50 ng/ml for 14 days (n=3for experiment, Western blot). Data are shown as the means ±SD(*P<0.05, **P<0.01, ***P<0.001)

Table 1. Four types of sequences predicted to promote adipocyte differentiation at the activation site of the plasminogen protein sequence were selected.

Name	Sequence	Mer.
PLP-1	LGAGSIEECA AKCEE	15
PLP-2	IEECA AKCEEDEEFT	15
PLP-3	LGAGSIEECA AKCEEDEEFT	20
PLP-4	VSRFV TWIEGVMRN	14

P < 0.001) in adipocyte differentiation in all the peptide-treated groups compared to that in the control groups at week 2, whereas at week 3, only the PLP-1-treated groups showed a significant increase (P < 0.001) in adipocyte differentiation (Figure 3(a)). The mRNA expression level of *PPARγ, ADIPOQ, FABP4*, and *CEBPA* was increased in the PLP-1 treatment group compared to the adipo control. The levels of increase were 210, 269 and 164%, respectively (Figure 3(b)). The protein expression level of FABP4, PPARγ and CEBPA was significantly increased in the PLP-1 treatment group compared to the adipo control (*P < 0.05, ***P < 0.001, Figure 3(c)).

PLP-1 promotes adipocyte differentiation in vitro

Among all the peptides, only PLP-1 and PLP-3 showed the potential to induce differentiation in preadipocytes *in vitro*. Therefore, we verified if PLP-1 and PLP-3 could promote adipogenesis *in vivo* using the fat grafting technique in mice. The grafted fat volume was significantly higher in the PLP-1-treated groups than that in the control groups (fat-only and fat + SVF groups) from days 7 to 28 (*P,0.05, **P < 0.01, ***P < 0.001, Figure 4(a)). The weight of fat obtained by biopsy at week 4 was higher in the PLP-1-treated groups (5 μg/kg of low concentration (L); 112%, 50 μg/kg of high concentration (H); 114%) than that in the control groups, but this increase was

