Biomaterials 30 (2009) 1098–1112

Contents lists available at ScienceDirect

Biomaterials

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

A three-dimensional model of vasculogenesis

Mani T. Valarmathi [a,*], Jeffrey M. Davis [a], Michael J. Yost [b], Richard L. Goodwin [a], Jay D. Potts [a]

[a] Department of Cell and Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina 29209, USA
[b] Department of Surgery, School of Medicine, University of South Carolina, Columbia, South Carolina 29209, USA

ARTICLE INFO

Article history:
Received 18 August 2008
Accepted 26 October 2008
Available online 22 November 2008

Keywords:
Bone marrow stromal cells
Mesenchymal stem cells
Vasculogenesis
Angiogenesis
Vascular tissue engineering

ABSTRACT

Postnatal bone marrow contains various subpopulations of resident and circulating stem cells (HSCs, BMSCs/MSCs) and progenitor cells (MAPCs, EPCs) that are capable of differentiating into one or more of the cellular components of the vascular bed in vitro as well as contribute to postnatal neo-vascularization in vivo. When rat BMSCs were seeded onto a three-dimensional (3-D) tubular scaffold engineered from topographically aligned type I collagen fibers and cultured either in vasculogenic or non-vasculogenic media for 7, 14, 21 or 28 days, the maturation and co-differentiation into endothelial and/or smooth muscle cell lineages were observed. Phenotypic induction of these substrate-grown cells was assayed at transcript level by real-time PCR and at protein level by confocal microscopy. In the present study, the observed upregulation of transcripts coding for vascular phenotypic markers is reminiscent of an in vivo expression pattern. Immunolocalization of vasculogenic lineage-associated markers revealed typical expression patterns of vascular endothelial and smooth muscle cells. These endothelial cells exhibited high metabolism of acetylated low-density lipoprotein. In addition to the induced monolayers of endothelial cells, the presence of numerous microvascular capillary-like structures was observed throughout the construct. At the level of scanning electron microscopy, smooth-walled cylindrical tube-like structures with smooth muscle cells and/or pericytes attached to its surface were elucidated. Our 3-D culture system not only induces the maturation and differentiation of BMSCs into vascular cell lineages but also supports microvessel morphogenesis. Thus, this unique in vitro model provides an excellent platform to study the temporal and spatial regulation of postnatal de novo vasculogenesis, as well as attack the lingering limit in developing engineered tissues, that is perfusion.

1. Introduction

Vasculogenesis is the process of blood vessel formation occurring by a de novo production of endothelial cells in an embryo (primitive vascular network) or a formerly avascular area when endothelial precursor cells (angioblasts, hemangioblasts, or stem cells) migrate and differentiate in response to local cues (such as growth factors and extra cellular matrix) to form new intact blood vessels [1], whereas angiogenesis refers to the sprouting of new blood vessels from the differentiated endothelium of pre-existing vessels. These vascular trees or plexuses are then pruned, remodeled and extended through angiogenesis to become larger caliber vessels [2].

The identification of bone-marrow-derived (hematopoietic and non-hematopoietic stem cells) and non-bone-marrow-derived (tissue-resident stem/progenitor cells – adipose, neural, heart, skeletal muscle; peripheral and cord blood-derived stem cells) endothelial progenitors cells (EPCs) has led to the realization of potential postnatal vasculogenesis [3]. Previous reports indicate that adult bone-marrow-derived mesenchymal stem cells (BMSCs/MSCs) and multipotent adult progenitor cells (MAPCs) can be differentiated into endothelial-like cells in vitro and contribute to neoangiogenesis in vivo [4–6], and are readily available. In addition, BMSCs can augment collateral remodeling and perfusion in ischemic models through paracrine mechanisms rather than by cellular incorporation upon local delivery [7].

For these reasons, embryonic, fetal and postnatal stem cells, as well as various types of endothelial progenitor cells, can be a potential cellular source for vascular tissue engineering [8]. However, the source for these early-stage developmental cells is problematic. Unlike embryonic stem cells (ES), obtaining autologous bone-marrow-derived stromal cells is feasible and can potentially be exploited to develop tissue-engineered blood vessel constructs for therapeutic purposes. Similarly, repeated isolation and rapid in vitro expansion of sufficient yield of autologous and/or allogenic non-bone-marrow-derived resident stem cells/progenitors, especially from vital organs for routine therapeutic purposes

* Corresponding author. Building 1 Room B-60, 6439 Garners Ferry Road, Department of Cell and Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina 29209, USA. Tel.: +1 803 733 3294; fax: +1 803 733 3212.
E-mail address: valarmathi.thiruvanamalai@uscmed.sc.edu (M.T. Valarmathi).

