Generation of Two Biological Wound Dressings as a Potential Delivery System of Human Adipose-Derived Mesenchymal Stem Cells

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Human adipose-derived mesenchymal stem cells (hADMSCs) are believed to be potential key factors for starting the regenerative process after tissue injury. However, an efficient method of delivering these regenerative cells to an external wound site is still lacking. Human amnion and pig skin have long been used as skin wound dressings for the treatment of burns and other skin lesions. Herein, we present the generation of two constructs using these two biomaterials as effective scaffolds for the culture of hADMSCs. It was found that hADMSCs seeded onto radio-sterilized human amnion and pig skin are viable and proliferate. These cells are able to migrate over these scaffolds as demonstrated by using time-lapse microscopy. In addition, the scaffolds induce hADMSCs to secrete interleukin-10, an important negative regulator of inflammation, and interleukin-1β, a proinflammatory protein. The interplay between these two proteins has been proven to be vital for a balanced restoration of all necessary tissues. Thus, radio-sterilized human amnion and pig skin are likely suitable scaffolds for delivery of hADMSC transplants that could promote tissue regeneration in skin injuries like patients with burn injuries. ASAIO Journal 2015; 61:718–725.

Key words: human adipose-derived mesenchymal stem cells, radio-sterilized human amnion, radio-sterilized pig skin, wound healing, wound dressing, cellular response, burn treatment

In 2013, the American Burn Association reported that in the United States, 400,000 people were hospitalized because of lesions related to burns or because of different kinds of accidents that could lead to burns by various other factors. Even though there are many skin substitutes in the market,1,2 patients with burn injuries in underdeveloped countries often do not have access to them because of their high cost. In the past few decades, amniotic membrane and pig skin have been used for the treatment of partial-thickness burns because of their favorable properties: avoiding loss of water, diminishing pain sensation, and decreasing the probability of acquiring an infection.3–5 During a second-degree skin burn, the epidermis and part of the dermis are completely lost, whereas in a third-degree burn, even the adipose tissue and muscle might be affected or lost. The use of a simple dressing for the treatment of these types of burn injuries does not aid in the restoration of damaged tissue. Hence, amnion and porcine skin have been used as skin substitutes in burns6,7 because they have different extracellular matrix components and could support the growth of fibroblasts, keratinocytes, or both. In recent years, the use of mesenchymal stem cells (MSCs) has revealed that these cells are helpful in wound healing because of their capacity to differentiate into the vascular lineage, fibroblasts, keratinocytes, and adipose tissue, altogether important components of the skin. MSCs are also capable of secreting interleukin (IL)-10, an important chemokine for immunoregulation. Several studies have shown that this chemokine can mark the difference between wound healing and regeneration.8–10 The treatment of patients with deep skin injuries such as third-grade burns requires the use of cells and a suitable scaffold for delivery of these cells to the site of wound or lesion. A good scaffold should allow cell adhesion and cell migration and, most importantly, must not be harmful to the cells that it supports. Because radio-sterilized human amnion (RHA) and radio-sterilized pig skin (RPS) are composed of extracellular matrix components such as type I collagen, type IV collagen, laminin, and fibronectin, they have been believed to be an excellent substrate for...
derived mesenchymal stem cell (hADMSC) culture. For this reason, we analyzed cytotolerance of hADMSCs cultured onto RHA and RPS evaluating viability, cell adhesion, proliferation, and migration. Our results show that both materials are suitable scaffolds promoting viability, adhesion, proliferation, and migration of hADMSC.

Materials and Methods

Human Adipose-Derived Mesenchymal Stem Cells Isolation and Culture

The consent and experimental protocols in this study were reviewed and approved by the ethics committee of the Instituto Nacional de Rehabilitación (México, D.F.). Subcutaneous adipose tissue was obtained from four aesthetic surgeries undergoing elective liposuction. Surgical procedures were performed using a liposuction needle with an internal diameter of 4 mm. Lipoaspirate samples were digested for 45 min at 37°C with shaking at 200 rpm in Dulbecco's Modified Eagle's Medium (DMEM) medium (GIBCO, Grand Island, NY) containing 0.1% type I collagenase (Worthington Biochemical, Lakewood, NJ). Cells were passed through a 70 µm strainer and centrifuged at 1,200 rpm for 5 min. Cells were seeded onto each scaffold at a concentration of 50,000 cells/cm². After 24 h, medium was changed, and the adherent hAMSCs were grown to confluence as passage zero cells. Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin (GIBCO).