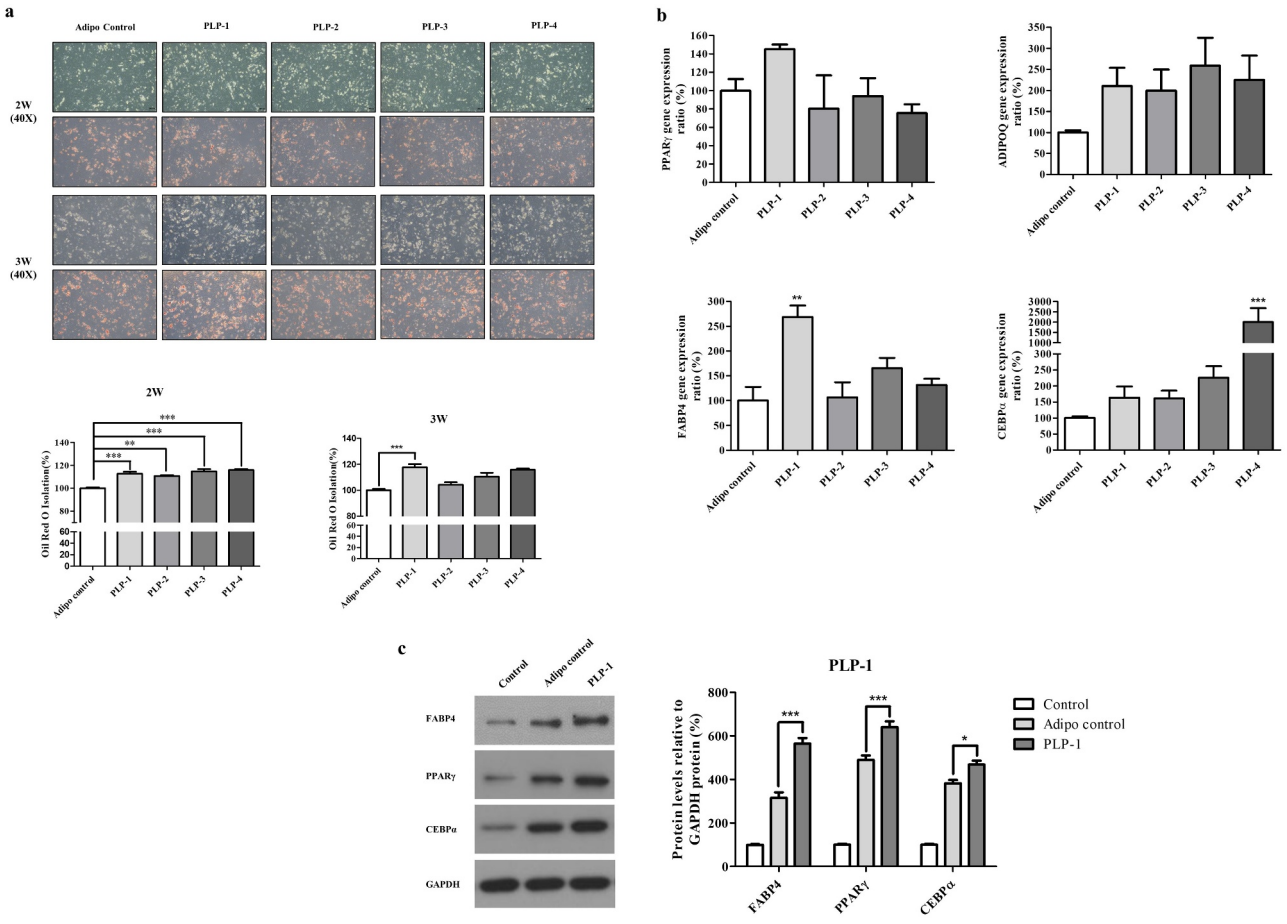

Figure 3. Confirmation of adipocytedifferentiation capacity of the selected four plasminogen peptides (PLP). (a)After 2 and 3 weeks of PLP treatment on preadipocytes, adipocytedifferentiation ability was confirmed by lipid droplet stain (n=3 forexperiment, Oil Red O). (b) After 2 weeks of PLP treatment on preadipocytes,RNA was extracted to confirm the expression of adipocytedifferentiation-specific genes (n=4 for experiment, Real time PCR). (c)Confirmation of adipocyte differentiation-specific protein expression after 2weeks of PLP treatment on preadipocytes (n=3 for experiment, Western blot). Dataare shown as the means ± SD (*P<0.05, **P<0.01, ***P<0.001).

