

Advanced Treatment of Burns and Skin Ulcers Using Tissue-Engineered Products

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Abstract

Three types of tissue-engineered products have been introduced in this review. The first product is an allogeneic cultured dermal substitute. This product is manufactured by incorporating allogeneic fibroblasts into a spongy sheet of hyaluronic acid and collagen. Allogeneic fibroblasts are rejected gradually in immune system. However, they are able to release some growth factors that are essential for wound healing. Hyaluronic acid and collagen also have a potential to promote wound healing. The clinical study demonstrated that this cultured dermal substitute is useful for the treatment of burns and skin ulcers. The second product is a functional wound dressing. This product is manufactured by freeze-drying an aqueous solution of hyaluronic acid, collagen, and epidermal growth factor. Epidermal growth factor has a potential to promote wound healing by enhancing both keratinocyte and fibroblast proliferation, and also by stimulating fibroblasts to synthesize vascular endothelial growth factor and hepatocyte growth factor that are essential for angiogenesis. The clinical study demonstrated that this wound dressing is useful for the treatment of burns and skin ulcers. The third product is a skin care product. This product is manufactured by freeze-drying an aqueous solution of hyaluronic acid, collagen, epidermal growth factor, and other ingredients for skin care. The experiment using a culture system demonstrated that this skin care product stimulates fibroblast to synthesize an increased amount of vascular endothelial growth factor and hepatocyte growth factor. This skin care product is aimed to use for post-treatment of chemical peeling and laser therapy used in aesthetic dermatology.

Keywords: Cultured dermal substitute; Wound dressing; Skin care product; Fibroblast; Epidermal growth factor

Introduction

In some cases, burns and skin ulcers fail to proceed through a normal wound healing process. One of the causes is deficiency of growth factors at the wound site. The application of growth factors is useful to improve such a wound condition. Several types of tissue-engineered products have been developed for the treatment of burns and skin ulcers [1,2]. Three types of skin substitutes using autologous or allogeneic cells are manufactured by some culture techniques. Cultured epidermal substitute (CES) is manufactured by using keratinocytes. Cultured dermal substitute (CDS) is manufactured by using fibroblasts and biomaterial. Cultured skin substitute (CSS) is manufactured by using keratinocyte, fibroblast, and biomaterial (Figure 1). The pioneering research of Green and co-workers demonstrated that it is possible to grow keratinocytes as a stratified sheet [3,4]. Autologous CES has been evaluated in many hospitals and proved very useful for the treatment of severe burns [5-11]. Another pioneering research of Bell and co-workers demonstrated that

CSS manufactured by using keratinocyte, fibroblast, and collagen gel sheet has an ideal skin-equivalent structure [12-14]. Allogeneic cells are rejected gradually in immune system. However, they are able to release some types of growth factors that are necessary for wound healing to promote. Allogeneic CSS has been evaluated in many hospitals and proved very effective for the treatment of skin ulcers [15].

As the first research strategy, we developed an allogeneic CDS. This product is manufactured by incorporating allogeneic fibroblast into a two-layered spongy sheet of hyaluronic acid (HA) and collagen (Col) [16-23]. The biomaterial itself has a potential to promote wound healing. This spongy sheet has an intermolecular cross-linking among HA and Col molecules, and therefore this CDS is able to be cryopreserved for a long period and then thawed prior to clinical application. This CDS can release various types of growth factors even after cryopreserving and thawing process. As the second research strategy, we developed a functional wound dressing that is composed of a spongy sheet of HA and Col containing epidermal growth factor (EGF) [24-26]. EGF is able to facilitate both keratinocyte and fibroblast proliferation. In addition, it is capable of stimulating fibroblast to synthesize an increased amount of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (Figure 2). In such a reason, EGF is considered as one of the ideal growth factors for

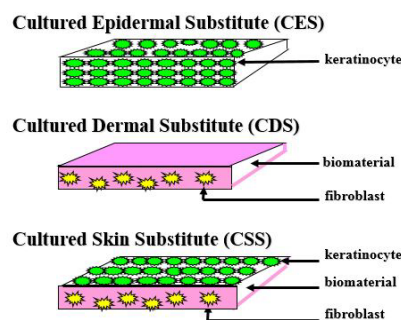


Figure 1: Structure of tissue-engineered products: CES manufactured by using keratinocyte, CDS manufactured by using fibroblast and biomaterial, and CSS manufactured by using keratinocyte, fibroblast and biomaterials.

