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Transdermal delivery of hyaluronic acid – Human growth hormone conjugate

Jeong-A Yang [a,1], Eung-Sam Kim [b,1], Jung Hee Kwon [c], Hyemin Kim [a], Ji Hye Shin [c], Seok Hyun Yun [d], Kwan Yong Choi [b,c,**], Sei Kwang Hahn [a,b,d,*]

[a] Department of Materials Science and Engineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea
[b] School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea
[c] Department of Life Science, Division of Molecular and Life Sciences, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea
[d] Wellman Center for Photomedicine, Harvard Medical School and Massachusetts General Hospital, 65 Landsdowne St. UP-523, Cambridge, MA 02139, USA

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ABSTRACT

Hyaluronic acid (HA) is one of the major components of extracellular matrix (ECM). Keratinocyte and fibroblast are known to have HA receptors in the skin. Fibroblast also has human growth hormone (hGH) receptors. In this work, HA–hGH conjugate was developed as a receptor mediated transdermal delivery system of protein drugs. HA–hGH conjugate was synthesized by specific coupling reaction between aldehyde modified HA and the N-terminal amine group of hGH. We could confirm the proliferative effect of HA on keratinocyte and fibroblast, and the biological activity of HA–hGH conjugate in fibroblast with an elevated expression level of phosphorylated Janus kinase 2 (p-JAK2). Interestingly, fluorescence microscopy clearly visualized the dramatically enhanced penetration of HA–hGH conjugate through the dorsal skin of mice after topical treatment with FITC labeled HA–hGH conjugate. According to pharmacokinetic analysis, HA–hGH conjugate appeared to be delivered through the skin into the blood stream possibly by the receptor mediated transdermal delivery. This work confirms the feasibility of using the HA–hGH conjugate as a model system for the receptor mediated transdermal delivery of protein drugs and their further exploitation for various cosmetic and tissue engineering applications.

1. Introduction

Recently, a number of transdermal drug delivery systems have been developed mainly due to their advantages such as effective systemic delivery bypassing digestive systems, patient compliance without painful injections, and easy control to terminate drug delivery [1–3]. Despite these benefits, a low bioavailability is one of the major disadvantages of transdermal drug delivery systems due to the poor skin permeability. The outermost layer of epidermis, stratum corneum, is the inevitable barrier consisting of highly ordered dead cells with intercellular lipids [4]. To circumvent this limitation, various methods have been developed using microneedle patch [5,6], iontophoresis [7,8], penetration enhancer [9,10], and ultrasound [11,12]. Microneedle patches employ an array of needles in a micron scale to create holes in stratum corneum for skin permeability enhancement. However, it requires multiple and complicate processes to develop microneedle arrays containing drugs, especially for the case of protein drugs [5,6]. Iontophoresis uses a continuous low voltage current to provide an electrical driving force for charged molecules and an electroosmotic flow for uncharged molecules to deliver drugs through stratum corneum without pain [7,8]. However, the application of iontophoresis has been limited mostly to the delivery of small molecules with a charge. Ultrasound disrupts the lipid structure of stratum corneum to enhance the skin permeability, which may cause deep tissue damages in some cases [11,12]. Currently, there are few reports on the efficient transdermal delivery systems of protein drugs.

* Corresponding author. Department of Materials Science and Engineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea. Tel.: +82 54 279 2159; fax: +82 54 279 2399.
** Corresponding author. School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea. Tel.: +82 54 279 2295; fax: +82 54 279 8290.
E-mail addresses: kchoi@postech.ac.kr (K.Y. Choi), skhanb@postech.ac.kr, skhanb@hanmail.net (S.K. Hahn).
[1] These authors contributed equally to this work.

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Hyaluronic acid (HA) is a linear polysaccharide in the body. More than 50% of HA is present in the skin tissue [13–15]. Despite the high molecular weight and hydrophilicity of HA, it is known to be delivered through the skin tissue in both mouse and human [16,17]. The mechanism for transdermal transport of HA has not been clearly verified yet, but there are some possible reasons for the positive effect of HA on transdermal delivery. First, HA is very hygroscopic and can hydrate the stratum corneum enhancing the permeability of skin. Second, the hydrophobic patch domain in HA chain can enhance the permeability of HA across the stratum corneum. Third, HA receptors distributed in the skin tissue may facilitate the localization of HA in the skin tissue [17–19]. Moreover, it is reported that HA can induce the proliferation, migration, adhesion, and differentiation of keratinocyte [20–23]. HA can also enhance the proliferation of fibroblast through CD44 receptors on the cell membrane [24]. Meanwhile, human growth hormone (hGH) has been widely used for the treatment of short stature by daily injection for months to years. It is well known that hGH receptors are distributed in the skin tissue and have important roles for cell proliferation, mitosis, and differentiation [25–27]. Especially, hGH promotes the synthesis of insulin-like growth factor I (IGF-1) in fibroblast [25] and the released IGF-1 can also enhance the proliferation of keratinocyte [26].

In this work, on the basis of possitive effect of HA on the transdermal delivery, HA–hGH conjugate was developed as a receptor mediated transdermal delivery system of protein drugs. HA–hGH conjugate was synthesized by coupling reaction of aldehyde modified HA (HA-ALD) with N-terminal primary amine group of hGH. The resulting HA–hGH conjugate was characterized by gel permeation chromatography (GPC) and circular dichroism (CD) spectroscopy. After confirmation of *in vitro* biological activity of hGH conjugated to HA from the elevated expression level of phosphorylated Janus kinase 2 (p-JAK2) in the fibroblast of Detroit 551 cell, the effect of HA and HA–hGH conjugate was investigated on the proliferatation of human keratinocyte and fibroblast. Then, *in vivo* skin penetration of HA–hGH conjugate was visualized by fluorescence microscopy after topical treatment of FITC labeled HA–hGH conjugate. Finally, pharmacokinetic (PK) analysis of topically delivered HA–hGH conjugate was carried out to confirm the feasibility of HA–hGH conjugate as a model system for the receptor mediated transdermal delivery of protein drugs with the discussion for their further exploitation for various cosmetic and tissue engineering applications.

