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Growth and endothelial differentiation of adipose stem cells on polycaprolactone

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Abstract: Adipose tissue is a readily available source of multipotent adult stem cells for use in tissue engineering/regenerative medicine. Various growth factors have been used to stimulate acquisition of endothelial characteristics by adipose-derived stem cells (ADSC). Herein, we study the growth and endothelial differentiation potential of ADSC seeded onto a porous polycaprolactone (PCL) scaffold. The objective of this study is to demonstrate that PCL is a good material to be used as a scaffold to support reconstruction of new endothelial tissue using adipose stem cells. We found that undifferentiated ADSC adhere and grow on PCL. We show that, after culture in endothelial differentiation medium, ADSC were positive to LDL uptake and expressed molecular markers characteristic of endothelial cells (CD31; eNOS and vWF). In addition, our study defines the time required for the differentiation of ADSC directly onto PCL. This study suggests that PCL can be used as a scaffold to generate endothelial tissue *in vitro*. PLC has excellent mechanical properties and a slow degradation rate. Moreover, based on our results, we propose that PCL could be used to graft scaffolds coated with endothelial cells derived from ADSC stem cells. Endothelial cells-coated PCL could find several applications to replace damaged area of the body; for example, a possible use could be the generation of vascular grafts. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 100A: 543–548, 2012.

Key Words: stromal cells, human adipose stem cells, endothelial differentiation, vascular grafts, stem cells

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INTRODUCTION

Endothelial damage is a prominent feature of a wide variety of diseases, including erectile dysfunction, coronary artery disease,[1] and diabetes mellitus.[2] One of the strategies to restore endothelial function is "therapy with stem cells," in which stem cells promote the formation of new endothelial tissue. However, cell proliferation and differentiation resulting in tissue regeneration for some diseases, is difficult without using a scaffold. Recent studies show that isolated cells are hardly able to organize themselves spontaneously to form complex tissue structures in the absence of a three-dimensional matrix that guide and stimulate their activities.[3] In many of the examined cases the cells in culture tend to multiply and to proliferate only in two dimensions. The three-dimensional tissue regeneration requires a support (scaffold) that emulates the extracellular matrix for cell organization into complex structures. The role of the scaffold is to induce tissue regeneration by providing a "temporary guide" for cell growth, under appropriate culture conditions for cell differentiation.[4]

In addition, for clinical applications the use of a scaffold could be very important for the following reasons: it works as an anchor for cells, decreasing the possibility of cell migration from the place of destination; it allows to solve structural problems (such as arterial bypass) and allows tissue growth and differentiation *in vitro*, with greater control by the operator and subsequent transplantation *in vivo*.

In this work we decided to use the polycaprolactone (PLC) for the study of endothelial differentiation because it has excellent mechanical properties and slow degradation; thus it might be an optimal candidate to be used *in vivo* for tissue transplantation.

The PCL is a highly biocompatible aliphatic polyester obtained by the polymerization to open-loop of ε-caprolactone. The PCL is a material that can support the creation *in vitro* of polymer-cell complex with subsequent implantation *in vivo*. This allows the organism the remodelling of the scaffold and the creation of the complex polymer/cell/tissue.

In this study, we used adipose tissue-derived stem cells (ADSC) isolated from the stromal vascular fraction (SVF). These cells were molecularly characterized with a panel of mesenchymal differentiation markers according to current literature.[5–7] Phenotypically, hMSCs have been defined as

