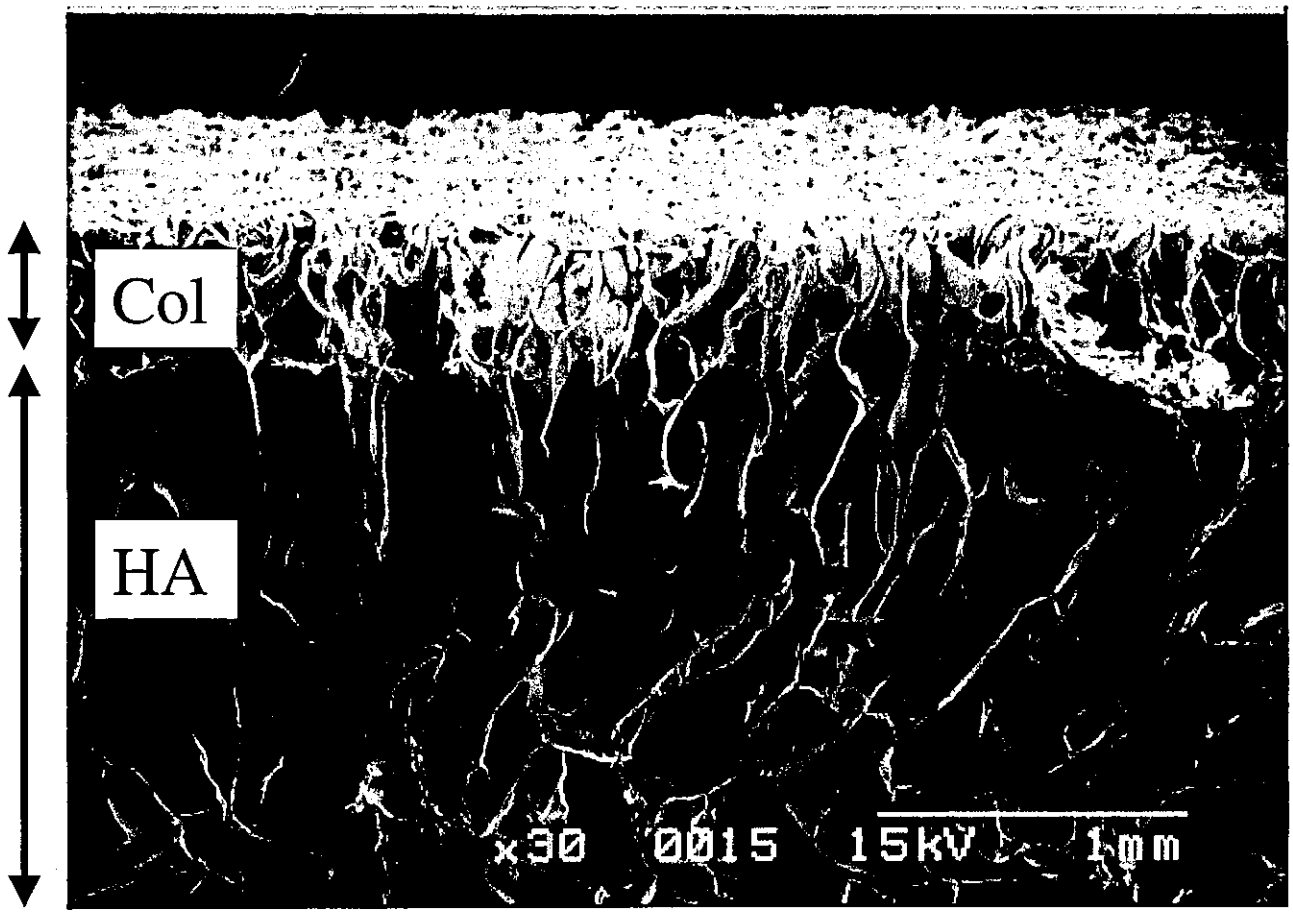

FIG. 2. Cross-sectional view of scanning electron microphotograph of a spongy matrix composed of HA and Col.

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

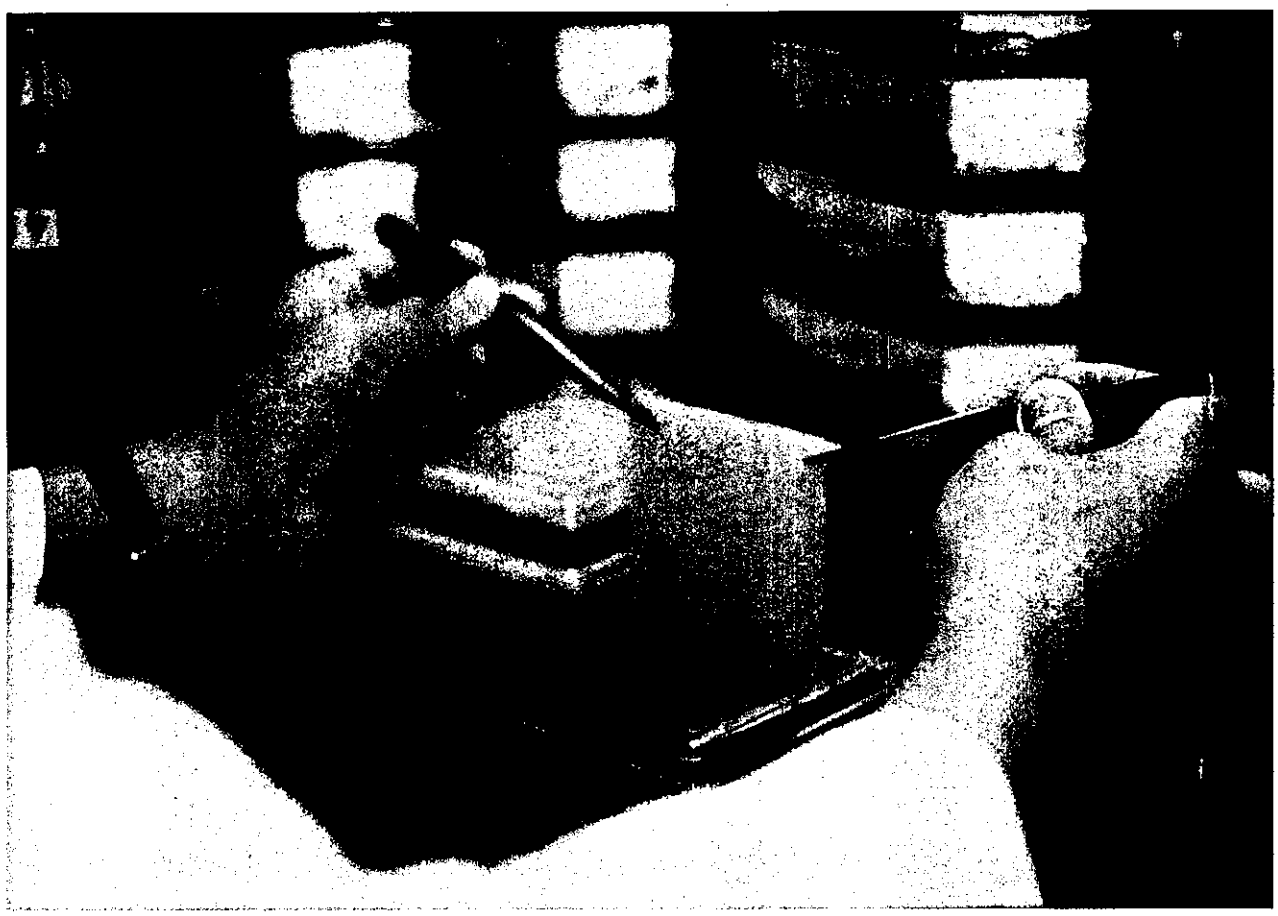

FIG. 3. Allogeneic CDS composed of fibroblasts combined with a two-layered spongy matrix of HA and Col.

