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Alloimmunity to Human Endothelial Cells Derived from Cord Blood Progenitors

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There is considerable interest in exploiting circulating endothelial progenitor cells (EPCs) for therapeutic organ repair. Such cells may be differentiated into endothelial cells (ECs) in vitro and then expanded for use in tissue engineering. Vessel-derived ECs are variably immunogenic, depending upon tissue source, and it is unknown whether ECs derived from cord blood EPCs are able to initiate an allogeneic response. In this study, we compare the phenotype and alloantigenicity of human cord blood progenitor cell-derived ECs with HUVECs isolated from the same donors. Human cord blood progenitor cell-derived ECs are very similar to HUVECs in the expression of proteins relevant for alloimmunity, including MHC molecules, costimulators, adhesion molecules, cytokines, chemokines, and IDO, and in their ability to initiate allogeneic CD4⁺ and CD8⁺ memory T cell responses in vitro and in vivo. These findings have significant implications for the use of cord blood EPCs in regenerative medicine or tissue engineering. *The Journal of Immunology, 2007, 179: 7488–7496.*

Both the regeneration of injured tissues and the construction, through bioengineering, of synthetic tissue substitutes depend upon the formation of new blood vessels (1–4). Endothelial cells (ECs) isolated from the luminal lining of mature blood vessels can spontaneously organize into tubes that may serve as the nucleus for new vessel development (3–6). However, vessel-derived ECs have only limited replication potential and rapidly senesce when expanded in vitro (7). ECs may also be differentiated from endothelial progenitors cells (EPCs) that are found in peripheral blood (8). Umbilical cord blood is a particularly rich source of EPCs that can be used for tissue engineering (9, 10). The EPCs found in human cord blood may themselves be heterogeneous. Some EPCs, when cultured in basal medium supplemented with FCS and vascular endothelial growth factor (VEGF), rapidly produce colonies of EC-like cells. These early outgrowth cells express some EC markers, like CD31 or von Willebrand factor, but also express markers of monocytes, like CD14 and CD45. Such cells have limited replicative capacities and do not form stable tubes when implanted in immunodeficient mice (11–13,15). Other EPCs give rise to EC-like colonies only after a delay of 7 days or more (10, 14, 16–21). Such late outgrowth cells have high replicative capacity, express EC but not monocyte markers, and form stable tubes in vivo (14). Hereafter we will refer to the differentiated progeny of late outgrowth EPCs as human cord blood derived ECs (HCBECs). Because of their greater replicative potential and their ability to form stable tubes in vivo, late outgrowth cells are a much more useful source of ECs for therapeutic purposes than early outgrowth EPCs.

Immunological rejection of allogeneic stem cells following differentiation represents a major hurdle for both organ regeneration and tissue engineering. This is likely to be particularly true for vascularized structures because some vessel-derived human ECs are capable of initiating an allogeneic immune response, directly presenting both non-self allelic forms of class I and class II MHC molecules to alloreactive memory CD8⁺ and CD4⁺ T cells, respectively (22, 23). Specifically, coculture of purified peripheral blood memory T cell subsets with allogeneic vessel-derived human ECs leads to expression of T cell activation markers, cytokine production, and proliferation (21, 22, 24, 25). Moreover, experiments using immunodeficient mouse hosts have revealed that human ECs are capable of triggering graft rejection by adoptively transferred alloreactive effectors or memory T cells (24). The majority of experiments involving vessel-derived ECs have used either cells isolated from human umbilical veins (HUVEC) or human dermal microvessels. It has been reported that ECs from other vascular beds may differ from HUVECs in their capacities to activate allogeneic T cells (26). It is unknown whether ECs derived from cord blood EPCs are able to activate allogeneic T cells, and if they can do so, how they compare with other types of ECs isolated from mature vessels.

In the present study, we have compared HCBECs with HUVECs in a variety of assays for immunological functions. In most instances, we have been able to isolate HCBECs and HUVECs from the same umbilical cord to control for genetic variations. Our key finding is that HCBECs are largely indistinguishable from HUVECs in their expression of molecules of relevance to allogeneic T cell activation and in their ability to stimulate an alloresponse, a result that has important implications for the use of HCBECs for regenerative medicine or tissue engineering.

