Regeneration of Skin Tissue Promoted by Mesenchymal Stem Cells Seeded in Nanostructured Membrane

C.M.C.O. Souza[a], L.A.F. Mesquita[a], D. Souza[a], A.C. Irioda[a], J.C. Francisco[a], C.F. Souza[b], L.C. Guarita-Souza[c], M.-R. Sierakowski[b], and K.A.T. Carvalho[a,*]

[a]Instituto de Pesquisa Pelé Pequeno Príncipe, [b]Universidade Federal do Paraná, and [c]Pontifícia Universidade Católica do Paraná; Curitiba Parana, Brazil

ABSTRACT

Background. The mesenchymal stem cell therapy has proven to be an effective option in the treatment of skin injuries. The combination of these cells with nanostructured membranes seems to be the future for tissues recovery. The aim of this project was to use biomolecules of polysaccharides to be incorporated on regenerated cellulose membranes and to prospect the improvement as bioactive wound dressings with mesenchymal stem cells.

Methods. The biocomposites were obtained after defibrillation with the use of never-dried bacterial cellulose to form a pulp, and, after the films were regenerated, in the presence of gellan gum with or without fluconazole. Membrane atomic force microscopy was performed for comparison of their structures.

Results. Adipose-derived mesenchymal stem cells were obtained from human adipose tissue liposuction in accordance with Zuk et al. The flow cytometric analysis and induction tests for adipocytes and osteocytes were performed. In vitro assays were performed on different membranes to evaluate the ability of these cells to adhere at 2 hours and proliferate at 7 days; the results were obtained by use of the MTT cell counting technique. In vivo testing allowed us to observe cell migration and participation in wound-healing by fluorescence labeling of the cells with BrdU. The bioactive curative, seeded with cells, was tested in skin burned in a murine model.

Conclusions. The bacterial cellulose with gelan gum membrane incorporated with fluconazole presented the best performance in adhesion and proliferation tests. The cells can be identified in burned host tissue after occurrence of the wound.

INTEREST in the treatment of burns has increased in the past years as a consequence of the increased number of victims of war, caused by fire and chemical agents [1]. Domestic accidents are the most prevalent causes in development countries [2].

Burns compromise the functional integrity of the skin and electrolytic and temperature disturbances, causing prejudice of flexibility and lubrication of the skin surface. The burned skin lesion occurs in dependence of the extension, and the depth of the wounds and burns are classified as first-, second-, or third-degree, depending on the depth of tissue affected [2,3].

On the other hand, the curative industry produces more than 2000 different types of wound and burn dressings that have various forms, from a covering with gauze, topical

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*Address correspondence to K.A.T. Carvalho, MD, PhD, Alameda Doutor Carlos de Carvalho, 655 - Conj: 1405, Curitiba/Paraná - Brazil. CEP: 80.430-180, Brazil. E-mail: katherinecarv@gmail.com

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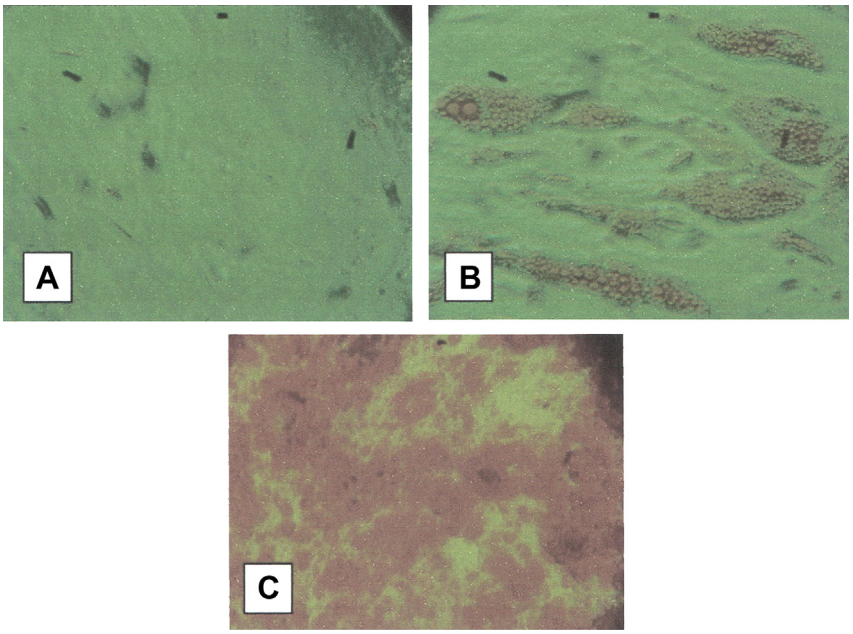