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

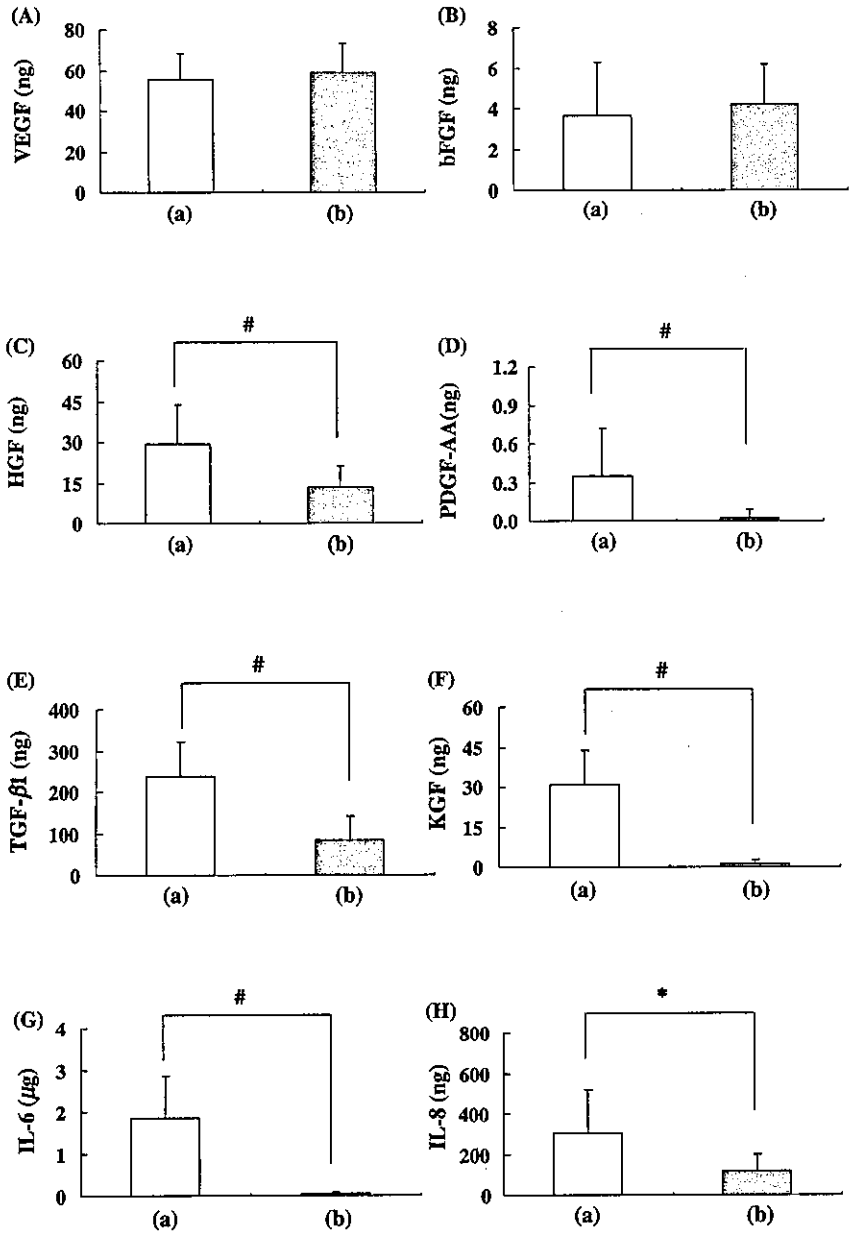

FIG. 4. Amount of cytokines released from fresh CDS over a cultivation period of one week. n = 5, means ± SD; $*P < 0.05$, $\#P < 0.01$. [A]: VEGF, [B]: bFGF, [C]: HGF, [D]: PDGF-AA, [E]: TGF-β1, [F]: KGF, [G]: IL-6, [H]: IL-8.

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

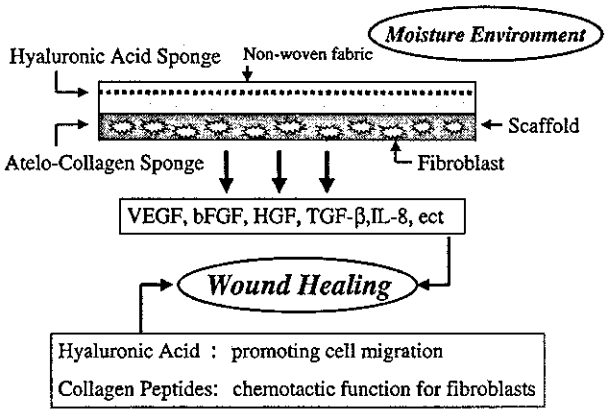

FIG. 5. Functions of allogeneic CDS composed of fibroblasts combined with a two-layered spongy matrix of HA and Col.

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

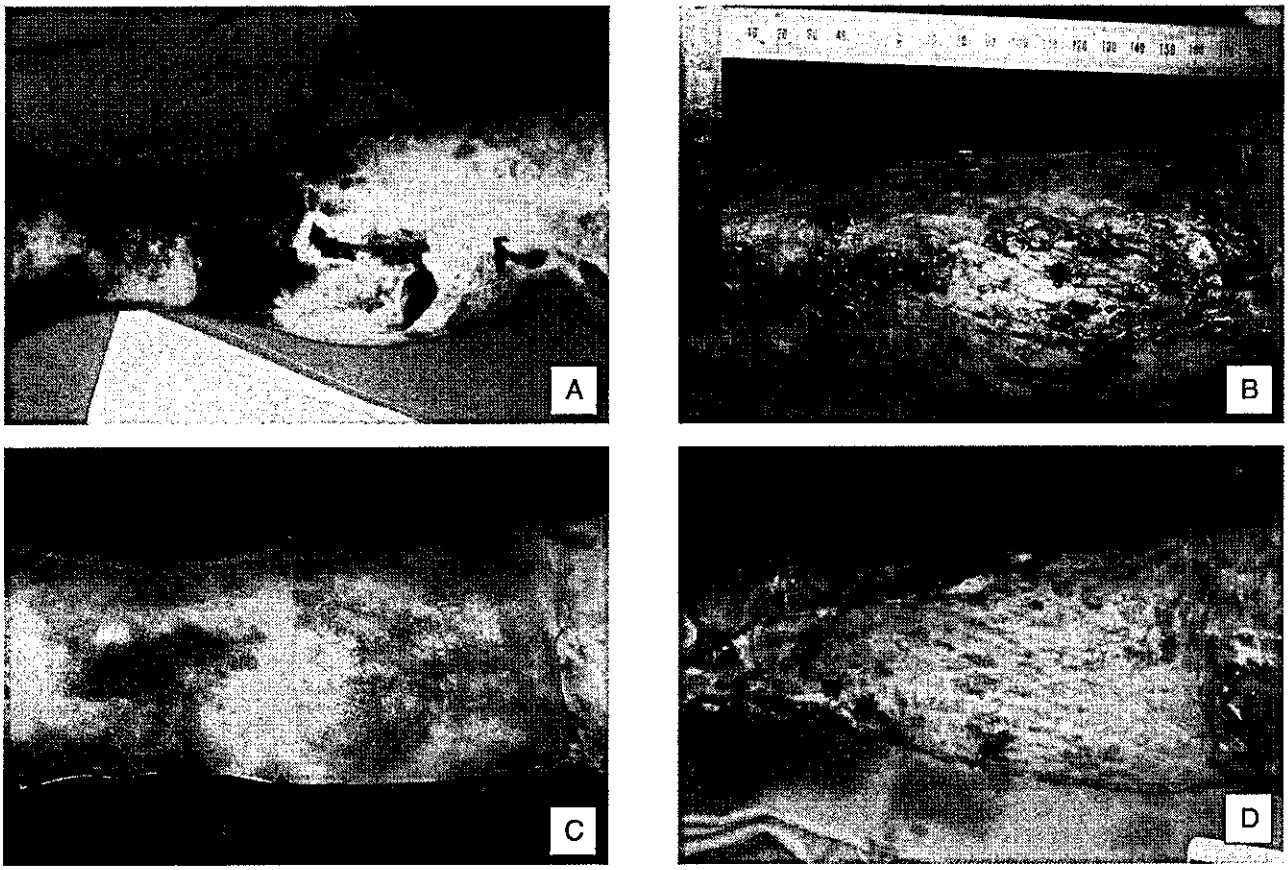

FIG. 6. An 81-year-old female suffered DDB and DB on the right lower leg (A). Sixfold extended mesh auto-skin fragments were applied to the debrided wound in sizes of 18 × 9 cm (B), and followed by the application of allogeneic CDS (C), on which a conventional ointment-gauze dressing and a dry gauze 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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Treatment of Full-thickness Skin Defect with Concomitant Grafting of 6-fold Extended Mesh Auto-skin and Allogeneic Cultured Dermal Substitute

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Abstract: The aim of this clinical study was to evaluate an allogeneic cultured dermal substitute (CDS) as a biological dressing for highly extended mesh auto-skin grafting. The subjects were five patients with extensive deep burn wounds. Allogeneic CDS was prepared by seeding fibroblasts on a spongy matrix of hyaluronic acid and atelocollagen. Six-fold extended auto-skin graft was applied to the debrided wound, on which allogeneic CDS was placed. A conventional ointment-gauze dressing was used to protect the CDS. The CDS was applied repeatedly at intervals of 5–7 days. In all cases, the wounds were closed by successful take of mesh auto-skin graft and prompt epithelization from the grafted skin. The skin on the grafted area had a cicatrix appearance, but was soft and thin, maintaining good quality. The application of 6-fold extended auto-skin graft in conjunction with allogeneic CDS is an effective method for treatment of extensive severe burn wounds. **Key Words:** Cultured dermal substitute—Fibroblasts—Hyaluronic acid—Collagen—Mesh skin grafting.