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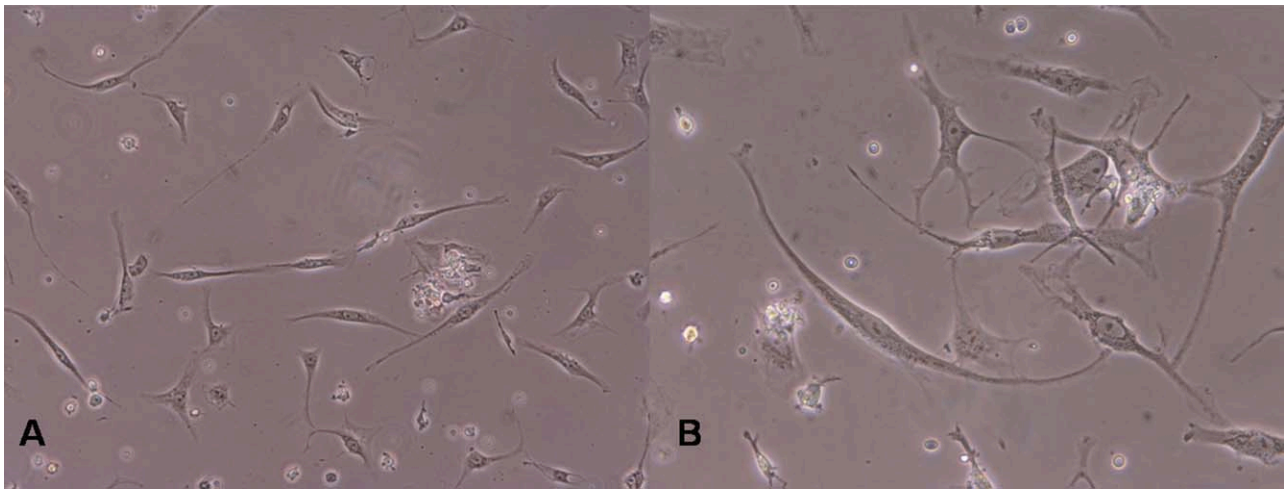

FIGURE 1. Panel show ADSC cells after 24 h (photo A, ×200) and (photo B, ×400). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$CD44^+$, $CD105^+$, and negative for hematopoietic lineage markers.[8] When cultured in the presence of endothelial growth factors, hMSC showed a strong increase in the expression of endothelial specific markers such as CD31, vWF, and eNOS.[9] ADSC are morphologically similar to hMSCs obtained from other tissues by isolation and culture.[5] It has been reported that human embryonic stem cells can differentiate in endothelial cells.[10] Some authors also showed that bone marrow-derived mesenchymal stem cells and adipose tissue-derived stromal cells had the same ability to differentiate in endothelial cells when cultured in differentiation medium.[11–15] The aim of this study was to demonstrate that PCL is a good material to use as a support (scaffold) for the reconstruction of new endothelial starting from adipose stem cells.

MATERIALS AND METHODS

Scaffold

Polycaprolactone (PCL) scaffolds (3D Insert-PCL) were obtained from Biotek (North Brunswick, U.S.A). The pore size was 300 μm (diameter). Round PCL dishes (diameter 10 mm, thickness 2 mm) were used in this study. Cells were seeded at a density of 2×10^5/PCL scaffold.

Selection of adipose tissue donors

Adipose tissue samples were obtained from the subcutaneous abdominal region of three women in the age range 30–40 years, undergoing elective abdominal surgery. Patients suffering from an inflammatory disease or tumors were excluded. The operations were carried out for the following reasons: one for herniotomy, two for cholecystectomy. All subjects were of normal weight as defined by guidance of the World Health Organization. Body mass index (IMC) was between 18.5 and 24.9 kg m^{-2}. All patients appeared normal at a physical examination and routine laboratory tests were within normal limits. None of the subjects was under a reduction diet at the time of the study.

ADSC cell isolation and expansion

Five grams of tissue were washed four to five times with phosphate-buffered saline (PBS), suspended in an equal volume of PBS supplemented with 1% bovine serum and 0.1% collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) and incubated at 37°C under continuous shacking for 60 min. The samples were then centrifuged for 5 min at 300*g* at room temperature. The supernatant, containing mature adipocytes, was discarded; the pellet was washed with PBS and centrifuged at 150*g* for 5 min at room temperature. Pellet was resuspended in DMEM-Ham's F-12 medium supplemented with 5% FBS [Gibco, Invitrogen], 100 U penicillin/100 μg, streptomycin/0.25 μg. Live nucleated cells were counted using the Trypan Blue exclusion Test followed by chamber counting. The cells were plated immediately in T25 flasks at a density of 1×10^5 cells cm^{-2}. After 48 h of incubation at 37°C and 5% CO_2, the cultures were washed with PBS to eliminate the nonadhesive cells; adherent cells were maintained in proliferation medium until they reached confluence (ca. 4 days). For culture expansion, cells were harvested with 0.05% trypsin 0.53 mM EDTA (Gibco-Invitrogen) and replated at a density of 5000 cells cm^{-2}.

