A Potato Peel Extract Stimulates Type I Collagen Synthesis via Akt and ERK Signaling in Normal Human Dermal Fibroblasts

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The ability of dermal fibroblasts to synthesize collagen decreases with ages. The integrity of collagen fibers severely decreases in aged skin, causing its characteristic morphological changes such as wrinkles and sagging. To prevent and improve skin aging, the stimulation of collagen synthesis in dermal fibroblasts is important. Potato peels contain many biofunctional compounds, but not much is known about their effects on human skin physiology. To characterize the potential effects of a potato peel extract (PPE) against skin aging, we examined its effects on the synthesis of type I collagen by normal human dermal fibroblasts (NHDFs). Treatment with the PPE significantly increased the expression of type I collagen mRNA in NHDFs and their secretion of type I collagen. To elucidate the mechanism involved, we examined the signaling pathway controlled by transforming growth factor-β (TGF-β), which regulates the synthesis of type I collagen. Treatment of NHDFs with the PPE significantly increased the expression of TGF-β receptor mRNA. TGF-β signaling involves Smad-dependent and Smad-independent pathways, like phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK). The PPE did not activate Smad, but significantly activated Akt and ERK. These results demonstrate that the PPE activates PI3K/Akt and MAPK/ERK signals via TGF-β receptors, which stimulate the synthesis of type I collagen in NHDFs. These results suggest that the PPE could be a novel and effective antiaging material.

Key words potato; collagen; dermal fibroblast; transforming growth factor-β; Akt; extracellular signal-regulated kinase (ERK)

INTRODUCTION

The dermis, which is located beneath the epidermis, is deeply involved in regulating the firmness and elasticity of the skin. The dermis is constructed of structural elements, including collagen fibers, elastin fibers and proteoglycans, that are produced by dermal fibroblasts. Among those, collagen fibers, which provide the structural skeleton of the dermis, play a key role in maintaining the mechanical strength and flexibility of the skin.1,2 In aged skin, a drastic depletion of collagen fibers at papillary regions has been recognized as a typical characteristic that results in wrinkling and sagging.1,4 The appearance of wrinkles and sagging strongly influences the QOL, because of their impact on visible appearance. Therefore, suppressing and/or improving wrinkling and sagging by stimulating collagen synthesis is an important approach to maintaining a high QOL. Collagen fibers in the dermis are primarily composed of types I and III collagen. Because type I collagen represents more than 90% of total collagen,5 it is an important target to prevent and improve skin wrinkling and sagging.

The synthesis of type I collagen has been reported to be controlled by two main growth factors, insulin-like growth factor-1 (IGF-1)6 and transforming growth factor-β (TGF-β),7,8 IGF-1 binds to the IGF-1 receptor, a transmembrane tyrosine kinase, and transmits signals by modulating the activity of its adaptor molecules (Insulin receptor substrate-1, Src homology 2 domain containing). The downstream signaling of this cascade is known to involve the phosphatidylinositol-3 kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway.9,10 On the other hand, TGF-β binds to a heterocomplex formed by two types of receptors, TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII) in order to transmit intracellular signals. TGF-β signal transmission is known to involve Smad-dependent and Smad-independent pathways.11 In the Smad-dependent pathway, TGF-β causes the phosphorylation of receptor-activated Smad (R-Smad). Phosphorylated R-Smad forms a complex with common mediator Smad (Co-Smad) after which it translocates into the nucleus, where it functions as a transcription factor that can upregulate the transcription of genes involved in type I collagen synthesis. On the other hand, the Smad-independent pathway involves stimulation by TGF-β causing the induction of signaling molecules like PI3K/Akt, MAPK and Rho-like guanosine 5′-triphosphatase (GTPase).12–13 These factors control the synthesis of type I collagen through a pathway distinct from the Smad-dependent pathway. However, IGF-1 and TGF-β are important growth factors in both pathways that control the synthesis of type I collagen in fibroblasts.

