Cell activity of human de-epidermized dermis and its characteristics of tissue structure*☆

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Abstract

BACKGROUND: Study confirmed that the de-epidermized dermis (DED) can be used as dermal substitute and may form epidermal structure after incubating keratinocytes. However, the cell biological activity, tissue structure characteristics and the basement membrane component analysis of dermal substitute have been reported less.

OBJECTIVE: To investigate the cell activity and the tissue structure characteristics of DED.

METHODS: Skin flap was treated with 56 ℃ phosphate buffered solution to remove the epidermis, and the dermal cell components were deleted by freezing and thawing with liquid nitrogen to obtain DED. The DED cell activity was detected with tissue culture method, hematoxylin nuclear staining was used to determine the DED cell nuclei, and vimentin immunohistochemistry was applied for fibroblast determinations. The basement membrane and its components were detected using Periodic Acid-Schiff staining and collagen type IV immunohistochemistry. Van Gieson stain, Weigart stain and those double staining were respectively used to determine DED collagen fibers and elastic fibers. The DED ultrastructure was observed under transmission and scanning electron microscope.

RESULTS AND CONCLUSION: Using tissue culture method, the cultured DED did not exhibit cell growth at 2 weeks. Hematoxylin-eosin staining showed no nuclear in DED, vimentin immunohistochemistry showed no vimentin expressed in DED. Van Gieson staining showed DED collagen fibers were stained as rose red, Weigert staining showed DED elastic fibers were stained as purplish black, double staining further demonstrated uniform arrangement of collagen fibers and elastic fibers. DED surface and the remaining appendages were strongly positive for Periodic Acid-Schiff staining, and type IV collagen expression was significant. Transmission and scanning electron microscope results showed that, the DED elastic fibers and collagen overlap arranged with pore intervals, they intercrossed into a network. There is no living cell component in DED, dermal matrix surface and appending organ luminal wall still retain glycogen, type IV collagen and other basement membrane components, dermal matrix is rich in collagen and elastic fibers, it is a three-dimensional collagen matrix similar to *in vivo* dermis.

INTRODUCTION

The artificial skin or tissue-engineered skin refer to the skin constructed by the seed cells compounded with artificial dermis (dermal substitute) *in vitro*, similar to *in vivo* skin regarding the structure and function. Artificial skin generally should have a two-layer structure of both epidermis and dermis, and have the biological characteristics of the skin[1-2]. With the seed cell culture and dermal substitute technological development, the artificial skin is not only used for trauma (such as burns), skin transplantation and repair for diabetic skin ulcers[3-5], but also applied to skin development, skin disease pathogenesis, drug development and cosmetic surgery, *etc.*[6-10].

The key of artificial skin technology is the development of dermal substitute, which should be rich in collagen so as that keratinocytes could develop and differentiated into full epidermis on a dermal matrix. As for the current developed and applied materials, dermal substitutes can be divided into two categories: artificial dermis and natural dermis. The former is polymerized by artificial or natural materials, such as polyglycolic acid, polylactic acid or chitin and glutin constituting network stents to combine with collagen, fibroblasts and other ingredients[2,11-12]; the later is animal derived or human derived dermal xenograft[13-14]. In 1981, Regnier *et al*[15] have described that a de-epidermized dermis (DED) with the removal of cellular components can be used as a dermal substitute, it can form epidermal structure after incubating keratinocytes on the surface. Because the allograft dermal substitutes have broader sources of materials and can be produced in a more simple way, it has been widely used in various applications[15-17]. However, according to previous reported literatures, human-derived acellular dermal matrix is produced with different ways, such as the separation of enzyme-polyethylene glycol octyl phenyl ether method, hypertonic saline-sodium dodecyl sulfate method and hypertonic salt-sodium dodecyl sulfate-trypsin method, *etc*[18-19]. No matter which approach, the aim is to remove the epidermis and antigenic living cell components in dermis, in order to obtain acellular dermal matrix similar to the dermal tissue composition *in vivo.* Previously the authors have reported that the surgically excised human skin was subjected to phosphate buffered solution (PBS) hydration to remove the epidermis, then repeated ultra-low temperature freeze-thaw inactivated dermal cells to obtain human-derived acellular DED, and it can be used as a dermal skin substitute to *in vitro* construct skins and achieve some progresses[16]. However, there have been less literatures reporting the cell biological activity, tissue structure characteristics and basement membrane component of dermal substitute produced by this method. Therefore, this paper was aimed to analyze the cell activity, tissue structure and components of the DED matrix by using of tissue culture, immunohistochemistry and electron microscope methods.