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doi:10.1016/j.biomaterials.2008.10.044

are highly constrained. On the contrary, to a certain extent autologous and/or allogenic bone-marrow-derived BMSCs are amenable for repeated isolation and rapid in vitro expansion from the patients.

Extracellular molecules initiate biological signals and play a critical role in the control of cellular proliferation, differentiation, and morphogenesis. Many parameters, such as the presence and amount of soluble factors such as hormones, growth factors, and cytokines or the insoluble factors such as the physical configuration of the matrix, which mediate the cell-cell and cell-matrix interactions, exert strong influence on the success of angiogenic processes in vitro and presumably in vivo [9,10]. The likelihood and ultimate success of in vitro cellular differentiation depends on how closely the cell-matrix relationship mimics that found during normal development or regeneration. In vascular tissue engineering, the application of these principles in vivo will be important to ensure that the matrix/scaffold to be implanted can support endothelial cell proliferation and migration resulting in endothelial tube formation [11]. The vital issue for realistic clinical application is whether these scaffolds with preformed endothelial tubes can survive implantation into tissue defects and subsequently be able to anastomose to the host vasculature [10].

We therefore hypothesized that under appropriate in vitro physicochemical microenvironmental cues (combination of growth factors and ECM) multipotent adult BMSCs could be differentiated into vascular endothelial and smooth muscle cell lineages. To test this hypothesis, we characterized the intrinsic vasculogenic differentiation potential of adult BMSCs when seeded onto a three-dimensional (3-D) tubular scaffold engineered from aligned type I collagen strands and cultured in both vasculogenic and non-vasculogenic growth media. In these culture conditions, BMSCs differentiated and matured into both endothelial and smooth muscle/pericyte cell lineages and showed microvascular morphogenesis. We also explored the potential of the 3-D model system to undergo postnatal de novo vasculogenesis.

Our results indicate that the 3-D tubular scaffold with its unique characteristics provides a favorable microenvironment that permits the development of in situ microvascular structures. Moreover, this is the first report that explicitly demonstrates that adult BMSCs under appropriate in vitro environmental cues can be induced to undergo vasculogenic differentiation culminating in microvessel morphogenesis. Our model recapitulates many aspects of in vivo de novo vasculogenesis. Thus, this unique culture system provides an in vitro model to investigate the maturation and differentiation of BMSC-derived vascular endothelial and smooth muscle cells in the context of postnatal vasculogenesis. In addition, it allows us to elucidate various molecular mechanisms underlying the origin of both endothelial and smooth muscle cells and especially to gain a deeper insight and validate the emerging concept of 'one cell and two fates' hypothesis of vascular development [12].

2. Materials and methods

2.1. Fabrication of tubular scaffold

The 3-D collagen type I tube served as a scaffold on which rat BMSC differentiation cultures were carried out. The details of the production and properties of the collagen tubes have previously been described [13]. Briefly, a 25 mg/ml solution of bovine collagen type I was extruded with a device that contained two counter-rotating cones. The liquid collagen was fed between the two cones and forced through a circular annulus in the presence of an NH_3-air (50–50 vol/vol) chamber. This process results in a hollow cylindrical tube of aligned collagen fibrils with an inner central lumen. The dimensions of tubes produced for this set of experiments had a length of 30 mm with a luminal diameter of 4 mm and an external diameter of 5 mm, leaving a wall thickness of 1 mm. The collagen tubes had a defined fiber angle of 18° relative to the central axis of the tube and had pores ranging from 1 to 10 μm. The rationale for the particular orientation of collagen fiber was based on our previous work on cardiovascular tissue engineering [13], when proepicardial (PE) cells were seeded onto this scaffold; they underwent maturation and differentiation and produced elongated vessel-like structures reminiscent of an in vivo-like phenotype [14]. The tubes were sterilized using gamma radiation 1200 Gy followed by Stratalinker UV crosslinker 1800 (Stratagene) and then placed in Moscona's solution (in mM: 136.8 NaCl, 28.6 KCl, 11.9 $NaHCO_3$, 9.4 glucose, 0.08 NaH_2PO_4, pH 7.4) (Sigma–Aldrich) containing 1 μl/ml gentamicin (Sigma–Aldrich) and incubated in 5% CO_2 at 37 °C until cellular seeding.