A typical engineered product is autologous cultured epidermal substitute (CES), which is composed of stratified keratinocytes (1–4). There are two types of allogeneic cultured dermal substitute (CDS) composed of fibroblasts on a scaffold (5–10). Another skin substitute is allogeneic cultured skin substitute (CSS), which is composed of keratinocytes and fibroblasts on a scaffold (11–15). Recently, however, the commercialization of these allogeneic products has been discontinued. There seem to be some problems in the design of these products.

Kuroyanagi et al. (16–19) developed an allogeneic CDS composed of a spongy collagen containing fibroblasts. 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, a new type of CDS was developed by culturing fibroblasts on a 2-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col) (20–24).

A multicenter clinical study on the use of allogeneic CDS was performed in 30 hospitals across Japan as the Regenerating Medical Millennium Project of the Ministry of Health, Labor and Welfare. Allogeneic CDS has been applied to debrided wound surfaces to prepare wound beds for split-thickness auto-skin graft, and reported results of clinical studies at other hospitals involved in this project indicate that use of CDS for this procedure has generally been successful (25–27).

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 (>3 weeks), the need to prepare the wound bed for CES, and reduced growth of keratinocytes derived from geriatric burn patients. The most practical treat-

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ment is mesh auto-skin grafting. Generally, a 1.5- or 3-fold extended mesh auto-skin graft is used because it usually results in successful epithelization. In practice, a mesh auto-skin graft is applied on the debrided wound surface, on which a conventional ointment gauze dressing was placed to protect the mesh auto-skin graft. When 6-fold 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 the present study, we evaluated allogeneic CDS as coverage for a 6-fold extended mesh auto-skin graft.

MATERIALS AND METHODS

Preparation of spongy matrix composed of hyaluronic acid (HA) and atelo-collagen (Col)

The spongy matrix was prepared using a method described in previous articles (21–23). An aqueous solution of HA with cross-linking agent was poured into a polystyrene dish (11 × 10 cm); a sheet of hydrated cellulose nonwoven fabric was attached to the bottom of the dish. The HA solution in the dish was frozen in a freezer at –85°C and then lyophilized to obtain the HA sponge. After the sponge was rinsed thoroughly with distilled water to remove free cross-linking agent, the hydrated HA sponge was frozen and lyophilized to obtain the purified HA sponge. The purified HA sponge was punched mechanically to produce many holes. The Col solution was poured into a polystyrene dish (11 × 10 cm). The HA sponge with many holes was carefully immersed in the dish containing Col solution, with a sheet of nonwoven fabric resting on the upper side of the HA sponge, and was then frozen and lyophilized to obtain a 2-layered sponge of HA and Col. Both surfaces of the 2-layered sponge were irradiated with ultraviolet light to produce intermolecular cross-linking of Col molecules. Each sponge was then packed in a bag and kept in a dry sterilizer at 121°C for 2 h.

Establishment of cell banking

A small piece of skin was obtained from a 3-month-old patient during surgical excision of an excrescence. This patient was free from infectious viruses such as HBV, HCV, HIV, and HTLV, and results of the treponema pallidum hemagglutination test (TPHA) were negative. 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 was 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. Then, fibroblasts were cultivated in culture medium to establish cell banking. The cells were checked for viruses such as HBV, HCV, HIV, HTLV, and Parvovirus (20,22).

Preparation of cultured dermal substitute

The CDS was prepared using a method described in previous articles (22–24). Prior to seeding of fibroblasts, the 2-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 and neutralize its acidity. The excess culture medium was carefully removed from the dish by suction. Fibroblasts obtained from successive cultivation of the cryopreserved cells were seeded onto the 2-layered sponge, by adding 5 mL of cellular suspension dropwise onto the collagen surface of the 2-layered sponge. The number of fibroblasts on the 2-layered sponge was adjusted to 1.0×10^5 cells/cm^2. The seeded sponge was kept in an incubator in a humidified atmosphere of 5% CO_2 at 37°C overnight, followed by addition of 50 mL of culture medium and culturing for 1 week.

Fibroblasts used in production of CDS were checked for mycoplasma. The culture medium used in production of CDS was checked for bacteria.

Cryopreserving and thawing of CDS

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 in the dish in a programmable freezer (AIR BLASTER, EBAC, Tokyo, Japan) at a gradient of –1°C/min from 4 to –60°C, and was then cryopreserved in a freezer at –152°C (23,24). The cryopreserved CDS (in the polystyrene dish) was placed in a foam polystyrene box containing dry ice, and was 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.

Clinical evaluation

The clinical evaluation of allogeneic CDS was conducted in compliance with the ethical guidelines of

TABLE 1. *Evaluation of efficacy according to four different conditions*

	Very good	Good	Medium	Poor	Very poor
Epithelization	35	27	18	9	0
Granulation tissue	35	27	18	9	0
Control of infection	15	13	8	4	0
Drainage condition	15	13	8	4	0

Kagawa Prefectural Central Hospital. The subjects were five patients with extensive deep burn wounds and one patient with necrotizing fasciitis. Skin fragments for grafting were collected from healthy skin on the back, at a split-thickness of 0.010–0.012 in. Six-fold extended mesh auto-skin graft was applied to the debrided wound, and was fixed using a stapler, with allogeneic CDS placed over the wound. A conventional ointment-gauze dressing was used to protect the CDS. The CDS was applied repeatedly at intervals of 3–5 days.

After treatment of full-thickness skin defects with concomitant grafting of 6-fold extended mesh auto-skin and allogeneic CDS, we clinically evaluated epithelization, granulation tissue formation, control of wound infection, and drainage conditions. Epithelization and granulation tissue formation were graded according to the following scale: very good, 35 points; good, 27 points; fair, 18 points; poor, 9 points; very poor, 0 points. Drainage conditions and control of wound infection were graded according to the following scale: very good, 15 points; good, 13 points; fair, 8 points; poor, 4 points; very poor, 0 points (Table 1). Safety was graded according to the following scale: A, very safe; B, mostly safe; C, problem with specific treatment; D, not safe.

Total evaluation consisted of judging both efficacy and safety (Table 2). Cases with a total point score >80 and a safety grade of A were assessed as excellent. Cases with a total point score ≥60 and ≤79 and a safety grade of A or B were assessed as good. Cases that fulfilled either of the following sets of criteria were assessed as fair: total point score ≥40 and ≤59, and safety grade of A, B, or C; total point score >60, and safety grade of C. Cases that fulfillled either of the following sets of criteria were assessed as poor: total point score ≥20 and ≤39, and safety grade of A, B, C, or D; total point score >40, and safety grade of D (26–28).

CASE REPORTS

Three representative cases are presented below.

Case 1: 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 6, when her general condition had improved, debridement of both lower legs was performed up to the fat layer, and 6-fold extended mesh auto-skin fragments were grafted onto the right lower leg, followed by application of allogeneic CDS (Fig. 1A–C). On day 13, debridement of the bilateral femoral and gluteal regions was performed up to the fat layer, and 6-fold extended mesh auto-skin fragments were grafted onto the left femoral region, followed by application of allogeneic CDS. Take and epithelization of the grafts were good on day 18 after grafting on the right lower leg (Fig. 1D) and on day 14 after grafting on the left femoral region (not shown). The application of CDS was continued for a period of 42 days at both sites. The patient had mild dementia and rejected bathing, but the epithelialized wound area seemed to itch less than the regions treated with conservative therapy, and good cicatrices were formed without scratch wounds. The patient was discharged wheelchair-bound 9 months after surgery, after gait training.

