

Establishment of a Primary Cell Culture of Human Fibroblast in Iran

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Abstract

Background: Human fibroblasts are the part of the dermis that secrete extracellular matrix for the purpose of tissue repair. Culturing fibroblasts, which leads to formation of a monolayer of these cells, is used for treating various conditions including thermal burns and other skin defects such as diabetic and varicose vein leg ulcers. Therefore, we aimed at developing a fibroblast bank to accomplish multiple goals including skin repair in defects such as burns and ulcers and also performing various research projects on these cells in order to further study of the mechanisms involved in wound healing, rejuvenation and medication effects.

Method: We initially developed primary cultures of skin fibroblasts in a DMEM medium. These primary cultures were formed by washing and trypsinizing foreskin specimens followed by separation of epidermis from dermis and cutting the dermis into small pieces. In about 10 days, a monolayer of fibroblasts was formed.

Result: We were able to develop the fibroblast bank successfully and to initiate other projects utilizing this bank.

Conclusions: With these cultured cells, we would be able to perform different research projects including studying the mechanisms of wound healing, rejuvenation, drug affects, inflammatory mediators, growth factors, etc. Moreover, further progress in this field will result in our independence from requesting these cells from external sources. (*Iran J Dermatol 2009;12: 4-8*)

Keywords: allograft, autograft, burn, cell culture, fibroblast

Introduction

Human skin, made up of keratinocytes on a bed of collagen and fibroblast cells, plays a critical role in homeostasis and the defense mechanism of body against environmental microorganisms.¹ Disruption of this protective barrier layer, for any reason, leads to malfunction of the entire vital system of an individual; which in severe cases may even lead to death.^{2,3} Clinical problems caused by the unavailability or insufficiency of tissues required for the repair of the damaged parts have led to the employment of cell culture methods in plastic surgeries. As a consequence, researchers partook in biosynthesis and multiplication of fibroblasts and keratinocytes as dermal biological substitutes.⁴ Nowadays, researchers are interested in employing fibroblasts placed on a scaffold enabling its relocation to the damaged skin sections patients,

including burns, open sores due to trauma, diabetic ulcers, variceal ulcers of legs, etc, as a primary dermal substitute which can perform skin's duties including infection control, prevention of body fluid loss and wound contracture.⁴ Moreover, fibroblast cultures are extensively used in laboratories to study wound contracture mechanisms, effect of beams on fibroblasts,⁵ inhibitory effects of chronic inflammatory mediators,⁶ biocompatibility of products used in wound repairing,⁷ cytotoxicity of products used in dentistry and ingredients used in topical antiseptic products.^{8,9} Therefore, various methods have been designed for fibroblast cell culturing.

Considering the prevalence of burn accidents, and untreatable wounds like leg ulcers due to varicose veins, traumatic wounds, diabetic wounds and their enormous economic costs and consequences for both the individual and the

society, this study was aimed at establishing fibroblast cell culture to produce fibroblasts exploitable for grafting purposes in order to save the patients and to decrease treatment costs. Furthermore, by creating a fibroblast bank, further investigations on wound repair and contracture mechanisms, biocompatibility of various products –i. e. antibiotics and products used in dentistry, etc. - and their cytotoxicity on wounds would be possible.

Materials and Methods

Preparing skin samples

In this study, we used skin samples obtained from the foreskins of infants; the samples were taken in a completely sterile condition and located on a DMEM medium containing antibiotics (penicillin, streptomycin, fungizone at a concentration 10 times more than that found in a cell culture medium). They were then transferred to the culture room. All steps of tissue separation from skin were carried out under a laminase hood using sterile appliances and material. It should be noted that prior to obtaining samples from infants, an informed written consent was acquired from their parents. This study, which included human fibroblast cell culturing and establishment of a fibroblast bank, was performed in Shahid Beheshti Medical University Skin Research Center.

Fibroblast culture

To make a fibroblast bank, initially the skin obtained from infants' foreskin was located on a DMEM medium containing penicillin, streptomycin and fungizone. To isolate cells, the sample was taken out of the DMEM solution and washed using

PBS (oxid). We were then able to separate epidermis from dermis by trypsinization. Thereafter, dermis was divided into pieces smaller than 1 millimeter, cultured in 50 milliliter culture flasks and finally located in a 37°C incubator with 5% carbon dioxide. A complete culture medium including a DMEM medium with 10% fetal bovine serum, L-glutamine, 100 units per milliliter penicillin, 100 microgram per milliliter streptomycin and 1 microgram per milliliter fungizone was added to each flask. Cultured cells were nourished every three days. After colonies had pasted together, fibroblasts were separated from the flask's base by short-term trypsinization and after centrifuging and creating a suspension, these fibroblasts were cultured in a new flask. After investigating the time of proliferation of cells and ensuring that the amount of live cells was equal to at least 10^7 cells per milliliter, they were relocated to an azoth tank for long-term preservation.

