



The Impressive Healing Power of Autologous Fibroblasts Isolated from Early Cultures of Skin Biopsies for the Treatment of Diabetic Foot Ulcers: Preliminary Results Regarding 2 Cases

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Authors' contributions

This work was carried out in collaboration between all authors. Author GT performed cultures, wrote the manuscript and researched data. Author IK performed cultures. Authors ID, PP, PT performed the skin biopsies. Authors EDV and AD assessed patients' follow-up. Author PS did the culture microbiology control. Author AI contributed to the discussion. Author CVL assessed patients' follow-up and reviewed/edited the manuscript. All authors read and approved the final manuscript.

Short Communication

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ABSTRACT

Aims: The purpose of this study was to investigate whether we can quickly, effectively, and with relatively low cost, heal long-standing (>8 months) diabetic foot ulcers using autologous skin fibroblasts.

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Voyatzoglou' Diabetic Foot Clinic, 'A. Fleming' General Hospital, Department of Biology, National and Kapodistrian University of Athens, Athens, Greece, between June 2011 and May 2012.

Study Design and Methodology: Early, autologous skin fibroblasts arisen in large numbers from small split-thickness skin biopsies, cultured in high concentration of fetal bovine serum, and dispersed in patients' own serum, were injected subcutaneously into the surrounding healthy tissue of uninfected diabetic foot ulcers of two type 2 diabetic patients without peripheral angiopathy.

Results: There was complete healing in 11 and 27 weeks in patients 1 & 2, respectively. The early cultured fibroblasts showed impressive healing power for diabetic foot ulcers. On the contrary, the power of the prolonged cultured fibroblast diminished steadily, while the fibroblasts undergone the freezing-thawing procedure were not effective.

Conclusion: The healing was complete, quick, safe, permanent, without scars or hyperkeratosis, and relatively inexpensive.

Keywords: Fibroblasts; ulcers; ulcer healing; diabetes mellitus; diabetic foot.

1. INTRODUCTION

Among the most serious complications of diabetes, the prevalence of which soars worldwide, is the diabetic foot ulcer, which can lead to amputation [1].

Fibroblasts play a predominant role in almost every cellular and molecular aspect of ulcers' healing process through the secretion of a) growth factors for the motivation-proliferation of other cells and b) other chemical substances like collagen for the extracellular matrix [2,3].

2. MATERIALS AND METHODS

2.1 Patients

Two women, age 64 and 66 years, with well-controlled (HbA1c 6,1 and 5,5%) type 2 diabetes (duration 29 and 15 years) in oral medication, with peripheral neuropathy (abnormal monofilament, biothesiometer and Neuropad® tests) and lack of peripheral angiopathy (normal Triplex arterial studies) were selected. They presented with neuropathic, uninfected ulcers of 1st toe (plantar surface) and heel, respectively, both caused by inappropriate footwear. Ulcers were long-standing (9,5 and 8,5 months), despite conservative treatment with frequent debridement, daily wound cleaning with normal saline and use of sterile simple gauze dressings. Special dressings (collagen dressings in Case 1 and alginate with silver in Case 2) had been used for the last 8 weeks, together with ulcer debridement, with unsatisfactory results. Patient 2 was formerly treated for Grade 2 infection (PEDIS classification [1]). Patient 1 had Grade 1 depth ulcer, diameter 1 cm, while patient 2 had Grade 2 ulcer, diameter 4 cm. There were neither systemic diseases affecting healing, nor corticosteroid /immunosuppressant use. Written informed consent was obtained from both patients.