2. Materials and methods

2.1. Materials

Sodium hyaluronate, the sodium salt of hyaluronic acid (HA), with a molecular weight of 100 kDa was obtained from Shiseido (Tokyo, Japan). Human growth hormone (hGH) was kindly provided by LG Lifesciences (Daejeon, Korea). Human serum, sodium periodate, sodium cyanoborohydride, ethyl carbazate, and tert-butyl carbazate were purchased from Sigma–Aldrich (St. Louis, MO). Human epidermal keratinocytes – neonatal (HEKn), EpiLife medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS) tablet were purchased from Invitrogen (Carlsbad, CA), and Bradford protein assay kit from Thermo Scientific (Rockford, IL). WST-1 reagent was purchased from Takara-Bio (Otsu, Shiga, Japan) and goat anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p-JAK2 antibody was purchased from Cell Signaling Technology (Danvers, MA) and horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Laboratories (West Grove, PA). A human skin fetal fibroblast cell line, Detroit 551, was purchased from ATCC (Manassas, VA). RPMI-1640 medium and DMEM were obtained from GIBCO (Grand Island, NY). The hGH ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). All reagents were used without further purification.

2.2. Preparation of aldehyde modified HA

HA-ALD was prepared as described elsewhere [28]. Briefly, 1 g of HA with an MW of 100 kDa was dissolved in 100 mL of water. Sodium periodate (1 M ratio of HA repeating unit) was added to the HA solution. After reaction in dark place for 2 h, 6 h, and 12 h, excess amount of ethylene glycol (1 g) was added to the solution for the reaction termination. Finally, the resulting solution was dialyzed against a large excess amount of water using a prewashed dialysis membrane tube (MWCO of 7 kDa) and lyophilized for 3 days. In order to analyze the degree of aldehyde modification, the HA-ALD was dissolved in sodium acetate buffer (5 mg/mL) at pH 5.2, which was reacted with tert-butyl carbazate in the presence of sodium cyanoborohydride both at 5 M ratio of HA repeating unit for 24 h. Then, the reaction solution was poured into the prewashed dialysis membrane tube (MWCO of 10 kDa) and dialyzed against a large excess amount of water. The solution was lyophilized for 3 days, which was characterized by ^{1}H NMR (DPX500, Bruker, Germany).

2.3. Synthesis of HA–hGH conjugate

HA-ALD with an aldehyde content of 10 mol% was dissolved in sodium acetate buffer (pH 5.5) at a concentration of 5 mg/mL. The number of hGH molecules per single HA chain in the feed was varied from 1, 3, 6, and 9. The conjugation reaction was performed at room temperature for 24 h with mild stirring. For the reduction of hydrazone bonds, 5 M excess of sodium cyanoborohydride to aldehyde group was added into the reaction solution. The unreacted aldehyde groups in HA-hGH conjugate were blocked with 5 M excess of ethyl carbazate in the presence of sodium cyanoborohydride at room temperature for another 24 h. Then, HA–hGH conjugate was purified by dialysis against a large excess amount of PBS for 2 days. For the following experiments, three kinds of HA–hGH conjugates were synthesized to contain 6 hGH molecules in a single HA chain using HA-ALD with 10, 20 and 35 mol% aldehyde contents, and represented as HA–hGH (10%/6), HA–hGH (20%/6) and HA–hGH (35%/6), respectively.

2.4. Characterization of HA–hGH conjugate

The successful synthesis of HA–hGH conjugate was assessed by GPC analysis measuring the retention time before and after conjugation of HA with hGH. The number of hGH molecules in HA–hGH conjugate was determined from the GPC peak area at 280 nm. The standard curve of hGH was prepared by several dilutions of the protein stock solution at a concentration of 1 mg/mL. The GPC analysis was carried out using the following systems: Waters 717 plus autosampler, Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, Ultrahydrogel™ 500 connected with Ultrahydrogel™ 250 column. The mobile phase was PBS at pH 7.4 and the flow rate was 0.5 mL/min. The detection wavelengths were 210 nm for HA and 280 nm for hGH, respectively. The blocking of the remaining aldehyde groups with ethyl carbazate was checked by ^{1}H NMR (DPX500, Bruker, Germany). The secondary structure of HA–hGH conjugate was analyzed by CD spectroscopy. CD spectra of hGH and HA–hGH conjugate were obtained with a spectrum-polarimeter (J-715, JASCO) at 20 °C under nitrogen. All scans from 250 nm to 200 nm for each sample were done in a quartz cuvette with a path length of 2 mm in triplicate. Raw data were acquired at a step size of 0.2 nm with a response time of 1.0 s. The residual ellipticity was obtained as an average of three scans and converted to molar ellipticity.

2.5. In vitro serum stability test of HA–hGH conjugate

The serum stability of hGH and HA–hGH (20%/6) conjugate was assessed by ELISA after incubation in human serum at a concentration of 0.5 mg/mL and 37 °C for up to 7 days. At the predetermined time intervals, each sample was immediately diluted by 10,000 times with PBS and stored at − 80 °C before the ELISA.

2.6. Effect of HA on the proliferation of skin cells

HEKn cells were maintained in EpiLife medium supplemented with 1% (v/v) human keratinocyte growth factors. Detroit 551 cells were cultured in DMEM supplemented with 10% (v/v) FBS. The proliferative activity of HA to skin cells was investigated by WST-1 assay. Prior to seeding cells, the culture medium of Detrioit 551 cell was replaced with a DMEM supplemented with 0.2% (v/v) FBS. The HEKn and Detroit 55l cells were resuspended at a concentration of 1×10^4 cells/mL in assay media and 100 μL of the cell suspension containing 3×10^3 cells was seeded on the flat bottom of 96 well tissue culture plate. After 24 h, the serial dilutions of the HA samples were prepared in assay media and added to the test wells in triplicate. The plates were incubated at 37 °C in a humidified 5% CO_2 tissue culture incubator for 48 h. Then, WST-1 reagent was added to each well and the plates were incubated at 37 °C in the tissue culture incubator for 1 h. The absorbance was measured at 450 nm using a microplate reader (SpectraFluor Plus, TECAN, Switzerland).