Case 2: A 79-year-old male fell into a bonfire and was burned. The injury was a mixture of DDB and DB accounting for 25% of the back, gluteal regions and both hands. On day 6, when the general condition had improved, debridement was performed on the bilateral gluteal regions up to the fat layer, and 6-fold extended mesh auto-skin fragments were grafted, followed by application of allogeneic CDS (Fig. 2A,B). Take and epithelization of the grafted skin were good on day 8 after grafting (Fig. 2C). Epithelization advanced smoothly on the granulation tissue under the CDS and is shown on day 22 after grafting (Fig. 2D). The application of CDS was continued for a period of 24 days. The patient was discharged 2 months after surgery, and he rides a motorcycle and visits the outpatient office once every few months. At 10 months after surgery, the cicatrices were thin and soft, maintaining good condition.

TABLE 2. *Total evaluation by judging both efficacy and safety*

Total evaluation	Efficacy	Safety
Excellent	80–100	A
Good	60–78	A or B
Fair	40–59	A, B, or C
	>60	C
Poor	20–39	A, B, C, or D
	>40	D

A, very safe; B, safe; C, with specific treatment; D, not safe.

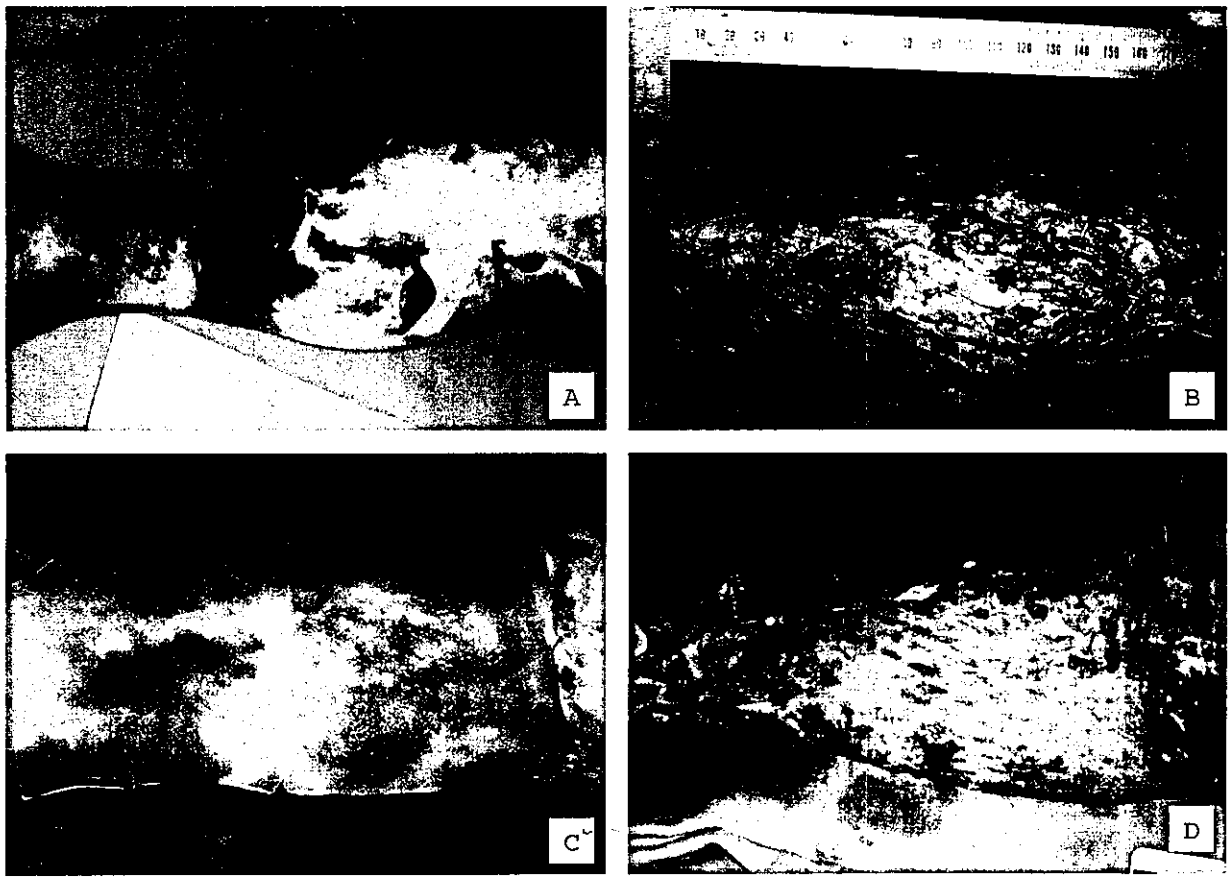

FIG. 1. Case 1: an 81-year-old female (Patient 1). The patient suffered DDB and DB on the right lower leg (A). Six-fold extended mesh auto-skin fragments were applied to the debrided wound (size 18 × 9 cm) (B), and followed by application of allogeneic CDS (C). The mesh skin took successfully and the area between strips of mesh skin were epithelized on day 18 after grafting (D).

Case 3: An 88-year-old female suffering from necrotizing fasciitis on the back lumbar region (size 39 × 28 cm) (Fig. 3A). Necrocectomy was performed up to the fat layer, and 6-fold extended mesh auto-skin fragments were grafted onto the debrided wound, followed by application of allogeneic CDS (Fig. 3B,C). Take and epithelization of the grafted skin were good on day 28 after grafting (Fig. 3D). The cicatrices were thin and soft, indicating good condition.

RESULTS

The mean duration of use of cultured allogeneic dermal substitute was 36 days, and the mean number of CDS exchanges was 7 (Table 3). In one case (Patient 4), the grafted area gave rise to infection at day 14 after grafting. Take of grafted skin was complete in all cases, and epithelization advanced smoothly in 6 cases. About 5 days after grafting, epithilization began in the area surrounding the grafted skin. Formation of good red-colored granulation between strips of mesh skin was smooth and flat, and epithelization advanced smoothly on that granulation tissue under the CDS. Healing was not slower than that of the 3-fold extended mesh skin grafts in the same patient. The duration of postoperative follow-up was 10–14 months, and cicatrices were soft and elastic. Stiffness was mild and did not disturb motor function.

DISCUSSION

A multicenter clinical study of allogeneic CDS has been conducted in 30 hospitals across Japan as part of the Regenerating Medical Millennium Project of the Ministry of Health, Labor and Welfare. This clinical study was designed to evaluate allogeneic CDS for treatment of severe wounds, including burn wounds (DDB, DB), skin ulcers, traumatic skin defects, and excise wounds from removal of giant pigmented nevus (26–28). The department of plastic reconstructive surgery of Kagawa Prefectural Central Hospital is a member of this project. In our

