14 Generation of Autologous Multipotent Endothelial-Like Cells from Lipoaspirates of Human Adipose-Derived Stem Cells and Polymer Microarrays Technology: Potential Cardiovascular Regeneration

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Abstract

Endothelial progenitor cells (EPCs) are a small population capable of self-renewal which participate actively in vasculogenesis, angiogenesis and arteriogenesis. Blood-derived EPCs or bone marrow (BM)-derived stem cells feature several drawbacks as far as their clinical utility is concerned, such as the extremely small number of circulating EPCs in the bloodstream and the low availability and harvesting difficulties of BM-derived stem cells. In contrast, human adipose-derived stem cells (hASCs) can be isolated in a greater number through a safe non-invasive liposuction procedure. We have developed a new approach of easy-to-derive large number of multipotent endothelial-like cells (ME-LCs) from human adipose tissue in culture for long periods. ME-LCs displayed increased expression levels of endothelial and hematopoietic lineage markers and EPC markers. Moreover, they formed tube-like structures when grown on 2-D coated Matrigel™ surfaces, secreted increased levels of SDF-1 and showed the ability to migrate attracted by cytokines. Importantly, ME-LCs retained the capacity to differentiate into cardiomyocyte-like cells.

An emerging bioengineering research is the development of synthetic biopolymer matrices as defined environments for EPC growth.

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A family of biopolymers capable of promoting adhesion and differentiation of human EPC was identified and used to coat a 3-D scaffolds for the generation of blood vessels *in vitro*. We showed a notable difference in the process of vascularization between the scaffolds coated with biopolymers than the gold standard matrix support. These results suggested a possible application of such biopolymers for remedying to ischemic injury allowing the endothelialization of artificial endoluminal vessel of intravascular prosthesis devices.

Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide. According to 2008 data from the World Health Organization died from this cause 17.3 million people, being 7.3 million due to heart attacks and 6.2 million to cerebrovascular accidents (Mendis et al. 2011).

Main strategies developed for healing or relief these CVD are based primarily on the use of pharmacological or surgical approaches. However, the discovery in 1997 of an adult population of CD34+/KDR+ hematopoietic progenitor cells, with ability to differentiate into endothelial cells *ex vivo* (Asahara et al. 1997), and the subsequent observation that these circulating endothelial progenitor cells (EPCs) could be derived from bone marrow (BM), have opened the possibility to use these cells, as promising candidates, in cell therapy for CVDs (Asahara et al. 2011).

Different studies show that the number, migratory activity and functionality of EPCs are significantly lower in subjects with hypercholesterolemia, hypertension or diabetes as well as smokers and elderly people (Asahara et al. 2011; Sen et al. 2011). In addition, it has been demonstrated an increase of circulating EPCs in the early stages after ischemic cardiopathy, after a cerebrovascular accident, or in a severe traumatic brain injury (Sen et al. 2011).

Currently, there are three main strategies for the clinical application of EPCs in CVD treatment:

(i) The development of treatments to improve EPCs mobilization, proliferation and/or functionality (Sen et al. 2011).

(ii) Cell transplantation, in fact several clinical trials based on EPCs transplantation have already be conducted providing evidence of their feasibility, safety and utility for the treatment of these pathologies (Asahara et al. 2011; Sen et al. 2011).

(iii) Tissue engineering through the implantation of biomaterials such as nanofibers, alginate or cardiac extracellular matrix (ECM) scaffolds to create a suitable environment for regeneration of damaged tissue, which is known as *in situ* tissue engineering. Another variant is based on the generation of tissue structures *in vitro*, where cells are growth on the biomaterial structures, and are subsequently implanted into the damaged tissue. For example, a two dimensional structure that mimic cardiac tissue characteristic have been generated and attached to the patient's beating heart without the need to use sutures or adhesives (Eschenhagen et al. 2012).

Although the use of EPCs to treat CVD seems to be a promising approach, there are some problems for their clinical use. EPCs are difficult to isolate from blood, due the reduced number of this cell population in the blood, and from BM due the invasiveness and discomfort of the procedure. In contrast, human adipose-derived stem cells (hASCs) can be easily isolated from lipoaspirates. hASCs can be expanded in culture and differentiate into different cell types, including endothelial cells, in a controllable and reproducible manner, and can be safely and effectively transplanted into an autologous or allogeneic host. Furthermore, the clinical potential of hASCs has been proved to be similar to the potential of BM derived mesenchymal stem cell (BM-MSC) (Strioga et al. 2012).

The term mesenchymal stem cells (MSCs) encompasses a heterogeneous group of plastic-adherent multipotent cells that share their capacity for self-renewal and differentiation into mesenchymal lineage cells, including bone, cartilage and adipose tissue. Moreover, MSCs are capable of differentiating into other cell types of mesodermal origin, such as muscle cells, endothelial cells or cardiomyocytes.

These cells reside *in vivo* in the vascular pericytes population present around the capillaries

and microvessels. In addition, MSCs show a transcriptome and a surface phenotype similar to pericytes, suggesting that they originate from pericytes. In normal physiological conditions MSCs appear as quiescent pericytes and may respond to tissue injury and contribute to the repair and regeneration. Likewise, it has been also postulated that MSCs may be related with the tunica adventitia of arteries and veins, supporting the notion that natural niche of the MSCs is the perivascular region (Corselli et al. 2012).

