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In Vivo Hair Growth Promotion Effects of Ultra-High Molecular Weight Poly-γ-Glutamic Acid from *Bacillus subtilis* (Chungkookjang)

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We investigated the effect of ultra-high molecular weight poly-γ-glutamic acid (UHMW γ-PGA) on hair loss *in vitro* and *in vivo*. 5-Alpha reductase is an enzyme that metabolizes the male hormone testosterone into dihydrotestosterone. By performing an *in vitro* experiment to analyze the inhibitory effects of UHMW γ-PGA on 5-alpha reductase activity, we determined that UHMW γ-PGA did in fact inhibit 5-alpha reductase activity, indicating the use of UHMW γ-PGA as a potential 5-alpha reductase inhibitor in the treatment of men with androgenetic alopecia. To evaluate the promotion of hair growth *in vivo*, we topically applied UHMW γ-PGA and minoxidil on the shaved dorsal skin of telogenic C57BL/6 mice for 4 weeks. At 4 weeks, the groups treated with UHMW γ-PGA showed hair growth on more than 50% of the shaved skin, whereas the control group showed less hair growth. To investigate the progression of hair follicles in the hair cycle, hematoxylin and eosin staining was performed. Histological observations revealed that the appearance of hair follicles was earlier in the UHMW γ-PGA-treated group than in the control group. The number of hair follicles on the relative area of shaved skin in the UHMW γ-PGA-treated group was higher than that observed on the shaved skin in the control group. These results indicate that UHMW γ-PGA can promote hair growth by effectively inducing the anagen phase in telogenic C57BL/6 mice.

Keywords: Ultra-high molecular weight poly-γ-glutamic acid (UHMW γ-PGA), hair growth, alopecia, 5-alpha reductase

Introduction

Alopecia is defined as hair loss. There are different types of alopecia that can cause hair loss on the scalp or body. Normal hair loss is about 50–100 hairs per day. Every hair follicle has four distinct phases that it cycles through on a regular basis: growth or anagen, transition or catagen, resting or telogen, and returning growth [21]. A full cycle can last anywhere from 2 to 5 years per follicle. Unusual hair loss and thinning occur when a follicle is stuck in the telogen or resting phase [17].

Hair loss can be attributed to multiple factors; these include hormonal deficiencies, diet or nutrient deficiencies, certain diseases, medications or treatments such as chemotherapy, and psychological issues such as stress and depression [16]. Apart from being attributed to external factors such as dandruff and sun exposure, hair loss may also be the result of factors such as consumption of junk food, use of styling tools, extreme weight loss, etc. [5]. Various treatments for hair loss are available; these include home remedies using natural products, pills, hormonal modifications, and even surgical options such as hair transplants [20]. Other treatments include hair restoration, hair extensions, stem cell treatment, platelet-rich plasma

therapy, wefts, and strand-by-strand procedures [7].

Androgenetic alopecia, or patterned alopecia, is the most common form of hair loss in both men and women and is characterized by a progressive loss of hair diameter, length, and pigmentation. The genetic inheritance of androgenetic alopecia is well known, although the causative genes have yet to be elucidated [20]. Minoxidil is a widely used hair growth-promoting drug among patients with androgenetic alopecia. It induces hair follicles in the telogen stage to undergo transition into the anagen stages [6]. However, the drug also causes adverse dermatological effects such as dryness, scaling, local irritation, and dermatitis [4, 19]. Finasteride has been reported to be efficacious for patients with androgenic alopecia; however, it is not recommended for female patients [3]. Therefore, the development of new hair growth-promoting drugs is needed.

Poly-γ-glutamic acid (γ-PGA) is an unusual anionic polypeptide in which D- and/or L-glutamate is polymerized *via* γ-amide linkages; therefore, it is an optically active polymer with a stereogenic center in every glutamate unit [2]. γ-PGA is a biopolymer for which a large range of applications has been suggested. It can be characterized by its molecular weight and the ratio of D- and L-glutamate monomers. The use of natural compounds and a biocatalyst in an aqueous solvent for synthesis of a polymer is clearly an appropriate approach for reducing the corresponding toxic chemicals used and formed during a production process [13]. Some strains of *Bacillus subtilis*, including the starters of *natto*, a traditional Japanese fermented food made from soybeans, and of *chungkookjang*, a traditional Korean fermented seasoning made from soybeans, produce DL-PGA as the main component of the extracellular mucilage. We reported that ultra-high molecular weight γ-PGA (UHMW γ-PGA) is effective as an antitumor agent, an antiviral agent, and a vaccine adjuvant [9, 10, 11, 12]. UHMW γ-PGA will be used for various industrial purposes, for example, in the production of medicines, functional food, cosmetics, etc. Therefore, improvements in UHMW γ-PGA production and applications will be of commercial interest to these industries.