Fig 1. The human adipose-derived mesenchymal stem cells (hAMSC) on culture flasks. **(A)** Standard cultivation medium; **(B)** Sample differentiated in adipocytes; **(C)** Sample differentiated in osteocytes.

agents, natural agents, and antiseptic solutions, to the most complex, so-called "intelligent" or bioactive dressings [4]. Among them are dressings made of cellulose, a biologically inert product, with low incidence of hypersensitivity, initially indicated as synthetic graft and temporary skin [5].

The cellulose produced by *Acetobacter xylinum* consists of indefinite-length microfibrils, distributed in random directions and interwoven, forming a loose gelatinous tissue and extremely fine texture, which, when applied as a dressing on the skin, provides to the injured area a protection against external contamination, assists in healing and allows gas exchange and monitoring of the progress of healing without removal of the dressing, and does not release waste [6].

The use of nanostructured films made of polymers can be an alternative to improve the healing of burned tissue. The advantage of nanostructured membranes is the large ratio of surface to volume, besides the presence of many pores, forming a 3-D structure that allows the application as scaffolding [7,8].

In the healing process, deeper burns treated with traditional dressings, bioactive curative or skin substitutes, wound contraction, and scar formation are inevitable [9]. To minimize these conditions, several studies have been used mesenchymal stem cell (MSC) therapy in burns, resulting in greater efficiency of healing [10].

MSC are adult pluripotent, being able to differentiate into a minimum of 2 cellular types different than its origin, characterized by immunophenotypic markers: CD105+, CD73+, CD34−, CD90+, and CD45−. It is expected that the transplanted MSC are capable of integrating into the host tissue, differentiating and promoting tissue regeneration [11,12].

In a search for more effective treatments to improve the quality of healing and fast recovery of burns, this study aims to compare nanostructured membranes as a dressing for second−degree burns [13].

METHODS

Cell Isolation

Adipose-derived MSC (AMSC) were obtained from human adipose tissue liposuction. The adipose tissue was processed in accordance with Zuk et al [11]. MSC were isolated and seeded in culture flasks. All cultures started with the same cell concentration (5×10^5/mL) were maintained in an incubator with 5% CO_2 at 37°C in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 1% antibiotic (100 g/mL streptomycin−100 g/mL penicillin), which was changed

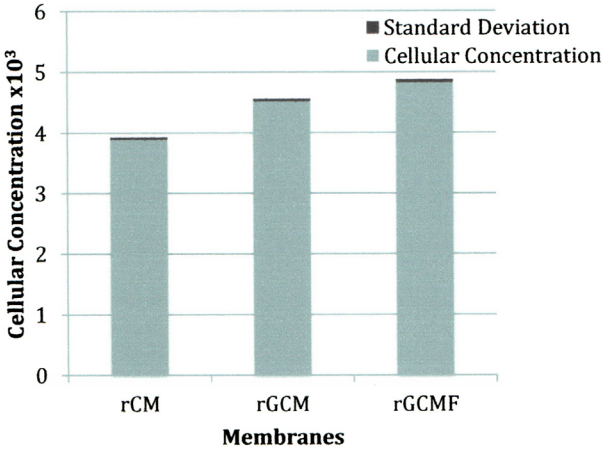

Fig 2. Graph showing cell adhesion rates.

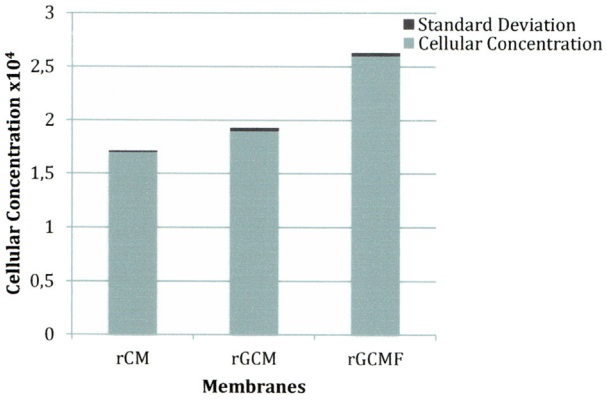

Fig 3. Graph showing cell proliferation rates.

every 48 hours for 21 days. The cell count was performed in a hemocytometer.