Flow Cytometry

To verify the presence of MSC markers, first passage (P1) MSCs were analyzed using a FACSCalibur flow cytometer (FACS; Becton Dickinson, San Jose, CA). We analyzed two positive and two negative markers for the mesenchymal stem cells. The mesenchymal stem cell markers were CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1). The hematopoietic markers were CD34 and CD45 (leukocyte common antigen). At 80% of confluence, cells at P1 were harvested from one 75 cm² flask, counted, and resuspended to a concentration of 2 × 10⁵ cells/ml. Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin (GIBCO).

Histological Analysis

Both RHA and RPS were fixed in 4% paraformaldehyde (Sigma, St Louis, MO) for 10 min, washed three times with PBS, and sections of 10 µm thick were stained with hematoxylin–eosin. The hADMSCs that were differentiated into the chondrocytic lineage were stained using alcian blue, and those differentiated into osteocytes were stained using Von Kossa staining.

Generation of Constructs by Seeding hADMSC onto Radiosterilized Human Amnion and Radiosterilized Pig Skin

Mesenchymal cells isolated from adipose tissue at P1 were cultured onto two different types of scaffolds, RHA and RPS. Under sterile conditions, fragments of approximately 0.5 cm diameter of RHA and RPS were cut and placed onto a 24 well culture plate. Mesenchymal cells previously counted and pelleted were seeded onto each scaffold at a concentration of...
30,000 cells/cm² and cultured in DMEM medium complemented with 10% FBS and 1% antibiotics. The constructs were kept for a week at 37°C and 5% CO₂.

Viability Assay

To confirm that RHA and RPS are feasible scaffolds for MSC isolated from adipose tissue, viability of hADMSCs cultured onto RHA or RPS was determined after 48 h using the LIVE/DEAD viability/cytotoxicity for mammalian cells Molecular Probes kit (Thermo Fisher Scientific, Rockford, IL). Following technical specifications established by the manufacturer, 1 µM calcein AM and 2 µM ethidium homodimer-1 (EthD-1) were diluted in Hank's medium. This solution was used to incubate constructs for 45 min at 37°C. Finally, constructs were washed with PBS and analyzed using a confocal microscope LSM 780 and ZEN 2010 Carl Zeiss software (Zeiss, Jena-Turingia, Germany).

hADMSC Proliferation

After 48 h, hADMSCs cultured onto RHA or RPS were washed and fixed in 4% paraformaldehyde/0.1 M PBS buffer (pH = 7.4). Cells were immunolabeled with primary antibody against human Ki67 (1:100, BioLegend, San Diego, CA) and incubated at 4°C overnight. The constructs were washed and incubated with Alexa Fluor 488 (1:500; Rockford, IL) secondary antibody Thermo Fisher Scientific for 2 h. Nuclei were stained using 1 mg/ml of 4′, 6-diamidine-2-phenylindole-dihydrochloride for 10 min. Images were obtained with an AxioVision Observer A.1 microscope (Zeiss).

Cell Migration

For examination of cell migration on the surface of RHA and RPS, cells were stained with Dil and seeded during 48 h onto the scaffolds; cells were photographed using “time series” function in an Axio observer microscope provided with a CO₂ incubation chamber during 6 h. Migration distance was calculated using AxioVision software.

ELISA

Conditioned medium from hADMSCs seeded onto RHA or RPS scaffolds was collected after the third day of culture. Enzyme-linked immunosorbent assays (ELISAs) to determine the presence of IL-10 and IL-1β were performed according kit supplier’s protocols (PeproTech, Inc., Rocky Hill, NJ, for IL-10 and Beckon Dickinson for IL-1β).

Confocal Images

For confocal microscope examination, fluorescently labeled cells were visualized and photographed using a laser scanning confocal microscope (LSM780, Carl Zeiss).

Statistical Analysis

All the experiments were repeated at least three times with hADMSC from three different donors. The materials were tested in parallel with the same batch of cells. The values are expressed as the mean ± standard error. The Student's t-test was used to determine statistically significant differences between experimental and control samples. Analysis of variance followed by Tukey's multiple comparison test were used for more than two populations. A p value less than or equal to 0.05 was considered significant.

