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Introduction of human β-defensin-3 into cultured human keratinocytes and fibroblasts by infection of a recombinant adenovirus vector

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ABSTRACT

Cultured epidermal autografts and cultured skin substitute are vulnerable to infection. Human beta defensin (HBD)-3 is an antimicrobial peptide that exhibits a wide-spectrum antimicrobial activity against gram-positive/negative bacteria and fungi. This study determined whether normal human keratinocytes (NHKs) and human dermal fibroblasts (HDFs) transfected with the HBD-3 gene secrete HBD-3 peptide with an antimicrobial activity. An adenovirus vector with an HBD-3 cDNA inserted downstream of the CMV promoter (ADhBD3) was created. The HBD-3 gene was introduced into NHKs and HDFs via ADhBD3 infection. HBD-3 gene expression in each type of transfected cells was evaluated by RT-PCR. The presence of HBD-3 peptide in the culture supernatants of each type of transfected cells was evaluated by Western blotting. The antimicrobial activities of the culture supernatants of each type of transfected cells against several bacterial strains were also measured. Both NHKs and HDFs infected with ADhBD3 expressed the HBD-3 gene and secreted HBD-3 peptide into culture supernatants. These supernatants exhibited a strong bacteriocidal activity against a *Staphylococcus aureus* reference strain and methicillin-resistant *S. aureus* (MRSA). NHKs and HDFs transfected with the HBD-3 gene secrete HBD-3 peptide with an antimicrobial activity against *S. aureus* and MRSA.

1. Introduction

Advances in the general management of severely burned patients have led to an improved survival rate in the early phase after injury. Nevertheless, treatment of extensive and deep burn injury is still difficult. Early burn excision and immediate skin grafting has recently been performed as a standard treatment for burns, and has been shown to prevent wound infection and reduce mortality and morbidity associated with severe burn [1]. However, in severely burned patients with an extensive burned area and limited availability of skin graft donor sites, it is often difficult to permanently cover post-excision wounds with autologous skin grafts [2]. To solve this problem, cultured epidermal autografts (CEAs) and

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cultured skin substitute (CSS) have been developed and used in clinical practice. The use of CEA or CSS in the treatment of severe burns has reduced the area of skin graft donor sites and mortality rate [3–6]. However, several problems remain unsolved. One of the disadvantages in using CEA or CSS is the instability of engraftment. This is thought to be partly because of the vulnerability of cultured cells to infection [5,6]. Common antibiotics used for infected burn wounds, such as silversulfadiazine, are highly cytotoxic and thus are not applicable in transplantation of cultured cells. Topical application of less cytotoxic antibiotics such as penicillins can promote the emergence of antibiotic-resistant bacteria; therefore, the long-term application of these drugs to infected wounds is not appropriate.

Antimicrobial peptides (AMPs) are molecules involved in natural immunity and are found in a wide range of organisms from plants to vertebrates. AMPs that have been identified in the human skin include human β-defensin (HBD)-1 to -4 and cathelicidin LL37 [7]. HBD-3 is an AMP with a wide-spectrum antimicrobial activity against gram-positive/negative bacteria and fungi. Unlike HBD-1, HBD-2, and HBD-4, whose antibiotic activities are affected by high salt concentrations (i.e., salt-sensitive), HBD-3 has been shown to be relatively insensitive to salt [7–9].

HBD-1 to -3 are expressed by CEA and CSS and localized mainly in the epidermal layer. However, their expression is inadequate to prevent post-transplantation infection and must be enhanced so that grafts can be less vulnerable to infection following transplantation. It has been shown that the application of IL-1α, TNF-α, IL-6 or IL-17 to CSS *in vitro* results in an increased expression of HBD-2 [11,12], but not HBD-3.

Attempts have been made to induce the overexpression of AMPs in human epidermal cells by introducing AMP genes [12,13]. Several studies have been conducted to introduce the HBD-3 gene into HaCaT cells, a human epidermal cell-derived cell line [14,15]. However, there has been no study introducing the HBD-3 gene into normal human keratinocytes (NHKs) or human dermal fibroblasts (HDFs) and demonstrating the secretion of a sufficient amount of HBD-3 that can produce antibiotic activity [15].