Materials and Methods

**Cell culture**

All human cell populations were obtained using protocols approved by the Yale Human Investigation Committee. HUVECs were released from cultured and perfusion-cleared umbilical veins, by collagenase digestion.

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3 Abbreviations used in this paper: EC, endothelial cell; EPC, endothelial progenitor cell; HUVEC, human umbilical veins; HCBEC, human cord blood progenitor cell-derived EC; VEGF, vascular endothelial growth factor; Uea-1, *Ulex europaeus* agglutinin 1; MFI, mean fluorescence intensity.

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Flow cytometric analysis of proteins of immunological significance in HUVECs and HCBECs isolated from the same donor.

Table I. Flow cytometric analysis of proteins of immunological significance in HUVECs and HCBECs isolated from the same donor

<table>
<thead>
<tr>
<th>Protein</th>
<th>HUVECs</th>
<th>HCBECs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IFN-γ (36 h)</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
<td>353</td>
<td>358</td>
</tr>
<tr>
<td>CD45</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>54</td>
<td>749</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>LFA-3 (CD58)</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>CD40</td>
<td>35</td>
<td>61</td>
</tr>
<tr>
<td>B7-1 (CD80)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B7-2 (CD86)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ICOS ligand</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>4-1BB ligand (CD137L)</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Ox40 ligand (CD134L)</td>
<td>337</td>
<td>331</td>
</tr>
<tr>
<td>GITR ligand</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>PD-L1 (CD274)</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td>PD-L2 (CD273)</td>
<td>13</td>
<td>54</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>ICAM-1 (CD54)</td>
<td>80</td>
<td>526</td>
</tr>
<tr>
<td>E-selectin (CD62E)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Cells were exposed to 10 ng/ml TNF, 50 ng/ml IFN-γ, or mock treated for the indicated times. Cells were harvested and labeled as described in Materials and Methods. The results are expressed in terms of corrected median fluorescence intensity (MFI), in arbitrary units of fluorescence, after subtracting the MFI of isotype control from the MFI of the specifically stained cells. Each cell type was analyzed from at least two separate cultures with similar results. ND, Not detected.
with EC. Following 1 wk of coculture, T cells were collected and stained with anti-CD4 or CD8 PE-labeled mAbs (Beckman Coulter), respectively, and analyzed using two-color FACS. Duplicate samples were labeled with anti-CD25 PE-labeled mAb, also from Beckman Coulter.

**In vivo analysis of EC alloantigenicity**

All animal protocols were approved by the Yale Institutional Animal Care and Use Committee. Human microvessels were generated and implanted in the s.c. position on the abdominal wall of C.B-17/Scid-beige mice as previously described (5). Briefly, HCBECs or HUVECs were suspended in a rat tail type I collagen-human plasma fibronectin gel and the cell suspension was gently poured into a single well of a 24-well tissue culture plate. The protein gel was polymerized at 37°C and an equal volume of EGM-2MV was added to the well. Twelve to 18 h after gel polymerization, the gels were removed, bisected, and implanted in the s.c. position on the abdominal wall. Three weeks after implantation, one third of the animals were euthanized and the grafts harvested for analysis of the human microvasculature. A second third of animals were given 3 \( \times 10^8 \) allogeneic human PBMCs by intraperitoneal inoculation. The remaining animals were injected with PBS and enrolled as nonreconstituted controls. Before the termination of the experiment, the number of circulating human T cells was evaluated. Heparinized retro-orbital venous blood samples were obtained and the erythrocytes were lysed using ammonium chloride. Leukocytes were incubated with PE-conjugated rat anti-mouse CD45 (BD Pharmingen) mAb and FITC-conjugated mouse anti-human CD3 mAb (Immunotech/Beckman Coulter) in PBS/1% BSA/5% NGS plus 2 \( \mu g/ml \)

**FIGURE 1.** Time course of surface expression of E-selectin, VCAM-1, and ICAM-1 after cytokine treatment on HCBECs and HUVECs. HUVECs (■) and HCBECs (□) isolated from the same donor were exposed to 10 ng/ml TNF (continuous line) or 50 ng/ml IFN-γ (dotted line) for the indicated times. Cells were harvested and labeled as described in Materials and Methods. The results are expressed in terms of corrected MFI, in arbitrary units of fluorescence, after subtracting the MFI of isotype control from the MFI of the specifically stained cells.

