Influence of Human Placenta Extracts on Prostanoids Production in Cultured Hair Follicle-Derived Keratinocytes: The Possibility of Pharmaceutical Regenerative Medicine

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(Received for Publication: March 19, 2020)

Abstract

It is known that human placenta extract (HPE), which contains numerous bioactive substances, can improve symptoms of hair loss. However, the mechanisms underlying this effect are unclear. It is reported that androgenetic alopecia (AGA) is induced and/or suppressed by arachidonic acid metabolites that affect hair follicle component cells. We investigated the effect of HPE on arachidonic acid metabolism using cultured hair follicle-derived keratinocytes. PG-E2, PG-F2α, PG-I2 (6-keto-PG-F1α), PG-D2 and TX-A2 (TX-B2) were measured in medium cultured with HPE. The cultured cells were used for the expression analysis of these five prostanoid synthase genes. The cultured keratinocytes produced all five types of prostanoids. However, the synthase genes of PG-D2 and PG-I2, i.e., PTGDS and PTGIS, did not express in cultured keratinocytes. In contrast, PG-E2 and PG-F2α synthase genes, i.e., PTGES and AKR1C3, could be detected. HPE enhanced the production of PG-E2 and PG-F2α, whereas that of PG-D2 was decreased. Minoxidil had no effect on arachidonic acid metabolism. HPE significantly increased the PG-E2/PG-D2 and PG-F2α/PGD2 ratios, whereas minoxidil showed no significant effect. HPE enhanced the production of PG-E2 and PG-F2α and suppressed that of PG-D2. The effects of HPE on hair growth could be mediated by its effects on arachidonic acid metabolism.

Key words

Hair growth, androgenic alopecia, PGD2, PGE2, PGF2α, hair follicle keratinocytes

Introduction

The male hormone dihydrotestosterone (DHT) causes hair follicle atrophy and hair loss that is characteristic of men⁹. This condition is called androgenetic alopecia (AGA) and occurs in young and middle-aged adults¹⁰. Because male hormones affect AGA, this condition mainly occurs in young men⁹. However, female pattern hair loss (FPHL) derived from male hormones can be observed in postmenopausal women⁸.

The systemic administration of the drugs finasteride and dutasteride, which inhibit DHT synthesis, have been used for AGA therapy⁶,⁸. In addition, minoxidil, which promotes hair growth by inhibiting ion channels, has been used in topical administration as an AGA treatment⁷. However, a stable effect has not been observed with these drugs, and the mechanism of AGA remains unclear. In addition, these drugs are restricted from use for FPHL patients or are not available. Therefore, hair loss therapy for women is difficult, and new therapeutic drugs are required.

PG-D2 and PG-E2, metabolites of arachidonic acid, were reported to be increased and decreased, respectively, in the skin and skin appendages of AGA patients⁸. PG-D2 is reported to promotes hair loss,
whereas PG-E2 suppresses hair loss\(^9\). Furthermore, among the arachidonic acid metabolites, PG-F2a can ameliorate alopecia of the eyelashes. Arachidonic acid metabolites and growth factors are physiologically active substances produced in somatic cells and are not only constantly produced but also induced in response to stimulation.

In recent years, platelet-rich plasma (PRP) has been used as new treatment for AGA. Takikawa et al. reported that PRP topical treatment on the scalp skin by subcutaneous injection enhanced hair shaft thickness\(^9\). PRP is also known to promote growth factor production and regulate the expression of signal proteins associated with the hair cycle in cultured dermal papilla cells\(^9\).

Meanwhile, Seo et al. reported that FGF7 in mouse skin was induced by human placenta extract (HPE)\(^11\), which has been suggested to contain various bioactive substances that affect hair growth\(^12\). As the hair cycle is regulated by various bioactive factors, the mechanism of hair growth cannot be explained by the function of FGF7 alone, and there is no single treatment for alopecia. It has been reported that arachidonic acid metabolites may affect AGA. In other words, arachidonic acid metabolites produced by keratinocytes might affect the hair follicle.

From these investigations, the drugs that regulate arachidonic acid metabolism, might be used for hair loss therapy as pharmaceutical regenerative medicine. Especially, these drugs might be able to be used for FPHL patients. These bioactive substances can stimulate the cascade of arachidonic acid metabolism in somatic cells. Therefore, HPE might affect various hair cells, including keratinocytes that can easily produce several types of prostaglandins upon chemical or mechanical stimulation.

