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Efficient differentiation of CD14$^+$ monocytic cells into endothelial cells on degradable biomaterials

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Abstract

Vascular tissue engineering aims at creating self-renewing, anti-thrombogenic, vascular grafts, which can be based on endothelial progenitor cells (EPC). EPC harbor essential features such as plasticity and longevity. Unfortunately, the archetype CD34$^+$ EPC is rare in peripheral blood. Monocytes, i.e. CD14$^+$ cells also have the ability to differentiate into endothelial-like cells and are by far more abundant in peripheral blood than are CD34$^+$ EPC. Therefore, CD14$^+$ cells would seem appropriate candidates for tissue engineering of small-diameter blood vessels. In this study, we investigated the differentiation of CD14$^+$ cells on three biodegradable biomaterials under angiogenic conditions. Morphological analyses, gene transcript analyses, endothelial marker (i.e. VE-Cadherin and eNOS) and macrophage marker (i.e. CD68 and CD163) expression analyses, revealed that a small fraction (15–25%) of cultured CD14$^+$ cells differentiated into macrophages after 21 days of culture. The majority of CD14$^+$ cells ($>75\%$) differentiated into endothelial-like cells (ELC) on all biomaterials used. The expression of endothelial markers was similar to their expression on HUVEC. Since CD14$^+$ cells are present in high numbers in adult peripheral blood, easy to isolate and because they easily differentiate into ELC on biomaterials, we conclude that CD14$^+$ cells are a suitable cell source for progenitor-based vascular tissue engineering.

Keywords: Cell culture; Endothelial cell; Monotype; Polycaprolactone; Polyurethane; RGD peptide

1. Introduction

Cardiovascular disease (e.g. coronary artery disease and peripheral artery disease) is the leading cause of mortality in the industrialized nations (www.who.org). Most common treatments are anti-thrombogenic, and anti-atherosclerotic therapies, surgical replacement of affected blood vessels and bypass surgery for treatment of coronary artery disease. For replacement surgery, the use of autologous vessels is the gold standard; however, many patients lack suitable vessels due to prior use or poor graft quality. In these patients, synthetic prostheses are used. Synthetic non-degradable polymers (e.g. pTFE) are applied successfully for replacement of large-diameter vessels. However, synthetic small-diameter (ID$\leqslant$6 mm) replacement vessels occlude thereby causing prosthetic failure [1,2].

The ideal small-diameter anti-thrombogenic replacement vessel would consist of a biodegradable scaffold (biomaterial) that is, on the luminal side, covered with biologically active endothelial cells (EC), in the middle layer covered with vascular smooth muscle cells (SMC), and has an outer fibroblast layer. The biomaterial ideally would support cell adhesion, cell function and growth, while degradation of the biomaterial, together with the generation of a new basal lamina by the EC and SMC, would convert the implantable vessel into a fully functional native vessel in vivo. However, a major limitation in the development of small-diameter vessels has been the application of autologous vascular EC. These EC are terminally differentiated and thus have a

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definite lifespan. This may cause premature vessel dysfunction due to detachment of EC, which causes coagulation. A more primitive source of EC would, therefore, be preferable for vascular tissue engineering [3–5].

Asahara et al. [6] described the isolation of putative endothelial progenitor cells (EPC) from human peripheral blood mononuclear cells (MNC). The use of undifferentiated progenitor cells with a strong capacity to proliferate in small-diameter blood vessel tissue engineering (BVTE) could facilitate the engineering of an adaptive small-diameter blood vessel that is anti-thrombogenic and grows and remodels over time.

The $CD34^+$ hematopoietic stem cell is often referred to as the archetype EPC, because it can contribute to the repair of vascular damage in vivo [7–9]. Recently, it has been shown that $CD14^+$ cells can also differentiate into an endothelial phenotype in vitro [10–13]. Because the $CD14^+$ cells are far more frequent in peripheral blood than $CD34^+$ cells (10–20% vs. 0.01–0.1% of the MNC fraction), $CD14^+$ cells would seem more appropriate candidates for small-diameter BVTE. However, $CD14^+$ cells are better known for their capacity to differentiate into macrophages in vitro and in vivo, also on biomaterials. As yet, it is unknown whether purified $CD14^+$ cells can also acquire an EC-like phenotype (ELC) when cultured on biomaterials under angiogenic conditions.

In the current study, we investigated the differentiation of $CD14^+$ cells into ELC on three different degradable biomaterials under angiogenic culture conditions (i.e. EndoCult™ Medium) in time. Novel supramolecular biomaterials based on ureido-pyrimidinone (UPy) end-functionalized oligocaprolactone (PCLdiUPy) with and without UPy-modified GRGDS-peptides (PCLdiUPy/UPy-GRGDS) [14,15], as well as PCL-based polyurethane (PU) [16], are non-toxic, non-immunogenic, and biodegradable. Comparison of these three materials can, therefore, give valuable information concerning material design and cellular behavior. To assay differentiation, *reverse transcriptase* polymerase chain reaction (RT-PCR) and immunofluorescence microscopy analysis for expression of EC markers eNOS and VE-Cadherin (CD144), and macrophage markers CD68 and CD163 were performed. Second, since cell–matrix adhesion, cell cycle control, cell survival, and endothelial differentiation is integrin dependent [17,18], we examined the expression of EC-related integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$.