Composition of proliferation and endothelial medium

Proliferation medium is composed of: DMEM-Ham's F-12 medium supplemented with 5% FBS [Gibco, Invitrogen], 100 U penicillin/100 μg, streptomycin 0.25μg, 1% sodium pyruvate, and 1% L-glutamine. Endothelial differentiation medium is composed of: RPMI 1640 medium supplemented with 2% FBS, 0.1% FGF-2, 0.1% ascorbic acid, 1% sodium pyruvate, and 1% L-glutamine (GIBCO).

Cell viability and proliferation

Cell viability and proliferation was studied by a modified MTT method according to the manufacturer's instructions (Dojindo Molecular Technologies-U.S.A). Quantification of mitochondrial dehydrogenase activity was obtained via the

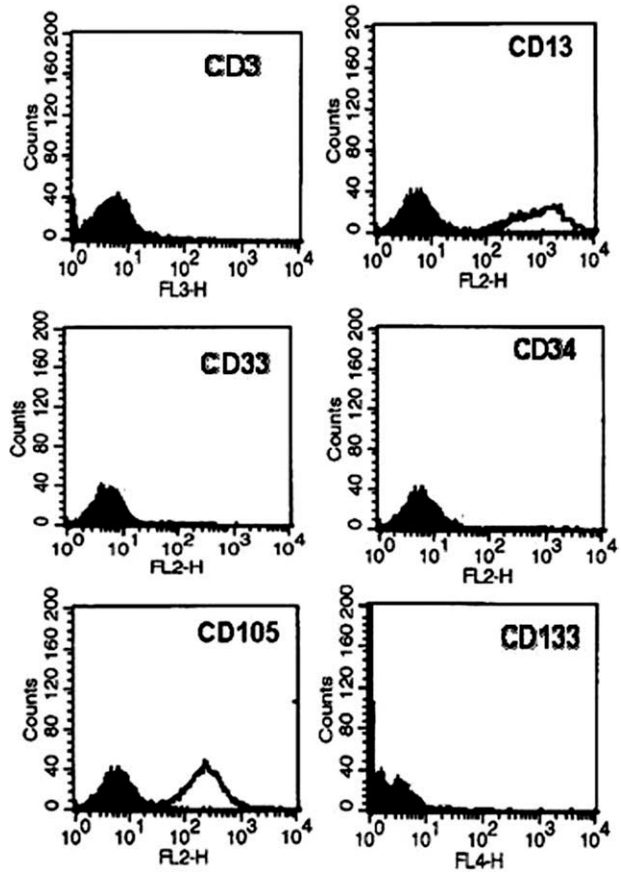

FIGURE 2. Panel shows flow cytometry of ADSC. The open histogram indicates the positive stained cells, whereas the filled histogram indicates the isotype-matched monoclonal antibody control.

enzymatic conversion of MTT tetrazolium water soluble salt to a coloured formazan product. Because reduction of MTT occurs only in metabolically active cells, the level of activity was a measure of cell viability. ADSC were seeded on PCL scaffolds at density of 2×10^5 cells/PCL and incubated in either proliferative or endothelial differentiative medium. Cell viability was detected at 3, 14, 21, and 28 days at 37°C and 5% CO_2. Each experimental point was performed in triplicate.

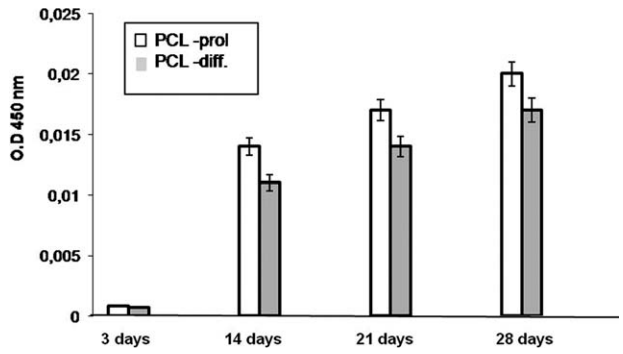

FIGURE 3. Viability assay of ADSC cells seeded on PCL in proliferation and differentiation medium.

