るプログラミングフリーザの使用が望ましい．しかし，容積が小さいため同種培養真皮の大量凍結には使用できない．そこで，血液バッグの大量凍結保存に使用する大型温度下降制御フリーザーを使用した（図7）．この装置では，氷結領域の温度下降を厳密にコントロールすることはできないが，凍結・解凍操作後の同種培養真皮の細胞生存率とVEGF産生能は高く維持されており，臨床使用において十分な効力を発現することが可能である．実際には，各医療施設において正しい方法で解凍操作を行うことが重要な鍵となる．

まとめ

本研究は，再生医療を具体的に推進するために企画された厚生科学再生医療ミレニアムプロジェクトの一つであり，将来，わが国の企業が細胞を組み込んだ培養皮膚などを製品化するためのパイロットスタディーである．本論文では，安全性を確保した同種培養真皮の製造と輸送，ならびに各施設における凍結保存と解凍など，多施設臨床研究を推進するための基盤整備について記載した．

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Munufacturing and Banking System for Allogeneic Cultured Dermal Substitute

(Regenerating Medical Millennium Project of the Ministry of Health, Labor and Welfare)

Yoshimitsu Kuroyanagi*, Kentaro Kubo*, Hiromichi Matsui*, Shizuko Kagawa*, Satoko Mori*, Hyun Jung Kim* and Yho Mabuchi*

Regenerative tissue engineering is moving rapidly from fundamental research to commercial applications. A number of skin substitutes have been produced by *in vitro* culture techniques. The U.S. Food and Drug Administration has already approved allogeneic skin substitutes. Recently, the Japanese Ministry of Health, Labor and Welfare published guide- lines for regenerative medicine. Kitasato University R & D Center for Artificial Skin headed by Yoshimitsu Kuroyanagi was charged with promotion of the Health and Welfare Ministry's Highly Advanced Medical Technology Research Project in 1998～99.

The center has been the heart of the Regenerating Medical Millennium Project (skin department) of the Ministry of Health, Labor and Welfare since 2000. This report includes the establishment of a banking system for multi-center's clinical study using the allogeneic cultured dermal substitute (CDS) composed of hyaluronic acid and collagen spongy matrix with fibroblasts. The master cells for massproduction of CDS were established with fibroblasts derived from skin samples about 1 cm^2 in size donated by three young donors who were free from infectious viruses such as HIB, HBV, HCV, and HTLV. In the period of 2001.4 to 2002.9, about 2700 sheets of CDS 100 cm^2 in size were produced in the R & D Center of Kitasato University and supplied to 30 hospitals across Japan.

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Guest Editorial

Studies on Regenerative Medicine in Japan

Tissue engineering is moving rapidly from fundamental research to commercial applications. Structural tissues, such as skin, cornea and cartilage, will most likely continue to dominate the first wave of success stories, thanks to their relative simplicity in structure as well as in cell source. The U.S. Food and Drug Administration has already approved a living skin product. The next tissue to be widely used in humans will most likely be cartilage for orthopedic and craniofacial applications. Regenerating Medical Millennium Projects of the Ministry of Health, Labor and Welfare have started in Japan since 2001. These projects cover many fields including skin, cornea, bone, cartilage, blood vessels, nerves and bone marrow.

Regenerative medicine is based on tissue engineering, a new field of science. In general, the tissue-engineered products include three prime constituents, i.e., cells, cell growth factors and materials (often referred to as scaffolds). In the first approach, cell growth factors, such as basic fibroblasts growth factor (bFGF), endothelial growth factor (VEGF) and bone morphogenic protein (BMP), are applied into a site of damaged tissues, together with a proper material to make it possible to release in a sustained fashion. In some cases, these factors are applied directly into a site of damaged tissues. These factors cause the patient's own cells to migrate into the desired area, turn into the correct type of cell and regenerate the lost tissue.

In the second approach, the usual procedure entails the proliferation of isolated cells in culture. These cells are seeded on or within a scaffold, such as biodegradable synthetic polymer or collagen, the protein that forms the natural support scaffolding of most tissues. As well as delivering the cells, the scaffold both creates and maintains a space for the formation of new tissue and guides its structural development. In the case of bone, bio-ceramic is used as a scaffold to maintain adequate mechanical strength. In this approach, the patient receives autologous cells that have been harvested previously and incorporated into a three-dimensional scaffold. The entire structure of cells combined with a scaffold is transplanted into the lost area of tissue, where the cells replicate, reorganize and form new tissue. When a biodegradable material is used as a scaffold, the scaffolding materials break down, leaving only a completely natural final product, a neo-organ in the body. In some cases, cell growth factors were preloaded into a scaffold to promote the formation of a neo-organ. According to this technique, allogeneic cells can also be combined with a scaffold. Although these cells are rejected gradually in the immune system, they are able to release some types of cell growth factors, and regenerate a damaged tissue as quickly as possible.

Of course, the holy grail of tissue engineering remains complete internal organs such as liver, pancreas, and kidney. A number of investigators have demonstrated that new liver-like tissues can be created in animals from transplanted liver cells, but the entire function of the organ has not yet been replicated. Some investigators have been attempting the implantation of encapsulated pancreatic islets isolated from pigs, the clusters of cells that contain the insulin-secreting components, to restore the proper pattern of insulin release. Some investigators are using kidney cells in animal tests to make a neo-organ that possesses the filtering capability of the kidney. Even the heart is a target for regenerative medicine. Some researchers have been trying the application of cardiac muscle cells derived from bone marrow to repair the damaged area of heart. It will likely take scientists ten to twenty years to learn how to grow an entire heart, but tissues such as heart valves and blood vessels may be available sooner.

The tissue-engineered products used in clinical applications are composed of human cells isolated from own tissue or a donor's tissue. The successful application is dependent on the cell source. The three products skin, cornea and cartilage can be prepared by using their cultured cells. On the contrary, other sophisticated organs with various functions should be prepared by using cells derived from bone

marrow or embryonic stem cells. This is why the study of these organs has remained at the fundamental step. The practical use of these organs depends on how to proliferate these cells in culture after isolation from bone marrow or the mass of embryonic stem cells.

This review includes studies on regenerative medicine in Japan, covering various organs, i.e., skin, cornea, cartilage, bone, blood vessels, liver, pancreas, kidney and heart.

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Establishment of Banking System for Allogeneic Cultured Dermal Substitute

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Abstract: Allogeneic cultured dermal substitute (CDS) was prepared by culturing fibroblasts on a two-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col). Allogeneic CDS can be cryopreserved and transported to other hospitals in a frozen state. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF)-AA, transforming growth factor (TGF)-β1, keratinocytes growth factor (KGF), interleukin (IL)-6 and IL-8 were contained in the culture medium which was used in preparing CDS over a cultivation period of one week (fresh CDS culture medium sample). After thawing a cryopreserved CDS, the CDS was recultured in a culture medium for one week. VEGF, bFGF, HGF, TGF-β1 and IL-8 were contained in the culture medium which was used in reculturing CDS for one week (cryopreserved CDS culture medium sample), although some cytokines were detected at a lower level than those before freezing. This finding suggests that the cryopreserved CDS retains its ability to release these cytokines.Clinical research on allogeneic CDS, which was newly developed at the R & D Center for Artificial Skin of Kitasato University, has been carried out in medical centers across Japan with the support of the Millennium Project of the Ministry of Health, Labor and Welfare. It was demonstrated that the allogeneic CDS functions as an excellent cell therapy for intractable skin ulcers as well as burn injuries. The spongy matrix itself, as well as the cytokines released from the allogeneic CDS, seemed to be beneficial for the treatment of intractable skin defect. **Key Words:** Cultured dermal substitute—Fibroblasts—Hyaluronic acid—Collagen—Cell growth factor—Tissue engineering.

A variety of skin substitutes have been developed (Table 1 and Fig. 1), and the following are commercially available in the U.S.A.: autologous cultured epidermal substitute (CES) composed of stratified keratinocytes (1–5) (Epicel); allogeneic cultured dermal substitute (CDS) composed of fibroblasts on a scaffold (6–11) (Dermagraft and Trans Cyte); and allogeneic cultured skin substitute (CSS) composed of keratinocytes and fibroblasts on a scaffold (12–16) (Apligraf). Allogeneic CDS and CSS require an appropriate matrix, i.e., a scaffold for fibroblasts and/or keratinocytes. Recently, however, the use of these allogeneic products has declined, possibly due to problems in material design.