This study was undertaken to investigate the effect of UHMW γ-PGA on hair growth *in vivo*. Pigmented C57BL/6 mice, preselected for their telogen phase of hair growth, were used. In this species, the truncal epidermis lacks melanin-producing melanocytes, and melanin production is coupled to the anagen phase of hair growth; these characteristics make this species highly desirable for trichology studies. UHMW γ-PGA was applied topically on these mice to evaluate telogen to anagen transition.

Materials and Methods

Preparation of UHMW γ-PGA

Bacillus subtilis subsp. Chungkookjang (KCTC 0697BP) (1% culture solution) was inoculated into 3 L of preparative Basic Medium (5% L-glutamic acid: glucose 5%, $(NH_4)_2SO_4$ 1%, KH_2PO_4 0.27%, $Na_2HPO_4 \cdot 12H_2O$ 0.42%, NaCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.3%, and vitamin solution 1 ml/l; pH 6.8), and incubated at 37°C for 72 h. The cells were removed by filtration. The filtered solution containing UHMW γ-PGA was precipitated after addition of 5 N HCl for 12 h. The precipitated UHMW γ-PGA was washed using a Nutsche filter, and the slugged UHMW γ-PGA was freeze-dried to obtain pure UHMW γ-PGA. The molecular mass of the above-mentioned UHMW γ-PGA was 1–15,000 kDa.

The molecular weight of UHMW γ-PGA was measured by gel permeation chromatography, using a $GMPW_{XL}$ column (7.8 mm × 30 cm; Viscotek, USA) and LR125 Laser Refractometer (Viscotek), which had been equilibrated with 0.1 M $NaNO_3$, at 40°C and run at a flow rate of 0.8 ml/min. Polyacrylamide was used as a standard material.

In Vitro 5-Alpha Reductase Activity

The effect of UHMW γ-PGA on 5-alpha reductase activity was assayed based on the method described by Sun and Tu [18, 22]. Briefly, 466 nM NADPH, 21.9 nM 5 alpha-reductase, 360 ng/ml testosterone, 25, 50, and 100 μg/ml UHMW γ-PGA, and 0.1 M Tris-HCl buffer (pH 7.2) were incubated together at 37°C, and the NADPH OD values were continually measured. Finasteride (3.7 μg/ml) was used as a positive control. One unit of enzyme converted 1.0 mmol of NADPH to $NADP^+$ per 1 min at 37°C. The unit specific activity is expressed as mmol/min/mg-protein (units/mg).

Experimental Animals and Studies with UHMW γ-PGA

Six- to nine-week-old female C57BL/6 mice (Central Lab. Animal Inc., Seoul, Korea) were housed in groups of 10 to 12 in stainless-steel wire cages under a controlled environment of 23°C ± 3°C, 55% ± 15% relative humidity, 10–20 changes of fresh filtered air per hour, and a 12/12 h light (268 Lux)/dark cycle. The mice had free access to water and mouse chow. The mice were in the telogen stage of the hair cycle when the experiment began. After a week of acclimation, the mice were randomly divided into three groups (n = 5). UHMW γ-PGA (30 mg/ml dissolved in PBS, 150 μl per mouse), 5% minoxidil (150 μl per mouse), or vehicle (placebo) was applied topically on the dorsal skin of C57BL/6 mice for 4 weeks. Visible hair growth was recorded weekly.

Histological Studies

Hair follicles in the C57BL/6 mouse model were histologically observed by referring to the method described by Adam *et al.* [1]. The dorsal skin was excised after topical application with γ-PGA at 3 weeks and 4 weeks. The dorsal skin was maintained in 4% paraformaldehyde at 4°C and embedded in paraffin blocks to obtain longitudinal and transverse sections. Sections (5 mm thick) were

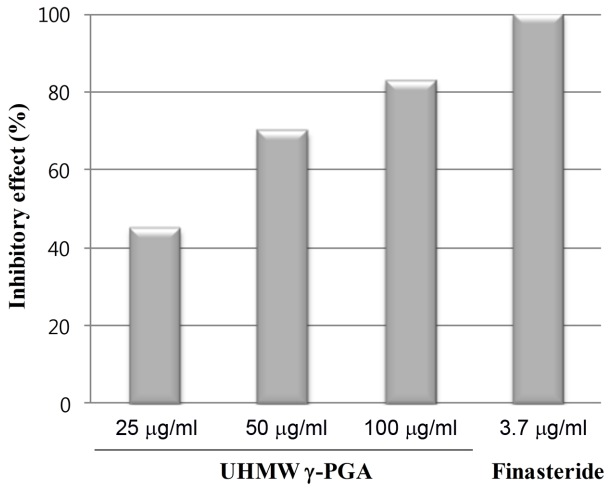

Fig. 1. Inhibitory effect of UHMW γ-PGA on 5-alpha reductase activity *in vitro*.

stained with hematoxylin and eosin. Digital photomicrographs were taken from representative areas at a fixed magnification of 40×.

