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ORIGINAL ARTICLE

Wound healing effects of gingival fibroblasts cultured in animal-free medium

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OBJECTIVE: The purpose of this study was to develop a graft material made of gingival fibroblasts cultured in animal-free medium (HFDM1).

METHODS: We examined the effects of human serum (HS) on cell growth and wound healing capability, demonstrated by cytokine production, of gingival fibroblasts cultured in HFDM1. Subsequently, the capability of fibroblasts cultured in HFDM1 with 2% HS to promote the healing of skin defects was evaluated using nude mice.

RESULTS: The proliferation of human gingival fibroblasts was increased when HS at a concentration of 0.5–2% was added to HFDM1. Wound healing cytokines, including transforming growth factor-β, keratinocyte growth factor, hepatocyte growth factor, vascular endothelial growth factor, and IL-6 produced by gingival fibroblasts were increased by adding 2% HS to HFDM1. In addition, gingival fibroblasts cultured in HFDM1 with 2% HS improved wound healing of mouse skin defects as well as those cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

CONCLUSION: Gingival fibroblasts cultured in HFDM1 with 2% HS may be useful as a graft material for reconstruction.

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Keywords: animal-free medium; HFDM1; wound healing

Introduction

Oral epidermal grafts derived from cells obtained from the treated patient have been applied clinically for reconstruction of extensive mucosal defects produced in the oral cavity, such as those resulting from extirpation of a malignant tumor (Teichgraeber *et al*, 1984). Further, clinical success has been achieved with the use of cultured oral epidermal grafts in other parts of the body in addition to the oral cavity. For example, tissue-engineered sheets produced from cultured oral mucosal epithelia were found to be useful in ocular surface reconstruction (Yang *et al*, 2007), while Ueda (1995) developed a method for skin repair using cultured oral epithelium. In the field of dermatology, oral epithelial sheets have an advantage over skin epithelial sheets, as it is difficult for a cultured epithelium preparation to produce an adequate amount of tissue in patients with burns covering a large body surface area. On the other hand, the oral cavity is a possible site of application. An advantage of epithelial segments is that they can be obtained for culturing without forming new scars (Ueda, 1995), thus they may be useful for skin defects caused by burns and ulcers. However, the hazards in the culture media when using oral mucosa cells are well recognized, the prominent being related to bovine spongiform encephalopathy, as bovine serum is considered to be a source of infectious prions (Eloit, 1999).

The existence of connective tissue is an important factor for success in cultured epithelial grafting, as differentiation and maintenance of epithelial grafting are directly dependent on the influence of the underlying mesenchymal tissue, and fibroblasts are necessary to maintain the epidermal architecture and sustain growth (Mackenzie and Hill, 1984). Various methods of grafting using cultured gingival fibroblasts have been presented (Simain-Sato *et al*, 1999; Hou *et al*, 2003; Prato *et al*, 2003), however, those cultures require the use of materials and extracts of animal origin, such as bovine serum and bovine collagen.

HFDM1 was recently introduced as a medium for culturing fibroblasts that contains no animal-derived materials. Gingival fibroblasts have been successfully cultured in HFDM1 using recombinant human collagen type 1 and 3 sponges with large numbers of fibroblasts observed on the surfaces of both types (Yamada *et al*, 2006). However, no known studies have demonstrated

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the efficacy of gingival fibroblasts cultured in HFDM1 with regard to wound healing. In this study, we first examined the proliferation and wound healing capability, as shown by cytokine production, of gingival fibroblasts cultured in HFDM1 supplemented with human serum (HS), to develop a graft material composed of gingival fibroblasts. Subsequently, we evaluated the effects of fibroblasts cultured in HFDM1 with 2% HS on wound healing of skin defects in nude mice.

Materials and methods

Cell lines

Primary fibroblasts were prepared as previously described (Kamata *et al*, 2004). Briefly, primary fibroblasts were obtained from healthy gingival tissues using a protocol approved by the Ethical Committee of Hiroshima University, then cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, MO, USA) with 10% fetal calf serum (FCS) or animal-free medium (HFDM1; Research Institute for the Functional Peptides, Yamagata, Japan) at 37°C in 5% CO_2. GT1, an immortalized human oral fibroblast cell line, was established by transfection of hTERT as described previously (Kamata *et al*, 2004; Ohta *et al*, 2008), and cultured in DMEM with 10% FCS or HFDM1 with various concentrations of HS at 37°C in 5% CO_2.

