IMPROVED MICROVASCULAR NETWORK FORMATION BY ADULT HUMAN BLOOD OUTGROWTH ENDOTHELIAL CELLS RELATIVE TO MATURE HUMAN ENDOTHELIAL CELLS

Alisha L. Sieminski (1), Robert P. Hebbel (2), Keith J. Gooch (1)

(1)Department of Bioengineering and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA
(2)Department of Medicine, University of Minnesota, Minneapolis, MN

INTRODUCTION

Endothelial cells (EC) have often been suggested as useful vehicles for efficient delivery of proteins to the circulation because of their ease of isolation, culture, and transfection in vitro, and most importantly, their direct contact with the flowing blood. When implanted in vivo, pre-formed microvascular networks (MVN) have the potential to incorporate with the host vasculature providing direct contact between genetically modified EC and the flowing blood, enabling efficient delivery of therapeutic proteins to the circulation. We previously demonstrated, using high-passage bovine aortic endothelial cells, that it was possible to prolong cell viability and delivery of hGH by including non-secreting fibroblasts in our system. However, the challenge remains to increase and prolong EC incorporation into blood vessels to facilitate efficient delivery of difficult to deliver proteins to the circulation.

Several groups have recently shown that implantation of EC that are either low-passage or genetically modified for survival advantage (e.g. with bcl-2 or telomerase) show improved survival, MVN formation in vitro, and incorporation into the host vasculature in vivo [1-3]. Another approach to improving cell survival and incorporation may be to use endothelial progenitor cells or circulating endothelial cells, which a number of groups have reported isolating from adult human peripheral blood or bone marrow. Blood outgrowth endothelial cells (BOEC), obtained from peripheral blood of adult humans, express endothelial markers while maintaining a high proliferative capacity (up to 10^{18} fold expansion) [4] not typically exhibited by mature human EC. These cells are attractive for use in cell-based therapies because, in theory, they could be expanded to obtain a therapeutically useful number of cells while maintaining the characteristics of "young" cells. Here we report on improved in vitro MVN formation by BOEC relative to other adult human EC.

To examine the potential of BOEC, we employed an in vitro model of angiogenesis to compare their survival, elongation, and lumen formation to three human endothelial cell lines at comparable population doublings. As with mature endothelial cells, serum concentration, presence of supplemental growth factors, and phorbol myristate acetate (PMA) modulate BOEC performance but under comparable conditions, BOEC consistently performed better than mature endothelial cells.

METHODOLOGY

BOEC, human umbilical vein endothelial cells (HUVEC), adult human dermal microvascular cells (adHDMVEC), and neonatal HDMVEC (neoHDMVEC) were cultured in EGM-2 media (Clonetics) with 10% fetal bovine serum (FBS) on collagen-coated flasks. Liquid type-I rat-tail collagen (Becton-Dickinson) was prepared as directed and mixed with cells. Gels of 200 μl were cast into 48-well plates and allowed to solidify at 37°C, leaving the cells suspended in 3 mg/ml collagen gels at 1 x 10^6 cells/ml. Gels were transferred to larger wells and cultured for 2 days in media containing either 2 or 10% FBS and supplemented with bFGF, VEGF, and PMA (all 50 ng/ml) as indicated. Samples were fixed and prepared for histology or stained with toluidine blue and mounted between a slide and coverslip. Images of mounted gels were taken with a CCD camera at 10X and to quantify MVN formation, images were thresholded, made binary, and skeletonized using Scion Image. The average skeletonized length was obtained for 3-4 gels per treatment. For mechanistic studies, integrin blocking antibodies (Chemicon) were added to culture media at 20 μg/ml; N-nitro-L-arginine methyl ester (L-NAME) was added at 0.5, 5, and 50 mM; and diethylenetriamine-nitric oxide (DETA) was added at 1 and 10 μM. TUNEL staining was performed on histological sections as directed by the manufacturer (Boehringer-Mannheim).

RESULTS

When cultured within a three-dimensional collagen gel in media supplemented with growth factors (bFGF and VEGF), within 2 days many individual BOEC elongated (Fig. 1a) and with further addition of PMA they formed interconnecting MVN (Fig. 1b) that were maintained for at least 6 days. Histological evaluation of BOEC in collagen with PMA revealed numerous lumen-like structures. Many individual HUVEC, adHDMVEC, and neoHDMVEC elongated in the presence of growth factors and PMA, but to a lesser extent than the

BOEC (Fig. 2). Without PMA many fewer of these cells elongated as frequently reported by others working with adult human EC [5]. Similar trends were seen with 10% FBS (not shown). While lumens were also frequently seen when HUVEC were cultured in the presence of PMA, the HDMVEC cells rarely formed them.