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Kuroyanagi and colleagues developed an allogeneic CDS composed of a spongy collagen containing fibroblasts (17–20). The efficacy of this allogeneic CDS on wound healing has been studied in animal tests and in preliminary clinical trials. On the basis of this technique, an advanced version of CDS was developed through the cultivation of fibroblasts on a two-layered spongy matrix of HA and Col (21–25). HA has a high capacity for hydration, resulting in a moist environment at the wound site. HA molecules

TABLE 1. *Type and function of tissue-engineered skin products*

(1) Autologous products: permanent coverage
Autologous cultured epidermal substitute*
Autologous cultured dermal substitute
Autologous cultured skin substitute
(2) Allogeneic products: cell therapy for wound healing
Allogeneic cultured epidermal substitute
Allogeneic cultured dermal substitute*
Allogeneic cultured skin substitute*

* U.S.A. products.

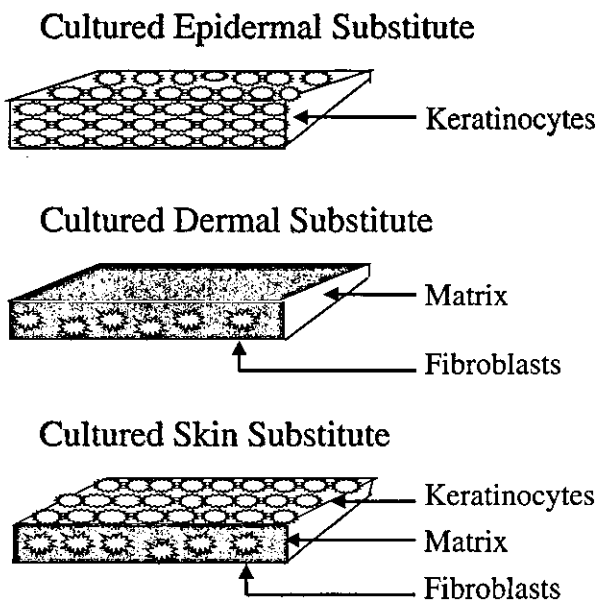

FIG. 1. Structure of tissue-engineered skin products.

play a critical role in several cellular functions, such as migration and proliferation, by promoting adhesion and disadhesion on the tissue substrate (26). Collagen and collagen-derived peptides act as chemoattractants for fibroblasts in vitro, and may have similar activity in vivo (27). The fibroblasts incorporated in this CDS can release cytokines and extracellular matrix (ECM) that are necessary for wound healing. This CDS is designed to promote wound healing by synergic effect of both the fibroblasts and the matrix.

Since April 2001, the R&D Center for Artificial Skin of Kitasato University has been the heart of the Regenerating Medical Millennium Project (skin department) of the Ministry of Health, Labor and Welfare. This center has established a banking system for cryopreserved allogeneic CDS, and distributed it to hospitals in a frozen state (28). A multicenter clinical study on the use of allogeneic CDS was performed in thirty hospitals across Japan. The results of a multicenter clinical study suggest that this type of allogeneic CDS can effectively promote the healing of full-thickness severe skin defects, such as chronic ulcers and burn injuries (29,30).

ESTABLISHMENT OF CELL BANKING

Cell banking was established by the procedure described in previous articles (22,23). A small piece of skin was obtained from patients younger than 1 year old during surgical excision of excrescence. These patients were free from infectious viruses such as HBV, HCV, HIV and HTLV, and also negative on the treponema pallidum hemagglutination test (TPHA). All procedures were in compliance with the ethical guidelines of St. Marianna Medical College. The sterilized piece of skin was immersed in Dulbecco's modified Eagle's medium (DMEM) containing dispase for 20 h at 4°C. After this enzymatic treatment, the epidermis was mechanically separated from the dermis. The dermis was minced and then treated with 0.5% collagenase in DMEM supplemented with 1% fetal bovine serum (FBS) for 2 h at 37°C to obtain the cellular suspension. Fibroblasts isolated by enzymatic treatment were cultivated successively in a culture medium to establish cell banking. The cultured fibroblasts were suspended in a cryo-tube containing DMEM supplemented with 10% DMSO and 20% FBS and then cryopreserved in liquid nitrogen according to a conventional procedure. The cells were checked to be negative for viruses including HBV, HCV, HIV, HTLV and Parvovirus.

PREPARATION OF CULTURED DERMAL SUBSTITUTE (CDS)

The CDS was prepared using a method described in previous articles (22,23). Prior to seeding of fibroblasts, the two-layered sponge of HA and Col (10.5 cm × 9.5 cm) was immersed in 50 mL of culture medium in a polystyrene dish (11 cm × 10 cm) to hydrate the sponge (Fig. 2). Excess culture medium was carefully removed from the dish by suction. The fibroblasts obtained from successive cultivation of the cryopreserved cells were seeded onto the two-layered sponge by adding 5 mL of cellular suspension dropwise onto the collagen surface of the sponge. The number of fibroblasts on the two-layered sponge was adjusted to 1.0×10^5 cells/cm^2. The cell-seeded sponge was kept in an incubator in a humidified atmosphere of 5% CO_2 at 37°C overnight, followed by the addition of 50 mL of cultured medium and culturing for one week (Fig. 3). The fibroblasts used in production of CDS were checked for mycoplasma and confirmed to be negative. The culture medium used in the production of CDS was checked for bacteria and confirmed to be negative.

CRYOPRESERVING AND THAWING OF CDS

Cryopreserving and thawing of CDS was performed according to the method described in previous articles (22,23). The CDS was turned upside down in a polystyrene dish and the culture medium was replaced with 30 mL of DMEM supplemented with 10% DMSO and 20% FBS. The CDS was frozen

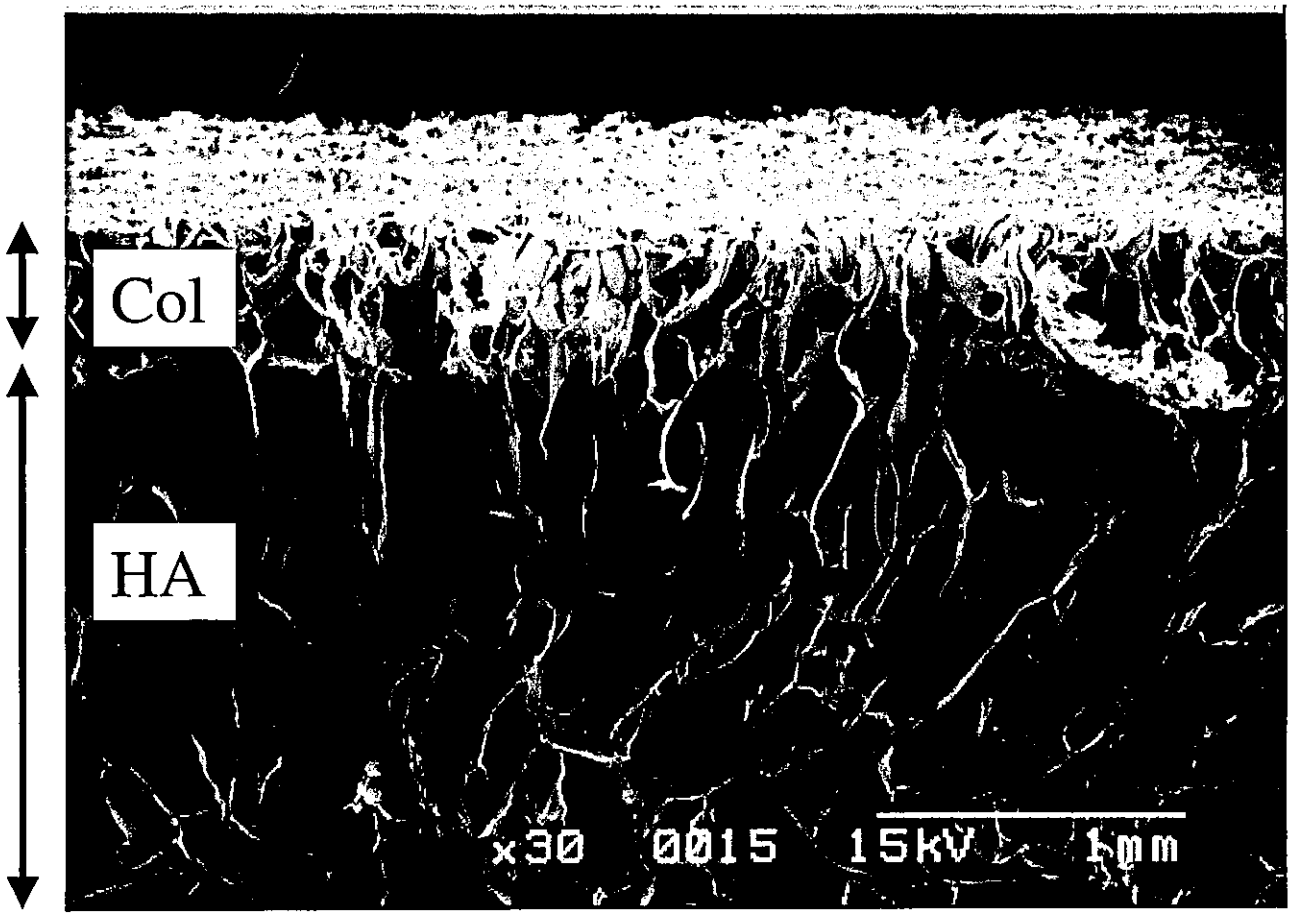

FIG. 2. Cross-sectional view of scanning electron microphotograph of a spongy matrix composed of HA and Col.

in the dish in a programmable freezer at a gradient of −1°C/min from 4°C to −60°C and then cryopreserved in a freezer at −152°C. The cryopreserved CDS (in the polystyrene dish) was placed in a foam polystyrene box containing dry ice and then shipped to hospitals, where it was preserved at −85°C. Prior to clinical application, the CDS (in the polystyrene dish) was placed in a foam polystyrene box at room temperature for 30 min and then floated in a water bath at 37°C, followed by rinsing with lactated Ringer's solution to remove DMSO and FBS.