2.2 Fibroblasts and Cultures

A split-thickness of 1 mm rhombus-shaped skin graft with diagonals 0,5cm and 1cm approximately was obtained from each patient. Fibroblasts were isolated using the method

described by Freshney [4] with one variation: in the end, the fibroblasts isolated from skin biopsies have been dispersed in a flask of 175 cm² with DMEM culture medium (Dulbecco's Modified Eagle Medium, Gibco), having high concentration (50%) of FBS (fetal bovine serum, Gibco) supplemented with 2mM glutamine, 1% non-essential aminoacids, 100IU/ml penicillin and 100mg/ml streptomycin. There, the cells have been left to converge and then the authors continued to subculture them for three passages in total (or approximately 10 doublings) until they managed to gather 30-40x10⁶ cells [5]. This procedure lasted approximately 25-30 days. These cells (early cultured cells) were considered superior to the cells from the subsequent seedings of the skin's pieces (prolonged cultured cells) [6] and were injected subcutaneously into the healthy tissue surrounding the wounds, dispersed in the patients' own serum. The wounds were then covered with sterile gauze soaked in dissolved penicillin and streptomycin powder.

As for the freezing-thawing procedures of the cells coming from the first and the subsequent seedings of the skin pieces, the methods described by Freshney [4] were also used.

2.3 Wound Care and Assessment

Ulcers were cleaned every second day with normal saline and covered with sterile gauze, which was soaked in pelicillin and streptomycin solution for the first 10 days following each cell infusion. Off-loading and tight glycemc control was asked from both patients.

Ulcers were assessed twice weekly for the first week after each infusion, then once weekly. Wound-healing progress was evaluated and documented with photography. Absence of infection was based on clinical evaluation.

3. RESULTS AND DISCUSSION

3.1 Patients

3.1.1 Patient 1

Two fibroblast infusions of 30-40x10⁶ cells each were performed, with an interval of 7 weeks. The first injection (early-cultured fibroblasts) was followed by an impressive healing procedure, decreasing ulcer both in size (the ulcer's diameter became 7 mm (Fig. 1a & 1b)) and in depth ten days after, but the administered cells showed signs of exhaustion after 50 days. Thus, a second injection (fibroblasts isolated from a second seeding of the skin pieces) was performed on day 51, followed by a minor improvement but steady healing procedure ten days after (Fig. 1c & 1d). Finally, ulcer was completely healed on week 11 (Fig. 1e).



1a



1b



1c



1d



1e

Fig. 1. Ulcer healing in patient 1

- a. Before fibroblast treatment. Ulcer diameter was 1 cm.
- b. Ten days after the 1st injection of fibroblast suspension (early cultured cells), ulcer size had considerably diminished: diameter was 7 mm.
- c. Healing process showed signs of exhaustion after 50 days and a second injection was performed, with fibroblasts isolated from a second seeding of the skin pieces and subsequently cultured until $30-40 \times 10^6$ cells were obtained.
- d. Compared to the 1st injection, there was minor improvement in the healing process 10 days after the 2nd injection.
- e. Ulcer was completely healed on week 11.

3.1.2 Patient 2

Three sessions in total ($30-40 \times 10^6$ cells each time) were performed. For the first and third sessions early cultured cells were used, while for the second frozen cells were employed. Ten days after the first injection (early cultured cells), ulcer' size and depth had impressively diminished (Fig. 2a & 2b). After 70 days, cells showed signs of exhaustion and a second injection (fibroblasts isolated from the first seeding of skin pieces and subsequently undergone freezing-thawing procedure and cultured until $30-40 \times 10^6$ cells were obtained) was performed (Fig. 2c). Interestingly, there was no improvement of the healing process 10 days after this second injection (Fig. 2d). Thus, a fresh skin biopsy was taken and another infusion of early cultured cells was applied, followed by a steady healing process. The wound closed completely after 27 weeks (Fig. 2e).



2a



2b



2c



2d



2e

Fig. 2. Ulcer healing in patient 2

- a. Before fibroblast treatment. Ulcer diameter was 4 cm.
- b. Ten days after the 1st injection of fibroblast suspension (early cultured cells), ulcer's size had impressively diminished: diameter was 30 mm.
- c. Cells showed signs of exhaustion after 70 days (ulcer's diameter 15 mm). Then a second injection was performed, with fibroblasts isolated from the first seeding of skin pieces and subsequently undergone freezing-thawing procedure and cultured until $30-40 \times 10^6$ cells were obtained.
- d. There was no improvement in the healing process 10 days after 2nd injection (freezing-thawing procedure).
- e. A fresh skin biopsy was taken and another infusion of early cultured cells was applied, followed by a steady healing process. The wound closed completely after 27 weeks.

