Table I. *Background of patients before the treatment with CDS*

Case	Age/Sex	Type*	Location	Tumor
1	68 M	1	Dorsum of foot	Eccrine poroma
2	49 M	2	Lower leg	SCC** on burn scar
3	43 F	3	Inner orbit	Malignant melanoma
4	59 M	1	Sole	Malignant melanoma
5	55 M	1	Finger	SCC
6	51 F	2	Chest	Dermatofibrosarcoma protuberance
7	50 F	1	Dorsum of foot	SCC on burn scar
8	27 F	1	Cheek	Nevoid basal cell carcinoma syndrome
9	73 F	1	Ankle	Malignant melanoma
10	41 F	1	Temporal head	Adenoid cystic carcinoma
11	87 F	1	Sole	Malignant melanoma in situ
12	87 F	1	Finger	SCC

** conditions of ulcer; 1: postoperative ulcer, 2: covering on mesh graft, 3: ulcer on orbital born that developed total resection of orbital tissue followed by split-thickness skin graft; ** squamous cell carcinoma.*

in size, with exposed tendons. Allogeneic CDS was applied to the skin defect. Granulation tissue formed rapidly *(Fig. 1b-1e)*. Skin grafting could be performed as soon as 10 days after tumor resection, and graft adaptation was excellent *(Fig. 1f)*. Imunohistochemical analysis of the entire excised tumor led us to make the diagnosis of benign eccrine poroma.

Case 7 was a 50-year-old woman with a moderately differentiated squamous cell carcinoma on the dorsum of her foot that had arisen on a burn scar *(Fig. 2a)*. Surgical resection of the tumor left a full-thickness skin defect of 14.0 cm × 8.0 cm in size, with exposed tendons *(Fig. 2b)*. CDS was then applied to the skin defect *(Fig. 2c)*. Granulation tissue rapidly developed *(Fig. 2d-2g)*. Seventeen days after beginning CDS application, a skin graft was successfully performed and graft adaptation was complete *(Fig. 2f)*. Observation for 1.5 years revealed no local recurrence of malignant tumor and no contracture of the foot joint after skin the graft.

Discussion

Autologous CES which is composed of stratified keratinocytes, is able to permanently take on the patient's own skin defect and to form the epidermis on the resulting neodermis. On the other hand, allogeneic CDS which is composed of fibroblasts and a material as a scaffold, fails to permanently take on a patient's skin defect. However, the cells in the CDS are able to 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 depends on the function of the cells and the function of the material that is used as a scaffold. Therefore, it is very important to use materials that have the ability to promote wound healing themselves. The two-layered spongy matrix of HA and Col was found to have higher potency for promoting wound healing, compared with a collagen spongy matrix, in a preliminary animal study [20]. When the CDS is applied on the wound

Table II. *Results of the treatment with CDS*

Case	Type*	Pre-size**(cm)	Post-size***(cm)	No. $ (days)	Granulation	Epithelization	Graft
1	1	9.3 × 7.8	9.3 × 7.8	3 (10)	+++	–	Good
2	2	22.0 × 15.7	0 × 0	5 (17)	++	++	Good
3	3	0.7 × 0.5	0.7 × 0.5	10 (38)	–	–	ND#
4	1	9.0 × 8.5	9.0 × 8.5	5 (21)	+++	–	Good
5	1	5.0 × 4.8	5.0 × 4.0	6 (21)	+++	+	Good
6	2	13.0 × 8.0	0 × 0	3 (7)	++	++	Good
7	1	14.0 × 8.0	14.0 × 8.0	5 (17)	+++	–	Good
8	1	4.1 × 3.8	3.8 × 3.5	5 (19)	+++	+	Good
9	1	8.0 × 8.0	8.0 × 8.0	4 (20)	+++	–	Good
10	1	10.0 × 7.0	10.0 × 7.0	4 (14)	+++	–	Good
11	1	8.1 × 5.7	8.1 × 5.7	5 (17)	++	–	Good
12	1	3.0 × 2.5	3.0 × 2.0	4 (15)	+++	+	Good

** conditions of ulcer; 1: postoperative ulcer, 2: covering on mesh graft, 3: ulcer on orbital born that developed total resection of orbital tissue followed by split-thickness skin graft; ** size of ulcer before the treatment; *** size of ulcer after the treatment; $ Number of CDSs applied (total number of days of CDS application); # ND: not done*

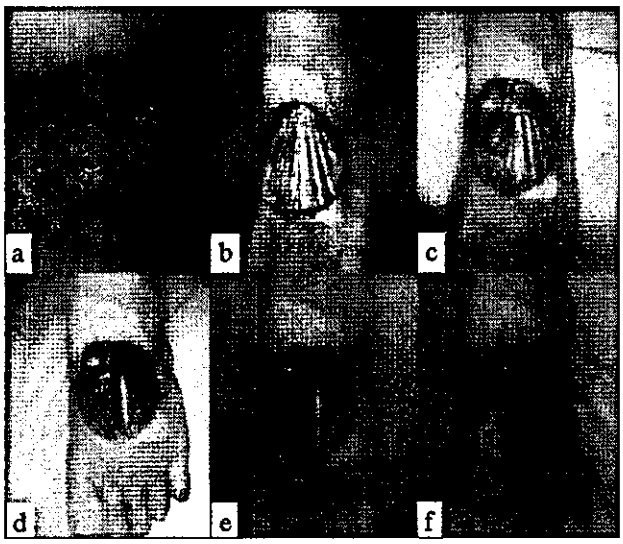

Figure 1. *Case 1. A 68-year-old man developed porocarcinoma on his left foot (a). Surgical resection left a full-thickness skin defect measuring 9.3 × 7.8 cm (b), on which the allogeneic CDS was applied. The development of granulation tissue at 3 days (c), 7 days (d), and 10 days (e), is shown. Skin grafting was performed on day 10, and grafted skin was completely adapted 7 days after reconstitution (f).*

surface in clinical use, the spongy structure degrades within about 1 week [20]. Both HA and Col molecules seem to be involved in the process of wound healing. Furthermore, fibroblasts also play multiple roles in the complex process of wound healing. They release a number of biologically active substances including growth factors and angiogenic factors. Neovascularization is essential for wound healing, in particular, for healing of chronic and poor-healing deeper wounds. VEGF plays a critical role in this process [28-30]. CDS releases a substantial amount of VEGF over a cultivation period of 1 week [21, 31]. Incubation with CDS enhanced proliferation of vascular endothelial cells in a dose-dependent fashion, and the addition of anti-human VEGF antibody reduced their proliferative activity [31]. Another important function of fibroblasts is production of extracellular matrix including collagen and fibronectin. Fibronectin serves several critical functions in effective wound healing. In practice, immunohistochemical staining for fibronectin in excised CDS showed substantial deposition of fibronectin on the spongy structure of CDS [21].

