European Review for Medical and Pharmacological Sciences 2021; 25 (1 Suppl): 49-55

In vitro cell culture of amniotic fluid keratinocytes on amniotic membrane: the ideal tissue for repairing skin ulcers

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Abstract. – OBJECTIVE: The amniotic fluid contains a large population of stem keratinocytes demonstrating minimal immunological rejection. Recent evidence suggests that stem cells from the amniotic fluid can be employed in the field of tissue engineering. In this work we identified precursors of the epithelial cells and expanded them *in vitro*.

MATERIALS AND METHODS: After collecting samples of amniotic fluid and separating the cells *via* centrifugation, we seeded a portion of these cells in selection media to analyze the proliferation of epithelial cells. The stem cells precursors of keratinocytes were identified through specific markers. The expression of these markers was evaluated by immunofluorescence and reverse transcription polymerase chain reaction (PCR).

RESULTS: The stem cells demonstrated 90% confluence, after undergoing proliferation in the selection medium for 15 days. Most of these cells tested positive for the keratinocyte-specific markers, but negative for stem cell specific markers. Of note, the identity of the keratinocytes was well established even after several subcultures.

CONCLUSIONS: These results suggested that it is feasible to isolate and expand differentiated cell populations in the amniotic fluid from precursor cells. Furthermore, amniotic membranes can be utilized as scaffolds to grow keratinocytes, which can be potentially exploited in areas of skin ulcer transplantation and tissue engineering interventions.

Key Words:
Amniotic fluid, Amniotic stem cells, Amniotic membrane, Keratinocytes, Wound healing, Tissue engineering.

Introduction

Amniotic fluid is a slightly yellowish clear liquid surrounding the fetus during pregnancy. Amniotic fluid is in constant circulation, through fetal inhalation and exhalation. Inhalation occurs through the fetal nose and mouth, and then, it is eliminated by the fetus through urination, thus

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enriching the amniotic fluid with several cells that are derived by the peeling of the external and internal surface of fetal organs[1].

Amniotic fluid comprises a heterogeneous population of fetal cells with different tissue origin. The majority of these cells come from the external and internal epithelial flaking. These amniotic fluid cells consist of mesodermal lineage committed stem cells, totipotent stem cells, and differentiated cells like keratinocytes, fibroblasts, neurons and glial cells[2]. Other cells in the amniotic fluid are epithelial, hematopoietic, trophoblastic and mesenchymal cells[3]. Amniotic fluid also contains hormones, nutrients and antibodies[1].

Skin wounds or ulcers may take a long time for healing and if not treated properly they could be infected by microorganisms. Research studies have revealed that placenta-derived tissues could stimulate angiogenesis and novel tissue formation, modulate inflammation, decrease formation of scar tissue and pain, and have anti-microbial effects[4]. The amniotic membrane is made of collagen, fibronectin, elastin and proteoglycans that provide a three-dimensional architecture to promote damaged tissue reconstruction and regenerative growth factors (GFs), like vascular endothelial GF, insulin-like GF, platelet-derived GF, transforming GF-ß and fibroblast GF, tissue repairing peptides and anti-inflammatory cytokines[5,6]. Human amniotic fluid and amniotic membrane are attractive sources of cells for the skin tissue engineering[7]. Amniotic cells can be harvested easily from the amniotic fluid that is frequently extracted during the diagnostic amniocenteses[3]. Amniotic fluid-derived stem (AFS) cells, such as human keratinocytes, are considered an attractive source of cells that could be used in regenerative medicine (repair, replacement and reconstruction of acute and chronic wounds, pressure ulcers, diabetic ulcers, traumatic injuries, venous and arterial ulcers, burns and surgical wounds) due to their intrinsic immunomodulatory activity, high proliferation ability, multipotency and absence of significant immunogenicity[8,9]. The AFS cells isolation is much simpler than the mesenchymal stem cells isolation, and greater numbers of amniotic fluid stem cells can be isolated and then expanded from just 2 ml of amniotic fluid. These cells rapidly proliferate and double in 30-36 hours. They do not require supporting feeder layers and were already used to create stem cell lines[4].

In patients with chronic wounds, ulcers of various types and burns, there is a great need to use either tissue transplants or inject stem cells to repair the damage. Keratinocytes derived from amniotic epithelial stem cells (hAESCs) are potentially suitable for this purpose. In a preliminary clinical trial safety was established that neither long-term nor acute toxic response is stimulated by the injection of hAESCs[10,11]. Furthermore, cryopreserved amniocytes remain functional and viable for decades. Therefore, autologous skin engineering might be possible for patient at any age[3]. The aim of the study was to better understand the potentialities of hAFS cells for regenerative medicine through the cell differentiation marker analysis and *in vitro* functional studies were used for the complete characterization of amniotic fluid cells. Moreover, our second purpose was to cultivate hAFS cells *in vitro* to expand and differentiate them.

