Technologies for *in vitro* cultivation of skin fibroblasts for patients with chronic ulcers treatment

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Abstract

Background: As many factors lead to chronic lower leg ulceration, an interdisciplinary approach to the systematic assessment of the patient is required, in order to ascertain the pathogenesis, definitive diagnosis, and optimal treatment. A number of researchers propose to use in ulcers treatment non senescent fibroblasts. By donating additional cells to the wound environment releasing extra growth factors and reversing the antiproliferative activity of chronic wound exudates can be achieved.

Material and methods: The main stages of fibroblasts' cultivation are: obtaining of skin biopsy, donors' serologic control, preparing culture flasks, separation of epidermis and dermis, proper cultivation, cells bacteriological control and application.

Results: The final cell density in primary culture was $4,5*10^4$ cells/cm^2. It's demonstrated that tissue-engineered human dermal fibroblasts can replace the dermis and provide essential stimulatory growth factors for wound healing.

Conclusions: The overall safety and lack of rejection reactions combined with the efficacy encourage the use of HFDD in addition to good wound care practices.

Key words: in vitro cultivation, fibroblasts, skin, trophic ulcers.

Abbreviations: CLU – chronic leg ulcers, HFDD – human fibroblast-derived dermal,
NMCF – Nutritive Medium for Cultivation of Fibroblasts

Introduction

Leg ulcers are debilitating and greatly reduce patients' quality of life. CLU are defined as a defect in the skin below the level of knee show no tendency to heal after 3 months of appropriate treatment or are still not fully healed at 12 months. The common causes are venous disease, arterial disease, and neuropathy. Less common causes are metabolic disorders, hematological disorders, and infective diseases [1, 2]. CLU affect 0.6–3% of those aged over 60 years, increasing to over 5% of those aged over 80 years. CLU are a common cause of morbidity, and its prevalence in the community ranges from 1.9% to 13.1% [3]. They remain a significant issue not only in specialist facilities but also in daily practice for family physicians and specialists across a wide variety of disciplines [4].

An ideal management plan for patients with CLU should involve an early strategic and coordinated approach to delivering the correct treatment option for each individual patient, based on accurate assessment of the underlying etiology and pathophysiology [5]. This is not always possible, and for these hard-to-heal ulcers there are new treatment options, such as growth factors, biological skin substitutes and spray formulation of allogeneic or autologic keratinocytes and fibroblasts.

The objective of this study was: (1) To develop a methodology to create fibroblast cell lines of patients with chronic wounds. (2) To describe and compare the cellular characteristics of fibroblasts taken from ulcers (wound-fb) with the fibroblasts of normal tissue and (3) To provide an overview of current data on main effects and advantages of HFDD for chronic ulcers treatment.

Material and methods

The procedure begins once the patients have read and signed a Consent Term.

1. Preparing Tissue. Initial processing of the skin specimens. The biopsy has to be performed under sterile conditions. The site of biopsy (underarm or inner side of the thigh) has to be disinfected with ethanol for 10 to 15 minutes. For disinfection the site of biopsy is wiped from the center in circular movements to the outer site using a sterile pad soaked with 70% ethanol. This procedure is repeated 10 times using a new ethanolic pad each time. With the help of dermatom we take a piece of skin 0,4-0,6 mm thick with surface area of 1,0-1,5 cm^2. The obtained sample is maintained in 50 ml conic tubes with 25,0 ml of **NMCF.** It should arrive in the lab in less than 48 hours after the biopsy has been taken.

2. The donors serologic control. Obligatory serological tests: HIV, HVC, HVB, lues, HTLV. Supplementary serological tests: ABO group, Rh-rezus, cytomegalovirus, toxoplasma, Ebstein-Barr, West Nile NAT Virus.

3. Preparing Gelatin-Coated Tissue Culture Flasks. For optimal cell attachment 0.1% Gelatin Solution is recommended for the coating of culture dishes or flasks on which HFDD are to be grown. Using aseptic technique and working in a laminar flow hood or biosafety cabinet, solution is added to the flasks (1,0 ml per 10 cm^2 of culture surface area).

4. Separation of Epidermis and Dermis. Mechanical, or Stretching Method. This is rapid, simple, and has few objections, except that a sizable piece of tissue is required. The skin is manually stretched to its limit over a slightly convex surface, and is anchored in place by means of thumbtacks. A razor blade or scalpel is used to scrape the epidermis free of the corium at one end, then the freed epidermis is grasped with a forceps and the whole epidermis is gently detached in a continuous sheet.

5. NMCF preparation. Main Characteristics. HiFibroXLTM Fibroblast Expansion Medium supplemented with an antibiotic-antimycotic mixture is used for fibroblasts cultivation. HiFibroXLTM Fibroblast Expansion Medium contains basal medium (Part A) and fibroblast growth supplement (Part B). Part A consists of inorganic, organic salts, amino acids, vitamins and sodium bicarbonate and is devoid of proteins, hormones, antibiotics and antimycotics. Part B consists of growth factors and nutrients necessary for growth of fibroblasts. Antibiotic-antimycotic mixture consists of Penicillin (100 U/ml), Streptomycin (0,01g/ml), Amphotericin B (0,25µg/ml).

6. Skin-derived Fibroblast Culture Procedure. The skin biopsy is cut into 3-4 smaller (1,0-1,5 mm^2) pieces with a sterile knife and then put on bottom of flask. 0,5 ml MCF are added very carefully. Flask is transferred to 37 degrees Celsius, 5% CO2 for a minimum of 3 days. On the third day is added carefully some extra medium (1,0 ml if cells start growing and 0,5 ml medium if there are no cells yet to prevent the skin pieces from starting to float). When T25 flask is completely filled with fibroblasts, fibroblasts should be transferred to T75 flask (0,5 ml trypsin + 9.5 ml medium).When the T75 flask is completely filled, a half of the cells can be frozen and the other half of the cells is put in a new T75 flask (this step is repeated a few times, until at least 4 vials from different passages are obtained).