Proliferation capability

Primary gingival fibroblasts and GT1 cells were seeded into 35 mm dishes (5×10^4 cells per well) in HFDM1 with various concentrations of HS, with the medium changed every 3 days. Cells were trypsinized with 0.25% recombinant trypsin and 1 mM EDTA solution, and the number of living cells was counted using the dye exclusion test with 0.15% trypan blue. The long-term growth of the cells in various media was evaluated by calculating the population-doubling time (PDT), as follows. Cells were seeded at a density of 1×10^5 cells cm^{-2} onto 12.5 cm^2 tissue culture flasks and harvested with trypsin, then subcultured for six passages by changing the medium every 5 days, after which PDT were determined.

ELISA

Primary gingival fibroblasts and GT1 cells were seeded into 48-well cell culture plates (5×10^4 cells per well) in HFDM1, until they reached approximately 80% confluence. The tissue-culture dishes were then incubated in the various media for 48 h. We also prepared those without cultured fibroblasts. The media were collected and centrifuged and, the supernatant fluids were stored at −80°C before the assays. The protein levels of wound healing cytokines, including transforming growth factor-β (TGF-β), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and IL-6, in the media were determined using an ELISA kit (R & D Systems, Minneapolis, MI, USA), according to the protocol recommended by the manufacturer.

Animal testing

The Committee of Animal Welfare of Hiroshima University approved the animal testing protocol. Twelve BALB/C-nude mice (20–30 g; Japan SLC, Inc., Hiroshima, Japan) were used as the graft recipients. First, the mice were anesthetized with a solution of ketamine and xylazine (Sigma Ltd, Tokyo, Japan). After disinfecting the skin with iodine, a full-thickness skin defect with a diameter of 1.5 cm was prepared on the back of the nude mice ($n = 4$ in each group). Temperature-responsible cell culture dishes (UpcellTM; CellSeed Inc., Tokyo, Japan) were prepared according to the manufacturer's instructions. Primary gingival fibroblasts were cultured to confluence on temperature responsible culture dishes with cell support (CellShifterTM; CellSeed Inc.) in DMEM with 10% FCS or HFDM1 with 2% HS, then harvested after reducing the temperature to 20°C for 30 min. Fibroblasts cultured with cell support were removed from the dishes and transplanted to the skin defects of the nude mice, with the cell support instrument carefully removed after 12 h. For the control group, we performed the same procedure without cultured fibroblasts. At 2, 6 and 24 days after grafting, the mice were euthanized and examined. Photographs of the wounds were taken before biopsy examinations to estimate the area of the wound surface, so that the extent of wound construction could be estimated using an area measuring instrument. All tissues were fixed overnight in 10% neutral formalin solution and then the fixed samples were embedded in paraffin and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin for routine microscopic examinations.

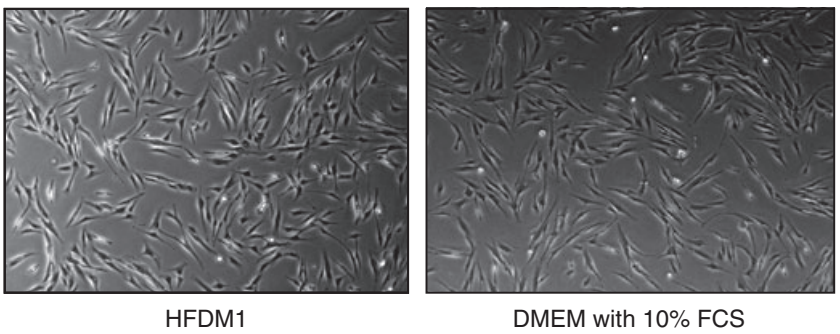

Figure 1 Microscopic images of primary gingival fibroblasts derived from gingival tissues generated in HFDM alone and Dulbecco's modified Eagle's medium with 10% fetal calf serum.