**FIGURE 2.** Chemokine secretion (IL-8 and IP-10), inflammatory cytokine expression (IL-1α), and the immunoinhibitory enzyme IDO expression in HUVECs and HCBECs isolated from the same donor. Cells were exposed to 10 ng/ml TNF (□), 50 ng/ml IFN-γ (●), or mock-treated (○) for 24 h. Supernatants were collected and the samples were assessed for IL-8 (A) or IP-10 (B) using ELISA as described in Materials and Methods. Cells were treated as indicated and cell lysates of cultured ECs were prepared as indicated in Materials and Methods. Lysates analyzed for expression of IL-1α (C) using ELISA and IDO (D) by Western blotting. Hsp-90 protein levels were used as loading control. For panels A–C, data represent mean ± SEM of quadruplicate determinations from one of two independent experiments with similar results. For panel D, blots are from one of two independent experiments with similar results.
Fc-Block (BD Biosciences) and analyzed by FACS. Three weeks following PBMC inoculation, all remaining mice were euthanized and the grafts harvested for analysis of human EC-lined microvessels and the degree of allogeneic PBMC infiltration. Recovered gels and surrounding soft tissue were fixed in 10% buffered formalin and embedded in paraffin. Sections (5-μm thick) were cut for immunostaining and for H&E staining. In some experiments, the harvested grafts were bisected and half of the specimen was fixed in 10% buffered formalin and half snap frozen in Tissue Tek OCT (Sakura Finetek), the latter used to prepare 6-μm cryosections. Sections were also stained with tetramethylrhodamine isothiocyanate-Uea-1-labeled lectin for detection of human EC-lined vessels within engrafted protein gels.

**Statistical analysis**

All data are expressed as means ± SEM. Statistical differences were measured by Student’s t test. A value of p ≤ 0.05 was considered as statistically significant.

**Results**

**Phenotypic analyses**

We first characterized multiple individual isolates of both HCBECs and HUVECs by extensive phenotyping. HCBECs were differentiated from EPCs in vitro as “late outgrowth” colonies and then serially expanded in culture (10). As we previously reported (10), these HCBECs formed a confluent monolayer of nonoverlapping polygonal cells containing cytoplasmic granules that appeared to be HDL in size. These HDLs showed a filopodial pattern of movement when examined by phase-contrast microscopy. By light microscopy, HCBECs uniformly bound Uea-1 and expressed CD34, VEGRF2 and CD31, all markers of differentiated ECs. The HCBECs used in these experiments were uniformly negative for myeloid markers CD133, CD45, CD18, and CD14 (data not shown), which may be coexpressed by “early outgrowth ECs” (14). This pattern of lineage marker expression was indistinguishable from that of HUVECs and defines HCBECs as true endothelium. However, compared with HUVECs, HCBECs grew faster (data not shown) and appeared capable of a greater number of population doublings without evidence of senescence, consistent with the growth characteristics previously reported for late outgrowth EPC-derived ECs (14, 16, 21). Because of their robust growth potential, HCBECs are of special interest to tissue engineering.

Having established that HCBECs appear to be true ECs, we proceeded to analyze the expression of surface proteins of immunological significance under basal conditions and after cytokine (TNF or IFN-γ) activation. There is considerable genetic variability among humans in EC expression of molecules of immunological relevance (29). Therefore, six independent isolates were compared and the results were generally consistent with some minor quantitative variations. This extent of variability among donors was comparable to the variability among HUVECs from different donors (data not shown). To compare HCBECs with HUVECs, we isolated three pairs from the same donor, avoiding donor-dependent variability. One representative data set from one of these experiments is shown in Table I. The expression levels in HCBECs were very similar to those in HUVECs, showing only low levels of the strongest stimulators of alloimmune response, class I and de novo induction of class II MHC molecules after IFN-γ treatment (30). Additionally, TNF induced-increases of class I MHC molecules (TNF or IFN-γ) was observed when compared with resting ECs (not pretreated with IFN-γ) or CD4+ T cells cultured in the absence of ECs. Data are mean ± SEM of quadruplicate determinations in one of two independent experiments with similar results. C, After 1 wk of cocultures, CFSE-labeled T cells were stained with anti-CD4 and anti-CD25 mAbs and subjected to FACS analysis. CD4+ T cells demonstrate significant proliferation in HUVECs or HCBECs cocultures of Donor I and Donor II, as judged by CFSE dilution, and CD25 expression was enhanced in proliferating cells (upper and lower panels, respectively). Data are from one of two independent experiments with similar results.