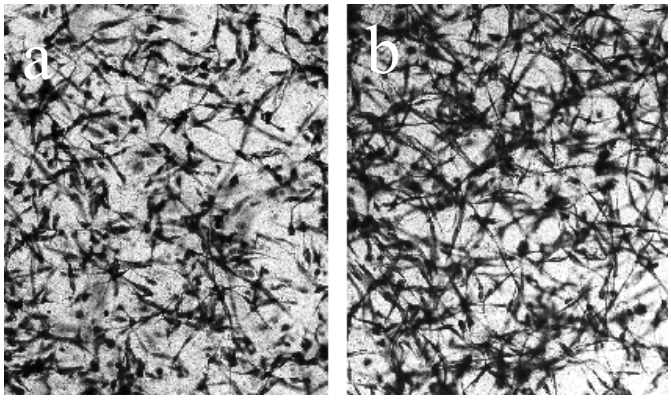

Figure 1. Images of fixed, toluidine blue stained BOEC cultured with GF (a) or GF and PMA (b) for 2 days.

To investigate three potential mechanisms for improved MVN formation by BOEC we assessed cell survival, integrin involvement and nitric oxide involvement. We first assessed cell survival under different conditions by performing a TUNEL stain on histological sections of collagen gels containing cells and cultured in media containing 10% FBS. High levels of cell death were seen for all cell types cultured in media only. The addition of growth factors significantly improved survival and further addition of PMA improved it only slightly for all cell types (not shown). Surprisingly the fraction of TUNEL-positive BOEC was similar to that of neoHDMVEC and greater than HUVEC.

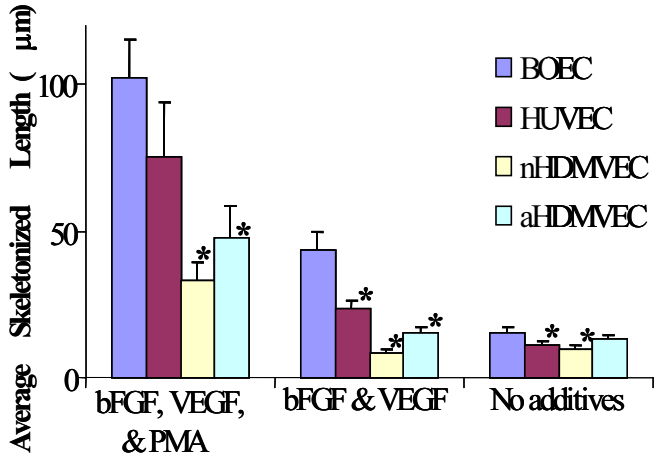

Figure 2. Quantification of MVN formed by cells cultured in 2% FBS. * Significantly different from BOEC under same conditions by Tukey-Kramer HSD test.

To next investigate the involvement of integrins in MVN formation, gels were incubated with various integrin-blocking antibodies. In a similar system, blocking $\alpha_2\beta_1$ but not $\alpha_v\beta_3$ integrins inhibited vacuole formation (a precursor to MVN formation) by HUVEC in collagen [6]. In our system, MVN formation by BOEC was dose-dependently inhibited by adding a blocking antibody to the β_1 integrin subunit. Further MVN formation by both BOEC and HUVEC in the presence of growth factors and PMA was inhibited by the addition of antibodies to β_1 or $\alpha_2\beta_1$ but not to $\alpha_v\beta_3$ (all at 20 μg/ml).

As nitric oxide (NO) has also been shown to play an important role in MVN formation [7], we explored the ability of an L-arginine analogue, L-NAME, to block MVN formation as well as the ability of an NO donor, DETA, to stimulate it. L-NAME dose-dependently inhibited MVN formation by BOEC in the presence of growth factors and PMA from 0.5 to 50 mM although toxicity was apparent at higher concentrations. Surprisingly in this system L-NAME did not block HUVEC MVN formation except at the higher, toxic, concentration. DETA appeared to slightly, but not significantly augment MVN formation by all 4 cell types in the presence of growth factors and PMA as well as increasing MVN formation by BOEC in the presence of just growth factors.

DISCUSSION

The challenge of cell-based gene therapy is to maintain endothelial survival and to achieve adequate incorporation into blood vessels to facilitate efficient delivery of difficult to deliver proteins to the circulation. Towards this end we have begun to explore the use easily obtainable adult human BOEC that may behave like low-passage cells as this may promote increased survival, MVN formation, and vascular incorporation without the use of oncogenes. Although the use of low passage cells may be beneficial, it is likely to be clinically desirable to expand cells in order to obtain a relevant quantity. For this reason the use of BOEC is very attractive. In an in vitro angiogenesis model BOEC show improved MVN formation over 3 lines of human adult endothelial cells under a variety of culture conditions. Exploration of the mechanism of improved MVN formation show that BOEC appear to be similar with respect to survival and integrin involvement in this system although they may be slightly more dependent on nitric oxide. Based on these favorable in vitro results in vivo exploration of BOEC appear to be warranted.

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