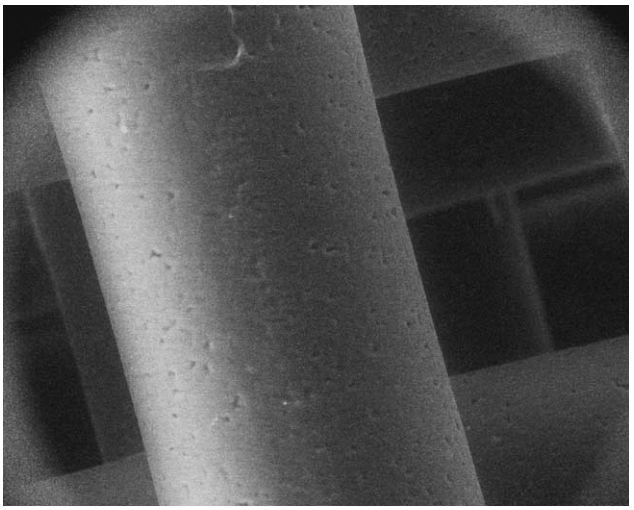

FIGURE 4. ESEM image of scaffold without cells (×200).

Flow cytometry

ADSC were cultured in proliferation medium for 20 days prior to analysis (16). Cells were detached using 10 m*M* EDTA in phosphate-buffered saline (PBS), counted and washed in 0.1% BSA in PBS. At least 200,000 cells (in 150 μL PBS/0.5% BSA) were incubated with fluorescent-labelled monoclonal antibodies (diluted 1/15, at 4°C for 30 min, in the dark). Cells were labeled with the following antibodies: CD3-APC, CD13-APC, CD33-APC, CD34-PE, CD45-FITC, (all from Becton Dickinson [BD], San Jose, CA); CD105 PE (Serotech, Kidlington, UK); CD133 PE (Miltenyi, Bergisch Gladbach, Germany); mouse isotype antibodies served as respective controls (BD). After washing steps, the labeled cells were analyzed by flow cytometry using BD FACS Calibur equipped with ModFit LT software.[5]

Environmental scanning electron microscopy

ESEM cell morphology studies were performed on cells seeded on the PCL matrix. ADSC were plated on PCL scaffolds (3D culture) at a density of 2×10^5 cells/scaffold (diameter 10 mm, thickness 2 mm) and incubated in either proliferation or endothelial differentiative medium for 7, 14, and 28 days at 37°C and under 5% CO_2 95% air. At the end of the experiments, cultures were rinsed gently three times in PBS, fixed with 2.5% glutaraldehyde/PBS, v/v) then rapidly dipped in deionized water to avoid crystal precipitation (PBS residue). Samples were immediately mounted onto holders, and observations were performed with an ESEM Quanta 200 FEI electron microscope. Environmental conditions in ESEM modality were 75% relative humidity, 5°C, and 4.90 Torr.

Immunocytochemistry and fluorescence microscopy

ADSC were plated on PCL scaffolds (3D culture) at a density of 2×10^5 cells/scaffold (diameter 10 mm, thickness 2 mm) and incubated in either proliferation or endothelial differentiative medium for 7, 14, and 28 days at 37°C and 5% CO_2. The next day, the cells were rinsed with PBS and fixed