Cytometric Analysis

Before AMSC were seeded in membrane, cells underwent cytometric flow analysis to characterize the immunophenotypic profile of cellular subsets, with the use of the antibody–anti-surface markers CD45, CD34, CD73, CD90, CD105, CD49d, and Annexin V. The specimens were appropriately identified. The cells were incubated with the specific monoclonal antibodies panel for 15 minutes at room temperature in a dark room and analyzed on a FACS Calibur with the use of Cyflogic v. 1.2.1 software. For the 2-color staining immunophenotyping analyses, we used the following human antibody conjugates, coupled with fluorescein isothiocyanate (FITC), phycoerythrin (PE); CD34-FITC (clone 581), CD45-PE (clone HI30), CD73-PE (clone AD2), CD90-FITC (clone 5E10), CD105-FITC (clone 266), CD49d-FITC (clone 9F10), and Annexin V–PE [11]. All antibodies were used at the concentrations recommended by the manufacturer.

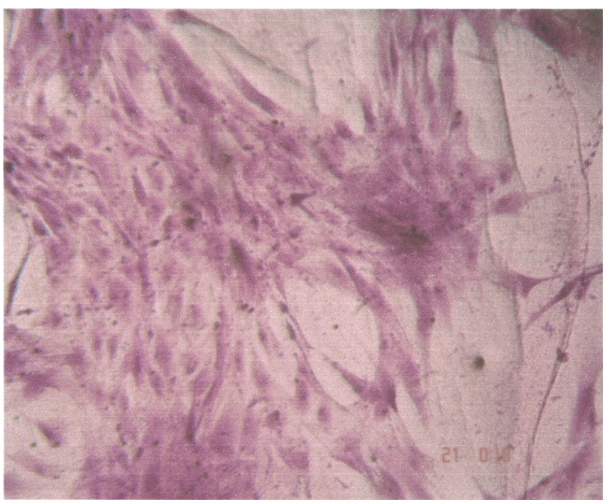

Fig 4. Membrane with human adipose-derived mesenchymal stem cells stained with crystal violet, observed in an optical microscope, ×100.

At the second passage (P2), cells were submitted to adipogenic- and osteogenic-induced medium, in accordance with Zuk et al [11].

Cellulose Matrix Proceedings

Membrane fabrication. Membranes were developed and provided by the BioPol Lab, Department of Chemistry, Federal University of Paraná. The nanocomposites used were obtained after defibrillation with the use of never-dried bacterial cellulose to form a pulp; films were then regenerated in the presence or not of hydrocolloid (10% wt/wt) with or without drugs (10% wt/wt). The total mass, based on dried films, was performed according to the commercial samples from MEMBRACEL. Membranes were seeded with stem cells incorporated with BrdU to track the cells after transplantation. Three modified membranes were tested: reconstituted cellulose membrane (rCM), gellan gum incorporated on cellulose pulp (GrCM), and GrCM with 10% (wt/wt) fluconazole (GrCMF) [14].

Adhesion and Proliferation Assays

The AMSC were transferred on 3 different types of modified membranes to evaluate the membrane efficiencies concerned with cell adhesion and proliferation. The seeded membranes were stained with crystal violet, and samples were analyzed in direct optical microscopy. Adhesion and proliferation assays are similar; cells were plated in 80% confluence of 1×10^4 cells/cm^2 concentration on membranes. After a period of 2 hours the adhesion test was performed and after 7 days, the proliferation test was performed. The analyses were then performed. Both tests were monitored by means of 3-4,5-dimethylthiazol-2-*yl*-2,5-diphenyltetrazolium bromide, known as MTT assay, and a standard curve was used to compare the number of cells and the absorbance in a photocolorimeter (Biotek Instruments Inc, ELX 800 Model, United States).

Imunocytochemistry Assay

The group of cells used for seeding the GrCMF membrane, used on the rat burned skin, was labeled with BrdU (Becton-Dickson, United States) as well as with DAPI (Becton-Dickson) to label the cell nuclei, both in accordance with the manufacturer, and the histological fragment of the treated wound was then revealed by FITC–anti-BrdU.

