

Development of an Allogeneic Cultured Dermal Substitute Using a Standard Human Fibroblast Bank

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Abstract

Background: Fibroblasts are mesenchymal cells that can be readily cultured in the laboratory and play a significant role in epithelial-mesenchymal interactions, secreting various growth factors and cytokines that have a direct effect on epidermal proliferation, differentiation and formation of extracellular matrix. They have been incorporated into various tissue-engineered and used for a variety of clinical applications, including the treatment of burns, chronic venous ulcers and several other clinical applications in dermatology and plastic surgery.

Method: Isolated fibroblasts by the enzymatic process from foreskin were cultivated successively in a culture medium to establish cell banking. Foreskin and the last subcultured cells were checked for HBV, HCV, HIV, HSV I, HSV II, HTLV I, HTLV II, EBV, CMV, Treponema Pallidum, Mycoplasma sp. and Clamydia. The 1st, 5th and 10th subcultured cells were processed for immunocytochemistry studies using a panel of monoclonal antibodies including antibodies to MHC class I & II antigens for ensuring the elimination of superficial cell antigens during cultivation. Subcultured cells were karyotyped to find any chromosomal abnormalities. The best passages were chosen for culturing on silicone sheets provided by the Iran Polymer and Petrochemical Institute.

Results: Evaluation for bacteria and viruses by molecular methods was negative. Karyotyping of cultured fibroblasts after the 10th passage showed some abnormalities. HLA expression was imperceptible in the cells obtained from the 10th sub-culture. The best passages were from 5th to 10th for banking and culturing on silicone sheets.

Conclusion: Expression of HLA on fibroblast surfaces was diminished during subculturing. To prevent chromosomal abnormalities in fibroblast passaging, we should select the best colony that is expected to be chromosomally stable with the least antigenicity. In our study, the 5th to 10th sub-cultures were the best cells for the purpose of grafting and acceleration of the wound healing. (*Iran J Dermatol* 2009;12: 111-116)

Keywords: fibroblast, cell bank, karyotyping, immunophenotyping

Introduction

Aside from being a sensory organ, the skin is the first line of defense against infections and dehydration and is to a large extent responsible for body temperature regulation. By altering the functions of this organ, wounds and ulcers can cause crucial problems for an individual. Wound healing, a series of complex and dynamic biological events, consists of processes such as inflammation, mitosis, synthesis, angiogenesis, chemotaxis, phagocytosis,

neocollagenesis, collagen degradation and remodeling of the extracellular matrix ¹. In fact this process is defined by three overlapping phases: hemostasis and inflammation, proliferation (including fibroblastic phase) and maturation or remodeling ². Fibroblastic phase which starts after the termination of inflammatory phase and lasts for a couple of weeks comprises a very critical phase of wound healing. Collagen fibers and collagen precursors, initially type III and then type I, are deposited in the wound milieu at this phase.

Fibroblasts, the key cells of the wound healing process, are accountable for the production of all the dermal connective tissue elements or their precursors. The manufacturing of collagen or its precursors by these cells has been demonstrated *in vitro*. Fibroblasts generate an early response to skin injuries by releasing pro-inflammatory and growth factors mediators³.

Any failure in the process of wound healing, as seen in diabetic foot ulcers, venous leg ulcers, pressure ulcers, surgical ulcers, burn ulcers imposes a significant burden on the patient and great costs on the health care system⁴. As a matter of fact, the prevalence of diabetic foot, venous leg ulcers and pressure ulcers ranges from 4.4% to 10.5% in diabetics, 0.18% to 1.35% in the general population and 5.0 to 8.8% in institutionalized patients, respectively. Each year near 82000 lower extremity amputations are performed due to diabetic foot ulcers⁵. Autografts and dermal equivalents have been utilized to treat these chronic non-healing wounds⁶.

Considering the significance and prevalence of chronic ulcers and the role of fibroblasts in wound healing and the need to perform various types of studies - cytogenetic, biotechnological, virological, etc.,- on these cells, we planned to establish a standard cellular bank to preserve cultivated fibroblasts.

Materials and Methods

Sample Obtaining

We performed this project in the Skin Research Center of Shahid Beheshti Medical University. Samples were obtained from the foreskin of the newborn babies under fully sterile conditions in the operation room. Therefore, prior to sample obtaining, parents were fully informed and a written consent was acquired from them. The samples were then transferred to the cultivation room in a DMEM medium (Sigma) supplemented with penicillin, streptomycin and fungizone (10 times the amount found in the cell culture medium). Tissue separation from the skin was performed under laminar hood employing sterile material, utensils and reagents.

