Abstract

Objectives: Human epidermal keratinocytes are currently established as a treatment for burns and wounds and have laboratory applications. Keratinocyte culture contamination by unwanted cells and inhibition of cell proliferation are barriers in primary keratinocyte culture. According to the recent literature, these cells are hard to culture. The present study was conducted to evaluate the efficacy of gelatin-coated surfaces in keratinocyte cultures.

Materials and Methods: After enzymatic isolation of keratinocytes from normal epidermis by trypsin, the cells were cultured on gelatin-coated flasks in serum-free medium. Another group of cells were cultured as a control group without gelatin coating.

Results: We showed positive effects of surface coating with gelatin on the primary culture of keratinocytes. Culture of these cells on a gelatin-coated surface showed better proliferation with suitable morphology. By using gelatin, adhesion of these cells to the surface was more efficient and without contamination by small round cells.

Conclusions: Successful primary culture of keratinocytes on a gelatin-coated surface may provide better yield and optimal number of cells for research and clinical applications.

Key words: Cell culture, Dermatology, Laboratory technique, Skin

Introduction

The technique for culture of keratinocytes, the main epidermal cells, with production of epithelial sheets for grafting was reported in 1975. In vitro cultivation of keratinocytes has a wide range of applications in many aspects of clinical medicine and research, such as the treatment of burns, treatment of chronic wounds, and biological studies. Successful isolation of pure keratinocytes from human epidermis is important for expansion of these cells in tissue culture. Therefore, culture systems were established to support keratinocyte growth and proliferation.

Application of different techniques such as density gradient centrifugation, specific cell surface antibodies, and attachment to specific substrates have been proposed in previous studies for achievement of high homogeneous primary cultures of keratinocytes. Many studies of adherent cell cultures have been performed with 2-dimensional cell culture without extracellular matrix (ECM). However, the ECM has an important role in cell and tissue function and proper tissue development. Matrix leads to the formation of basement membrane and epithelial cell polarization.

Some studies have been performed to find a suitable material as an ECM for culture of keratinocytes. Researchers indicated that collagen for murine keratinocyte adhesion and proliferation was better than gelatin and collagen hydrolysate. Proliferation of human keratinocytes is possible on fibronectin-coated dishes, and with this method, suitable cell growth was achieved with initial seeding rate 10% of the amount necessary for cells cultured on collagen-coated dishes and 5% for cells cultured on dishes not coated with collagen.

Gelatin and collagen scaffolds have comparable properties. In this study, gelatin was selected to find
an appropriate biomaterial for culture of keratinocytes on a coated surface. Collagen is one of the most abundant proteins of ECM, and gelatin is a collagen derivative. Gelatin has advantages such as biocompatibility and low cost.11

In response to these benefits and the limited number of studies on this topic, we selected gelatin to coat the substrate for keratinocyte culture. The purpose of this experiment was to evaluate the biological effects of a gelatin-coated surface on human epidermal adhesion, purification, and proliferation.

**Materials and Methods**

**Keratinocyte isolation**

Most materials were purchased (Gibco/BRL, Grand Island, NY, USA) except for specific items noted. According to the ethical guidelines of the 1975 Declaration of Helsinki, all studies involving human subjects were approved by the ethics committee of Shiraz University of Medical Sciences, and a consent form was obtained from patients.

Separation of the epidermis layer from the skin and cultivation of keratinocytes were performed according to published methods with slight variations.12 Skin pieces (n = 10) were provided by the plastic surgery department, with informed consent of patients who had reduction mammoplasty. The skin pieces were transferred to the cell culture laboratory in defined keratinocyte serum-free medium (DKSFM) supplemented with 5 μg/mL gentamicin. The skin was washed in 70% isopropanol and put in Dulbecco phosphate buffered saline (DPBS) containing 20 μg/mL gentamicin for 1 hour. Skin pieces (size, 0.5-1 cm²) were placed in Dispase solution (10 mg/mL) in DKSFM with 5 μg/mL overnight at 4ºC to 6ºC. On the next day, the epidermis was separated from the dermis, and the epidermal layer was digested with 0.05% trypsin/ethylenediaminetetraacetic acid for 5 to 10 minutes at 37ºC. After dissociation of cells, trypsin was inactivated with 10 mg/mL soybean trypsin inhibitor in DPBS. The cell suspension was centrifuged at 338 ×g for 5 minutes. The pellet was rinsed with DPBS and resuspended, and cells were counted with a hemocytometer and trypan blue.

**Preparation of gelatin-coated surface and keratinocyte culture**

Gelatin (1%) (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared in distilled water. The gelatin solution was placed on the surface of flasks and incubated for 30 minutes at 37ºC. Excess solution was removed, and keratinocytes were cultured (density, 4-6 × 10⁴ cells/cm²) in flasks with coated and noncoated surfaces.

