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Evaluation of Epidermal Growth Factor-Incorporating Skin Care Product in Culture Experiment Using Human Fibroblasts

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Abstract

This study aimed to investigate the potential of a skin care product composed of hyaluronic acid (HA) and collagen (Col) sponge containing epidermal growth factor (EGF), vitamin C derivative (VC), glucosylceramide (GC), poly(γ-glutamic acid) (PGA), and argentine (Arg). High-molecular weight HA aqueous solution, hydrolyzed low-molecular weight HA aqueous solution, and heat- denatured Col aqueous solution were mixed, into which each aqueous solution containing EGF, VC, GC, PGA, or Arg were added, followed by freeze-drying to obtain a spongy EGF-incorporating skin care product (EGF-skin care product). In order to evaluate the first efficacy of EGF, fibroblast proliferation was assessed after 6 days of cultivation in the conditioned medium prepared by dissolving EGF-skin care product in a conventional culture medium. The fibroblast densities increased more effectively in conditioned medium with EGF than in control medium without EGF. In order to evaluate the second efficacy of EGF, the amount of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) produced by fibroblasts were assessed in a wound surface model. A fibroblast-incorporating Col gel sheet (cultured dermal substitute: CDS) was elevated to the air-medium interface, onto which a spongy sheet of EGF-skin care product was placed and cultured for 7 days. The condition covered with or without EGF-skin care product is divided into (+) EGF or (−) EGF, respectively. Fibroblasts in the CDS released 3.7 times more VEGF and 25 times more HGF in (+) EGF compared with (−) EGF. In another experiment, an aqueous solution of EGF-skin care product was added onto CDS and cultured for 7 days. Aqueous solutions were prepared and stored at 4˚C or 37˚C for a different period of 1 day, 2 weeks, and 4 weeks, respectively. Fibroblasts in CDS under different condition released similar amount of VEGF and HGF. This result indicated that the efficacy of EGF was maintained even after preservation at 37˚C for 4 weeks. These findings suggest

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that EGF-skin care product can be used on damaged skin surface by placing its spongy sheet or its solution.

Keywords

Skin Care Product, Hyaluronic Acid, Collagen, Epidermal Growth Factor, Vitamin C Derivative

1. Introduction

It is well known that EGF is a potent stimulator of cell proliferation of various cells including keratinocytes, fibroblasts and vascular endothelial cells [1]-[3]. In addition, EGF stimulates fibroblasts to synthesize increased amount of VEGF and HGF [4]. VEGF and HGF are known to be the most potent cytokines for the promotion of wound angiogenesis [5] [6]. Recent research has demonstrated that simultaneous administration of HGF and VEGF synergistically promotes new blood vessel formation, as compared to administration of each factor alone [7]. HGF also facilitates proliferation of epithelial cells as well as vascular endothelial cells. Thus HGF is considered to be one of the key cytokines for both epithelialization and angiogenesis. It is well known that VC is known to accelerate deposition of several basement membrane proteins, such as type IV and VII collagens at the dermal-epidermal junction [8]. VC has the potential for enhancing tissue repair [9]. In addition, VC has the potential to synergistically enhance HGF secretion by fibroblasts, when VC is applied together with growth factors such as EGF, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [10]. Thus the combination use of EGF and VC has a higher potential to stimulate fibroblasts to release increased amount of VEGF and HGF.

Considering these efficacies of EGF and VC, we developed a functional wound dressing composed of HA and Col containing EGF [11]-[14]. This EGF-incorporating wound dressing showed the potential to facilitate granulating tissue formation associated with angiogenesis, and to accelerate epithelialization in rats and diabetic mice. In another previous study [15], a functional wound dressing composed of HA and Col containing EGF and VC was prepared. The efficacy of this wound dressing was investigated by *in vitro* and *in vivo* experiments assessing various parameters of wound healing. This EGF and VC-incorporating wound dressing showed a higher potential to facilitate production of VEGF and HGF by fibroblast *in vitro*, and enhance granulation tissue formation associated with angiogenesis and collagen deposition *in vivo*, compared with the EGF-incorporating wound dressing. Thus the combination of EGF and VC is promising for the functional wound dressing.

Taking these results into consideration, we developed a functional skin care product for application after chemical peels [16]. This skin care product is composed of HA containing EGF and VC, supplemented with GC and PGA as cosmetic ingredients. The results in the animal experiment using nude mice demonstrated that this skin care product was more promising to facilitate wound healing after chemical peels using 50% tri-chloroacetic acid.

Base on these results, we developed a novel skin care product composed of HA and Col containing EGF and VC, supplemented with GC, PGA, and Arg as cosmetic ingredients (EGF-skin care product). The present study aimed to investigate the efficacy of EGF in this skin care product on fibroblasts proliferation and fibroblast cytokine production in culture experiments using human fibroblasts.