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wound healing. As the third research strategy, we developed EGF-incorporating skin care product. This skin care product is aimed to use for post-treatment of chemical peeling and laser therapy.

Materials and Methods

Cultured dermal substitute using cell and biomaterial

Properties of biomaterial used in cultured dermal substitute: HA is a useful biomaterial for wound healing, as it has various biological activities [27-31]. HA is able to create an excellent wound healing environment. HA also stimulates cell migration and angiogenesis. Col is also a useful biomaterial for wound healing. Col-derived peptides are able to act as a chemoattractant for fibroblasts *in vitro* and may have a similar activity *in vivo* [32].

Preparation of allogeneic CDS: Allogeneic CDS (10 cm × 10 cm) was manufactured by seeding fibroblasts on the Col layer of two-

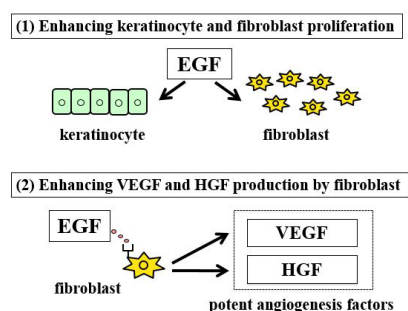


Figure 2: Functions of EGF: (1) Enhancing keratinocyte and fibroblast proliferation (2) Enhancing VEGF and HGF production by fibroblast.

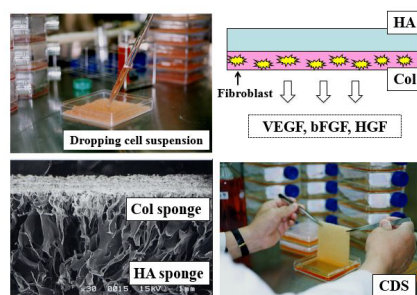


Figure 3: CDS manufactured by seeding and culturing fibroblasts within the Col layer of two-layered spongy sheet of HA and Col, followed by cryopreserving. Prior to clinical application, the cryopreserved CDS is thawed and rinsed with lactated Ringer's solution. Fibroblasts in CDS synthesize and release VEGF, bFGF and HGF.

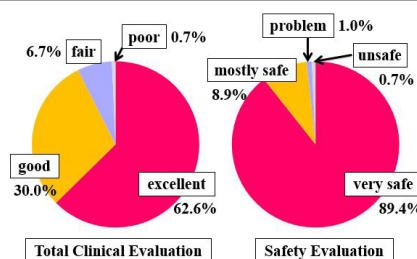


Figure 4: Results of total clinical evaluation and safety evaluation in 415 clinical cases, including severe burns and intractable skin ulcers. It was observed that excellent and good results came to 92.6% in all, also very safe and mostly safe results came to 98.3% in all.

layered spongy sheet of HA and Col, followed by culturing for 1 week (Figure 3). This spongy sheet has an intermolecular cross-linking among HA and Col molecules, and therefore this CDS is able to be cryopreserved for a long period and then thawed prior to clinical application. When this CDS is applied on a wound surface, this spongy sheet is biodegraded within 1 week. In practice, this spongy sheet is manufactured by a two-step freeze-drying method. As a first step, a spongy sheet of chemically cross-linked HA is manufactured by freeze-drying an aqueous solution of HA containing cross-linking agent. As a second step, this spongy sheet of cross-linked HA is soaked into an aqueous solution of Col, followed by freeze-drying to obtain a two-layered spongy sheet of HA and Col. Finally, Col spongy side is irradiated with an ultraviolet lamp in order to induce intermolecular cross-linking among Col molecules [17].