To facilitate the MSC characterization the International Society for Cellular Therapy (ISCT) proposed three minimum criteria for identification: (i) must be plastic-adherent, (ii) the cell population must submit expression levels ≥95% of CD105, CD73 and CD90, and lack of expression of the CD45, CD34, CD14 or CD11b, CD79α, CD19 or HLA class II markers, and (iii) the cells must be able to differentiate *in vitro* into several cell types under differentiation conditions.

Mesenchymal stem cells are used in regenerative medicine mainly based on their ability to differentiate into multiple cell types and their specific production capacity of soluble factors that promote the regeneration of damaged tissue by various mechanisms. 5-azacytidine (5-aza) has been proved to be a potent agent to induce *in vitro* differentiation of MSCs toward a cardiac phenotype. The combination of several factors such as insulin, dexamethasone and ascorbic acid have been also employed in various assays for cardiac differentiation of human MSCs (Kuraitis et al. 2011). In addition, co-culture of MSCs with cardiomyocytes and the use of cell extracts obtained from human cardiomyocytes are other successfully strategies to obtain differentiated cardiomyocytes from MSCs (Peran et al. 2011).

Recent studies demonstrated that MSCs are involved in postnatal vascularization, especially during ischemia of tissues and tumor development. It has been identified the signaling mechanism which vascular endothelial growth factor (VEGF) regulates mobilization, recruitment to sites of neovascularization and differentiation of MSCs into vascular cells. Although MSCs do not express VEGF receptors (VEGFR), they present many platelets derived growth factor receptors (PDGFR) which are stimulated by VEGF (Ball et al. 2010).

Mesenchymal stem cell differentiation toward vascular-like cells has been shown *in vitro* using medium supplemented with certain factors. Especially, VEGF induces endothelial differentiation of MSCs, with the ability to form capillary-like structures and to express endothelial lineage specific markers (Ball et al. 2010). Another methodologies consist in the co-culture of MSCs with mature endothelial cells (ECs) or under hypoxic conditions.

Endothelial Progenitor Cells: Characterization and Differentiation

Endothelial progenitor cells are a small population of circulating endothelial lineage cells found in the peripheral blood. These cells are poorly differentiated, are capable of self-renewal and actively participate in vasculogenesis, angiogenesis and arteriogenesis that occur during postnatal neovascularization, both physiological and pathological processes (Asahara et al. 2011). Upon vascular injury, EPCs are mobilized from the BM, by cytokine secretion such as vascular VEGF and SDF-1, in order to migrate and regenerate the damaged tissue (George et al. 2011; Kim and von Recum 2008).

Endothelial progenitor cells were identified for the first time by Asahara et al. (1997), calling them "angioblasts", and postulated a possible common origin with the hematopoietic stem cells (HSCs) (Asahara et al. 1997). There are different theories about the origin of EPCs and its relation with HSC: (i) the hemangioblast model, which proposes the existence of a common progenitor endothelial and hematopoietic, called hemangioblast, which would be originated from the mesodermal germ layer (ii) the hemogenic endothelium model, which postulates that the endothelium itself can undergo asymmetric divisions and originate both HSCs and EPCs, which would be released into the peripheral blood; (iii) or the hybrid model, which defends the existence of a hemangioblast that originate so HSCs as a hemogenic endothelium, which, in turn, serve as

a source for the generation of both EPCs and HSCs (Bautch 2011).

The characterization of EPCs is still controversial and, at present, there is no consensus with respect to phenotype of this cell population. EPCs were first isolated from human peripheral blood, based on their ability to express the surface markers CD34, and grown in culture surfaces coated with fibronectin to promote its adhesion (Asahara et al. 1997). One of the most important aspects to consider when identifying the EPCs is to distinguish them from the ECs, as both have a similar phenotype, and from the HSCs, that show some similarities due to their common origin. In this sense, like in HSCs, EPCs can be characterized as a population of cells expressing the surface markers CD34, CD133, CXCR4, KDR, Tie-2, FLT1 and CD105, among others (Asahara et al. 2011; Kim and von Recum 2008); but, unlike them, EPCs show low expression or a complete loss of the hematopoietic antigen CD45 and express endothelial cell-specific markers such as VE-cadherin and CD146. In addition to these markers, also the endothelial lineage is usually determined by the binding capacity to certain lectins such as agglutinin-1 of Ulex europaeus (UEA-1), the uptake of acetylated low density lipoprotein (Ac-LDL), or the intracellular expression of vWF (Kim and von Recum 2008).

Although these cells have been identified in BM, peripheral blood or umbilical cord; however, recent studies reveal that they can also be obtained from organs such as the liver, intestine, spleen, adipose tissue or the walls of blood vessels. Currently it is postulated that EPCs might be divided into two main categories depending on their origin: (i) hematopoietic EPCs derived from the BM and, in turn, can stimulate various cellular components of the blood causing a heterogeneous cell population composed of colony forming EPCs, no colony forming EPCs, myeloid EPCs and angiogenic cells, (ii) and non-hematopoietic EPCs, which are not related to HSCs and derive from different tissues or blood vessels from organs (Asahara et al. 2011).

It has been shown that the non-hematopoietic EPC type, known as endothelial outgrowth cells, do not originate from hematopoietic precursors CD45+ or CD133+. It has also been demonstrated that EPCs mobilized from organs, such as the liver or gut, to the blood derive from precursor cells with c-KIT+/CD45− phenotype residents in these tissues (Asahara et al. 2011). All these studies show the difficulty to establish a standard phenotypic characterization of EPCs, because it can be affected by both the origin and their maturation state.