Results

In Vitro 5-Alpha Reductase Activity

To investigate the mechanism by which UHMW γ-PGA promotes hair growth, the effect of UHMW γ-PGA on 5-alpha reductase activity was evaluated *in vitro*. As shown in Fig. 1, UHMW γ-PGA inhibited the activity of 5-alpha reductase in a dose-dependent manner. In this study, we confirmed that UHMW γ-PGA is an inhibitor of 5-alpha reductase. These results provide a rationale for the use of UHMW γ-PGA as a 5-alpha reductase inhibitor in the treatment of men with androgenetic alopecia.

Effect of UHMW γ-PGA on Hair Growth

Black pigmentation is a sure indication of the transition

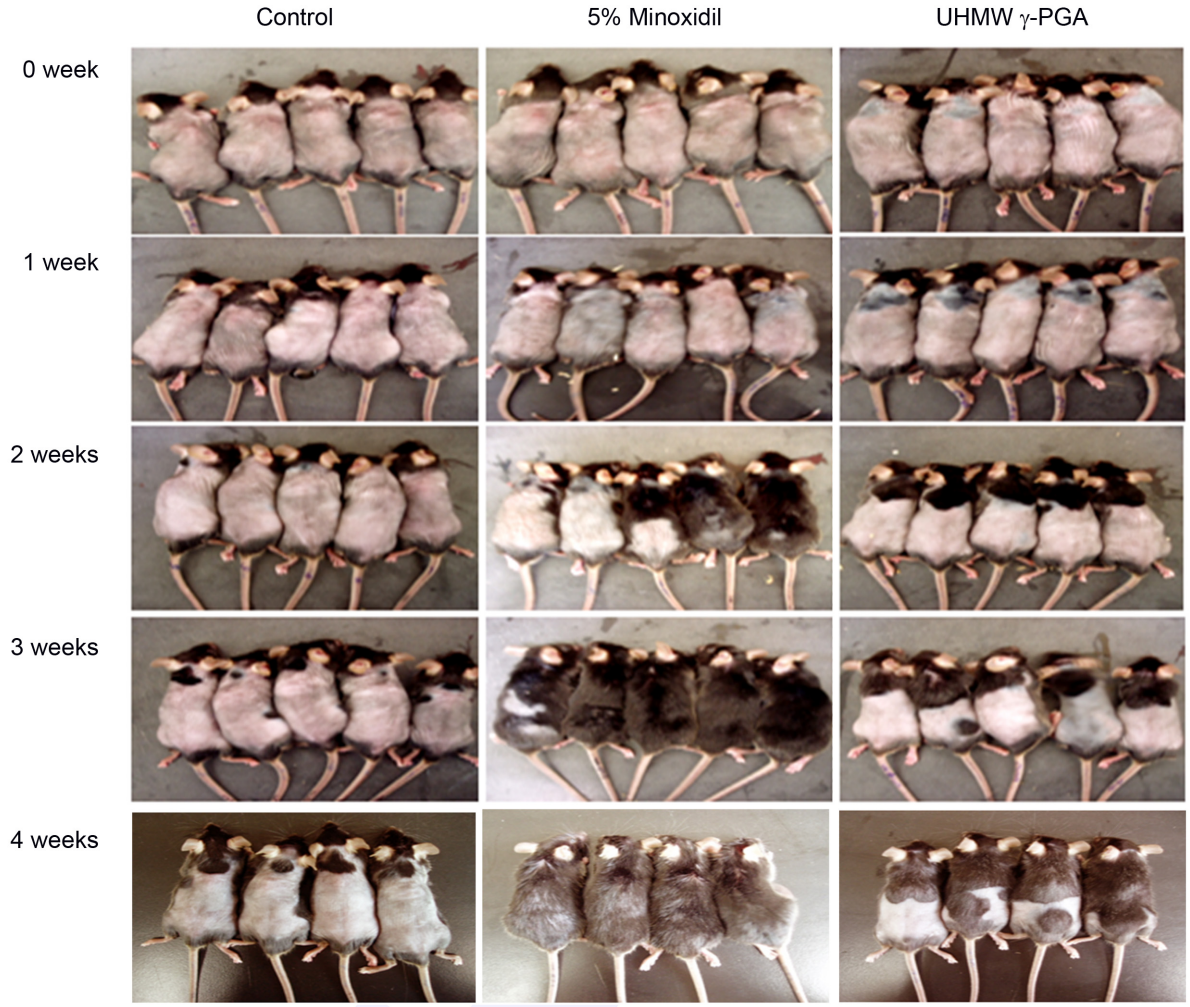

Fig. 2. *In vivo* hair-growth promotion the UHMW γ-PGA in C57BL/6 mouse models of alopecia.

of hair follicles from the telogen to the anagen phase. To evaluate the hair growth-promoting activity of UHMW γ-PGA, we topically applied UHMW γ-PGA and minoxidil on the shaved dorsal skin of telogenic C57BL/6 mice for 4 weeks. At each week, we evaluated the degree of hair growth by observing the skin color.

At 2 weeks, C57BL/6 mice treated with UHMW γ-PGA showed significant black pigmentation of the shaved skin. The minoxidil-treated group showed the most marked black pigmentation, and the least visible hair growth and black pigmentation was observed in the control group (Fig. 2). At week 4, UHMW γ-PGA-treated groups showed hair growth on more than 50% of the shaved dorsal skin, whereas the control group showed relatively less hair growth. These results indicate that UHMW γ-PGA promotes hair growth by effectively inducing the anagen phase in telogenic C57BL/6 mice.

Effect of UHMW γ-PGA on the Number of Hair Follicles