To date, CDS has been used in 12 patients at our hospital. Although CDS application was discontinued in one patient, 11 patients had an excellent response to CDS application. Our clinical results suggest that CDS is extremely useful for the treatment of deep wounds such as full-thickness skin defects. Upon application of CDS, all 9 cases with surgical skin defects due to resection of a malignant tumor showed rapid granulation tissue formation acceptable for secondary skin grafting. Granulation tissue formation upon treatment with CDS appeared to occur faster than that upon conventional ointment therapies. In the two cases in which CDS was applied to cover a mesh graft, quick epithelial formation was observed, and CDS seems to contribute to epithelial formation.

There were no adverse effects and no allergic reaction to CDS treatment was noticed in any patient. After application of a CDS, allogeneic fibroblasts in the CDS are gradually rejected depending on the wound condition. However, repeated application of CDS might lead to an allo-specific immune response and the materials that remain in the culture medium of CDS also could induce immune reactions. No patient complained of itchiness, pain nor any other sensation. In addition, we had paid special attention to signs of infection of the wound during the clinical course, because CDS can serve as an infectious basement. Although sufficient debridement should be performed before starting treatment with CDS, some cases showed bacterial colonization on their ulcers before and during the CDS treatment. We continue to use the CDS and no patient has had to discontinue this treatment due to bacterial infection. The resistance of CDS against bacterial infection remains to be elucidated.

The topical basic fibroblast growth factor (b-FGF) product has been used for a variety of skin ulcers in Japan, and its

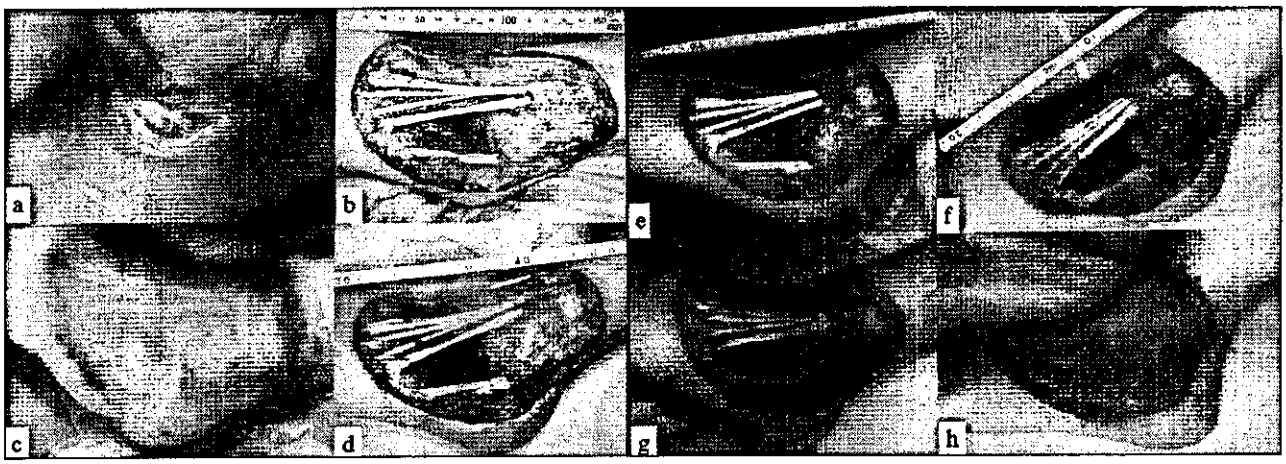

Figure 2. *Case 7. A 50-year-old woman developed moderately differentiated squamous cell carcinoma on the dorsum of her foot which had arisen on a burn scar (a). Surgical resection left a full-thickness skin defect measuring 14.0 × 8.0 cm (b), on which the allogeneic CDS was applied (c). The development of granulation tissue at 4 days (d), 7 days (e), 11 days (f), and 19 days (g), is shown. Skin grafting was performed 19 days after tumor resection, and grafted skin was completely adapted 15 days after reconstitution (h).*

effectiveness was confirmed [32]. However, this product cannot be used for skin defects after tumor resection, because b-FGF could serve as a growth factor for the malignant tumor. However, upon application of CDS on postoperative ulcers after resection of malignant tumor, none of our patients developed local recurrence of tumor during an observation period ranging 1 to 3 years.
This study showed that CDS is useful for the treatment of intractable skin ulcers, and CDS could be a safe and powerful tool for the treatment of postoperative skin ulcers. ■

Acknowledgements. The present study was conducted with the support of the Regenerating Medical Millennium Project of the Ministry of Health, Labor and Welfare of Japan.

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[Artificial Organs in press]

A study of cytokines released from fibroblasts in cultured dermal substitute (CDS)

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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). CDS can be cryopreserved and transported to other hospitals in a frozen state. The present study was designed to analyze amounts of cytokines released from fibroblasts in fresh or cryopreserved CDS. The culture medium used in preparing CDS over a cultivation period of 1 week (fresh CDS culture medium sample) contained 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, keratinocyte growth factor (KGF), interleukin (IL)-6 and IL-8. After thawing of cryopreserved CDS, the CDS was re-cultured in culture medium for 1 week. The culture medium used in re-culturing CDS for 1 week (cryopreserved CDS culture medium sample) contained VEGF, bFGF, and HGF in the same level before freezing, and TGF-β1 and IL-8 in a half level before freezing. Levels of PDGF-AA, KGF, and IL-6 were significantly less than before freezing. This finding suggests that the cryopreserved CDS retains its ability to release VEGF, bFGF, and HGF that are essential for wound healing.

INTRODUCTION

We have developed an allogeneic CDS by cultivating fibroblasts on a two-layered spongy matrix of HA and Col [1-4]. HA has a high capacity for hydration, resulting in a moist environment at the wound site [5]. Collagen and collagen-derived peptides act as chemoattractants for fibroblasts in vitro, and may have similar activity in vivo [6]. The authors designed a matrix using biomaterials that can promote wound healing [1]. The fibroblasts incorporated in this CDS can release VEGF and fibronectin that are necessary for wound healing [3]. The resultant CDS is designed to promote wound healing by synergic effects of the fibroblasts and matrix.

Wound healing is a complex process involving intricate interplay among a variety of cells, fibrous proteins, proteinases, cytokines such as endogenous chemoattractants, growth factors, and angiogenic factors. Chronic wounds including diabetic ulcers, pressure ulcers, and venous ulcer fail to proceed through an orderly and timely healing process. These wounds ultimately may result from deficiency of cytokines or inhibition of their function. 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 cytokines including PDGF [7-9], bFGF [8-10,12], EGF [11], VEGF [12], HGF [13] and TGF-β 1 [14] have been applied to chronic ulcers in animal or clinical studies. However, effects of topical 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 extracellular matrix (ECM) components such as collagen and fibronectin, and are an important source of several cytokines including growth factors and angiogenic factors.

CDS containing fibroblasts can provide more effective treatment than conventional methods for deeper wounds including chronic ulcers. Clinical application of fresh allogeneic CDS has disadvantages due to practical problems in mass-production, preservation and transportation. Therefore, if the cryopreserved allogeneic CDS would maintain the potency to release some vital cytokines such as VEGF, bFGF, and HGF that are essential for wound healing after thawing, it would be more useful for clinical applications. In the present study, we analyzed amounts of various cytokines released from fibroblasts in fresh or cryopreserved CDS.