Cell banking was established in accordance with the procedure described in our article [16]. A small piece of skin (1 cm × 1 cm) was obtained from patients younger than one-year-old during surgical excision of excrescence. The skin donors were checked to be free from infectious viruses such as HBV, HCV, HIV and HTLV, and also negative on the treponema pallidum hemagglutination test. Fibroblasts were isolated from a skin and then cultured successively. These fibroblasts were suspended in a cryopreservation medium and then cryopreserved in liquid nitrogen according to a conventional procedure. Prior to manufacturing CDS, a part of these cryopreserved fibroblasts was thawed and then cultured successively to get an adequate number of cells. Cryopreserving and thawing CDS were performed in accordance with the method described in the article [17]. The amounts of VEGF, basic fibroblast growth factor (bFGF), and HGF were measured [18,19]. This experiment showed that the cryopreserved CDS is able to keep the potential to release VEGF, bFGF, and HGF at appreciable levels.

Clinical study using allogeneic CDS: A multi-center clinical study using allogeneic CDS was conducted at 31 hospitals as a project of Regenerating Medical Millennium Projects of the Ministry of Health, Labour and Welfare. During this period of the project, 4700 sheets of allogeneic CDS were manufactured and cryopreserved at -153°C at the R&D Center for Artificial Skin, School of Allied Health Sciences, Kitasato University. The clinical study was carried out in compliance with the ethical guidelines of the participating hospitals. The cryopreserved CDS were delivered to the participating hospitals using a box filled with dry ice and then continuously cryopreserved in a freezer at -85°C. Prior to clinical application, the cryopreserved CDS was thawed and rinsed with lactated Ringer's solution.

In practice, wound surface was debrided as necessary and rinsed with physiological saline solution. CDS was placed cell-seeded side down on the wound surface. CDS was applied to the wound surface, on which a conventional ointment-gauze dressing was placed to fix the CDS. This treatment using CDS together with ointment-gauze dressing was applied repeatedly at an interval of 5 to 7 days for a period of 6 weeks, or longer if necessary. The efficacy of this treatment was assessed in 415 cases, including severe burns and intractable skin ulcers. Excellent result was assessed in 62.6%, good result was assessed in 30.0%, fair result was assessed in 6.7%, and poor result was assessed in 0.7% of the all cases in total clinical evaluation. Very safe result was assessed in 89.4%, mostly safe result was assessed in 8.9%, problem result was assessed in 1.0%, and unsafe result was assessed in 0.7% of the all cases in safety evaluation (Figure 4). The representative clinical results have been reported in the articles [20-23].

The representative clinical results are shown here:

A 95-year-old woman with an intractable skin ulcer was treated with

allogeneic CDS. The wound condition improved 1 month later, showing a reduction in wound size associated with a good granulation tissue formation. The skin ulcer healed mostly 2 months later (Figure 5).

A 90-year-old woman with a squamous cell carcinoma was treated with allogeneic CDS. CDS was applied to the debrided wound surface. The wound condition improved, showing a good granulation tissue formation, and the debrided wound surface healed completely 28 days later (Figure 6).

A 65-year-old man with a necrotic lesion on the right foot was treated with allogeneic CDS. At 39 days, a favourable wound bed was prepared, on which a split-thickness auto-skin graft was performed. At 83 days after auto-skin grafting, the condition of grafted skin was excellent (Figure 7).

A 60-year-old man with an intractable ulcer on the left leg was



Figure 5: A 95-year-old woman with an intractable skin ulcer was treated with allogeneic CDS. CDS was applied on a debrided wound repeatedly at an interval of 5 to 7 days. The wound condition improved 1 month later. The skin ulcer healed mostly 2 months later.

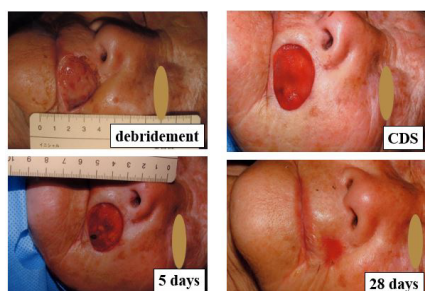


Figure 6: A 90-year-old woman with a squamous cell carcinoma was treated with allogeneic CDS. CDS was applied on a debrided wound repeatedly at an interval of 5 to 7 days. The wound was improved, and then healed 28 days later.



Figure 7: A 65-year-old man with a necrotic lesion on the right foot was treated with allogeneic CDS. CDS was applied on a debrided wound repeatedly at an interval of 5 to 7 days. At 39 days, a favourable wound bed was prepared, on which a split-thickness auto-skin graft was performed. After 83 days of auto-skin grafting, the condition of grafted skin was excellent.