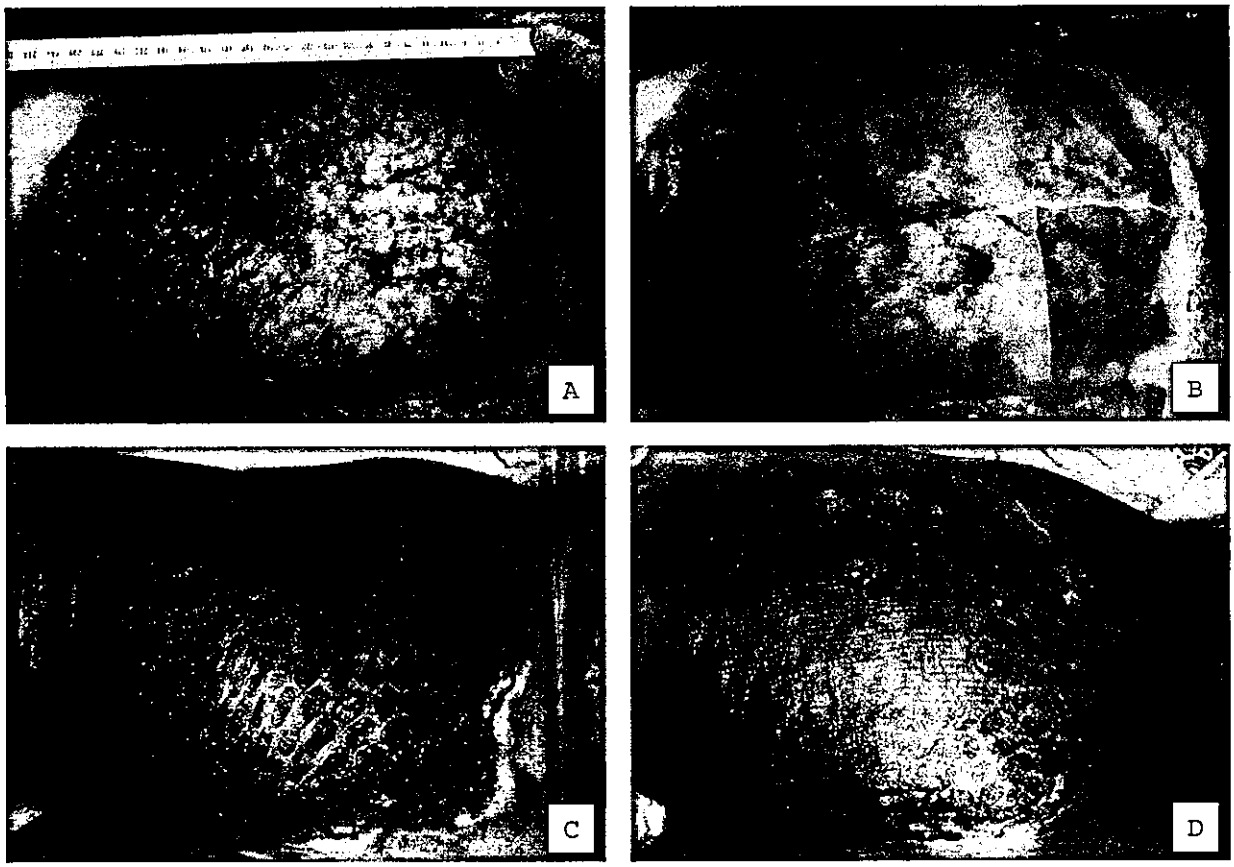

FIG. 2. Case 2: a 79-year-old male (Patient 2). The patient suffered intermingled DDB and DB on the bilateral gluteal regions. Debridement was applied up to the fat layer, and 6-fold extended mesh auto-skin fragments were grafted on the wound (A), on which the allogenic CDS was placed (B). On day 8 after grafting, the grafted skin took successfully (C). On day 22 after grafting, epithelization was advanced (D).

hospital, the focus of this clinical study was application of CDS for coverage of 6-fold extended auto-skin graft.

Allogeneic CDS fails to take permanently on the wound surface, but is able to produce cell growth factors (e.g., vascular endothelial growth factor [VEGF]) and extracellular matrix components (e.g., fibronectin), which are necessary for wound healing (22,25). The constituents of the spongy matrix of CDS promote wound healing. Hyaluronic acid has a high capacity for hydration, is involved in adherence/detachment of cells and substrates, and

TABLE 3. *Information on patients given concomitant grafting of 6-fold extended mesh auto-skin and allogeneic CDS*

	Patient 1	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	81	81	79	39	44	54	88
Sex	M	M	M	F	M	M	F
Indication	DDB/DB	DDB/DB	DDB/DB	DDB/DB	DDB/DB	DDB/DB	Necrotiz. fasciitis
Region	Right lower leg	Left femoral region	Left gluteal region	Right forearm	Right left arm	Right lower leg	Left lumbar region
Size (cm × cm)	18 × 9	15 × 9	24 × 17	14 × 7 7 × 6	30 × 10 40 × 10	27 × 10	27 × 13
Duration (days)	42	42	22	46	28	26	47
Exchange (number)	8	7	6	7	7	7	7
Efficacy	96	96	98	88	75	82	88
Safety	A	A	A	A	C	A	A
Total evaluation	Excellent	Excellent	Excellent	Excellent	Fair	Excellent	Excellent

CDS, cultured dermal substitute; DDB, deep dermal burns; DB, deep burns.

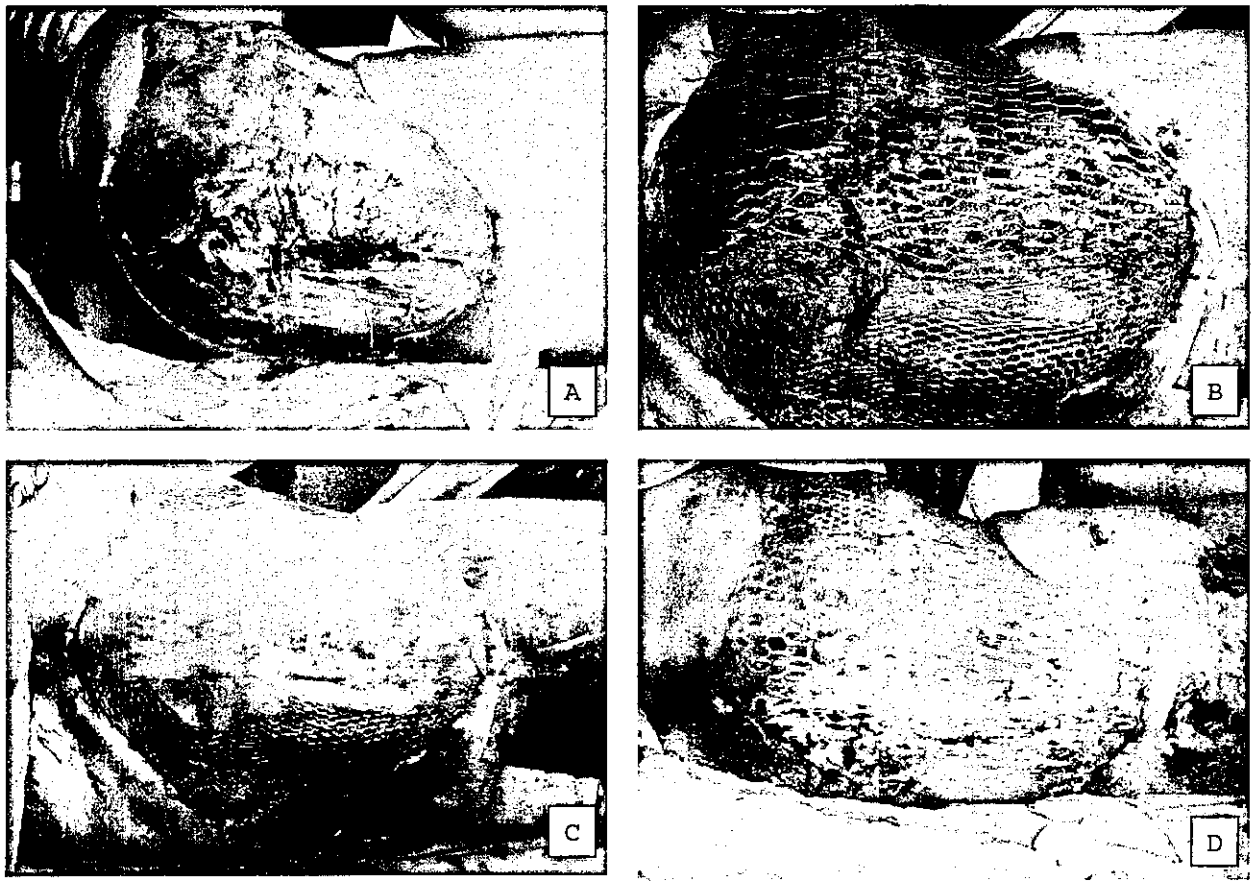

FIG. 3. Case 3: An 88-year-old female (Patient 6). The patient suffered necrotizing fasciitis on the back lumber region (A). Necrosectomy was performed, and 6-fold extended mesh auto-skin fragments were grafted (B), on which the allogeneic CDS was placed (C). On day 28 after grafting, the grafted skin took successfully and epithelization was advanced (D).

promotes cell migration (29,30). Collagen-derived polypeptide acts as a chemotactic factor for fibroblasts (31).