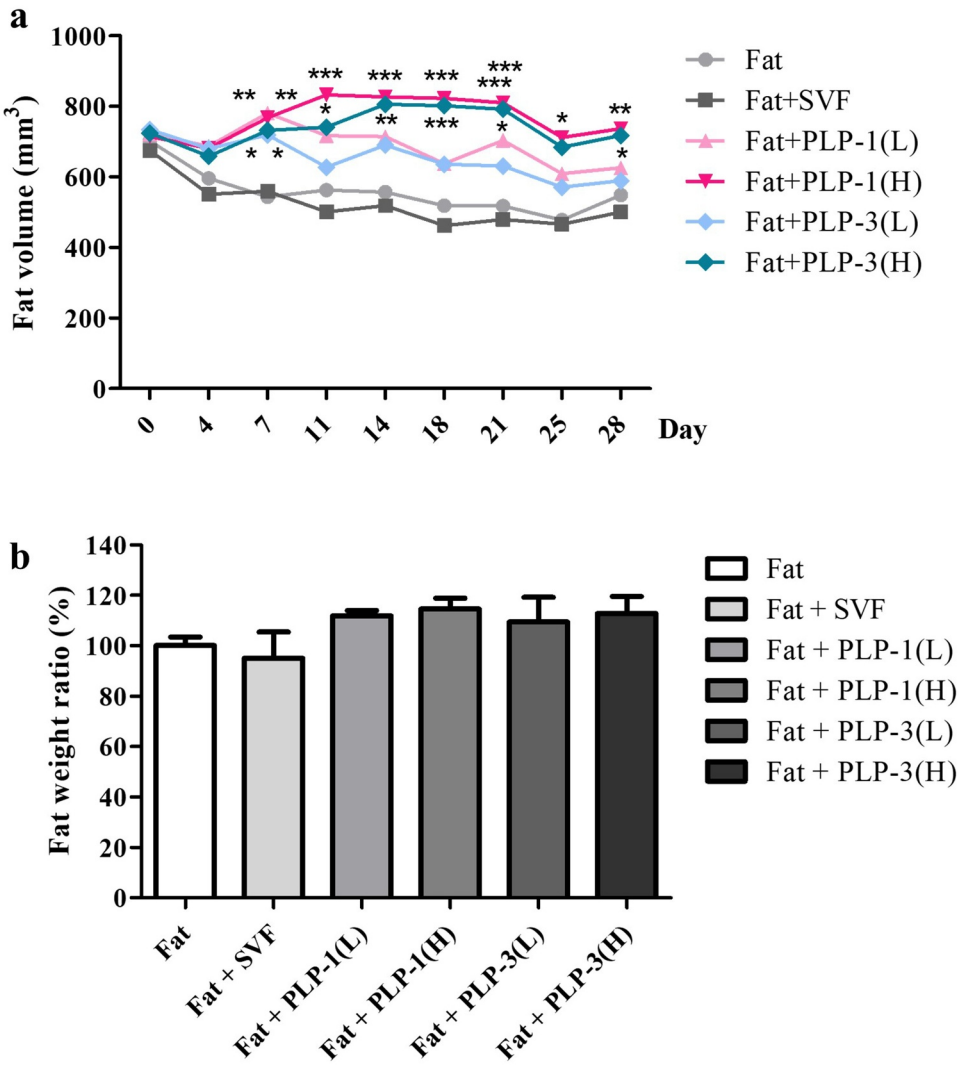

Figure 4. After injecting human-derived fat, fat+SVF, and fat+peptide in a total volume of 400ul, theexternal size and fat weight were confirmed. (a) Measurement of external volumefor 4 weeks, twice a week, immediately after fat injection (n=4~8 for experiment). (b) Measurement of the weight offat collected by biopsy 4 weeks after fat injection (n=4~5 for experiment).Dataare shown as the means ± SD(*P<0.05, **P<0.01,***P<0.001).

not significant (Figure 4(b)). Oil red O staining showed that the rate of differentiation was elevated in the PLP-1-treated groups (Figure 5(a)). Immunohistochemical analysis revealed that cluster of differentiation 31 (CD31), an angiogenic factor, was expressed in the PLP-1-treated groups, but not in the control and the PLP-3-treated groups (Figure 5(b)). Tumour necrosis factor-α (TNF-α), an inflammatory factor, was not expressed in any of the groups (Figure 5(c)).

Discussion

Soft tissue defects caused by the loss of adipose tissue result in various conditions, congenital diseases, and ageing, which represent considerable challenges in clinical practice. The fat grafting technique has been widely used to treat such defects. And several studies have demonstrated its positive effects [16]. For example, fat grafting is commonly performed to restore soft tissue defects in cases of breast reconstruction after mastectomy [14,15]. However, the absorption rates of grafted fat are highly variable, leading to the low level of efficacy of this method (20–80%) [7]. Thus, various enrichment strategies for fat grafting have been studied, most of which are related to growth factors and ADSCs [17]. Platelet-rich plasma has also been shown to improve the clinical outcomes of fat grafting [18]. However, these strategies involve complex and expensive procedures. Many studies have focused on increasing the survival rates of grafted fat tissues; however, these methods remain unsatisfactory. Research on stem cell differentiation via peptide regulation has become increasingly important for the development of