We checked the progression of hair follicles by H&E staining. Histological observations revealed that the hair follicles in UHMW γ-PGA-treated mice appeared earlier than those in the control group (Fig. 3). Furthermore, the number of hair follicles in the UHMW γ-PGA-treated group was higher than that in the control group. Application of minoxidil resulted in the maximum number of hair follicles. These data indicate that UHMW γ-PGA could promote hair growth by inducing the anagen phase of hair follicles.

Discussion

Androgenetic alopecia is often exacerbated by conditions that can induce telogen effluvium, including drugs, acute stressors, weight loss, and partum. To measure hair growth, relevant, easy, and inexpensive experimental models are essential. To be effective in both *in vivo* and *in vitro* conditions, these models have to reflect the major regulatory processes. The laboratory mouse has been a favorite subject for trichology studies, and the pigmented C57BL/6 and C3H mice are the most commonly used strains. The rationale for choosing these mice is that their truncal pigmentation is entirely dependent on their follicular melanocytes, and their truncal epidermis lacks melanin-producing melanocytes. Because pigment production is active only during the follicle growth (anagen) phase, the skin darkens only when hair growth occurs. Therefore, by assessing skin color, one can also access the follicle growth phase. Another feature of the mouse system is that the growth phase of its follicles can be synchronized, allowing the investigator to isolate and analyze follicles of certain phases after hair growth induction.

γ-PGA is a biopolymer traditionally produced during the fermentation of soy-based nutrients by *Bacillus subtilis*, a naturally occurring microorganism that is fundamental to the production of fermented soy foods such as *natto* in Japan and *chungkookjang* in Korea. These foods have been consumed for centuries; hence, there is strong evidence to support the fact that these foods are safe for consumption.