Fibroblast cultivation

To isolate the cells, the skin samples initially placed in antibiotic containing DMEM were washed using PBS solution (oxidod) and then trypsinized. After separating the epidermis from dermis, we divided the dermis into sections smaller than 1mm and then cultivated these pieces in 50 mL cultivation flasks.

These flasks were situated in 37°C incubator with 5% dioxide carbon. The complete cultivation medium added to the flask consisted of cultivation medium DMEM with 10% fetal calf serum, 100 units per mL penicillin, 100 micro grams per mL streptomycin and fungizone. Cultivated cells were feed once every 3 days. When the colonies were connected, a short-term trypsinization was performed to separate fibroblasts from other sorts of cells. Once the suspension was centrifuged and prepared, collected fibroblasts were cultivated in a new flask. During the next 3 months, cultivation was continued until the 12th sub-culture and all sub-cultures were preserved in cryovials with precise descriptive labels at -80°C. For the purpose of long-term preservation, cells were removed from the freezer, checked twice, subjected to live cell count (minimum number of 10⁷ cells per mL) and then were carefully transferred to a nitrogen tank.

Fibroblast karyotyping

To detect any possible chromosomal abnormalities, we karyotyped the fibroblasts obtained from the 1st to 12th subcultures. To do so, we treated the cells with colchicine and then fixed and assessed the metaphase spreads.

Immunophenotyping to assess MHC molecule condition

Considering the fact that one of the major goals in creating this fibroblast bank was to use it for developing an allogeneic cultured dermal substitute (AlloSkin), we had to test the cells obtained from the 1st, 5th and 10th sub-cultures for superficial antigens. Therefore, we employed immunocytochemistry tests with monoclonal anti HLA class I Fitc, monoclonal anti HLA DQ-Fitc and monoclonal anti HLA Dr-Fitc (sigma, cat No: F 5662, F1777, F 1902).

Non-specific mouse immunoglobulin M was used as negative control (negative control, sigma cat no: 5284), and human cultivated macrophages were utilized as positive control. Initially, we dispensed the cells on a 4-hole slide chamber (No: C6932 Nunc Lab-tek, cat). When a monolayer of cells was formed employing a combination of acetone and cold ethanol, the cells were fixed during 8 minutes in a two-to-three ratio. To wash the cells, 10 micro liters of antibodies were added to the diluted buffer comprised of bovine serum albumin, sodium azide and PBS and the cells were then immediately checked by an immunofluorescence microscope (Nikon).

Assessing microbial contaminations

To assess the possible contaminations found in the cell cultivation medium, we appraised the

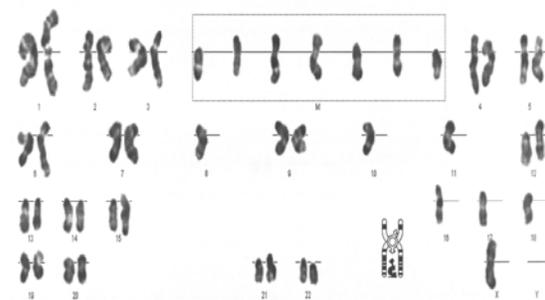


Figure 1. Chromosomal abnormalities in the 12th sub-cultured fibroblasts.

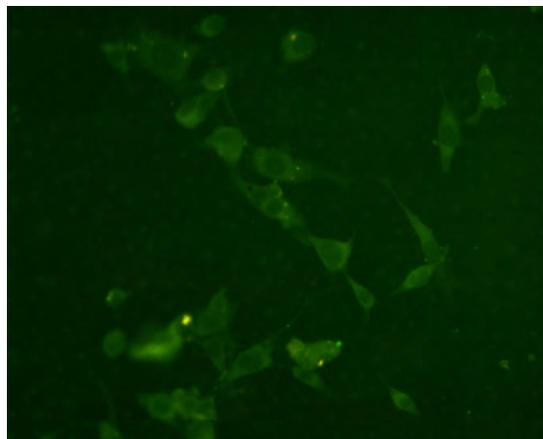


Figure 2. Positive expression of fibroblast HLA class I antigen on the 1st passage.

