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Wound dressing material containing lyophilized allogeneic cultured cells ☆

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ABSTRACT

Although topical application of a single growth factor is known to accelerate wound healing, treatment with allogeneic cultured cells is more efficient and physiological, because they release various mediators that interact and regulate the wound healing mechanism. However, in clinics, the cells must be cryopreserved until use. To overcome this inconvenience, we designed novel wound dressing materials containing lyophilized allogeneic cultured epithelial cells and/or fibroblasts. This study aimed to confirm growth factor release from those lyophilized products. The results revealed that the cultured cells retained their morphology even after lyophilization and released growth factors. When fibroblasts were used alone, they released growth factors in significantly higher concentrations after lyophilization than after cryopreservation. In particular, bFGF release was almost a hundredfold higher in the lyophilized group compared to the cryopreserved group. When epithelial cells and fibroblasts were co-cultured, both bFGF and VEGF were released in higher concentrations by the cryopreserved dressing material than by the lyophilized dressing material. The growth factors' release was probably regulated by interaction between epithelial cells and fibroblasts. We speculate that repeated application may be necessary for treating difficult wounds with the lyophilized product, because the lyophilized cells release the mediators in a single, transient burst.

Introduction

O'Connor [21] reported the first cases of extensive burns successfully treated with autologous cultured epithelium in 1981, and cultured epithelial autografting soon became an essential tool for burn treatment. In the late 1980s, the possibility of using allogeneic cultured epithelium for treatment of burn wounds was explored, because it takes at least 2–3 weeks to prepare autologous cultured epithelium. Various investigators subsequently demonstrated the effectiveness of cultured allogeneic epithelium in enhancing wound healing, such as at split-thickness skin donor sites [10,22], burn wounds [4–6] and chronic leg ulcers [7]. When allogeneic cultured epithelium was grafted to the wound site, it was progressively replaced by recipient cells, without any apparent sign of rejection [2,11]. That suggests that the enhanced wound healing with allogeneic cultures is not due to permanent take of the grafts but due to stimulation of the wound healing mechanism, mediated by growth factors released from the grafted cultures. More recently, several studies showed that allogeneic cultured fibroblasts have similar potential to keratinocytes [12,15,16].

Allogeneic cultured cells, such as epithelial cells and fibroblasts, have had to be cryopreserved for clinical use. Therefore, surgeons have to thaw and rinse the cryoprotective agent out of the cultures prior to application to wounds. To improve the convenience of supply, banking and utilization of cell cultures, we designed a novel wound dressing material containing lyophilized cultured epithelial cells and/or fibroblasts. This study was designed to preliminarily evaluate the amounts of two cytokines, bFGF and VEGF, released from the lyophilized dressing materials.

Material and methods

The study was conducted with the approval of the Ethics Committee of Tokyo Women's Medical University.

In vitro culture of epithelial cells and fibroblasts

Epithelial cells and dermal fibroblasts were isolated from a single human skin sample obtained from a patient undergoing surgical excision of a nevus. The dermis and epidermis were enzymatically separated with dispase. For isolation and culture of

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fibroblasts, the dermis was cut into small pieces and placed in culture dishes containing DMEM (Dulbecco's modified Eagle's medium; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Epithelial cells were cultured according to the "3T3 feeder layer technique" described by Rheinwald and Green [24]. Briefly, the epidermis was disaggregated in 0.25% trypsin to form a single-cell suspension that was inoculated into culture dishes containing an irradiated 3T3 fibroblast feeder layer. The culture medium, a mixture of F-12 (flavin adenine dinucleotide medium; Gibco) and DMEM (Gibco) at a 1:3 ratio, was supplemented with adenine (24.3 mg/L; Sigma, USA), hydrocortisone (0.4 mg/L; Sigma), insulin (5 mg/L; Sigma), transferrin/triiodo-thyronine (10 μg/L; Sigma), cholera toxin (10 μg/L; Sigma), human recombinant epidermal growth factor (10 μg/L; Gibco) and 10% FBS (Gibco). All isolation and culture steps were performed in an incubator maintained at 37 °C, 5% CO_2.

Experimental protocol

Preparation of wound dressing material containing cultured cells

Cultured dermal fibroblasts and epithelial cells were enzymatically detached from the culture dishes after one-passage culture of the epithelium and 2-passage culture of the fibroblasts, respectively, and immediately seeded onto a dressing material. The dressing material consisted of sodium carboxymethylcellulose primary wound dressing (Hydrofiber™ dressing, Aquacel®, 3 × 3 cm; Convatec, Inc., USA). Seeding was performed by pipetting the cell suspension onto the material at a seeding density of 1.0×10^5 cells. The dressing materials seeded with cultured cells were then incubated at 37 °C in an atmosphere of 5% CO_2 for 1 week.