In the present study, we investigated the effect of HPE on arachidonic acid metabolism in hair follicle-derived keratinocytes.

**Materials and methods**

**Culture of human follicle-derived keratinocytes**

After obtaining informed consent, excess hair follicles from the scalp were collected microscopically during head surgery of three male patients (17–67 years old and without malignant diseases). After enzymatic digestion of the hair follicles, as described by Kobayashi et al.\(^13\), epidermal basal cells (referred to as keratinocytes) were cultured as described by Boyce and Ham\(^14\). Briefly, the enzyme-digested specimens were treated with 600 U/mL dispase for 60 min at 37°C and then with 0.25% trypsin for 16 h at 4°C. Then, the samples were centrifuged for 5 min at 200 \(\times\) g and 4°C, and the obtained cells were cultured in EpiLife (Kurabo Co., Kurashiki, Japan) in 5% CO\(_2\) at 37°C. The medium was changed twice a week until confluence, after which the cultured keratinocytes were stored in liquid nitrogen according to standard methods.

This study was approved by the Clinical Study Group of the Ethics Committee of St. Marianna University School of Medicine (No. 1548).

**Measurement of prostanoids in keratinocytes**

The keratinocytes were seeded at 2500 cells/cm\(^2\) onto 6-well plates and cultured in 5% CO\(_2\) at 37°C until confluence. Following this, new culture medium was added to the cells with or without the drug HPE (final concentrations of 10–500 \(\mu\)g/mL) and then cultured for 2 to 24 h. Minoxidil (final concentrations of 1–100 \(\mu\)g/mL) was used as a drug for comparison. The concentrations of HPE and minoxidil that did not cause cell toxicity were used as the maximum doses based on the evaluation of cell toxicity of HPE and minoxidil with an LDH assay using NIH-3T3 cells in a previous study\(^15\).

After the indicated time points, 10\(^5\) M indomethacin was added to stop the production of arachidonic acid derivatives, and then the culture medium of each well was collected. Total RNA was extracted from the cells using ISOGEN (Nippon Gene Co., Toyama, JAPAN). Prostanoids in the culture supernatants (PG-E\(_2\), PG-F\(_2a\), PG-D\(_2\) and PG-I\(_2\) [stable metabolite: 6keto-PG-F\(_1a\)], and TX-A\(_2\) [stable metabolite: TX-B\(_2\)]) were measured using an EIA kit (Cayman Chem Co., Ann Arbor, MI, USA).

**Expression of arachidonic acid metabolite synthase genes in keratinocytes**

cDNA was synthesized from the obtained RNA using an iScript cDNA Synthesis kit (#1708891; BioRad Lab. Co., Shinagawa, Japan). PTGES (PG-E\(_2\) synthase gene), PTGFS (AKR1C3: PG-F\(_2a\) synthase gene), TBXAS1 (TX-A\(_2\) synthase gene), PTGIS (PG-I\(_2\) synthase gene), PTGDS (PG-D\(_2\) synthase gene) and COX2 (PG-H\(_2\) synthase gene) were analyzed by real-time quantitative PCR using a Rotor-Gene SYBR Green PCR Kit and Rotor-Gene Q (QIAGEN, Hilden, Germany). The primer sequences used for Rotor-Gene Q system are as follows. PTGIS (Cat. No. QT00047747), PTGDS (Cat. No. QT01006901), PTGES Cat. No. QT00209607), AKR1C3 (Cat. No.
Hair growth and placenta extracts

Results

First, production of the five prostanoids and expression of the synthase genes were investigated qualitatively. As a result, TX-B2, a stable metabolite of TX-A2, and 6keto-PG-F1a, a stable metabolite of PG-I2, were not detected in the cultured keratinocytes. However, the synthase genes PG-E2, PG-F2a and PG-H2 (COX-2) could be detected. Based on these results, the authors selected the detectable prostanoids and their synthetic genes for further experiments.

Effects of HPE on gene expression in cultured keratinocytes

The effects of minoxidil and HPE on the gene expression levels of the prostanoid synthases were evaluated, with the results shown in Figures 1–3. The expression of COX2, which encodes for a rate-limiting enzyme in the production of various prostanoids, was not affected by HPE. However, minoxidil significantly inhibited COX2 expression after 2 h of treatment.

The addition of HPE increased PTGES gene expression at 6 h after treatment in a dose-dependent manner, but minoxidil had no effect on the expression of this gene. HPE significantly increased AKR1C3 gene expression in a dose-dependent manner after 6 h.