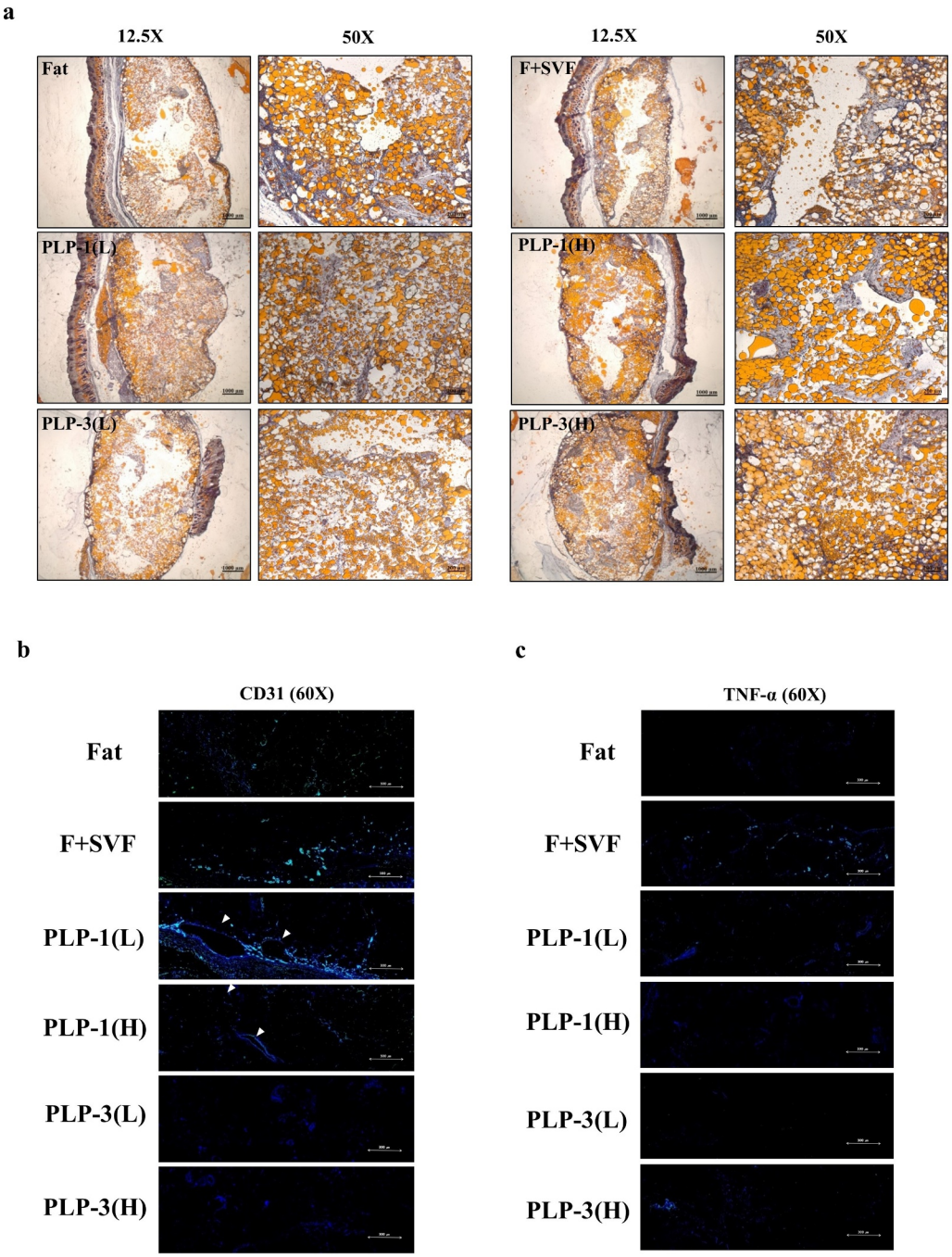

Figure 5. After fat injection into mice,biopsy was performed at 4 weeks and the injected fat was colleced and lipiddroplet and tissue immunological tests were performed. Representative image.(a) Lipid droplet staining to confirm the formation of lipid droplet (Oil RedO). (b) Identification of the angiogenic factor CD31 (green), which is animportant factor in the engraftment rate of injected fat. (c) Identification ofTNF-a (green) as an immune response factor in injected adipose tissue. Nucleicacid; blue stain.

molecular medicine and cell therapeutic approaches. Short peptides (tetrapeptides) are signalling molecules that can interact with DNA and histone proteins [19,20]. Many studies have demonstrated the effect of short peptides on the proliferation and differentiation of stem cells [21,22]. This suggests that it may affect the proliferation and differentiation of adipocytes. Short peptides, which consist of no more than 20 amino acids, show great potential as cost-effective therapeutics [11,13,20]. Numerous peptides were shown to regulate biological processes and have the potential to treat various diseases [23,24]. Incretin mimetics are peptide drugs that mimic the action of glucagon-like peptide-1 and have been approved for the treatment of type 2 diabetes mellitus owing to their antihyperglycemic effects [25,26]. Administration of pNaKtide, a peptide designed to inhibit

Na^+/K^+-ATPase amplification, hindered gain in body weight and improved insulin sensitivity in mice [27]. A novel peptide derived from the L-lactate dehydrogenase A chain was shown to prevent the adipogenic differentiation of preadipocytes [28]. Thus, these compounds may be useful for the treatment of obesity and diabetes.