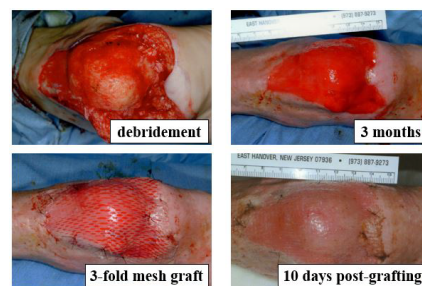


Figure 8: A 60-year-old man with an intractable ulcer on the left leg was treated with allogeneic CDS. CDS was applied on a debrided wound repeatedly at an interval of 5 to 7 days. At 3 months, a favourable wound bed was prepared, on which a 3-fold extended mesh split-thickness auto-skin graft was performed. At 10 days after auto-skin grafting, the condition of grafted skin was excellent.

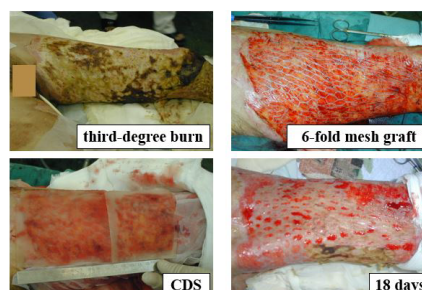


Figure 9: An 81-year-old woman who suffered a third-degree burn on the left leg was treated with an auto-skin graft and allogeneic CDS. In this case, a 6-fold extended mesh split-thickness auto-skin graft was applied, on which CDS was placed. CDS was applied repeatedly at an interval of 5 to 7 days. This extended mesh graft took successfully, and showed excellent epithelialization 18 days later, despite poor wound conditions.

treated with allogeneic CDS. At 3 months, a favourable wound bed was prepared, on which a 3-fold extended mesh split-thickness auto-skin graft was performed. At 10 days after auto-skin grafting, the condition of grafted skin was excellent (Figure 8).

An 81-year-old woman who suffered a third-degree burn on the left leg was treated with an auto-skin graft. In this case, a 6-fold extended mesh split-thickness auto-skin graft was applied, on which CDS was placed. This extended mesh graft took successfully and showed excellent epithelialization 18 days later; despite poor wound conditions in elderly patient with severe burns (Figure 9).

An 88-year-old woman with a necrotizing fasciitis was treated with an auto-skin graft. In this case, a 6-fold extended mesh split-thickness auto-skin graft was applied, on which CDS was placed. This extended mesh graft took successfully and showed excellent epithelialization 28 days later; despite poor wound conditions (Figure 10).

Wound dressing using growth factor and biomaterial

Property of EGF: EGF has some useful effects for wound healing. EGF is able to enhance both keratinocytes and fibroblasts proliferation, thus facilitating granulation tissue formation as well as epithelialization [33,34]. In addition, EGF is able to stimulate fibroblasts to synthesize an increased amount of VEGF and HGF that are important to facilitate angiogenesis. Another research demonstrated that simultaneous administration of VEGF and HGF is able to promote synergistically new blood vessel formation compared with administration of each factor alone [35]. HGF has a useful effect for angiogenesis as well as epithelialization [36]. Because of these reasons, EGF is a promising factor in order to promote wound healing.

Preparation of EGF-incorporating wound dressing: EGF-incorporating wound dressing was manufactured by freeze-drying an aqueous solution of high molecular weight HA, low molecular weight HA, and Col containing EGF. High molecular weight HA is useful to create an excellent wound healing environment, while low molecular weight HA is useful to promote angiogenesis. Both side of spongy sheet was irradiated with an ultraviolet lamp in order to induce intermolecular cross-linking among Col molecules [24-26]. This wound dressing (5 cm × 8 cm) contains EGF at a concentration of 2.0 µg/cm².