QUANTITATIVE ANALYSIS OF CYTOKINES

It is well known that fibroblasts have a potency to release some types of cytokines, but the amount of cytokine is dependent on the culturing conditions. We measured the amounts of the eight cytokines VEGF, bFGF, HGF, PDGF-AA, TGF-β1, KGF, IL-6 and IL-8 (31). The culture medium used in preparing CDS (fresh CDS culture medium sample) was collected and stored at −30°C. After the cryopreserved CDS was thawed, it was recultured in a culture medium for one week. The culture medium used in reculturing cryopreserved CDS (cryopreserved CDS culture medium sample) was collected and stored at −30°C. The amount of cytokines in these culture medium samples was measured by enzyme-linked immunosorbent assay (ELISA).

Figure 4 shows the amount of each cytokine in the medium before freezing (fresh CDS culture medium sample), and after thawing and reculturing for one week (cryopreserved CDS culture medium sample). The amounts of VEGF and bFGF in the cryopreserved CDS culture medium were the same as those of the fresh CDS culture medium (Fig. 4A,B). The amounts of HGF, TGF-β1 and IL-8 in the cryopreserved CDS culture medium were slightly lower than those of the fresh CDS culture medium (Fig. 4C,E,H). The amounts of PDGF-AA, KGF and IL-6 in the cryopreserved CDS culture medium were considerably lower than those of the fresh CDS culture medium (Fig. 4D,F,G). The results shown in Fig. 4 demonstrate that the cryopreserved CDS can

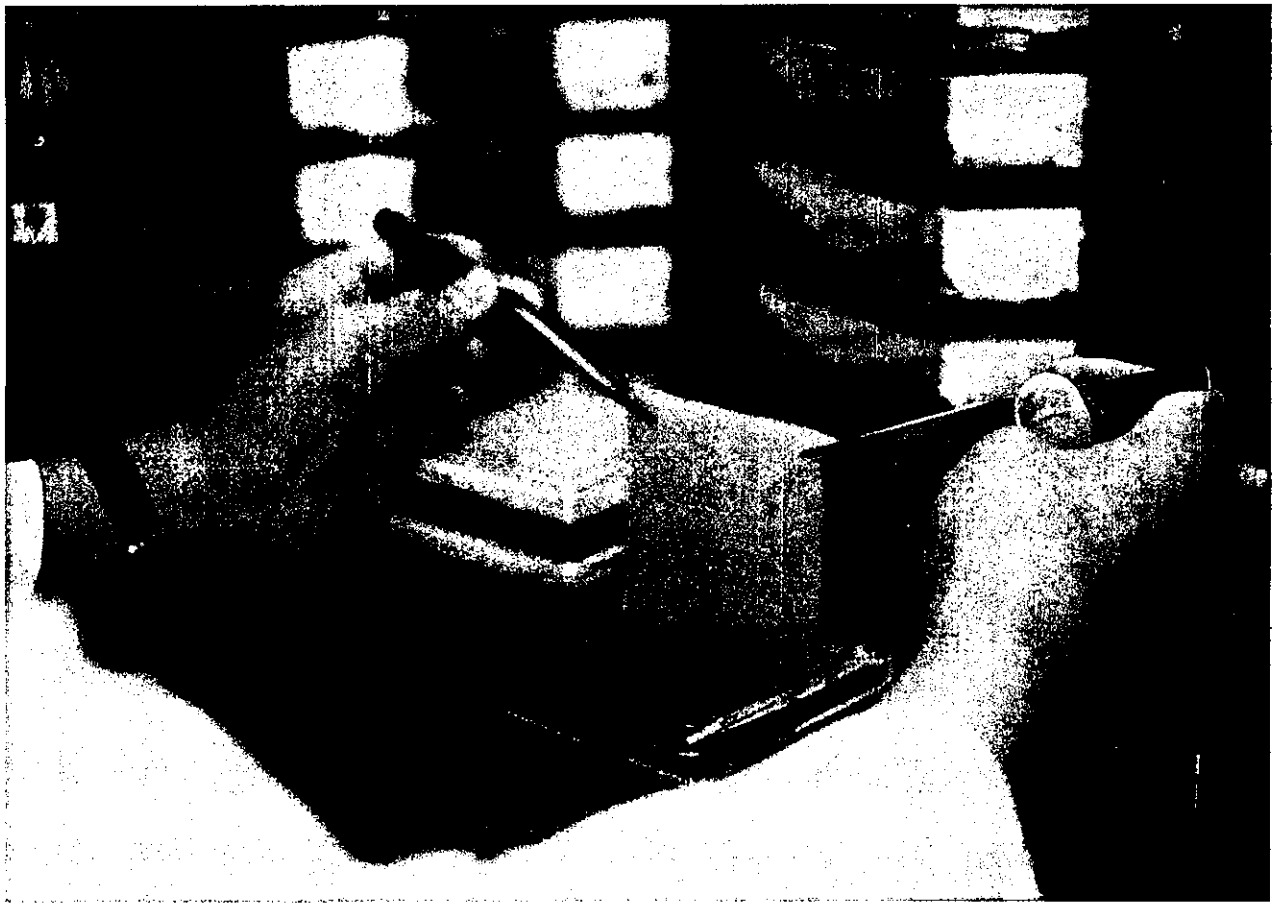

FIG. 3. Allogeneic CDS composed of fibroblasts combined with a two-layered spongy matrix of HA and Col.

release VEGF, bFGF, HGF, TGF-β1 and IL-8 at appreciable levels. The number of fibroblasts in cryopreserved CDS increased when recultured for one week after thawing, reaching about 150% of the number of fibroblasts in fresh CDS. These findings indicate that the surviving fibroblasts can proliferate markedly and release considerable amounts of several types of cytokines.

FUNCTION OF CYTOKINES IN WOUND HEALING

Wound healing is a complex process involving the intricate interplay among a variety of cells, fibrous proteins, proteinases, cytokines (such as endogenous chemoattractants), growth factors and angiogenic factors. The normal acute wound healing process is a coordinated and predictable series of cellular and biochemical events. PDGF, TGF-α, TGF-β, IL-1, IL-6, IL-8, bFGF and EGF are present in acute wound fluid (32,33). Orderly and efficient progression of events through the wound healing process is regulated by these cytokines in wound fluid. However, chronic wounds, including diabetic ulcers, pressure ulcers and venous ulcers, fail to proceed through an orderly and timely healing process because certain pathophysiologic conditions and metabolic factors involved in these ulcers can alter cellular function and reduce ability to prevent infection. These wounds may ultimately result from a deficiency of cytokines or an inhibition of their function. Such a deficiency may be partly the result of elevated levels of proteinases that degrade growth factors and ECM components at the wound site (34–38). Fibroblasts isolated from chronic ulcers have little ability to proliferate, and chronic wound fluids inhibit normal fibroblast proliferation (39,40). Such decreased proliferation may be partially responsible for the delay in healing of chronic ulcers. In such cases, proliferation can be stimulated by cytokines such as bFGF, EGF and IL-1β (41). Thus, it may be possible to induce a healing response in chronic wounds by adding exogenous growth factors or by inhibiting proteinase activity at the wound site. A variety of