In this study, we introduced an HBD-3 cDNA into NHKs and HDFs via a recombinant adenovirus vector and examined the expression of HBD-3 in the transfected cells, secretion of HBD-3 peptide into culture supernatants and its antibiotic activity against several bacterial strains.

2. Materials and methods

This study was carried out with the approval of the Clinical Research Review Committee of Tokai University.

2.1. Cell culture

Primary culture of human epidermal cells and fibroblasts was carried out as previously described [16]. Human skins derived from surgical specimens were washed with sterile saline and then treated with dispase II (2000 U/ml) at 37 °C for 2–3 h to separate the epidermal layer from the dermis. The separated epidermis was treated with 0.25% trypsin at 37 °C for 5 min and then pipetted to disperse epidermal cells. The epidermal cells were seeded in culture vessels at a density of $5 \times 10^4/cm^2$ in a defined SFM® (Invitrogen®) medium supplemented with 20% fetal bovine serum (FBS) and cultured for 24 h. Then, the medium was exchanged for a defined SFM medium without FBS and the culture was continued until subconfluency and the cells were trypsinized for subculturing. Cells after second passage were trypsinized, collected and stored frozen until used for experiments as NHKs. The dermis obtained from the separation of epidermis was washed with saline and cultured in MEM-α medium (Invitrogen®) supplemented with 20% FBS for about 1 week. Proliferating fibroblasts were subcultured twice and stored frozen until used for experiments as HDFs.

2.2. Bacteria

A *Staphylococcus aureus* reference strain (ATCC25923), an *Escherichia coli* reference strain (ATCC25922) and a *Pseudomonas aeruginosa* reference strain (ATCC27853) were purchased from Microbiologics Corp.®. Strains of methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *P. aeruginosa* (MDRP) isolated and cultured from burn patients admitted to our hospital were used in this study. Bacteria of these strains were centrifugally cultured in a heart infusion BROSS® (10 g of beef heart infusion + 10 g of peptone + 5 g of NaCl, Code 05505, NISSUI Pharmaceutical Corp.®) at 37 °C for 24 h before being used for experiments.

2.3. Antimicrobial activities

An HBD-3 reference substance (PEPTIDE INSTITUTE®) was dissolved in sterile distilled water. Bacteria of each strain were centrifuged and pelleted, and culture supernatant was removed. The pellet was washed three times with phosphate buffered saline (PBS) (−) and resuspended in either defined SFM (containing 134 mM Na, 0.09 mM Ca, and 1.6 mM Mg) or 10-fold diluted PBS containing Ca^{++} and Mg^{++} (1:10 PBS (+), containing 14.5 mM Na, 0.09 mM Ca, and 0.5 mM Mg) to prepare a 10^6 cfu/μl bacterial suspension. To 50 μl of this bacterial suspension, 50 μl of each dilution of the HBD-3 reference substance dissolved in distilled water was added and incubated under 5% CO_2 at 37 °C for 1 h. The sodium concentrations of the mixture of each bacterial suspension prepared with defined SFM or 1:10 PBS (+) and each HBD-3 standard solution were 67 and 7.25 mM, respectively. Then, each bacterial suspension was applied to soft agar plates prepared with 10 g of beef heart infusion, 10 g of tryptose, 5 g of sodium chloride, and 15 g of agar (Heart Infusion Agar®, Difco®), incubated at 37 °C for 24 h and subjected to colony counting. Colony inhibition rate (%) was calculated according to the following formula: (No. of colonies of negative control − No. of colonies of each plate)/No. of colonies of negative control × 100.

2.4. Construction of recombinant adenoviral vectors

Cloning of the HBD-3 cDNA was carried out as previously described by Erdag et al. [11]. NHKs were cultured in a defined SFM medium supplemented with 100 ng/ml IL-1α for 48 h.