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Co-culturing epidermal keratinocytes and dermal fibroblasts on nano-structured titanium surfaces

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ABSTRACT

Long-term success of percutaneous implants depends mostly on the stable connection between the soft tissue and implant surface because bacterial invasion and infection can be prevented by a proper seal between the skin and implant. The percutaneous seal is affected by responses of keratinocytes and/or fibroblasts to the implant. Herein, the *in vitro* functionality of fibroblasts and keratinocytes on titania nanotubes (TNT) and polished titanium (pTi) surfaces was investigated by different culture methods. Adhesion, proliferation, morphology, and differentiation were evaluated by cell viability assay, fluorescence microscopy, real-time quantitative polymerase chain reaction (RT-PCR), and indirect immunofluorescence. Single cultured fibroblasts on the TNT surface showed increased adhesion, proliferation, and differentiation, while these cellular properties were decreased in single cultured keratinocytes. In non-contact co-culture with keratinocytes, fibroblasts presented better orientation, continuous proliferation, and increased gene expression on TNT. However, decreased adhesion and proliferation were observed for keratinocytes in non-contact co-culture with fibroblasts. Furthermore, keratinocytes presented high abilities to proliferate and differentiate in contact co-culture on fibroblasts adhering on the TNT surface. The gene expression results of contact co-culture model suggested that the nano-structured titanium surface promoted the maturation of fibroblasts and the formation of dermal matrix through secreting collagen I and transforming growth factor-β1 (TGF-β1), and indirectly facilitated the proliferation of keratinocytes and the formation of the basement membrane by stimulating fibroblasts to secrete keratinocyte growth factor (KGF), nidogen, and collagen IVα-1. Meanwhile, keratinocytes secreted TGF-β1 to promote fibroblast differentiation. Moreover, the enhanced proliferation and differentiation of keratinocytes were favorable for skin-implant integration.

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1. Introduction

Percutaneous devices are widely used in the field of surgical implantation. Their successful applications require the creation of an effective seal at the skin-implant interface to prevent pathogens from entering the body. Lack of complete integration between the skin and implant may cause infection, tissue morbidity, implant removal, and even mortality [1,2]. The skin consists of the epidermis, dermis, and hypodermis. Keratinocyte is the main cell of the epidermal layer and dermal fibroblast is the principal cell of the dermal layer. Moreover, the epidermis is tightly linked to the dermis by the basement membrane [3]. When a percutaneous device is embedded into the body, the injury of the skin initiates a cascade of events, including inflammation, new tissue formation, and tissue remodeling [4].

The process of wound healing is peculiar as the implant intervened. The skin cells can only migrate around the implant surface, go down to the deep connective tissue, and tightly attach to the implant surface [5]. The process is immediately initiated after the implant is inserted into the body, and the wound is filled with blood clots. After 48 h, the dermal fibroblasts migrate to the implant surface, proliferate, and secrete large amounts of extracellular matrix. Simultaneously, the epidermal keratinocytes migrate and proliferate at the wound edge. If the implant is biocompatible, the keratinocytes anchor to the implant surface and proliferate to form a complete epidermis [6,7]. The skin tissues in the whole process of wound healing need to closely attach to the implant surface and finally form a biological seal at the interface.

Titanium (Ti) and titanium-based alloys are the most widely used percutaneous devices due to their mechanical strength, biocompatibility, non-toxicity, and corrosion resistance [8–11]. Many studies showed that the nano-structured titanium surface can enhance the functionality of different cell types [12,13]. Springer et al. assessed viability and

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proliferation of human epidermal keratinocytes and dermal fibroblasts on several Ti6Al4V surfaces: solid polished commercial, solid polished electron beam melting (EBM), solid unpolished EBM, and porous unpolished EBM. Fibroblast proliferation was the highest on the commercial and EBM polished surfaces, and that of keratinocytes was the highest on commercial polished surfaces [14]. An *in vitro* study evaluated the functionality of human dermal fibroblasts and epidermal keratinocytes on titania nanotube arrays (diameter 70–90 nm), the results indicated that titania nanotube arrays increased dermal fibroblast and decreased epidermal keratinocyte adhesion and proliferation [15]. The process of implant-skin integration involves the interaction between the titanium implant surface, epidermal keratinocytes, and dermal fibroblasts, thus co-culturing keratinocytes and fibroblasts on titanium surface can simulate the *in vivo* situation and can allow us to investigate their interaction.

Co-culture systems can be applied with the intent of using one cell type to provide a desired stimulus to a second cell type. This strategy takes advantage of the naturally occurring cross-talk between cell types, either through soluble factors or direct cell-cell contact [16–18]. Co-culture systems for skin regeneration were extensively developed in the 90's and they predominately involved co-cultures of fibroblasts and keratinocytes [19–21]. A study showed that the proliferative and migratory potential of fibroblasts was significantly enhanced in co-culture of fibroblasts and keratinocytes using a Transwell system [22]. Biomaterial scaffolds are popularly used for co-culture of keratinocytes and dermal fibroblasts such as chitosan, hydrogels, fibers, membranes, and nanocomposites [19,23]. A remarkably well-structured and differentiated squamous epithelium developed and formed a nearly complete basement membrane after 3 weeks in co-culture of keratinocytes on top of collagen gels containing dermal fibroblasts [24]. Mazzone et al. prepared plastically compressed collagen hydrogels and fibrin gels containing fibroblasts, and the keratinocytes were seeded onto these dermal constructs. A stratified epidermis covered the entire surface of the collagen gel, but did not form a physiologically organized epidermis on fibrin gels [25]. However, the *in vitro* study seldom referred to the co-culture of fibroblasts and keratinocytes on titanium surfaces [26].