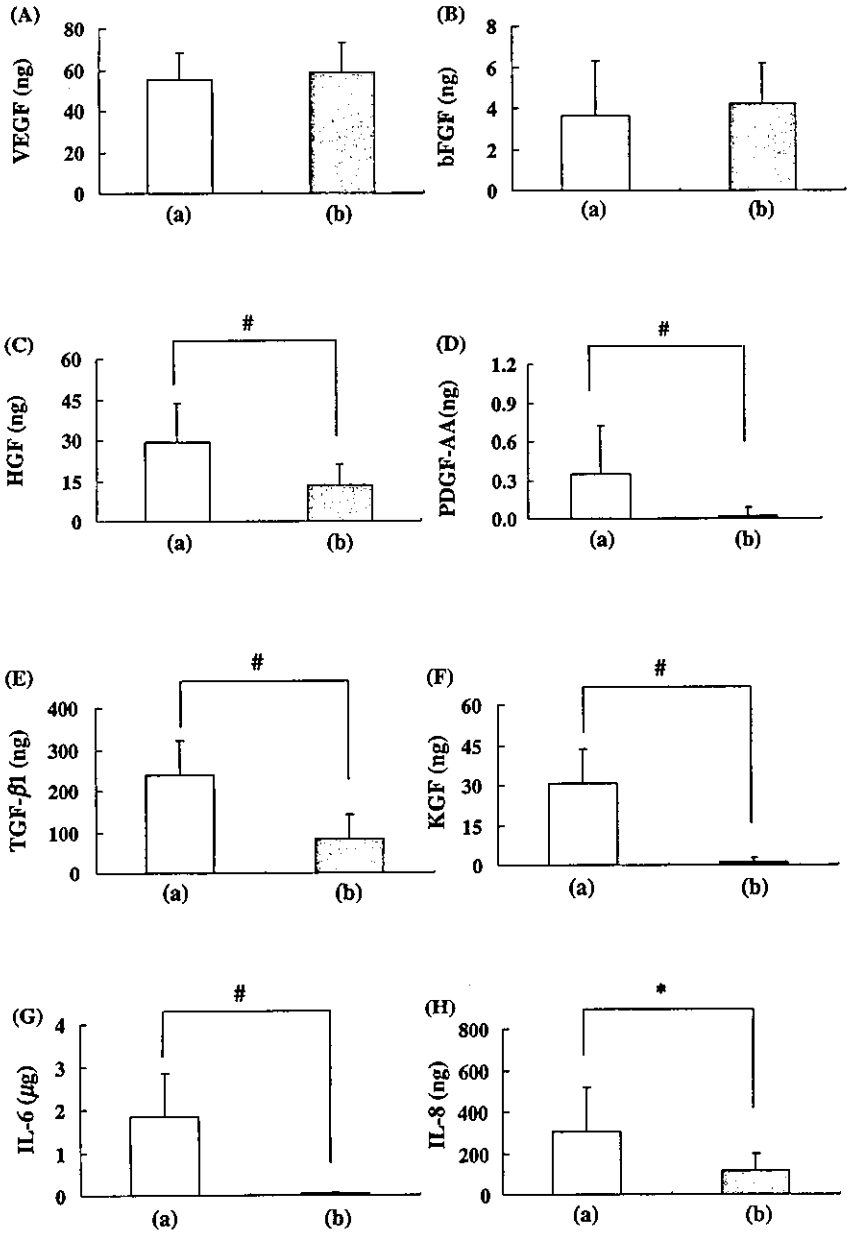

FIG. 4. Amount of cytokines released from fresh CDS over a cultivation period of one week. n = 5, means ± SD; $^*P < 0.05$, $\#P < 0.01$. [A]: VEGF, [B]: bFGF, [C]: HGF, [D]: PDGF-AA, [E]: TGF-β1, [F]: KGF, [G]: IL-6, [H]: IL-8.

cytokines, including PDGF (42–44), bFGF (43–45), EGF (46), VEGF (47), HGF (48) and TGF-β (49), have been applied to chronic ulcers in animal or clinical studies. However, results of the local application of these cytokines are unclear because the biology of wound healing is much more complex than predicted from in vitro activities. Fibroblasts have several roles in wound healing. They can produce ECM components such as collagen, and are an important source of several cytokines, including growth factors and angiogenic factors.

Various endogenous cells migrate into wound sites in response to cytokines. PDGF (50,51), bFGF (50,51), TGF-β (50–52) and KGF (53) are considered to be growth and chemoattractive factors for fibroblasts. Fibroblasts play a pivotal role in granulation tissue formation due to their ability to release various cytokines and ECM components. PDGF is a fibroblast-specific mitogen, and has no effect on the growth of keratinocytes or endothelial cells because they lack its receptor, which is different from that of bFGF. PDGF and TGF-β stimulate fibroblasts in an autocrine manner to amplify their proliferation and ECM synthesis. TGF-β plays an important role in the formation of granulation tissue. It promotes the deposition of ECM components such as collagen, fibronectin, hyaluronic acid and protease inhibitor. VEGF (54,55), bFGF (53,55), HGF (55,56) and IL-8

(57,58) are considered to be growth and chemoattractive factors of endothelial cells.

Re-epithelialization of wounds begins within hours after injury, and involves the movement of keratinocytes from the free edge of the tissue across the defect (59). The activation of keratinocytes requires loss of contact with the basement membrane, and depends on the exposure of these cells to cytokines in the wound site (60). EGF (61), TGF-α (62), IL-6 (63,64), KGF (53), bFGF (45) and TGF-β (65) are involved in this phase. TGF-β inhibits keratinocyte proliferation in vitro. However, in vivo, TGF-β alters the expression of integrin, so that keratinocytes migrate into the wound site and synthesize their own matrix.

FUNCTION OF ALLOGENEIC CDS IN WOUND HEALING

Autologous CES composed of stratified keratinocytes can take permanently on a skin defect and form epidermis on the resulting neodermis. In contrast, allogeneic CDS composed of fibroblasts and scaffold materials fails to take permanently on skin defects. However, cells in CDS can produce a variety of biologically active substances, including cell growth factor and extracellular matrix, which are necessary for wound healing. The efficacy of allogeneic CDS is dependent on both cell functions and the scaffold materials. It is very important to use materials that promote wound healing.

A biodegradable synthetic polymer mesh, Vicryl, has been used as a matrix for Dermagraft. However, this matrix was not designed to facilitate granulation tissue formation. In addition, a commercially available wound dressing, Biobrane, composed of a silicone film combined with a nylon mesh has been used as a matrix for Trans Cyte. This wound dressing adheres firmly to the debrided wound surface, but was not designed to facilitate granulation tissue formation.

To develop more efficacious allogeneic CDS for the treatment of severe wounds such as full-thickness skin defects, we designed a two-layered spongy matrix composed of an HA spongy layer and a Col spongy layer. In a preliminary animal test (21), this two-layered spongy matrix exhibited stronger promotion of wound healing than a collagen spongy matrix. This two-layered spongy matrix is designed so that HA molecules are cross-linked by a cross-linking agent, and so that Col molecules are cross-linked by UV radiation. This matrix maintains its spongy structure during the manufacture of CDS, cryopreservation, thawing and rinsing. When this CDS is applied to a wound surface in a clinical setting, the spongy structure degrades within about one week. Both HA and Col seem to function biologically in the process of wound healing. The molecular design of this CDS was derived from the results of a series of animal tests. Spongy matrix designed to degrade on the wound surface within about one week was found to be more effective than spongy matrix that failed to degrade within about one week due to strong cross-linkage. The preliminary animal study suggests that free HA and Col play a pivotal role in wound healing. The spongy matrix of the CDS is not just scaffolding for cultivating fibroblasts, but is made of materials that promote healing.

The cryopreserved CDS is able to release a variety of cytokines. The amounts of VEGF and bFGF released by cryopreserved CDS after it was recultured for a week after thawing were the same as those of fresh CDS. This finding appears to be related to clinical results showing that CDS induces the formation of an excellent wound bed with highly vascularized granulation tissue (29,30).

The amount of cytokines released from CDS tends to be decreased by freezing and thawing. However, clinical application of fresh allogeneic CDS has disadvantages due to practical problems in mass production, preservation and transportation. Therefore, cryopreserved allogeneic CDS is more useful for clinical applications.

CLINICAL STUDY

The clinical evaluation of allogeneic CDS was conducted in compliance with the study protocol of this Millennium Project and the ethical guidelines of each University Hospital. In a general procedure, the wounds were debrided and resulted in full-thickness skin defects, and were then rinsed with saline solution. The allogeneic CDS that had been rinsed with lactated Ringer's solution after thawing was placed cell-seeded side down on the full-thickness skin defects, and a conventional ointment-gauze dressing was used to protect the CDS. The CDS was applied repeatedly at intervals of three to five days.