Materials and Methods

Amniotic Fluid

The amniotic fluid used was derived from amniocenteses made at 14 weeks and sent to the prenatal diagnostic center. The karyotype was normal and for every sample used in this experiment the indication for amniocentesis was the age of the mother (over 35 years).

Isolation and Culture of hAFS Cells

After the amniotic fluid sample collection cells were separated through centrifugation, part of those cells was seeded in the selection media for the growth of epithelial cells. When the cell confluence reached 90%, they were subcultured for the identification of markers.

RT-PCR Analysis

For the identification of stem cells precursors of keratinocytes through certain specific markers, RNA samples were extracted following the standard protocol, and then, they were examined using the process of reverse transcription, following the random hexamer protocol. Each sample underwent a control reverse transcription (RT)-PCR with GAPDH as target; after that, the samples were analyzed by PCR with a specific set of primers for a list of markers (Table I) specific for cell types that can be found in the amniotic fluid.

Table I. List of markers analyzed by PCR to identify cell types in the amniotic fluid.

Marker	Specificity
OCT4	Totipotent stem cells
ALPL	
ABCG2	
VMN	Neuronal and glial stem cells
NES	
SOX1	
GFAP	
MBP	
ENO2	
P63	Keratinocyte stem cells
ITGA6	
K15	
K19	
CD34	Blood stem cells
CD15	
CD133	
MYF5	Muscular stem cells
MYF6	
MYOG	
COL1A1	Fibroblasts
PPARG2	Fat cells
AFP	Endodermal stem cells

Flow Cytometry Analysis/Immunofluorescence

The method of analysis was methanol fixation and subsequent incubation with primary antibodies directed against the antigen that needed to be tested. After that, the cells were washed and incubated with fluorescent secondary antibodies. The nuclei were colored with DAPI. Details of the experiment are summarized in Figure 1.

Immunohistochemical Analysis

Immunohistochemical analysis was performed with Pan-Cytokeratin antibody cocktail recognizing acidic and basic cytokeratins (CK1, 3-6, 8, 10, 14-16, 19) after 20 days in selection medium and after their differentiation for keratinocytes.

Results

Characterization of Amniotic Fluid Cells

RT-PCR analysis of amniotic fluid cells have been done for keratinocytes before and after 20 days of culturing them in the selective medium using several specific cell markers (Figure 2), followed by the flow cytometry that is carried out to characterize the expression of immune-related markers in hAFS cells. We identified cells expressing some specific epithelial markers like Pan-Cytokeratins K15, K19, CD49 and P63 (Figure 2) indicating that hAFS cells have the po-