The maturation of EPCs towards a differentiated lineage shows that during this process there is an increase of CD31, VE-cadherin and vWF expression, while occurs a progressive loss of expression in CD133 and CD34 antigens (Schatteman et al. 2007). It has also been determined that TIE-2 or CCR7 genes stop expressing, while DLL4 and CXCR4 gene expression is maintained and CDK2 gene increases (Igreja et al. 2008). This allows distinguishing different EPCs populations according to their state of differentiation, which are known as early EPCs and late EPCs (George et al. 2011).

One of the main features of the EPCs is their ability to EC differentiation. In this regard, it is known that VEGF, in addition to mediate mobilization and expansion of EPCs, is the main inducer of endothelial differentiation. This differentiation is favored *in vitro* using scaffolds or culture surfaces coated by an ECM (Mooney and Vandenburgh 2008). Studies also demonstrate that the *in vitro* application of a shear stress, defined as the force per unit area that the blood flow exerts on the vascular wall, causes the endothelial differentiation of EPCs. It has been observed that the application of this force to cultures suppresses the expression of genes encoding CD34 and CD133, and at the same time increases the mRNA expression of the genes coding for CD31 and vWF (Suzuki et al. 2012).

Besides the ability to differentiate EPCs to ECs, some studies indicate that these cells are capable of transdifferentiate to smooth muscle cells through a process of endothelial-mesenchymal transition (EndMT) when treated with TGF-β1 (Imamura et al. 2010). Furthermore, there is also evidence that EPCs might express cardiac markers *in vitro* by co-culturing with cardiomyocytes. Also, *in vivo*

experimental assays have demonstrated that EPCs can transdifferentiate into cardiomyocytes and participate in cardiac tissue regeneration after injury (Feng et al. 2012).

Regarding the relationship of EPCs with cardiomyopathy and vascular disease, studies showed that the number of circulating EPCs is inversely correlated with cardiovascular risk and they can be used as a prognostic marker of potential cardiovascular events (Sen et al. 2011). When a vascular accident occurs there is a decreased flow of oxygen in the tissue, that is detected by ECs through a protein prolyl hydroxylase domain (PHD) which hydroxylate different hypoxia inducible factors (HIF). These cells produce HIF-1α which in turn induces the production of factors such as VEGF and SDF-1 cytokines. The binding of SDF-1 to its receptor CXCR4 causes activation of ERK signaling pathway of phosphatidylinositol 3 kinase (PI3K)/AKT and increased eNOS expression, which are responsible for the migration of EPCs to ischemic site (George et al. 2011).

Furthermore, the main function of VEGF is to induce proliferation and differentiation of EPCs to EC in neovascularization sites. Other factors such as granulocyte-colony stimulating factor (G-CSF) or the granulocyte-macrophage colony stimulating factor (GM-CSF) also mediate migration of EPC to sites of new vessel formation (George et al. 2011). Moreover, EPCs migrating to these tissues do not always participate in the formation of blood vessels, but may also be resident in the interstitial tissue, releasing cytokines and pro-angiogenic growth factors such as VEGF, ANG-1, SDF or HGF 1. These cytokines act in a paracrine manner to promote local angiogenesis, proliferation and mobilization of ECs into the living tissues to restore homeostasis (Asahara et al. 2011).

Human Adipose-Derived Stem Cells

From all possible sources to obtain MSCs in the adult, the adipose tissue has attracted more interest since its discovery earlier this century. The plastic-adherent multipotent cells in this tissue have received different nomenclatures such as processed lipoaspirates cells, stromal cells derived from adipose tissue, fat-derived MSCs or human multipotent adipose-derived stem cells. So in 2004 the International Society Fat Applied technology agreed to use the "human adipose-derived stem cells" (hASCs) term to define this cell population.

These cells show certain advantages that make them more attractive from the point of view of potential clinical application respect to those obtain from BM: (i) adipose tissue is abundant and is found in all individuals; (ii) hASCs can be isolated in a greater number, up to billions of cells, through a safe non-invasive routinely liposuction procedure under local anesthesia; (iii) from the enzymatic disintegration is possible to obtain stromal vascular fraction containing different cell types including hASC, fibroblasts, endothelial cells, pericytes, smooth muscle cells and other circulating cells such as immune system cells and HSCs. The hASCs are isolated based on their capacity for adhesion to the culture plastic surface, showing a fibroblastic morphology when grow *in vitro*. These hASCs can also be expanded in culture and differentiated into different cell types, including ECs, in a controllable and reproducible manner, and can be safely and effectively transplanted into an autologous or allogeneic host (Strioga et al. 2012). Therefore, hASCs might be a promising source of progenitor cells that could be used for regeneration of vascular and/or heart lesions. Also, hASCs secrete multiple potentially synergistic pro-angiogenic growth factors including VEGF, HGF and chemokine SDF-1 which are likely to play a pivotal role for the hASC-mediated angiogenesis.

These cells meet the minimum requirements set by the ISCT to be considered MSCs, although they show some differences in their immunophenotype, proliferative capacity, differentiation potential, their transcriptome, proteome and their immunomodulatory activity compared to those obtained from bone marrow, being partly due to differences in the protocols used for isolation and cultivation (Strioga et al. 2012).

