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Biological Compatibility of the Lando® Artificial Dermal Regeneration Matrix and Normal Human Epidermal Cells

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Abstract

Objective: To determine the biocompatibility of the Lando® Artificial Dermal Regeneration Matrix (LADRM) and normal human epidermal cells *in vitro*.

Methods: Human epidermal sheets obtained from foreskin tissues were cultured on the LADRM and were observed daily using an inverted microscope. On the 36th day, the LADRM sheets containing epidermal cells were freeze-sectioned and stained with HE and immunofluorescence. Antibodies against CK15 and gp100 were used to identify Keratinocytes (KCs) and Melanocytes (MCs), respectively. The types, morphologies and distribution of epidermal cells cultured on the LADRM were observed.

Results: Epidermal cells grew well on the LADRM and over time, they continued to proliferate. On the 36th day, HE staining showed that epidermal cells grew in multiple layers on the surface and inside of the LADRM and they were inclined to attach and grow along the collagen fibers. Fluorescence staining with antibodies against CK15 and gp100 indicated that a small number of MCs were scattered among a large number of KCs, and MCs were evenly dispersed among KCs, most of which were dividing and showed high levels of proliferation.

Conclusion: The LADRM and epidermal cells have good biocompatibility and this biological material is conducive to the stratification, proliferation and differentiation of epidermal cells.

Keywords: Biocompatibility; Lando® Artificial Dermal Regeneration Matrix (LADRM); Keratinocytes; Melanocytes

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Introduction

The skin covers the entire surface of the body and defects are readily caused due to external damage. When the skin loses its function of protecting the underlying tissues, the damaged areas are prone to infections and disfiguring scars [1]. Therefore, the wounds must be repaired as soon as possible, especially large and deep injuries. Rheinwald and Green established the epidermal cell culture technology in 1975 [2], which opened a new route for the clinical application of the patient's own skin to repair large areas of burns and wounds [3]. However, that method is difficult to use in clinical practice due to the following disadvantages: 1) Epidermal cells take a long time to culture *in vitro* and require a highly suitable environment to be transplanted successfully; 2) Owing to the lack of dermal support, epithelial tissue has poor elasticity and poor resistance to friction and tends to shrink and rupture during wound healing. Therefore, dermal substitutes play an important role in the process of skin reconstruction [4,5]. The development of artificial skin provides new approaches for the repair of skin defects. Tissue engineered dermis stimulates or induces new dermis, induces fibroblast infiltration in wound surfaces, forms new blood vessels and dermal-like granulation tissue to improve the quality of healing [6]. When the biomaterials are pre-seeded or have cells incorporated within their matrix, they are classified as cellular artificial skin grafts. Tissue engineered skin uses cultured keratinocytes and dermal substitutes to construct a full-thickness skin structure to complete the simultaneous repair of the dermis and epidermis to reduce contractures and scar hyperplasia, to improve the appearance of wounds after repair and to improve the quality of life of patients [7]. At present, there are two kinds of artificial dermis used clinically in China: Pelnac (GUNZE, Japan) [8] and the Lando® artificial dermal regeneration matrix (LADRM, also called bilayer artificial skin) (Shenzhen Lando Biomaterials Co. Ltd, China). The clinical application and animal models of LADRM have been reported in the literature [9]. However, there have been few reports on the biological compatibility of the LADRM with normal human epidermal cells

cultured *in vitro*. This study aimed to explore the biocompatibility of the LADRM and to observe the growth of epidermal cells on or within in its layers in order to provide more experimental evidence for future clinical applications.

Materials and Methods

Preparation of epidermal sheets and biological materials

A foreskin was obtained from a healthy 12 year-old boy who underwent a circumcision in the Third Affiliated Hospital of Soochow University, after obtaining informed consent of the patient's parent. The specimen obtained was immediately immersed in 0.5% iodine solution for 5 min and was then washed three times with Phosphate Buffered Saline (PBS) containing 400 U/ml penicillin and 400 U/ml streptomycin to remove the iodine residue and to prevent possible bacterial contamination. After trimming and removing the subcutaneous fat and fascia, the remaining tissue was cut into small pieces (approximately 0.5 cm × 0.5 cm) and incubated in Dispase II solution for 2 h to separate the epidermis from the dermis. A sheet of LADRM (Registration Certificate Number: 20173461356) was cut into small pieces (1 cm × 1 cm) that were placed onto the bottom of a 6-well plate, with a silica gel layer underneath and a collagen sponge layer on top.