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 a previous article [1]. Briefly, an aqueous solution of HA and cross-linking agent was poured into a polystyrene dish, in which a sheet of hydrated non-woven fabric was attached to the bottom. The dishes were kept at 50°C to promote the cross-linking reaction and concentration. A combination of HA sponge and the non-woven fabric was obtained by lyophilization, followed by thorough rinsing with water to remove unreacted cross-

linking agent, and lyophilization to obtain the purified HA sponge. The purified HA sponge was soaked in an aqueous solution of Col, followed by lyophilization to obtain a two-layered sponge. The two-layered sponge was treated with UV irradiation to induce cross-linking of Col.

Preparation of cultured dermal substitute (CDS)

The CDS was prepared using a method described in previous articles [2-4]. Briefly, cryopreserved fibroblasts were thawed and then successively cultured to obtain an adequate number of fibroblasts. The fibroblasts were seeded on the atelo-collagen surface of the two-layered spongy matrix at an initial seeding density of 1.0×10^5 cells/cm^2, followed by culturing for 1 week in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Human dermal fibroblasts were isolated from a piece of skin with a size of 0.5cm x 1.0cm which donated from a 3-months old patient in the surgical excision of excrescence.

Cryopreserving and thawing procedures

CDS was cryopreserved using a method described in a previous article [4]. Briefly, the CDS was cryopreserved in DMEM supplemented with 10% DMSO and 20% FBS. A dish containing CDS was frozen in a programmable freezer (AIR BLASTER, EBAC, Tokyo, Japan) at a rate of -1°C/min from 4°C to -60°C, and then cryopreserved at -152°C. Cryopreserved CDS was thawed by placing a polystyrene dish containing cryopreserved CDS in a foam polystyrene box at room temperature for 30 min, and then floating it in a water bath at 37°C, followed by rinsing of the CDS with lactated Ringer's solution 3 times to remove DMSO and FBS.

Quantitative analysis of cytokines released from CDS

We measured the amounts of 8 cytokines: VEGF, bFGF, HGF, PDGF-AA, TGF-β1, KGF, IL-6 and IL-8. The culture medium used in preparing CDS (fresh CDS culture medium sample) was collected over a cultivation period of 1 week and stored at –30°C. After cryopreserved CDS was thawed, it was re-cultured in culture medium for 1 week. The culture medium used in re-culturing 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). The standard for each cytokine and the culture medium sample were added to each well of a 96-well microplate in duplicate. The amount of cytokines was measured using commercial kits (R&D Systems Inc., Minneapolis, MN, USA), according to the package insert. The absorbance of cytokines in wells was measured with a microplate reader at 450 nm. Amounts of cytokines were determined by plotting on a calibration curve. In order to determine the net amount of cytokine released from the CDS, background corrections were made on all measurements using the amount of cytokine contained in the original medium (DMEM supplemented with 10% FBS). Each experiment was repeated 5 times, and each sample was assayed in duplicate. The data was expressed as mean and standard deviation, and was statistically analyzed using a two-tailed Student's t-test.

RESULTS

The amount of each cytokine measured, as a function of cultivation time, is plotted in Figure 1. The amount of VEGF increased after a cultivation period of 5 days, and reached a level of about 60 ng (Fig.1 A). Levels of bFGF and HGF were low: bFGF ranged from 2 to 4 ng (Fig.1 B); HGF ranged from 3 to 20 ng (Fig.1 C). The amount of PDGF-AA was less than 0.6 ng (Fig.1 D). The amount of TGF-β 1 increased with increasing cultivation time, and reached a level of about 300 ng (Fig.1 E). The amount of KGF also increased with increasing cultivation time, and reached a level of about 35 ng (Fig.1 F). The amounts of IL-6 and IL-8 were relatively high: IL-6 peaked at >2 μ g (Fig.1 G); IL-8 ranged from about 200 to 400 ng (Fig.1 H).

Figure 2 shows the amount of each cytokine in the medium before freezing (fresh CDS culture medium sample), and after thawing and re-culturing for 1 week (cryopreserved CDS culture medium sample). The amounts of VEGF and bFGF in cryopreserved CDS culture medium were the same as those of fresh CDS culture medium (Fig.2 A and B). The amounts of HGF, TGF-β 1, and IL-8 in cryopreserved CDS culture medium were found to be in a half level of those measured in fresh CDS culture medium (Fig.2 C, E, and H). The amounts of PDGF-AA, KGF and IL-6 in cryopreserved CDS culture medium were considerably lower than those measured in fresh CDS culture medium (Fig. 2 D, F, and G). The results shown in Figure 2 demonstrate that the cryopreserved CDS can release VEGF, bFGF, HGF, TGF-β 1 and IL-8 at appreciable levels. The number of fibroblasts in cryopreserved CDS increased when re-cultured for 1 week after thawing, reaching about 150% of the number of fibroblasts in fresh CDS [3]. These findings indicate that the surviving fibroblasts can proliferate markedly and release considerable amounts of several types of cytokines, but fail to release useful amounts of some types of cytokines.

DISCUSSION

In parallel with the clinical study, the authors conducted the basic research on the ability of cryopreserved CDS to release cytokines. In another study, we confirmed that VEGF released from both fresh and cryopreserved CDS promotes proliferation of vascular endothelial cells in vitro, and that the CDS also releases other cytokines that stimulate proliferation of vascular endothelial cells [15]. In the present study, we confirmed that the fresh CDS is able to release VEGF, bFGF, HGF, PDGF-AA, TGF-β 1, KGF, IL-6, and IL-8. On the other hand, the cryopreserved CDS was found to releases VEGF, bFGF, HGF, TGF-β 1, and IL-8. The reason why the amount of PDGF-AA, KGF, and IL-6 decreased remarkably is not clear in the present study.

Cytokines released at wound sites regulate complex wound healing process. Various endogenous cells migrate into wound sites in response to cytokines process. PDGF [16-18], bFGF [16,17,19], TGF β [16,17,20], KGF [19] and TGF-β [19] are considered to be growth and chemoattractive factors for fibroblasts. VEGF [21-23], bFGF [8,21,23], HGF [23,24] and IL-8 [23,25,26] are considered to be growth and chemoattractive factors of endothelial cells. TGF-β plays an important role in formation of granulation tissue. It promotes deposition of ECM components such as collagen, fibronectin, hyaluronic acid and protease inhibitor. It is thought that healing of chronic ulcers depends primarily on improvement in blood supply to the wound site. The cryopreserved CDS is able to release cytokines such as VEGF, bFGF, HGF, and TGF-β that are essential for wound healing. This finding seems to be related to clinical result. Cryopreserved allogeneic CDS has been found to be useful in the treatment of severe burn and intractable skin ulcers [27-34]. In the period of April 2001 to March 2005, multi-center clinical study has been conducted by the support of the Regenerative Medical Millennium Projects of the Ministry of Health, Labor and Welfare [35,36]. In 412 of clinical cases, 90% of various wounds were

evaluated as achieving good or excellent results.