The procedure to isolate hASCs from adipose tissue begins with several washed using phosphate-buffered saline (PBS) to remove, as far as possible,

the blood present in the sample. Then, it is digested with type I collagenase and after centrifugation it can be obtained a high density cell pellet without mature adipocytes which is plated on flask cultures. The characterization of these cells shows expression of CD105 (>99%), CD90 (>90%) and CD73 (>99%) surface markers and a lacked expression for both hematopoietic and EC markers CD45, CD34, CD133, CXCR4 and KDR (Marchal et al. 2012).

Multipotent Endothelial-Like Cells: Isolation and Expansion

Recently, our group has developed a new approach of easy-to-derive large number of ME-LC through a series of consecutive stages (Marchal et al. 2012). Firstly, hASCs were cultured in DMEM-FBS (stage I), and then, culture medium was replaced by serum-free medium for 3 weeks (stage II), which resulted in the appearance of sphere cluster formations (SCF) that increased in number and size throughout the subsequent culture stages. After that SCF were cultured in SFO3 medium (stage III) composed by RPMI-1640: DMEM: F12, 0.1% bovine serum albumin (BSA) and 2-mercaptoethanol and in EC medium (stage IV) composed by endothelial basal medium-2 (EBM-2, Lonza) containing 5% FBS, and human recombinant vascular endothelial growth factor (VEGF), hydrocortisone, human recombinant epidermal growth factor (rhEGF), human recombinant long R insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human recombinant basic fibroblast growth factor (rhFGF) and gentamicin sulfate-amphotericin-B EGM-2 (Fig. 14.1). Previously, Hirashima et al. (2003) demonstrated that a chemically defined serum-free culture system, including 2-mecarptoethanol, have the ability to support the proliferation of endothelial cells and their progenitors from mesoderm cells (Hirashima et al. 2003). Cells cultured in stage III and IV were considered as ME-LC because they increased the expression of EPC and hematopoietic markers (CD34, CD133, KDR, CXCR4, and CD45). The coexistence of hematopoietic and endothelial markers in the ME-LC is indicative of a phenotype resembling early vascular progenitors since it has been reported the existence of the hemangioblast, capable of giving rise to both hematopoiesis and vascular endothelium (Bautch 2011). In contrast to these cells, HUVEC mature endothelial cells were negative for CD34 and CD133 progenitor markers and strongly positive for CD31. Both CD34 and CD133 antigens are lost upon differentiation of endothelial progenitors to endothelium (Schatteman et al. 2007).

High levels of expression of the MSC markers CD105, CD73 and CD90 were observed throughout the different stages of the ME-LC isolation procedure. Interestingly, the CD90 MSC marker, which expression is practically absent in HUVEC, was the marker which expression most significantly decreased when ME-LC were cultured in SOF3 medium (stage III), suggesting endothelial differentiation. In agreement, it has been shown a loss of CD90 antigen expression on MSCs by angiogenic stimulation *in vitro* induce angiogenic differentiation without experimenting a complete endothelial maduration (Marchal et al. 2012).

All together, these data suggest that ME-LC isolated from hASCs cultures express markers resembling a vascular progenitor phenotype. Gene expression profile confirmed the endothelial progenitor phenotype with the expression and maintenance of specific genes involved in self-renewal, cell cycle promotion and antiapoptotic such as has been recently showed in cord blood (CB)-derived EPC. CXCR4 and CD133 vascular genes were expressed at similar levels throughout the several culture stages. Nevertheless, genes expressed in differentiated EPCs such as *Cdk2* and *Flt-1* (Igreja et al. 2008) showed a weak expression level in ME-LC cultured in SOF3 medium. Another interesting result was the disappearance of *Tie-2* gene expression, only detected at stage I, which has been previously reported as an angiogenic factor clearly induced in the differentiated ECs (Furuhata et al. 2007). In addition to endothelial cells, Tie2 is expressed in a subpopulation of HSC being, in part, responsible of maintaining a quiescent state in the bone marrow niche.