MATERIALS AND METHODS

Design

Sample tissue sample observation.

Time and setting

The experiment was performed in the laboratory of Dermatology Department at Guiyang Medical College and China Medical University from August 2006 to October 2008.

Materials

Skin flap was taken from healthy adult female breast skin specimens undergoing surgery at Department of Plastic Surgery, Guiyang Medical College. All donors have given informed consents.

The main reagents and instrument used are as follows:

Reagent and instrument	Source
Mouse anti-human vimentin IgG monoclonal antibody, mouse anti-human type IV collagen IgG monoclonal antibody, immunohistochemistry kit	Dako, Denmark
Horse anti-mouse IgG, horse serum	Vector Labs, USA
DMEM culture medium (low sugar), F_{12} nutrient solution, penicillin, streptomycin	Sigma, USA
Cell incubator	Thermo Forma, USA
Light microscope	Olympus, Japan
Anatomical microscope	Leica, Germany
Ultramicrotome	Type 8800, LKB, Sweden
Transmission electron microscope	Hitachi H-6000, Japan
Scanning electron microscope	JSM-6360LV, Japan

Methods

Preparation of DED tissue

Skin flap was added with appropriate PBS and preserved in 4 ℃ refrigerator for further use. According to the previously reported methods, the DED was treated[16]. Samples were washed several times with PBS and placed in sterile containers with the specimens epidermis faced down and dermis faced up, the fat and subcutaneous tissues were carefully removed with the eye curved scissors under an anatomical microscope, to about 2.5 mm thick, during which tissues were moistened with PBS repeatedly. Drawn with 10-mm biopsy device, the specimens were immersed in PBS, and then placed in 56 ℃ water bath for 30 minutes, transferred to aseptic boxes and gently separated epidermis and dermis using flat tweezers, removing the epidermis. The dermis was placed in petri dish at room temperature for about 1 hour to dry, and later placed in polyethylene plastic bottle (6 specimens in each bottle), adding a drop of DMSO, closed bottle and quickly placed in liquid nitrogen (-120 ℃ to -160 ℃) for about 5-7 minutes, taken out and preserved at room temperature for 30 minutes, and then placed in liquid nitrogen. This procedure was repeated 10 consecutive cycles in order to kill the cellular components of DED.

Tissue culture method

The DED was washed with Hank's solution and cut into sizes of approximately 2 mm × 5 mm, cultured in petri dishes containing a small amount of tissue culture fluid (3:1 ratio preparation of DMEM: F_{12}, 10% volume fraction of fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin), each dish was about 6-8 tissue mass, at the interval of about 0.5 cm, incubated at 37 ℃, 5% CO_2 for 3 hours, added 5 mL tissue culture fluid for culture, the culture medium was abandoned 4-5 days later, adding an equal volume of culture medium to continue culture, sub-culture medium was renewed every 3 days, continuous culture for 2 weeks. Each time before the medium was changed, the cell growth was observed under inverted microscope. The culture was repeated for more than three times.

DED conventional and immunohistochemical staining

The conventional paraffin sections (3-6 μm). Periodic Acid-Schiff staining was applied to detect basement membrane, Van Gieson staining of collagen fibers, Weigert staining of elastic fibers, and Van Gieson with Weigert double staining of collagen fibers and elastic fibers. Type Ⅳ collagen immunohistochemical was used to determine basement membrane components, vimentin immunohistochemical staining was used to determine vimentin, the former primary antibody was mouse anti-human collagen type Ⅳ IgG monoclonal antibody (1: 150), secondary antibody was biotin labeled horse anti-mouse IgG (1: 100), the later primary antibody was mouse anti-human vimentin IgG monoclonal antibody (1: 200), secondary antibody was biotin labeled horse anti-mouse IgG (1: 200). Blank control adopted PBS as primary antibody. ABC method was used for incubation, DAB coloration, specimens fixed.

Scanning electron microscope observation

The tissues were removed from paraffin blocks, xylene dewaxing, acetone replacement xylene (50%, 70%, 90%, 100%), isoamyl acetate replacement acetone (50%, 70%, 90%, 100%), critical point drying instrument dried, ion sputtering instrument coatings, scanning electron microscope observed.

Transmission electron microscope

The tissues were removed from paraffin block, xylene dewaxing, ethanol gradient dehydration, PBS immersion, 4% glutaraldehyde fixed 24 hours (4 ℃ refrigerator), PBS soaking 15 minutes, 1% osmium tetroxide fixed 1 hour, acetone replacement xylene (50%, 70%, 90%, 100%), Epon 812 soaking, epoxy resin embedding medium and 100% acetone soaking in a 1:1 ratio of 24 hours, Epon 812 epoxy resin embedding medium embedded, repairing block machine repaired and positioned, a section by ultra-thin slicing machine, uranyl acetate and lead nitrate double staining, transmission electron microscope observed.