*FIGURE 3.* Activation of CD4+ T cells by allogeneic HUVECs and HCBECs isolated from the same donor (donors I and II). HUVECs and HCBECs were treated with IFN-γ to up-regulate MHC class I/II or mock-treated and subsequently cocultured with CD4+ T cells. A and B, Medium were collected for ELISA from HUVECs and HCBECs cultured with CD4+ T cells for 24 h. Significant production of both IFN-γ (A and B, left panels) and IL-2 (A and B, right panels) was observed when compared with resting ECs (not pretreated with IFN-γ) or CD4+ T cells cultured in the absence of ECs. Data are mean ± SEM of quadruplicate determinations in one of two independent experiments with similar results. C, After 1 wk of cocultures, CFSE-labeled T cells were stained with anti-CD4 and anti-CD25 mAbs and subjected to FACS analysis. CD4+ T cells demonstrate significant proliferation in HUVECs or HCBECs cocultures of Donor I and Donor II, as judged by CFSE dilution, and CD25 expression was enhanced in proliferating cells (upper and lower panels, respectively). Data are from one of two independent experiments with similar results.
FIGURE 4. Activation of CD8+ T cells by allogeneic HUVECs and HCBECs isolated from the same donor. HUVECs and HCBECs were cocultured with CD8+ T cells. A and B, Medium were collected for ELISA from HUVECs and HCBECs cultured with CD8+ T cells for 48 h. CD8+ T cells cultured with both HUVECs and HCBECs showed significant production of both IFN-γ (A) and IL-2 (B) when compared with CD8+ T cells cultured in the absence of ECs. Data are mean ± SEM of quadruplicate determinations from one of three independent experiments with similar results. C, After 1 wk of cocultures, CFSE-labeled T cells were stained with anti-CD8 or anti-CD25 mAbs and subjected to FACS analysis. CD8+ T cells demonstrate significant proliferation in HUVECs and HCBECs cocultures, as judged by CFSE dilution (4.3% and 3.5%, respectively), and the CD25 expression was enhanced in proliferating cells (upper and lower panels, respectively). Data are from one of three independent experiments with similar results.

and glucocorticoid-induced TNF receptor ligand, but not CD80 or CD86 (Table I). The levels of expression were generally comparable between HCBECs and HUVECs. The single notable difference being that basal expression of CD40 was consistently lower on HCBECs than on HUVECs, although comparable expression levels were seen after cytokine activation (32). The expression of the negative signaling molecules PD-L1 and PD-L2 on HCBECs were also very similar to HUVECs. We next compared the expression levels of the TNF-induced leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1. No significant differences were observed between HCBECs and HUVECs. The pattern and level of expression of these adhesion molecules following either TNF or IFN-γ treatment was analyzed in a time course experiment and found to be similar between both cell types (Fig. 1). Collectively, these data suggest that HCBECs express the same pattern of surface molecules relevant for interactions with the immune system as HUVECs.

Many soluble factors secreted by ECs may also contribute to allogeneic responses. Therefore, we analyzed the EC secretion of some of these proteins including cytokine-induced proinflammatory chemokines (IL-8 and IP-10) and the inflammatory cytokine (IL-1α) (in our hands HUVECS synthesize very little IL-β and do not synthesize TNF (33) and unpublished data). As shown in Fig. 2, A and B, the secretion of IL-8 and IP-10 in response to TNF and IFN-γ, respectively, was very similar in both cell types and among different donors. The levels of IL-1α after TNF treatment were also very similar in both cell types but varied more highly among donors (Fig. 2C). We also examined the expression of the immunoinhibitory enzyme IDO, which may play a role in the regulation of adaptive immune responses (26, 34). The level of expression of IDO was undetectable under basal conditions but was induced to comparable levels in both cell types following IFN-γ treatment (Fig. 2D). Additionally, when the surface expression of CD95L (FasL) was analyzed under basal conditions and after cytokine treatment, no detectable expression was found in either cell type (data not shown).