Observation and staining of the tissue culture

The epidermal sheet obtained was placed on the LADRM for routine tissue culture. The medium contained complete M254 and Epilife culture medium at a ratio of 1:1 and was changed every two days. The growth of the epidermis on the LADRM was observed using a microscope and was photographed. On the 36th day, the LADRM containing epidermal cells was taken out to prepare frozen sections. In addition to conventional HE staining, we also performed fluorescent staining of keratinocytes and melanocytes. The frozen sections were fixed with 4% paraformaldehyde at room temperature for 15 min followed by washing three times with PBS, and then were permeabilized with 0.2% Triton X-100 (diluted with PBS) for 15 min at room temperature. The primary antibodies used were antibodies against CK15 in keratinocytes and gp100 in melanocytes, respectively, namely, the rabbit monoclonal antibody (PR1614Y) and the mouse monoclonal antibody (HMB45). The corresponding secondary antibodies were goat anti-rabbit IgG H&L (DyLight 594) (1:200) and goat anti-mouse IgG (H+L) (Alexa Fluor 488) (1:200). The nuclei were then stained with DAPI (1:500) for 5 min. Photos were taken under a fluorescence microscope.

Results

Daily observation of tissue culture

Since the tissue culture was three-dimensional, the number and morphology of cells at different levels was also diverse. Therefore, these observations had certain limitations but still have some scientific significance. When viewed using an inverted microscope, the epidermis on the sheet of LADRM displayed cloud-like areas. Within about 24 days, the number of cells gradually increased with prolonged culture time (Figure 1), which indicated the biocompatibility of the epidermal cells and the LADRM. There were two distinct phenotypes of epidermal cells observed, one of which was light and translucent, presumed to be keratinocytes, and the other was tan-black, presumed to be melanocytes. The distribution of those two kinds of cells was scattered, without any obvious structural significance.

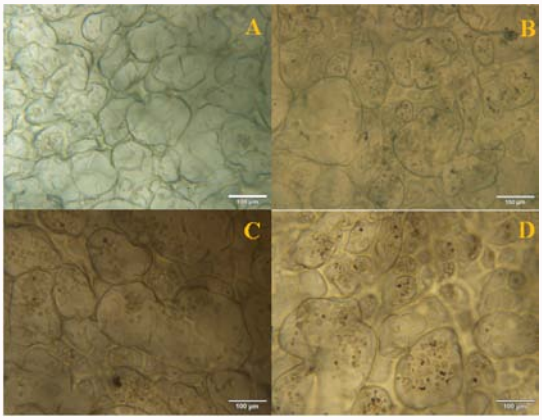

Figure 1: Observation of tissue cultures at different time points, including Days 6 (A: 200x), 12 (B: 200x), 24 (C: 200x) and 36 (D: 200x). Scale bars =100 μm.

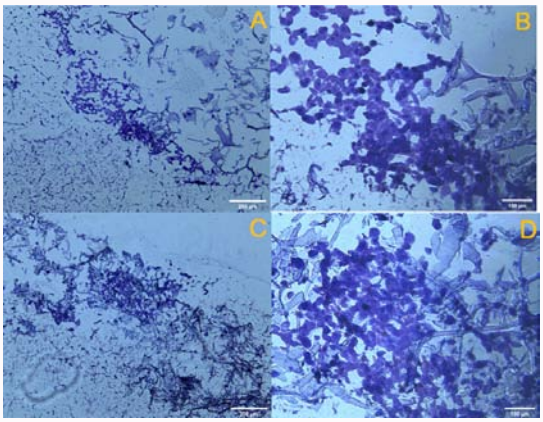

Figure 2: The results of HE staining of the cultured tissues. There were 2 to 6 layers of cells growing on the surface of the artificial skin. A (40x), B (100x), C (40x), D (100x). Scale bars (A/C=200 μm. Scale bar (B/D) =100 μm.

Interactive distribution of epidermal cells and biological materials

On the 36th day, the cultured tissues were cut into frozen sections and stained with HE or immunofluorescence. In the HE stained images, strips stained light blue can be seen that represent the fiber filaments of the dermis layer of the artificial skin (Figure 2A, 2B). On the surface of the artificial skin, there were 2 to 6 layers of cells, which were round or polygonal and were uniform in size and color (Figure 2C, 2D). Within the dermal layer containing interlaced collagen fibers, there were also large numbers of cells, whose morphology was not different from the cells on the surface of the artificial skin. This suggested that cells that had dropped down or migrated from the epidermal sheet can directly enter the dermal layer of the artificial skin and can grow well there. It was also obvious that these cells are more inclined to attach to the fibrous filaments made of collagen. Fluorescent staining showed that most cells on the surface and inside the artificial skin were keratinocytes expressing CK15, and only a few melanocytes were seen that expressed gp100 (Figure 3). It is worth mentioning that the distribution of melanocytes in the LADRM was scattered and even. That distribution is not exactly similar to normal epidermis, where melanocytes are only located in the basal layer, and the ratio of melanocytes to basal layer keratinocytes is about 1:10. This further suggested that those proliferating cells were no longer inherent cells in the epidermal sheets, but were cells and their progeny

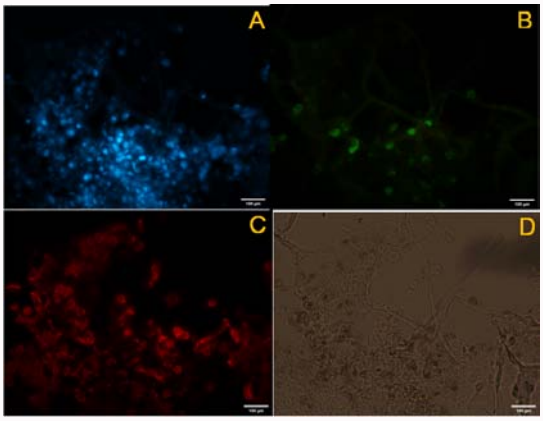

Figure 3: Fluorescence staining of the cultured tissue. A: Cell nucleus stained by DAPI (100x). B: Melanocytes expressing gp100 (100x); C: Keratinocytes expressing CK15 (100x). D: Observation by ordinary optical microscopy (100x). Scale bars =100 μm.