Atomic Force Microscopy

Atomic force microscopy analyses were performed with the use of an Agilent 5500 microscope (Agilent Technologies, Santa Clara, California, United States), with the use of Pico Image software (Agilent Technologies). The tapping mode images were obtained with the use of Vistaprobes (Nanoscience Instruments, Inc, Phoenix, Arizona, United States) silicon tips (nominal spring of 48 N/m and resonance frequency of ~180 kHz), and the scanning was performed over 5.0 × 5.0 μm. The data treatment and presentation were accomplished with the use of Gwyddion Software (Czech Metrology Institute).

Burn Protocol in Rats

To obtain the second-degree burns, one male rat of the species *Rattus norvegicus albinus* Wistar (weight, 300 g) was anesthetized with ketamine (10 mg/kg) and xylazine (50 mg/kg) intraperitoneally. The rat's dorsum had been shaved, and its skin was exposed to an iron bar (heated at 100°C) for 30 seconds. The membrane seeded with MSC was placed on the burn area. At the 15th day, the rat was euthanized, and histological sections were performed for analysis of

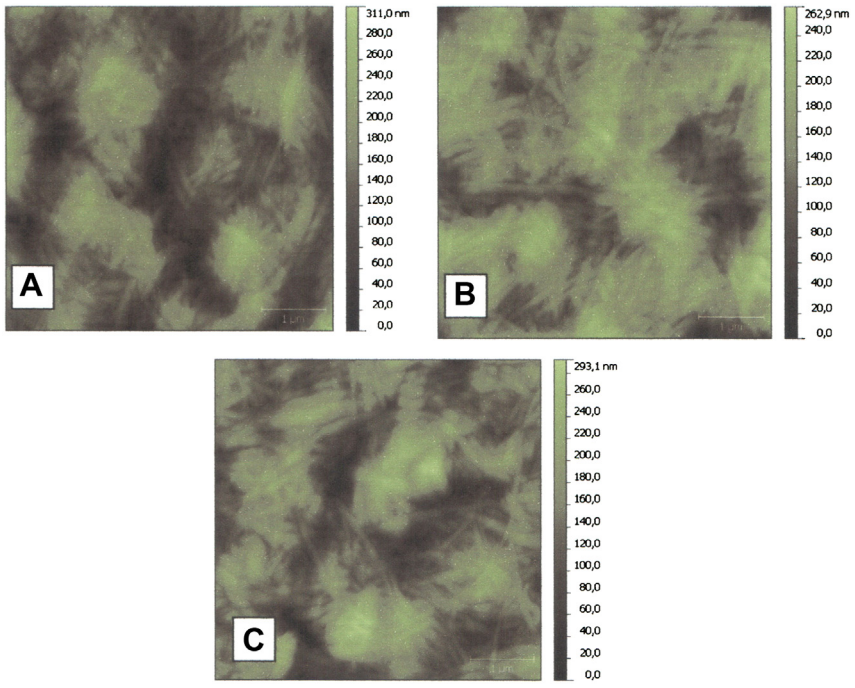

Fig 5. Image of membranes obtained from the atomic force microscope. The color scale on the right represent the differences in height. **(A)** reconstituted cellulose membrane (rCM); **(B)** gellan gum incorporated on cellulose pulp (GrCM); **(C)** GrCM with 10% (w/w) fluconazole (GrCMF).

fragments. This procedure follows the guidelines of the Brazilian Ethics Committee on Use of Animal Subjects and is approved by the Pequeno Príncipe Hospital Complex Ethics Committee, No. 015-2012.

RESULTS

Flow cytometry demonstrated that the isolated cells were CD45−, CD 34+, CD73+, CD90+, CD 105+, and CD49d+, and the cells were differentiated into adipocytes and osteocytes (Fig 1).

Among the tested membranes, that with gellan biocompound membrane showed the best performance in adhesion (4.8×10^3 cells) and proliferation (2.6×10^4 cells) assays (Fig 2 and Fig 3), respectively. GrCMF membrane was 65.4% more efficient than was the commercial membrane. The adhesion graphic indicates an adhesion rate of >50% from the inoculum.