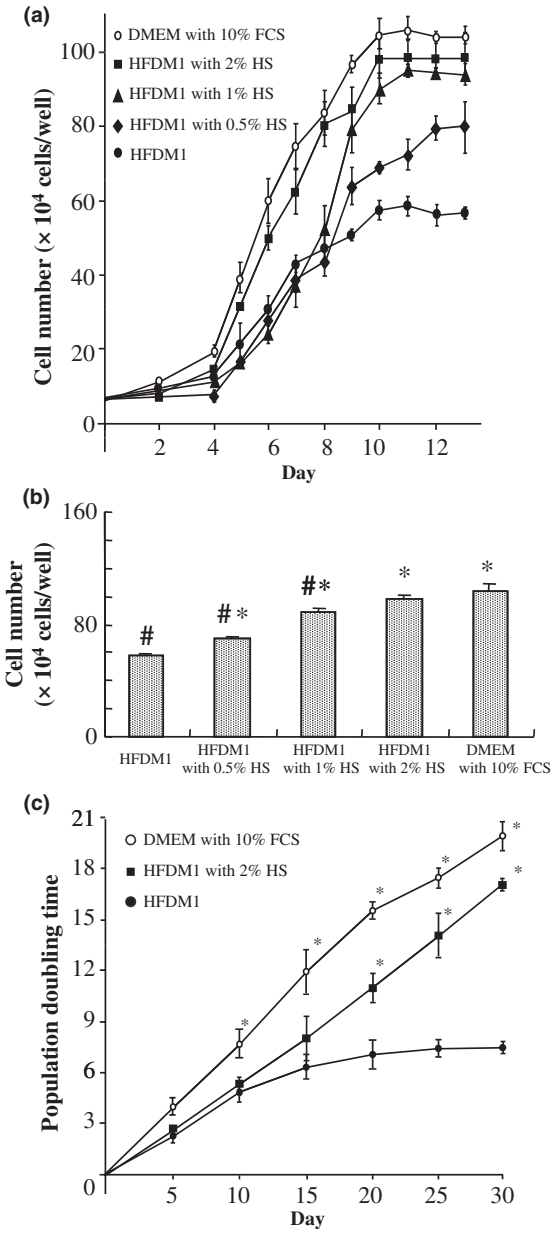

Figure 2 (**a**) Growth curves of primary gingival fibroblasts determined by counting the number of cells after culturing in HFDM1 with 0%, 0.5%, 1% and 2%, or in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Growth curves were determined by measuring the cell number in HFDM1 alone, HFDM1 with 0.5% HS, HFDM1 with 1% HS, HFDM1 with 2% HS, and DMEM with 10% FCS. (**b**) Differences in proliferation of primary gingival fibroblasts cultured in various media for 10 days. *Significantly different from HFDM1 alone (Dunnett's test; $P < 0.05$). #Significantly different from DMEM with 10% FCS (Dunnett's test; $P < 0.05$). (**c**) Proliferation of primary gingival fibroblasts during long-term cultures in HFDM1 alone, HFDM1 with 2% HS, and DMEM with 10% FCS. Subcultures were repeated six times every 5 days in the various media. *Significantly different from HFDM1 alone (Dunnett's test; $P < 0.05$)

Immunohistochemistry

Deparaffinized sections were immersed in 1% H_2O_2 in methanol to eliminate endogenous peroxidase activity. The sections were incubated in primary antibody anti-Ly6g rat polyclonal antibody (BD Pharmingen, Tokyo, Japan) (diluted 1:100) for 60 min at room temperature and then with the secondary antibody anti-rat IgG antibody produced in rabbit (Sigma Ltd) (diluted 1:200) for 60 min at room temperature. Thereafter, immune complexes were visualized using HistoGreen (Linaris-Biologische Produkte, WB, Germany) according to the manufacturer's instructions. Ly6g positive cells were counted in 10 different fields in each wound site, and the number of neutrophils per square millimeter was determined (Swift *et al*, 2001).