Results

Phenotype Determination and Differentiation of hADMSC

Multiple studies have demonstrated the existence of a small population of mesenchymal stem cells in adipose tissue. The hADMSCs were obtained from lipoaspirates of patients undergoing cosmetic surgery under signed informed consent. We found that after passing adipose tissue through a size 4 needle, we obtained approximately five times the total number of cells than when we disaggregated the tissue using just a scalpel as it has been reported in previous study. Once the tissue was minced (in cases where adipose tissue was collected in big pieces), it was incubated with type I collagenase for 1 h at 37°C and 200 rpm shaking. One characteristic of MSCs is their ability to adhere to the culture dish surface; so we counted the number of cells and seeded them at 50,000 cells/cm² during 24 h. Cells that did not attach to the culture dish were washed with PBS. Attached cells reached confluency about 1 week later. At the onset of the cultures, we observed cells with varying morphology: a portion of them had a spiculated shape like fibroblasts and others had a rounded morphology and formed colonies (Figure 1). During the culture, cells gradually acquired a fibroblast phenotype. As reported in other studies, MSCs express CD90 and CD73 but show negative expression of CD34 and CD45. We detected the expression of CD90 (84.8%), CD73 (99.9%), CD45 (0.15%), and CD34 (1.6%) in our cultures (Figure 1). These data along with the aforementioned cell morphology phenotypes suggest that we obtained a population of hADMSCs; this was further supported by other investigations that prove the isolation of MSCs in adipose tissue. A third characteristic of MSCs is their multipotent capacity of differentiation and thus, as described in the Materials and Methods, we differentiated MSCs into chondrocytes, osteocytes, and adipocytes. To analyze the presence of chondrocytes after the differentiation process, we performed an immunofluorescence for type II collagen and alcian blue staining; for osteocytes, we analyzed the presence of Runx2 and performed Von Kossa staining. Finally, for adipocytes detection, we performed Oil red staining. Our data suggest that we have a population of hADMSCs in our cultures, and this population is capable of differentiating into other cell types (Figure 2).

hADMSCs Show High Viability in Constructs Made by RHA or RPS

Once the cells were isolated and characterized, we performed histological analysis of the RHA and RPS. These biomaterials have been used for a long time for treating burn patients without any reported problems caused by these wound dressings. Although both materials are pretreated with hypochlorite, they still contain a remaining population of cells from the epithelium in the case of the RHA or the complete epidermis (and a few cells from the dermis) in the case of the RPS (Figure 3). There were generated two constructs by seeding hADMSCs over RHA or RPS. We performed a viability test to confirm that
mesenchymal cells isolated from adipose tissue remained viable and can be supported by the scaffolds. This test allows simultaneous determination of live and dead cells using two probes that measure recognized parameters of cell viability—intracellular esterase activity and plasma membrane integrity, using the calcein AM and EthD-1 as principal components. We compared both scaffolds by counting the number of living cells using the software Image J; the 10X and 20X fields and different focal planes were analyzed to count the cells in the whole area of the scaffold. Viability of mesenchymal cells seeded onto RHA was 92.7% and onto RPS was 90.5%. Despite the viability on both scaffolds being very similar, we observed that the number of cells supported by the RPS was larger than that of the RHA. This evidence suggests that this pair of wound dressings could work as good scaffolds and possess a potential application for future autologous or heterologous cell transplants in patients with burn injuries or for other skin problems such as ulcers.

**Radiosterilized Human Amnion and Radiosterilized Pig Skin Promote Cell Proliferation in the Constructs**

Previous investigations have described that human mesenchymal stem cells are multipotent, have the capacity to differentiate into various cell types, and possess an extensive self-renewal capacity in vitro. The nuclear protein Ki67 plays an important role during the replication phase of the cell cycle and has been a useful tool to evaluate cell proliferation. To analyze the self-renewal capacity of hADMSCs, a proliferation assay was performed to determine whether these cells were able to proliferate after 4 day culture onto radiosterilized human amniotic membrane or RPS. As determined, hADMSCs cultured onto RHA showed an increased proliferation (49.5%) when compared with those cultured onto RPS (27.7%). Cells clearly displayed a fluorescent signal inside the nuclei where Ki67 was actively participating in cell division (Figure 4). The increase in cell proliferation is a good parameter because we want to use these materials as carriers of autologous cells to promote new tissue formation in wounds.