7. **Sampling cells for bacteriological control.**

8. Application of cultured cells to the wound. There are different methods of cells transfer and application to the ulcers site: injection, direct application, grafting, spray systems; but, unfortunately, none of them satisfies completely clinicians' requirements.

Results

The cells' density in the primary culture on the 24^{th} day was $4,5*10^4$ cells/cm^2 (Figure 1). If we compare the cellular characteristics of fibroblasts taken from venous ulcers (wound-fb) with the fibroblasts of normal tissue (normal-fb), two major differences can be noted: (1) normal-fb replicate more rapidly than wound-fb; and (2) the morphologic features of wound-fb are different. Normal-fb are compact and tapered, with well-defined nuclear morphologic features. Wound-fb are larger and polygonal in shape, with less uniform nuclear morphologic features [6]. In conclusion, it is demonstrated that wound-fb proliferate at a slower rate and are morphologically distinct from normal-fb. These characteristics are typical of aged or senescent cells.

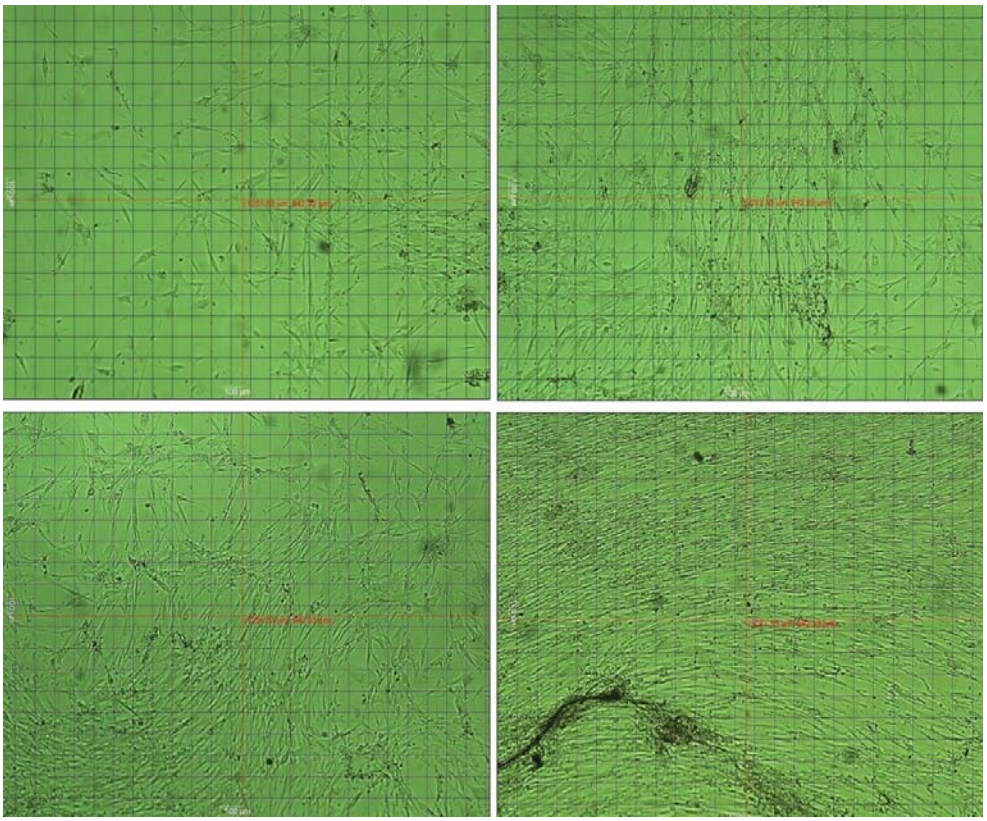

Fig. 1. Primary culture: a – 10^{th} day ($1,0*10^4$ cell/cm^2), b – 14^{th} day ($2,5*10^4$ cell/cm^2), c – 19^{th} day ($3,0*10^4$ cell/cm^2), d – 24^{th} day ($4,5*10^4$ cell/cm^2).

Tissue-engineered human dermal fibroblasts are designed to replace the dermis and to provide essential stimulatory growth factors for wound healing. Live, nonsenescent fibroblasts are capable to colonize the wound bed and persist in situ for several weeks. They secrete a number of cytokines and growth factors, including platelet-derived growth factor, insulin-like growth factors I and II, heparin-binding epidermal growth factor, vascular endothelial growth factor, transforming growth factors a and b, and keratinocyte growth factor. Growth factors are known to stimulate fibroblasts, granulation tissue, matrix deposition, angiogenesis, and skin cell maturation. The fibroblasts also produce matrix proteins like collagen types I and III, fibronectin, and tenascin, as well as glycosaminoglycans, which bind growth factors and enhance their activity [7, 8].

Conclusions

The therapy for chronic ulcers has not changed significantly in decades and the need for new therapies is justified. HFDD are effective, well tolerated, and can be used in the treatment of hard-to-heal chronic ulcers of various origins (e.g. venous, pressure, diabetic foot ulcers).Retrospective analysis in different scientific centers has shown that healed HFDD patients achieve wound closure significantly faster than CT patients. [9]. So, it was demonstrated that venous leg ulcers can be healed with a spray formulation of allogeneic neonatal keratinocytes and fibroblasts at an optimum dose of $0,5*10^6$ cells per ml every 14 days [10]. The overall safety and lack of rejection reactions combined with the efficacy encourage the use of HFDD in addition to good wound care practices [11, 12].

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