2.7. Biological activity of HA–hGH conjugate to skin cells

The biological activity of HA–hGH conjugate to skin cells was investigated by Western blot for the expression level of p-JAK2, the hGH receptor mediated signaling pathway product. The cells treated with hGH and HA–hGH conjugate were solubilized in a lysis buffer. The cell lysates were centrifuged at 15,000 g and 4 °C for 30 min to recover the supernatants. The total protein of 50 μg was separated in an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The

membrane was blocked with 4% (w/v) skimmed milk in a transfer buffer and then probed sequentially with a primary antibody and an HRP-conjugated secondary antibody for each target protein. The chemo-luminescence from the enzymatic reaction of peroxidase in the presence of an ECL solution was detected with an X-ray film. The chemically developed bands on the X-ray film were quantified with the NIH Image/J program (available at http://rsbweb.nih.gov/ij/). Then, the proliferative activity of hGH and HA–hGH conjugate to skin cells was investigated by WST-1 assay as described above.

2.8. Fluorescene microscopy for the transdermal delivery of HA–hGH conjugate

To visualize the effect of HA on skin penetration, amine modified HA, hGH, and HA–hGH conjugate were fluorescently labeled with FITC. FITC was dissolved in PBS at pH 9 and mixed with amine modified HA, hGH, and HA–hGH conjugate solutions at a molar excess ratio of 10. The conjugation reaction was performed at room temperature for 3 h with mild stirring. The FITC labeled HA, hGH, and HA–hGH conjugate were purified using PD 10 desalting columns. The degree of substitution was calculated by measuring the absorbance at 280 nm and 495 nm. Then, FITC labeled HA, hGH, and HA–hGH conjugate were topically administered to the dorsal skin of female hairless Balb/c mice at an age of 5 weeks. The dose was 5 nmol hGH with 2 nmol FITC. After 1 h, the skin was harvested for cryosection. The skin sample was embedded using optimal cutting temperature (OCT) compound on dry ice. The specimen was sliced into 5 μm thickness section and observed by fluorescence microscopy. We have complied with the POSTECH institutional ethical use protocols for animals.

2.9. Pharmacokinetic analysis of hGH and HA–hGH conjugate

Female Sprague Dawley (SD) rats at an age of 5 weeks weighing approximately 200 g were housed under a standard condition of a 12 h light/dark cycle with free

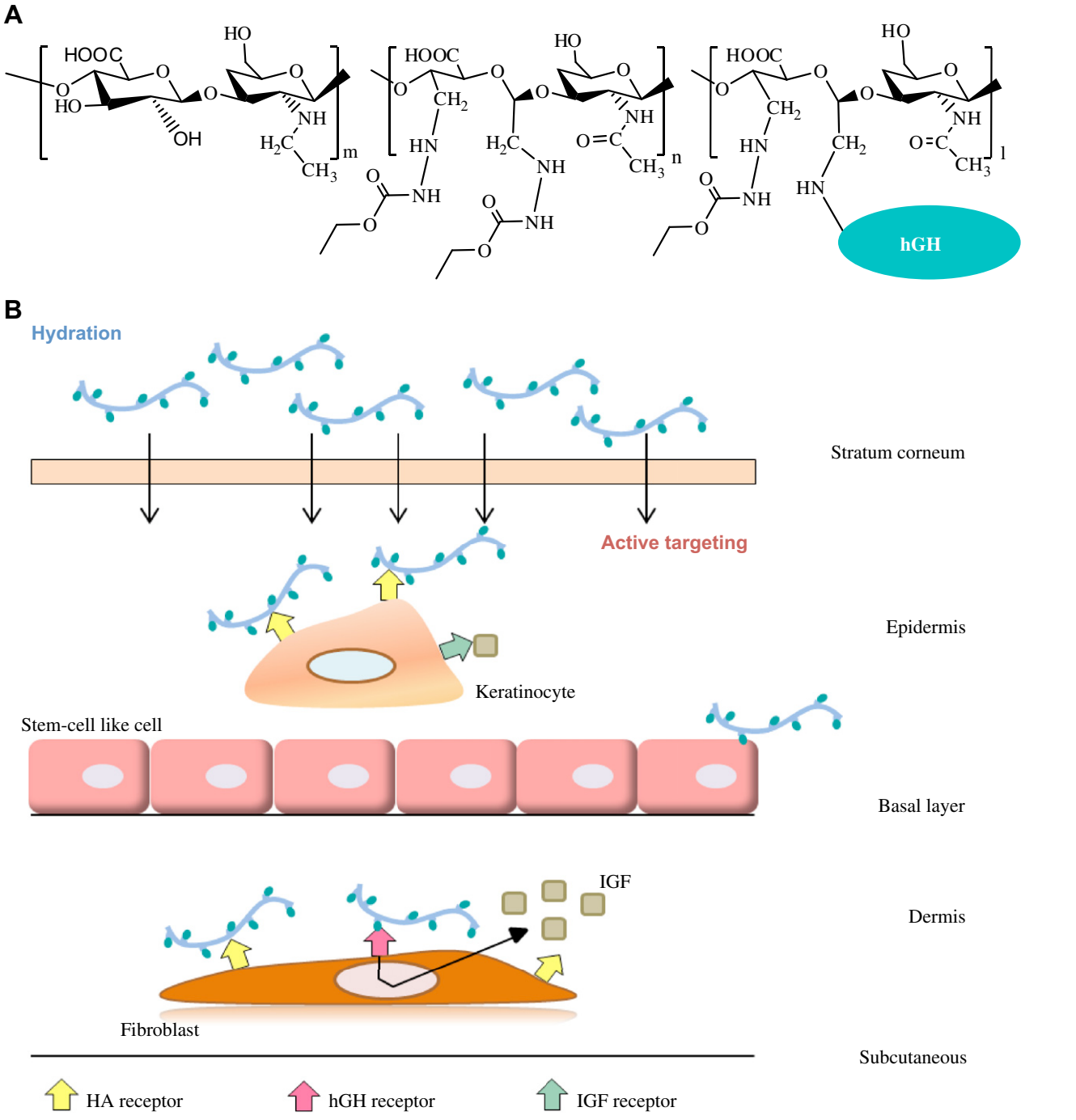

Fig. 1. (A) The chemical structure of hyaluronic acid–human growth hormone (HA–hGH) conjugate. (B) Schematic representation for the transdermal delivery of HA–hGH conjugate across the skin tissue.