In this study, we examined a novel PLP that promoted adipogenic differentiation. The plasminogen system plays a role in the process of primary fibrinolysis, which is responsible for dissolution of clots after thrombosis. Although some studies suggest that plasminogen regulates stem cell mobilization, the role of the plasminogen system in adipose tissue differentiation has not been widely reported [29,30]. However, some papers have shown that the degree of plasminogen binding is significantly increased during differentiation of 3T3-L1 cells [30]. We found that plasminogen levels increased during preadipocyte differentiation, and that plasminogen itself can induce adipogenic differentiation. These findings suggested that PLPs (plasminogen-derived peptide) may promote adipogenic differentiation. Plasminogens are single-chain glycoproteins that are composed of five triple-loop kringle domains and a serine protease domain. On the basis of the active sites of plasminogen, four peptides were selected as candidates, out of which PLP-1 showed adipogenic differentiation potential, both *in vitro* and *in vivo*.

The conversion of preadipocytes into mature adipocyte cells requires a confluence of various molecular events and signalling pathways. For example, peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer binding proteins, and sterol regulatory element-binding transcription factor 1 are early-acting factors that induce the expression of adipocyte-specific genes [30,31]. In our study, treatment using PLP-1 significantly increased the transcript levels of ADIPOQ, PPARγ, and FABP4. FABP4 is known to promote lipid storage in adipocytes. FABP4, also known as aP2, is highly expressed in adipocytes and is regulated by PPARγ agonists and insulin [32]. Therefore, an increase in FABP4 expression level suggests an increased fat weight. And the elevation in PPAR-γ expression level was likely related to the increased rate of adipocyte differentiation.

We observed a significant increase in adipogenesis and adipogenic differentiation potential after treatment of the cells with PLP-1 both in vitro and in vivo. In conclusion, we identified a novel peptide, PLP-1, with adipogenic potential that may be useful in the development of therapeutic strategies to treat adipose tissue defects. But further studies are required to elucidate the underlying mechanisms. may be useful in the development of therapeutic strategies to treat adipose tissue defects. Clinical use of PLP-1 may be possible after conducting studies for validation of its safety.

Materials and methods

Cell culture and adipogenic differentiation in vitro

Human bone marrow mesenchymal stem cells, ADSCs, and preadipocytes (catalogue numbers PT-2501, PT-5006, and PT-5020; Lonza) were cultured in Dulbecco's modified Eagle medium (DMEM; catalogue number 11,995–065; Gibco BRL). The cells were supplemented with 10% heat-inactivated foetal bovine serum (FBS; catalogue number 16,000–044; Gibco) and 1% penicillin streptomycin (P/S; catalogue number 15,140–122; Gibco) in DMEM. The cells were incubated at 37°C in 5% CO_2.

To validate the proteins' and peptides' ability to induce cell differentiation, preadipocytes were seeded in 12-well plates at a density of 1×10^5 cells/well. After cell attachment, the samples were incubated in the adipocyte differentiation medium to induce preadipocyte differentiation. The StemPro Adipogenesis Differentiation Kit (catalogue number A1007001; Gibco) was used to induce adipocyte differentiation.

Co-culture model

For the co-culture experiments, the preadipocytes were seeded in 12-well plates at a density of 1×10^5 cells/well, and BM cells and ADSCs were seeded on transwell inserts (1 μm pore size; catalogue number 353,010; Falcon) at a density of 1×10^5 cells/well. The cells were allowed to adhere overnight. To confirm their adipogenic differentiation capacity, preadipocytes seeded in the 12-well plate were cultured in the adipogenic differentiation medium. The cells (BMs and ADSCs) seeded in the transwell plates were cultured in differentiation or growth medium, depending on the conditions. The cell culture period was either 14 or 21 days.