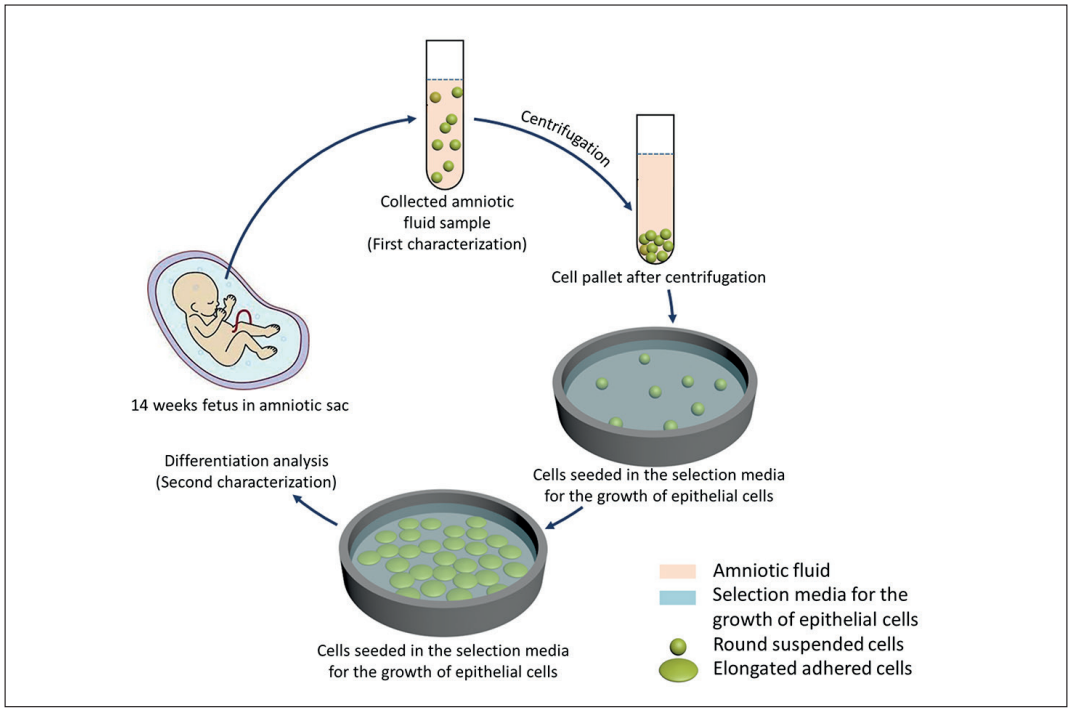

Figure 1. Flowchart of the analysis performed.

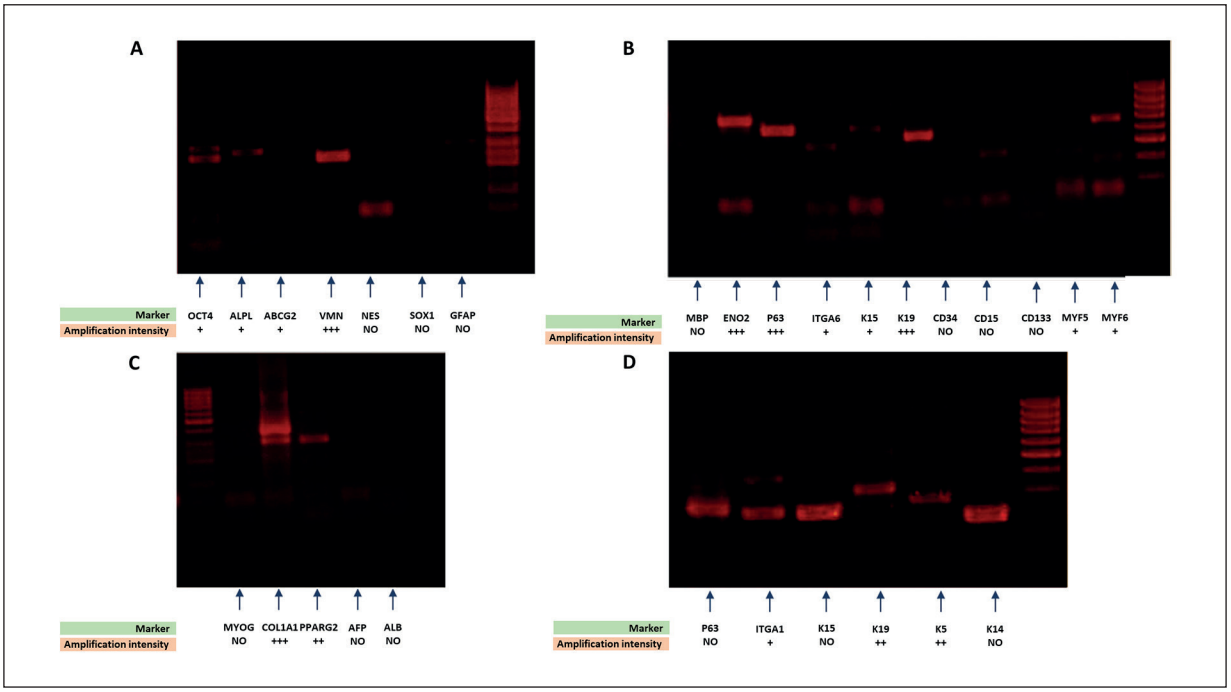

Figure 2. A-C, RT-PCR results of the amniotic fluid cells before the culture in the keratinocytes selective medium. **D,** RT-PCR results of the amniotic fluid cells after 20 days in selection medium, where they have differentiated as keratinocytes.

tential to differentiate into keratinocytes, which represents a type of epithelial cell. The criteria used to evaluate the expression of these markers were immunofluorescence and the presence of mRNA *via* RT-PCR. After 20 days in selection medium most of these cells tested positive for the keratinocytes specific markers but tested negative for stem cells specific markers. Control cultures did not result positive for keratinocyte differentiation markers.

Morphology and Phenotype Identification

To stimulate differentiation into keratinocytes, hAFS cells were grown in the selection medium for 15-20 days and after 20 days the stem cells confluence percentage was 90%.