Fig. 1  Influence of human placenta extract (HPE) and minoxidil on gene expression of PTGHS (COX2)
HPE did not influence COX2 gene expression, but minoxidil significantly decreased the expression of COX2 gene at 2 h after treatment. Data are shown as mean ± SD; p < 0.0125 compared with the control.

Statistical analysis

All experiments were carried out with specimens obtained from 3 patients (N=3). The data are shown as means ± standard deviation (SD), and comparisons with the control group (without drug) were analyzed using the Student paired t-test. In the case of prostanoid production, the primary endpoints were PG-D2 and PG-E2, and the secondary endpoint was PG-F2a. For statistical analysis, the major analysis was performed a total of 6 times (2 groups and 3 dosages) with respect to the control group. In the case of prostanoid synthase gene expression, the major analysis was performed 4 times (2 groups and 2 dosages) with respect to the control group. The multiplicity of the analysis was compensated for by Bonferroni’s method. In other words, although the p-value was statistically significant at p < 0.05, this statistical significance was compensated to p < 0.0083 (prostanoids product) and p < 0.0125 (gene expression) by Bonferroni’s method. JMP statistical analysis software (SAS Institute Japan Co. Tokyo, Japan) was used for statistical analysis.

QT00013692), PTGHS2 ([COX2] Cat. No. QT00040586), TBXAS1 (Cat. No. QT00090307) and β-actin (ACTB Cat. No. QT00095431) were obtained from Qiagen (Hilden, Germany). Gene expression was quantified using the comparative ΔΔCt method.
Fig. 2  Influence of human placenta extract (HPE) and minoxidil on PTGES gene expression in cultured keratinocytes
PTGES gene expression was not changed at 2 h by HPE, although a slight insignificant increase was observed at 6 h. Minoxidil did not affect the expression of PTGES. Data are shown as mean ± SD.

Fig. 3  Influence of human placenta extract (HPE) and minoxidil on AKR1C3 gene expression in cultured keratinocytes
AKR1C3 gene expression was not changed at 2 h by HPE, although it significantly increased at 6 h. Minoxidil did not affect the expression of AKR1C3. Data are shown as mean ± SD; p < 0.0125 compared with the control.

As the spontaneous expression levels of the PTGDS, PTGIS and TBXAS genes were extremely weak, quantitative measurement of these genes could not be performed.

Effects of minoxidil and HPE on prostanoid production
HPE significantly increased, and minoxidil suppressed, PG-E2 production (significantly at 100 μg/mL) in cultured keratinocytes in a dose-dependent manner (Figure 4). In contrast, PG-D2 production
was suppressed by both HPE (not significant) and minoxidil (significantly at 100 μg/mL) (Figure 5). PG-F2a production was significantly increased in a dose-dependent manner upon HPE treatment, but it was significantly suppressed by minoxidil in the same manner at concentrations of 10 μg/mL and

![Graph showing PG-E2 production](image1)

**Fig. 4** Influence of human placenta extract (HPE) and minoxidil on PG-E2 synthesis in cultured keratinocytes
PG-E2 production increased significantly in a dose-dependent manner upon application of HPE, but not of minoxidil. Data are shown as mean ± SD; p < 0.0083 compared with control.

![Graph showing PG-D2 production](image2)

**Fig. 5** Influence of human placenta extract (HPE) and minoxidil on PG-D2 synthesis in cultured keratinocytes
PG-D2 production in keratinocytes decreased upon application of HPE and of minoxidil. Data are shown as mean ± SD; p < 0.0083 compared with the control.
100 μg/mL (Figure 6).

**PGE2/PGD2 and PGF2α/PGD2 ratios**

HPE treatment at 100 μg/mL and 500 μg/mL significantly increased the PG-E2/PG-D2 production ratio of the cultured keratinocytes (Table 1). The PGF-2α/PG-D2 ratio also increased upon HPE treatment, albeit not significantly. Neither the PG-E2/PG-
D2 nor the PG-F2a/PG-D2 ratio was significantly changed by the addition of minoxidil.

Discussion

HPE has long been used as a drug to improve liver function. In some countries, its effects on fatigue recovery and the promotion of wound healing are also known. However, neither the pharmacological mechanism of its function nor the effective components of the product are understood. Furthermore, HPE has been reported to affect the proliferation of pigment cells. In particular, Pal et al. detected components that reacted with ET-1 and ACTH antibodies as structural elements of HPE. The HPE used for this investigation was a soluble fraction obtained by enzymatic degradation of the placenta. Furthermore, the soluble fraction was sterilized in an autoclave. Therefore, although almost the entire protein component was denatured, it is considered that low molecular peptides such as ET-1 were intact. In this study, HPE significantly increased the production of PG-E2 and PG-F2a in hair follicle-derived keratinocytes, whereas PG-D2 production was significantly reduced.