The potato (Solanum tuberosum L.) is among the five most significant food crops of the world. The edible portion of the plant, the tuber, contains many nutritional ingredients, including ascorbic acid,14 folic acid15 and potassium.16 Although the peel of each potato is removed during processing, the interest of reducing industrial byproducts like the potato peel to aim for zero emissions is currently becoming a global fashion. Thus, we considered how to effectively use a potato peel since some functional ingredients, including phenolic compounds such as chlorogenic acid, and flavonoids such as catechin, have been identified in potato peels.17–19 However,
there have been relatively few reports on the functionality of those ingredients on the skin. For instance, previous research studies have reported that ascorbic acid21–23 and some phenolic compounds and flavonoids24 influence the synthesis of type I collagen in the dermis. Ascorbic acid is well known to stimulate collagen synthesis by contributing to the formation of its triple helix structure by promoting the hydroxylation of proline and lysine in procollagen.20 On the other hand, phenolic compounds and flavonoids have been reported to suppress the synthesis of matrix metalloproteinases (MMPs) through their antioxidative activities.22–24 Those reports suggest that a PPE also might be capable of preventing and/or improving skin aging through effects on collagen.

Thus, the purpose of this study was to investigate the potential of a potato peel on the synthesis of type I collagen using normal human dermal fibroblasts (NHDFs) expecting to achieve zero emission and to propose an effective use of this byproduct.

MATERIALS AND METHODS

Materials NHDFs were obtained from Kurabo (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). Trypsin–ethylenediaminetetraacetic acid (EDTA) was purchased from Gibco™ Invitrogen Corporation (Carlsbad, CA, U.S.A.). Human type I collagen was obtained from Kyowa Pharma Chemical (Toyama, Japan). Anti-collagen I was obtained from Cosmo Bio (Tokyo, Japan). Anti-β-actin (Tyr1135) (DA7A8) Rabbit mAb, Smad3 (C67H9) Rabbit mAb, Phospho-Akt (C67E7) Rabbit mAb, Phospho-Smad3 (C25A9) Rabbit mAb, Akt (pan) (C67E7) Rabbit mAb, Phospho-Akt (Ser473) (D9E) XP Rabbit mAb, p44/p42 MAPK (Erk1/2) (137F5) Rabbit mAb, Phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb, β-Anti Antibody, Anti-rabbit immunoglobulin G (IgG) and HRP-linked Antibody, U0126 were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). GAPDH (GAPDH) was measured as an endogenous control.

Western Blotting NHDFs were cultured in DMEM containing 0.5% FBS with or without the PPE at various concentrations for 24h. Afterwards, cells were lysed with lysis buffer (50 mM Tris–HCl, 1 mM EDTA-2Na, 1% protease inhibitor cocktail, 1% PhosSTOP, 0.1% TritonX-100 and 0.1% sodium dodecyl sulfate (SDS)) to extract proteins. The proteins were separated using 8–16% Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad), after blocking with 2% BSA in Tris-buffered saline (TBS-T) at room temperature, the membranes were incubated with the primary antibody at 4°C for 24h and then were further incubated with the secondary antibody for 1h. Antibodies bound to the membrane were visualized with an ABTS coloring solution, and absorbances were measured at 405 nm using a microplate reader (Spark 10M, Tecan Japan, Kanagawa, Japan). Levels of TGF-β1 and IGF-1 were measured using commercial ELISA kits as follows; TGF-β1 (Quantikine, 2nd Generation (96 well), R&D Systems, Minneapolis, MN, U.S.A.), IGF-1 (Quantikine (96 well), R&D Systems, Minneapolis, MN, U.S.A.). Amounts of each protein, type I collagen, TGF-β1 and IGF-1, are expressed as values normalized against the protein content of cells determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Quantitative PCR Analysis (qPCR) NHDFs were cultured in DMEM containing 0.5% FBS with or without the PPE at various concentrations for 24h. Total RNA was extracted using a SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo, Osaka, Japan) and cDNA was synthesized. qPCR was accomplished using THUNDERBIRD SYBR qPCR mix (Toyobo). A Fast Real-Time PCR System (7900HT, Applied Biosystems, Waltham, MA, U.S.A.) was used to detect qPCR values, and relative quantifications were carried out using the ΔΔCt method. The various mRNAs (COL1A1, COL1A2, TGF-βRII, TGF-βRI, TPH2 and their primers (Prime Time qPCR primers, Integrated DNA Technologies, Coralville, IA, U.S.A.) are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an endogenous control.

