Biomaterials 150 (2018) 49–59

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Biofabricating atherosclerotic plaques: *In vitro* engineering of a three-dimensional human fibroatheroma model

Anna Mallone [a, *], Chantal Stenger [a], Arnold Von Eckardstein [b], Simon P. Hoerstrup [a], Benedikt Weber [a]

[a] Institute for Regenerative Medicine (IREM), University of Zurich, Zurich, Switzerland
[b] Institute of Clinical Chemistry, University Hospital Zurich, Zurich, Switzerland

ARTICLE INFO

Article history:
Received 2 August 2017
Received in revised form
11 September 2017
Accepted 27 September 2017
Available online 28 September 2017

Keywords:
Bioengineering
Biofabrication
Disease modeling
Tissue engineering
vi-SNE
Atherosclerotic plaque

ABSTRACT

Atherosclerotic plaques are cholesterol-induced inflammatory niches accumulating in the vascular sub-endothelial space. Cellular and extracellular composition of human plaques is maneuvered by local inflammation that leads to alterations in the original vascular microenvironment and to the recruitment of an invading fibrous layer (fibroatharoma). In the present study we introduce a bioengineered three-dimensional model of human fibroatheroma (ps-plaque) assembled with a tailored hanging-drop protocol. Using vi-SNE based multidimensional flow cytometry data analysis we compared the myeloid cell-populations in ps-plaques to those in plaques isolated from human carotid arteries. We observed that plasmacytoid and activated dendritic cells are the main myeloid components of human carotid plaques and that both cell types are present in the biofabricated model. We found that low-density lipoproteins affect cell viability and contribute to population polarization in ps-plaques. The current work describes the first human bioengineered *in vitro* model of late atherosclerotic lesion for the investigation of atherosclerosis aetiopathogenesis.

1. Introduction

Atherosclerosis is a life threatening vascular pathology characterized by the accumulation of a fatty plaque in the vascular sub-endothelial space [1]. Atherosclerotic plaque formation is influenced by the synergistic interplay of different risk factors such as sex, age, genetic predisposition, high blood pressure and high blood levels of low-density lipoprotein (LDL) cholesterol [2,3]. The formation of a well-structured plaque microenvironment results from the interplay of cholesterol-rich lipoproteins, endothelial cells, monocytes, macrophages, dendritic cells and fibroblasts in a complex matrix milieu [4]. The process of plaque formation is progressive and reversible [3,5] and can be counteracted by a reduction of the modifiable risk factors mentioned above or promoted by their persistence. Plaques are classified according to their stage of development and cellular components [4]. Among different plaque stages, the thin-cap fibroatheroma is the one most prone to rupture and to potentially cause thrombus formation and vessel obstruction [6]. The fibroatheroma is characterized by the presence of a necrotic core, macrophage-derived foam cells and dendritic cells all being enriched with cholesterol and embedded in a collagenous matrix surrounded by a thin layer of fibrotic cells [4,5]. The cellular mechanisms underlying plaque formation and regression have been investigated *in vivo* in both small and large animals, predominantly in hypercholesterolemic mice with knock-out of either Apoe or LDL-receptor [7–9] or non-human primates [10,11]. Despite the stunning contribution to the field, major differences in anatomy, lipoprotein profiles and inflammatory mechanisms hampered the translation of these results to the human pathophysiology. To overcome the translational gap, human cell-based co-culture *in vitro* models have been established and provided a first glimpse into the initial events of plaque deposition in humans [12–15]. To our knowledge, no human model of late stages of atherosclerotic plaque development does exist. In the present study we introduce a bioengineered *in vitro* model of fibroatheroma that we call pseudo plaque (ps-plaque). The ps-plaque architecture is characterized by a spheroid core of monocytes, macrophages and dendritic cells embedded in a collagenous and lipid-rich matrix, surrounded by a thin layer of myofibroblasts. In order to investigate

* Corresponding author. Institute for Regenerative Medicine (IREM), University of Zurich, Wagistrasse 12, WAD M180, Schlieren, Zurich CH-8952, Switzerland.
E-mail address: anna.mallone@irem.uzh.ch (A. Mallone).

https://doi.org/10.1016/j.biomaterials.2017.09.034

a possible source for model up-scaling we biofabricated ps-plaques using either blood-derived myeloid cells or cells from the thp-1 cell line. We explored the similarities in surface antigen expression patterns of myeloid populations from ps-plaques and human carotid plaques isolated from patients undergoing carotid endarterectomy. Additionally, we compared the expression levels of key pro-inflammatory and remodeling gene-targets in both plaque types. We found similarities in population distribution and gene expression profiles between ps-plaques and human samples. Finally, we used the ps-plaque to investigate the role of LDL in plaque population remodeling and cell viability. With this work we introduce a new technology for investigating atherosclerosis pathomechanisms, aiming at providing a novel human cell-based platform for drug design and high-throughput screening.

2. Materials and methods

2.1. Isolation of myeloid cells from blood

Myeloid cells were isolated from human blood using a double gradient centrifugation. The blood was provided by the Zurich blood bank (Blutspende Zürich – Nr.6676) and maintained at room temperature in slow rocking motion until processing. First, 20 ml of blood from each donor were diluted 1:2 with 1 × Phosphate Buffer Saline (PBS, Sigma) at room temperature and layered onto a Ficoll solution (1.077 g/ml, Sigma). Samples were then centrifuged at 400 g for 30min without break. Second, a 46% iso-osmotic Percoll gradient was performed to separate the lymphocytes from the peripheral blood mononuclear cells (PBMCs) as previously described [16]. Briefly, the buffy coat was re-suspended in 20 ml of xVivo15 chemically defined medium (Lonza) without red phenol and carefully layered on top of a Percoll solution prepared with 50% Roswell Park Memorial Institute (RPMI) medium with red phenol (Sigma), 46% Percoll (GE Healthcare) and 4% 1 × PBS (Sigma). The second gradient was centrifuged at 550 g for 30min without break and the white cell ring at the interphase was collected for further processing.