2. Materials and Methods

2.1. Experimental Materials

HA (Bio Sodium Hyaluronate HA 20; molecular weight, 2000 kDa) was purchased from Shiseido (Tokyo, Japan). Col (NMP Collagen PS) was purchased from Nippon Meat Packers (Osaka, Japan). EGF (recombinant human, rh-EGF) was purchased from Shanghai Haohai Biological Technology (Shanghai, China). VC was purchased from Showa Denko (Tokyo, Japan). GC and PGA were purchased from Ichimaru Pharcos (Gifu, Japan). Arg was purchased from JUNSEI (Tokyo, Japan).

2.2. Preparation of EGF-Skin Care Product

HA powder was dissolved in distilled water (DW) (pH 6.8) to prepare a high-molecular weight HA solution.

Another portion of HA powder was dissolved in DW (pH 6.8), and then autoclaved at 120˚C for 40 min to obtain a partially hydrolyzed low-molecular weight HA solution. Col powder was dissolved in DW (pH 2.8), and then warmed at 105˚C for 5 min to obtain a heat-denatured Col solution. Arg was dissolved in DW, and adjusted to pH 8.7 using HCl. Each solution was mixed with stirring, and adjusted to pH 7 using HCl, and then autoclaved at 105˚C for 20 min to obtain a sterilized mixture (A). GC was dissolved in DW, and then autoclaved at 105˚C for 10 min for sterilization. PGA was dissolved in DW, and then autoclaved at 105˚C for 10 min for sterilization. VC was dissolved in DW, and then sterilized using a micro-filter. Sterilized GC solution and sterilized PGA solution were added into a sterilized mixture (A), in which sterilized VC solution was added with stirring to obtain a sterilized mixture (B). EGF was dissolved in DW, and sterilized using a micro-filter, and then added into a sterilized mixture (B) to prepare final mixture (C). A given portion of mixture (C) was poured into a dish (8 cm × 5 cm), and stored overnight in a refrigerator at 4˚C and then frozen at −85˚C for 1 day, followed by freeze-drying to obtain a spongy EGF-skin care product (8 cm × 5 cm, 200 mg, containing 10 μg EGF). This EGF-skin care product was packed into a bag and kept in a dry sterilizer at 110˚C for 30 min for sterilization.

2.3. Fibroblast Proliferation in Conditioned Media

In order to demonstrate the first function of EGF, the fibroblasts proliferation was measured in the conditioned medium prepared by dissolving EGF-skin care product in a conventional culture medium, *i.e.*, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Each conditioned mediums was prepared by dissolving 1/2, 1, or 2 sheets of EGF-skin care product in 100 mL of conventional culture medium, respectively divided into Group I, Group II, and Group III. Each conditioned medium contains EGF in concentration of 5, 10, 20 μg/100mL, respectively. Prior to this experiment, cryopreserved human-derived fibroblasts were thawed and cultured in a conventional culture medium for one passage to obtain an adequate number of cells. The cell suspension was prepared by mixing a certain amount of fibroblasts in a conventional culture medium, and then 15 mL of cell suspension was poured into each flask (75-cm^2), followed by cultivation at 37˚C for 1 day. In practice, fibroblasts were seeded into flasks at a density of 1×10^4 cells/cm^2. The density of attached cells after 1 day of cultivation was calculated using a conventional method. After 1 day of cultivation, the cultured medium was exchanged by each conditioned medium, respectively Group I, Group II, Group III. On the other hand, the cultured medium was exchanged by a conventional culture medium (control medium). The cell number was measured after cultivation at 37˚C for 7 days. The cell proliferation experiments were conducted eight times (n = 8).

2.4. Preparation of CDS for Wound Surface Model

In order to demonstrate the second function of EGF, the culture experiment using a wound surface model was conducted. CDS was prepared by the method described previously [4]. Briefly, Col powder was sterilized in a dry sterilizer at 110˚C for 30 min. DW was autoclaved at 120˚C for 20 min for sterilization. Collagen powder was dissolved in sterilized DW at a concentration of 1% (pH 2.8). Prior to this experiment, cryopreserved human-derived fibroblasts were thawed and cultured in a conventional culture medium for two passages to obtain an adequate number of cells. A double concentrated DMEM was prepared at a double concentration of conventional one. Fibroblasts were suspended in double concentrated DMEM supplemented with 20% FBS at a density of 160×10^5 cells/12mL. Fibroblast suspension (12 mL) was mixed with Col aqueous solution (12 mL) at a temperature below 10˚C. The mixture was then poured into a sterilized dish (8 cm × 5 cm), and a sterilized nylon mesh sheet (8 cm × 5 cm) was incorporated into the mixture to provide a mechanical support. The mixture was placed in a 5% CO_2 incubator at 37˚C for 1 day in order to allow jellification, followed by culturing in a conventional culture medium for 6 days to obtain CDS (incorporating cell density: 4×10^5 cells per 1 cm^2 of collagen gel sheet).