Preparation of the Potato Peel Extract (PPE) Potato (Solanum tuberosum L. c.v. Kitahime) peels were collected and dried at 60°C, after which they were soaked in 50% ethanol for 1 week. After filtration, the solvent was evaporated using a dried at 60°C, after which they were soaked in 50% ethanol and were incubated at 4°C for 24h until used.

Preparation of the Potato Peel Extract (PPE) NHDFs were cultured in DMEM containing 0.5% FBS with or without the PPE at various concentrations for 24h. Afterwards, the culture supernatants were collected and amounts of type I collagen, TGF-β1 and IGF-1 were quantified using ELISAs. The ELISA for type I collagen was conducted as follows: the culture supernatants were coated in 96-well plates and were incubated at 4°C for 24h. After blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)(−) at room temperature, the plates were incubated with the primary antibody at 4°C for 24h and then were further incubated with the secondary antibody for 1h. Antibodies bound to type I collagen on the plate were visualized with an ABTS coloring solution, and absorbances were measured at 405 nm using a microplate reader (Spark 10M, Tecan Japan, Kanagawa, Japan). Levels of TGF-β1 and IGF-1 were measured using commercial ELISA kits as follows; TGF-β1 (Quantikine, 2nd Generation (96 well), R&D Systems, Minneapolis, MN, U.S.A.), IGF-1 (Quantikine (96 well), R&D Systems, Minneapolis, MN, U.S.A.). Amounts of each protein, type I collagen, TGF-β1 and IGF-1, are expressed as values normalized against the protein content of cells determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

| Gene   | Primer sequence                                      |
|--------|------------------------------------------------------|
| COL1A1 | sense: 5'-GACATGGTTCAGGTGTTGGAC-3'                  |
|        | antisense: 5'-TTCTGTTACGAGGAGTGGTGAAGGTTG-3'        |
| COL1A2 | sense: 5'-GTTAGAGAGGGATGGTTGGAC-3'                  |
|        | antisense: 5'-CCTTACAATCCATGACGACCAT-3'             |
| T/R1   | sense: 5'-CCCTGTTTCCATTTCCAAC-3'                    |
|        | antisense: 5'-GCCAGCTCTAAGCTGCGAAT-3'               |
| T/R2   | sense: 5'-TGAACAGAGAACAAAACTCCCATGAC-3'             |
|        | antisense: 5'-CTGCTTCTAGTGTATGTTCTCGA-3'            |
| GAPDH  | sense: 5'-AGGTTGTTGGAGCCTCAT-3'                     |
|        | antisense: 5'-TGAGTGTGGCAGGGACT-3'                  |

Table 1. Target Genes and Primer Sequences Used for qPCR
Japan) and levels were measured using a Gel Doc Ez imager (Bio-Rad). β-Actin was measured as an endogenous control.

Statistical Analysis Measured values are expressed as means ± standard error of the mean (S.E.M.), and Student’s t-test was used to identify statistically significant differences. A p-value <0.05 is considered the threshold for significance.

RESULTS

Effects of the PPE on the Synthesis of Type I Collagen in NHDFs The effects of the PPE on the synthesis of type I collagen were examined. NHDFs treated with the PPE for 24h showed a significant upregulation of mRNA expression levels of COL1A1 and COL1A2 compared to the untreated control (Fig. 1A). Furthermore, treatment of NHDFs with the PPE increased the amount of type I collagen protein in a PPE concentration-dependent manner (Fig. 1B).