Clinical study using EGF-incorporating wound dressing: The

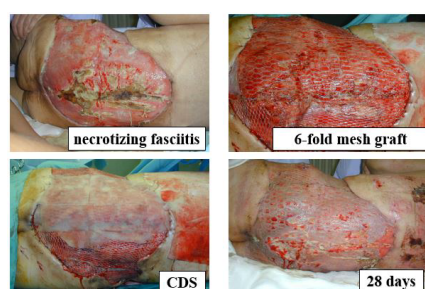


Figure 10: An 88-year-old woman with a necrotizing fasciitis was treated with an auto-skin graft and allogeneic CDS. In this case, a 6-fold extended mesh split-thickness auto-skin graft was applied, on which CDS was placed. CDS was applied repeatedly at an interval of 5 to 7 days. This extended mesh graft took successfully, and showed excellent epithelialization 28 days later, despite poor wound conditions.



Figure 11: A 74-year-old woman with a traumatic ulcer on the left leg. The wound was debrided, on which EGF-incorporating wound dressing was applied twice a week. Wound size decreased significantly 6 weeks later. The wound healed approximately 3 months later.



Figure 12: A 65-year-old woman with a skin ulcer on the right knee. The wound was debrided, on which EGF-incorporating wound dressing was applied twice a week. Wound condition improved and a favorable wound bed was prepared 6 weeks later, on which a full-thickness auto-skin graft was applied and taken well. At 2 months after auto-skin grafting, the condition of grafted skin was excellent.

fundamental study showed an excellent result [24,25]. Based on this result, the efficacy of EGF-incorporating wound dressing was assessed in 16 clinical cases, including burns, donor sites, traumatic skin defects, and intractable skin ulcers. Wound surface was debrided as necessary and rinsed with physiological saline solution. As basic usage, EGF-incorporating wound dressing was placed on the wound site, on which a commercially available polyurethane film dressing was placed as a top dressing. In the case of wounds with excess amounts of exudate, a conventional ointment-gauze dressing was used as a top dressing. This treatment using EGF-wound dressing together with each top dressing was applied repeatedly at an interval of 3 to 5 days for a period of 6 weeks, or longer if necessary. Good or excellent result was assessed in 93.7% of the all cases in total clinical evaluation [26].

The representative clinical results are shown below:

A 74-year-old woman with a traumatic ulcer on the left leg was treated with EGF-incorporating wound dressing, basically twice a week. Wound size decreased significantly 6 weeks later. The wound healed mostly 3 months later (Figure 11).

A 65-year-old woman with a skin ulcer on the right knee was treated with EGF-incorporating wound dressing, basically twice a week. At 6 weeks, a favourable wound bed was prepared, showing a reduction in wound size associated with a good granulation tissue formation, on which a split-thickness auto-skin graft was performed. At 2 months after auto-skin grafting, the condition of grafted skin was excellent (Figure 12).

Skin care product using growth factor and biomaterials

Properties of ingredients for skin care: Arginine (Arg) has useful biological properties. One report demonstrated that Arg is necessary for T lymphocyte maturation [37]. Another report demonstrated that Arg is a major substrate for the production of nitric oxide, which is toxic to tumors and infected cells. Other reports indicated that Arg and Arg-derived nitric oxide are useful for wound healing [38-42]. Vitamin C (VC) has useful biological properties. One report demonstrated that VC has a potential to enhance HGF secretion by fibroblasts, when VC is applied together with EGF [43]. For this reason, the combination uses of EGF and VC have a higher potential to stimulate fibroblasts to synthesize an increased amount of VEGF and HGF [44]. Poly-γ-glutamic acid (PGA) has a potential to enhance production of natural moisturizing factors. PGA has a superior film-forming property. By such reason, PGA is able to keep moisture conditions by coating a skin surface. Glucosyl ceramide (GC) has a potential to connect epidermal keratinocytes themselves, and enhancing moisture-keeping environment by controlling water evaporation from skin. Because of these reasons, EGF-incorporating skin care product was manufactured by freeze-drying an aqueous solution of high molecular weight HA, low molecular weight HA, Col, Arg, VC-derivative, GC, PGA, and EGF. This skin care product is water-soluble, because of no intermolecular cross-linking among HA and Col. This skin care product (5 cm × 8 cm) contains EGF at a concentration of 1.0 µg/cm² [45].