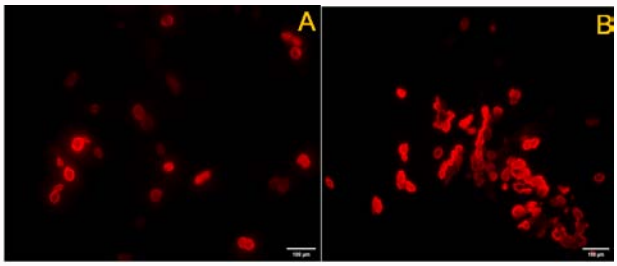

Figure 4: Keratinocytes that are dividing and proliferating. A (100x), B (100x). Scale bars =100 μm.

that had migrated from the epidermis. Under higher power, it can be seen that the size and fluorescent brightness of keratinocytes was also very different. This may be due to the three-dimensional distribution and the different differentiation states of those cells. On the 36th day of culture, the images in which cells were stained with the antibody against CK15 showed that many cells were dividing or had completed dividing but their two daughter cells were still close (Figure 4). This phenomenon indicated that the rate of cell proliferation was good.

Discussion

Skin substitutes are used as bioactive wound dressings and can promote wound healing, not just cover the injured area. They are roughly divided into two categories, namely acellular and cellular artificial skin grafts [10]. The most common types used in the clinic are acellular dermal scaffolds, which do not have cells. When the biomaterials are pre-seeded or have cells incorporated within their matrix, they are classified as cellular artificial skin grafts. The ideal dermal scaffold should be a temporary biodegradable three-dimensional framework with good biocompatibility and an appropriate porous microstructure to facilitate cell adhesion, migration, proliferation and differentiation. Their main roles are to supply oxygen through oxygen permeation, to keep the wound from dehydration, to prevent microbial infections and to promote healing [11]. The LADRM is a 3D culture frame structure that has a double-layer structure [9]. The upper layer is a semi-permeable medical silica gel layer that provides mechanical support and prevents excessive water loss and bacterial invasion in the early stages of wound healing. The lower layer is a wet collagen sponge layer that includes cross-linked type I collagen fibers extracted from bovine Achilles Tendon (AT) and Chondroitin Sulfate (CS) [12]. Type I collagen fibers mainly play a role in maintaining the frame structure and are conducive to cell attachment and growth. CS promotes tissue regeneration, intracellular signal transduction, cell proliferation, cell adhesion and inhibits cell apoptosis. Several studies have reported that the combination of those two substances enables the rapid repair of damaged skin, which has been proven in the clinic. AT and CS have an excellent biological compatibility and eventually they are absorbed by the human body [13]. The microstructure of dermal scaffolds, including their porosity, pore size, pore shape, permeability, etc., are key factors affecting cell adhesion, movement, contraction, proliferation, gene expression and overall biological activity [14]. A proper pore size range is favorable for cell growth. A small pore size may inhibit cell migration, while a large pore size may affect cell adhesion. The LADRM has an irregular circular structure, with different depths of internal pores [15]. The pore sizes of the LADRM are 20 μm to 120 μm in diameter and connect with each other. This structure not only enables cells in the surface layer to obtain nutrients and cells in the middle layer to obtain oxygen but is also beneficial for cells to migrate from the periphery to the middle or *vice-versa* [16]. In this study, we observed that epidermal cells on the surface and those within the layers grow well. Moreover, those cells tended to attach to filaments first, then proliferate and expand to the surrounding area. Daily observations made using a microscope found that the epidermal cells gradually formed a multilayer structure. This finding was confirmed by HE staining of tissue sections on the 36th day. According to the distribution of the cells, it can be inferred that they migrated from the epidermal sheet and proliferated on the surface or inside the biomaterial matrix, and the original epidermal sheet had fallen off. The results of immunofluorescence staining showed that most of the epidermal cells were keratinocytes, accounting for more than 95% of cells, and only a few were melanocytes. Many of the keratinocytes were dividing. The location of melanocytes and the ratio of melanocytes to keratinocytes did not strictly follow the normal physiological state of the epidermis [17]. In the entire process of wound repair, in addition to the most basic repair of appearance, there are a series of other functional repairs that need to be considered, including the regeneration of blood vessels and nerves, hair follicles and sweat glands [18]. Therefore, planting pluripotent stem cells on artificial dermal scaffolds may be a reasonable strategy, which needs further research.

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