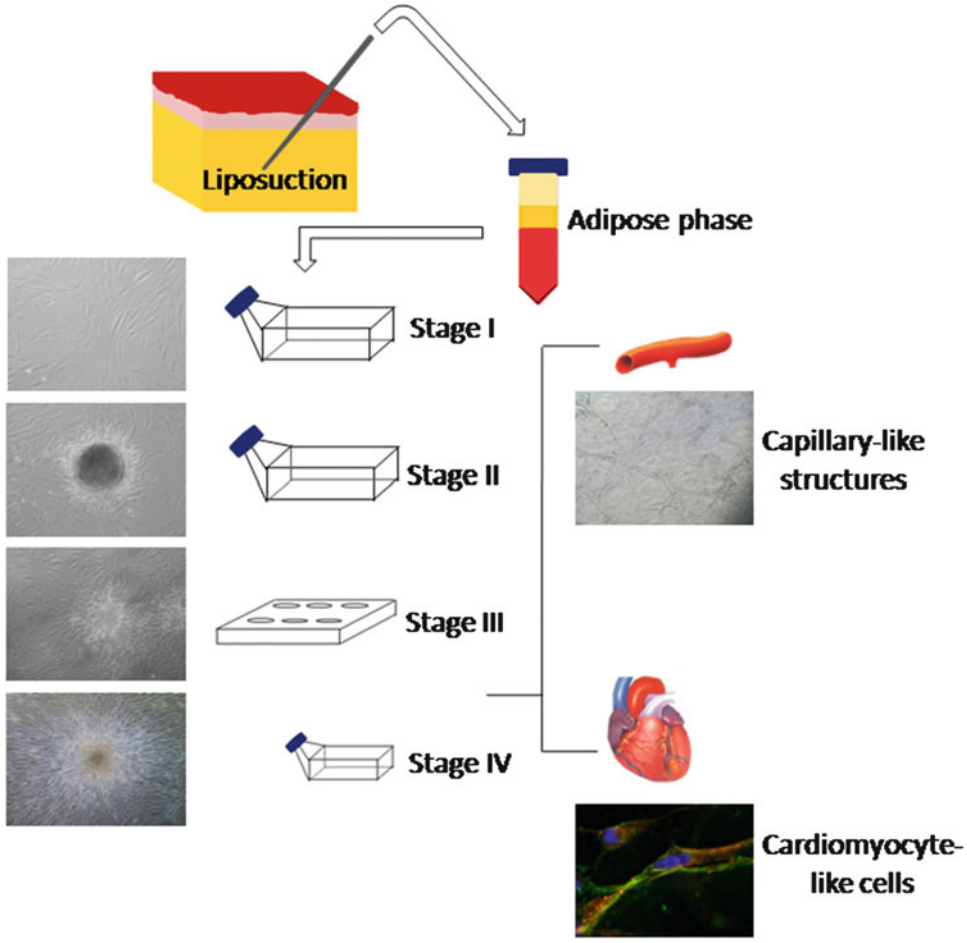

Fig. 14.1 Culture and differentiation of ME-LCs. hASCs were obtained from liposuction procedure. These cells were cultured along different stages in diverse media: DMEM-FBS (stage I), DMEM free-serum (stage II), SFO3 medium (stage III) and EC medium (stage IV). Cells obtained from stage III and stage IV were considered ME-LCs. These cells are capable to form capillary-like structures similar in comparison with the data rendered by HUVEC and differentiated to cardiomyocyte-like cells with the acquisition of a cardiogenic phenotype and the expression of cardiomyocyte-specific markers such as troponin T (*red*) and desmin (*green*)

Ability of Multipotent Endothelial-Like Cells to Enhance Functional Capillary-Like Structures Formation

The ability to form capillaries of ME-LCs was assayed using cells obtained from each stage and were cutured independently on Matrigel™ plates and EGM-2 medium. Cells from cultures at stage I and II were not able to form any capillaries over a 7 days period. On the other hand, cells previously grown in SFO3 or EGM-2 (stages III and IV, respectively) displayed a large number of capillary-like structures as early as 4 h after being seeded and the appearance of capillary tube-like structures increased overtime. After 7 days in culture a well-established cellular network was present in all cell cultures. By stage IV, the number of capillary-like structures was similar in comparison with the data rendered by HUVEC, used as positive control (Marchal et al. 2012).

Recent studies have been shown that VEGF stimulation of HUVEC induces DLL4

expression which reduced vessel sprout length in a 3D tubulogenesis assay, confirming that DLL4 signaling inhibits angiogenesis. DLL4 expression seems to acts as a switch blocking endothelial cell proliferation and allowing induction of a more mature differentiated phenotype (Harrington et al. 2008). Isolated ME-LCs showed a down regulation of this ligand, which correlated with the early formation of large number of capillary-like structures and the late formation of a vascular network.

Moreover, ME-LCs showed an increased secretion of SDF-1 in comparison with hASCs, even when they were seeded for 2 weeks on Matrigel™. In addition, the role of SDF-1 to induce migration of CD133+/CD34+/KDR+ cells has been shown (Peichev et al. 2000). Results demonstrated how the ME-LCs, which express the SDF-1 receptor CXCR4, were able to migrate towards a SDF-1 gradient (Marchal et al. 2012). These data suggest the potential homing of ME-LCs to sites of vascular injury for tissue repair.

Ability of Multipotent Endothelial-Like Cells to Differentiate into a Cardiac Phenotype

The potential of ME-LCs to differentiated toward a cardiomyocyte phenotype following exposure to 5-aza has been tested. These cells were seeded on Matrigel™-coated plate in EGM-2 medium. This culture medium was replaced 2 weeks later by EBM-2 containing 5–10 or 15 μM of 5-aza for 24–48 h and changed back to EGM-2. Then these cells are cultured for 3–4 weeks.

Following exposure to 5-aza ME-LCs retained the capacity to differentiate into cardiomyocyte-like cells with the acquisition of a cardiogenic phenotype and the expression of cardiomyocyte-specific markers. Morphological changes consisted in the appearance of wide, branching and multinucleated cells resembling cardiac muscle cells. The expression of typical cardiomyocyte markers such as troponin-T and α-sarcomeric actinin and the presence of a desmin filaments network, which take part in regulating the mesodermal specification into cardiomyocytes (Fig. 14.1) (Labovsky et al. 2010), support the cardiomyocyte differentiation potential of ME-LCs.