Involvement of IGF-1 Signaling and TGF-β Signaling on the PPE-Induced Synthesis of Type I Collagen in NHDFs IGF-1 is one of the primary growth factors that induces the synthesis of type I collagen. Thus, in order to investigate the mechanism involved in the induction of type I collagen synthesis by the PPE, we examined the contribution of IGF-1 signaling. First, we examined whether the PPE stimulates IGF-1 synthesis. After treatment of the PPE 24h, the amount of IGF-1 in culture supernatants was measured. NHDFs treated with the PPE 5μg/mL slightly increased the IGF-1 production, whereas treatment of NHDFs with the PPE 10, 15μg/mL did not have increased in a PPE concentration-dependent manner (Fig. 2A). Then, we examined whether the PPE causes the phosphorylation of IGF-1 receptor (IGF-1R), but increase in the phosphorylation of IGF-1R was not detected (Fig. 2B). From these results, it was thought to be unlikely that the IGF-1 signaling pathway is involved in the PPE-induced synthesis of type I collagen.

Thus, we examined other possible mechanisms that might be involved in the effects of the PPE on NHDFs. It has been reported that TGF-β is also a stimulatory factor for type I collagen synthesis. Thus, we examined the possible contribution of TGF-β signaling on the PPE-induced synthesis of type I collagen. First, we examined whether the PPE stimulates TGF-β synthesis. NHDFs treated with the PPE for 24h did not have increased levels of TGF-β (Fig. 3A). We then examined whether the PPE increases levels of the TGF-β receptor subunits (TβRI and/or TβRII) from the aspect of their mRNA expression levels. Treatment with the PPE significantly upregulated the mRNA expression levels of TβRI and TβRII (Fig. 3B). This result indicated that the PPE stimulated the synthesis of type I collagen through TGF-β signaling due to the upregulation of the TGF-β receptor.

PPE-Induced TGF-β Signaling in NHDFs Smad-dependent and Smad-independent pathways are involved in regulating TGF-β signaling. Thus, in order to identify the...
The precise mechanism involved, we examined whether the PPE causes the phosphorylation of Smad3, but no increase in the phosphorylation of Smad3 was detected (Fig. 4). That result indicated that the PPE does not function through Smad signaling in collagen synthesis. Previous studies reported that TGF-β promotes the production of the extracellular matrix in the dermis through PI3K/Akt and MAPK/ERK signaling. Thus, we examined the possible involvement of PI3K/Akt and MAPK/ERK signaling in the PPE-induced synthesis of type I collagen. First, we examined whether the PPE causes the phosphorylation of Akt and ERK1/2, the results showed that the PPE significantly increased the phosphorylation of Akt and ERK1/2 (Figs. 5A, B) in NHDFs. Furthermore, we assessed whether LY294002, a specific inhibitor of PI3K that phosphorylates Akt, or U0126, a specific inhibitor of MEK1 and MEK2 that phosphorylates ERK1/2, inhibits PPE-induced type I collagen synthesis. LY294002- or U0126-treated NHDFs significantly inhibited PPE-stimulated increase of type I collagen (Fig. 5C). From these results, we concluded that the PPE promotes type I collagen synthesis by activating PI3K/Akt and MAPK/ERK signaling.

**DISCUSSION**

Collagen produced in dermal fibroblasts forms fibers in the dermis, which provides the mechanical strength of the skin. In aged skin, which is characterized by wrinkles and sagging and the loss of viscoelasticity, a drastic loss of collagen fibers in the papillary dermis is observed as a typical histological alteration. Thus, it is currently accepted that the loss of collagen fibers in the dermis is one cause of wrinkling and sagging of the skin. The loss of collagen fibers in aged skin is considered to be due, at least in part, to an imbalance of collagen synthesis and degradation by MMP-1. Thus, the promotion of collagen synthesis in dermal fibroblasts is considered as an effective approach to prevent and/or improve the symptoms of skin aging. In this study, we examined the effects of a PPE on collagen synthesis as a candidate anti-aging material, because potato peels contain phenolic compounds and flavonoids and also several other compounds that have been reported to enhance collagen synthesis in fibroblasts.