access to food and water throughout the study period. Twenty female SD rats were randomized into 4 treatment groups ($n = 5$) for PK anlaysis after intravenous (iv) injections as follows: the control, hGH, HA–hGH (10%/6), and HA–hGH (20%/6). Each group received a single iv injection of the samples at a hGH dose of 0.5 mg/kg through a 26-gauge needle. In addition, twelve female SD rats were randomized into 4 treatment groups ($n = 3$) for PK analysis after transdermal delivery as follows: transdermal hGH, transdermal HA–hGH (10%/6), subcutaneous HA–hGH (10%/6), and intravenous hGH for comparision. The dose of hGH was 300 μg/rat. At predetermined time intervals, blood samples were collected via the tail vein for up to 2 days. After centrifugation, the serum was collected and stored after freezing at −80 °C before the analysis of hGH with human hGH ELISA kits.

2.10. Statistical analysis

The data are expressed as means ± standard deviation from several separate experiments. Statistical analysis was carried out via the two-way analysis of variance (ANOVA) test using the software of SigmaPlot12.0 and a value for $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of HA–hGH conjugate

Fig. 1 shows the schematic representations for the chemical structure of HA–hGH conjugate and the possible mechanism for its receptor mediated transdermal delivery in the skin tissue. It has been previously reported that the receptors of HA and hGH are distributed in the skin tissue, and HA can penetrate even to the dermis [16]. The HA–hGH conjugate binds to HA and hGH receptors on keratinocytes in the epidermis and fibroblasts in the dermis promoting cell proliferation and the synthesis of IGF-1 [27,29]. In addition, the released IGF-1 can also stimulate the proliferation of

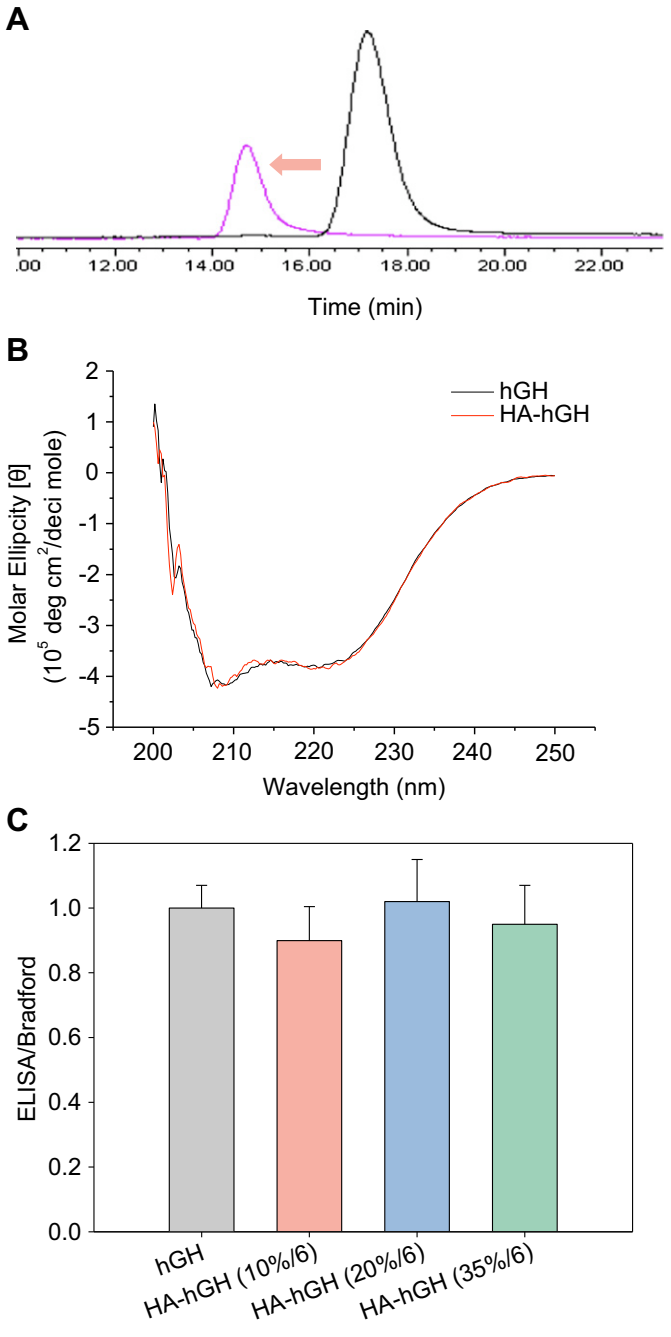

Fig. 2. (A) Gel permeation chromatograms (GPCs) of human growth hormone (hGH) (right peak in black) and hyaluronic acid (HA)–hGH conjugate (left peak in pink) synthesized using 10 mol% modified HA–aldehyde to contain 6 hGH. (B) Circular dichroism spectra of hGH and the HA–hGH conjugate. (C) The ratios of hGH concentrations in HA–hGH conjugates determined by ELISA and Bradford assay. HA–hGH conjugates were synthesized using 10, 20, and 35 mol% modified HA–aldehyde to contain 6 hGH ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