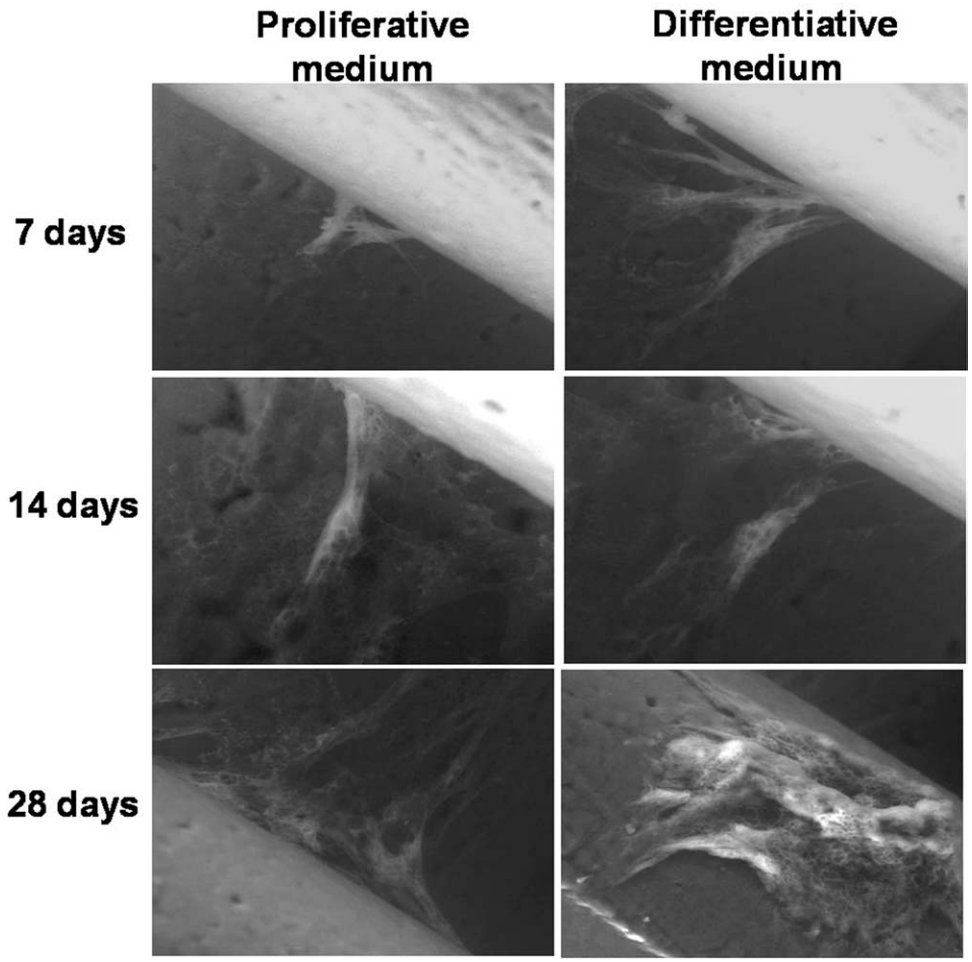

FIGURE 5. Panel show ADSC cells on scaffold after 7–14–28 days growth in proliferation medium and differentiation medium.

with ice-cold methanol for 5 min. After washing with PBS, cells were permeabilized with 0.1% Triton-X100 in PBS for 10 min. After another PBS rinse, cells were blocked with 1% BSA in PBS for 20 min. After appropriate washes, cells were incubated with anti-CD31 monoclonal antibody (180-820, Ancell Corporation, USA), anti vWF antibody (AB7356 Millipor, MA) and anti-eNOS polyclonal antibody (210-505/1-R100, Alexis, Axxora, USA) diluted 1/200 in 1% BSA for 1 h. After three washes in PBS (5 min each), alexa fluor 488 goat anti-mouse IgG (A11001, Molecular Probes) and Texas Red goat anti-rabbit IgG (T-2767, Molecular Probes) diluted 1/1000 in 1% BSA were added for 1 h. For nuclei staining, cells were washed with PBS and incubated with To-Pro 3 iodide (T-3605, Invitrogen) diluted 1/1000 in 1% BSA, for 1 h. Stained cells were examined with Nikon Confocal Microscope C1 equipped with a EZ-C1 Software for data acquisition.

Low-density lipoprotein (LDL) uptake

ADSC were plated on PCL scaffolds (3D culture) at a density of 2×10^5 cells/scaffold (diameter 10 mm, thickness 2 mm) and incubated in either proliferation and endothelial differentiation medium for 7, 14, and 28 days at 37°C and under 5% CO_2 95% air. The next day, 10 mg mL^{-1} of acetylated low-density lipoprotein DiI complex (DiI AcLDL, Invitrogen corporation, Carlsbad, CA) was added to the culture medium. The next day, after the medium was removed, the cells were washed three times, examined by phase-contrast and fluorescence microscopy, and photographed.

RESULTS

Characterization of ADSC cells by flow cytometry and fluorescence microscopy

Five grams of adipose tissue from the subcutaneous abdominal region was used to isolate ADSC. After collagenase digestion and 24 h of incubation in proliferation medium, ADSC cells were obtained (Fig. 1). A cell aliquot was cultured for 20 days (nine passages) for immunophenotype stabilization[16] and observed daily to assess morphological homogeneity. Immunological characterization, using a panel of surface markers, revealed that ADSC were negative for CD3, CD14, CD33, CD34, CD45, and CD133 while they were positive for CD13, CD44, and CD105 (Fig. 2).

