Cytotherapy, 2011; 13: 606–617

informa
healthcare

Effect of cell-seeding density on the proliferation and gene expression profile of human umbilical vein endothelial cells within ex vivo culture

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Abstract

Background aims. Characterization of endothelial cell–biomaterial interaction is crucial for the development of blood-contacting biomedical devices and implants. However, a crucial parameter that has largely been overlooked is the cell-seeding density. *Methods.* This study investigated how varying cell-seeding density influences human umbilical vein endothelial cell (HUVEC) proliferation on three different substrata: gelatin, tissue culture polystyrene (TCPS) and poly-L-lactic acid (PLLA). *Results.* The fastest proliferation was seen on gelatin, followed by TCPS and PLLA, regardless of seeding density. On both TCPS and gelatin, maximal proliferation was attained at an initial seeding density of 1000 cells/cm^2. At seeding densities above and below 1000 cells/cm^2, the proliferation rate decreased sharply. On PLLA, there was a decrease in cell numbers over 7 days of culture, below a certain threshold seeding density (*c.* 2500–3000 cells/cm^2), which meant that some of the cells were dying off rather than proliferating. Above this threshold seeding density, HUVEC displayed slow proliferation. Subsequently, quantitative real-time polymerase chain reaction (RT-qPCR) analysis of eight gene markers associated with adhesion and endothelial functionality (VEGF-A, integrin-α5, VWF, ICAM1, ICAM2, VE-cadherin, endoglin and PECAM1) was carried out on HUVEC seeded at varying densities on the three substrata. A significant downregulation of gene expression was observed at an ultralow cell-seeding density of 100 cells/cm^2. This was accompanied by an extremely slow proliferation rate, probably because of an acute lack of intercellular contacts and paracrine signaling. *Conclusion.* Hence, this study demonstrates that seeding density has a profound effect on the proliferation and gene expression profile of endothelial cells seeded on different biomaterial surfaces.

Key Words: *endothelial, gelatin, gene expression, poly-L-lactic acid, polystyrene, seeding density*

Introduction

A major challenge faced in the development of blood-contacting biomedical devices and implants is the propensity for platelets to adhere and deposit on the device/implant surface, which consequently results in activation of the thrombogenic pathway (1,2). Endothelial coverage of blood-contacting devices such as coronary stents has attracted considerable interest in recent years as a possible strategy for ensuring hematocompatibility, by preventing platelet adhesion/deposition and resultant thrombogenesis (3,4). Hence the characterization of endothelial attachment and growth on biomaterial surfaces is of utmost importance. This is given further impetus by the increasing recognition of the vast potential of endothelial cells and their progenitors in tissue engineering and cell transplantation therapy (5,6).

Nevertheless, a crucial parameter that has largely been overlooked in studying endothelial adhesion and proliferation on the biomaterial interface is the cell-seeding density. Within the scientific literature, there is abundant evidence that demonstrates that intercellular contacts and paracrine signaling (i.e. secreted cytokines and growth factors from neighboring cells) are crucial for maintaining cellular homeostasis in a diverse array of different somatic lineages (7,8). Hence it is plausible to speculate that endothelial cells are reliant on intercellular contacts and paracrine signaling for optimal proliferation and maintenance of their differentiated phenotype. The extent

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Received 3 May 2010; accepted 12 November 2010)

ISSN 1465-3249 print/ISSN 1477-2566 online © 2011 Informa Healthcare
DOI: 10.3109/14653249.2010.542455

of intercellular contacts and paracrine signaling encountered by individual cells is in turn dependent on the density in which they were seeded on the biomaterial surface. This is particularly significant in certain clinical applications where biomaterials are exposed to limited number of cells, such as in cardiovascular prostheses for *in situ* endothelialization, a process that involves capture of circulating endothelial progenitor cells within the blood stream together with the migration and proliferation of neighboring cells.

We investigated how varying the cell-seeding density influences the proliferation rate of endothelial cells on three different substrata: tissue culture polystyrene (TCPS), gelatin adsorbed on TCPS, and poly-L-lactic acid (PLLA). Human umbilical vein endothelial cells (HUVEC) were chosen for this study because they are a well-characterized and commonly used model for studying endothelial cell biology and physiology *in vitro* (9,10). Moreover, discarded human umbilical cord is a relatively abundant source of these cells (9,10), so that HUVEC can be readily purchased from various commercial sources. Additionally, it must be noted that several studies have previously demonstrated that immortalized endothelial cell lines are inadequate for modeling the physiology and biology of primary explanted endothelial cells such as HUVEC (11–13).

The expression of various gene markers pertaining to endothelial functionality and cellular adhesion (Table I) by HUVEC seeded at various densities on these three substrata were characterized by quantitative real-time polymerase chain reaction (RT-qPCR). It was hoped that the data generated would enable us to determine the optimal cell-seeding density for attaining maximal proliferation of endothelial cells on biomaterial surfaces, while at the same time enabling retention of their endothelial functionality and phenotype. Additionally, the data generated should be useful for optimizing *in vitro* culture protocols for the *ex vivo* expansion of endothelial cells and their progenitors in cell transplantation and tissue engineering applications.

Methods

Cells, culture media and reagents

HUVEC were purchased from Lonza Inc. (Walkersville, MD, USA). Unless otherwise stated, all reagents and chemicals were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), all culture media and supplements from Lonza Inc., and all labware consumables from Techno Plastic Products Inc. (Trasadingen, Switzerland).

Ex vivo expansion of HUVEC

Primary explanted HUVEC were shipped in cryovials under dry ice and stored immediately in liquid nitrogen upon arrival. Subsequently the cells were thawed and cultured within T-75 flasks. The culture medium utilized was endothelial growth media (EGM; catalog number CC-3121; Lonza Inc.) supplemented with 0.4% (v/v) bovine brain extract (catalog number CC-4133; Lonza Inc.). Upon reaching confluence, the cells were detached with 0.25% (w/v) bovine trypsin and 1 mM ethyelene diamine tetra acetic acid (EDTA; Gibco-BRL Inc., Franklin Lakes, NJ, USA), and cultured on fresh T-75 flasks at a passage split ratio of 1:3. The HUVEC were propagated for six to seven passages (P6–P7) prior to being utilized for experiments.

Preparation of cell culture substrata

Gelatin (porcine type A, catalog number G1890; Sigma-Aldrich Inc.) was reconstituted in de-ionized water at a concentration of 1 mg/mL and subsequently

Table I. Gene markers examined by RT-qPCR.

	Gene marker	Putative function	Reference
Endothelial-specific adhesion markers	VE-cadherin (CD144)	Integrity of intercellular junctions between endothelial cells	(36)
	PECAM1 (CD31)	Cell adhesion and signaling molecule that is enriched at the endothelial cell junctions	(37)
Other endothelial-specific markers	VEGF-A	A key cytokine/growth factor involved in the regulation of angiogenesis	(38)
	VWF	Platelet adhesion to wound sites, binding to factor VIII	(33)
	Endoglin (CD105)	Endothelial cell proliferation, angiogenesis and vascular remodeling	(30)
Other generic adhesion markers	Integrin-α5	Focal adhesion points	(39)
	ICAM1 (CD54)	Cell migration	(32)
	ICAM2	Expressed at the endothelial junctions, also mediates angiogenesis	(31)

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