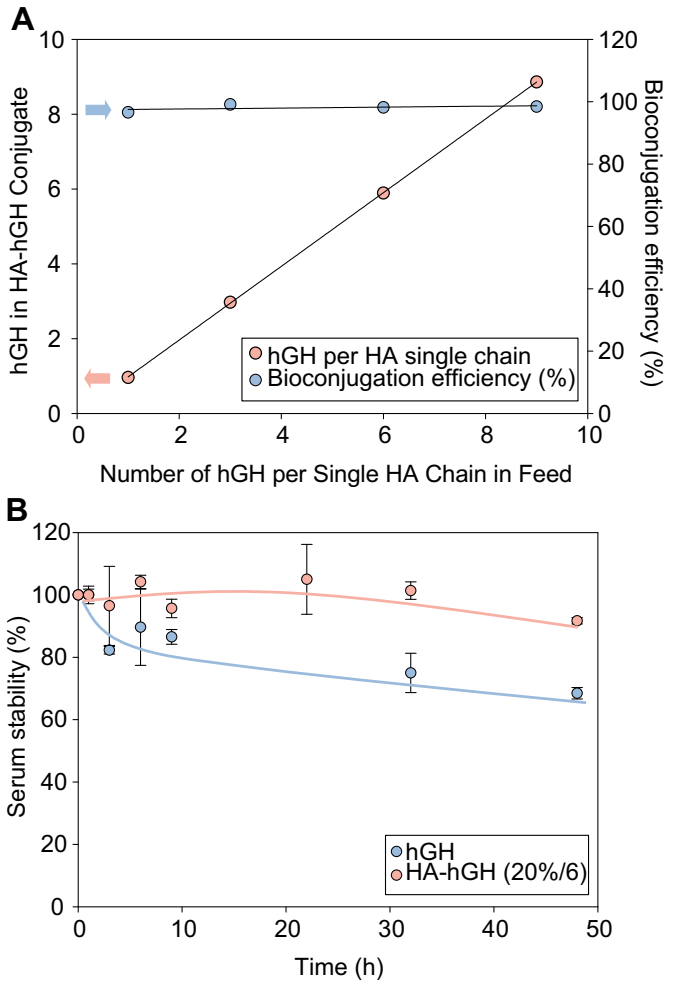

Fig. 3. (A) The number of human growth hormone (hGH) in hyaluronic acid (HA)–hGH conjugate and the corresponding bioconjugation efficiency (%). (B) *In vitro* stability of hGH and HA–hGH conjugate in human serum. The HA–hGH conjugate was synthesized using 20 mol% modified HA–aldehyde to contain 6 hGH. The remaining immunobiological activity of hGH to interact with antibody was determined by ELISA ($n = 3$).

keratinocyte in the epidermal tissue. On the basis of this hypothesis, HA–hGH conjugate was successfully synthesized for transdermal delivery via the coupling reaction between aldehyde groups of HA–ALD and N-terminal primary amine groups of hGH. The remaining aldehyde groups were blocked with ethyl carbazate to prevent the adverse effect of unreacted remaining aldehyde groups in HA–hGH conjugate. The successful synthesis of HA–hGH conjugate was confirmed by GPC analysis (Fig. 2A). The retention time of native hGH with an MW of 22 kDa was *ca.* 17 min, which was shortened to *ca.* 15 min after conjugation to 10 mol% aldehyde modified HA with a high MW of 100 kDa. As shown in Fig. 2B, the CD spectrum of HA–hGH conjugate was not different from that of intact hGH reflecting the maintenance of the secondary structure of hGH after conjugation. Fig. 2C shows the ratios of hGH concentrations in HA–hGH conjugate determined by ELISA and Bradford assay. The hGH in HA–hGH conjugate maintained the immunobiological activity comparable to intact hGH. The hGH content in HA–hGH conjugate was determined from the GPC peak area detected at 280 nm. The number of hGH molecules per single HA chain could be controlled in the range between 1 and 9. The hGH contents in HA–hGH conjugates increased with the feeding ratio of hGH to single HA chain. Regardless of the protein contents in feed, hGH was conjugated to HA–ALD with a bioconjugation efficiency higher than 95% (Fig. 3A). The bioconjugation efficiency (%) represents the molar ratio of hGH in the HA–hGH conjugate to the total hGH added initially for the conjugation reaction. Three kinds of HA–hGH conjugates were prepared to contain 6 protein molecules per single HA chain using 10 mol%, 20 mol% and 35 mol% modified HA–ALD. In order to investigate the serum stability of hGH after conjugation to HA, native hGH and HA–hGH (20%/6) conjugate were incubated in human serum at 37 °C for 2 days. The intactness of hGH in samples was assessed by ELISA. As shown in Fig. 3B, hGH was degraded and lost 30% of the immunobiological activity in human serum within 2 days. On the other hand, hGH in the HA–hGH conjugate maintained the immunobiological activity almost completely in human serum for longer than 2 days (Fig. 3B).

3.2. *In vitro* biological activity of HA–hGH conjugate to skin cells

Keratinocytes in the epidermis have HA and IGF-1 receptors, and fibroblasts in the dermis have HA and hGH receptors [19,26]. First, we investigated the effect of HA and HA–hGH conjugate on the proliferation of human keratinocytes (HEKn cells) and fibroblasts (Detroit 551 cells) using WST-1 assay (Fig. 4). HA significantly increased the proliferation of HEKn cells and Detroit 551 cells (Fig. 4A and B). In case of Detroit 551 cells, however, the cell proliferation was significantly inhibited with increasing HA concentration (Fig. 4B), as reported elsewhere for melanoma cells [21] and dermal fibroblasts [24]. On the other hand, while HA–hGH (10%/6) conjugates had little effect on the proliferation of HEKn cells (Fig. 4C), they effectively stimulated the proliferation of Detroit 551 cells which have both HA and hGH receptors (Fig. 4D).