Statistical analysis

Cell proliferation data were statistically evaluated using one-way ANOVA followed by Dunnett's multiple comparison test and the results are presented as mean ± standard deviation. The difference between means was considered significant at $P < 0.05$.

Results

Primary gingival fibroblasts derived from gingival tissue were generated in HFDM alone, and seen as spindle-shaped fibroblast-like cells, similar to those cultured in DMEM medium with 10% FCS (Figure 1). However, as the number of passages increased (4–5 passages), the fibroblasts cultured in HFDM1 became elongated, and showed a decrease in proliferation (data not shown).

Primary fibroblasts plated in HFDM1 with various concentrations of HS showed increased rates of proliferation after 10 days when HS was increased from 0.5% to 2%, with the numbers of cells cultured in HFDM1 with 2% HS similar to those of cells cultured in DMEM with 10% FCS (Figure 2a,b). Adding more than 5% HS to HFDM1 resulted in inhibition of fibroblast proliferation (data not shown). GT1 cells showed the same response as primary fibroblasts, when various HS concentrations were added to the HFDM1 (data not shown). As for the long-term growth of fibroblasts cultured in HFDM1, primary gingival fibroblasts cultured in HFDM1 alone stopped proliferation after four passages, while the addition of 2% HS to HFDM1 caused an increase in PDT (Figure 2c).

We examined wound healing-related cytokine production by primary gingival fibroblasts cultured in HFDM1, HFDM1 with 2% HS, and DMEM with 10% FCS. Gingival fibroblasts cultured in each medium produced VEGF, HGF, TGF-β, KGF, and IL-6 proteins (Figure 3). The addition of 2% HS to HFDM1 increased KGF, IL-6, and HGF protein release from fibroblasts, to levels comparable to those from fibroblasts cultured in DMEM with 10% FCS (Figure 3). None of those cytokine proteins were detected in any of the media without fibroblasts (data not shown). In addition, cytokine production by GT1 cells cultured in HFDM1 with 2% HS was similar to that by those cultured in DMEM with 10% FCS (data not shown).

The effects of cultured fibroblasts in HFDM1 with 2% HS on wound healing of skin defects in nude mice were also evaluated. Gingival fibroblasts cultured in HFDM1 with 2% HS and in DMEM with 10% FCS