The following 3 experimental groups were established. Group 1, epithelial cell group ($n = 12$): Only epithelial cells (1.0×10^5 cells) were seeded onto the dressing material and cultured in epithelial cell culture medium containing 7 kinds of supplemental factors and 10% FBS. Group 2, fibroblast group ($n = 12$): Only fibroblasts (1.0×10^5 cells) were seeded onto the dressing material and cultured in DMEM supplemented with 10% FBS. Group 3, co-culture group ($n = 14$): Both epithelial cells (1.0×10^5 cells) and fibroblasts (1.0×10^5 cells) were seeded onto the dressing material and cultured in the same culture medium as Group 1.

Cryopreservation and lyophilization procedure and histological examination

After one week of cultivation, each group was divided into two subgroups: a cryopreservation group (Groups 1C ($n = 6$), 2C ($n = 6$), 3C ($n = 7$)) and a lyophilization group (Groups 1L ($n = 6$), 2L ($n = 6$), 3L ($n = 7$)). For cryopreservation, specimens were placed in flasks containing a cryoprotective medium consisting of Roswell Park Memorial Institute (RPMI) 1640 solution (Gibco) with 10% glycerol and 10% FBS (Gibco) and cooled to −80 °C at a rate of 0.4 °C/min. The flasks were stored at −135 °C. Lyophilization was performed by the ordinary technique. Briefly, the specimens were pretreated by irrigating with phosphate buffered saline to remove the culture medium and supplemental factors. Then the specimens were snap-frozen with liquid nitrogen and placed in a vacuum below −50 °C for 12 h to allow the frozen water in the materials to sublimate directly from the solid state to the gas phase. After lyophilization, the specimens were stored at room temperature. One specimen from each of Group 3C and Group 3L was microscopically examined to confirm the existence of the co-cultures inside the dressing materials. Cross-sections were prepared from the central area of each specimen and stained with hematoxylin and eosin (H&E).

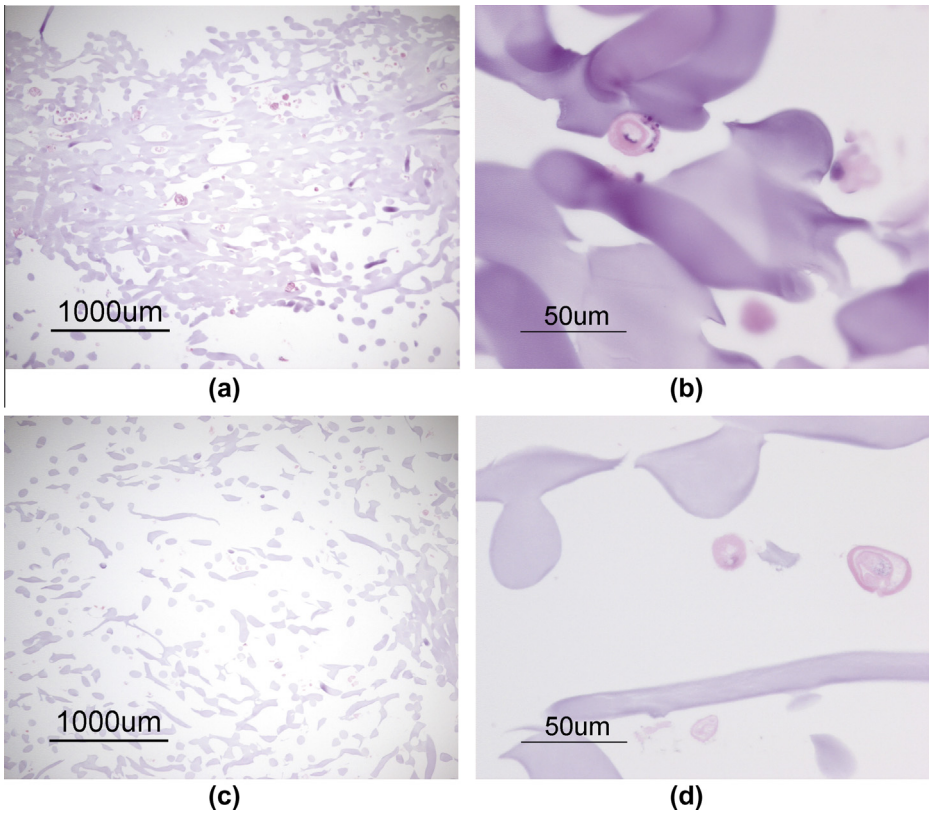

Fig. 1. Magnified images of the wound dressing materials. (a and b) After cryopreservation. (c and d) After lyophilization. Hematoxylin–eosin stain. Scale bars represent 1000 μm in a, c and 50 μm in b, d.