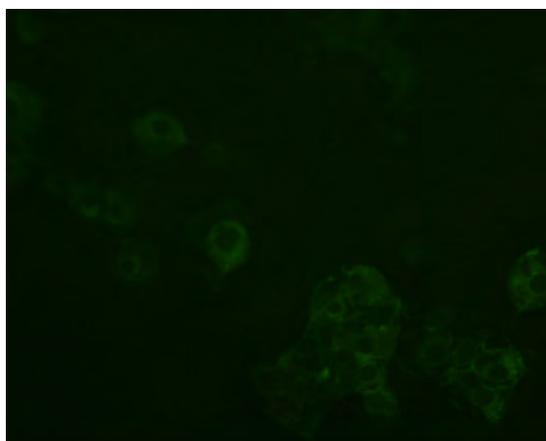


Figure 3. Weak positive expression of fibroblast HLA class I antigen on the 5th

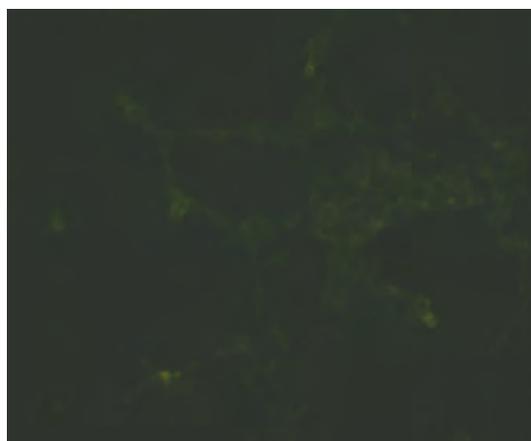


Figure 4. Very weak positive expression of fibroblast HLA class I antigen on the 10th passage.

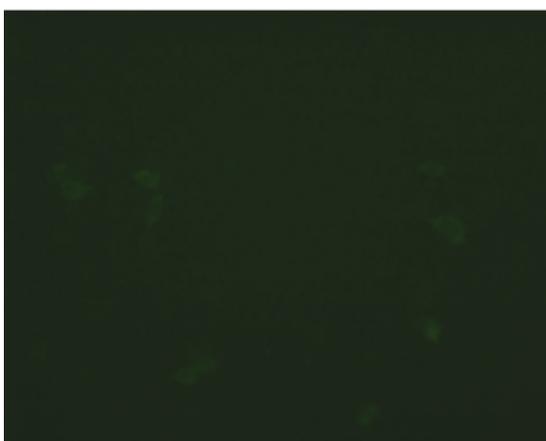


Figure 5. Negative control (Fibroblasts)

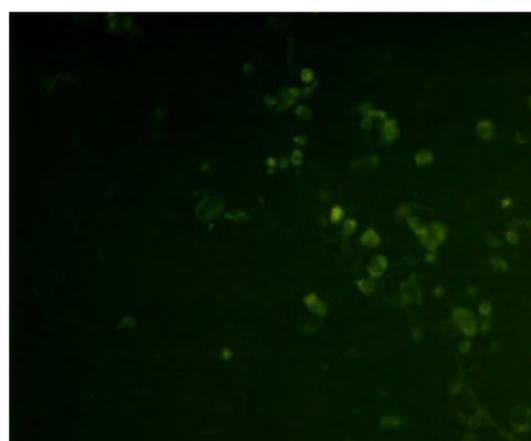


Figure 6. Positive control (Macrophages)

fibroblasts from the 1st and the last sub-cultures by performing DNA analysis to detect CMV, EBV, HSV I, HSV II, HBV, Treponema pallidum, Mycoplasma

sp. and Chlamydia and RNA analysis to identify HCV, HTLV I, HTLV II and HIV microorganisms.

In order to perform PCR on the samples for HBV and HCV, we used AMPLICOR HBV v2.0, (Roche

Diagnostics, USA) MONITOR® Test, v2.0 and APLICOR® HCV Test, V2.0, (Roche Diagnostics, USA) kits. As for the rest of the microorganisms, the DNA and RNA were extracted using DNeasy Tissue kit (QIAGEN, cat.No.:69504, Crawley, UK) and then subjected to PCR using special primers.

Preparation of the cultured dermal substitute (Alloskin)

The silicone sheet was prepared at the Iran Polymer and Petrochemical Institute. Fibroblasts obtained from successive cultivation of the cryopreserved cells (5th to 10th passages) were seeded by adding 10 mL of cellular suspension onto the silicone sheet in a Petri dish. The number of fibroblasts on the silicone sheet was adjusted to 1×10^5 cells/cm². The seeded sheet was kept in an incubator in a humidified atmosphere of 5% CO₂ at 37°C overnight. Then, it was ready to be used for grafting.

Results

Karyotyping

The fibroblasts acquired from the 1st to the 12th sub-cultures were karyotyped. Following harvest, metaphase spreads were analyzed and photographed. Various metaphase spreads from the 11th and 12th sub-cultures demonstrated multiple centromeric fissures. Moreover, subsequent to the complex rearrangements and deletions, it was not possible to identify several chromosomes. The photo of the metaphase spread and karyotype of the 12th passage is demonstrated in figure 1.