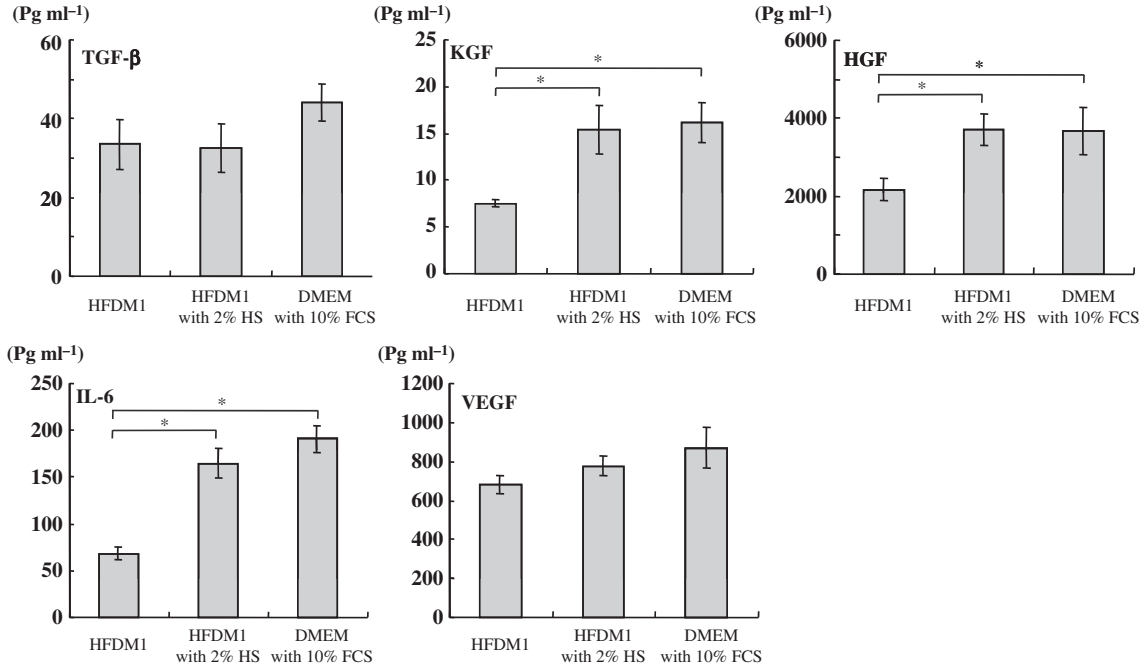

Figure 3 Transforming growth factor-β (TGF-β), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), IL-6 and vascular endothelial growth factor (VEGF) protein production from gingival fibroblasts cultured in HFDM1, HFDM1 with 2% HS and DMEM with 10% FCS. Primary gingival fibroblasts were cultured as described in Materials and methods, then incubated in the various media for 48 h. *Significantly different among differential media (Dunnett's test; $P < 0.05$)

increased wound healing faster than controls without fibroblasts (Figure 4a,b). At 6 days after injury, neutrophils in the wound sites that received transplanted fibroblasts cultured in HFDM1 with 2% HS and those cultured in DMEM with 10% FCS were decreased compared with wound sites without fibroblasts (Figure 5). In addition, dermis surfaces covered by epithelial cells were observed in wound sites with transplantation of cultured fibroblasts (Figure 6). Twenty-four days after injury, keratinization with multiple layers was

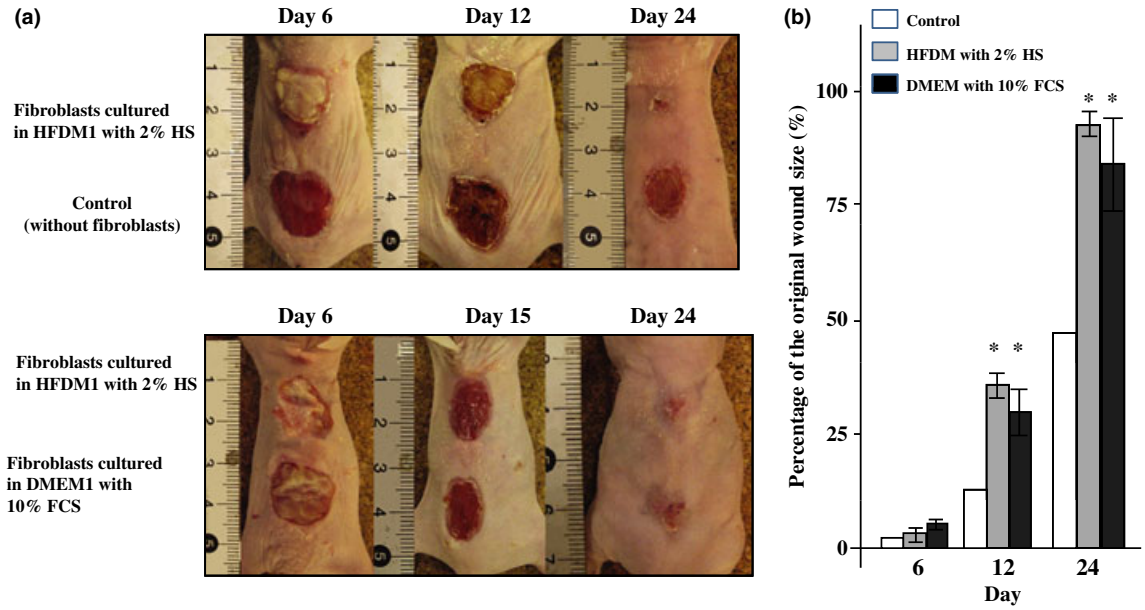

Figure 4 (**a**) Representative photographs of skin defects produced on the backs of nude mice prior to transplantation. Fibroblasts cultured in HFDM with 2% HS and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) were transplanted to the skin defects, while control mice received the same preparations without cultured fibroblasts. (**b**) Changes in wound healing rates in the skin defect areas following transplantation with cultured fibroblasts in HFDM with 2% HS and DMEM with 10% FCS. *Significantly different from the control (Dunnett's test; $P < 0.05$)