In fact, the results show that the PPE stimulates type I collagen synthesis in NHDFs. Type I collagen synthesis has been demonstrated to be regulated by various growth factors, cytokines and chemokines. IGF-1 and TGF-β have been reported as predominant factors that promote collagen synthesis. Treatment of NHDFs with the PPE did not affect the expression of TGF-β but showed a significant upregulation of Tj/RI and Tj/RII mRNA levels (Fig. 3). The involvement of the TGF-β pathways on collagen synthesis have been
reported to involve the Smad-dependent pathway and the Smad-independent pathway by the activation of PI3K/Akt and MAPK/ERK.\textsuperscript{11,13} To demonstrate the precise mechanism, the involvement of TGF-β signaling on the PPE-induced synthesis of type I collagen was examined. The Smad pathway is well-known to involve TGF-β signaling in the stimulation of collagen synthesis.\textsuperscript{2,7} However, because the PPE failed to stimulate the phosphorylation of Smad (Fig. 4), the contribution of the Smad pathway was ruled out regarding the stimulation of collagen synthesis.

Thus, we shifted to investigating another mechanism, the Smad-independent pathway. It has been reported that TGF-β also promotes collagen synthesis by activating PI3K/Akt signaling.\textsuperscript{13} Knock-down of Akt in dermal fibroblasts decreased collagen synthesis and increased MMP-1.\textsuperscript{25} Although MAPK/ERK signaling is also controlled by TGF-β, the response to TGF-β is interestingly different depending on the cell type, epithelial cells and mesenchymal cells.\textsuperscript{8,11,13} TGF-β activates ERK signaling in dermal fibroblasts, which are mesenchymal cells, but suppresses it in keratinocytes, which are

Fig. 3. Effects of the PPE on TGF-β Signaling in NHDFs

NHDFs were incubated with or without various concentrations of the PPE (0–15 µg/mL) for 24 h. (A) The secretion of TGF-β was analyzed by ELISA. (B) mRNA expression levels of TβRI and TβRII were analyzed by qPCR, and were normalized to the expression of GAPDH. Values represent means ± S.E.M. of three independent experiments. *p < 0.05, **p < 0.01.

Fig. 4. Effects of the PPE on the Phosphorylation of Smad3

Serum-starved NHDFs were treated with or without the PPE for 1 h. Cell lysates were analyzed by Western blotting. The densities of Smad3 and phospho-Smad3 were quantified, and expression levels were normalized to β-actin. Values represent means ± S.E.M. of three independent experiments.
This difference in responses is understood by differences in the expression levels of TGF-β receptors. TGF-β receptors are composed of TβRI and TβRII. Whereas the expression of TβRI does not depend on the cell type, TβRII is expressed at a higher level in dermal fibroblasts compared with keratinocytes. On the other hand, the TGF-β-mediated activation of ERK signaling promoted collagen synthesis in human mesangial cells and human osteoblasts. It has also been reported that a velvet antler extract, which is known to activate ERK, promotes type I collagen synthesis in human dermal fibroblasts. In our study, treatment with the PPE increased expression levels of TβRII mRNA (Fig. 3B). PPE-treated NHDFs showed increased phosphorylation of Akt and ERK1/2 (Fig. 5), which indicated that the PPE induced stimulation of collagen synthesis occurred through the activation of PI3K/Akt and MAPK/ERK. PI3K/Akt and MAPK/ERK
signaling is in the Smad-independent pathway, but there have been few reports on the stimulation of collagen synthesis via these pathways.\(^{3,33}\) Given this, we believe that the PPE is an incredibly useful substance.

It is known that PI3K/Akt and MAPK/ERK signaling is not only involved in regulating collagen synthesis but also affects all aspects of skin aging.\(^{32}\) Decreases of MAPPK/ERK signaling are observed in the skin of elderly humans and such declines are particularly evident in photoaged skin.\(^{33,34}\) Furthermore, it has been reported that blocking PI3K/Akt signaling occurs in the senescent phenotype in skin-derived precursor cells. These facts suggest that PI3K/Akt signaling plays a critical role to prevent the aging process.\(^{35}\)

Gathering these facts, we conclude that the PPE, which stimulates collagen synthesis in NHDFs through the activation of PI3K/Akt and MAPK/ERK signaling, will be a novel and effective antiaging material.

**Conflict of Interest**  Mari Suto, Hirofumi Masutomi and Katsuyuki Ishihara are employees of Calbee, Inc. Hitoshi Masaki has no conflict of interest.

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