Results

The results obtained from various separation methods demonstrated the fact that separating fibroblasts from keratinocytes can be performed more easily by using a higher concentration of trypsin, increasing the incubation period and also using a combination of trypsin and EDTA instead of trypsin alone. At the time of cell culture, we reached the conclusion that it was best to locate skin pieces in a limited culture medium with increased fetal bovine serum (FBS) concentration- even up to 40%- to increase cellular proliferation. No specific culture medium was needed, as fibroblast cells proliferated at a high speed and we succeeded at

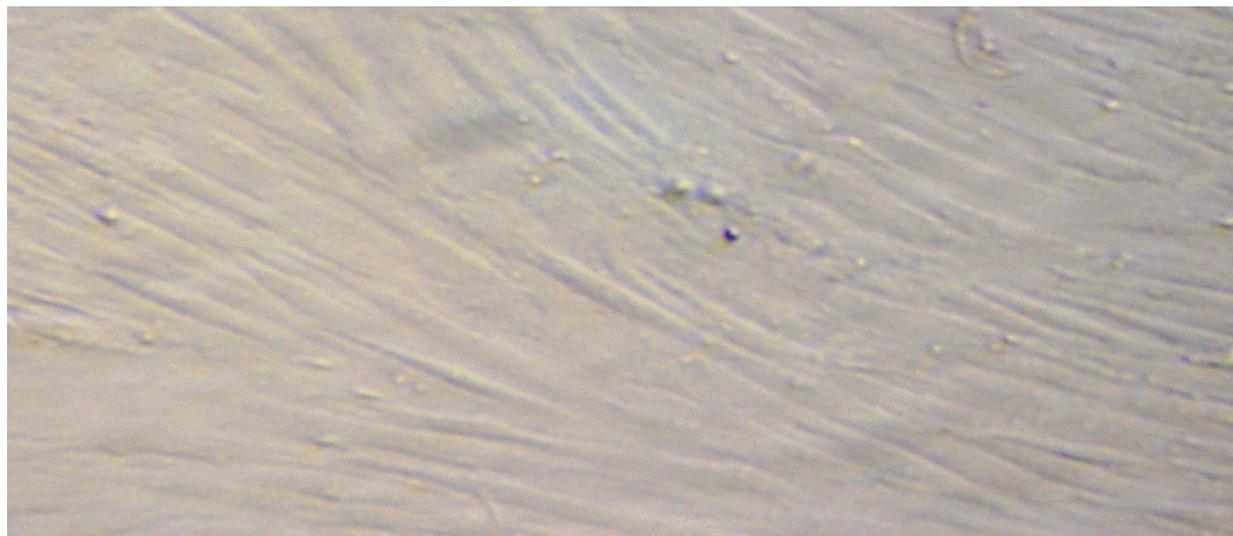


Figure 1: Fibroblast layer observed via inverted microscope.

culturing them in most mediums; however, the only required substance was FBS which helped greatly in their growth. Different methods used for fibroblast biocompatibility showed an increased proliferation speed of fibroblast in a DMEM medium.

Locating tissue specimens in a limited culture medium led to pasting of the tissue to the base of the flask; the cells were separated after 24 hours and attached to the base of the flask. These cells then started to reproduce and formed colonies which attached to each other which led to the filling of the flask after 10 days and as a result, a fibroblast cell layer could be observed via inverted microscope. (Figure-1)

We tested two methods to separate the fibroblast layer: 1- using 0.5% trypsin. 2- employing a glucose and EDTA solution. In the first method, we covered the cellular layer with 0.5% trypsin enzyme for 5 minutes after the flask was filled with fibroblast cells. Cellular separation started at the periphery and ended at the center and as a result, the whole fibroblast layer was separated. The second method was the same except for employing a glucose and EDTA solution.

Discussion

Skin acts as the body's protective barrier against loss of fluids and invasion of chemical and microbial agents.¹ Nowadays, considering the need to manufacture skin as a graft for ulcers resulting from burns, traumas, diabetes and variceal leg ulcers, the necessity to perform investigations for tissue engineering of the lost skin becomes clear.⁴ To construct the skin, with its bi-layer arrangement including dermis and epidermis which form a complete structure, keratinocytes should be located on a structure made up of a collagen scaffold and fibroblasts similar to dermis so that with the aid of this collagen scaffold, both types of cells become able to reproduce.