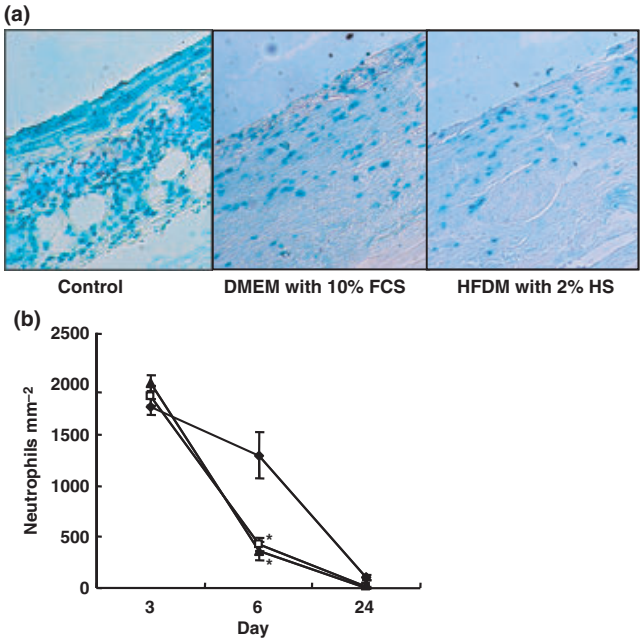

Figure 5 (**a**) Immunohistochemical staining with the neutrophil marker anti-Ly6g in wound areas following transplantation of fibroblasts cultured in HFDM1 with 2% HS and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), or without fibroblasts (control) at 6 days after injury. (**b**) Infiltration by neutrophils in wound areas following transplantation with fibroblast cultured in HFDM1 with 2% serum and DMEM with 10% FCS, or without fibroblasts (control). *Significantly different from the control (Dunnett's test; $P < 0.05$)

observed in dermis that underwent transplantation with cultured fibroblasts (Figure 6).

Discussion

In an attempt to eliminate various hazards associated with animal-based products, HFDM1 was developed as serum-free medium for the culturing of fibroblasts. In the present study, we demonstrated the wound healing utility of fibroblasts cultured in HFDM1 in 2% HS when applied as a graft material. First, we examined the effects of various HS concentrations on the proliferation of gingival fibroblasts cultured in HFDM1. Primary gingival fibroblasts derived from gingival tissues could be generated in HFDM1 alone. However, following a few passages (4–5), a decrease in proliferation was seen. Yamada *et al* (2006) evaluated the proliferation of gingival fibroblasts on recombinant collagen sponges in HFDM1 with 2% HS or DMEM with 10% FCS, and found that the growth rate after 9 days was equal between those media types. In agreement with that report, we found that the proliferation rates of gingival fibroblasts increased when HS from 0.5% to 2% was added to HFDM1, whereas proliferation was inhibited when adding more than 5% HS to HFDM1. Therefore, when using gingival fibroblasts cultured in HFDM1 as tissue-engineered materials, serum derived from patients may be required to achieve a culturing performance comparable to DMEM with 10% FCS, although the optimum concentration may be as low as 2%.

Fibroblasts release cytokines in an extracellular matrix that provide wound healing effects when applied to epithelial grafting (El-Ghalbzouri *et al*, 2002; Aoki *et al*, 2004; Wong *et al*, 2007). HGF is an important fibroblast-derived factor that plays a central role in re-epithelialization of wounds (Ono *et al*, 2004, 2007). TGF-β released by fibroblasts has both autocrine and paracrine effects which promote collagen synthesis (Igarashi *et al*, 1993), while the paracrine activity of KGF produced by fibroblasts has effects on keratinocyte growth and differentiation (Werner and Smola, 2001). VEGF is important for regulating vascular and lymphatic endothelial cell proliferation through specific receptors (Trompezinski *et al*, 2004), and IL-6, which is produced in wounds by fibroblasts and macrophages, affects multiple processes related to wound healing (Mateo *et al*, 1994). Each of these soluble factors is released from fibroblasts and important for grafting success. In the present study, fibroblasts cultured in HFDM1 secreted those cytokines to influence fibroblasts and keratinocytes in both autocrine and paracrine manners, while the addition of 2%HS to HFDM1 increased the levels of KGF, IL-6, and HGF released from the fibroblasts. Gingival fibroblasts cultured in HFDM1 may promote epidermis formation and facilitate wound healing in wound sites by producing these soluble factors, and the addition of 2% HS may positively influence the acceleration of wound repair caused by their increase.