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Legends

Figure 1.
Amount of cytokines released from fresh CDS over a cultivation period of 1 week. n = 5, means±SD; * $P<0.05$, # $P<0.01$. [A]:VEGF, [B]:bFGF, [C]:HGF, [D]:PDGF-AA, [E]:TGF-β, [F]:KGF, [G]:IL-6, [H]:IL-8

Figure 2.
Amount of cytokines released from fresh CDS before freezing (a); after thawing in incubator at 37°C, followed by re-culturing for 1 week (b). n=7, means±SD; * $P<0.05$, # $P<0.01$. [A]:VEGF, [B]:bFGF, [C]:HGF, [D]:PDGF-AA, [E]:TGF-β, [F]:KGF, [G]:IL-6, [H]:IL-8

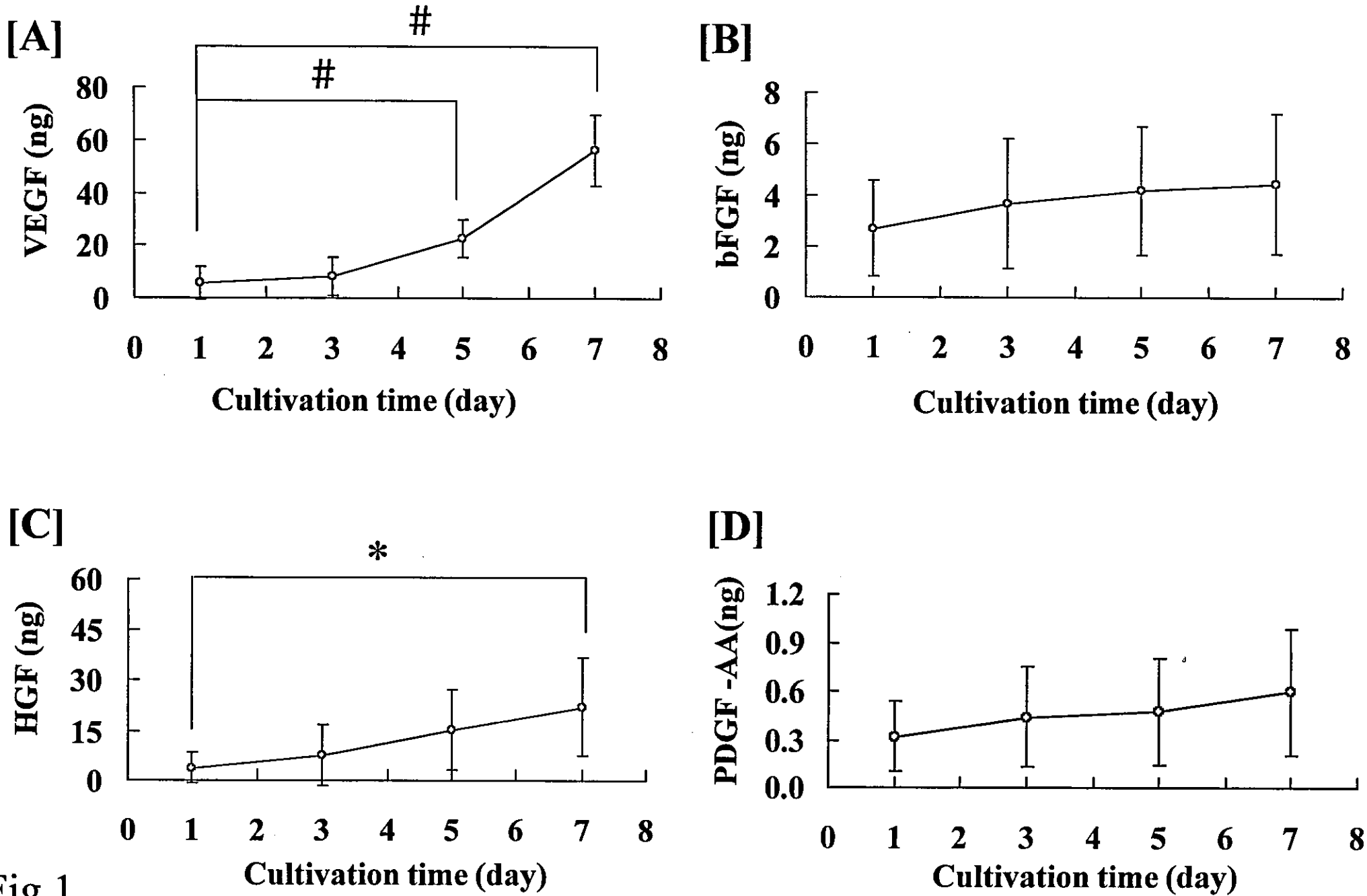
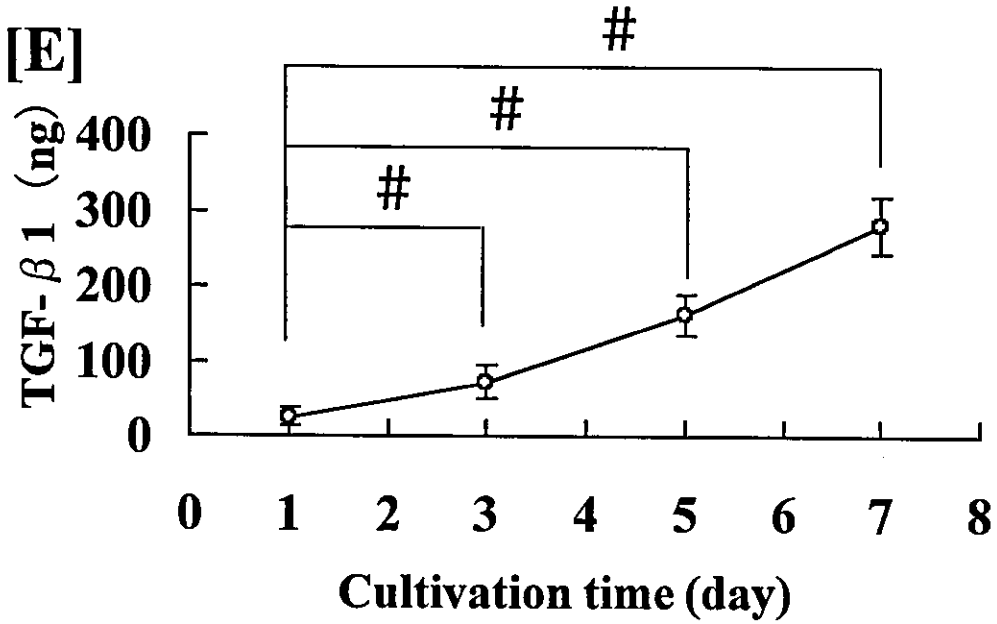
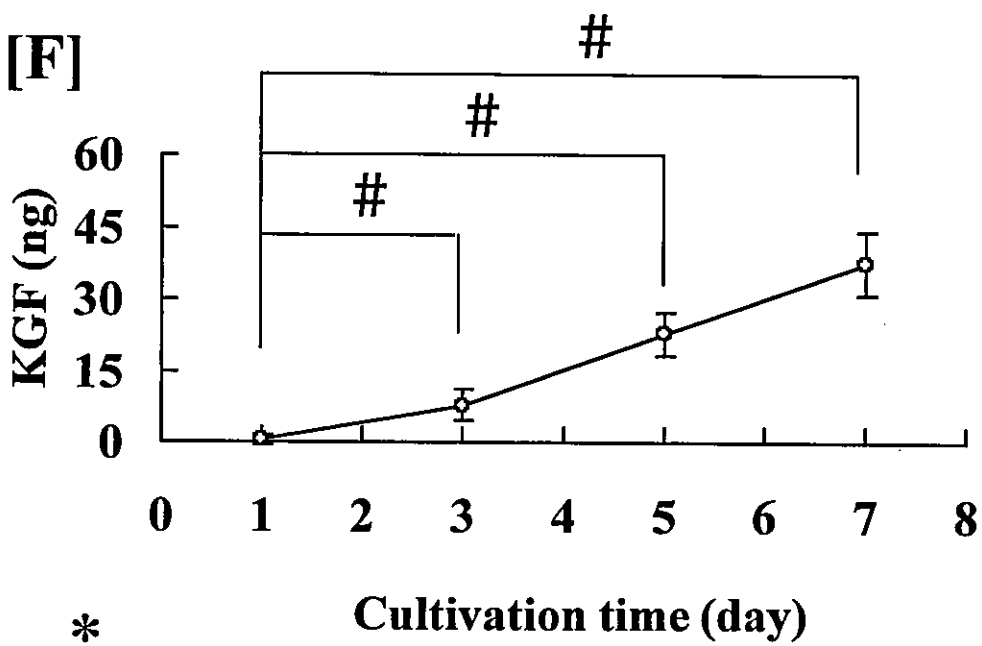