Polymer Microarrays – Powerful Technology to Identify New Synthetic Matrices for Endothelial Progenitor Cells

Since the discovery of circulating ECs in peripheral blood a decade ago (Asahara et al. 1997), regenerative stem-cell-based therapeutic strategies have aimed at both developing novel therapies and improving current treatments by restoring ischemic tissues (Chan and Mooney 2008). Traditional methods of direct cell infusion or injection (which represent the most commonly utilized cell delivery strategy in the clinic – for example, BM transplants) often lead to big issues such as massive cell death, extremely poor homing/engraftment efficiency to targeted tissues and loss of control over the fate of transplanted cells (Karp and Leng Teo 2009). The introduction of biomaterial scaffolds as cell carriers provide a solution to these problems, due to their templating capability for guiding the formation of new tissue and for promoting engraftment within the host (Eschenhagen et al. 2012; Hench and Polak 2002). An emerging area of bioengineering research is, therefore, the development of synthetic biopolymer matrices as defined environments for EPC growth, providing sites of adhesion together with signals that can control EPC propagation and synchronize their differentiation (Mooney and Vandenburgh 2008).

This chapter describes a high throughput screening of polymer microarrays (Pernagallo and Diaz-Mochon 2011) for the identification of new polymer matrices which contain the necessary signals that not only promote adhesion and propagation of EPCs but also, synchronize their endothelial specialization. Due to the complexity and diversity of biocompatibility testing, it has been difficult to find a universal method which permits high throughput determination of *in vitro*

properties. It is impossible to theoretically predict cellular response, and thus every time a new material is generated it is essential to test its biocompatibility using cells with which it will come into contact. To address this issue the development of a new system of screening, based on microarray technology has been developed. Polymer microarrays accelerate the process to find new biomaterials while minimizing expenditures regarding time and money. Furthermore, as these polymers are synthetic animal-free materials which can be manufactured to GMP standards they overcome one of the biggest issues, in terms of regulation and human health safety, of current industry standards, such as Matrigel™, which have animal-source origins. Therefore, they can provide clinically useful materials for the construction of extra-corporeal devices and facilitate novel studies in modern vascular surgery.

Pre-formed and fully characterized polymer libraries were printed using a robotic printer onto agarose coated glass-slide following a defined xy pattern (Pernagallo and Diaz-Mochon 2011). These arrays were then used to identify new substrates for attachment, promotion, and propagation of EPCs which at the same time would keep cell functionality (Fig. 14.2 a, b).

Cord blood (CB) products (50 mL) were aspirated from umbilical placental veins from normal caesarean deliveries and collected into heparin (Pernagallo et al 2012). Mononuclear cells (MNCs) were isolated by buoyant density centrifugation and cultured over 3 weeks in endothelial basal medium-2 (EBM-2). By that time, spindle-shaped EPCs started to emerge. These cells express a number of endothelial markers comparable to a mature EC and human umbilical vein endothelial cells (HUVECs) and they are able to form new blood vessels in Matrigel™ (Skovseth et al. 2007). Thereafter cells were passaged until incubation with the polymer microarrays. As control HUVECs, which are specialized ECs, were also maintained in cell culture. At that point EPCs and HUVECs were removed from their substrate and plated onto two identical polymer microarrays containing 345 polyurethanes and polyacrylates, printed in quadruplicate given rise to 1,345 feature arrays. After 3 days of culturing, under standard cell culture conditions, cells were fixed and stained with a nuclear stain (Hoechst 33342), and conjugated monoclonal mouse anti-human CD31-PE antibody (Fig. 14.2c, d). CD31 (Anti-PECAM-1) recognizes the platelet/endothelial cell adhesion molecule-1 (PECAM-1), a 130–140-kDa single-chain integral membrane glycoprotein that is a member of the immunoglobulin gene superfamily (Pinter et al. 1997). The CD31 antigen is expressed on ECs, functions as a vascular cell adhesion molecule and is involved in the process of leukocyte migration through the intercellular junctions of vascular ECs (Delisser et al. 1997). Following fixing and staining, arrays were scanned using a High Content Screening Platform (Nikon 50i fluorescence microscope (×20 objective) with an automated XYZ stage, equipped with the Pathfinder™ software package – IMSTAR) fit with two fluorescence channels (DAPI and Cy3). Cell numbers on spots were determined by analyzing images captured using DAPI channel (Hoechst 33342ex/em 355/465 nm) while endothelial phenotype was assessed via Cy3 channel (R-PE ex/em 545/575 nm).

Primary screening identified 6 polyurethanes and 12 polyacrylates that supported attachment of EPCs providing over 150 cells/mm^2. After looking at initial data with more detail it was found out that 3 out of those initial 18 hits (3AA7, 8g7 and 9g7) provided to be excellent substrates, with over 200 cells/mm^2, for both EPCs and HUVECs. These results supported the idea that 3AA7, 8g7 and 9g7, as well as enabling high cell adhesion could enhance endothelial maturation of EPCs towards specialized cells such as HUVECs. In order to study the endothelial function in further detail and confirm the results of these experiments, the three selected polymers (3AA7, 8g7 and 9g7) were scaled-up and spin-coated onto glass cover slips which were then used to grow both EPCs and HUVECs for flow cytometry analysis. EPCs and HUVECs cultured on polymer – coated surfaces, expressed endothelial cells surface markers vWF, VEGFR2 (also referred to as Flk-1 or KDR), endothelial nitric oxide, synthase (eNOS), CD31, CD34, CD105 and CD146 but not the hematopoietic cell surface