Oil red O staining and triglyceride assays

The differentiated cells were washed with phosphate-buffered saline (PBS; catalogue number 10,010–023; Gibco) and fixed with 4% paraformaldehyde for 40 min. Subsequently, the fixed cells were washed with 60% isopropanol. The samples were stained with 1% oil red O dye (catalogue number O0625; Sigma-Aldrich) for 0.5 h and washed three times with PBS. Images were captured using an Olympus microscope (CKX53, Olympus). For quantification, the retained dye was solubilized in isopropanol and its absorbance was measured at 500 nm using a microplate reader (EPOCH2; BioTek).

Table 2. Primer for real-time PCR to identify factors related to adipocyte differentiation.

Target	Forward(5′-3′)	Revers (5′-3′)
GAPDH	ACA GTT GCC ATG TAG ACC	TTT TTG GTT GAG CAC AGG
PPARγ	GGT TGA CAC AGA GAT GCC ATT CTG	GAG TTG GAA GGC TCT TCA TGA GGC
ADIPOQ	GGA GAT CCA GGT CTT ATT GGT CC	GCA CCT TCT CCA GGT TCT CC
FABP4	CAT CAG TGT GAA TGG GGA TG	GTG GAA GTG ACG CCT TTC AT
CEBPa	TCG GTG GAC AAG AAC AGC AA	TTG TCA CTG GTC AGC TCC AG

RNA isolation and quantitative polymerase chain reaction (qPCR)

RNA was isolated from the cells using TRIzol reagent (catalogue number 15,596,026; Invitrogen). The RNA concentration was assessed using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific). RNA (1 μg) was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (K1622; Thermo Fisher Scientific).

qPCR was performed using the Power SYBR Green Master Mix (4,367,659; Applied Biosystems). The mixture contained 1× SYBR-Green Master Mix and 500 nM primers. Template DNA (15 ng) was then added to the mixture.

The reactions were performed using an Applied Biosystems ViiA7 qPCR System. The fold-change in target gene expression was calculated used the $2^{-\Delta\Delta Ct}$ method. Expression levels of the control housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used to normalize the data obtained. The primer sequences are listed in Table 2.

Protein array analysis

Protein array analysis was performed by culturing preadipocytes alone or co-culturing preadipocytes and mesenchymal stem cells in the medium. Preadipocytes were allowed to differentiate into adipocytes by culturing them in the adipogenesis differentiation medium; factors whose concentration increased in the co-cultured medium compared to that in the single-cultured medium were analysed. Analysis of the samples was outsourced to Ebiogen Inc. The experiments were performed in accordance with the manufacturer's instructions.

The purified samples were analysed using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology). The purity of the sample was confirmed using UV spectroscopy. Antibody array analysis was performed using an antibody array (RayBiotech). The slides were scanned using a GenePix 4100A scanner (Axon Instrument). After obtaining the scanned images, they were gridded and quantified using GenePix Software (Axon Instrument). The analysed proteins were annotated using UniProt database (DB). Data mining and graphic visualization were performed using ExDEGA (Ebiogen Inc.). The experiments were performed according to the manufacturer's (Ebiogen Inc.) instructions.

Cell viability analysis

The ability of the protein to promote cell proliferation was confirmed using the CCK-8 assay (Dojindo). For the CCK-8 analysis, preadipocytes (seeded in 24-well plates) were treated with the protein whose ability to stimulate cell proliferation was being examined; subsequently, the extent of cell proliferation was assessed on days 3, 6, and 9 using the CCK-8 kit. The absorbance of each well was measured at 450 nm wavelength using a microplate reader (EPOCH2; BioTek).

Peptide synthesis

The peptides were synthesized by Peptron Co. Each peptide was synthesized using a standard solid-phase peptide synthesis protocol. At each step, the resin was washed twice with dimethylformamide (DMF) and methanol. When the desired sequence was completed, the crude peptide was cleaved from the resin. The solution was precipitated using cold ether and air dried. The crude peptide was dissolved in distilled water (DW)and purified by reverse-phase high-performance liquid chromatography (HPLC, Shimadzu Prominence HPLC) using a C18 column (250 mm × 22 mm i.d., 10 μm particle size). Elution was carried out using a water-acetonitrile linear gradient (10–75% [v/v] acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. The pure peptide was collected and lyophilized using a freeze-dryer (FDT-12012; Operon). The peptide's purity (95%) and sequence were confirmed by liquid chromatography-mass spectrometry (LC-MS, Shimadzu LC-MS-2020 series). The experimental methods were provided by Peptron Co.