Functional analyses in vitro

Cultured HUVECs can present both class I and class II MHC molecules in a manner that results in the activation of allogeneic memory T cells (25, 35). We analyzed the responses of multiple T cell isolates from adult human peripheral blood to multiple different cultures of HCBECs and HUVECs and found generally similar responses to both cell types (data not shown). Once again, to avoid differences attributable to different donor origin we focused our experiments on the response of T cells to pairs of HCBECs and HUVECs isolated from the same EC donors, and the results from two independent donors are shown in Fig. 3. In these experiments with CD4+ T cells, HUVECs and HCBECs were first treated with IFN-γ for three days (to induce HLA-DR expression) or mock-treated and then cocultured with allogeneic, CFSE-labeled CD4+ T cells. Untreated (HLA-DR−) HUVECs and HCBECs from both donor I and II were less able to induce CD4+ T cells to secrete either IFN-γ or IL-2 (Fig. 3, A and B). In contrast, both types of ECs that had been pretreated with IFN-γ induced significant production of these two cytokines in cocultures with allogeneic CD4+ T cells (Fig. 3, A and B). The overall degree of secretion of both cytokines by CD4+ T cells was higher in response to donor I than in donor II. Although cytokine secretion by T cells stimulated with EC from donor I was slightly less in HCBEC cocultures than in HUVEC cocultures (Fig. 3A, left and right panels), this difference was not observed in cocultures donor II (Fig. 3B, left and right panels). Consistent with IL-2 secretion, CD4+ T cells demonstrate both proliferation and activation in response to IFN-γ-pretreated HUVECs or HCBECs from both donor I and II, as judged by CFSE dilution and CD25 expression in proliferating cells, respectively (Fig. 3C, upper and lower panels). Again, the degree of the T cell response was higher in cocultures with ECs from donor I.
and HCBEC cocultures from this donor were less able to activate CD4⁺ T cells than HUVEC cocultures from this same donor. This difference was less clear in ECs from donor II. These results show that HCBECs are roughly comparable to HUVECs in their ability to activate allogeneic CD4⁺ T cells.

We next compared the capacity of HCBECs to activate allogeneic CD8⁺ T cells. As shown in Fig. 3, resting HCBECs were able to induce CD8⁺ T cells to secrete IFN-γ and IL-2 (Fig. 4, A and B) and to proliferate (Fig. 4C). As expected, similar percentages of proliferating cells were found when activated cells were analyzed (CD25high and CFSElow) (Fig. 4C, lower panels). The CD8⁺ T cell response to HCBECs was similar to the response to HUVECs in the same experiments.

HUVECs preferentially activated allogeneic memory T cells compared with naive T cells (22, 23). To assess whether HCBECs also share this characteristic, allogeneic CD4⁺ and CD8⁺ T cells were first separated into memory (CD45RO⁺) and naive (CD45RA⁻) T cells and then separately cocultured with HCBECs or HUVECs (Fig. 5). As shown in Fig. 4, A and B, CD4⁺ T cells (memory and naive) did not respond to HUVECs or HCBECs that were not pretreated with IFN-γ, but IFN-γ-pretreated HUVEC and HCBECs were both able to induce memory (CD45RO⁺) but not naive (CD45RA⁻) CD4⁺ T cells to secrete IFN-γ and IL-2. By CFSE dilution and FACS analysis, purified memory CD4⁺ T cells, but not naive CD4⁺ T cells, proliferated in cocultures with IFN-γ-pretreated HUVECs and HCBECs (Fig. 5C). Similarly, only memory CD8⁺ T cells were induced to secrete IFN-γ- and IL-2 (Fig. 5, D and E) and to proliferate significantly (Fig. 5F) in response to allogeneic HUVECs or HCBECs, but in this case IFN-γ pretreatment was not required. The overall magnitude of T cell responses

![Image](http://www.jimmunol.org/)

### Figure 5
HUVECs and HCBECs activate CD4⁺ and CD8⁺ T cells memory but not naive T cells. A–C, HUVECs and HCBECs were treated with IFN-γ as previously indicated and subsequently cocultured with CD4⁺ T memory (CD45RO⁺) or naive (CD45RA⁻) T cells. A and B, Media were collected for ELISA from HUVECs and HCBECs cultured with either memory or naive CD4⁺ T cells for 24 h. Memory CD4⁺ T cells cultured with both HUVECs and HCBECs showed significant production of both IFN-γ (A) and IL-2 (B) when compared with naive CD4⁺ T cells or when compared with resting ECs (not pretreated with IFN-γ) or memory and naive CD4⁺ T cells cultured in the absence of ECs. Data are mean ± SEM of quadruplicate determinations from one of two independent experiments with similar results. C, After 1 wk of cocultures, purified CFSE-labeled CD4⁺ memory and naive T cells were stained with anti-CD4 mAb and subjected to FACS analysis. Purified memory CD4⁺ T cells showed significantly greater proliferation than their purified naive counterparts in HUVECs and HCBECs cocultures. Data are from one of two independent experiments with similar results.

D–F, HUVECs and HCBECs were cocultured with CD8⁺ memory (CD45RO⁺) or naive (CD45RA⁻) T cells. D and E, Media were collected for ELISA from HUVECs and HCBECs cultured either memory or naive CD8⁺ T cells for 48 h. Memory CD8⁺ T cells cultured with both HUVECs and HCBECs showed significant production of both IFN-γ (A) and IL-2 (B) when compared with naive CD8⁺ T cells or memory and naive CD8⁺ T cells cultured in the absence of ECs. Data are mean ± SEM of quadruplicate determinations from one of two independent experiments with similar results. E, After 1 wk of cocultures, CFSE-labeled T cells were stained with anti-CD8 mAb and subjected to FACS analysis. Purified memory CD8⁺ T cells showed greater proliferation than their purified naive counterparts in HUVECs and HCBECs cocultures. Data are from one of two independent experiments with similar results.
to HCBECs were somewhat less than those elicited by allogeneic HUVECs, but differences did not reach statistical significance in multiple comparisons. To determine whether the variably lesser degree of stimulation induced by HCBECs from donor I correlated with the slightly higher level of expression of the IFN-γ-induced IDO (Fig. 2D), we either inhibited IDO activity with 1-methyl-DL-triptophan (200 μM) or supplemented the cocultures with tryptophan (200 μM). Neither of these strategies increased the ability of HCBECs (or HUVECs) to further stimulate T cell proliferation (data not shown).

Functional analyses in vivo

In a final series of experiments, we evaluated the interactions between allogeneic lymphocytes and human EC-lined microvessels in vivo using a previously described model. Specifically, human ECs were suspended in proteins gels formed from rat tail type I collagen and human plasma fibronectin (5, 36, 37) and then implanted into the abdominal wall of C.B-17/SCID-beige mouse. Consistent with previous reports, implanted HUVECs and HCBECs will form tubes that inosculate with the host vasculature and are perfused with mouse blood (Fig. 6A) (5, 14). Perfused tubes are established by 3 wk and persist for as long as 6 wk (Fig. 6A). To detect T cell-mediated alloreactivity in vivo, 3 wk after implantation of collagen-fibronectin gels containing HUVECs or HCBECs, some of the animals were inoculated i.p. with $3 \times 10^8$ PBMC allogeneic to the HUVECs or HCBECs lining the developed human microvessels. The remaining animals were injected with saline as control groups. All grafts were harvested 3 wk later. Grafts containing either HCBECs or HUVECs showed a plexus of fused, microvessels were formed at 3 wk postimplantation and the appearance at 6 wk was largely unchanged in animals inoculated with saline (Fig. 6A, left and center panels). Inoculation with PBMCs led similar changes in grafts formed from HCBECs or HUVECs, namely loss of tubes, a sparse inflammatory infiltrate comprised of mononuclear cells, and focal areas of calcification (Fig. 6A, right panels). Immunostaining identified only rare human CD45$^+$ cells within the gel, although focal accumulations were noted at the edge of the gel in some specimens (data not shown). Staining of human ECs with Uea-1 lectin confirmed previous results with HUVECs that the perfused tubes within the gels of control animals were lined with human ECs and that human ECs disappeared in animals receiving allogeneic human PBMCs (5, 36, 37). Quantitation of Uea-1 staining revealed that the number of human EC-lined tubes increased between 3 and 6 wk in saline inoculated animals, but were decreased in animals receiving PBMCs (Fig. 6B). Significantly, no differences were observed in this in vivo model of human graft rejection between gels containing HCBECs or gels containing HUVECs.