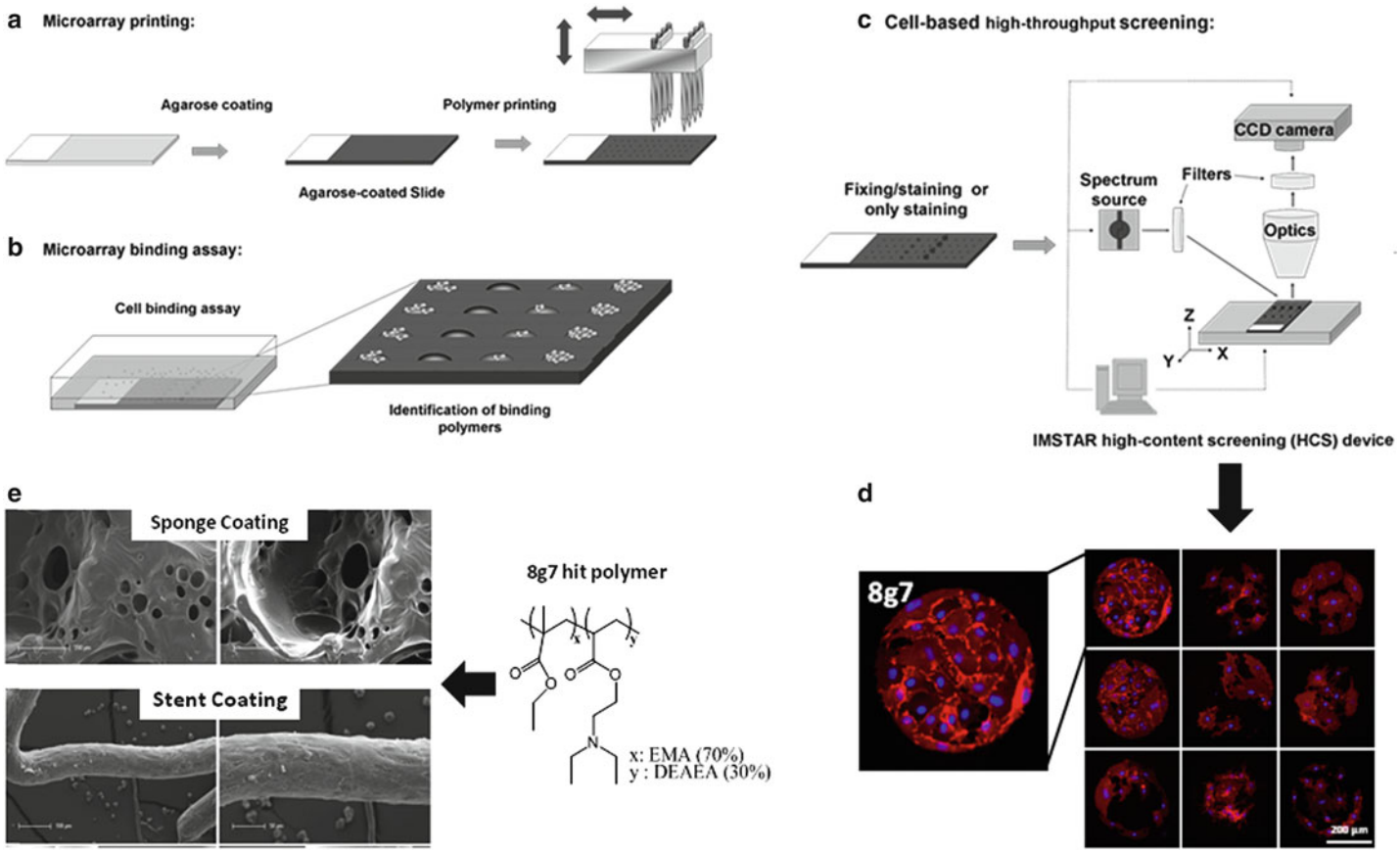

Fig. 14.2 Finding novel synthetic materials compatible with endothelial progenitors cells and their direct application for coating extra-corporeal devices. (**a**) Polymer microarray preparation; (**b**) cell-based microarray binding assay; (**c**) cell-based high-content screening; (**d**) fluorescence images from a polymer microarray incubated with EPCs and stained with Hoechst 33342 and CD31-PE antibody and (**e**) extra-corporal devices – sponges and stent – coated with a polymer – 8g7(see *insert*) – identified via polymer microarray approach (**a**–**d**) incubated with EPCs

antigens CD45. Therefore, flow cytometry results confirmed HTS data, thus demonstrating that new substrates, identified via polymer microarray approach, enhance specialization of EPCs (Pernagallo et al. 2012).

Generation of Coated Devices to Make Them Biocompatible with Endothelial Progenitor Cells

Further studies were undertaken by cultivating EPCs on pieces of sponges coated with a solution of 8g7 (Pernagallo et al. 2012). The ultimate aim of studying sponge as a cell scaffold was to generate functional 3-D tissues outside of the organism, which might then be implanted into the organism. In an ideal scenario 8g7 coated sponges would have the ability to adhere EPCs in a *in vitro* fashion before being implanted into animals so that they can either promote the growth and the specialization of those cells previously anchored to the sponge as well as recruit distant BM-derived progenitor cells for restoring vascularization at the implanted scaffold/host tissue interface. One cm^3 sponges were dip-coated in a 1% w/w solution of 8g7 in THF and further sponges were also dip-coated in the current gold standard, Growth Factor Reduced Matrigel™ (GFR-MG). Polymer coated sponge showed a smoother uniformly coated layer allowing a friendlier environment for cells to grow. The coated sponge was impregnated with either EPCs or HUVECs in complete EBM-2 medium that supports endothelial cultivation. After overnight incubation, cells were fixed and SEM analysis carried out. SEM images clearly showed that only sponges pre-coated with polymer allowed tube formation while sponges pre-coated with GFR-MG lacked tube formation features (Fig. 14.2d). It was also clear that by increasing number of EPCs adhered to the 8g7 sponge, a major release of both cytoprotective and pro-angiogenic factors in a paracrine manner to promote the survival and proliferation of endothelial cells happened (Gnecchi et al. 2008). The functional 3-D tissue generated *in vitro* was tested for its ability to promote cell growth and recruit distant BM-derived progenitor cells for promoting engraftment with the host, after implantation. Therefore, sponges were pre-treated with 8g7 and loaded with EPCs *in vitro*. Following initial incubation, these sponges were implanted into animals. Each animal had a EPCs-loaded 8g7 impregnated sponges on one side and a control EPCs loaded native sponge on the other. Twenty days after implantation, mice were sacrificed and sponges were excised and analyzed for vessel formation. Sponges were divided into three pieces and analyzed as follows: SEM imaging; Chalkley count and hemoglobin assay (Pernagallo et al. 2012).