Main outcome measures

The cell activity and tissue structural characteristics of the DED.

Design, enforcement and evaluation

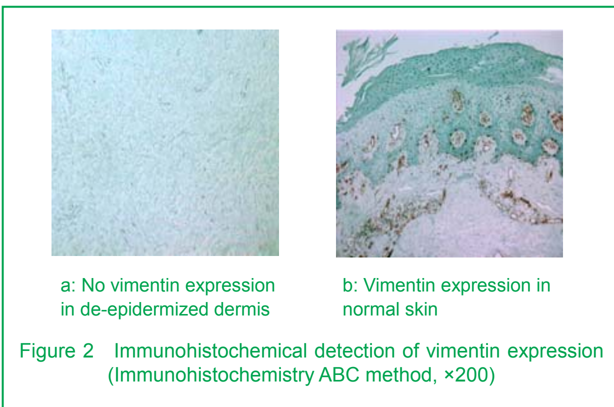
All the authors completed this study.

RESULTS

Cell activity determination of the DED

By tissue culture method, there was no cell growth occurred at 2 weeks. The hematoxylin staining showed no cell nuclear staining in DED. Vimentin immunohistochemistry results revealed no vimentin expression in the DED, but can be seen in the dermal superficial lamella of normal skin tissues (Figures 1, 2).

Figure 1 Hematoxylin staining shows no cell nuclei in de-epidermized dermis (×100)



a: No vimentin expression in de-epidermized dermis

b: Vimentin expression in normal skin

Figure 2 Immunohistochemical detection of vimentin expression (Immunohistochemistry ABC method, ×200)

Histological test results of the DED

Periodic Acid-Schiff staining displayed that purple chromatobar was observed in the uneven DED surface and DED residual sudoriferous duct (Figure 3).

Figure 3 Periodic Acid-Schiff staining showed the de-epidermized dermis surface was purple, and the stain was also observed in de-epidermized dermis matrix residual sudoriferous duct (×400)

Type Ⅳ collagen is expressed in the DED surface and matrix residual sudoriferous duct (Figure 4). Van Gieson staining showed the DED collagen fibers were uniformly colored as red; while elastic fibers as purplish black coloring by Weigert staining; double staining showed the DED matrix collagen fibers and elastic fibers were evenly arranged (Figure 5).

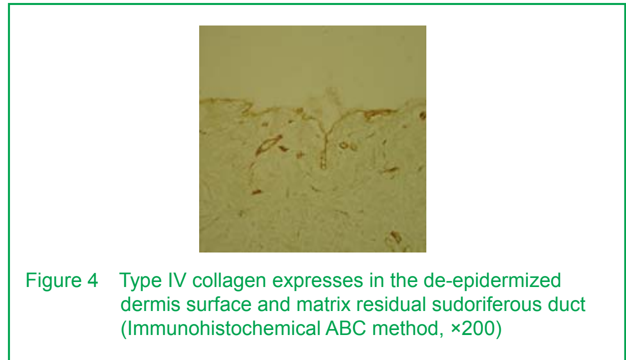
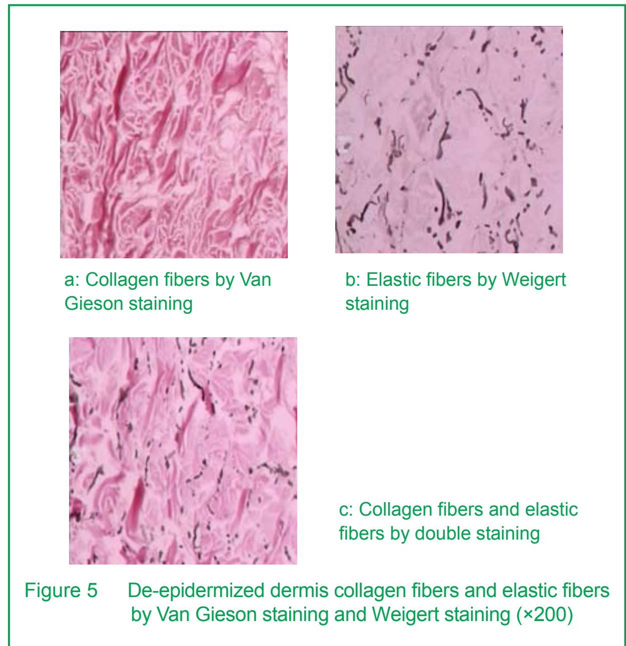