Garza et al. suggested that PG-D2 promotes AGA and reported the possibility of treating AGA with PG-D2 receptor antagonists. Nieves and Garza also stated that arachidonic acid metabolites affect the hair cycle and that PG-E2, PG-F2a and their analogs are involved in hair growth. It was reported that HPE promotes hair growth by expressing FGF-7 or via GSK-3β or some hair growth-related genes in hair papilla tissue. However, the effect of HPE on hair follicle arachidonic acid metabolism remains unknown.

Keratinocytes produce large amounts of vascular endothelial growth factor (VEGF) in the presence of PG-E2. Majima et al. reported that PG-E2 promotes angiogenesis through VEGF production. VEGF is involved in the transition from resting to active phase during the hair cycle and prolongs the active phase. Minoxidil is known to directly affect the dermal papilla and promote the production of cytokines such as VEGF via the K+ channel and thereby promote hair growth. Based on these findings, the enhancement of PG-E2 production by HPE might be related to hair growth via VEGF.

It has been reported that PG-D2 production is involved in hair follicle atrophy. In the present study, HPE suppressed PG-D2 production. Garza et al. reported that the PG-E2/PG-D2 ratio in AGA patients decreased significantly compared with that in normal scalp and that the total cutaneous content of PG-E2 decreased in the AGA patients. Thus, the PG-E2/PG-D2 ratio may affect AGA progression, and increasing this ratio may improve hair loss or suppress AGA progression. In this study, the molecular ratios of both PG-E2/PG-D2 and PG-F2a/PG-D2 increased significantly in the cultured keratinocytes by HPE treatment. Based on the molecular ratio of prostanoids associated with hair loss/hair growth, these results suggested that HPE may lead to hair growth.

This regulation of arachidonic acid metabolites by HPE is thought to originate from the expression of PG-E2 and PG-F2a synthase genes. In this study, the PTGES gene tended to increase at 6 h after culture but not significantly so. However, the enzymatic product, PG-E2, did increase significantly. The discrepancy here is that the enzymatic product is a cumulative amount for 24 h, but the gene expression values indicates the condition at measurement time and not the accumulated amount. For these reasons, there was no significant difference in gene expression, and it was considered that the reaction product increased significantly. In the future, PTGES gene expression may need to be measured over time. Expression of the PGD2 synthase gene was extremely weak and could not be quantified. PG-D2 is known to be involved in the differentiation and maturation of skin connective tissue-type mast cells. It has also been suggested that PG-D2 is involved in skin diseases such as atopic dermatitis and the induction of epidermal differentiation. Although it was difficult to quantify the gene encoding PG-D2 synthase, it was possible to measure PG-D2 by EIA. AKR1C3, a PG-F2a synthase, can metabolize PG-D2 to PG-F2a and PG-E2 to PG-F2a. In keratinocytes, the possibility that some of the other prostanoids produced were converted to PG-D2 during metabolic processes cannot be denied.

The behavior of the minoxidil used in this comparative study was completely different from that of HPE. The minoxidil had no effect on the prostanoid synthesis-related genes in keratinocytes. From these results, the expression of COX2, the rate-limiting enzyme involved in the synthesis of stimuli-responsive prostaglandins, was suppressed by minoxidil. However, Kwon et al. reported that the combined treatment of HPE and minoxidil had a synergic effect on hair growth. It was suggested that the hair growth effect of minoxidil is a mechanism that is independent of arachidonic acid metabolism.
The hair cycle is regulated by crosstalk between epithelial stem cells (bulges) and dermal papilla cells. Increased production of epithelial PG-E2 will affect dermal papilla and other hair follicle component cells as a paracrine effect via EP2 receptors, which may affect VEGF production in dermal papilla and thus hair growth. These results of HPE might also affect VEGF production indirectly. As the use of HPE is not limited in women, HPE might be a new target of pharmaceutical regenerative medicine in the therapy of FPHL. Currently, we are investigating the interaction between arachidonic acid metabolites produced from keratinocytes treated with HPE and VEGF production in dermal papilla cells.

Conflicts of interest and funding sources
None of the authors have conflicts of interest to report. This work did not receive any funding.

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