Fat sampling

Human adipose tissue was obtained from three patients who underwent mastectomy. All the patients were women, with ages ranging from 28 to 54 years. Informed consent was obtained from all patients who underwent mastectomy before fat harvesting, and the experimental protocol was approved by the Institutional Review Board of Seoul National University Bundang Hospital (permit number B-2004-608-301).

The collected human adipose tissue was washed with PBS containing 1% penicillin-streptomycin. The washed fat was cut into small pieces and fitted into an 18-gauge needle. A mixture of the cut fat (0.3 mL) and the test sample (0.1 mL) was placed in a 1-ml Luer-Lock syringe and injected into mice using an 18-gauge needle.

Preparation of stromal vascular fraction (SVF)

Human adipose tissue was washed with PBS to remove blood cells. After the washed tissue was chopped with scissors, it was incubated with 0.1% collagenase solution (C0130; Sigma-Aldrich) at 37°C for 1–2 h to completely dissolve the tissue. The incubated cells were centrifuged at 400 × *g* for 3 min, and half of the supernatant was removed except for the pellet. The remaining supernatant containing the pellet was filtered using a cell strainer (352,360; Falcon) and transferred to a new tube. The collagenase was inactivated by adding an equal amount of 10% FBS (in DMEM containing 1% P/S). The treated cells were centrifuged at 400 × *g* for 30 min and the supernatant was removed; the pellets was resuspended in 10 ml of DMEM and the cells were counted. SVF was prepared for treatment with PBS (3×10^5 cells/20 μL PBS).

Experimental design of the fat graft

Eight-week-old male nude mice (Orient Bio) weighing approximately 25 g were used for the experiment to prevent the rejection of human fat tissue grafts. The control and the test groups were placed on the backs of the same mouse to minimize variance between the experimental results. The isolated SVF and the plasminogen peptides (PLP-1 and PLP-3) were added to the adipose tissue. Then, 20 μL of SVF and peptides were added to 80 μL of PBS. One hundred microlitres of the test sample (SVF and peptide mixture) were mixed with 300 μL of human fat tissue. After gentle mixing, the fat with the test sample was transferred into 1-mL Luer-Lock syringes for placement of the graft. Each mixture (total volume 0.4 mL) was injected using an 18-gauge needle. Six mice were sacrificed four weeks after transplantation, and the grafted tissues were harvested.

Macroscopic analysis of grafted fat tissue

We evaluated the volume of these grafts to analyse the results of fat grafting. For the evaluation, the external size (3/4π[height × width × length]/2) of the injected fat was measured weekly before conducting the biopsy. After the biopsy, the weight of the retrieved fat was measured and compared between the groups.

Histological analysis of skin

Adipose tissues were fixed in 10% formaldehyde. Owing to the characteristics of adipose tissues, the freezing process was performed in a cryostat at −40°C. Inside the cryostat, the sections were cut to a thickness of 12 μm and air-dried on slides. The samples were fixed in 10% buffered formalin and briefly washed with tap water. The frozen sections were oil red O- (O0625; Sigma-Aldrich) or immunohistochemistry (IHC) stained (CD31, ab24590; TNF-α, ab1793; Abcam) to assess their fat content. Images were obtained using a slide scanner (Pannoramic 250 Flash III, 3DHISTECH).

Statistical analyses

The differences between the results obtained for the analysed groups were determined by Student's t-test and one-way analysis using GraphPad Prism (GraphPad software). The results were analysed using the Kruskal-Wallis test. The statistical significance was set at $P < 0.05$. All the values were expressed as mean ± standard deviation (SD).

Declarations

Ethical approval

Human subcutaneous preadipocytes and mesenchymal stem cells were purchased from Lonza (PT-5020, lot number 0000409278 and PT-2501, lot number 0000636886, respectively). Animal studies were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (IACUC approval no. BA-2005-296-047-04). Informed consent was obtained from all the patients who participated in this research.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Korean government (MSIT) [No. 2017M3A9E9073680].

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