Surgical closure with auto-skin grafting is the gold standard for treatment of victims with extensive deep dermal burns (DDB) or deep burns (DB). However, if the donor site is limited, an alternative treatment may be required. Although autologous CES can serve as an alternative material, its use raises practical problems, including the long preparation process (more than three weeks), the need to prepare the wound bed for CES, and the reduced growth of keratinocytes derived from geriatric burn patients. The

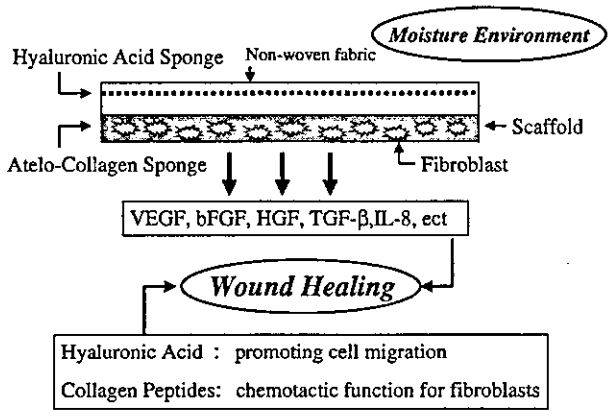

FIG. 5. Functions of allogeneic CDS composed of fibroblasts combined with a two-layered spongy matrix of HA and Col.

most practical treatment is mesh auto-skin grafting. Generally, a 1.5-fold or threefold extended mesh auto-skin graft is used because it usually results in successful epithelization. When sixfold extended mesh auto-skin graft has been applied to a wound surface in poor condition, the mesh skin graft has failed to take. With highly extended skin grafting there is a risk of poor epithelization. To overcome this problem, excellent biological dressing is required. In order to establish a new cell therapy, we evaluated allogeneic CDS as coverage for a sixfold extended mesh auto-skin graft.

Clinical evaluation of the cryopreserved allogeneic CDS has been performed. A total of 255 clinical cases with various wounds were evaluated; 63% (163/255) achieved excellent results and 30% (77/255) achieved good results. These results suggest that this allogeneic CDS is effective in promoting healing of partial- and full-thickness skin defects.

In conclusion, the cryopreserved CDS can release various cytokines that regulate complex wound healing. Various cytokines released from CDS may play a pivotal role in the balance between stimulatory and inhibitory effects during wound healing. Successful healing in severe skin defects, such as chronic ulcers, is considered to be dependent on the control of cell function by these cytokines. As well as the cytokines released from the allogeneic CDS, the spongy matrix

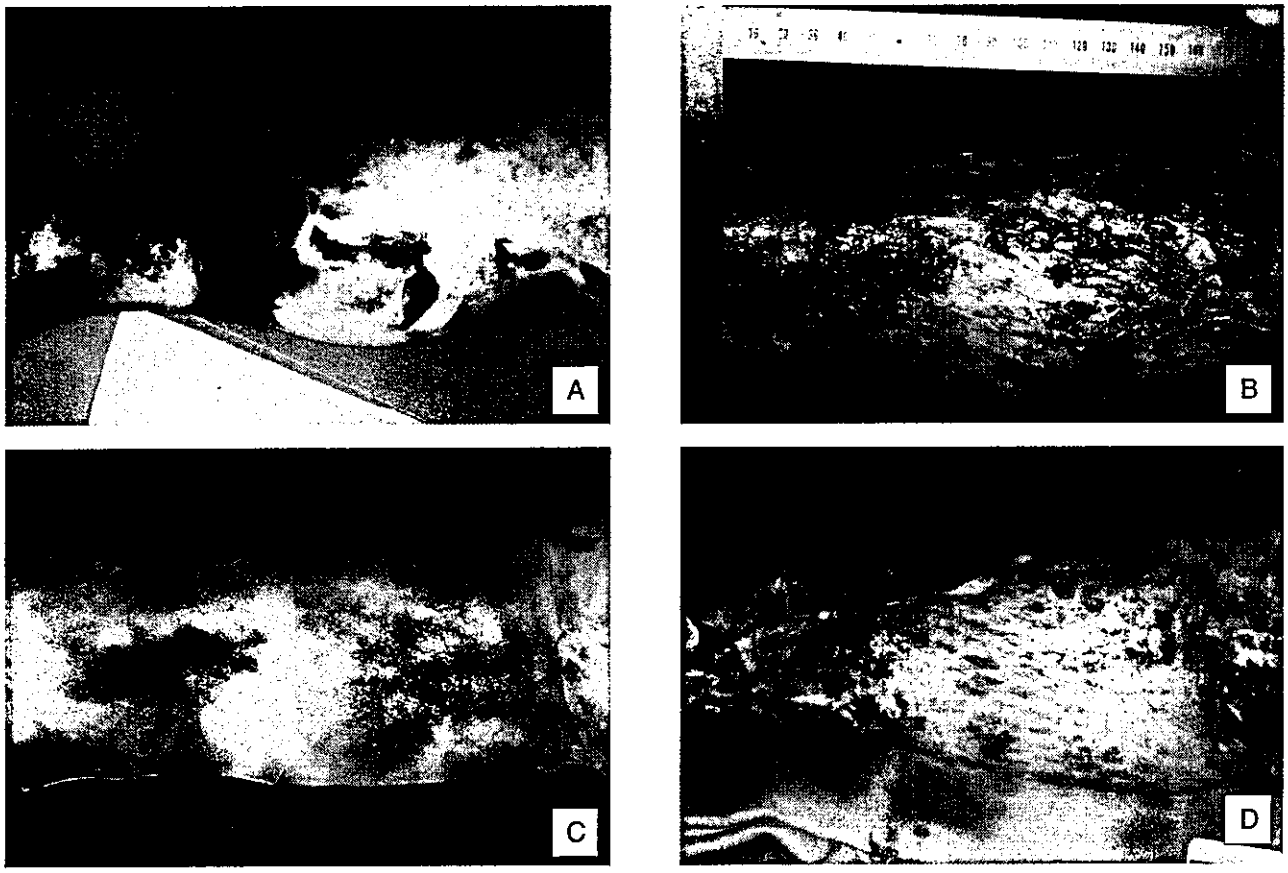

FIG. 6. An 81-year-old female suffered DDB and DB on the right lower leg (A). Sixfold extended mesh auto-skin fragments were applied to the debrided wound in sizes of 18 × 9 cm (B), and followed by the application of allogeneic CDS (C), on which a conventional ointment-gauze dressing and a dry gauze dressing were applied to protect the CDS. The mesh skin took successfully and the areas between strips of mesh skin were epithelized on day eighteen after grafting (D).

composed of HA and Col is beneficial for the treatment of intractable skin defects (Fig. 5).

REPRESENTATIVE CLINICAL CASE

An 81-year-old female was injured when her clothes caught fire while she was burning dry grass as part of farm work. The injury was located in the lumbar-gluteal region over the bilateral lower limbs, and a mixture of DDB and DB accounted for 36% of the injured region. On day six, when her general condition had improved, debridement of both lower legs was performed up to the fat layer, and sixfold extended mesh auto-skin fragments were grafted onto the right lower leg, followed by the application of allogeneic CDS (Fig. 6A–C). Take and epithelization of the grafts were in good condition on day eighteen after grafting on the right lower leg (Fig. 6D) (66).

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Development of a Cultured Dermal Substitute Composed of a Spongy Matrix of Hyaluronic Acid and Atelo-collagen Combined with Fibroblasts: Cryopreservation

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Abstract: An allogeneic cultured dermal substitute (CDS) was prepared by cultivating fibroblasts on a two-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col). The ability of fibroblasts to secrete cytokines is dependent on the conditions of freezing and thawing. The first experiment was designed to investigate the effects of supplements in a cryoprotective medium, that is, dimethylsulfoxide (DMSO), glycerol, and fetal bovine serum (FBS). In each experiment we measured the cell viability after thawing and the cell growth in CDS recultured after thawing. In addition, the amount of vascular endothelial growth factor (VEGF) released from the CDS recultured for one week after thawing was measured. The highest values of cell viability, cell growth, and the amount of VEGF released were obtained when CDS was frozen in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% DMSO and 40% FBS, and then thawed quickly in a water bath at 37°C. However, due to the high cost of FBS, in clinical applications CDS is usually frozen in DMEM supplemented with 10% DMSO and 20% FBS. In practice, however, physicians often cannot use CDS immediately after thawing, depending on clinical conditions. Therefore, in the second experiment we investigated cell viability at different time points after thawing. In addition, we investigated cell growth and the amount of VEGF released from fibroblasts in CDS at different time points after thawing under different conditions, and after further reculturing for one week. We recommend that CDS be rinsed with lactated Ringer's solution immediately after thawing, and that it be used within 4 h after thawing. **Key Words:** Cultured dermal substitute—Fibroblast—Hyaluronic acid—Collagen—Vascular endothelial growth factor.

Two types of allogeneic cultured dermal substitute (CDS), both of which are composed of fibroblasts and a scaffold of biodegradable or nonbiodegradable synthetic materials, have been developed in the U.S.A. (1–6). Recently, however, their commercialization has ceased. There appear to be problems in the design of scaffolds for fibroblasts. Kuroyanagi et al. developed a CDS using a spongy collagen matrix, and reported that it promoted wound healing in animal tests (7,8) and clinical studies (9–11). Based on their technique, a new version of CDS has been developed by cultivating fibroblasts on a two-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col) (12–13). HA molecules play a critical role in several cellular functions, including migration and proliferation, by promoting adhesion and disadhesion on tissue substrates (14–19). Col molecules play a pivotal role in wound healing; they act as chemoattractants for fibroblasts in vitro, and may have similar activity in vivo (20). The present authors have designed a matrix that is not just a scaffold for cultivating fibroblasts, but is made of biomaterials that promote wound healing.