**Radiosterilized Human Amnion and Radiosterilized Pig Skin Allow Migration of hADMSCs**

One of the integral characteristics of a good scaffold is that it should support cell migration, which is important for tissue organization. To analyze whether these biomaterials were good candidates with regard to cell migration, we captured time-lapse images using an Axio Observer microscope equipped...
with a CO₂ incubation chamber. Cells were stained with Dil before they were cultured onto each material to identify them by fluorescence, (see Supplemental Digital Content 1, 2, and 3, http://links.lww.com/ASAIo/A70, http://links.lww.com/ASAIo/A71, http://links.lww.com/ASAIo/A72) then we measured how far the cells could displace within 6 h. The migration distance was quantified using the program AxioVision. The hADMSCs were capable of migrating over the surface of both biomaterials. We did not find significant differences between cell migration over these two scaffolds (Figure 4).

hADMSCs Secrete Interleukin-10 and Interleukin-1β When Seeded onto Radiosterilized Human Amnion and Radiosterilized Pig Skin

The regulation of inflammation is a very important process during wound healing. Different studies have shown that when inflammation is diminished, the healing is more akin to a regeneration event; and when inflammation is exacerbated, the healing can give rise to hypertrophic scars. However, it is still reported that without inflammation, the healing does not carry on.20,21 The MSCs typically secrete IL-10, which is a cytokine that negatively regulates inflammation;22 conversely, IL-1β is one of the most important positive regulators of inflammation that determines whether wound healing will be exacerbated. We analyzed by ELISA whether our cells secrete these two important cytokines after they were seeded onto the scaffolds. We did not find significant differences in the concentration of IL-10 secreted by hADMSCs when they were seeded over RHA or RPS. Interestingly hADMSC secrete more IL-1β when they are grown on RPS rather than RHA (Figure 5). This result shows that RPS promotes IL-1β secretion, and this is possibly related to its xenogeneic origin.

Discussion

During recent years, there has been increased interest in mesenchymal stem cell research from the field of clinical medicine. The advances in knowledge of MSC biology have generated an important evolution in our perception of their nature and in their clinical application. The advantages for the use of MSC are as follows: we could treat patients with their own cells; in patients with burn injuries, adipose tissue is more probable to remain than epidermis and dermis; MSC could differentiate into fibroblasts that are the principal cellular component in the dermis; they could differentiate to vascular lineages and promote the growth of new tissue; and finally...
their capacity to release IL-10 promote immunoregulation of the wounded area.23–26 Adipose tissue is a feasible source of hADMSCs because aesthetic surgeries are performed in high quantity and adipose tissue is considered biological waste. This kind of tissue is not only a good source to obtain a population of mesenchymal stem cells (with the appropriate legal documents and the consent of the patient); but also, adipose tissue is a great supply for autologous cell transplants as we propose here using RHA and RPS. Herein, we describe that when we isolate cells from adipose tissue derived from liposapires by passing it through a size 4 needle, the quantity of cells was higher than when the tissue was disaggregated using only a scalpel. This result may occur because the needle helps to obtain tinier fragments that, in effect, increase the total surface area, making it easier for the collagenase to digest the extracellular matrix and release the cells. In our research, these cells can be cultured onto the scaffolds previously used as skin coverages, showing a good anchoring to the scaffolds and they also have high viability. In our cultures, cells from P1 formed small colonies and others were extended; however, after determined passages, most of them tended to look like fibroblasts, which is a characteristic of MSCs. The presence of hADMSCs was corroborated using two important MSC surface markers: CD90 and CD73; we also observed a low percentage of CD34- and CD45-positive cells in flow cytometry assays. The capacity of our isolated cells to differentiate into chondrocytes, osteocytes, and adipocytes supports the idea that in the mixed cell population, we have a fraction of hADMSCs, and additional evidence shows that these cells are capable of releasing IL-10, which is also a property of MSC. We wanted to analyze the histology of the biomaterials before seeding the cells and we found that, as in many protocols, our procedure of dehydrating and radiosterilizing the human amnion and pig skin did not release the structural components of the cells. Some studies have shown that when biomaterials still contain cells, it is difficult for the new cells to adhere onto the scaffold, but even when there were cell remnants in both materials, hADMSCs were able to attach to them.27 Viability assays