Our previous work demonstrated that the nano-structured titanium surface enhanced the skin-implant integration *in vivo* [27]. In addition, modified nano-topographic titanium surface can modulate macrophage behavior to prevent infection and encourage tissue remodeling [28]. In order to better understand the mechanisms of skin cell-implant interactions, we co-cultured keratinocytes and fibroblasts on titania nanotubes (TNT) and polished Ti (pTi) surfaces. Non-contact and contact co-cultured models were used to evaluate the cell-cell and cell-material interactions.

2. Materials and methods

2.1. Titanium sample preparation

Titanium discs (10 mm diameter, 0.5 mm thickness) were used for *in vitro* analysis, TNT samples with 100 nm in diameter were prepared by anodization on titanium surfaces and pTi was used as the control. The electrolyte was a miscible liquid of H_3PO_4 (2 M) and HF (0.15 M). The voltage was set at 20 V and the anodization was carried out for 1 h to obtain TNT with a diameter of 100 nm and length of 800 nm [27].

2.2. Cell co-culture model

The human epidermal keratinocytes (HaCat) and human dermal fibroblasts (HSF) were cultured in DMEM supplemented with 10% FBS (Hyclone) and 100 U/mL of penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Medium was refreshed every two days during cell culture. The substrates were placed in culture plates and divided into five groups:

1) K: HaCat (K) cells were seeded at the density of 5×10^4 cells/mL on TNT or pTi surfaces.
2) F: HSF (F) cells were seeded at the density of 5×10^4 cells/mL on TNT or pTi surfaces.
3) K/F: HaCat (K) cells on material surfaces were non-contact co-cultured with HSF (F) cells. HaCat cells were seeded at a density of 5×10^4 cells/mL on TNT or pTi surfaces. Transwell chambers were inserted into the culture plates, and HSF cells were seeded at a density of 5×10^4 cells/mL on a polyester membrane with 0.4 mm pore size and 4.5 cm^2 area of the Transwell chamber (Corning Incorporated Life Sciences, Acton, MA, USA).
4) F/K: HSF (F) cells on material surface were non-contact co-cultured with HaCat (K) cells. HSF cells were seeded on TNT or pTi surface, and then HaCat cells were seeded on the Transwell polyester membrane. The cell density and membrane specifications were the same as those in 3.
5) Co: The two types of cells were contact co-cultured on material surfaces. HSF cells were seeded at a density of 1×10^5 cells/mL on TNT or pTi surface for 5 d, and then HaCat cells were seeded at a density of 1×10^5 cells/mL on top of HSF cells.

2.3. Cell proliferation and adhesion assays

Cell proliferation was evaluated by the alamarBlue assay. After culture for 1, 3, and 7 d, the culture medium was replaced by Medium 199 (GIBCO, Carlsbad, CA, USA) supplemented with 10% FBS and 10% alamarBlue. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 4 h. The optical density (OD) of the medium was read at 570 nm in a microplate reader (MQX200).

Cell morphology was observed under a fluorescence microscope (FM, Leica DM5500) after 1, 3, and 7 d of culture. The medium was removed and the cells were fixed in a solution containing 2.5% glutaraldehyde for 4 h. The samples were then transferred to a new culture plate and stained with FITC-Phalloidin (1:40 in 5% FBS/0.1 Triton X-100 in phosphate buffer saline (PBS), Sigma, St Louis, MO, USA) for 30 min to visualize F-actin filaments as well as a 4,6-diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma) for 5 min to visualize the cell nucleus.

2.4. Gene expression assay

Total RNA was extracted (TRIZOL, Invitrogen, Carlsbad, CA, USA) after 4, 24, and 72 h and reverse transcription was carried out using a PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan). Quantitative analysis of mRNA expression was performed using a PIKORed 96 real-time fluorescence quantitative polymerase chain reaction (RT-PCR, Thermo Fisher, Waltham, MA, USA). Design of primers for inflammatory cytokines and chemokines was performed using the Primer Premier web-based software. RT-PCR was carried out with the following primers for: laminin-β3 (Forward (fwd): 5′-CTCCAGCGGACCAGGCAGATGATT-3′, reverse (rev): 5′-TGGATTAGGAGCCGTGTGCGTCTG-3′), collagen IVα-1 (fwd: 5′-AGGAGACTTCGCCACCAAGGGAGA-3′, rev:5′-GGGTTTGCCTCTGGGTCC TGGTTT-3′), nidogen (fwd: 5′-AGGAACCTTCCGCTGCGAGTGT-3′, rev: 5′-ACATCGGCTTGGCTGGCATTCA-3′), transforming growth factor-β1 (TGF-β1, fwd: 5′-CCGACTACTACGCCAAGGAGGTCAC-3′, rev: 5′-CGGTGGCT GAGGTATCGCCAGGAAT-3′), and keratinocyte growth factor (KGF, fwd: 5′-AGCCCTGAGCGACACACAAGAAGT-3′, rev: 5′-TGCCACAATTCCAACT GCCACTGT-3′). β-actin (fwd: 5′-GAAGATCAAGATCATTGCTCCT-3′, rev: 5′-TACTCCTGCTTGC TGATCCA-3′) was used to verify the equal amounts of RNA used for amplification. The relative mRNA expression level was calculated by the value of $2^{-\Delta\Delta CT}$. Threshold cycle (CT) value for each test sample was analyzed using Sequence Detection software version 1.2.3 software (Applied Biosystems, Foster City, CA, USA).

2.5. Immunofluorescence assay

The medium was removed and cells were gently rinsed with PBS. The cells were fixed in 3% paraformaldehyde in PBS for 2 h at 4 °C and

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