The banking system of cryopreserved allogeneic CDS is very useful in emergencies, because the CDS is shipped frozen and then used immediately or stored temporarily, in a manner similar to that used with the banking system of cadaver skin. In clinical applications, cryopreserved CDS is thawed and then rinsed to remove cryoprotectants and fetal bovine serum (FBS). Allogeneic CDS has been used to provide effective therapy for patients with various

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wounds, including burns, chronic ulcers, traumatic skin defects, and giant pigmented nevi. The purpose of the present study was to evaluate the functions of cryopreserved CDS, particularly cell viability and the amount of vascular endothelial growth factor (VEGF) released from the CDS, because VEGF released from living cells in the CDS plays an important role in wound healing processes such as angiogenesis (21–24).

MATERIALS AND METHODS

Preparation of a spongy matrix composed of HA and Col

Hyaluronic acid powder (sodium salt, MW = 2 000 000; Shiseido Co. Ltd, Yokohama, Japan) was dissolved in distilled water at a concentration of 1%. This HA solution was adjusted to pH 3.5 with 1 N HCl. The HA solution was kept in a refrigerator at 4°C for 2 h. Ethylene glycol diglycidyl ether (EX810, Nagasekasei Co. Ltd, Hyogo, Japan) was used as a cross-linking agent for HA molecules. EX810 was mixed with distilled water at a concentration of 10%, and this solution was kept in a refrigerator at 4°C for 2 h. The aqueous solution of EX810 was then added dropwise to the HA. The weight ratio of EX810 to HA was adjusted to 1 : 5. This mixed solution (50 g) was poured into a polystyrene dish (11 cm × 10 cm) and a sheet of hydrated cellulose nonwoven fabric was attached to the bottom of the dish. The dish was kept at 50°C for 5 h to promote cross-linking between HA and EX810. The HA solution was reduced to about one-half of its initial volume, kept in a refrigerator at 4°C for 2 h, quickly frozen in a freezer at –85°C, and then lyophilized to obtain the HA sponge. The sponge was rinsed thoroughly with distilled water to remove the free cross-linking agent. After rinsing, the hydrated HA sponge was placed in a dish and kept in a refrigerator at 4°C for 2 h, and was then quickly frozen and lyophilized to obtain the purified HA sponge. The HA sponge was punched mechanically to produce many holes (diameter 0.5 mm) separated by a distance of 4 mm.

The Col powder (Koken Co. Ltd, Tokyo, Japan) was prepared by enzymatic cleavage of telopeptides located at both ends of type I collagen molecules derived from bovine hide dermis. It was dissolved in distilled water at a concentration of 0.5%. This Col solution was adjusted to pH 3.5 with 1 N HCl. The Col solution (40 g) was then poured into a polystyrene dish (11 cm × 10 cm). The HA sponge with many holes was carefully immersed in the dish containing Col solution, and a sheet of nonwoven fabric was placed on its upper side. The holes of the HA sponge were filled with Col solution. The hydrated HA sponge was kept in a refrigerator at 4°C overnight, and was then quickly frozen and lyophilized to obtain a two-layered sponge of HA and Col. Both surfaces of the two-layered sponge were irradiated with a 15 W ultraviolet lamp at a distance of 15 cm for 30 min, to produce intermolecular cross-linking of Col molecules. Each sponge was packed in a bag and kept in a dry sterilizer at 121°C for 2 h.

Establishment of cell banking

A piece of skin (of size 0.5 cm × 1.0 cm) was obtained from a 3-month-old patient during surgical excision of excrescence. The patient was free from infectious viruses such as HBV, HCV, HIV, and HTLV, and the results of the treponema pallidum hemagglutination test (TPHA) were negative. All procedures were performed in accordance with the ethical guidelines of St. Marianna Medical College.

The donated skin was immersed in Hanks' solution containing antibiotic–antimycotic agents (100 U/ml penicillin G sodium, 100 U/ml streptomycin sulfate, 0.25 μg/mL amphotericin B; Gibco, NY, U.S.A.) at room temperature for 30 min. The sterilized piece of skin was immersed in Dulbecco's modified Eagle's medium (DMEM) containing dispase (Godo-shusei Co. Ltd, Tokyo, Japan) at a concentration of 1000 PU/mL for 20 h at 4°C. After this enzymatic treatment, the epidermis was mechanically separated from the dermis using sterilized stainless steel tweezers. The strips of dermis were cut into small pieces and minced using sterilized stainless steel scissors. The minced dermis was treated with 0.5% collagenase in DMEM supplemented with 1% FBS for 2 h at 37°C to obtain the cellular suspension. Following this enzymatic treatment, Hanks' solution was added to the cellular suspension. This diluted cellular suspension was centrifuged for 5 min at 1500 rpm. The resulting cellular pellet was suspended in a culture medium (DMEM supplemented with 10% FBS), followed by stirring for 10 min at room temperature. The cellular suspension was centrifuged for 5 min at 2000 rpm to obtain a cellular pellet. The cultivation of fibroblasts was then initiated in the culture medium. Over the cultivation period, some fibroblasts were collected for cryopreservation to establish a master cell bank. The fibroblasts were suspended in DMEM supplemented with 10% dimethylsulfoxide (DMSO) and 20% FBS, and adjusted to a concentration of 2.0×10^6 cells/mL. Then, 2 mL of the cellular suspension in each tube was cryopreserved. These tubes were placed in a foam polystyrene case, kept in a freezer at –85°C overnight and then cryopreserved in liquid N_2.

Preparation of CDS

Prior to seeding fibroblasts on the two-layered sponge of HA and Col, 50 ml of culture medium was poured into a polystyrene dish (11 cm × 10 cm), and the two-layered sponge (10.5 cm × 9.5 cm) was immersed in the culture medium and kept in an incubator in a humidified atmosphere of 5% CO_2 at 37°C for 2 h, to hydrate and neutralize the acidic two-layered sponge. The excess culture medium was removed carefully from each dish by suction. Fibroblasts, obtained by successive cultivation from cryopreserved cells, were seeded onto the two-layered sponge by adding 5 ml of cellular suspension dropwise onto the collagen surface of the two-layered sponge. The number of fibroblasts on the two-layered sponge was adjusted to 1.0×10^5 cells/cm^2. This sponge was kept in an incubator in a humidified atmosphere of 5% CO_2 at 37°C overnight, followed by the addition of 50 ml of culture medium and culturing for one week.

Freezing of CDS

The CDS (of size 10.5 cm × 9.5 cm) was turned upside down, and then the culture medium was replaced with 30 mL of cryoprotective medium. Cryopreservation was performed under seven different conditions: in DMEM supplemented with 10% DMSO and 20% FBS; in DMEM supplemented with 10%, 20%, or 40% glycerol and 20% FBS; and in DMEM supplemented with 10% DMSO and 0%, 10%, 20%, or 40% FBS. The dish containing CDS was frozen using a programmable freezer (AIR BLASTER, EBAC, Tokyo, Japan). Prior to freezing, the dish containing CDS was kept in a refrigerator at 4°C for either of two periods (to achieve equilibrium): 30 min when using DMSO as the cryoprotectant, and 2 h when using glycerol as the cryoprotectant. The dish was then rapidly transferred into the programmable freezer and frozen. The temperature in the freezer decreased at a rate of −1.0°C/min from 4°C to −60°C, and was then maintained at −60°C for 2 h. The dish was then rapidly transferred into a freezer at −152°C for cryopreservation. The freezing rate was measured using a sensor connected to a thermometer (TR-52, T&D Corporation, Tokyo, Japan).

Thawing of CDS

Prior to thawing the CDS, the dish containing CDS was placed in a foam polystyrene box for 20 min to prevent physical damage to the CDS due to a rapid change in temperature. Then, the dish containing CDS was kept in an incubator at 37°C until the CDS was completely thawed. The thawing rate was measured by placing a sensor in each sample. After thawing, the CDS was rinsed with 50 ml of lactated Ringer's solution three times to remove cryoprotectant and FBS.

Quantitative analysis of living cells in the CDS

The cell viability through the processes of freezing and thawing was measured by an MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After the cryopreserved CDS was thawed at 37°C, a sample of the CDS (4 cm × 3 cm) was transferred into another dish, which contained 6 mL of culture medium (containing 3 mg of MTT); it was then kept in an incubator at 37°C for 2 h, and subsequently cut into small pieces. This sample was immersed in 5 ml of DMSO, followed by stirring for 10 min at room temperature. The absorbance of the resulting blue–violet solution was measured with a microplate reader at 570 nm. Also, the number of fibroblasts in the CDS that had been recultured for one week after thawing was measured by an MTT assay using the above procedure.