[A]
#
#
VEGF (ng)
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Cultivation time (day)
[B]
bFGF (ng)
8
6
4
2
0
0 1 2 3 4 5 6 7 8
Cultivation time (day)
[C]
*
HGF (ng)
60
45
30
15
0
0 1 2 3 4 5 6 7 8
Cultivation time (day)
[D]
PDGF -AA(ng)
1.2
0.9
0.6
0.3
0.0
0 1 2 3 4 5 6 7 8
Cultivation time (day)

Fig.1


[E]
TGF-β1 (ng)
400
300
200
100
0
#
#
#
0 1 2 3 4 5 6 7 8
Cultivation time (day)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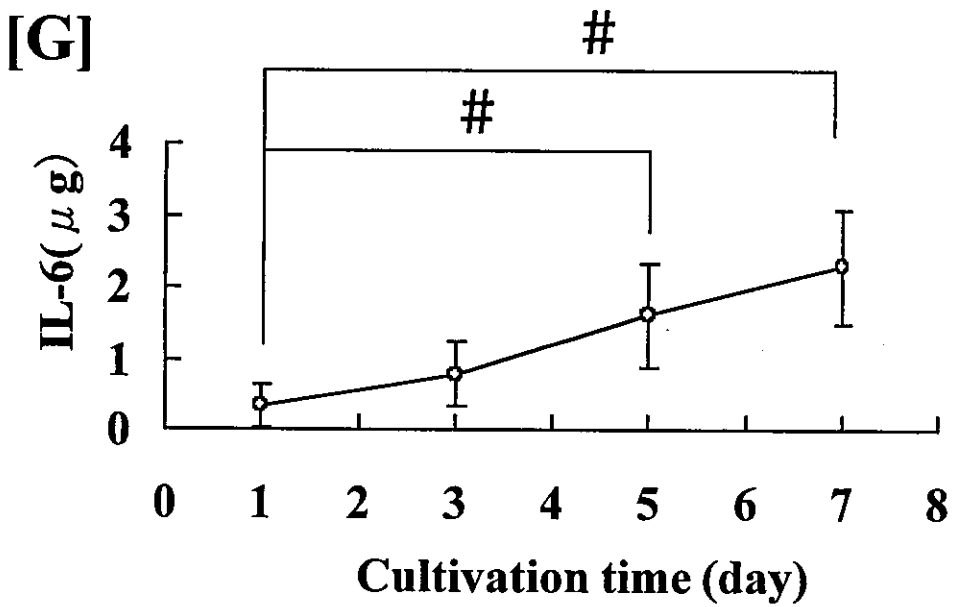
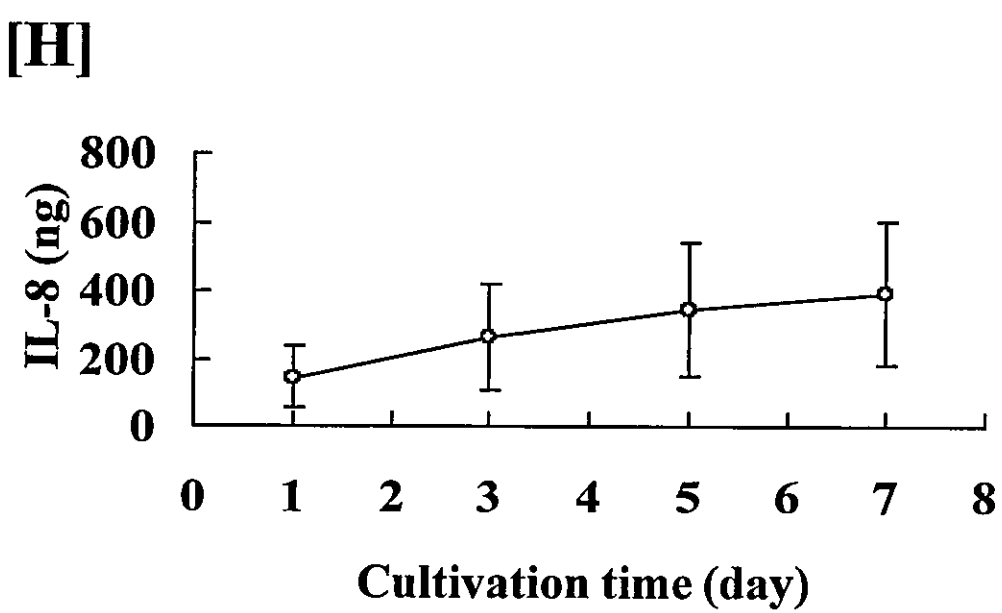
KGF (ng)
60
45
30
15
0
#
#
#
0 1 2 3 4 5 6 7 8
Cultivation time (day)

*


[G]
IL-6(μg)
4
3
2
1
0
#
#
0 1 2 3 4 5 6 7 8
Cultivation time (day)


[H]
IL-8 (ng)
800
600
400
200
0
0 1 2 3 4 5 6 7 8
Cultivation time (day)