2.2. BMSCs isolation, expansion and maintenance (nonclonal BMSCs culture)

The procedures were performed in accordance with the guidelines for animal experimentation by the Institutional Animal Care and Use Committee, School of Medicine, University of South Carolina. Rat BMSCs were isolated from the bone marrow of adult 300 g Sprague Dawley®™ SD®™ rats (Harlan Sprague Dawley, Inc.). Briefly, after deep anesthesia, the femoral and tibial bones were removed aseptically and cleaned extensively to remove associated soft connective tissues. The marrow cavities of these bones were flushed with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) and combined. The obtained marrow plugs were triturated, and passed through needles of decreasing gauge (from 18 gauge to 22 gauge) to break up clumps and cellular aggregates. The resulting single-cell suspensions were centrifuged at 200 g for 5 min. Nucleated cells were counted using a Neubauer chamber. Cells were plated at a density of $5 \times 10^6 - 2 \times 10^7$ cells per T75 cm^2 flasks in basal media composed of DMEM supplemented with 10% fetal bovine serum (FBS, lot-selected; Hyclone), gentamicin (50 μg/ml) and amphotericin B (250 ng/ml) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 7 days. The medium was replaced, and changed three times per week until the cultures become ~70% confluent (between 12 and 14 days). Cells were trypsinized using 0.05% trypsin-0.1% EDTA and re-plated at a density of 1×10^6 cells per T75 cm^2 flasks. After three passages, attached marrow stromal cells were devoid of any non-adhering population of cells.

2.3. Clonal BMSCs culture

Single-cell suspensions of BMSCs prepared as described above were plated in 150 mm × 20 mm Petri dishes at a low density of $1–4 \times 10^3$ nucleated cells/cm^2. After 24 h of incubation, the cultures were thoroughly washed with complete DMEM to remove nonadherent cells. The cultures were continued as described above for 12–14 days. Colonies that were well defined and separated from neighboring colonies were washed with Moscona's solution, pH 7.4 and individual colonies were isolated using cloning cylinders (Sigma–Aldrich). Cells were trypsinized and re-plated onto individual wells of a six-well culture plates. At approximately 80% confluency, the subcultured cells were transferred to T25 cm^2 followed by T75 cm^2 (passage 3) flasks and expanded to confluency.

2.4. Phenotypic characterization of BMSCs by flow cytometry and confocal microscopy

Qualitative evaluation for various cell surface markers was performed on cells grown in the Lab-tek™ chamber slide system™ (Nunc) using a Zeiss LSM 510 Meta confocal scanning laser microscope (Carl Zeiss, Inc.) and quantitative analysis of the same set of markers was performed by single-color flow cytometry using a Coulter® EPICS® XL™ Flow Cytometer (Beckman Coulter, Inc.). Briefly, the passage 3 maintained BMSCs were trypsinized, pelleted at 200 g for 5 min and washed twice with Moscona's solution, pH 7.4. Cells were re-suspended in staining buffer (1.5% bovine serum albumin in PBS, pH 7.4) (BSA, Sigma–Aldrich) and incubated for 30 min at 4 °C with appropriate dilutions of FITC-conjugated mouse anti-rat CD11b, CD31, CD44, CD45, CD90 and OX43 monoclonal antibodies for direct immunostaining (Table 1). Similarly, the cells were incubated with FITC-unconjugated mouse anti-rat CD34, CD73, CD106, Flk1, VE-cadherin, α-SMA, calponin and rabbit anti-rat Flt1 monoclonal antibodies followed by incubation with appropriate FITC-conjugated anti-mouse or, anti-rabbit secondary antibodies for indirect immunostaining (Table 1). Cells were briefly permeabilized in PBS, pH 7.4 containing 0.1% Triton X-100 (Sigma–Aldrich) and 0.5 mM glycine (Sigma–Aldrich) at 4 °C for the intracellular staining of α-SMA and calponin. FITC-labeled mouse anti-rat-IgGs and FITC-labeled rabbit anti-rat-IgG antibodies served as the isotype controls. The stained cells were washed twice with Moscona's solution and either acquired immediately or fixed in ice-cold 0.5% paraformaldehyde (Sigma–Aldrich) and stored in the dark at 4 °C until acquired in flow cytometry. The obtained data were analyzed using Expo32 ADC software (Beckman Coulter, Inc.).

2.5. Purification and enrichment of BMSCs by magnetic cell sorting

Passage 3 adherent populations of BMSCs were further purified by indirect magnetic cell labeling method using an autoMACS™ Pro Separator (Miltenyi Biotech). The cells were subjected to CD90 positive selection by incubating the cells with FITC-labeled anti-CD90 antibodies (BD Pharmingen), followed by incubation with anti-FITC magnetic microbeads (Miltenyi Biotech), and passed through the magnetic columns as per the manufacturer's instructions. The resultant enriched $CD90^+/CD34^-/CD45^-$ fractions were expanded by subcultivation and subjected to flow cytometric analysis.