Treatment of deep burn wounds requires surgical closure with auto-skin grafting. However, in cases of massive burn injury, the donor area is insufficient. To reduce the area of donor skin surface needed, the area of harvested split-thickness skin can be increased by preparing mesh skin grafts. Although mesh skin grafting has disadvantages with regard to functional and esthetic aspects of cicatricial skin, it is a good method for closing wounds at an early stage, to improve the general condition of the patient. It seems that successful application of mesh skin grafting extended to more than 3-fold is difficult, because of insufficient epithelization between the strips of meshed skin. Successful application of highly expanded mesh skin grafting requires excellent biological dressing, which can promote granulation tissue formation and epithelization on the resulting granulation tissue. The present study was designed to evaluate the ability of allogeneic CDS to promote granulation tissue formation and epithelization when used as a biological dressing for 6-fold extended mesh auto-skin grafting. In all present cases, the 6-fold extended mesh auto-skin took successfully and most of the wound surface between the strips of meshed skin epithelialized within 3 weeks. In addition, the quality of final cicatrices was good. The present results suggest that allogeneic CDS is highly suitable as a biological dressing for 6-fold extended mesh auto-skin grafting. This application has promise as a therapeutic method for patients with massive severe burn injuries.

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Digital gangrene associated with idiopathic hypereosinophilia: treatment with allogeneic cultured dermal substitute (CDS)

In the present case study, the patient was a 65-year-old man who suddenly developed purpuric and necrotic lesions with severe pain in his fingers and toes. Laboratory investigations revealed marked eosinophilia (77.9%), but there was no evidence to support a diagnosis of parasitic infections, allergic disease, neoplasm or connective tissue disorder. The histopathological findings did not show any distinct vasculitis, but there were obliterative changes of the arterioles. The digital gangrene gradually progressed and was unresponsive to corticosteroid therapy. The patient eventually underwent amputation of the distal phalanges. We applied allogeneic cultured dermal substitute (CDS) to the skin defect. The allogeneic CDS was prepared by culturing fibroblasts on a two-layered sponge of hyaluronic acid and atelo-collagen. This CDS is able to release a number of cytokines including VEGF. The present case had a good clinical result.

Key words: allogeneic cultured dermal substitute, gangrene, hypereosinophilic syndrome, vasculitis

Hypereosinophilic syndrome (HES) is characterized by peripheral blood eosinophilia and the infiltration of eosinophils into many organs, including the skin. The most common cutaneous involvements are pruritic maculopapular and nodular lesions, or urticarial and angio-edematous plaques [1].

Recently, Jang *et al.* described a HES patient presenting with multiple erythematous patches and plaques on the lower extremities complicated by digital gangrene [2]. Histopathological examination of the cutaneous lesions in that patient revealed necrotizing eosinophilic vasculitis. They speculated that necrotizing eosinophilic vasculitis may be one of the cutaneous manifestations of HES, and that digital gangrene may be less rare as one of its manifestations than has previously been thought.

In the present case study, we investigated the efficacy of a new approach in which cultured cells are used to treat severe skin defects. A number of skin substitutes containing keratinocytes and/or fibroblasts have been developed [3-6]. These products can provide effective therapy for patients suffering from burns, diabetes ulcer or pressure sores [7-12]. Kuroyanagi *et al.* developed an allogeneic cultured dermal substitute (CDS) composed of a spongy matrix of collagen containing fibroblasts [13]. To improve wound management, they have developed another CDS by culturing fibroblasts on a two-layered spongy matrix of hyaluronic acid and collagen; this is the CDS that was used in the present case [14,15]. Hyaluronic acid has a high capacity for hydration, resulting in a moist environment at the wound site. This molecule plays a critical role in several cellular functions, including migration and proliferation, by promoting adhesion and disadhesion on tissue substrate [16]. Collagen and collagen-derived peptides act as chemoattractants for fibroblasts *in vitro*, and may have similar activity *in vivo* [17]. The fibroblasts contained in the present CDS can release biologically active substances such as VEGF and extracellular matrix components such as fibronectin, which are necessary for wound healing (Kubo K, Kuroyanagi Y. unpublished data). Since April 2001, the R&D Center for Artificial Skin of Kitasato University has been the main participating institution of the Regenerating Medical Millennium Project (skin department) of the Ministry of Health, Labor and Welfare of Japan. This center has established a banking system for cryopreserved allogeneic CDS, and has distributed these products to 30 hospitals across Japan in a frozen state. The results of a multi-center clinical study suggest that the present type of allogeneic CDS can provide an excellent wound bed with highly vascularized granulation tissue suitable for autologous split-thickness skin graft [18, 19]. Here, we describe use of this CDS in a case of idiopathic hypereosinophilia with digital gangrene.

Case report

A 65-year-old man noticed a cold sensation, pain, and purpuric patches on the fingers of both hands on May 10, 2002. Although vasodilators and anti-inflammatory drugs were prescribed by his doctor, the symptoms did not improve. Because the patient subsequently presented with similar lesions on both soles, he visited our hospital on June 4, 2002, and was admitted 2 days later. Although his previous and family history was unremarkable, he had smoked 1 or 2 packs of cigarettes per day for 45 years.

The patient was 162 cm in height and 56.3 kg in weight. There had been no weight loss during the previous month.

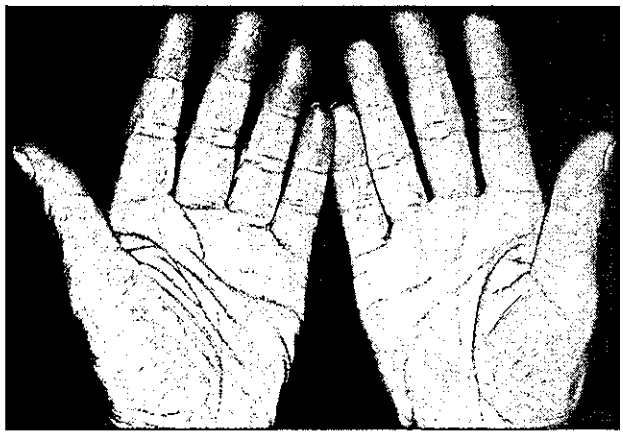

Figure 1. *Purpuric patches on the fingertips.*

On admission, his blood pressure was 110/72 mmHg, his pulse rate was 68/min, and his body temperature was 36.5 °C. There were purpuric patches on the tips of the fingers *(Fig. 1)* and soles. The distal area of the first toe of the left foot was necrotic. Laboratory investigations revealed the following: white blood cell count, 30700/mm^3 (77.9% eosinophils); IgE, 6366U/l; anti-nuclear antibody (ANA), x 160. All other immunological screening results were negative or normal; these included anti-ds DNA, anti-SS-A, anti-SS-B, anti-Jo-1, anti-cardiolipin antibody, anti-neutrophil cytoplasmic autoantibody (ANCA), and complement components (C3, C4, CH50) Coagulation parameters, including protein C and S, were normal. Stool samples were negative for parasites. No abnormalities were found by thoracoabdominal X-ray, CT scans or echocardiographic examinations. The karyotype of peripheral blood cells was normal (46, XY). A skin biopsy specimen obtained from a lesion of the right sole showed a perivascular infiltrate composed predominantly of lymphocytes and eosinophils in the superficial dermis. In the deep dermis, thickening of the small blood vessels and obliterative changes were found *(Fig. 2)*, but without any distinct vasculitis or panniculitis.

The patient was treated with vasodilators, anti-platelets, and oral prednisolone (60 mg/ day). Although the peripheral blood eosinophilia improved within a week, his pain and numbness persisted and digital gangrene developed day by day *(Figs. 3a, 3b)*. Beginning on August 13, 2002, oral ciclosporin (200 mg/day) was prescribed, but was not effective. After amputation of the distal phalanges and debridement of the lateral side of the right foot on September 13, 2002 *(Fig. 4)*, trafermin was applied to the skin defects. Due to persistent pain, we applied the allogenic CDS to the skin defects *(Fig. 5)*, and a conventional ointment-gauze dressing was used to protect the CDS. The CDS was replaced with fresh CDS at intervals of 3 to 5 days for 6 weeks. During CDS treatment, the pain almost disappeared, and there was no other complaint about the wound area. Because healthy granulation tissue had successfully formed we covered the skin defects with split-thickness skin grafts harvested from the thigh on November 11, 2002 *(Fig. 6)*. The treatment with prednisolone and ciclosporin was gradually discontinued, and the patient was discharged from our hospital on December 11, 2002.