Identification by Immunohistochemical/ immunofluorescence Staining

Immunohistochemical/immunofluorescence staining with DAPI (4′,6-diamidino-2-phenylindole) of amniotic fluid cells with Pan-Cytokeratin and Anti-ITGA6 antibody after 20 days in selection medium revealed their differentiation into keratinocytes (Figure 3).

Discussion

In the present work, we were able to test *via* RT-PCR the cells from the amniotic fluid for a large number of cell markers (Figure 1); the results show that the amniotic fluid contains cells expressing the markers specific for epithelial stem cells (Figure 2). Whereas, by immunofluorescence we identified the presence of cells expressing markers specific for keratinocytes like p63, CD49, K15, K19 (Figure 3). Out of these cell markers, p63 is strongly expressed in the basal layer of epidermis with great clonogenic and proliferative capacity and is able to induce hyperproliferation[12]. P63 also plays the role of a molecular switch needed for epithelial stratification initiation during embryogenesis. Moreover, p63 might sustain the basal keratinocytes proliferative potential in mature epidermis[13]. Similarly, K15 marker also seems to be expressed in the epidermal stem cells and in the basal cells[14]. hAFS cells also express the genes of epithelial stem cell marker K19 and β1-integrin, thus indicating their epithelial origin. K19 and K15 in combination are significant markers for the routinely monitoring of epidermal homeostasis and the indirect analysis of the engineered skin self-renewing potential[15]. Moreover, Davydova et al[16], in their

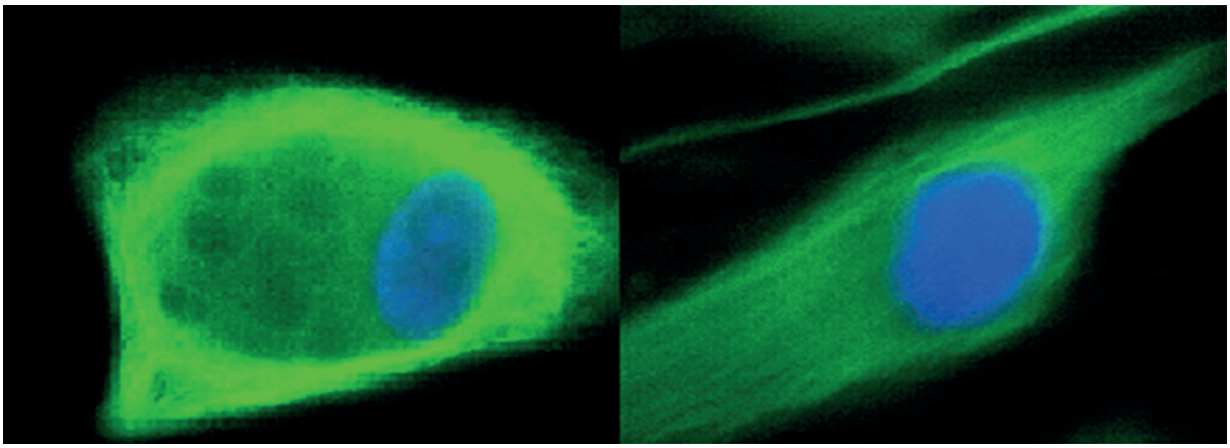

Figure 3. Immunohistochemical/immunofluorescence staining with DAPI results of amniotic fluid cell with Pan-Cytokeratin and Anti-ITGA6 antibody after 20 days in selection medium and after their differentiation for keratinocytes.

research study, isolated the AFSCs from 3 amniotic fluid samples and reported that these putative progenitor/stem cells from amniotic fluid have the ability of continuous proliferation *in vitro*. AECs also express CD49 adhesion marker, typically expressed in mesenchymal stem cells. Previous research studies have revealed that after culturing the subset of cells expressing the CD49 marker for 35 days, they still retained their regenerative abilities in *in vivo* xenotransplants. These studies established the existence of a subpopulation of multipotent cells that maintain the strong epithelial induction[17]. Thus, the presence of these specific markers in amniotic fluid cells in this study establishes the existence of the precursors of the epithelial cells that differentiates into keratinocytes and could be used in tissue engineering for the treatment of wounds[17].

Furthermore, in this study we have cultivated the cells in selection medium for the growth and differentiation of keratinocytes and we managed to obtain a culture that reached the confluence in 15-20 days, thus enabling us to develop many subcultures and suggesting that large quantities could be produced. Thus, there could be enough cells for clinical treatment of skin injuries. Taken together, the present study identifies AFS cells as a source of keratinocytes (Figure 4) that are able to form an epidermis and make these cells a potential resource for patients requiring urgent treatment of a large area of damaged skin. These results have provided very compelling evidence about the ample presence of AESCs in amniotic fluid which could further be differentiated into keratinocytes (Figure 4). The immunomodulatory and the high proliferation capacity of AFS cells suggests that they could be used as an "off-the-shelf" cell therapeutic product for healing wounds[7].