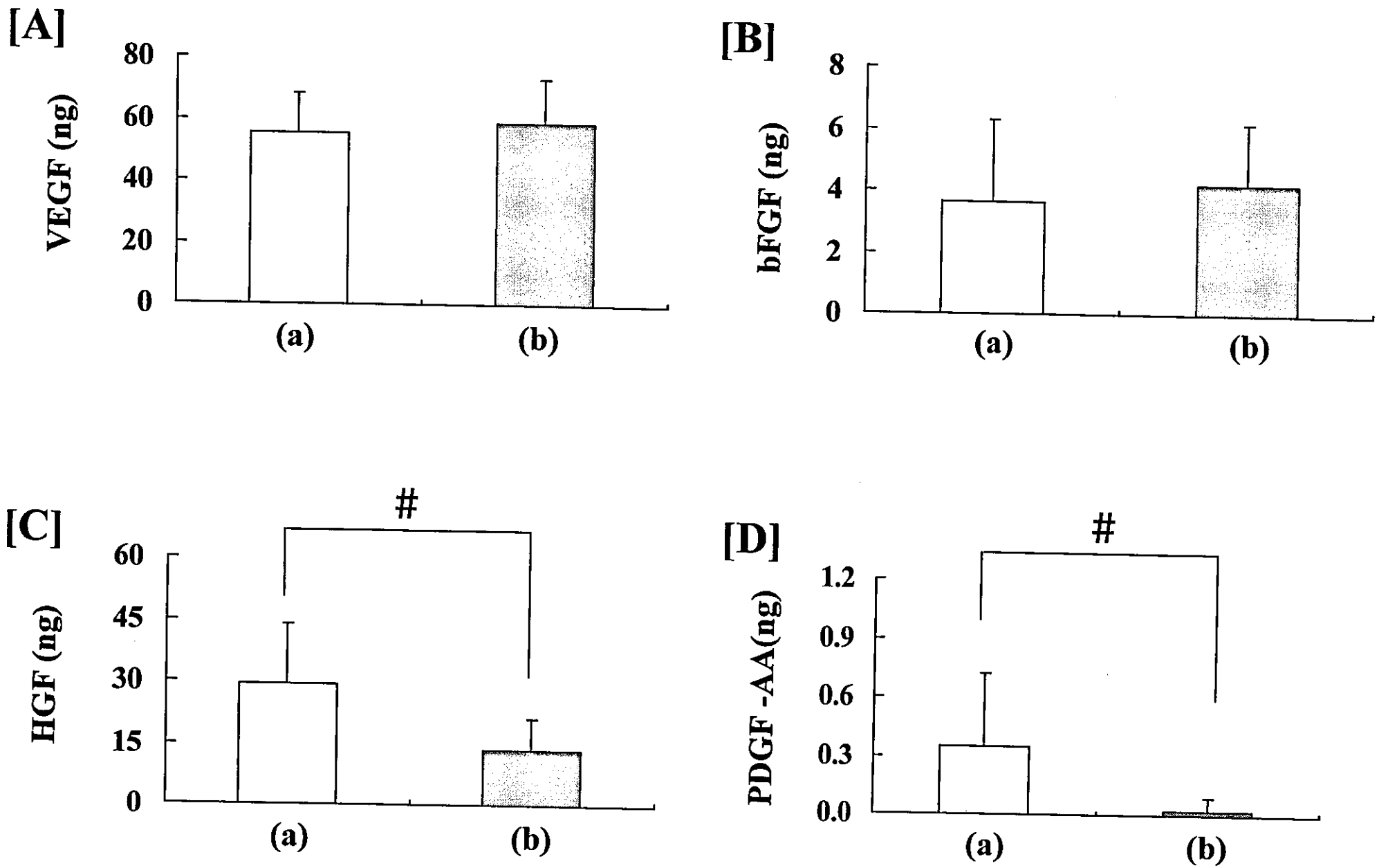
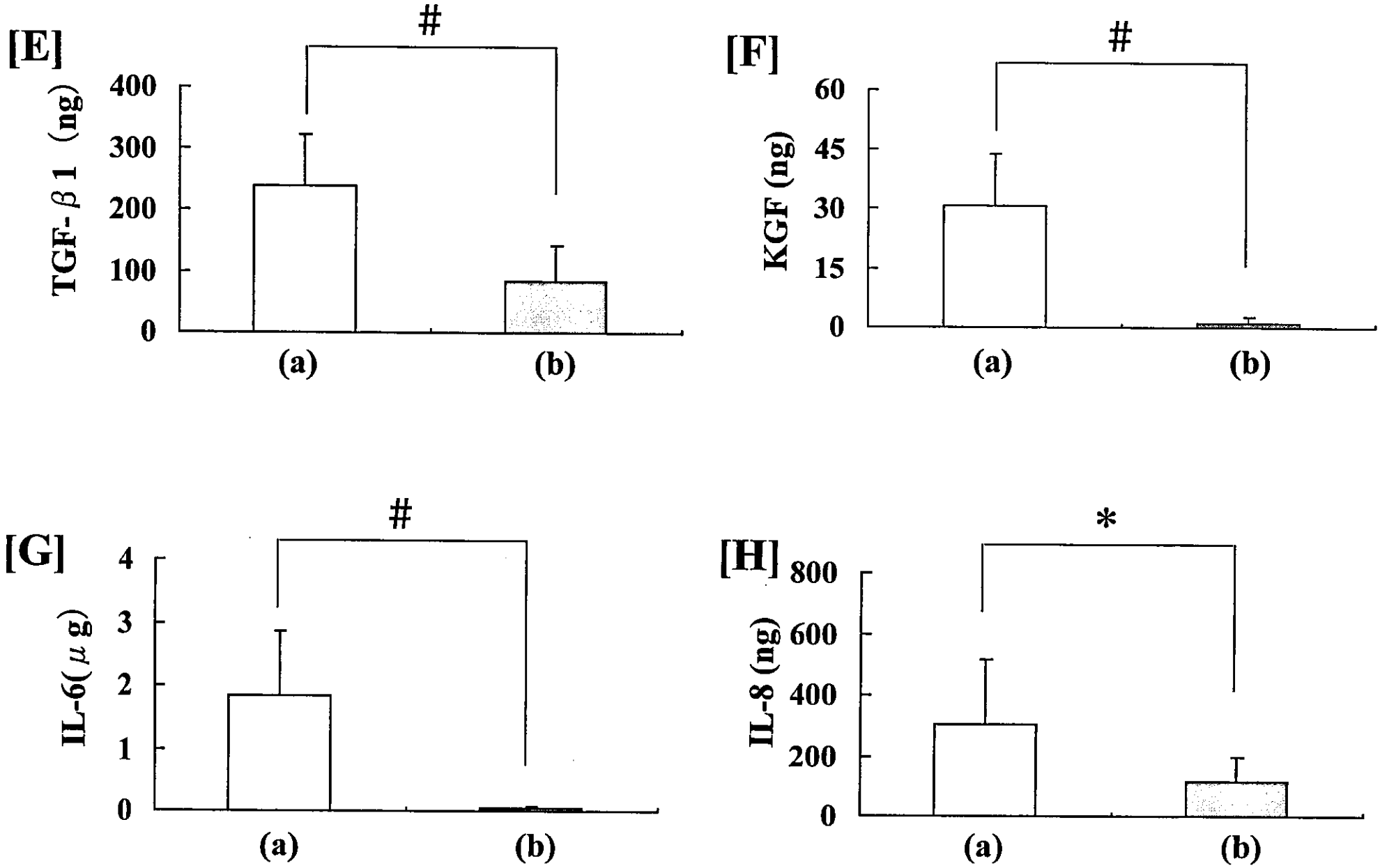