Figure 4 Type IV collagen expresses in the de-epidermized dermis surface and matrix residual sudoriferous duct (Immunohistochemical ABC method, ×200)



a: Collagen fibers by Van Gieson staining

b: Elastic fibers by Weigert staining

c: Collagen fibers and elastic fibers by double staining

Figure 5 De-epidermized dermis collagen fibers and elastic fibers by Van Gieson staining and Weigert staining (×200)

DED ultrastructure

Scanning electron microscope showed that, collagen fibers overlapped in DED, the fibers of reticular formation had a certain porosity (Figure 6), transmission electron microscope showed collagen fibers were arranged as dense bundles (Figure 7).

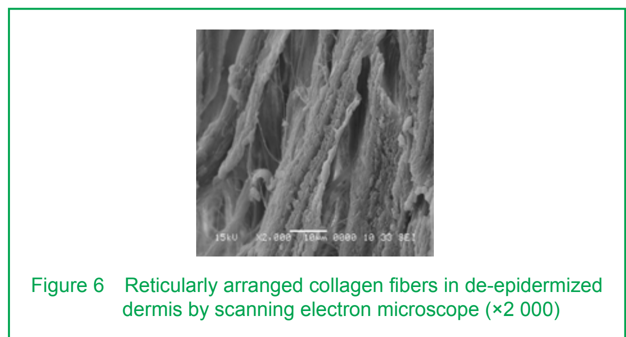


Figure 6 Reticularly arranged collagen fibers in de-epidermized dermis by scanning electron microscope (×2 000)

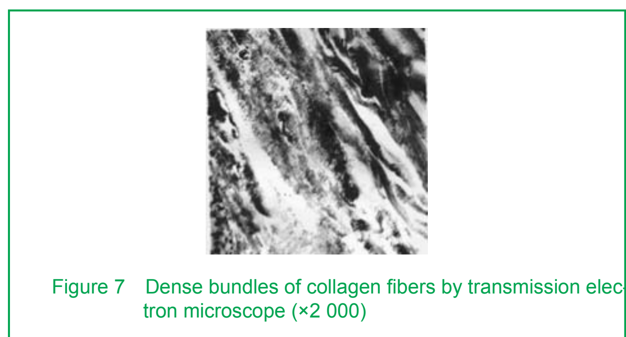


Figure 7 Dense bundles of collagen fibers by transmission electron microscope (×2 000)

DISCUSSION

Previously, skin seed cells (such as keratinocytes) cultured in petri dishes for tissue culture, although the monolayer cells can be observed, but without epidermal hierarchical structure and cuticle skin. However, the seed cells were seeded in dermal substitutes, combined with air-liquid culture technology, will receive a complete epidermis, and be able to express keratin, envelope protein and other components[20]. It can be seen, the dermal substitute not only provides the three-dimensional environment for the growth and development of seeded cell culture, the dermal substitute tissue matrix protein components also play a key role on inducing cell differentiation. Salag *et al*[21] mixed the cultured fibroblasts with collagen gel according to certain percentages, this mixture formed gel within a few minutes in the petri dish. A few days later, the gel became more dense and similar to dermal tissue structures, and then inoculated with keratinocytes on surface, to obtain a composite skin of epithelial-like structure by culture. Later, Coulomb[22] applied this dermal matrix to study epidermal development, but the dermal matrix is obviously insufficient: if the collagen gel would severely contract by 70%-80%; collagen gel is poorly resist to wound collagenase digestion and degradation; collagen gel brittleness is relatively large, with weak compression resistance or traction resistance. In order to overcome the shortcomings of the collagen gel skin substitute, someone tries to use collagen-glycosaminoglycan sponges as scaffolds to incubate fibroblast as dermal substitutes, and then inoculating keratinocytes to form a composite skin substitute[23]. Recently, people use synthetic or natural hybrid stents mix collagen and fibroblasts as a dermal substitute. Artificial materials commonly used should have good histocompatibility, easy degradability, good surface activity and certain adhesion, such as polyglycolic acid, polylactic acid, L-polylactic acid, as well as the polymers of above two kinds of materials, also adding into collagen sponge by the use of good biocompatibility and biodegradable properties of chitosan. Apligraf is a dermal substitute produced by type I bovine collagen adding to neonatal foreskin fibroblasts, and then incubating keratinocytes, some people use it to treat epidermolysis bullosa syndrome and can shorten healing time[24].