2.5. Potential of EGF-Skin Care Product for Fibroblast Cytokine Production

Cytokine production was evaluated by the method described previously [4]. Briefly, in order to create a wound surface model, a half sheet of CDS (4 cm × 5 cm) was elevated to the air-medium interface in a 100-cm^2 dish containing a conventional culture medium (60 mL). The condition covered with or without EGF-skin care product is divided into to (+) EGF or (−) EGF, respectively. In practice, a half sheet (4 cm × 5 cm) of EGF-skin care

product was placed on the CDS. Production of VEGF and HGF by fibroblasts in the CDS at the air-medium interface was measured using ELISA. Culture media were collected after 6 days of cultivation, and were stored at −30˚C. Samples of culture media and standards for each cytokine were added to duplicate wells of a 96-well micro-plate, and ELISA (R & D Systems, Minneapolis, MN) was performed according to the manufacturer's instructions [Experiment A] (**Figure 1**). The cytokine production experiments were conducted 14 times (n = 14).

In a similar manner, another experiment was conducted using aqueous solution of EGF-skin care product. Prior to this experiment, a sheet of EGF-skin care product was dissolved in sterilized accompanying solution (10 mL) and stored at 4˚C or 37˚C for a different period of 1 day, 2 weeks, and 4 weeks, respectively. This accompanying solution is prepared by mixing butylene glycol and hydrolyzed *Prunus domestica* into DW. Each aqueous solution (5 mL) was added on the CDS. This added aqueous solution (5 mL) contains 5 μg EGF. In control, no aqueous solution was added on the CDS. Production of VEGF and HGF by fibroblasts in CDS at the air-medium interface was measured using ELISA according to the method described above [Experiment B] (**Figure 1**). The cytokine production experiments were conducted 4 or 6 times (n = 4 or 6).

2.6. Statistical Evaluation

Data are expressed as means ± standard error (SE). Statistical analysis was performed using Student's t-test for comparison between two groups and Tukey-Kramer test for comparison among more than three groups.

3. Results

3.1. Preparation of EGF-Skin Care Product

When a Col aqueous solution at acidic pH was added into a HA aqueous solution at neutral pH with stirring, a precipitation was caused by forming an aggregation of HA molecules and rod-like helical Col molecules. The Col molecule in an aqueous solution changes from a rod-like helical structure to a random coil structure by warming at a temperature over 37˚C. Col aqueous solution was warmed at 105˚C for 5 min to obtain a heat-denatured Col solution having a random coil structure. When the heat-denatured Col aqueous solution was added into the HA aqueous solution, no precipitation was caused. This is a significant process to prepare a clear mixture without any precipitation. Thus a spongy skin care product was prepared from a clear aqueous solution composed of HA and Col containing other ingredients by freeze-drying.

3.2. Fibroblast Proliferation in Conditioned Media

EGF can enhance fibroblasts proliferation. In order to demonstrate the efficacy of EGF-skin care product,

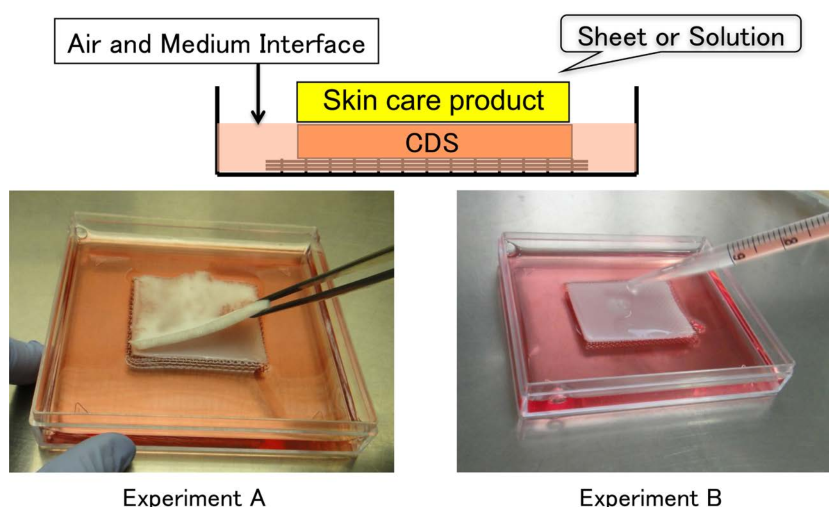


Figure 1. Culture system using wound surface model: a sheet of EGF-skin care product is placed on CDS [Experiment A] and an aqueous solution of EGF-skin care product is placed on CDS [Experiment B].