The success of fibroblast transplantation with tissue grafts has been reported in the clinical situations. Mohammadi *et al* used cultured fibroblast grafts in nine patients with insufficiently attached gingiva, and found that the amount of attached gingiva after applying the cultured grafts was higher than that obtained with periosteal fenestration technique for gingival augmentation (Mohammadi *et al*, 2007). Fibroblasts cultured in HFDM1, which contains no animal-derived materials, are thought to augment tissue repair following aesthetic and reconstructive surgery, and contribute to patient safety. Thus, animal studies have been performed to demonstrate the wound healing efficacy of fibroblasts cultured in HFDM1 with 2%HS. To graft a carrier-free fibroblast culture onto the backs of nude mice, we applied temperature-responsive culture disks (Murakami *et al*, 2006) in this study. By covalently immobilizing a temperature-responsive polymer (*N*-isopropylacyrlamide) onto commercially available tissue culture plastics, these modified culture surfaces undergo transitions between hydrophobic and hydrophilic states depending on the temperature (Okano *et al*, 1995; Yang *et al*, 2005). Under typical *in vitro* culture conditions at 37°C, various cell types adhere, then spread and proliferate similarly onto normal tissue culture polystyrene. However, after simply reducing the temperature to 20°C, all cultured cells spontaneously detach along with their deposited extracellular matrix, without the need for proteolytic enzymes such as trypsin, which are generally required to harvest cultured epithelial grafts (Kushida