Therefore, as the first step in producing a dermal tissue, culturing fibroblast cells becomes of great importance. In this study, the foreskins obtained from infants were used and the samples were taken to the laboratory in sterile conditions and in a medium composed of DMEM and antibiotics. Connective tissue fibroblasts were cultured in a DMEM medium containing 10% FBS and after 10 days, when the flask was filled with fibroblasts, they were trypsinized for detachment purposes and then transmitted to a new flask. These cells underwent several passages and were reserved to be used in the establishment of the fibroblast bank for second and third degree burns, as a base in the

construction of a three dimensional skin⁴ and finally as the substance for performing various studies.⁵⁻⁹

Different studies have proposed different methods for culturing the dermis including culturing fibroblast cells on a reticular collagen scaffold with various hybrids recommended by many authorities¹⁰⁻¹⁴ Another method consists of a collagen scaffold bearing pores on which fibroblast cells grow very well. This method was invented by Lie Ma et al. who used lysine amino acid to prevent collagen degeneration. The collagen scaffold which did not lead to any biocompatibility problems was composed of glycine, glutamic acid and lysine amino acids adjuvant to a water soluble carbodiimide-1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide- and N-hydroxy succinimide (NHS). These fibroblasts and the accompanying collagen scaffold can be grafted successfully on humans.¹⁵ In the method employed by Glushchenko et al. in 1993 and 1994, cultured fibroblasts were used for third degree burns. This method was very successful for grafting limited areas.^{16,17} In 1994, these authors used the healthy skin of burnt patients as an autograft. They prepared a mesh out of the healthy skin and created pores which were 6 times bigger than the previous ones, and then they placed this mesh on the debrided burn wound and covered the pores with fibroblasts. After a week, the fibroblasts had proliferated and the wound edges were healed. They conducted this method successfully in 184 patients.¹⁸ In 1999, Tumanov and colleagues reported a 10-year experience of grafting cultured fibroblasts on 517 patients from different hospitals of Russia. They demonstrated the ability of these fibroblasts in DNA, collagen, fibronectin and glycosaminoglycan synthesis.¹⁹

In a method reported by Lee et al., a scaffold composed of gelatin, beta-glucan and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride was prepared which had pores measuring 90-150 micrometers. This scaffold was used to prepare a three dimensional skin and fibroblasts and keratinocytes were simultaneously cultured on this scaffold. In vivo investigations demonstrated that the grafted skin was completely rehabilitated.²⁰

In 1999, Hafemann and colleagues reported using a mixture of collagen and elastin as a scaffold to construct dermis and thereafter culturing the fibroblasts of the burnt patients on this scaffold. Almost 3 weeks post-grafting, the membranes were filled with vessels and various cells had formed colonies. After 6 weeks, the old collagen fibers were replaced by the new collagen fibers;

however, elastin fibers remained for the next 20 weeks. In the next step, cultured keratinocytes were located on the membranes and 8 to 11 days after the regrafting, 47% of the grafted area had epithelial coverage. On day 6 post grafting, a multi-layered epithelium containing lymphocytes and langerhans cells was observed. Basal layer was completed after 10 days and the result was a three dimensional skin.²¹

Llames et al. used plasma clot obtained from patients as a matrix to culture human fibroblasts. Then, they placed human keratinocytes on this scaffold. After 24 to 26 days, these keratinocytes expanded 1000 times and created a 3 dimensional skin which was grafted to 2 burnt patients. The results were excellent after 2 years of follow-up.²² In all the mentioned cases, fibroblast culture on a polymer or various hybrids was used in burnt patients to prevent fluid and electrolyte loss and in primary stages to prevent the risk of death in these patients.

In this study, we aimed at establishing a standard bank by culturing fibroblasts in order to be used to accelerate wound healing in burns and other deep wounds. Moreover, we look forward to use this product in various laboratory studies in the future.

Maintaining fibroblasts and their transferring to absorbable or non-absorbable polymers as a substitute for dermis in a laboratory environment can be of great assistance in treating second and third degree burns for preventing from infections and fluid loss and therefore saving the patients' life. Moreover, a standard bank could be established using these fibroblasts which can be used immediately in cases of allogenic grafting for burnt patients. Additionally, this bank makes it possible to perform laboratory studies on wounds and the effective factors in their healing.

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