fibroblasts proliferation was measured in three conditioned media and in a control medium. The phase-contrast microphotographs of fibroblasts are shown in **Figure 2**. The photograph (a) shows well-attached fibroblasts day 1 in a conventional culture medium. The photograph (b) shows the confluent fibroblasts day 7 in a control medium (Control). The photograph (c) shows the confluent and partially overlapped fibroblasts day 7 in conditioned medium (Group I). Fibroblasts proliferation was enhanced more increasingly in Group I (EGF; 5 μg/100mL) compared with Control without EGF. This result on cell proliferation experiment demonstrates that fibroblast proliferation is effectively enhanced in a conditioned medium containing EGF in a concentration of 5 μg/100mL. In addition, fibroblasts proliferation was enhanced more increasingly in Group II (EGF; 10 μg/100mL) and Group III (EGF; 20 μg/100mL) compared with Group I. However, there was no difference between Group II and Group III. This finding indicates that EGF promoted fibroblasts proliferation in a dose-dependent manner at a range from 5 μg/100mL to 10 μg/100mL (**Figure 3**).

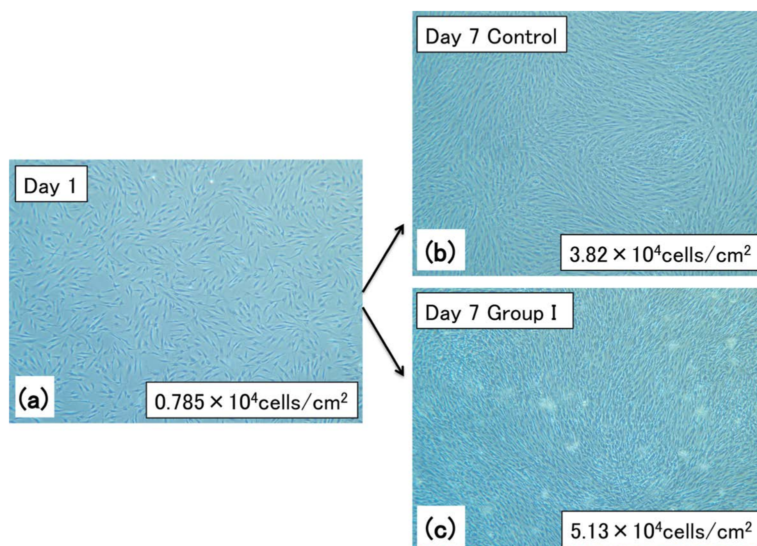


Figure 2. Phase-contrast microphotographs of fibroblasts: (a) Day 1, (b) Day 7 in Control, (c) Day 7 in Group I.

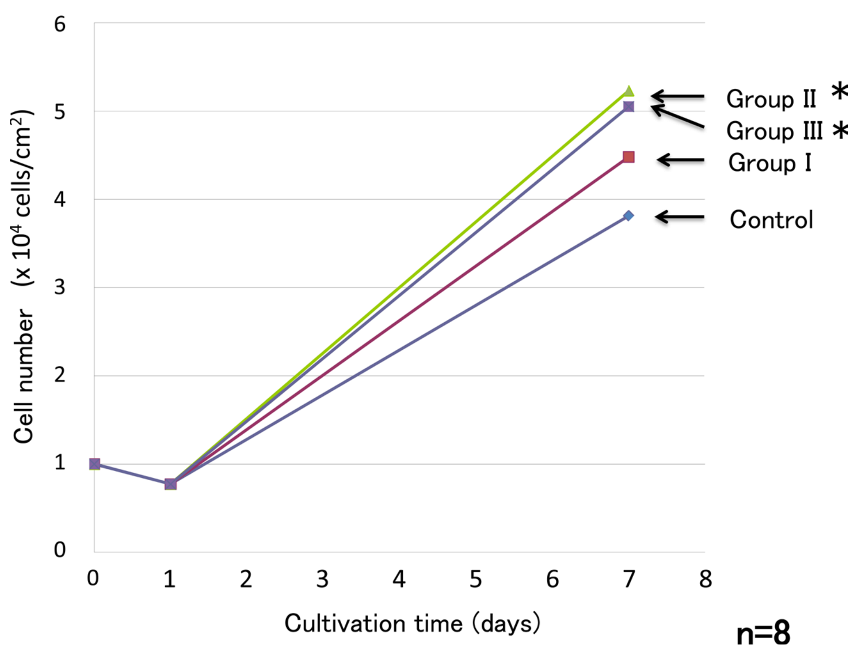


Figure 3. Measurement of fibroblast proliferation in conventional medium (Control) and conditioned media (Groups I. II. III). $^{*}p < 0.01$ vs. Control [Tukey-Kramer test].

3.3. Preparation of CDS for Wound Surface Model

The Col aqueous solution (pH 2.8) changes from acidic pH to neutral pH by mixing with a double concentrated cultured medium (pH 7.4), and thereby allowing jellification. The CDS used in this experiment was prepared by mixing a fibroblast cell suspension in double concentrated cultured medium and a collagen aqueous solution, and by allowing jellification within 1 hour at 37˚C and then culturing for 7 days. In general, a fibroblast-incorporating Col gel sheet causes a remarkable contraction in size after jellification. The resulting CDS, however, caused no contraction and had strength enough to handling because of incorporating nylon mesh sheet into a Col gel (**Figure 4**). This is a significant process to prepare a CDS for wound surface model.