Figure 4. hADMSCs show high cell viability and proliferation when they are seeded onto RHA and RPS. Top images show viability assay where the live cells are stained with calcein (green) and dead cells are positive for ethidium homodimer-1 (red), and we did not find significant differences between RHA and RPS in this test. Bottom images show the presence of proliferation marker Ki67 (green) of hADMSCs seeded onto RHA or RPS. Cells growing on RHA have a greater proliferative capacity in comparison with RPS. Nuclei were stained with DAPI (blue). Student's t-test, **p < 0.001. DAPI, 4′,6-diamidine-2-phenylidole-dihydrochloride; hADMSCs, human adipose-derived mesenchymal stem cells; RHA, radio-sterilized human amnion; RPS, radio-sterilized pig skin.

Figure 5. RHA and RPS scaffolds promote cell migration and cytokine release. Left graph shows differences in cell migration. RHA and RPS did not show a difference in migration distance; however, both induced more cell migration compared with cells grown over culture dish. Analysis of variance, Tukey test, **p < 0.001. Center graph reveals IL-10 release by hADMSCs in both biomaterials tested by enzyme-linked immunosorbent assay. There were no significant differences between groups. Right graph represents IL-1β concentrations in conditioned medium, and we found that RPS induced more secretion of this cytokine than RHA. ANOVA, Tukey test, **p < 0.001. CTL, Control; hADMSCs, human adipose-derived mesenchymal stem cells; IL, interleukin; RHA, radio-sterilized human amnion; RPS, radio-sterilized pig skin.
proved that hADMSCs could attach to both surfaces even in the presence of cellular remnants, and they also demonstrated that these remnants do not affect viability of the cells. By definition, MSCs are undifferentiated cells with the potential for long-term self-renewal, mainly influenced by growth factors; however, it also has been described that matrix interactions are crucial for cell differentiation and proliferation. In our study, we report that hADMSCs isolated from liposuction aspirates possess the capacity to attach to the surface of the flask within 24 h. Our results demonstrate that interactions established between RHA and hADMSCs favored proliferation 4 days postisolation and suggest that the self-renewal capacity is maintained in these cells. Similar results have shown that porcine dermal matrix promotes the growth and differentiation of human keratinocytes, while Huang et al. used the amniotic membrane to promote the rapid expansion of epidermal keratinocytes. In our case, both biomaterials induced cell proliferation and cell migration, excellent features to function as cell carriers into skin wound sites; we may also be able to increase the bioactivity of these biomaterials by removing the remaining cellular components. Another study using a rat model has revealed that mesenchymal stem cells seeded onto porcine dermis were able to induce neovascularization. We know little about the mechanism by which both biomaterials induce the biological activity of hADMSCs seeded onto them; however, we believe it is because of the extracellular matrix structural proteins present in the original tissue. The list of proteins in the acellular dermis matrix includes type I, IV, and VII collagen; laminin; fibronectin; cytokines; vascular endothelial growth factor that can induce angiogenic activity; and tumor growth factor-β, a potent mitogen that can induce collagen production. The amniotic membrane includes proteins such as NGF, HGF, KGF, bFGF, TGF-β1, and EGF, and these proteins create a microenvironment niche for the stem cells similar to the native tissue. Many of these proteins can interact with cell membrane receptors and induce growth, proliferation, migration, adhesion, etc. Finally, cells were able to produce IL-10, which is important for diminishing inflammation, but surprisingly, RPS also induced the release of IL-1β that could be detrimental when found in increased amounts. This effect could be related to the presence of pig proteins that are contained in the cell remnants. Although it could be interpreted as a bad indicator, it is important to remember that there is also evidence showing the need to have inflammation to start a regenerative process. Another important note is that these biomaterials have been used in patients with burn injuries for a long time and are considered good apposite for healing wounds. For these reasons, our laboratory has started to analyze the effects of these skin substitutes using animal models. It will be important to further analyze how these substitutes and stem cells help to heal wounds caused by burns.

**Conclusion**

This work shows that these economical and accessible materials could represent a good option to carry out autologous cell transplants using hADMSCs, a tissue more likely to remain during burn incidents. These long-standing biomaterials could be promising scaffolds to be used with new treatments involving human adipose-derived mesenchymal stem cells.

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