Natural dermal substitutes have been conducted early research and application, its production methods and processes is relatively simple, therefore used widely. Such dermal substitute materials are mainly derived from animal and human skin, in order to avoid allogeneic or xenograft rejection following graft into the receptor, all cells in the epidermis and the dermis should be removed and killed, to form acellular dermis[9,15,25]. Some studies have described a dermal substitute derived from the human skin, because human-derived dermal substitute tissue composition is closest to the autologous skin, the human seed cells (keratinocytes or epidermal stem cells) have homology[22]. In recent years, it is commonly used in research and clinical treatment of artificial skin and seed cell development[9,18,26]. However, human acellular dermal substitute is produced in different methods according to previous reports, the names are also different, such as Alloderm used hypertonic saline and sodium lauryl sulfate to remove human cadaver skin epidermis and dermis cell components[27]. There are also reports of acellular dermal matrix can be obtained epidermal separation with the enzymes treated by Triton X-100[18]. Although the literature dealing with different methods, the obtained acellular dermis all retain the previous fiber composition and the basic tissue structure, and parts of the basement membrane components still exist at the acellular dermal surface, so as to provide a guarantee for seed cells *in vitro* development[28-30]. In this study, histology and immunohistochemistry of vimentin staining results showed the active cells have been missing in DED, the dermis is rich in collagen fibers and elastic fibers, scanning electron microscope and transmission electron microscope demonstrated dermal collagen three-dimensional structure arranged bundles in the dermis, this structure is conducive to infiltrate the culture medium in the DED matrix and to nourish seed cell development. In addition, the dermis has a certain inter-fiber pore size, which is conducive to fibroblasts and capillary ingrowth in post-transplant tissues, thus facilitating wound healing. Previous research data show that the basement membrane and its components play an important role in the epidermal proliferation and differentiation. The existence of dermal substitute basement membrane components, or components of dermal substitutes can produce basement membrane components, is the key of seed cells developing into a full skin structure[31]. In this experiment, the Periodic Acid-Schiff staining and immunohistochemistry have proved that the DED surface and appendages catheter retained rich mucopolysaccharides and type IV collagen, which were consistent with the basement membrane components previously reported in the human-derived acellular dermal matrix surface[18-19]. The DED basement membrane components, in particular the presence of type IV collagen, can provide a micro-environmental conditions for inducing epidermal development and differentiation[15-16,31], it could serve as a biomimetic dermal scaffold for tissue-engineered skin construction[9,16]. In addition, it provides a good tissue model for the study addressing the *in vitro* tumor invasion.

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人去表皮真皮细胞活性及组织结构特征*☆

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摘要

背景：研究证实，去表皮的真皮可以作为真皮替代物，在其上接种角质形成细胞后形成表皮结构。但有关真皮替代物细胞生物活性、组织结构特点及基底膜成分分析的研究报道较少。

目的：观察人去表皮真皮细胞活性及组织结构特征。

方法：将健康成人皮瓣用 56 ℃ PBS 溶液处理以去除表皮，用液氮连续冻融处理去除真皮中细胞成分，获得去表皮真皮。以组织块培养法观察去表皮真皮细胞活性。以苏木精染色检测去表皮真皮细胞核，以波形蛋白免疫组化检测去表皮真皮成纤维细胞成分。以 PAS 染色及Ⅳ型胶原免疫组化检测基底膜及其成分。以 VG 染色检测去表皮真皮胶原纤维，Weigert 染色检测弹力纤维，VG 与 Weigert 双染色检测胶原纤维及弹力纤维，透射及扫描电镜观察去表皮真皮超微结构。

结果与结论：用组织块培养方法培养的去表皮真皮 2 周无细胞生长。苏木精-伊红染色显示去表皮真皮中无细胞核、波形蛋白免疫组化显示去表皮真皮中无波形蛋白表达。VG 染色显示去表皮真皮胶原纤维染成玫瑰红色，Weigert 染色显示去表皮真皮弹力纤维染成紫黑色，双染色进一步显示胶原纤维与弹力纤维均匀排列。去表皮真皮表面及附属器残留部位 PAS 反应强阳性，Ⅳ型胶原表达明显。透射及扫描电镜下观察到去表皮真皮中胶原、弹力纤维交错排列，间有孔隙，相互交织成网。去表皮真皮无活细胞成分，真皮基质表面及附属器管腔壁仍保留糖原、Ⅳ型胶原等基底膜成分，真皮基质中富含胶原及弹力纤维，是一种类似在体真皮的三维胶原基质。

关键词：去表皮真皮；真皮替代物；细胞活性；组织结构特征；皮肤组织工程

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