3.4. Potential of EGF-Skin Care Product for Fibroblast Cytokine Production

The experiment A can create the practical condition when a spongy sheet of EGF-skin care product is placed on a wound surface. The amount of VEGF was measured in order to evaluate the efficacy of EGF. Fibroblasts in the CDS released 3.7-times more VEGF in (+) EGF compared with (−) EGF (**Figure 5**). This finding demonstrates that EGF-skin care product effectively stimulated fibroblast to synthesize an increased amount of VEGF. In addition to this, the amount of HGF was measured. Fibroblasts released 25 times more HGF in (+) EGF compared with (−) EGF (**Figure 6**). This finding demonstrates that EGF-skin care product more effectively

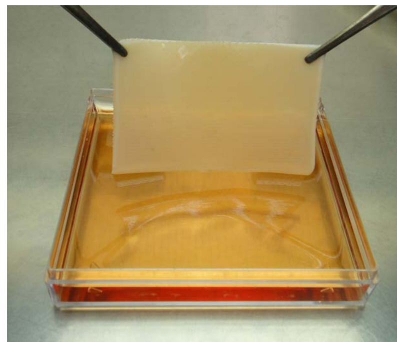


Figure 4. Fibroblast-incorporating Col gel sheet (cultured dermal substitute: CDS) having strength enough to handling because of incorporating nylon mesh sheet into a collagen gel.

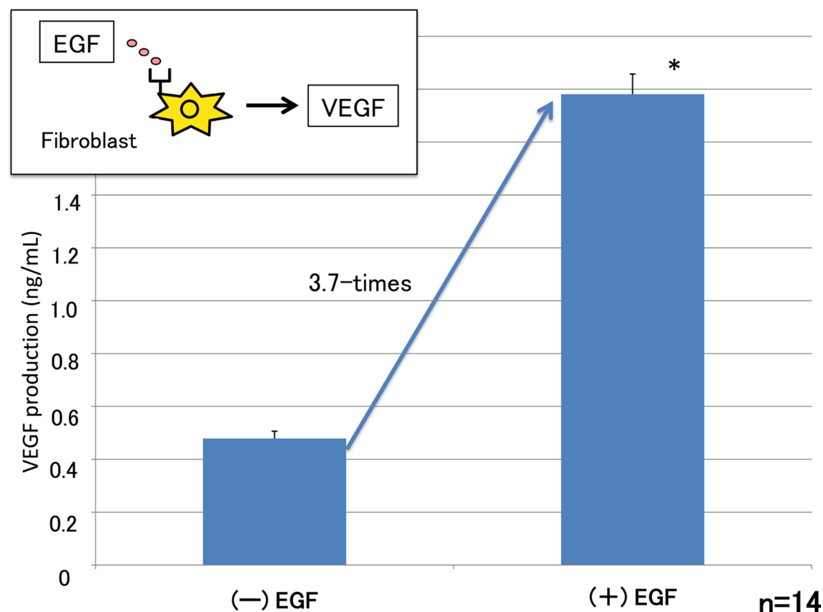


Figure 5. Results on quantitative analysis of VEGF production by fibroblast in CDS covered with or without EGF-skin care product; (+) EGF or (−) EGF. *$p < 0.01$ vs. (−) EGF [Student's t test].

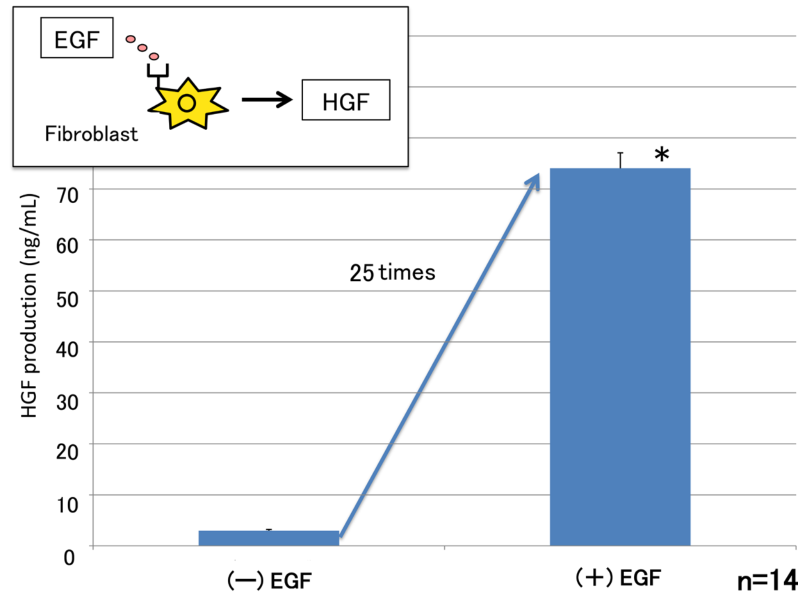


Figure 6. Results on quantitative analysis of HGF production by fibroblast in CDS covered with or without EGF-skin care product; (+) EGF or (−) EGF. $^{*}p < 0.01$ vs. (−) EGF [Student's t test].