Quantitative analysis of VEGF released from the CDS

The amount of VEGF released from the CDS was measured by an enzyme-linked immunosorbent assay (ELISA). VEGF standards and culture medium were added into each well of a ninety-six-well microplate in duplicate. The amount of VEGF was measured according to the package insert of commercial kits (R & D Systems Inc., Minneapolis, MN, U.S.A.). The absorbance of VEGF in wells was measured with a microplate reader at 450 nm. The amounts of VEGF were determined by plotting on a calibration curve. The calibration curves for VEGF ranged from 15.6 to 1000 pg/mL. Each measurement was repeated six times, and each sample was assayed in duplicate, to obtain the data with a standard deviation. The data were statistically analyzed by means of a two-tailed Student's *t*-test.

Functions of CDS at different times after thawing

The CDS (of size 10.5 cm × 9.5 cm) was frozen in 30 mL of cryoprotective medium (DMEM supplemented with 10% DMSO and 20% FBS) using a programmable freezer. The cryopreserved CDS was thawed in a water bath at 37°C. After thawing, the CDS underwent one of two different procedures: either it was kept in cryoprotective medium at room temperature for 1, 2, 4, or 8 h; or it was rinsed with 50 mL of lactated Ringer's solution three times, and then kept in lactated Ringer's solution at room temperature for 1, 2, 4, or 8 h. The number of living cells

in the CDS at each time point after thawing was measured. In addition, the number of cells in the CDS after further reculturing for one week was measured. The amount of VEGF released from the CDS that was recultured for one week after thawing and then underwent one of the above post-thawing procedures was also measured.

RESULTS

Measurement of freezing and thawing rates

The freezing rate of the CDS in the programmable freezer was measured by a thermometer. The freezing rate had four phases: a first phase of –0.5°C/min (10.2°C to –3.4°C); a second phase of –0.05°C/min (–3.4°C to –4.7°C); a third phase of –1.0°C/min (–4.7°C to –49.0°C); and a final phase of –0.2°C/min (–49.0°C to –54.5°C). The CDS was cryopreserved in a freezer at –152°C after completion of the final phase.

The thawing rate of the CDS in a water bath at 37°C was measured by a thermometer. A dish containing CDS that was cryopreserved in a freezer at –152°C was placed in a foamed polystyrene box for 20 min. During this process, the temperature of the CDS increased to about –60°C. In a water bath at 37°C, the thawing rate had three phases: a first phase of 12°C/min (–60.0°C to –11.0°C); a second phase of 2.0°C/min (–11.0°C to –0.5°C); and a final phase of 5.5°C/min (0.5°C to 20.0°C). Using this method, the CDS was thawed within about 10 min.

Evaluation of conditions for cryopreservation

We determined the number of living cells in the CDS cryopreserved under one of the seven different cryoprotective conditions and then thawed. The number of cells in CDS recultured for one week after thawing was also measured. Figure 1 shows the number of cells in the CDS that was cryopreserved in DMEM supplemented with 10% DMSO and 20% FBS, and also in DMEM supplemented with 10%, 20% or 40% glycerol and 20% FBS. The number of living cells in the CDS after thawing was about 50% of the number in fresh CDS before freezing. The number of living cells in the CDS recultured for one week after thawing ranged from 125% to 150% of the number in fresh CDS. There was no significant difference in the number of cells in the CDS between the three different concentrations of glycerol in cryoprotective media supplemented with 20% FBS. Figure 2 shows the amount of VEGF released from CDS recultured for one week after thawing. There were significant differences in the amount of VEGF released. The amount of VEGF released was highest for CDS cryopreserved in DMEM supplemented with 10% DMSO and 20% FBS. This indicates that DMSO is a more effective cryoprotectant than glycerol.

In the second experiment, we attempted to determine the optimum concentration of FBS. Figure 3 shows the number of cells in CDS cryopreserved in 10% DMSO and 0%, 10%, 20%, or 40% FBS. The number of living cells in the CDS after thawing was

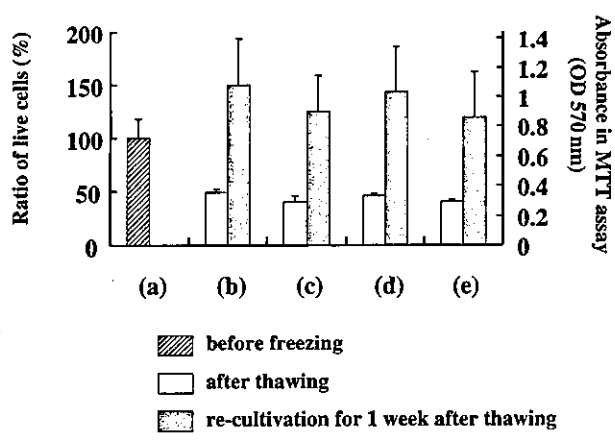

FIG. 1. Number of cells in the CDS after freezing under different conditions (b–e) and then thawing, and after reculturing for one week ($n = 3$, means ± SD): (a) fresh CDS before freezing; (b) in 10% DMSO and 20% FBS; (c) in 10% glycerol and 20% FBS; (d) in 20% glycerol and 20% FBS; (e) in 40% glycerol and 20% FBS.

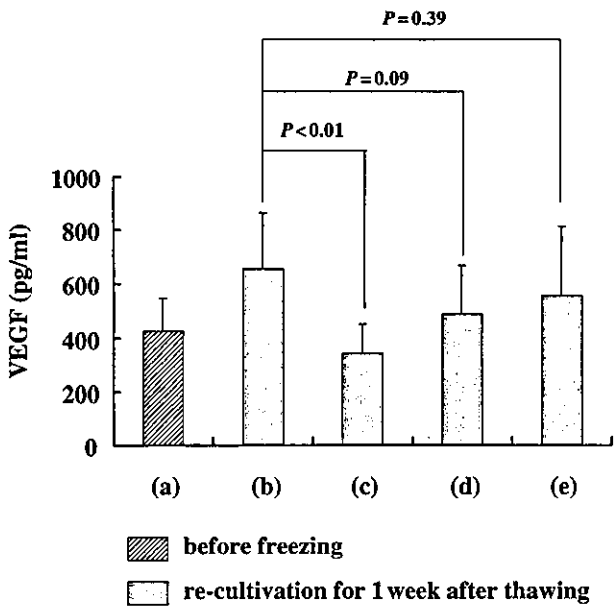

FIG. 2. Amount of VEGF released from the CDS after freezing under different conditions (b–e) and then thawing, and after reculturing for one week ($n = 3$, means ± SD): (a) fresh CDS before freezing; (b) in 10% DMSO and 20% FBS; (c) in 10% glycerol and 20% FBS; (d) in 20% glycerol and 20% FBS; (e) in 40% glycerol and 20% FBS.

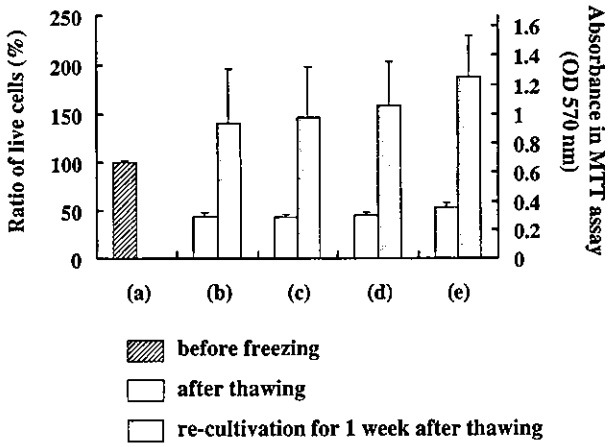

FIG. 3. Number of cells in the CDS after freezing under different conditions (b–e) and then thawing, and after reculturing for one week ($n = 3$, means ± SD): (a) fresh CDS before freezing; (b) in 10% DMSO and 0% FBS; (c) in 10% DMSO and 10% FBS; (d) in 10% DMSO and 20% FBS; (e) in 10% DMSO and 40% FBS.