[A]
VEGF (ng)
80
60
40
20
0
(a)
(b)
[B]
bFGF (ng)
8
6
4
2
0
(a)
(b)
[C]
HGF (ng)
#
60
45
30
15
0
(a)
(b)
[D]
PDGF -AA(ng)
#
1.2
0.9
0.6
0.3
0.0
(a)
(b)

Fig.2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TGF-β1 (ng)
400
300
200
100
0
#
(a)
(b)
[F]
KGF (ng)
60
45
30
15
0
#
(a)
(b)
[G]
IL-6(μg)
4
3
2
1
0
#
(a)
(b)
[H]
IL-8 (ng)
800
600
400
200
0
*
(a)
(b)

[Artificial Organs in press]

The possibility of long-term cryopreservation of cultured dermal substitute

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Abstract

Allogeneic cultured dermal substitute (CDS) was prepared by cultivating fibroblasts on a two-layered spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col). CDS can be cryopreserved and transported to other hospitals in a frozen state. To evaluate cell viability, cell growth, and release of VEGF after long-term cryopreservation, the CDS was cryopreserved at –85°C or -152°C for a given period. We measured cell viability immediately after thawing and cell growth in CDS that was re-cultured for 1 week after thawing. In addition, the amount of vascular endothelial growth factor (VEGF) released from CDS that was re-cultured for 1 week after thawing was measured. The cell viability and cell growth of control CDS that was thawed within 3 weeks after freezing was 56.2% and 132.7%, respectively. The cell viability and cell growth of the CDS that was cryopreserved at -85°C for 6 months was 43.4% and 119.7%, respectively. When cryopreserved at -152°C for 1 year, the cell viability and cell growth was 52.0% and 110.8%, respectively. These values were comparable to those of the control. The amount of VEGF released from CDS cryopreserved at -85°C for 6 months (491.0 pg/ml) or at -152°C for 1 year (586.8 pg/ml) was comparable to that of the control CDS (587.3 pg/ml). In contrast, the amounts of VEGF released from CDS cryopreserved at -85°C for 1 year (322.5 pg/ml) or at -152°C for 2 years (210.8 pg/ml) were low, with a marked decrease in cell viability and cell growth. These findings suggest that CDS cryopreserved at -85°C for 6 months or –at 152°C for 1 year maintains sufficient cell viability and the ability to proliferate and release a significant amount of VEGF. The release of VEGF from CDS after long-term cryopreservation is a useful therapeutic effect, and is important for clinical use.

INTRODUCTION

Tissue engineering is moving rapidly from fundamental research to commercial applications. A number of skin substitutes have been developed by *in vitro* culture techniques [1-3]. Several types of cultured dermal substitutes (CDS) have been developed. Various materials were used as a matrix for CDS. A biodegradable synthetic polymer mesh, Vicryl®, was used as a matrix for Dermagraft®. In addition, a commercially available wound dressing, Biobrane® was used as a matrix for TransCyte®. The U.S. Food and Drug Administration has already approved these allogeneic cultured CDS [4, 5]. These matrices, however, were not designed to facilitate wound healing. The matrix should be designed by using materials which are able to promote wound healing. The authors developed an allogeneic CDS composed of fibroblasts combined with a spongy matrix of hyaluronic acid (HA) and atelo-collagen (Col) [6]. HA plays a critical role in several cellular functions, including migration and proliferation, by promoting adhesion and disadhesion on tissue substrate [7]. Collagen also plays a pivotal role in wound healing. Besides providing structural support and strength to new tissue, Col can have profound effects on the cells within and on its matrix. Col and Col-derived peptides act as chemoattractants for fibroblasts in vitro, and may have similar activity in vivo [8]. The R & D Center for Artificial Skin of Kitasato University was charged with the promotion of the Health and Welfare Ministry's Highly Advanced Medical Technology Research Project in 1998 [9]. This center has been the heart of the Regenerating Medical Millennium Project (field of skin) of the Ministry of Health, Labor and Welfare since 2000. A banking system of allogeneic CDS was established for multi-center clinical study by 30 hospitals across Japan [10, 11]. The results of a multi-center clinical study suggest that this type of allogeneic CDS can provide effective therapy for patients with various wounds, including burns, chronic ulcers, traumatic skin defects, and giant pigmented nevus [12-16]. The banking system of cryopreserved allogeneic CDS is very useful from the standpoint of clinical application. CDS can be transported to a hospital in a frozen state and then used immediately or stored temporarily in a freezer until clinical application, in a manner similar to that used with the banking system of cadaver skin. Thus, there is a need for investigation of the potency of cryopreserved CDS. We determined the practical conditions for cryopreservation of CDS, including the composition of cryoprotective medium and the procedure of freezing, thawing and rinsing [17]. The highest levels of cell viability and ability to release cytokines such as VEGF were retained after freezing and thawing under these practical conditions. Establishment of a more practical banking system of allogeneic CDS requires that potency of CDS is maintained after long-term cryopreservation. Several studies have been conducted to determine the optimum conditions for cryopreservation of cultured skin cells [18-21]. Cryopreserved cultured skin substitutes applied to clinical research and provided effective therapy [22, 23]. However, there have been a few studies of long-term cryopreservation of cultured skin substitutes [24]. There have been no studies of the properties of CDS after a long-term cryopreservation. The purpose of the present study is to evaluate the functions of CDS after long-term cryopreservation.

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 [6, 30]. Briefly, an aqueous solution of HA and cross-linking agent was poured into a polystyrene dish, in which a sheet of hydrated non-woven fabric was attached to the bottom. The dishes were kept at 50°C to promote the cross-linking reaction and concentration. The HA sponge combined with non-woven fabric obtained by lyophilization was rinsed thoroughly with water to remove unreacted cross-linking agent, followed by lyophilization to obtain the purified HA sponge. The purified HA sponge was soaked in an aqueous solution of atelo-collagen, followed by lyophilization to obtain a two-layered sponge. The two-layered sponge was treated with UV irradiation to induce cross-linking of atelo-collagen.

Preparation of cultured dermal substitute (CDS)

The CDS was prepared using a method described in previous articles [6,11]. Briefly, cryopreserved fibroblasts were thawed and then successively cultured to obtain an adequate number of fibroblasts. The fibroblasts were seeded on the atelo-collagen surface of a two-layered spongy matrix at an initial seeding density of 1.0×10^5 cells/cm^2, and were cultured for 1 week in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Human dermal fibroblasts were isolated from a piece of skin with a size of 0.5cm x 1.0cm which donated from a 3-months old patient in the surgical excision of excrescence in accordance with the study protocol and the ethical guidelines of St. Marianna Medical University Hospital and Kitasato University Hospital.