Immunophenotyping

By means of immunophenotyping of the sub-cultured cells, we were able to demonstrate that fibroblast surface HLA expression diminished during the sub-culturing process and consequently HLA expression was in fact imperceptible in the cells obtained from the 10th sub-culture (Figures 2-6). Compared to macrophages (positive controls), HLA class I antigen expression was determined to be 80%, 40% and 10% on the 1st, 5th and 10th sub-cultures, respectively. HLA DQ and HLA DR antigens expression were also diminished during the sub-culturing process.

Microbial contamination

Assessing fibroblasts obtained from the 1st and the last sub-cultures by means of PCR, we did not detect HBV, HCV, HIV, HSV II, HSV I, HTLV I, HTLV II, EBV, CMV, Treponema pallidum, Mycoplasma sp and Chlamydia.

Preparation of the cultured dermal substitute (Alloskin)

The microscopic photo of the cultured dermal substitute (Alloskin) is shown in Figure 7. The cultured dermal substitute (Alloskin) was composed of a silicone sheet and the best sub-cultured fibroblasts (5th to 10th subcultured fibroblasts) that were able to attach to it. The cell number was counted to be $0.85-0.97 \times 10^5$ cells/cm² on day 1.

Discussion

Confronting the limitations associated with the use of autografts, attempts have been made to produce tissue-engineered skin ⁷. Allogeneic skin substitutes have been manufactured using cultivated fibroblasts. By releasing extracellular matrix (ECM), different growth factors and cytokines fibroblasts provide an excellent condition for wound healing ⁸. Prior investigations have proven the efficacy of cultured dermal substitutes (CDS) primarily composed of fibroblasts in treating partial and/or full-thickness skin defects ⁹⁻¹¹. Allogenic fibroblasts can be used for the purpose of biological dressing or even to precondition the wound bed before applying permanent grafts ¹². Numerous benefits are achieved when employing cultured dermal substitutes; the allogeneic cultured dermal substitute is immediately available for use (the delay caused by the time needed for autologous cell isolation and multiplication is avoided), no biopsies need to be performed, donor site issues are avoided and last but not least they can be cryopreserved and banked ¹³⁻¹⁵. Immunological impact and possible rejection have been the major concerns when employing allogeneic cultured fibroblasts; however, large trials have not demonstrated any such adverse incidents. Furthermore, by synthesizing prostaglandins, leukotrienes and several cytokines, fibroblasts contribute significantly to the normal function of the healthy skin and the epithelial-mesenchymal interactions ^{12, 16}.

Genetic alterations due to anarchy in chromosomal structure are occasionally observed in cells. Any such irregularities and aberrations can be identified by the means of chromosomal counting and analysis of the karyotype. Sub-culturing of the cells predisposes them to an increased possibility for the incidence of chromosomal abnormalities. This phenomenon can even be observed in the initial sub-cultures which are more stable genetic-wise. In fact, as the quantity of sub-cultures increases, more heterogeneity can be detected in cells and therefore, cells demonstrate different amounts of growth.



Figure 7. (left) microscopic cultured fibroblasts on the silicone sheet, (right) Alloskin

In this study, we did not detect any chromosomal aberrations in the fibroblasts of the 1st to the 10th sub-cultures; however, continuing the sub-culturing process from the 10th passage resulted in cells harboring, multiple centromeric fissions and chromosomal abnormalities observed in the metaphase spreads. Multiple centromeric fissions and the consequent numerous abnormalities in the 12th sub-culture caused rearrangements and deletions of chromosomes.

For years, the common approach has been to use the cultivated fibroblasts obtained from the 5th to the 10th sub-cultures for the purpose of grafting and acceleration of wound healing. Employing this method, the fibroblast surface antigenicity is gradually eliminated as the number of sub-cultures increases. In our study HLA class I & II antigen expression was determined to be 80%, 40% and 10% on the 1st, 5th and 10th sub-cultures, respectively. Consequently, this low-cost simple technique yields fibroblasts that are suitable for allogenic cell grafting purposes and can be beneficial for a wide range of clinical practices.

To achieve a cellular bank with lower surface antigenicity while maintaining the normal chromosomal content, the 5th to 10th passages should be taken into account.

In our study, the 5th to 10th allogenic fibroblast sub-cultures were the best ones. They were chromosomally stable with the least antigenicity and were suitable for cell banking and culturing on silicone sheets for grafting and accelerating wound healing.

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