Post-treatment of chemical peeling and laser therapy: A partially damaged skin is capable of healing through the repair mechanisms of skin. Based on this regenerative potential, chemical peel has been widely used in aesthetic dermatology. Chemical agents cause exfoliation of the skin. This treatment leads to the skin rejuvenation through a normal wound healing process. However, the wound conditions depend on the concentration of the acid and the duration of exposure [46-48]. This skin damage is similar to a superficial chemical burn. Therefore, excellent skin care products are needed for post-treatment of chemical

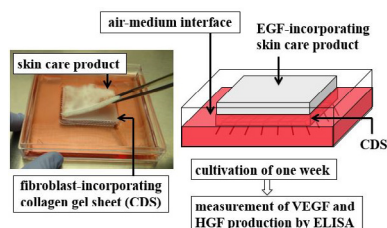


Figure 13: Measurement method of VEGF and HGF production by fibroblast using wound surface model: EGF-incorporating skin care product is placed on a wound surface model. After cultivation of 1 week, the amount of VEGF and HGF was measured by ELISA.

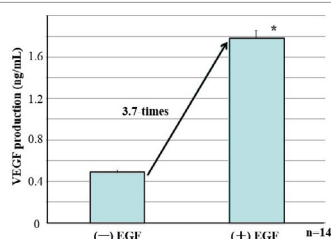


Figure 14: Amount of VEGF production by fibroblast in collagen gel sheet, covered with or without EGF-incorporating skin care product; (+) EGF or (-) EGF, * $p < 0.01$ vs. (-) EGF [Student's t test].

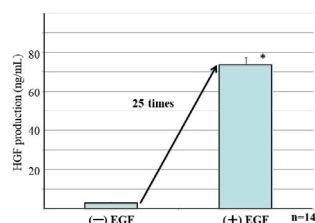


Figure 15: Amount of HGF production by fibroblast in collagen gel sheet, covered with or without EGF-incorporating skin care product; (+) EGF or (-) EGF, * $p < 0.01$ vs. (-) EGF [Student's t test].

peeling. As well as chemical peeling, laser therapy is widely used in order to improve skin condition. Laser therapy also results in tissue damage. This treatment leads to the skin rejuvenation through a normal wound healing process. When laser treatment is strong, a deep wound like burn injury is caused. Therefore, excellent skin care products are needed for post-treatment of laser therapy. The common issue for chemical peeling and laser therapy is possible to obtain a satisfactory clinical result without an undesirable skin contraction and scar formation.

Fundamental study using EGF-incorporating skin care product:

In order to evaluate the efficacy of EGF-incorporating skin care product, the amount of VEGF and HGF production by fibroblasts was measured by ELISA using a wound surface model (Figure 13). A fibroblast-incorporating collagen gel sheet was elevated to the air-medium interface. This culture system is similar to a wound surface model. A spongy sheet of EGF-incorporating skin care product was placed on this wound surface model (+EGF group) and cultured for 7 days. In control group, no skin care product was placed (-EGF group). The result demonstrated that fibroblasts in collagen gel sheet released 3.7-times more VEGF (Figure 14) and 25-times more HGF in +EGF group compared with -EGF group (Figure 15). EGF-incorporating

skin care product has a potential to stimulate effectively fibroblast to synthesize an increased amount of VEGF and HGF [45].

Discussion and Conclusion

The application of allogeneic fibroblast is useful, because this cell is able to synthesize various types of growth factors capable of enhancing wound healing. The application of HA and Col is also useful, because these biomaterials have a potential to facilitate wound healing. The clinical study demonstrated that allogeneic CDS is useful for the treatment of burns and skin ulcers. Another clinical study showed that EGF-incorporating wound dressing is useful for the treatment of burns and skin ulcers. The experiment using a culture system demonstrated that EGF-incorporating skin care product can enhance the VEGF and HGF production by fibroblast. This skin care product is promising for post-treatment of chemical peeling and laser therapy used in aesthetic dermatology.

Future Perspectives

As future clinical research, it is worth examining the efficacy of allogeneic CDS combined with EGF-incorporating skin care product. As basic usage, CDS was applied to the wound surface, on which a conventional ointment-gauze dressing was used to fix the CDS. In future clinical study, CDS is applied to the wound surface, on which EGF-incorporating skin care product is placed, and more a conventional ointment-gauze dressing is used as a top-dressing. This additional EGF can enhance VEGF and HGF production by fibroblasts in CDS.

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