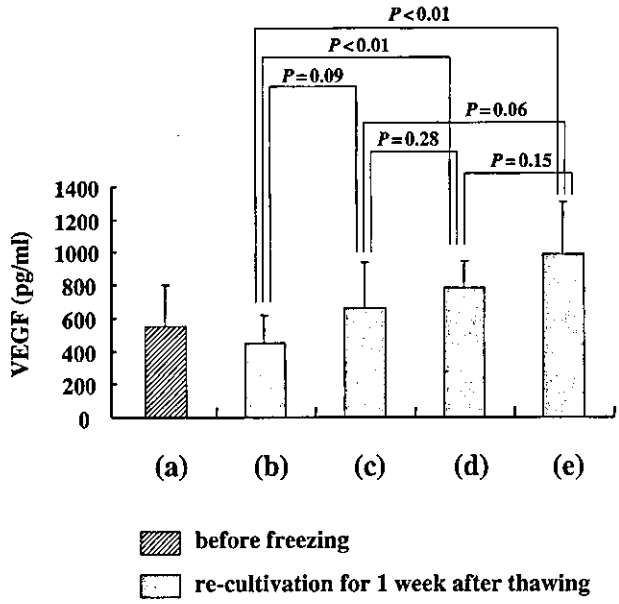

FIG. 4. Amount of VEGF released from the CDS after freezing under different conditions (b–e) and then thawing, and after reculturing for one week ($n = 3$, means ± SD): (a) fresh CDS before freezing; (b) in 10% DMSO and 0% FBS; (c) in 10% DMSO and 10% FBS; (d) in 10% DMSO and 20% FBS; (e) in 10% DMSO and 40% FBS.

about 50% of the number in fresh CDS before freezing. The number of cells in the CDS recultured for one week after thawing ranged from 150% to 200% of the number in fresh CDS. The number of cells increased as the concentration of FBS increased. The amount of VEGF released from CDS recultured for one week after thawing is shown in Fig. 4. The amount of VEGF released increased in proportion to the concentration of FBS. This indicates that the concentration of FBS in a cryoprotective medium should be higher than 20%. From these results, optimum conditions for cryopreservation were determined. The amount of VEGF released from CDS was highest when the CDS was cryopreserved in DMEM supplemented with 10% DMSO and 40% FBS, thawed quickly in a water bath at 37°C, and recultured for one week after thawing.

Functions of CDS at different times after thawing

We assessed different methods for treatment of the CDS prior to clinical application. Figure 5 shows the number of living cells in the CDS at one of several

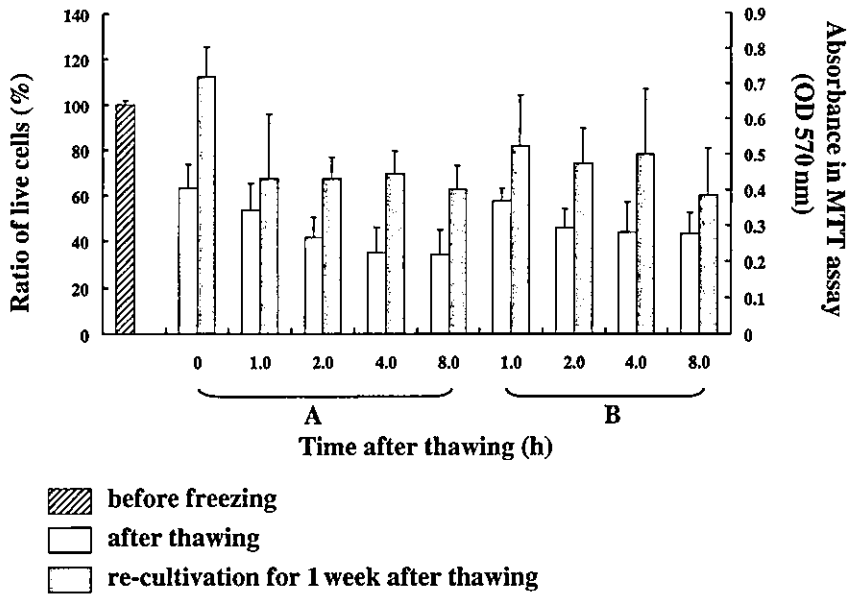

FIG. 5. Number of cells in the CDS at each period after thawing, and after reculturing for one week ($n = 3$, means ± SD): (A) in 10% DMSO and 20% FBS; (B) in lactated Ringer's solution after rinsing.

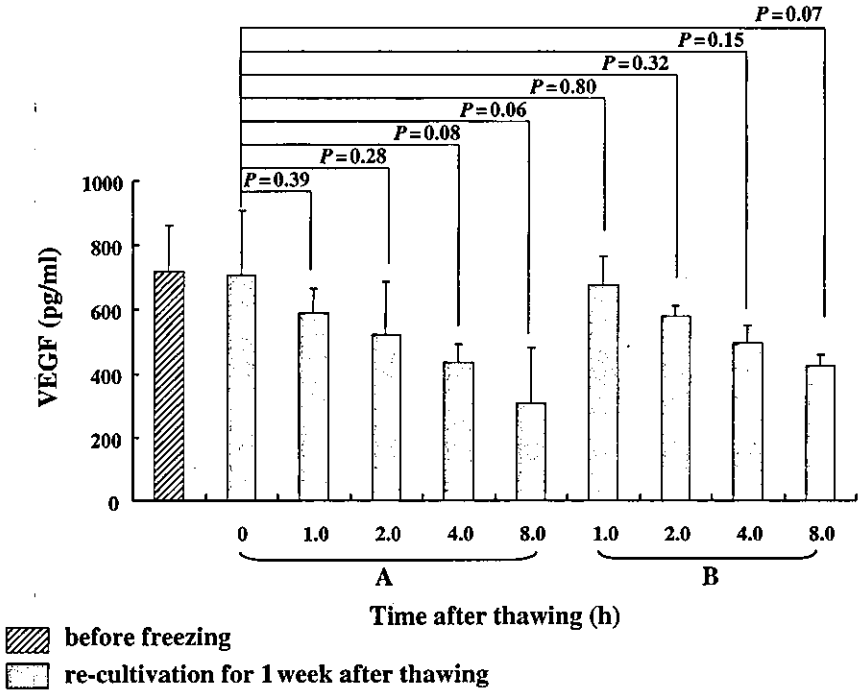

FIG. 6. Amount of VEGF released from the CDS immediately after thawing, at various times after thawing, and after reculturing for one week ($n = 3$, means ± SD): (A) in 10% DMSO and 20% FBS; (B) in lactated Ringer's solution after rinsing.

different time points after thawing, and the number of cells in CDS recultured for one week after thawing. The number of cells tended to decrease as a function of time after thawing, in the cryoprotective medium and the in lactated Ringer's solution. The amount of VEGF released from the CDS that was recultured for one week after thawing and then underwent one of the above post-thawing procedures (see the Materials and Methods section) is shown in Fig. 6. The amount of VEGF released tended to decrease as a function of time after thawing. Thawing followed by rinsing with lactated Ringer's solution produced the greatest release of VEGF. These results suggest that physicians should use CDS within 4 h after thawing, followed by rinsing with lactated Ringer's solution.

DISCUSSION

In clinical applications of CDS, it is important to maintain cell viability and the ability to release cytokines such as VEGF after freezing and thawing. Several studies have been conducted to determine the optimum conditions for cryopreservation of cultured skin cells and fibroblasts in collagen gels (25–27).

The aim of the present study was to determine the optimum conditions for freezing CDS. Fibroblasts in the CDS can release cytokines that are useful in promoting wound healing. The ability of fibroblasts to secrete cytokines is dependent on the conditions of freezing and thawing. The first experiment was designed to investigate the effects of supplements in the cryoprotective medium, that is, DMSO, glycerol and FBS. In each experiment we measured cell viability after thawing and cell growth in the CDS recultured after thawing. In addition, the amount of VEGF released from the CDS recultured for one week after thawing was measured. The optimum conditions for freezing CDS were determined from the results of these experiments. The highest values of cell viability, cell growth and the amount of VEGF released were obtained when the CDS was frozen in DMEM supplemented with 10% DMSO and 40% FBS, and then thawed quickly in a water bath at 37°C. However, due to the high cost of FBS, in clinical applications the CDS is usually frozen in DMEM supplemented with 10% DMSO and 20% FBS.

Ideally, immediately prior to clinical application, cryopreserved CDS should be thawed and then rinsed with lactated Ringer's solution to remove cryoprotectants such as DMSO and FBS. In practice, however, physicians often cannot use CDS immediately after thawing, depending on clinical conditions. Therefore, in the second experiment we investigated cell viability at different time points after thawing. In addition, we investigated cell growth and the amount of VEGF released from fibroblasts in the CDS at different time points after thawing under different conditions, and after further reculturing for one

week. We recommend that CDS be rinsed with lactated Ringer's solution immediately after thawing, and that it be used within 4 h after thawing.

In the present study, we determined the optimal procedure for cryopreservation of CDS. Cryopreserved CDS has advantages in mass production, preservation and transportation. Determining the optimal procedure for cryopreservation of CDS is necessary for the development of clinical applications. Clinical research on allogeneic CDS, which was newly developed by the R&D Center for Artificial Skin at Kitasato University, has been carried out in medical centers across Japan with the support of the Millennium Project of the Ministry of Health, Labor and Welfare. According to the protocol based on the results in the present study, 259 clinical trials were performed. The spongy matrix itself, as well as the vascular endothelial growth factor (VEGF) released by the allogeneic CDS, seemed to be beneficial for the treatment of intractable skin ulcers. Allogeneic CDS functions as an excellent biological dressing, and could dramatically change the treatment of intractable skin ulcers.

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