After implantation in mice, the sponges impregnated with 8g7 promoted better engraftment within the host, showing clearly the formation of a vascular network in contrast with the blank (uncoated) sponge. Notably, uncoated sponge resulted in a poor and incomplete engraftment within the host, showing the native sponge without cells at several points. It seems evident that the large number of EPCs adhered to the 8g7 sponge, allowed not only a large quantity of local cell growth but also made possible a major release of cytokines which recruited distant BM-derived progenitor cells that effectively mediated vascularization at the implanted scaffold/host tissue interface. Histological analysis revealed that significantly increased numbers of vessels formed on the polymer coated sponge compared with the control (sponge uncoated). One part of the sponge was fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with haemaoxylin/eosin for identification of blood vessels. Sponge vessel density was determined by using the mean of triplicate Chalkley counts on two sections per sponge. Histology showed vascularization by a number of blood vessels but in polymer coated sponges had significantly more blood vessels compared to non polymer coated sponges. A further study focused on measuring hemoglobin levels of the sample. This is an excellent method of determining the frequency of blood vessels, as they are proportional to the blood hemoglobin concentration. Polymer coated sponges had significantly more hemoglobin, displaying ~twofold increase compared to uncoated polymer sponges.

A clear application was the coating of stent used commonly to treat ischemia related pathologies. A notable step forward in the treatment of ischemia related pathologies occurred in the1990s with the introduction of metal scaffolds, stents, capable of giving the stenotic vessel the necessary mechanical support to avoid collapse (Fanelli and Aronoff 1990). The introduction into medical practice of such devices revolutionized the field of interventional cardiology, bringing notable improvements in prevention of restenosis. In spite of the fact that stents are capable of eliminating elastic recoil of the stenotic vessel, however, their implantation and the consequent trauma to the vessel walls initiates an excessive proliferation of neointima and, therefore, an in-stent restenosis.

An initial negative reaction following implant of the stent is due to the raised thrombogenicity of the metal link of which it is made. Therefore, a first intuitive approach is aimed at improving the biocompatibility of the implant through there coating of the stent with polymer film (Tepe et al. 2006).

In order to study adhesion of endothelial outgrowth cell to the polymer-coated stent, 8g7 was used (Pernagallo et al. 2012). A coronary stent was dip-coated in the 8g7. The control was a coronary stent in its native form. EPCs were removed from their substrate and re-incubated onto both, a 8g7-coated stent or native stent and cultured over-night in conditions that support EC survival. In order to assess cellular adhesion and the utility of 8g7, following incubation cells on both stents were fixed and SEM performed to asses cell morphology. EPCs maintained on the uncoated stent showed poor cell attachment, whereas EPCs maintained on the 8g7-coated stent exhibited a consistent adhesion with around 80% of confluence (Fig. 14.2d). These data exemplify the value of 8g7 and EPCs in an alternative setting for improvement in angioplasty therapy.

Conclusions

In summary, these data indicate that subcutaneous adipose tissue may be a useful source of autologous ME-LCs with the capacity to maintain their vascular progenitor properties. Future studies should explore further the functional *in vitro* and *in vivo* mechanisms of ME-LCs along with their therapeutic potential, since these data suggest that these cells may prove to be a valuable tool for vascular and cardiac repair.

Moreover, results presented in this chapter suggest a possible application *in vivo* of polymer technology as a potential method for remedying ischemic injury. Artificial vessel prostheses generated *in vitro* coated with 8g7 was implanted in mice with a notable difference in the re-endothelialization in contrast to the native sponge. The reason for this is unclear but might have been due to the fact that there was already a large quantity of cells growing on the sponge. Alternatively, it might have been caused by the fact that those cells attracted BM-derived progenitor cells through the release of cytokines. The capacity of 8g7 to bind EPCs suggests the possibility of using this polymer for coating stents. These cells might be isolated from blood from the patient and used to adhere and cover the stent pre-coated with the selected polymer *in vitro*. At this point the stent could be reintroduced into the patient avoiding the problems mentioned above. It was demonstrated that a stent can easily be coated with 8g7, and, as a consequence of cell adhesion, an EPCs layer was generated *in vitro*. This method might, therefore, offer a future means of reducing the process of thrombogenesis and in-stent restenosis and improving angioplasty therapy. Finally, actually studies are ongoing to analyze the ability of 8g7 to bind ME-LCs obtained from lipoaspirates which could have interesting clinical application.

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