The origin of hAFS cells and their biological characteristics suggest that they possess unique epidermal regenerative potential. The presence

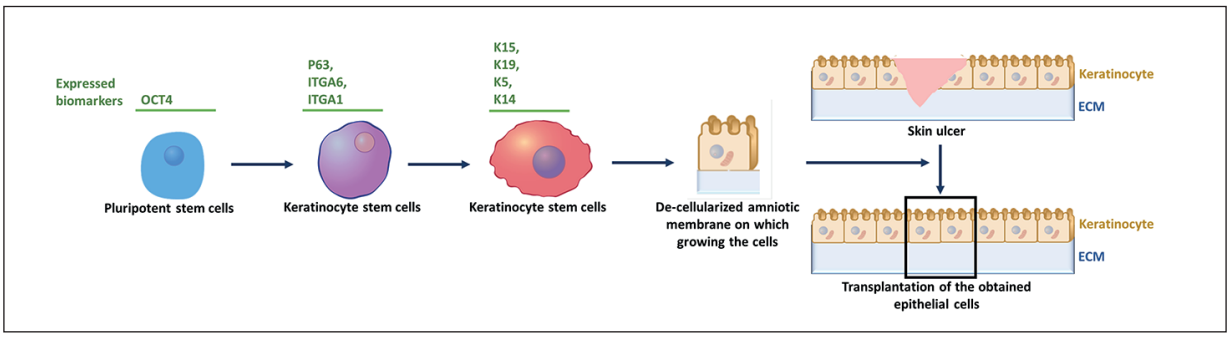

Figure 4. Differentiation steps from pluripotent stem cells to keratinocyte transplantation for wound repair.

of cells derived from the skin and from the fetal respiratory, gastrointestinal and urinary tracts implicates that hAFS could be used for seeding the skin during wound repair. In addition, it has been established that hAFS cells could generate ectodermal cells expressing the epithelial stem cell marker genes like K19 and β1-integrin[18,19]. Interestingly, in the context of skin transplantation, hAECs exercise several immunosuppressive activities[20] that may be associated with anti-rejection properties for the transplanted skin. In fact, the cells in the amniotic fluid may possess an immune-privileged status, as fetal cells during development retain mechanisms to escape the destruction by the maternal immune system and maintain the feto-maternal tolerance during the pregnancy[8]. For instance, they inhibit the proliferation of B-and T-cells and suppress the pro-inflammatory properties of macrophages, monocytes, natural killer cells, neutrophils and dendritic cells, while stimulating the induction of regulatory T-cells and the anti-inflammatory macrophages M2[20]. Further studies have demonstrated that cultured hAECs could be transplanted successfully to the damaged rabbit cornea without tissue rejection during ten days of observation. This may be due to the fact that hAECs does not express MHC class I antigens. This confers a reduced rejection rate by the host immune system[21]. On the other hand, hAECs express immunoregulatory molecules such as HLA-G and CD95[22]. Thus, AECs secreted factors inhibit the innate and the adaptive immune systems. The inflammation modulation by amniotic cells is considered a key element to initiate the tissue repair, by reducing the proinflammatory signals and increasing the anti-inflammatory immune components like M2-macrophages[23]. In addition, hAFS cells are able to reduce IL-6, IL-1β, Cox2, Mac3 and TNF-α expression, leading to moderate inflammatory response and producing a better microenvironment that facilitates wound repair[24].

Conclusions

The characteristics of hAESCs and amniotic membrane, including low immunogenicity, high proliferative potential, high differentiation potential, decreased inflammatory cytokine production, easy accessibility and non-invasive application procedure make them a potentially ideal cell type for utilization in regenerative medicine. Furthermore, these hAFS cells could be easily grown on decellularized matrix of amniotic membrane and transplanted for the treatment of skin wounds and burns[10] (Figure 4).

In conclusion, in the present study we were able to isolate committed stem cells towards keratinocytes from the amniotic fluid and found that they can be expanded in culture maintaining the stemness status for a sufficient time for possibly recreating artificial tissues for wound and ulcers repair. In the future, collecting amniotic liquid and membrane at birth could allow to store in biobanks the tissue and use it when needed for *in vitro* and auto-transplantation treatment of skin wounds.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We appreciate the assistance in scientific services provided by Dr. Narendra Kale.

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