3.2 Discussion

Although many attempts have been made during the last two decades to promote wound healing using growth factors, fibroblasts (autologous or heterologous), keratinocytes (alone or in combination with fibroblasts), and cell-free derivatives from mesenchymal stem cells [7], the success in all attempts does not seem to be complete or permanent. The reason is that exploiting substances and cells in the right time with the proper order and the suitable quantity, is not well-defined [8], since the stages of the wound healing (hemostasis, inflammation, proliferation and remodeling) are many times overlapping. Unlike acute wounds, in the case of chronic wounds the efficient and orderly process of the prementioned four stages is lost and the healing process is arrested in one of the above mentioned stages; usually, chronic ulcers are "locked" in inflammatory stage [9]. The role of fibroblasts in normal wound healing begins after inflammatory phase. The early, stimulated fibroblasts in our culture's conditions (50% FBS) were able to successfully perform their task when they were injected into the wound's surrounding healthy area, re-uptaking the role they normally perform in wound healing after inflammatory phase. Thus, by fibroblast injection, inflammatory phase was terminated and it was succeeded by proliferative phase. Thereafter, wound healing continued normally from this point on. Of course, fibroblasts' time culture and the way of maintaining (frozen or not) before the administration is important, as we also observed in our two cases. At proliferative stage, fibroblasts need plenty of nutrients and for this reason they had been suspended in the patients' own serum.

Our lab has sufficient experience in treating burns, surgical wounds, and decubitus ulcers with autologous skin fibroblasts [5]. Diabetic foot ulcers, though, might present a different healing behavior. Patients are usually older, with slower proliferation rate of their skin fibroblasts in culture. Thus, issues like the optimum concentration of FBS in fibroblast cultures, the most suitable way to administer fibroblasts to the wound area, the number of required injections and number of cells each time had to be addressed. The question whether cultured cells, after some passages or doublings and/or freezing-thawing procedure, lose their capacity to perform their task in healing process had to be answered.

Certainly, studies with large samples are needed to establish the usefulness of fibroblasts in diabetic foot ulcer treatment. But with our patients, the authors had the opportunity to assess that the healing process was similar to healing of other kinds of wounds, and that frozen fibroblasts lost entirely their healing capacity, while, as already noted by Wagner [6], the ability of the prolonged cultured fibroblasts to communicate with other cells and to produce the necessary substances was diminished steadily and was not regained even when the cells found themselves in their natural environment again. It seems that fibroblasts orientated their metabolic activity solely in yielding binding factors like fibronectin, which helped them to adhere on the flask's surface and to survive as long as they can [6]. This may be caused by epigenetics procedure for energy conservation reasons [10,11]. Obviously, these phenomena arouse controversial discussions and reservations concerning the safety of therapeutic approaches using cultured cells. However, no malignancies aroused from such cultures are reported up to now [12,13].

4. CONCLUSION

Based on our preliminary results, early, autologous skin fibroblasts arisen in large numbers from small split-thickness skin biopsies, cultured in high concentration FBS and dispersed in patients' own serum, consist a promising method to effectively treat non-healing diabetic foot

ulcers. This relatively simple method produced a considerable amount of cells from old people's small split-thickness skin biopsies in a short time, and seems superior to culture methods with additives like growth factors [14,15], cultured allogeneic fibroblasts and keratinocytes [16,17], and fibroblasts cultured on scaffolds [18]. Healing was quick, permanent, safe, without scar or hyperkeratosis, and relatively inexpensive.

CONSENT

All authors declare that 'written informed consent was obtained from the patients'.

ETHICAL APPROVAL

Not applicable.

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None.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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