Growth of ADSC cells on PCL

Figure 3 shows that proliferation of ADSC cells on PCL scaffolds increases over time. We observed an increase of cell viability both in cells cultured in proliferation media and in cells

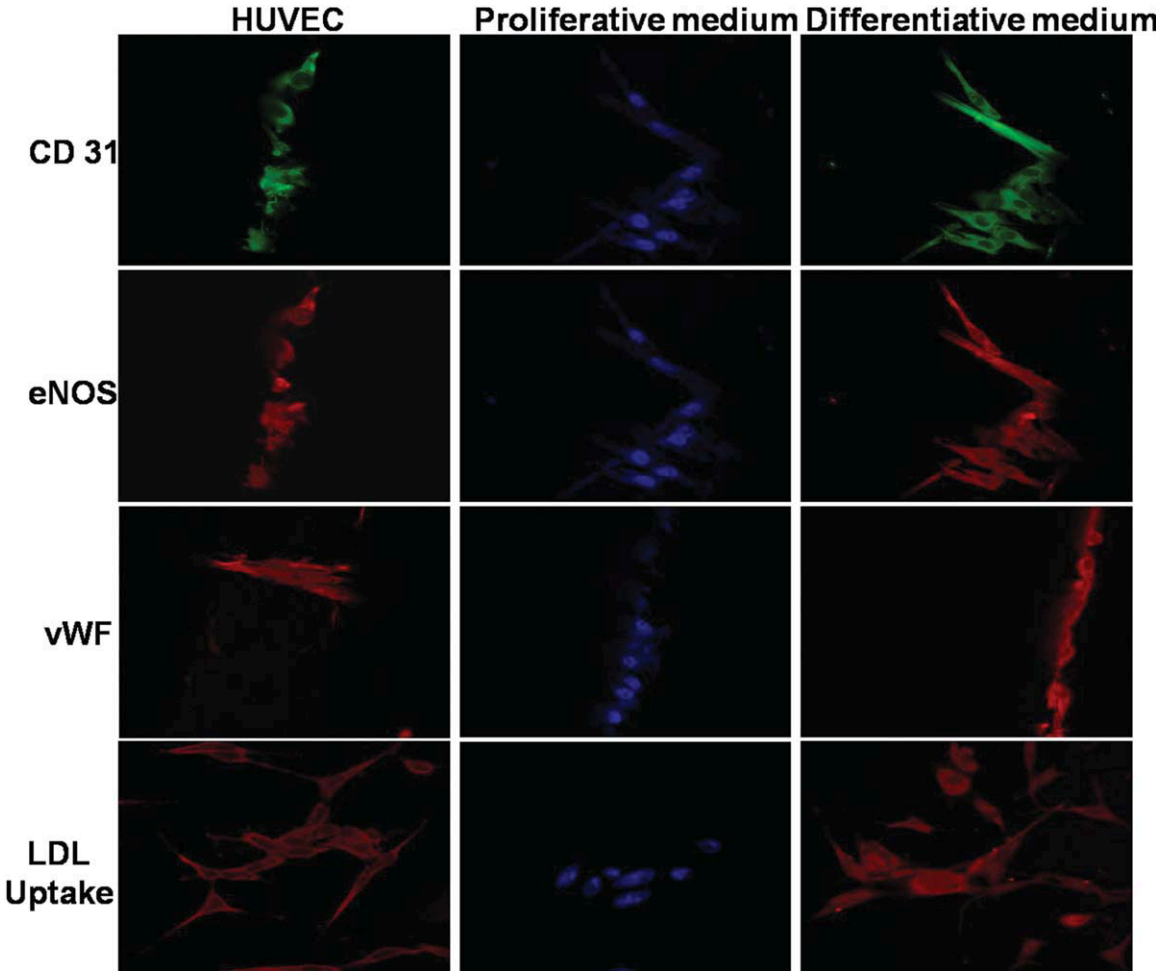

FIGURE 6. Panel shows expression of endothelial markers and LDL uptake. HUVEC (human umbilical vein endothelial cells) were used as positive control. ADSC cells grown in proliferation medium are negative for CD31, vWF eNOS, and LDL uptake, while cells grown in differentiation medium and HUVEC show the expression of CD31,vWF eNOS, and also LDL uptake. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cultured in endothelial medium. At various time points, all data showed that cells are metabolically active and grow on PCL.