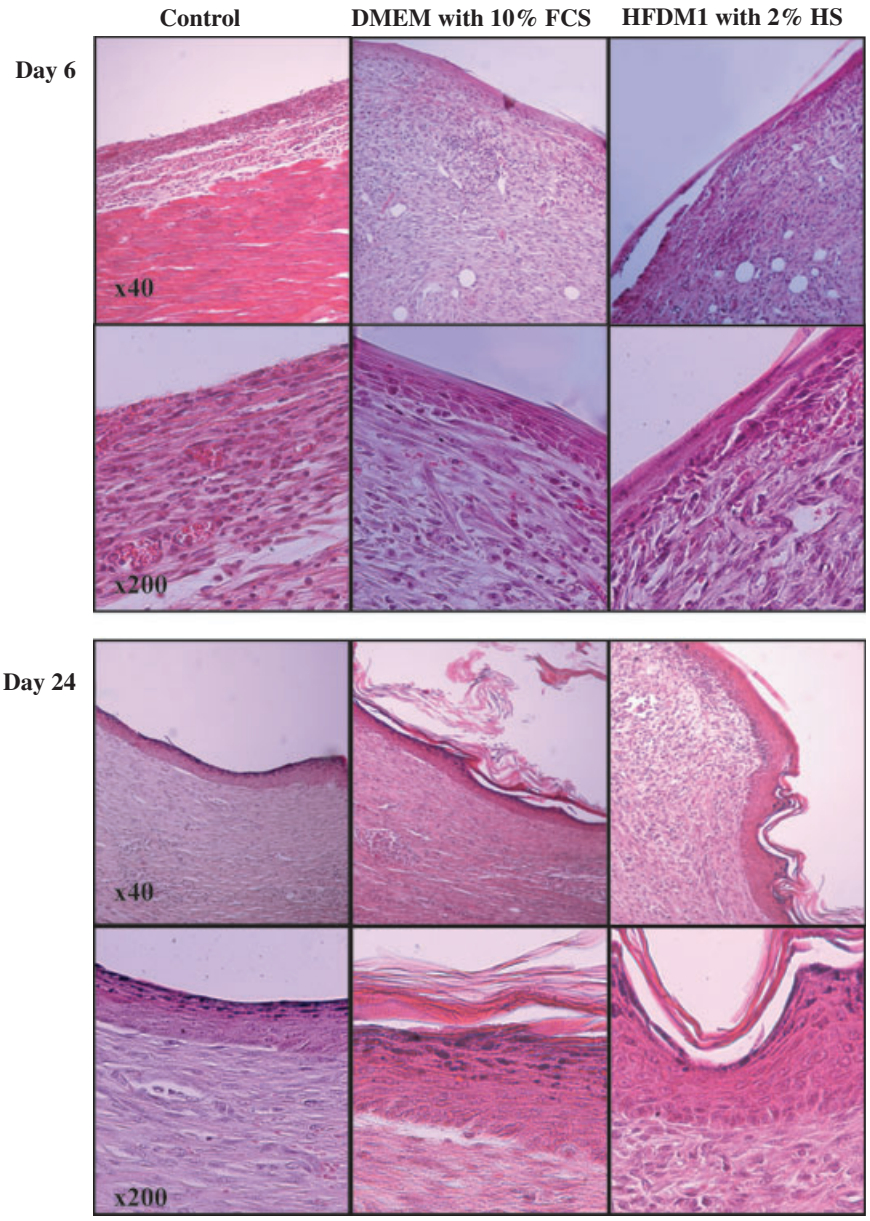

Figure 6 Histological findings in wound areas following transplantation of fibroblasts cultured in HFDM with 2% HS and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), or without fibroblasts (control) (H&E staining). (**a**) Six days after injury. (**b**) Twenty-four days after injury. Top panel, original magnification (top), ×200 (bottom); bottom panel ×40(top) and ×200 (bottom)

et al, 1999). Yang *et al* (2007) reported successful clinical outcomes for patients who had tissue-engineered cell sheets composed of an autologous oral mucosal epithelium obtained from the their own oral cavity applied to the surface of a denuded corneal stroma using a temperature-responsive culture dish. Herein, we transplanted gingival fibroblasts cultured in HFDM1 with 2% HS in temperature-responsive culture dish to skin defects on the back of the nude mice. Those cultured in HFDM1 with 2% HS improved wound healing in a manner similar to gingival fibroblasts cultured in DMEM with 10% FCS. Thus, graft materials constructed by culturing gingival fibroblasts in HFDM1 with 2% HS in temperature-responsive culture dishes could contribute to wound healing.

The normal wound healing process is composed of three phases: inflammation, wound closure, and tissue remodeling (Werner and Grose, 2003). Neutrophils, markers of the hallmark of the inflammatory phase of wound healing, migrate to wound sites during the acute inflammatory phase, while, neutrophil influx diminishes in the later inflammatory phase, and fibroblasts and keratinocytes begin to cover and fill the wound area to restore tissue integrity (Swift *et al*, 2001). Neutrophils are thought to prevent re-epithelization by accelerating keratinocyte differentiation in the wound sites (Dovi *et al*, 2004). In this study, neutrophils in wound sites that received transplantation with fibroblasts cultured in HFDM1 with 2% HS as well as those cultured in DMEM with 10% FCS were decreased compared with controls without fibroblasts at 6 days after injury, while dermis surfaces covered by epithelial cells were observed in the wound sites with transplanted fibroblasts. Previous study have shown that soluble factors produced by

fibroblasts diffuse to the overlying epidermis, which influence fibroblasts and keratinocytes in both autocrine and paracrine manners (Aoki *et al*, 2004). Acceleration of wound healing by cytokines released from cultured fibroblasts may lead to early reduction of inflammation following neutrophil infiltration. Therefore, fibroblasts cultured in HFDM1 with 2% HS may be useful as a graft material for reconstructing oral mucosal and skin defects.

In conclusion, the use of HFDM1 with 2% HS as culture medium for gingival fibroblasts resulted in good cell proliferation and led to release of wound healing cytokines. In addition, those cultured fibroblasts were useful as graft material of wounds. In future, we intend to construct three-dimensional cultured dermis structures using fibroblast scaffolding as a base to improve the utility of our method for clinical application.

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