**Cell features**

After keratinocytes were seeded, cells were observed every day and evaluated for morphology, surface adhesion, proliferation, and homogeneity using an inverted microscope. We used Papanicolaou stain as a multichromatic staining cytologic technique for evaluation of keratinocytes. For this technique, keratinocytes were transferred to a slide and fixed in acetic acid-alcohol fixative for 15 minutes. The cells were stained with hematoxylin, Orange G, and Eosin Azure.

**Results**

Investigation of keratinocyte morphology showed epithelial morphology (also known as pavement stone) in keratinocytes, which were confirmed by Papanicolaou stain (Figure 1). The cultured cells in gelatin-coated flasks were compared with cells cultured in conventional polystyrene flasks (control...
The adhesion of cells in primary culture was analyzed every day. The cells cultured on gelatin showed a marked increase in adhesion rate compared with the control group. In the gelatin group, cells attached to the surface showed normal morphology of epithelial cells, but contamination with round attached cells was observed in the control group (Figure 2). Evaluation of proliferation after primary seeding showed that 7 of the 10 samples cultured on gelatin developed a monolayer appearance, but only 3 samples in the control group had this appearance (Figure 3).

Discussion

Cell culture is a complex process with cell proliferation under controlled conditions. Animal cell culture has become a frequent laboratory approach for maintaining fresh cells separated from their native tissue environment. There are many clinical indications for application of cells, and cell expansion methods made it possible to investigate the clinical potential of these methods. Therefore, cell therapy in skin problems has been considered. In the past decade, treatment of skin injuries by keratinocyte culture and tissue engineering has focused the attention of researchers.

The first development in skin tissue engineering was the in vitro culture of keratinocytes from separated epidermis. A major improvement in keratinocyte culture was made in the 1970s, when Rheinwald and Green cultivated human primary epidermal cells on murine fibroblasts (3T3 cells). Numerous researchers applied the method of cultured keratinocytes clinically for the treatment of severely burned patients. However, application of 3T3 cells for keratinocyte expansion is associated with the risk of contamination with infections and animal antigens. At present, the focus of research is to discover an optimal protocol for keratinocyte culture.

Despite the importance of human keratinocytes in cell therapy, limited studies have been conducted in the field of keratinocyte culture in Iran. Shokerzadeh and associates cultured rat keratinocytes in serum-free medium, and they applied an explant method for the culture of keratinocytes. Reiisi and coworkers reported the isolation and culture of newborn mouse epidermal stem cells by rapid adherence on a composite matrix made of type I collagen and fibronectin. In another experiment, researchers investigated different methods for separation of epidermal cells from skin, and concluded that the sodium bromide (4N) method...
yielded more live cells and had less toxicity than trypsin application.²¹

Successful cultivation of keratinocytes and achievement of the appropriate number of cells are first steps toward application of these cells as a therapeutic approach for patients who have severe burn injuries and wounds. The purpose of this study was to isolate, culture, and improve the proliferation of human epidermal keratinocytes.

The skin epidermal layer is a stratified squamous epithelium composed of multiple layers of keratinocytes. There are difficulties in keratinocyte culture, including cell senescence, differentiation, apoptosis, and contamination with unwanted cells. Cultivation of keratinocytes under unsuitable conditions causes loss of proliferation potential and the occurrence of early differentiation.²² In the present study, we applied coated tissue culture surfaces with gelatin for primary keratinocyte culture as a simple and rapid method.

The ECM provides a suitable microenvironment for cells and affects their behavior and function.²³ Conventional adherent cell culture is performed on rigid surfaces. The differences between in vitro and in vivo conditions lead to the impossibility of generalizing the results of laboratory studies to the natural conditions in the body. Some synthetic matrices have been evaluated to mimic the ECM.²⁴ Various compounds are present for coating the substrate with ECM proteins to aid cell attachment. To find a suitable biomaterial for coating the culture surface in this study, gelatin was selected because it is obtained by partial hydrolysis of collagen, which is the major protein of ECM in connective tissue. Gelatin is a soluble compound that does not have antigenicity and has lower cost than collagen.¹¹,²⁴ Gelatin is widely used for pharmaceutical and clinical purposes. The biocompatibility of gelatin has been identified from its long-standing clinical purposes. Gelatin is a suitable material for use as a biomaterial that promotes cell adhesion and proliferation.

In this experiment, we observed positive effects of gelatin on primary culture of epidermal keratinocytes. In this method, contamination with round cells was removed. The results demonstrated that the ability of keratinocytes to reach confluence was significantly better when cultured on a gelatin-coated surface. Cell morphology, an important biomarker, was represented by a high nucleus-to-cytoplasm ratio that indicated less differentiated keratinocytes in early passages. However, with continued keratinocyte culture, it was observed that the morphology of keratinocytes was altered and the nucleus-to-cytoplasm ratio decreased.

Keratinocytes are a great source of epithelial cells for many cell biology research studies and medical applications.²⁷ The requirement to achieve rapid wound dressing in patients who have skin injuries necessitated the development of cellular expansion of keratinocytes in vitro. In this study, it was concluded that gelatin, as a biological polymer, can be effective for expansion of keratinocytes for research and clinical purposes.

References