ADSC cells on PCL observed with ESEM

ESEM is an electron optical instrument which enables the examination of the surface of soft, hydrated, unfixed, uncoated, and electrically insulated specimens with a depth of field and magnifications that are typically achieved by CSEM. Using ESEM technology, we studied the interactions of ADSC with the PCL matrix. Electron micrographs show, PCL scaffold without cells (Fig. 4) and interaction of ADSC with the PCL surface after 7, 14, and 28 days of incubation in proliferation and differentiation media (Fig. 5). These images show that ADSC cells adhere to the PCL and remain adherent for 7, 14, and 28 days in proliferation and differentiation medium.

Endothelial characteristics

Fluorescence microscopy showed that ADSC grown on PCL, in endothelial differentiation medium, express endothelial specific markers: CD31, vWF, and eNOS (Fig. 6) at 14 day. The same results were obtained after 28 days (data not shown). LDL uptake assay showed that ADSC grown on PCL were capable of LDL uptake at 14 day (Fig. 6). The endothelial specificity of these two assays was supported by positive results with HUVEC cells (Fig. 6) and negative results with ADSC grown in proliferation medium (Fig. 6).

DISCUSSION

Endothelial damage is a prominent feature of a wide variety of diseases, including erectile dysfunction, coronary artery disease,[1] and diabetes mellitus.[2] One of the strategies to restore endothelial function is "therapy with stem cells," in which stem cells promote the formation of new endothelial tissue. However, cell proliferation and differentiation, resulting in tissue regeneration for some diseases, are difficult without using a scaffold. Recent studies show that isolated cells are not able to organize themselves spontaneously to form complex tissue structures in the absence of a three-dimensional matrix that guide and stimulate their activities.[3] In many of the examined cases, cells in culture tend to multiply and to proliferate only in two dimensions. The

three-dimensional tissue regeneration requires a support (scaffold) that emulates the extracellular matrix for the organization of cells into complex structures. The role of the scaffold is to induce tissue regeneration by providing a "temporary guide" for cell growth, under appropriate culture conditions for cell differentiation.[4]

Immunophenotypical characterization clearly revealed the stem origin of ADSC: in fact, these cells are strongly $CD13^+/CD44^+/CD105^+$ and $CD3^-/CD14^-/CD33^-/CD34^-/CD45^-/CD133^-$ in agreement with literature.[5] The capacity to differentiate toward an endothelial phenotype is a characteristic of stem cells[11–13,15] and our results confirm these data. However, the main purpose of this study was the evaluation of the endothelial potential of ADSC seeded onto PCL scaffolds. First, we verified the endothelial potential of ADSC in 2D culture conditions both in proliferation and differentiation media (data not shown). In proliferation medium, no endothelial differentiation was observed during the entire experimental time (28 days), further confirming the stem origin of isolated cells. In differentiation medium, fluorescence microscopy confirmed the endothelial potential of these cells.

The data obtained allowed us to determine the time required for the differentiation of ADSC in endothelial cells on PCL. We made several experimental time points (7, 10, 12, 14 day) and observed positive findings for endothelial markers at 14 days of incubation.

This study demonstrates, for the first time, that ADSC cells can be induced to differentiate in endothelial cells directly on PCL matrix.

Finally, cell-scaffold interactions were studied using ESEM technology. ESEM is an electron optical instrument which permits the examination of the surfaces of soft, hydrated, unfixed, uncoated, and electrically insulating specimens with a depth of field and with magnifications comparable to conventional SEM.[17] The high water vapor pressure in the ESEM analysis chamber allows high resolution imaging of wet or hydrated specimens, obviating the need for sample drying and the associated artifacts.[18] Using this technology, we demonstrate that ADSC cells interact with the PCL surface when grown in proliferation and differentiation media, and that their morphology is typical of metabolically active cells.

CONCLUSIONS

This study suggests that PCL can be used as scaffold for the creation of endothelial tissue *in vitro*. PLC has excellent mechanical properties and a slow degradation. On the basis of our results, we suggest its use for the creation of scaffolds coated with endothelial cells derived from ADSC stem cells. Reconstruction of PCL-endothelial tissue could find several applications involving implantation in the damaged area of the body. A possible use could be for example the creation of vascular grafts.

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