Cryopreserving and thawing procedures

We have determined the practical conditions for freezing and thawing CDS, as described in previous articles [11, 17]. Briefly, the CDS was turned upside down in a polystyrene dish, and the culture medium was exchanged with 30 ml of DMEM supplemented with 10% DMSO and 20% FBS. The dish containing CDS was frozen in a programmable freezer (AIR BLASTER, EBAC, Tokyo, Japan) at a rate of -1°C/min from 4°C to -60°C. One group was transferred to a freezer at -152°C, and the other group was transferred to a freezer at -85°C. The CDS was cryopreserved at -85°C for 6 months or 1 year, or at -152°C for 1 year or 2 years. These long-term cryopreserved samples were compared with a control that was thawed within 3 weeks after cryopreserving at -152°C. After a given period of cryopreservation, cryopreserved CDS was thawed by placing a polystyrene dish containing cryopreserved CDS in a foam polystyrene box at room temperature for 30 min, and then floating it in a water bath at 37°C, followed by rinsing of the CDS with lactated Ringer's solution 3 times to remove DMSO and FBS.

Viability of fibroblasts in long-term cryopreserved CDS

Cell viability through the processes of freezing and thawing was measured by MTT assay using 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in a manner similar to that described in a previous article (17). The cryopreserved CDS was thawed at 37°C using the method described above. A sample of CDS (4 cm x 3 cm) was transferred into another dish, which contained 6 ml of culture medium (containing 3 mg of MTT), and was then incubated in an incubator in a humidified atmosphere of 5% CO_2 at 37°C for 2 hr, followed by cutting 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. The number of cells in CDS re-cultured for 1 week after thawing was measured by MTT assay. Each measurement was repeated 5 times, and each sample was assayed in duplicate to obtain data with standard deviation. Only the CDS sample cryopreserved at -152°C for 2 years was measured twice.

Quantitative analysis of VEGF released from CDS

The amount of VEGF released from CDS was measured by enzyme-linked immunosorbent assay (ELISA) according to the method described in a previous article (17). Each measurement was repeated 5 times, and each sample was assayed in duplicate to obtain data with standard deviation. Only the CDS sample cryopreserved at -152°C for 2 years was measured twice. The data were statistically analyzed using a two-tailed Student's t-test.

Scanning electron microscopy (SEM) of long-term cryopreserved CDS

For SEM examination, a piece of CDS (2 cm x 3 cm) was fixed with 20% glutaraldehyde and 2% osmic acid. After dehydration in graded alcohols, the CDS sample was dried by lyophilization and then coated with palladium.

RESULTS

Viability of fibroblasts in long-term cryopreserved CDS

The cell number was evaluated indirectly by measuring the absorbance in MTT assay. Figure 1 shows the number of surviving cells in CDS thawed after cryopreservation under four different conditions: at -85°C for 6 months, at -85°C for 1 year, at -152°C for 1 year, and at -152°C for 2 years. In the MTT assay, percentage of surviving cells was expressed as the ratio of absorbance for cryopreserved CDS to absorbance for fresh CDS. Percentage of surviving fibroblasts for the CDS that was thawed within 3 weeks after cryopreservation at -152°C was 56.2%, and was increased to 132.7% by re-culturing for 1 week after quick thawing (B). This indicates that the CDS retains its ability to proliferate after thawing. These data were used as a control for comparison with other data. There was a slight difference in number of cells between the control (B) and CDS cryopreserved at -85°C for 6 months (C) or at -152°C for 1 year (E). In contrast, the number of surviving cells in CDS cryopreserved at -85°C for 1 year (D) or at -152°C for 2 years (F) was markedly reduced, and the surviving cells failed to proliferate after thawing.

Quantitative analysis of VEGF released from CDS

Figure 2 shows the amount of VEGF released from fresh CDS (A) and CDS re-cultured for 1 week after thawing (B to F). The amount of VEGF released from CDS was dependent on the number of cells in CDS after thawing followed by re-culturing for 1 week. The amount of VEGF released from fresh CDS was 572.3 pg/ml (A). The amount of VEGF released from the control CDS was 587.3 pg/ml (B). This indicates that the CDS retains its ability to release VEGF after thawing. For CDS cryopreserved at -85°C for 6 months, the amount of VEGF released from CDS was 491.0 pg/ml. For CDS cryopreserved at -152°C for 1 year, the amount of VEGF released from CDS was 586.8 pg/ml. These levels were comparable to those of fresh (A) and control (B) CDS. For CDS cryopreserved at -85°C for 1 year and CDS cryopreserved at -152°C for 2 years, the amount of VEGF released from CDS was 322.5 pg/ml and 210.8 pg/ml, respectively. This indicates that the CDS fails to maintain its ability to release VEGF at the original level under these conditions.

Scanning electron microscopy (SEM) of long-term cryopreserved CDS

A scanning electron micrograph of the CDS is shown in Fig 3. This CDS has a porous structure (size, 100 to 300 nm) on the Col surface and unique structure composed of a spongy layer and an upper layer of non-woven fabric. The non-woven fabric was firmly attached to the surface of the HA sponge. The original structure and thickness of CDS was well preserved by freezing, thawing, and rinsing under four different conditions as described above.

DISCUSSION

In order to move from fundamental research to clinical application, the properties of cryopreserved CDS must be clarified. Cryopreservation is prerequisite for a banking system of allogeneic CDS. In general, cryopreserved CDS is considered to be less effective in wound healing than fresh CDS, because viability of fibroblasts tends to be decreased by freezing and thawing. However, clinical application of cryopreserved allogeneic CDS has advantages due to issues of mass-production, preservation and transportation. In clinical application of allogeneic CDS, cell viability and potency to release cytokines such as VEGF need to be retained at the original level after freezing and thawing. The optimal cryopreserving condition is achieved using a programmable freezer with liquid nitrogen, which can precisely control the rate of freezing. However, most programmable freezers have a small chamber for cell suspension contained in cryo-tubes. This type of programmable freezer is not suitable for cryopreservation of many 10 x 10-cm CDS sheets. Therefore, an electrical programmable freezer with a large chamber was used in the present study. This freezer is used for cryopreservation of blood in clinical settings. Although this electrical programmable freezer does not allow precise control of the freezing temperature (6), it has a large chamber (80 cm x 60 cm x 50 cm).

We have already determined some practical conditions for cryopreservation of CDS, including the composition of cryoprotective medium and the procedure of freezing, thawing and rinsing [17]. Using these practical conditions, cell viability of CDS that was thawed within 3 weeks after freezing was 56.2% of the value of fresh CDS. It has been reported that physical damage and loss of cell viability occur in thawing of cryopreserved cadaver skin even under well-controlled conditions [31-33]. Taking into account the decrease in cell number after thawing, we have designed a practical seeding density of fibroblasts on the two-layered sponge; i.e., double seeding density of cells might be required for clinical use [6, 11].

In a recent study, we confirmed that fibroblasts in cryopreserved CDS retain their ability to proliferate and release cytokines (VEGF, bFGF, HGF, TGF-β1 and IL-8) after thawing (in submission). The cytokines, in particular, VEGF solved in the medium obtained by re-culturing the CDS after thawing induce proliferation of vascular endothelial cells *in vitro* [34]. In the present study, amount of VEGF released from CDS re-cultured for 1 week after thawing was measured, because VEGF, which is released from living cells in CDS, plays an important role in wound healing processes such as angiogenesis [35-37].

Currently, allogeneic CDS is available at 30 hospitals across Japan in a frozen state