2. Materials and methods

2.1. Preparation of polymer films

The UPy-modified PCLdiUPy ($M_n = 2100$ g/mol), as well as the UPy-GRGDS peptide were synthesized as described by Folmer et al. [19] and Dankers et al. [14]. The polymer films were prepared according to the following procedure. A PCLdiUPy solution in THF was drop cast on glass coverslips, after which the polymer films were dried at 37 °C (PCLdiUPy). The bioactive polymer–peptide film was prepared by subsequent drop casting of the peptide solution (4 mol%) on the dried polymer film. The bioactive film was dried at 37 °C. Additionally, 50 μL THF was put on the polymer–peptide film, which was dried at 37 °C again. All blends on the glass cover slips were dried in vacuo for 2–3 days at 35–40 °C. The samples were sterilized under UV for at least 3 h, prior to use. Synthesis of the PCL-based PU (M_n(PCL) = 1600 g/mol) was performed as described previously [16]. Coverslips were prepared by dissolving PU in dioxane at 80 °C for 2 h. The polymer solution was cast on Thermanox® coverslips (Nunc Brand Products, Roskilde, Denmark) and the films were dried overnight at room temperature. The PU films were placed in a Soxhlet extraction apparatus utilizing hexane as the extraction solvent in order to extract low molecular weight compounds. After 15 h of extraction, the films were dried in vacuo for 12 h at 40 °C. After casting of the polymers, fibronectin (Fn) coating was performed. In short, coverslips were incubated with 1% recombinant human Fn (Sigma, St. Louis, MO) in PBS at room temperature for 1 h. Thereafter, Fn was fixed using a 0.5% glutaraldehyde (GA) solution at room temperature for 15 min. Incubation was followed by extensive washing with culture medium to remove remaining GA.

2.2. Cell isolation and culture

Human umbilical vein EC (HUVEC) were a kind gift from Prof. Dr. G. Molema (Dept. Med. Biol., UMC, Groningen, The Netherlands). MNC were isolated from buffy coats obtained from healthy donors (Sanquin, Groningen, The Netherlands) using density gradient centrifugation on lymphoprep (Nycomed Pharma, Roskilde, Norway). Monocytic cells (i.e. $CD14^+$ cells) were isolated by magnetic bead separation. Briefly, 1×10^7 MNC were labeled with 20 μL MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated in a volume of 80 μL PBS supplemented with 0.5% fetal calf serum (FCS) and 2 mM EDTA on ice for 15 min. Cells were washed with buffer and resuspended in 500 μL PBS. The cell suspension was separated on a LS^+/VS^+ column placed in a strong magnetic field. $CD14^+$ cells were retained in the column, washed extensively, and flushed out with buffer after removal of the column from the magnet. Aliquots of 1×10^6 cells were labeled with PE-conjugated mAb to human CD14 and with FITC-conjugated mAb to human CD34 (both 1:10 in 2% BSA/PBS; IQ Products, Groningen, The Netherlands) on ice for 15 min. Flow cytometric analysis revealed an average purity of $95.4 \pm 3.9\%$ ($n = 4$) for isolated $CD14^+$ cells. HUVEC (30,000 cells/cm^2) and $CD14^+$ cells (150,000 cells/cm^2) were seeded on Thermanox® coverslips coated with polymer and 1% Fn. Control coverslips were coated with 1% Fn only. Cells were cultured in EndoCult™ Medium supplemented with 20% FCS (StemCell Technologies Inc., Vancouver, Canada). Medium was refreshed every 3rd day, and non-attached cells were removed from culture at day 7.

2.3. Analysis of cell adhesion and growth

After 24 h, 3 and 7 days of cell culture, non-adherent cells were removed by extensive washing with PBS. Subsequently, cells were fixed in 2% paraformaldehyde (PFA) in PBS at room temperature for 15 min. Fixed cells were stained with mAb to human Ki-67 as noted below (Section 2.5). Slides were mounted in Citifluor AP1 (Agar Scientific, Essex, UK) and examined by immunofluorescence microscopy using a Leica DMRXA microscope and Leica Software (Leica Microsystems, Wetzlar, Germany).

2.4. RNA isolation and RT-PCR

At days 0, 3, and 21, total RNA was isolated from approximately 150,000 cells using the Absolutely RNA Microprep Kit (Stratagene, Cedar Creek, TX), in accordance to the manufacturers' protocol. In short, a lysate was made of 150,000 $CD14^+$ cells and diluted with an equal volume of ethanol (70%). RNA was collected on an RNA binding filter by centrifugation. DNase treatment was performed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed