



Morphology and vasoactive hormone profiles from endothelial cells derived from stem cells of different sources



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ARTICLE INFO

Article history:

Received 21 October 2014

Available online 10 November 2014

Keywords:

Stem cells

Endothelial cells

Prostacyclin

Endothelin-1

Shear stress

ABSTRACT

Endothelial cells form a highly specialised lining of all blood vessels where they provide an anti-thrombotic surface on the luminal side and protect the underlying vascular smooth muscle on the abluminal side. Specialised functions of endothelial cells include their unique ability to release vasoactive hormones and to morphologically adapt to complex shear stress. Stem cell derived-endothelial cells have a growing number of applications and will be critical in any organ regeneration programme. Generally endothelial cells are identified in stem cell studies by well-recognised markers such as CD31. However, the ability of stem cell-derived endothelial cells to release vasoactive hormones and align with shear stress has not been studied extensively. With this in mind, we have compared directly the ability of endothelial cells derived from a range of stem cell sources, including embryonic stem cells (hESC-EC) and adult progenitors in blood (blood out growth endothelial cells, BOEC) with those cultured from mature vessels, to release the vasoconstrictor peptide endothelin (ET)-1, the cardioprotective hormone prostacyclin, and to respond morphologically to conditions of complex shear stress. All endothelial cell types, except hESC-EC, released high and comparable levels of ET-1 and prostacyclin. Under static culture conditions all endothelial cell types, except for hESC-EC, had the typical cobblestone morphology whilst hESC-EC had an elongated phenotype. When cells were grown under shear stress endothelial cells from vessels (human aorta) or BOEC elongated and aligned in the direction of shear. By contrast hESC-EC did not align in the direction of shear stress. These observations show key differences in endothelial cells derived from embryonic stem cells versus those from blood progenitor cells, and that BOEC are more similar than hESC-EC to endothelial cells from vessels. This may be advantageous in some settings particularly where an in vitro test bed is required. However, for other applications, because of low ET-1 release hESC-EC may prove to be protected from vascular inflammation.

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1. Introduction

Endothelial cells on vessels release key vasoactive hormones, which act to regulate vascular tone and platelet reactivity. These are (i) prostacyclin [1], (ii) nitric oxide [2] and (iii) endothelin (ET)-1 [3]. Prostacyclin has a myriad of protective effects on the cardiovascular system including induction of vasodilation, inhibition of platelet aggregation and inhibition of vascular smooth muscle

proliferation [4]. ET-1 on the other hand causes vasoconstriction and smooth muscle proliferation [3,5], and exists in balance with prostacyclin.

In vessels, endothelial cells are exposed to shear stress, which is the frictional force of blood flow on the luminal surface. The degree and direction of shear stress that endothelial cells are exposed to has profound effects on their morphology and function. For example, endothelial cells lining vessels at the site of directional, laminar shear stress are elongated and aligned in the direction of flow and protected from inflammation whilst those exposed to non-directional, turbulent shear stress are cobblestone shaped and primed for inflammation [6,7].

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Stem cell derived endothelial cells have a growing number of applications in clinical medicine and biomedical research and will be critical in any organ regeneration programme. There are three cardinal sources of stem cell derived endothelial cells, which are (i) embryonic stem cells, (ii) induced pluripotent stem cells and (iii) progenitor cells circulating in the blood. Stem cell derived endothelial cells have been rigorously characterised in terms of lineage markers but their ability to release hormones critical to endothelial cell function in vessels, and their ability to align under conditions of complex shear stress has not been fully assessed.

With this in mind, we have compared directly the ability of endothelial cells derived from two key stem cell sources, including embryonic stem cells (human embryonic stem cell derived endothelial cells; hESC-EC) and adult progenitors in blood (blood outgrowth endothelial cells, BOEC) with those cultured from mature vessels to release the vasoconstrictor peptide ET-1, and the cardioprotective hormone prostacyclin, and to align appropriately under conditions of complex shear stress. Endothelial cells also fulfil crucial immune functions by responding to pathogens and cytokines. Work from our group has shown that in culture endothelial cells, including those derived from embryonic stem cells, release the ubiquitous chemokine/cytokine CXCL8 [8,9]. Thus, in this study, in order to assess how inflammatory hormones might compare with vasoactive hormone release from endothelial cells we have measured CXCL8 from each cell type in parallel.

2. Methods

2.1. Isolation and culture of hESC-EC

Differentiation of hESC (H7 cell line) into hESC-EC was carried out as described previously [10]. Briefly, cells were dissociated into clumps and plated on ultra-low attachment plates (Nunc, Denmark) with Lonza-EGM2 with 2% foetal bovine serum (FBS) to allow formation of embryoid bodies. After 4 days embryoid bodies were re-plated on gelatin (1%; Sigma–Aldrich, USA) coated 6-well plates in Lonza-EGM2 with 2% FBS. After 13 days clusters were dissociated and stained for the endothelial cell marker CD31 using an AlexaFluor 488 fluorescence dye labelled anti-CD31 antibody (BD Biosciences, Oxford, UK). Cells were sorted using a FACS Aria II cell sorter (BD Biosciences, Oxford, UK) and expanded in Lonza-EGM2 media with 2% FBS for further use. Cells were maintained in Lonza-EGM2 media with 2% FBS and grown on gelatin (1%; Sigma–Aldrich, USA) coated 75 cm² flasks. Cells were used between passages 2–8.

2.2. Isolation and culture of BOEC

BOEC were isolated using a well established protocol [11] with minor modifications [8,12,13]. Briefly, peripheral blood mononuclear cells were isolated from blood and expanded on tissue culture plates for up to 22 days in Lonza-EGM2 with 10% FBS on collagen coated plates (5.2 µg/cm²; BD Biosciences). Typically, endothelial cell colonies emerged between 5 and 22 days in culture and were expanded for use in experiments. Cells were maintained in Lonza-EGM2 media with 2% FBS on collagen coated 75 cm² flasks. Cells were used between passages 2–8.

2.3. Endothelial cells from vessels

Human umbilical vein endothelial cells (HUVEC) were a gift from Caroline Wheeler-Jones (Royal Veterinary College, London) and were maintained in Lonza-EGM2 media with 2% FBS. Cells were at passage 2 on arrival and used for experiments between passages 2–8.

Human lung microvascular endothelial cells (HMVEC), and human aortic endothelial cells (HAEC) were purchased from Lonza or Promocell as cryopreserved cells. Cells were grown and maintained in Lonza-EGM2 media with 2% FBS and grown on gelatin (1%; Sigma–Aldrich, USA) coated 75 cm² flasks. Cells were used between passages 2–8.

2.4. Cell plating

For all experiments, cells were plated in identical conditions in Lonza EGM2 media with 10% FBS on gelatin (1%; Sigma–Aldrich, USA) coated tissue culture plates. For experiments where hormone (ET-1, prostacyclin or CXCL8) release was measured, cells were plated at a density of 10,000 per well (100 µl) in 96 well culture plates. At this seeding density cells generally reached confluence after 48 h, after which media was replaced with fresh media and cells incubated for a further 24 h. In some experiments IL-1β was added to the media in order to further activate prostacyclin pathways and release. Conditioned media was then collected for measurement of ET-1, prostacyclin or CXCL8. For shear stress protocols cells were plated on 6-well plates at a density of 100,000 cells per well (2 ml) for 48 h under static conditions and allowed to reach confluence. Media was then replaced with fresh media and cells either exposed to shear stress by placing plates on the orbital shaker [7] (as described below) or incubated under 'normal' static culture conditions for a period of 4 days.

2.5. Assessment of the ability of stem cell derived endothelial cells to align under shear stress

Endothelial cell alignment under shear stress was determined using a model previously defined by our group [7]. Briefly, cells were grown to confluence in 6 well plates as described above and placed on an orbital shaker. The movement of the shaker results in a wave of media that transverses around the well resulting in a complex pattern of shear stress with directional (laminar) shear towards the edge of the well, and non-directional (turbulent) shear at the centre. Alignment of cells was visualised by light microscopy. Quantification of cell alignment and elongation was carried out using a blind scoring system. Users were blinded to the identity of all images captured at the centre and edge wells from cells cultured under static and shear stress conditions and asked to score alignment (0–4) and elongation (0–4). Data were collected from 5 separate scorers. Elongation and alignment were defined and explained to blind scorers as follows: 'elongation' means how stretched the cells appear. A cell that looks like a square or cobblestone scores zero. An image with cells that look stretched/elongated = 4 with 1, 2 and 3 being intermediate. 'Alignment' means whether the cells are aligned in a similar direction. Remember elongated cells might not be aligned. Cells that are strongly aligned in one direction score 4 and cells not aligned and that are randomly arranged (regardless of elongation or not) score 0 with 1, 2 and 3 as intermediates.

2.6. Measurement of ET-1, prostacyclin and CXCL8 by specific ELISA

ET-1, CXCL8 (R and D Systems, UK) and prostacyclin (measured as its breakdown product 6-keto-PGF_{1α}; Cayman Chemical, UK) were measured in supernatants by specific ELISA according to manufacturer's instructions. Optical density was determined using a microplate reader (Dyne, Magellen Biosciences, UK).

2.7. Statistical analysis

Data are the mean ± SEM for *n* separate observations as described in each figure legend. Data was analysed according to

the information in each figure legend. Unless otherwise stated, all experiments were carried out on three independent occasions with separate cell isolations.

3. Results

3.1. Hormone release by endothelial cells derived from different types of stem cells

ET-1 release was detected in high amounts of similar magnitude from all endothelial cell types except for hESC-EC (Fig. 1A). The low ET-1 release from hESC-EC was reflected by relatively low levels of gene expression when compared to HUVEC (Fig. 1B). Prostacyclin release, under control culture conditions or after stimulation with IL-1β, was detectable from all endothelial cell types studied, with BOEC releasing the highest levels and hESC-EC releasing low/negligible amounts (Fig. 1C and D). By contrast to the relatively low levels of ET-1 and prostacyclin, hESC-EC released similar levels of CXCL8 as other endothelial cells types (Table 1).

3.2. Morphology and response to shear stress of endothelial cells derived from different types of stem cells

As with endothelial cells grown from the vasculature (HAEC) endothelial cells from blood progenitors (BOEC) were cobblestone in appearance when grown under static culture conditions (Fig. 2A). As we have described previously for porcine aortic endothelial cells [7], using a simple orbital shaker method, BOEC and HAEC changed morphology when cultured under shear stress for 4 days. Both BOEC and HAEC elongated and aligned when exposed to directional shear stress (Fig. 2B, edge) but remained

Table 1
CXCL8 release from different stem cell derived endothelial cells versus endothelial cells from vessels. Release was measured as following 24 h incubation. Data are mean ± SEM for *n* separate experiments, except for data HAEC, which is triplicate incubations, derived from a single isolation of cells. Human embryonic stem cell derived cells (hESC-EC; *n* = 8), blood outgrowth endothelial cells (BOEC; *n* = 7), human lung microvascular endothelial cells (HMVEC; *n* = 3), human umbilical vein endothelial cells (HUVEC *n* = 7). Statistical significance was determined by one-way ANOVA followed by Bonferroni's post-test to compare all cell types. No significant differences in any of the cell types tested were detected (*p* > 0.05).

Cell type	CXCL8 (pg/ml)
hESC-EC	1870.6 ± 495.2
BOEC	3018.0 ± 1251.6
HMVEC	2849.2 ± 856.9
HUVEC	2291.7 ± 710.0
HAEC	836.1 ± 56.7

cobblestoned in morphology when exposed to non-directional, turbulent shear stress (Fig. 2B, centre). By contrast, hESC-EC isolated in our laboratory had a clearly different morphology being elongated (rather than cobblestone) when cultured under static or shear stress conditions (Fig. 2). Quantification of cell elongation and alignment by blind scoring showed statistically significant elongation and alignment of both HAEC and BOEC cultured under directional shear stress and that for all conditions hESC-EC did not appear to respond to shear stress (Fig. 3).

4. Discussion

Endothelial cells derived from stem cells are currently the subject of investigation for both therapeutic and experimental uses. Re-endothelialisation of engineered organs has recently been

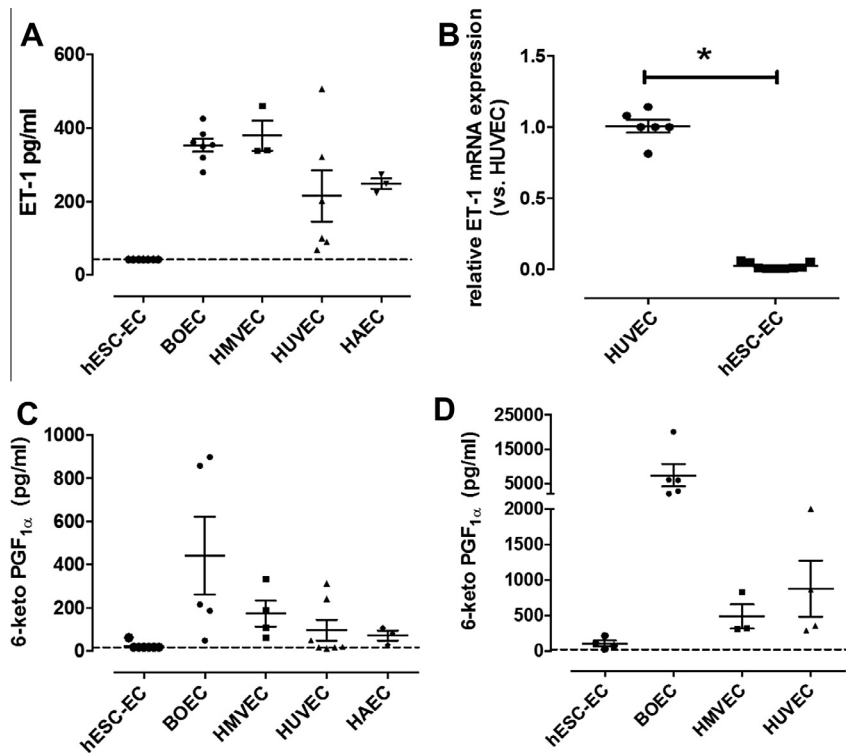


Fig. 1. Hormone release from different types of endothelial cells. Panel (A) shows endothelin (ET)-1 release and panel (B) shows expression of ET-1 mRNA versus HUVEC. Panels (C and D) show prostacyclin (measured as the breakdown product 6-keto PGF_{1α}). Cells were cultured in media alone (A–C) or with IL-1 (1 ng/ml; D) for 24 h. Data are mean ± SEM for *n* separate experiments, except for data HAEC, which is triplicate incubations, derived from a single isolation of cells. Human embryonic stem cell derived cells (hESC-EC; A; *n* = 7, B; *n* = 6, C; *n* = 7 and D; *n* = 4), blood outgrowth endothelial cells (BOEC; A; *n* = 7 and C and D; *n* = 5), human lung microvascular endothelial cells (HMVEC; A and C; *n* = 3 and D; *n* = 4), human umbilical vein endothelial cells (HUVEC; A; *n* = 6 and C; *n* = 7 and D; *n* = 4). Minimum detection limits of the assays are represented by a dotted line. Statistical significance in panel (B) was determined by one-sample *t*-test versus HUVEC (*n* = 6) (**p* < 0.05).

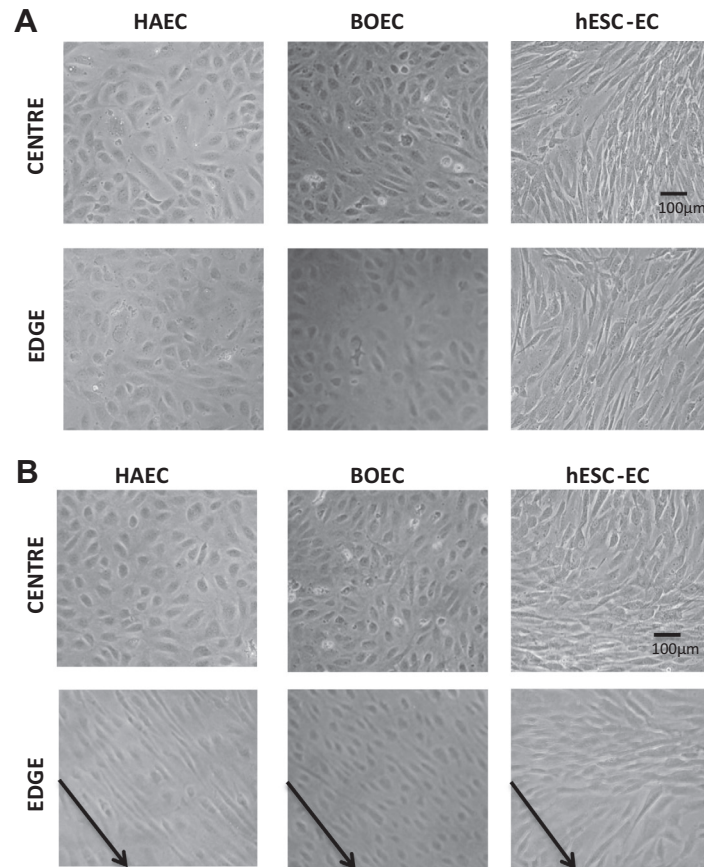


Fig. 2. Responses of different stem cell derived endothelial cells to shear stress. Human aortic endothelial cells (HAEC), blood outgrowth endothelial cells (BOEC), and human embryonic stem cell derived endothelial cells (hESC-EC) (left to right) after 4 days cultured under either (A) static conditions or (B) under shear stress. Images were taken at the edge of the well, where shear stress is unidirectional and cells align, and at the centre of the well where shear stress had no preferred direction. Black arrows on shear plate edge images indicate the direction of shear stress. Images are from cells of individual experiments and representative of observations made from $n = 3$ –8 experiments which are quantified in Fig. 3.

demonstrated and shown to reduce thrombogenicity and improve function of engineered heart tissue [14]. However, our knowledge of how endothelial cells derived from different sources of stem cells function compared to cells derived from vessels is still incomplete. Thus, the identification of the optimum stem cell choice to generate endothelial cells for various scenarios is critical. Consequently, the ability of endothelial cells generated from different stem cell sources to release vasoactive hormones is important for us to know. For example, if endothelial cells are to be incorporated into engineered organs or vessels they must have the potential to release prostacyclin in order to provide an antithrombotic surface. Without this ability thrombi would likely form in the transplanted organ resulting in failure. Similarly the ability of derived endothelial cells to release ET-1, which is associated with cardiovascular disease, will impact on the function of grafted cells. Another important feature of endothelial cells is that they are acutely sensitive to shear stress, presumably as an adaption to being exposed to the physical forces applied by blood passing over them in the circulation. Specifically endothelial cells align in the direction of shear stress and so appear elongated in areas of directional shear, which occurs where vessels are straight and uniform whilst appearing cobblestone in appearance in areas of non-directional shear such as branch points. This morphological response is thought to be important in vascular biology since elongated and aligned endothelial cells are protected from inflammation and atherosclerosis [6]. The premise of this study therefore was to assess the ability of endothelial cells derived from the cardinal stem cell sources to release ET-1 and prostacyclin and to align under conditions of shear stress.

We found that all endothelial cell types were able to release the protective hormone prostacyclin. Of note, we found that hESC-EC released very low levels, but that BOEC released high amounts, superior not only to endothelial cells derived from hESC, but also endothelial cells cultured from mature vessels. Since prostacyclin is a powerful anti-thrombotic hormone that also limits vascular smooth muscle remodelling and cholesterol accumulation [4], as low releasers, hESC-EC may represent a poorer choice of cell type for therapy. However, we also found that, whilst hESC-EC released low levels of prostacyclin, they equally released low levels of the endothelial hormone ET-1. ET-1 is associated with cardiovascular disease and considered to be a prime mediator of conditions such as pulmonary hypertension [15]. Thus, a reduced capacity to synthesise ET-1 may well provide an advantage over endothelial cells from other sources in therapy. It should be noted that hESC-EC released comparable levels of CXCL8 to other endothelial cell types tested, suggesting that the low release of ET-1 and prostacyclin from hESC-EC was not due to an intrinsic inability of these cells to produce and release mediators.

BOEC, had an identical morphology to endothelial cells from human aorta (HAEC) and also aligned identically to HAEC when grown under directional shear stress. This is in line with what others report for BOEC [16,17]. By contrast we found endothelial cells derived from hESC, in our hands, and isolated using CD31 positive sorting, to have a different morphology to other endothelial cells. Specifically we found hESC-EC produced in this way to have an elongated, randomly orientated morphology that was retained under conditions of shear stress. This observation is in contrast

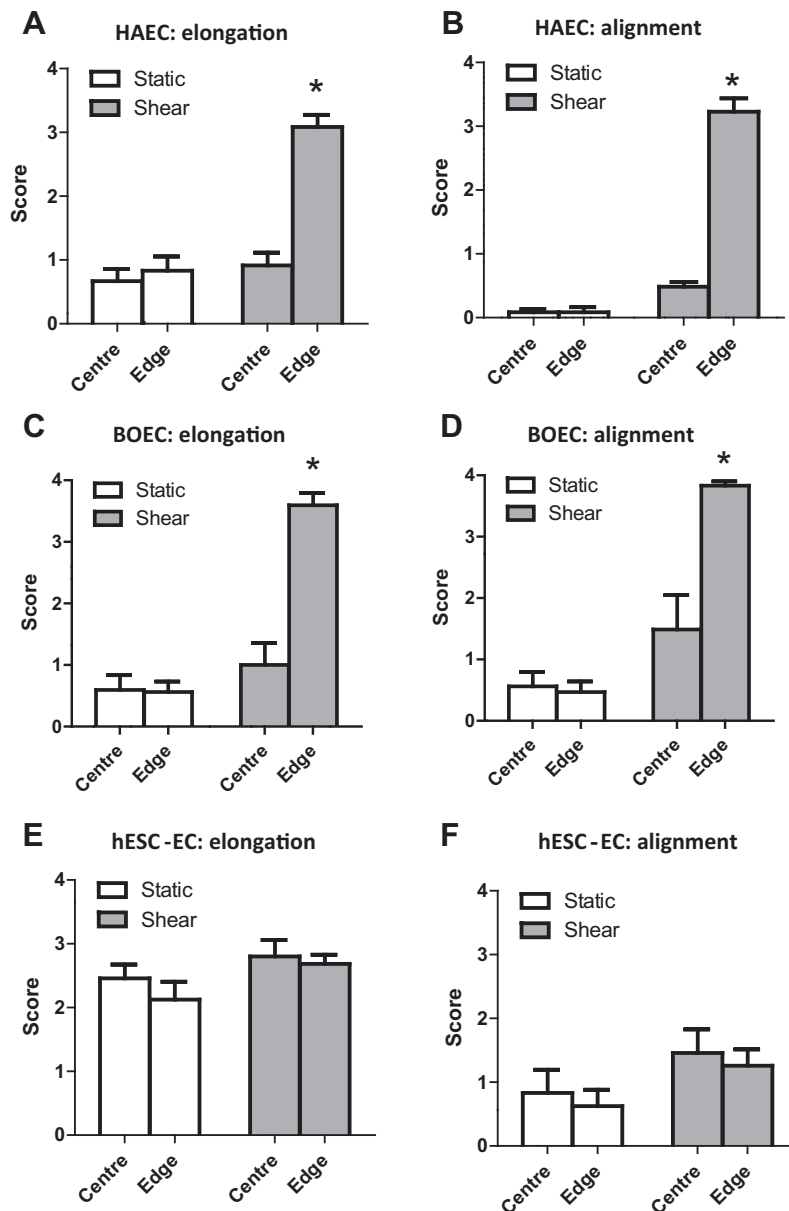


Fig. 3. Quantification of elongation and alignment of stem cell derived endothelial cells in response to 4 days of shear stress. HAEC (A and B), BOEC (C and D) and hESC-EC (E and F) were scored for elongation (A, C and E) and alignment (B, D and F) from images at the centre and edge of the well under static and shear stress conditions. Scoring (0–4) was carried in using a blind and is the average of five independent scores. Data are mean \pm SEM for n separate wells (HAEC; $n = 6$ –7, BOEC; $n = 8$, hESC-EC; $n = 6$ –7) derived from 3 to 8 separate experiments. Statistical significance between centre and edge scores for each cell and condition was determined by paired t -test (* $p < 0.05$).

to isolations of hESC-EC made by other groups which had a cobblestone morphology [18,19] and align in the direction of shear stress [20] but suggest that endothelial cells from embryonic stem cells may have multiple morphologies that, unlike other types of endothelial cells, emerge as non-reversible phenotypes. Indeed it is relevant to note that we have observed that the elongated and aligned morphology that endothelial cells take on after culture under directional shear stress reverts to a cobblestone phenotype after around 24 h of static culture (unpublished observations). Nevertheless, taken together these observations could indicate that BOEC are closer to 'authentic' endothelial cells and would therefore perform better as an in vitro test bed and/or as source for tissue regeneration and therapy. However, the elongated morphology of hESC-EC is consistent with cells that would be 'protected' from inflammation [21]. The idea that hESC-EC are resistant to some types of inflammatory stimuli is in line with results recently

published by our group showing that they are protected from activation with the Toll-like receptor (TLR)-4 agonist LPS [8,10].

In summary, we have shown that BOEC, like endothelial cells from vessels, release ET-1 and prostacyclin and have a typical cobblestone morphology when cultured under static conditions. hESC-EC, in our hands, released comparably less prostacyclin but also less ET-1 and had a different morphology than other endothelial cell types. Others have shown hESC-EC to have a more classical endothelial cell morphology that is responsive to shear stress [20]. Taken together this suggests that, unlike endothelial cells from blood progenitors, those from embryonic stem cell sources may emerge as specific, and variable pre-set phenotypes. Whilst this is not a typical feature of endothelial cells grown from vessels, which emerge as consistent phenotypes across isolations, it appears that endothelial cells from separate isolations of embryonic, and possibly other stem cell sources, will need to be

individually tested for morphology and vasoactive hormone release. Our data suggests that in some settings, such as those designed to model endothelial cell biology or patient phenotype, BOEC may perform better, but that in others, specific hESC-EC phenotypes may prove to be protected from inflammation. However, whether different isolations of hESC-EC can result in varied types of endothelial cells and how ET-1 and prostacyclin release from other clonal lines profile remains to be determined. The impact of these observations functionally in transplanted tissues is beyond the scope of this study, but we suggest that they provide a starting point for additional, but critical types of cell assessment for future consideration in the field of stem cell testing, therapy and organ regeneration.

Acknowledgments

This work was funded by an MRC doctoral training award (DMR) and by grants awarded by the BHF and Wellcome Trust.

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