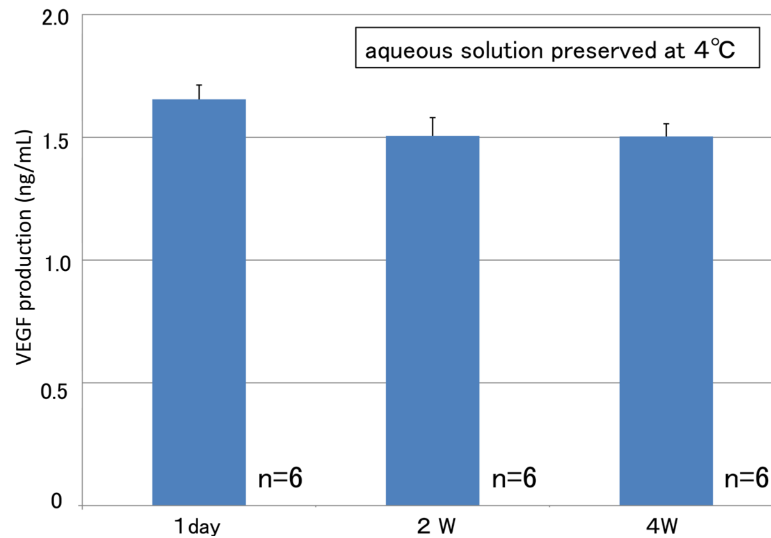


Figure 7. Results on quantitative analysis of VEGF production by fibroblast in CDS coated with aqueous solution of EGF-skin care product preserved at 4˚C for 1 day, 2 weeks, and 4 weeks.

stimulated fibroblast to synthesize an increased amount of HGF. It is noteworthy that HGF production was significantly higher than VEGF production.

The experiment B can create the practical condition when an aqueous solution of EGF-skin care product is coated on a wound surface. The efficacy of EGF was evaluated using the aqueous solution of EGF-skin care product. After preserving this aqueous solution at 4˚C or 37˚C for 1 day, 2 weeks, and 4 weeks, the efficacy of EGF was evaluated in the culture experiment using a wound surface model. There was no significant difference in VEGF production among conditions of 1 day, 2 weeks, and 4 weeks preservation at 4˚C or 37˚C (**Figure 7** and **Figure 8**). There was a slight difference in HGF production among conditions of 1 day, 2 weeks, and 4 weeks preservation at 4˚C (**Figure 9**). However, there was no significant difference in HGF production among conditions of 1 day, 2 weeks, and 4 weeks preservation at 37˚C (**Figure 10**). These findings indicate that the efficacy of EGF was maintained even after preservation at the most severe condition, *i.e.* at 37˚C for 4 weeks.

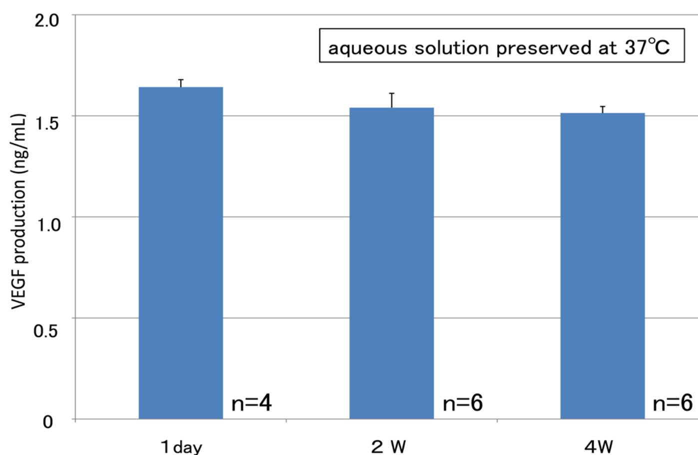


Figure 8. Results on quantitative analysis of VEGF production by fibroblast in CDS coated with aqueous solution of EGF-skin care product preserved at 37˚C for 1 day, 2 weeks, and 4 weeks.