2.2. Myofibroblasts isolation

Human umbilical vein myofibrobalsts (HUVM) were isolated from human umbilical cords. The tissues were processed in accordance to the ethical permit released by the Kantonale Ethikkommission Zürich (KEK-Stv-21-2006). Briefly, umbilical cords were stored after labor at 4 °C in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) prepared with 10% Fetal Bovine Serum (FBS, Gibco), 1% GlutaMax (Gibco) and 1% penicillin/streptomycin (Penn/Strep, Gibco) for maximum 2 h prior to processing. The umbilical vein was carefully extracted from the umbilical cord and the inner lumen was flashed twice with 1 × PBS. The adventitia layer was peeled off with the help of forceps and scalpel. The intima layer was removed by incubating the inner lumen for 30min in a 1 mg/ml collagenase/dispase (Roche) solution in 1 × PBS. The remaining endothelial cells were washed out from the lumen with 1 × PBS. The remaining media layer was minced into small pieces of approximately 2 mm length and let adhere for 10min on the bottom of a petri dish. The tunica media fragments were then covered in DMEM medium and maintained at 37 °C, 5% CO_2 and 95% humidity. The medium was replaced every 48 h. After about 20 days myofibroblasts sprouting from the minced pieces reached about 80% confluence and were ready for sub-culturing.

2.3. Cell culture

HUVM were cultured in DMEM medium with 10% FBS and 1% GlutaMax and the medium was replaced every 48–72 h. For sub-culturing, HUVM were detached using trypsin 0.5% (Sigma) for 4 min and seeded at a cell density of 4000 cells/cm^2. HUVM were expanded up to passage 5 prior to use for the experiments in this study. Human monocytic leukaemia cell line (thp-1) isolated from the peripheral blood of a 1-year-old human male with acute monocytic leukemia, were purchased from Sigma. Thp-1 cells were cultured in suspension in xVivo15 medium and the medium was replaced every 2–3 days. Thp-1 cells were seeded at a density of 100,000 cells/ml and sub-cultured at a density of 800,000 cells/ml.

2.4. Ps-plaque biofabrication

The pseudo-plaque production pipeline encompasses three steps: differentiation, priming and hanging-drop. First, fresh blood-derived myeloid cells or thp-1 cells were seeded onto petri dishes for 72 h and differentiated in chemically defined xVivo15 medium with 10% FBS in order to achieve a macrophage/dendritic cell phenotype. To induce thp-1 differentiation 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) were added to the culture medium. Second, a priming step was performed to obtain heterogeneous macrophage/dendritic cell populations with both pro-inflammatory and remodeling phenotypes. For this purpose the differentiated cells were rinsed in 1 × PBS and treated for 1 h in xVivo15 medium with 10% FBS and 10 ng/ml lipopolysaccharide (LPS, Sigma). Finally, the primed cells were transferred in hanging-drop culture. Briefly, adhesive myeloid-derived cells were mechanically detached by 20min incubation in 5 mM Ethylenediaminetetraacetic acid (EDTA, Life Technologies) in 1 × PBS at 4 °C and gentle scraping. Cells were re-suspended at a cell density of 2.4 × 10^6 cells/ml in presence of LDL 50 μg/ml (LEE Biosolutions) in xVivo15 medium with 10% FBS. Droplets of 10 μl were pipetted on the lead of a 10 cm diameter petri dish and kept in hanging-drop culture for 48 h. To the core of myeloid-derived cells assembled during the 48 h incubation, an external layer of HUVM was added. HUVM were prepared at a cell density of 4 × 10^5 cells/ml in DMEM medium, with or without 50 μg/ml LDL. 10 μl of the cell suspension were carefully added to each pre-existing drop and cultured in hanging-drop for further 48 h.

2.5. 2D co-culture

Primed myeloid cells from blood or thp-1 origin were seeded in 24 well plates maintaining the same cell ratio used for the ps-plaque biofabrication (myeloid cells:myofibroblasts 6:1). In detail, about 300′000 primed myeloid cells were seeded with or without 50 μg/ml LDL in each well in order to achieve a confluent cell layer. Cells were kept in xVivo15 medium with 10% FCS and in 2D culture for 48 h. 50′000 fibroblasts/well in DMEM medium (with or without 50 μg/ml LDL) were then added on top of the myeloid cell layer and incubated for 48 h to obtain a stratified 2D co-culture system.

2.6. Flow cytometry

Biopsies of carotid branches were obtained from patients undergoing carotid endarterectomy and shunting, secondary to vascular stenosis (Ethik Kommission der Universität Witten/Herdecke – Nr.79/2012). Carotid plaques and biofabricated ps-plaques were digested with 1 mg/ml collagenase/dispase solution in 1 × PBS for 15 min at 37 °C. Cells were gently pipetted through a cell strainer with the mesh size of 40 μm (Falcon) and incubated for 5 min at 4 °C with magnetic beads coated with anti CD45 antibodies, according to the provider instructions (MACS Miltenyi Biotec). $CD45^+$ cells were magnetically sorted and stained with