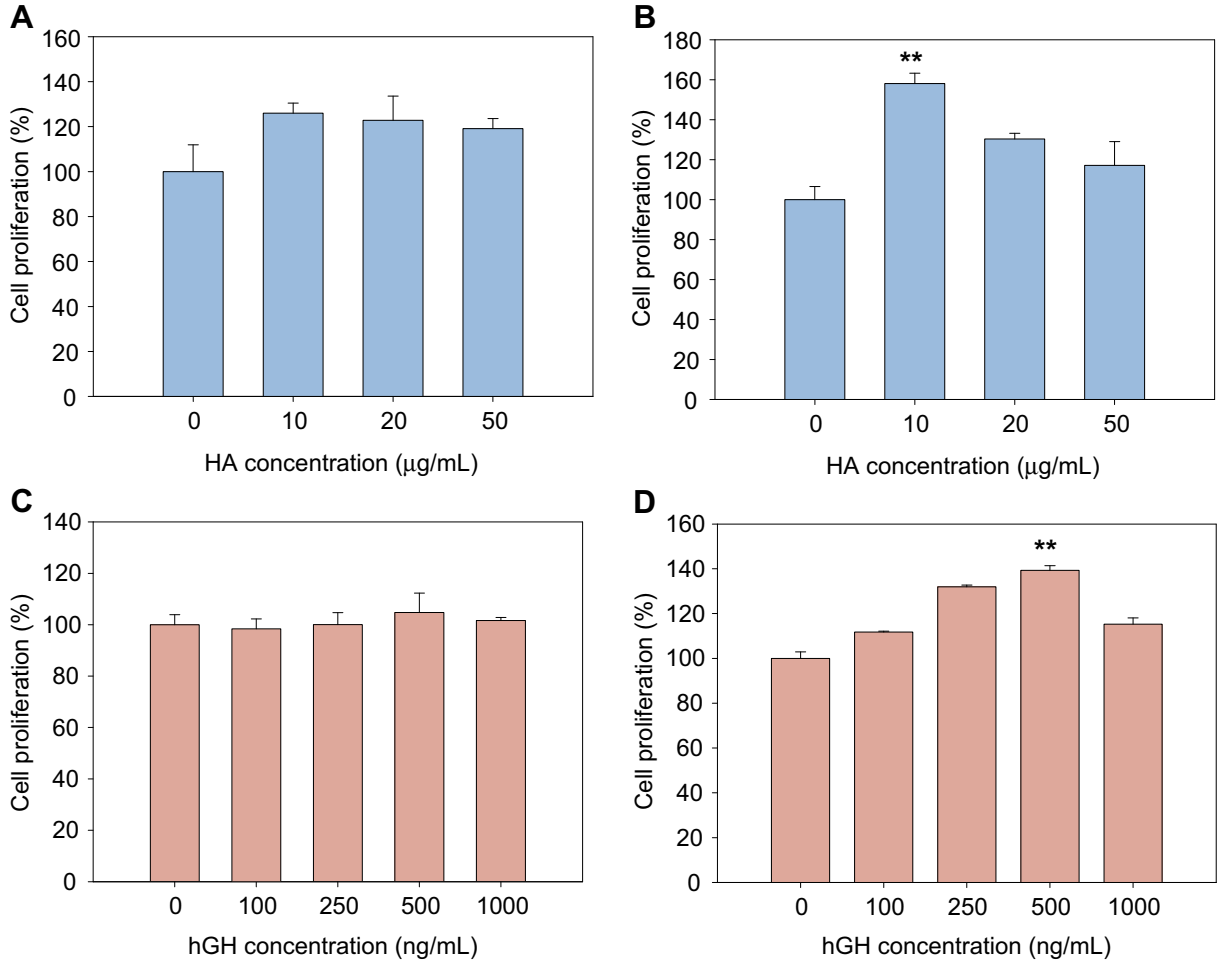

Fig. 4. *In vitro* proliferative activity of hyaluronic acid (HA) in (A) HEKn cells and (B) Detroit 551 cells. The proliferative activity of HA–human growth hormone (hGH) conjugate synthesized using 10 mol% modified HA–aldehyde to contain 6 hGH in (C) HEKn cells and (D) Detroit 551 cells (**$P < 0.01$ versus the control group).

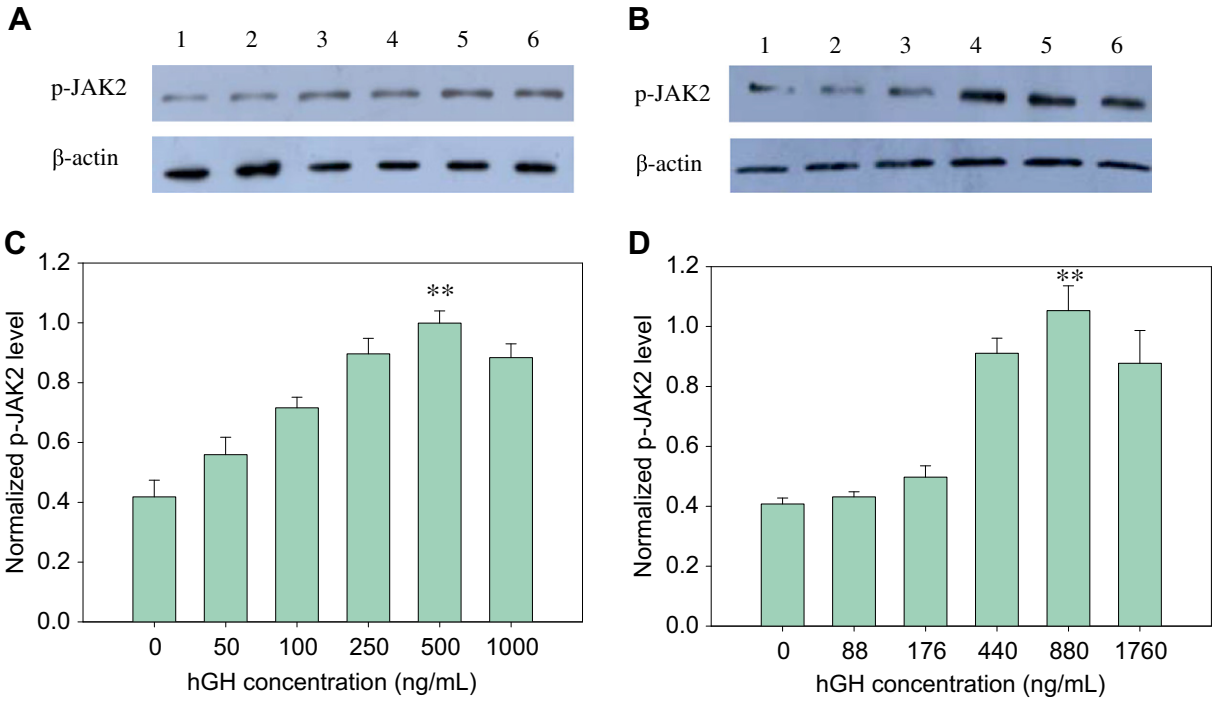

Fig. 5. Western blot analysis of phosphorylated Janus kinase 2 (p-JAK2) in Detroit 551 cells after treatment with (A) human growth hormone (hGH) (1: 0 ng/mL, 2: 50 ng/mL, 3: 100 ng/mL, 4: 250 ng/mL, 5: 500 ng/mL, 6: 1000 ng/mL) or (B) hyaluronic acid (HA)–hGH conjugate synthesized using 10 mol% modified HA–aldehyde to contain 6 hGH (1: 0 ng/mL, 2: 88 ng/mL, 3: 176 ng/mL, 4: 440 ng/mL, 5: 880 ng/mL, 6: 1760 ng/mL). Quantification of the expression levels of p-JAK1 in Detroit 551 cells after treatment with (C) hGH and (D) HA–hGH (10%/6) conjugate by the densitometric analysis ($n = 3$, $^{**}P < 0.01$ versus the control group).