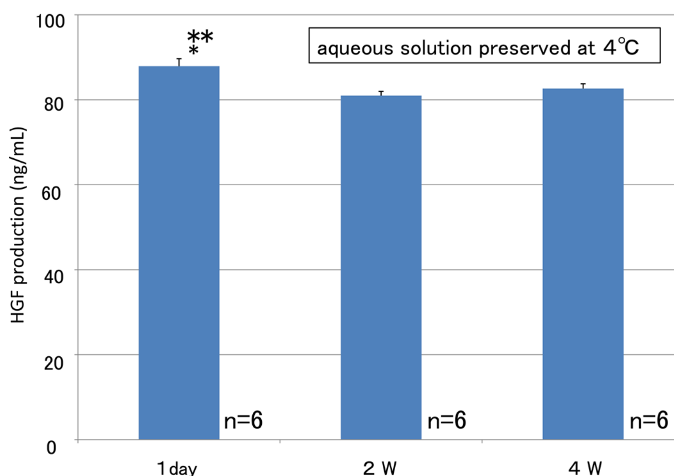


Figure 9. Results on quantitative analysis of HGF production by fibroblast in CDS coated with aqueous solution of EGF-skin care product preserved at 4˚C for 1 day, 2 weeks, and 4 weeks. *$p < 0.01$ vs. 2 weeks **$p < 0.05$ vs. 4 weeks [Tukey-Kramer test].

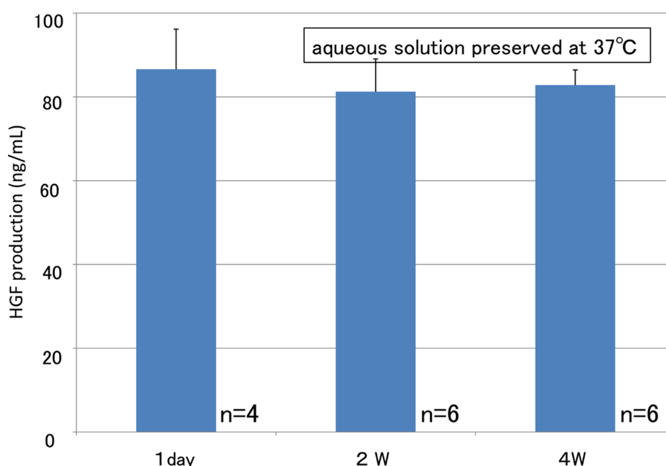


Figure 10. Results on quantitative analysis of HGF production by fibroblast in CDS coated with aqueous solution of EGF-skin care product preserved at 37˚C for 1 day, 2 weeks, and 4 weeks.

4. Discussion

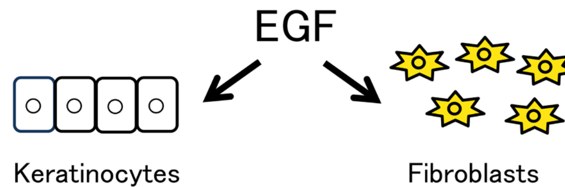
In the present study, we developed EGF-skin care product composed of HA and Col containing EGF and VC, supplemented with GC, PGA, and Arg as cosmetic ingredients. It is generally accepted that incorporation of HA is beneficial in tissue repair products. HA stimulates cell migration, angiogenesis and reduces inflammation [17] [18]. Especially low-molecular weight HA is capable of providing a superior environment for vascular endothelial cell regeneration [19]-[21]. HA has a high moisture-keeping effect because of a superior performance to combine with water molecules. High-molecular weight HA is useful to maintain moisture-keeping environment. Considering these aesthetic medical efficacies, both high-molecular weight and low-molecular weight HA are used as the main ingredient of EGF-skin care product. In addition, Col plays a pivotal role in wound healing. Col and Col-derived peptides act as a chemo-attractants for fibroblasts *in vitro* and may have a similar activity *in vivo* [22]. The medical efficacy is known as following. The polypeptide derived from biodegradation of collagen molecule in a body serves as an attracting factor that can enhance fibroblasts migration from normal area to wound area. Taking this medical efficacy into consideration, medical-grade collagen is used as the ingredient of skin care product.

EGF has a medical efficacy capable of enhancing keratinocyte and fibroblast proliferation, and also stimulating fibroblasts to synthesize increased amount of VEGF and HGF that are potent angiogenesis factors. Taking these medical efficacies into consideration, EGF is used as the ingredient of EGF-skin care product. EGF is known as human oligopeptide-1 by the cosmetics name that is an ingredient of cosmetics approved by Ministry of Health, Labor and Welfare of Japan. Water-soluble Vitamin C derivative, VC is converted into Vitamin C by enzyme reaction in a body. The derivatization is able to increase the external stability and is capable of increasing internal absorption. VC is capable of controlling the occurrence of redness and pigmentation left by an acne, suppressing a sebaceous secretion, and enhancing a collagen synthesis. Considering these aesthetic medical efficacies, VC is used as the ingredient of EGF-skin care product.