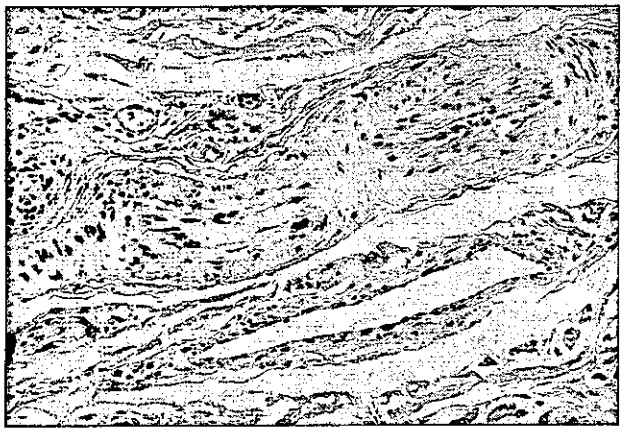

Figure 2. *Obliterative changes of the arterioles in the deep dermis (hematoxylin, eosin staining, x 200).*

Discussion

HES generally involves marked eosinophilia of unknown etiology and damage to multiple organs. The criteria for diagnosis of HES include the following: peripheral blood eosinophilia with eosinophil counts > 1 500/mm^3 for at least 6 months; no evidence of parasitic, allergic or other known causes of eosinophilia; and presumptive signs and symptoms of multiple organ involvement [1]. Because we

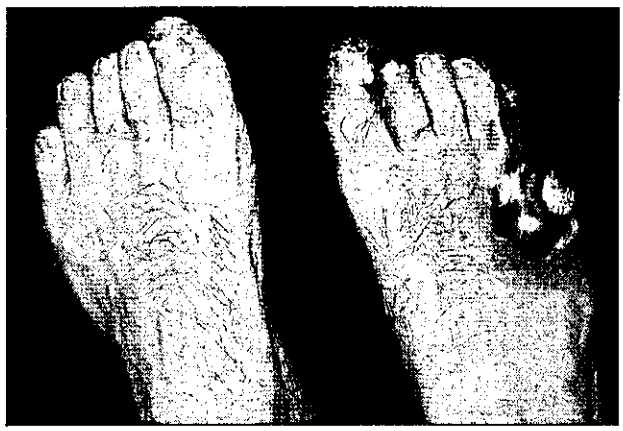

Figure 3a. *Necrotic lesions on the feet.*

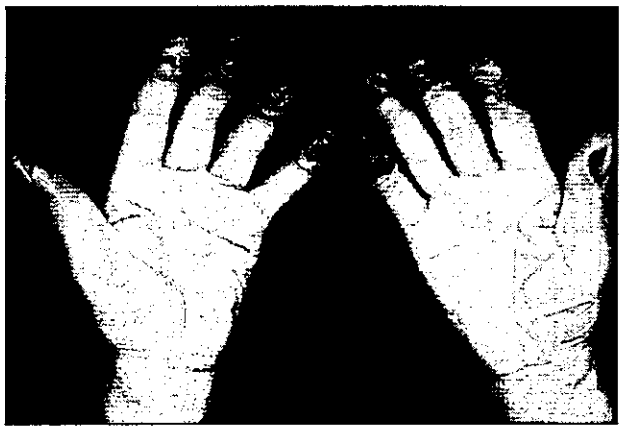

Figure 3b. *Necrotic lesions on the fingertips.*

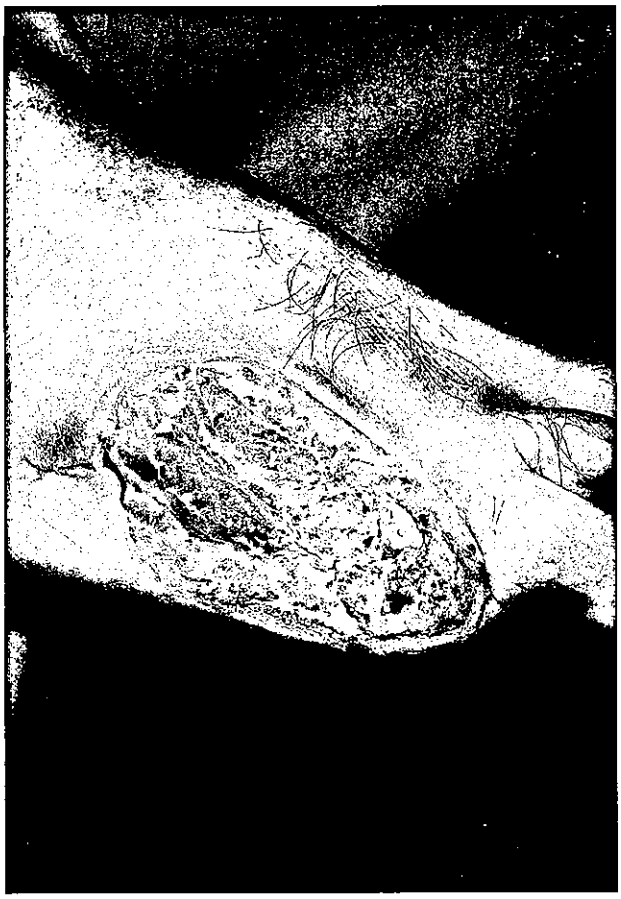

Figure 4. *Debridement of the lateral side of the right foot.*

began medication as soon as the laboratory investigations revealed hypereosinophilia (eosinophil counts, 23 900/mm^3), it is not clear whether the first criteria was fulfilled in the present case. However, the symptoms and laboratory results generally indicated HES.

Cutaneous manifestations occur in more than 50% of HES patients [1]. However, digital gangrene in HES has very rarely been reported [2, 20, 21]. As mentioned above, Jang

Figure 5. *Application of CDS to the skin defects of the right foot.*

Figure 6. *Right foot at 6 weeks after starting the CDS treatment.*

et al. described a HES patient presenting with cutaneous vasculitis complicated by digital gangrene [2]. In contrast, Oppliger *et al.* reported a HES patient with digital gangrene associated with occlusion of medium-sized arteries, but without cutaneous vasculitis [20]; this suggests that digital gangrene in HES can develop independently of cutaneous vasculitis. In the present case, cutaneous vasculitis was not detected by histopathological analysis. Instead, some blood vessels were almost obliterated, whereas others were extremely dilated, probably due to compensation. Elastica van Gieson staining showed that these obliterated vessels were arterioles. Angiography of the extremities was not performed, because we were unable to obtain approval from the patient; thus the condition of his medium-sized arteries was unclear. However, it is possible that his long-term smoking induced thromboangiitis obliterans (TAO). Ferguson *et al.* found marked eosinophilic infiltration of the thrombus and vessel wall in a patient with TAO associated with idiopathic hypereosinophilia. It is possible that, in some TAO patients, eosinophils are involved in pathogenesis of TAO [22]. In fact, other cases of hypereosinophilia with arterial occlusion have been reported [23]. Although the precise pathomechanism responsible for thrombosis in patients with hypereosinophilia is unknown, it has been suggested that eosinophilic granule protein impairs thrombomodulin function [24, 25].

In the present patient, prednisolone reduced the absolute eosinophil count. However, the severe pain in his fingers and toes was uncontrollable. Takekawa *et al.* have suggested that tumor necrosis factor alpha (TNF-α) may play a role in the etiology of necrosis of the fingertips in HES [21]. Further investigations are needed to determine if any other interventions, such as anti-TNF antibody, suppress the necrosis more effectively than corticosteroids. In addition, cases of HES, that respond well to imatinib, have recently been reported [26, 27].

In the present case, we used the allogeneic CDS prepared by Kuroyanagi *et al.* [14], at the sites where amputation and debridement were performed.

This CDS has been found to release a number of biologically active substances, including VEGF, bFGF, HGF, KGF, PDGF, TGF-β, IL-6, and IL-8 (Kubo K, Kuroyanagi Y. unpublished data), and to reduce wound size more rapidly than ordinary ointment. In the near future, CDS may be commonly used to improve skin defects. ■

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Clinical evaluation of allogeneic cultured dermal substitutes for intractable skin ulcers after tumor resection

Clinical research on allogeneic cultured dermal substitute (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 of Japan. Allogeneic CDS was prepared by cultivation of fibroblasts on a two-layered spongy matrix of hyaluronic acid and atelocollagen. This paper reports the clinical results of application of allogeneic CDS in 12 patients with full-thickness skin defects after surgical resection of skin tumors. In 9 of 10 patients, healthy granulation tissue developed immediately, allowing us to perform split-thickness skin grafts at an early stage. In two cases, allogeneic CDS was used to cover an expanded mesh skin graft that had been applied to treat a large ulcer, and rapid epithelization was observed. No patient developed local infection nor local tumor recurrence after treatment with CDS. 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.