The photomicrograph in Fig 4 shows the morphology of human adipose-derived MSC seeded on the cellulose membrane after 7 days of proliferation.

The structure revealed by atomic force microscopy shows morphological similarity between the membranes, which, despite being composed of different biopolymers, are based on cellulose (Fig 5). We observed that the incorporation of gellan gum made this hydrocolloid act as a cementing substance, coating the cellulose microfibrils.

Some of the most important information in this study is shown in Fig 6, which is the fluorescence microscopy of a burned area section that was treated with the GrCMF membrane seeded with MSC. The cells expressing green fluorescence (FITC-BrdU) and blue fluorescence (DAPI) simultaneously are the stem cells that migrated from the dressing to the skin.

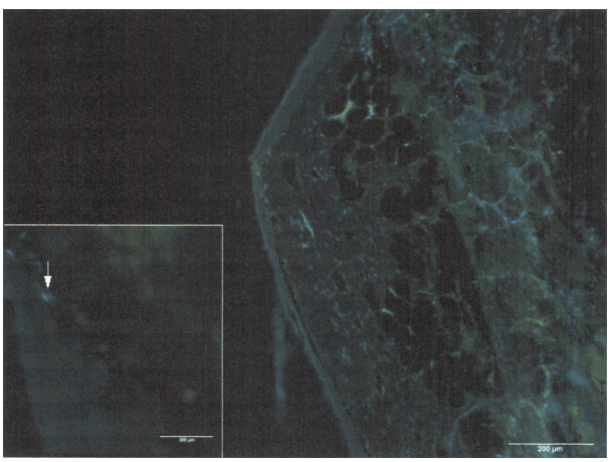

Fig 6. Photomicrography of burn skin treated with GrCMF membrane seeded with stem cell after 15 days, observed in fluorescence microscope, ×100. The arrow points to a marked stem cell, ×400.

DISCUSSION

Flow cytometry analysis demonstrated that the isolated cells were MSC, showing the same result obtained by De Ugarte et al [15], and the ability of these cells to differentiate into

osteocytes and adipocytes reinforces this result, according to Bunnel et al [16].

The membranes appear to be a suitable substrate for MSC. In morphological point of view, these cells appear as adherent cells, similar in aspect while cultured in polypropylene flasks. The differences between the membrane compositions interfere with cell expansion, but, in all cases, the cells were adherents and capable of proliferation. This shows that the interactions between the biopolymers that compose the membranes promote a greater or lesser stimulus to cellular growth.

Many cellulose-based dressings are used for treatment of burns because cellulose is inert, having low rejection of the injured tissue, and forms a porous mesh that allows gas exchange.

In this study, the healing rate was higher when compared with the control (treatment without membrane). This can be explained in part by the micro-modulation environment, promoting wound contraction, which would be the activation the myofibroblast, a special type of fibroblast that has ultrastructural characteristics of muscle cells [5].

The integration between AMSC and the membrane was satisfactory, allowing cell expansion and delivery of these cells to the injured tissue, which indicates that dressings made of cellulose are an alternative to bioactive curatives. It is important to observe the cell's ability to migrate from the membrane surface to the injured area, so that the cells can participate in tissue recovery, and it points to the use of these membranes as a system for cell delivery. Furthermore, this system is efficient and has low cost.

The regenerative potential of AMSC is already known. These cells are considered to be of low immunogenicity because they have a low ability to generate the immune response. The AMSC produce a large number of bioactive molecules such as adhesion molecules, extracellular matrix proteins, cytokines, and growth factors receptors, which may be involved in the immunomodulatory response and in its migration function [17]. These molecules modulate the inflammatory response, angiogenesis, and cell mitosis involved in the tissue repair process [18]. In an experiment conducted in 2003, MSC transplanted into the injured tissue differentiated into vascular endothelial tissue, forming new blood vessels, improving healing, and reducing burn area [19].

The first allogeneic transplant of MSC was conducted in 2005, in Russia, with a female patient who had extensive burns (40% of the total area burned). It showed that angiogenesis stimulated by stem cells, reduction of healing time, and rehabilitation [18–20].

CONCLUSIONS

The results suggest that biofilms made of bacterial cellulose but enhanced with gellan gum in its composition, when associated with AMSC, could be powerful dressings for burns and sores and could also be used in diverse tissues as a cellular patch to enhance tissue repair.

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