EGF-skin care product contains GC, PGA, and Arg as cosmetic ingredients. GC is one of glycolipids. It is the combined product prepared by combining a ceramide and a glucose derived from rice. GC is capable of enhancing moisture-keeping environment by controlling water evaporation from skin. In addition, it can keep a barrier function and prevent an invasion of allergen causing skin trouble. Considering these aesthetic medical efficacies, GC is used as the ingredient of EGF-skin care product. PGA has a function to enhance production of natural moisturizing factors. It is capable of maintaining skin homeostasis and keeping moisture conditions, and has a moisture-keeping effect equal to HA. Moreover, it has a superior film-forming property. Therefore, it is capable of keeping moisture conditions by coating skin surface. Considering these aesthetic medical efficacies, PGA is used as the ingredient of EGF-skin care product. Arg is one of the amino acids produced in a body. The production of Arg decreases gradually with increasing years. Arg is capable of inhibiting glycosylation of protein, and thereby preventing spots and wrinkles and is useful for anti-aging. Moreover, Arg is useful to enhance immunogenicity and thereby facilitating wound healing process. In a previous study, we reported on the development of a wound dressing composed of a HA sponge containing Arg [23]. Taking these aesthetic medical efficacies into consideration, Arg is used as the ingredient of EGF-skin care product.

Additional ingredients are included in EGF-skin care product. GC is water-insoluble. The surface of minute particle of GC is coated with water-soluble alpha-glucan in order to convert it into water-soluble product. In addition, D-mannitol is used as stabilizer for freeze-dried EGF. Therefore, alph-glucan and D-mannitol are included in EGF-skin care product.

The enhancement of keratinocyte proliferation is essential for the treatment of superficial wound. In addition, the enhancement of fibroblasts proliferation and vascularization is essential for the treatment of deeper wound. The application of EGF is promising, because the EGF has both functions to enhance the keratinocyte and fibroblasts proliferation and also stimulate fibroblasts to release vascularization factors (**Figure 11**). The present study aimed to investigate the efficacy of EGF in this skin care product on fibroblast proliferation and fibroblast cytokine production in culture experiments using human fibroblasts. As the first function, EGF can enhance fibroblasts proliferation. The result on cell proliferation experiment demonstrates that fibroblast proliferation is effectively enhanced in a conditioned medium containing a certain amount of EGF-skin care product. As the second function, EGF stimulates fibroblasts to synthesize an increased amount of VEGF and HGF that can enhance strongly vascularization. The finding in this experiment demonstrates that EGF-skin care product effectively stimulated fibroblast to synthesize an increased amount of VEGF and HGF. It is noteworthy that EGF-skin care product can maintain the efficacy of EGF after placing in a dry sterilizer at 110˚C for 30 min for



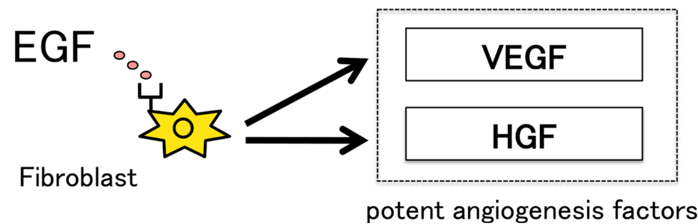


Figure 11. Two main functions of EGF: enhancing keratinocyte and fibroblast proliferation and enhancing fibroblast cytokine production.

sterilization. This is very important performance because EGF-skin care product does not contain any antiseptics in order to be adapted for aesthetic medical use. In addition, the efficacy of EGF was evaluated using an aqueous solution of EGF-skin care product. There was no significant difference in VEGF and HGF production between both conditions of 1 day and 4 weeks preservation at 37˚C. In practice, however, the aqueous solution of EGF-skin care product is recommended to use within 4 weeks under preservation in a refrigerator because of containing no antiseptics.

5. Conclusion

The present study aimed to investigate the efficacy of EGF-skin care product on fibroblasts proliferation and fibroblast cytokine production in culture experiments using human fibroblasts. The result on cell proliferation experiment demonstrates that fibroblast proliferation is effectively enhanced in a conditioned medium containing a certain amount of EGF-skin care product (EGF; 5 μg/100mL). The experiment using wound surface model demonstrated that fibroblasts in the CDS covered with EGF-skin care product released 3.7 times more VEGF and 25 times more HGF compared with control without EGF-skin care product. The efficacy of EGF was maintained even after placing in a dry sterilizer at 110˚C for 30 min for sterilization. In addition, the efficacy of EGF was evaluated using the aqueous solution of EGF-skin care product. The efficacy of EGF was maintained even after preservation at 37˚C for 4 weeks. This performance of skin care product is promising for practical use. The further research is designed to investigate the efficacy of this EGF-skin care product on enhancing the activity of cells damaged by chemical pealing agent in culture experiments using a wound surface model. In addition, the final research is designed to evaluate the potential of this EGF-skin care product in clinical study, especially focused on the skin care after laser treatment.

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