Key words: allogeneic cultured dermal substitute, fibroblast, full-thickness skin ulcer, VEGF

A variety of skin substitutes have been developed and some of them are commercially available in the United States. A representative engineered product is autologous cultured epidermal substitute (CES), which is composed of stratified keratinocytes (Epicel®; Genzyme Tissue Repair, Cambridge, MA, USA) [1-4]. Two types of allogeneic cultured dermal substitute (CDS) (Dermagraft® and Trans Cyte®; Advanced Tissue Sciences, La Jolla, CA, USA) are composed of fibroblasts on a scaffold [5-10]. Another skin substitute is allogeneic cultured skin substitute (CSS) (Apligraf®; Organogenesis, Canton, MA, USA), which is composed of keratinocytes and fibroblasts on a scaffold [11-13]. Allogeneic CDS and CSS require an appropriate matrix, i.e., a scaffold for fibroblasts and/or keratinocytes. Recently, however, the commercialization of these allogeneic products was discontinued. There seem to be some problems in the design of these products.

Kuroyanagi and colleagues [16-19] developed an allogeneic CDS composed of a spongy collagen containing fibroblasts. The efficacy of this allogeneic CDS on wound healing has been assessed in animal studies and in preliminary clinical trials [20, 21]. Based on this concept, a second version of the CDS was developed through cultivation of fibroblasts on a two-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col). The HA molecule plays a critical role in several functions such as migration and proliferation of various types of cells by promoting adhesion and disadhesion on the tissue substrate [23-26]. The collagen molecules also play a pivotal role in the wound healing process. Collagen and collagen-derived peptides act as chemoattractants for fibroblasts *in vitro* and may have a similar activity *in vivo* [27].

A multi-center clinical study on the use of allogeneic CDS was performed in 30 hospitals across Japan as the Regenerating Medical Millennium Project of the Ministry of Health, Labor and Welfare of Japan. The Department of Dermatology of Kyushu University Hospital is a member of this project. This clinical study was designed to evaluate the usefulness and efficacy of allogeneic CDS in the treatment of full-thickness skin defects after the resection of skin tumors.

Materials and methods

Preparation of spongy matrix composed of hyaluronic acid (HA) and atelo-collagen (Col)

Spongy matrix was prepared by the method described previously [20, 21]. An aqueous solution of HA with cross-linking agent was poured into a polystyrene dish (11 cm × 10 cm), in which a sheet of hydrated, cellulose, non-woven fabric had been attached at the bottom. The HA solution in the dish was frozen and then lyophilized to obtain the HA sponge. After the sponge was thoroughly rinsed with distilled water to remove free cross-linking agent, the hydrated HA sponge was frozen and lyophilized to obtain the purified HA sponge. The purified HA sponge was punched mechanically to produce many holes. The Col

solution was poured into a polystyrene dish (11 cm × 10 cm). The HA sponge with many holes was carefully immersed into the dish containing Col solution, keeping the sheet of non-woven fabric on the HA sponge facing upward. The hydrated HA sponge was frozen and lyophilized to obtain a two-layered sponge of HA and Col. Both surfaces of the two-layered sponge were irradiated with an ultraviolet lamp to induce intermolecular cross-linking of Col molecules.

Establishment of cell banking

Cell banking was established by the procedure described previously [21, 22]. A small piece of skin was donated from a 3-month-old patient during the surgical excision of excrescence. The patient was free from infectious viruses such as HBV, HCV, HIV, and HTLV, and also negative on the treponema pallidum hemagglutination test (TPHA), in compliance with the ethical guidelines of St. Marianna Medical College (Kawasaki, Kanangawa, Japan). Fibroblasts were isolated by enzymatic treatment. The successive cultivation of fibroblasts was initiated in culture medium to establish cell banking. The cells were tested for viruses including HBV, HCV, HIV, HTLV, and Parvovirus, and were found to be negative.

Preparation of cultured dermal substitute (CDS)

The allogeneic CDS was prepared by the method described previously [21, 22]. 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 to hydrate and neutralize the acidic two-layered sponge. Excess culture medium was carefully removed from each dish by suction. The fibroblasts that had been obtained in successive cultivations from the cryopreserved cells were seeded on the two-layered sponge. Five ml of the cellular suspension was added dropwise on 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 placed in an incubator overnight, and then 50 ml of culture medium was added, followed by culturing for 1 week. The fibroblasts used in each CDS that was produced, were previously tested for mycoplasma and confirmed to be negative. The culture medium used in each CDS that was produced, was previously tested for bacteria and confirmed to be negative.

Cryopreserving and thawing procedures

Cryopreservation of CDS was performed according to the method described previously [21, 22]. The CDS was frozen in DMEM supplemented with 10% DMSO and 20% FBS from 4 °C to – 60 °C at a rate of – 1 °C/min, and then cryopreserved in a freezer at – 15 °C. The cryopreserved CDS was placed in a foam polystyrene box containing dry ice, shipped to hospitals, and then preserved at – 85 °C. Prior to clinical application, the polystyrene dish containing CDS was placed in a foam polystyrene box at room temperature for 30 min and then floated in a water bath at 37 °C; then, the CDS was rinsed with lactated Ringer's solution to remove DMSO and FBS.

Clinical study

A clinical study on the use of allogeneic CDS was conducted in accordance with the study protocol and the ethical guidelines of Kitasato University Hospital and Kyushu University Hospital. Twelve patients aged 27-87 years (mean age, 57.5 years) with skin defects after the resection of skin tumors were included in this study. Informed consent for CDS treatment was obtained from all patients. *Table I* shows the background of the 12 patients. Application of allogeneic CDS was performed for the following conditions, (1) postoperative ulcer (n = 9), (2) covering on a mesh graft (n = 2), (3) other (n = 1; ulcer on orbital born that developed after total resection of orbital tissue and split-thickness skin graft). The wound surface was 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 wound surface, and conventional ointment-gauze dressing was used to protect the CDS. A new CDS was applied every 3 to 5 days. Clinical evaluation was performed according to the study protocol. Epithelization and granulation tissue formation were graded according to the following scale: excellent (+ + +), good (+ +), fair (+), poor (–). The size of the ulcers was presented as long diameter x short diameter (cm). In all cases with postoperative ulcer (type 1), skin graft was performed on the resultant healthy granulation tissue.

Results

The clinical results of application of allogeneic CDS in patients with skin ulcers are shown in *Table II*. In 11 of the 12 cases, healthy granulation tissue developed rapidly. Full-thickness skin defects, especially those in which tendon and/or bone is exposed after surgical resection of a malignant tumor, are not suitable for immediate free skin graft. In cases of delayed surgery, it takes a long time for granulation tissue to form before secondary skin grafting can be performed. The application of CDS, however, resulted in rapid granulation tissue formation acceptable for skin graft in 10 cases (Cases 1, 4, 5, 7-12). The mesh skin graft is very useful for unhealthy and/or non-flat graft beds. Although a highly expanded mesh graft shows good adaptation, completion of epithelization takes a long time. In Cases 2 and 6, rapid epithelization was observed after CDS was applied on the mesh skin graft that had been placed over a large postoperative ulcer. Case 3 was an unusual case in which the patient had undergone total orbital tissue resection followed by split-thickness skin graft for a malignant melanoma on her conjunctiva. A dry ulcer with exposed bone was found on the orbital cavity and was subjected to CDS treatment. Although this dry ulcer was small, its size did not change even after applying CDS ten times. Treatment with CDS was discontinued. This case suggests that for a bone-exposing ulcer surrounding a thin skin graft CDS application may not induce the production of a sufficient number of endogenous fibroblasts to form granulation tissues.

Application of CDS was found to be useful in the majority of cases. Allergic reactions to the CDS treatments such as erythema, itchiness around the ulcer or anaphylactic shock, were not observed in any patient. The healing process in two representative cases who underwent allogeneic CDS is described.

Case 1 was a 68-year-old man with eccrine poroma on the dorsum of his foot *(Fig. 1a)*. Preoperative biopsy suggested malignant transformation, and surgical resection of the tumor left a full- thickness skin defect of 9.3 cm × 7.8 cm