The concentration of HA in the tested HA–hGH conjugate was below the threshhold for HA to stimulate cell proliferation (Fig. 4C). Then, the biological activity of HA–hGH (10%/6) conjugate was assessed by the analysis of hGH receptor mediated signaling to human keratinocytes and fibroblasts (Fig. 5). After treament with hGH and HA–hGH (10%/6) conjugate, the p-JAK2 expression level in the skin cells was measured by immunoblotting analysis. The hGH receptors on the cell surface are activated by dimerization, which promotes the receptor transphosphorylation by JAK2 tyrosine kinase [30]. The expression level of p-JAK2 was negligible in

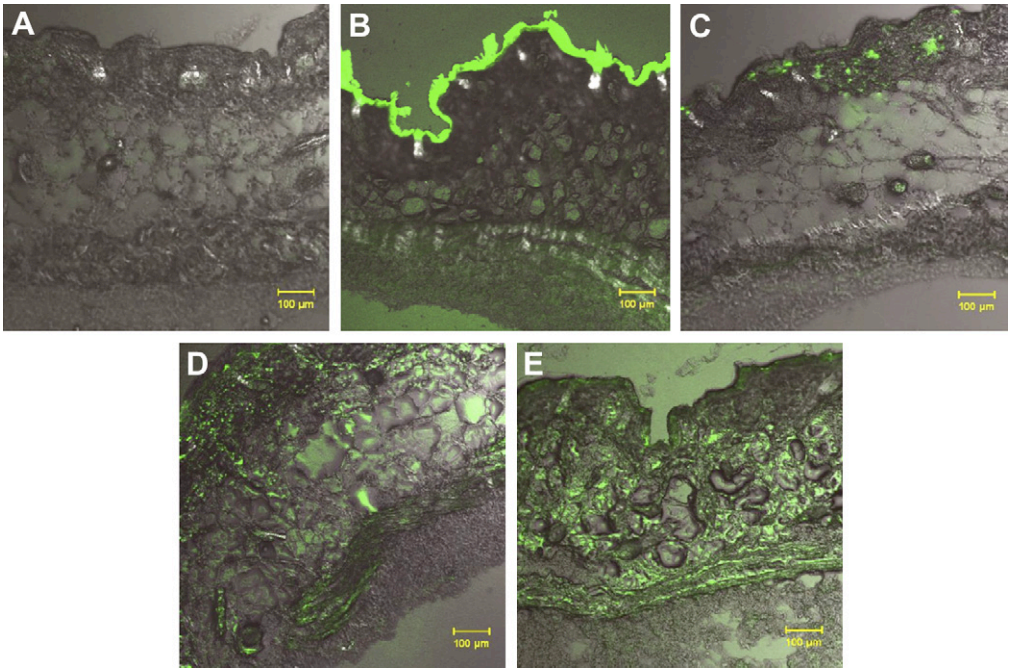

Fig. 6. Fluorescence microscopic images of mouse dorsal skin tissues after transdermal delivery of (A) PBS, (B) FITC, (C) FITC labeled hGH, (D) FITC labeled HA, and (E) FITC labeled HA–hGH conjugate prepared with 10 mol% modified HA–aldehyde to contain 6 hGH.

HEKn cells treated with hGH and HA–hGH (10%/6) conjugate. HEKn cells have much less hGH receptors than Detroit 551 cells. In contrast, the expression level of p-JAK2 was highly elevated in Detroit 551 cells showing a maximum at a hGH concentration of 500 ng/mL for the case of hGH treatment (Fig. 5C) and 880 ng/mL for the treatment with HA–hGH (10%/6) conjugate (Fig. 5D). The relatively high expression of p-JAK2 and the proliferation in Detroit 551 cells by the treatment with the HA–hGH (10%/6) conjugate might be attributed to the synergistic effect of HA and hGH on their receptors. From the results, we could confirm the presence of HA and hGH receptors on the skin cells, and the positive effect of HA and hGH on the proliferation of skin cells.

3.3. Bioimaging for the transdermal delivery of HA–hGH conjugate

The effect of HA on the transdermal delivery of FITC labeled HA–hGH conjugate was visualized by fluorescence microscopy 1 h after its topical treatment. The HA and hGH receptors on keratinocytes and fibroblasts were thought to facilitate the transdermal delivery of HA–hGH conjugate. As shown in Fig. 6, the HA–FITC and HA–hGH–FITC conjugates were evenly distributed in the skin tissue, and effectively delivered even to the dermis. Interestingly, the model protein drug of hGH with a dimension of *ca.* 4 nm appeared to be significantly delivered through the skin possibly due to the hydration of stratum corneum by hygroscopic HA and the HA receptor mediated penetration. On the other hand, FITC and hGH–FITC conjugate could not penetrate the skin tissue mostly remaining on the surface of stratum corneum. The facilitated penetration and distribution of HA–hGH (10%/6) conjugate in the skin tissue might be beneficial for various cosmetic and tissue engineering applications including wound healing treatment.