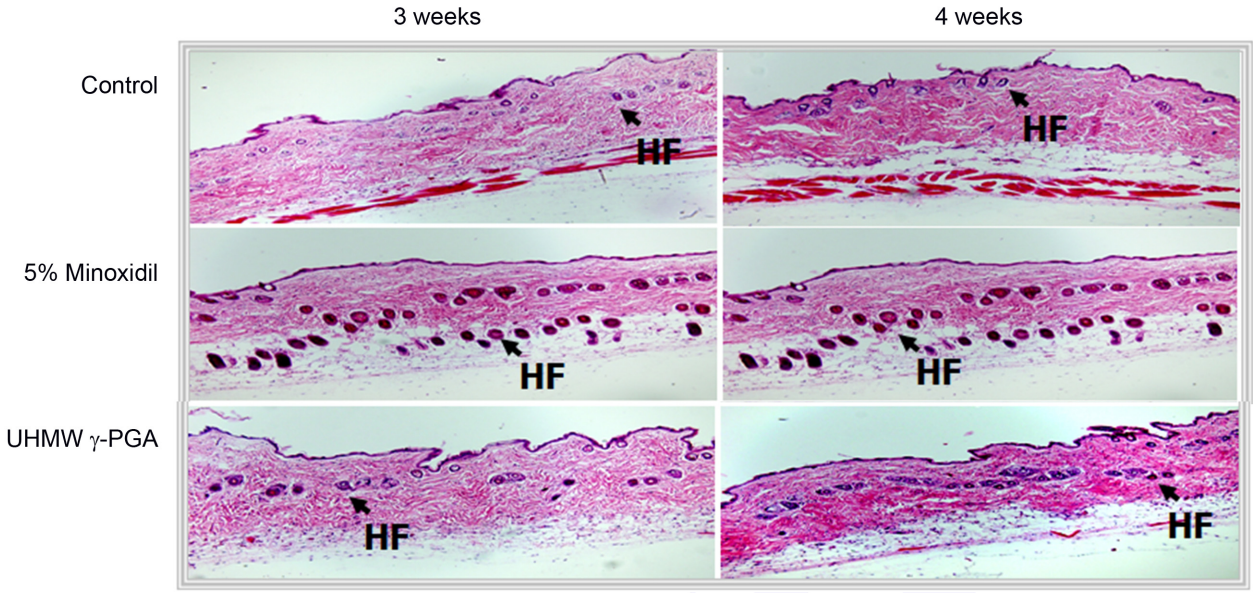

Fig. 3. Histological observation of hair follicles in a male C57BL/6 mouse model of alopecia after topical application of samples for 3 and 4 weeks.
HF, hair follicle.

The polymeric γ-PGA, which gives these foods their unique sticky texture, is essentially a chain of gamma-linked D- and L-glutamic acid peptides. The gamma linkage within γ-PGA is different from the alpha-linked peptide bonds in regular proteins. This makes γ-PGA more resistant to proteases that are found on the skin, which can rapidly break down proteins and other peptide ingredients that are added to many skin-care products. γ-PGA imparts superior skin hydration through both extrinsic and intrinsic modes of action that can provide immediate and long-term moisturizing effects, depending on its molecular weight. For these reasons, we expected that UHMW γ-PGA would influence the hair and scalp, as well as the skin. First, we investigated whether UHMW γ-PGA is an inhibitor of 5-alpha reductase, an enzyme produced in the prostate, adrenal glands, and scalp, which metabolizes the male hormone testosterone into DHT. DHT, a metabolite of testosterone produced by the enzyme 5-alpha reductase, has been implicated as the specific androgen in the pathogenesis of androgenic alopecia. 5-Alpha reductase has been detected in scalp hair follicles, and balding scalps contain increased 5-alpha reductase activity and DHT levels [8]. UHMW γ-PGA effectively inhibited the activity of 5-alpha reductase in a manner similar to that of finasteride, a synthetic drug for the treatment of male pattern baldness.

The above-mentioned *in vivo* efficacy study was performed using C57BL/6 mice. After 4 weeks, UHMW γ-PGA-treated groups showed hair growth of more than 50% on the shaved skin, whereas the control group showed relatively less hair growth. Although the hair growth of minoxidil-treated groups was faster than that of UHMW γ-PGA-treated groups, we must consider the safety issues regarding minoxidil because the drug product contains a high concentration of minoxidil: 5%.

Several studies have found that the number and size of hair follicles increase during anagen phase induction [15]. The growth density of hair follicles is proof that the hair cycle progresses from the telogen to anagen phase [14]. Therefore, we have also investigated the influence of UHMW γ-PGA on hair follicles and the hair growth cycle. Histological observations showed that the hair follicles of the UHMW γ-PGA-treated group appeared earlier than did those of the control group, and the number of hair follicles on the shaved skin in the UHMW γ-PGA-treated group was relatively higher than that observed on the shaved skin in the control group, consistent with the results pertaining to hair growth. The hair growth cycle describes the changing morphology of the shaft and follicle. Starting with anagen, the follicle and its shaft pass through catagen, telogen, and finally exogen phases [17, 20]. All body hairs undergo this cycle, although the duration of the cycle, the duration of the individual phases, and the length of the individual shafts vary dramatically from site to site. Alopecia includes a group of conditions characterized by inflammation and subsequent destruction of the hair follicle, resulting in irreversible hair loss [5, 16]. In this study, we found that UHMW γ-PGA promotes hair growth and increases the number of hair follicles. Taken together, our data suggest that γ-PGA initiates anagen during hair follicle cycling and encourages the use of UHMW γ-PGA for anagen modulation in skin affected by hair growth disorders, all of which are characterized by the elongation of the telogen and the shortening of the anagen phases.

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