Discussion

Previous reports have shown that human EPCs isolated from peripheral blood or umbilical cord blood can be differentiated to mature ECs and expanded in vitro, providing a robust source of ECs for cell transplantation and for promoting vascularization of tissue engineered organs and tissues (38–44). In theory, the expansion of vascular cells from blood progenitors could allow patients to serve as their own (autologous) stem cell donors, minimizing the potential for immunological rejection. However, most indications for the use of allogeneic sources of ECs will of necessity be derived from allogeneic stem cell sources, e.g., in the treatment of acute ischemic events or in the prefabrication of bioengineered tissues for clinical use. Moreover, cord blood EPCs appear more robust than EPCs from peripheral blood of adults and most adult recipients will not have access to autologous cord blood. Differentiation and expansion of ECs from allogeneic EPCs will give rise to alloantigenic differences between donor and recipient. Before this study, the significance of such differences for graft survival was unknown. It has been demonstrated that differentiated ECs isolated from mature blood vessels, such as HUVECs, are immunogenic,

**FIGURE 6.** HUVECs and HCBECs are subjected to T cell-mediated rejection in vivo. A, H&E staining of collagen-fibronectin gels containing either HCBECs or HUVECs. Stable, perfused, microvessels were formed at 3 wk by either HCBECs or HUVECs in protein gels following s.c. implantation in immunodeficient mice (left panels) and were found to persist for at least 6 wk (middle panels). Three weeks after implantation, the remaining animals were reconstituted with PBMCs or given saline. Three weeks after reconstitution, vessel destruction was noted in gels containing either HCBECs or HUVECs in animals receiving PBMCs (right panels), but not saline injected controls (central panels). B, Quantification of human vessel density, calculated as Uea-1 positive staining per gel area in arbitrary units. For each animal, 2–4 randomly selected fields (×20) from two sections were quantified; n = 3. *, p < 0.05.
stimulating nonself (allogeneic) memory T cells to proliferate, differentiate, and produce effector cytokines (25, 35). Remarkably, there are no previous data comparing the capacities of circulating EPC-derived ECs with their counterparts isolated from mature blood vessels with regard to stimulating alloreactive T cells (45), the principal mediators of allograft rejection. The present study was conducted to address this question.

We (10) and others (14, 16, 21) have demonstrated that “late outgrowth” EPCs from cord blood efficiently differentiate into ECs, and HCBECs appear to behave in a manner, except from their greater proliferative potential, that is indistinguishable from ECs isolated from a vessel wall such as HUVECs assessed by morphology, lineage markers, and tube formation capacity in vivo. In the present study, we show that HCBECs express essentially the same pattern of immunologically relevant molecules as HUVECs, including class I and II MHC molecules, costimulators, adhesion molecules, cytokines, and chemokines under basal conditions and after cytokine stimulation. We also found that HCBECs are generally comparable to HUVECs in their ability to activate allogeneic memory CD4+ and CD8+ T cells, assessed by cytokine production and proliferation, although HCBECs may be slightly less efficient than HUVECs at eliciting these T cell responses in vitro. The IDO-mediated immunoinhibitory response was not responsible for this difference. We confirm that HCBECs possess a similar capacity as HUVECs to generate perfused human microvessel-like structures when incorporated in a collagen-fibrinogen-gel and implanted in the s.c. position on the abdominal wall of immunocompromised mice. We did confirm that when mice that had received microvascular grafts prepared from HUVECs were reconstituted with human PBMCs allogeneic to the cells used to generate the human microvascular tissue, the vessels were subjected to PBMC-mediated vessel destruction (37). Unpublished studies (J. S. Poer and A. L. M. Bothwell) using T cells primed by coculture with allogeneic ECs suggested that vessel destruction in this model is likely the result of an allogeneic response. In the present study, we found that vessel destruction was indistinguishable when the grafts were constructed with HCBECs or with HUVECs. Cumulatively, these findings establish that ECs differ in their ability to stimulate allogeneic memory T cells and after cytokine stimulation. Our findings suggest that EPC-derived ECs with their counterparts isolated from mature human umbilical vein endothelial cells. Proc. Natl. Acad. Sci. USA 91: 1559–1563.