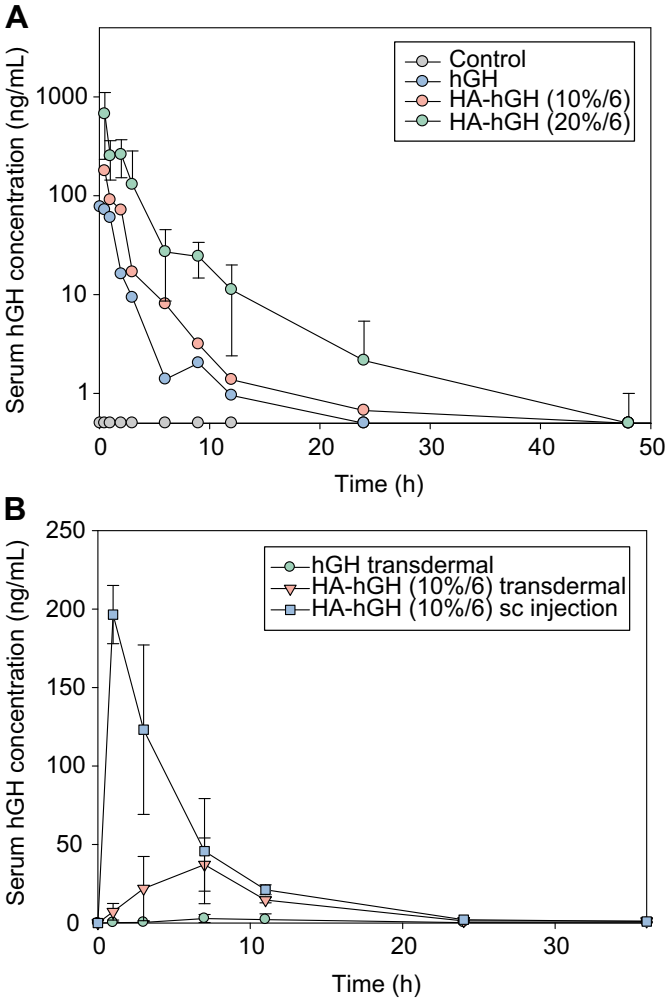

Fig. 7. (A) Pharmacokinetic (PK) analysis of human growth hormone (hGH) and hyaluronic acid (HA)–hGH conjugates prepared with 10 mol% and 20 mol% modified HA–aldehyde to contain 6 hGH after intravenous injection ($n = 5$). (B) PK analysis of hGH and HA–hGH (10%/6) conjugate after transdermal delivery and subcutaneous injection ($n = 3$).

Table 1
Pharmacokinetic analysis of hGH and HA–hGH conjugate after administration to SD rats.

	Groups			
	hGH *iv* injection	hGH topical delivery	HA–hGH topical delivery	HA–hGH *sc* injection
Dose [μg]	300	300	300	300
C_{max} [ng/mL]	1147 ± 298	2.8 ± 2.5	37.2 ± 16.9	196.5 ± 18.5
t_{max} [h]	1	7	7	1
$t_{1/2}$ [h]	2.2 ± 0.14	10.4 ± 3.5	9.6 ± 1.5	3.2 ± 0.2
AUC[a] [h ng/mL]	2415 ± 689	37.0 ± 43.3	384.0 ± 33.5	968.9 ± 164.2
Bioavailability [%]	100 ± 28.5	1.53 ± 1.79	15.9 ± 1.38	40 ± 6.8

[a] AUC: area under the curve.

3.4. Pharmacokinetic analysis of HA–hGH conjugate

After confirmation of the effective penetration through the skin, PK analysis of HA–hGH conjugate was carried out to assess the receptor mediated transdermal delivery of hGH. Four kinds of samples, PBS, hGH, HA–hGH (10%/6), and HA–hGH (20%/6) conjugates, were intravenously injected to SD rats at a hGH dose of 0.5 mg/kg. Fig. 7A shows the PK profiles of hGH and HA–hGH conjugate determined by hGH ELISA. The single administration of HA–hGH (20%/6) conjugate resulted in the prolonged circulation of hGH for up to 48 h, whereas that of hGH led to the rapid clearance within 10 h. The area under the curve ($AUC_{0-48\ h}$) for HA–hGH conjugate was higher than that for hGH despite using the same dosage of hGH (Table 1). The bioconjugation of hGH to HA was thought to effectively reduce the renal clearance and enzymatic degradation in the body resulting in the enhanced bioavailability (AUC/dose) of hGH. Then, PK analysis of transdermally delivered HA–hGH conjugate was carried out in SD rats after a topical treatment of three kinds of samples, PBS, hGH, and HA–hGH (10%/6) conjugate at a hGH dose of 300 μg/rat. For comparision, HA–hGH conjugate was subcutaneously injected to SD rats at the same dose. As shown in Fig. 7B, PK analysis of topically delivered HA–hGH (10%/6) conjugate revealed the effective delivery of HA–hGH conjugate through the skin into the blood stream. The $AUC_{0-36\ h}$ for topically delivered HA–hGH conjugate was much higher than that for hGH. The bioavailability (AUC/dose) of transdermally delivered HA–hGH (10%/6) conjugate was as high as 16% in comparison to that of intravenously injected hGH (Table 1). Considering all these results, we could confirm the feasibility of HA–hGH conjugate as a model system for the transdermal delivery of protein drugs, and their further cosmetic and tissue engineering applications.

4. Conclusions

HA–hGH conjugate was successfully synthesized by the coupling reaction of HA–ALD with N-terminal primary amine group of hGH for receptor mediated transdermal delivery. GPC analysis confirmed the successful synthesis of HA–hGH conjugate. The number of hGH molecules in HA–hGH conjugate could be controlled in the range from 1 to 9 by changing the amount of hGH

in the feed with a bioconjugation efficiency higher than 95%. CD analysis revealed the maintenance of the secondary structure of hGH even after conjugation to HA. In addition, we could confirm the proliferative effect of HA on keratinocyte and fibroblast, and the biological activity of HA–hGH conjugate not in keratinocyte but in fibroblast with elevated p-JAK2 levels. In addition, fluorescence microscopy successfully visualized the effective penetration of topically delivered HA–hGH–FITC conjugate to the epidermis and even to the dermis in mice. Finally, PK analysis of topically applied HA–hGH conjugate revealed the effective delivery of HA–hGH conjugate through the skin into the blood stream.

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