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Human Umbilical Cord Blood Endothelial Progenitor Cells Decrease Vein Graft Neointimal Hyperplasia in SCID Mice

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Abstract

Aims—Vein graft endothelial damage is a key step in the development of neointimal hyperplasia, leading to vein graft failure. We sought to determine whether exogenous endothelial progenitor cells could promote vein graft re-endothelialization, and thereby ameliorate neointimal hyperplasia.

Methods and Results—Carotid artery interposition grafting was performed with syngeneic inferior vena cavae in mice with severe combined immunodeficiency (SCID). Lineage-negative human umbilical cord blood (hUCB) cells (or medium alone) were injected into vein-grafted mice intraoperatively and 2 weeks postoperatively. In vein grafts from hUCB cell-injected mice, we found human HLA-expressing endothelial cells, as well as increased levels of VEGF and FGF-2. Furthermore, hUCB cells secreted VEGF and FGF-2 *in vitro*. The markedly enhanced endothelial regeneration, likely resulting from both direct engraftment and paracrine actions of hUCB cells, inhibited inflammatory response, diminished intimal cell proliferation, and reduced neointimal hyperplasia in the vein grafts.

Conclusions—hUCB cells may accelerate vein graft re-endothelialization via both direct differentiation into endothelial cells and release of paracrine factors to enhance endothelial regeneration and reduce inflammation. These data highlight a potential therapeutic role for cellular therapy in vessel injury.

Keywords

endothelial progenitor cells; umbilical cord blood; and vascular repair

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[§]These authors made equal contributions to this work.

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Introduction

Vein grafts are commonly used for revascularization procedures. Graft remodeling is initiated by perioperative ischemia and hemodynamic injury to the endothelium. Distended by exposure to arterial pressure after implantation, the vein graft is too large to be surfaced by its own endothelium; moreover, there is evidence for profound post-operative endothelial cell (EC) loss (1). The substantial compromise of the endothelial barrier attracts and activates inflammatory cells and platelets, which secrete cytokines, chemokines, and other bioactive substances (2) that activate smooth muscle cells (SMCs) to proliferate, migrate into the sub-endothelial (intimal) space, and secrete extracellular matrix in a process termed neointimal hyperplasia. Although non-atherosclerotic at its onset, the neointimal hyperplasia appears to facilitate the acceleration of subsequent vein graft atherosclerosis, a common cause of vein graft failure (3). In light of this prevailing view of vein graft disease, vein graft re-endothelialization is thought to play an important role in limiting neointimal hyperplasia (4). Specifically, the intact endothelial layer prevents accumulation of inflammatory cells, and nitric oxide and prostacyclin secreted by ECs inhibit SMC migration and proliferation.

Vascular repair/re-endothelialization involves circulating endothelial progenitor cells (EPCs) derived from the bone marrow (BM) (5). Numerous reports have documented the existence of blood-borne EPCs originating from the BM (6, 7). Indeed, levels of circulating EPCs inversely correlate with lesion severity in acute and chronic vascular diseases (8, 9). Human umbilical cord blood (hUCB) cells have been shown to produce EPCs more efficiently and in greater numbers than peripheral blood or BM *in vitro* (10). Furthermore, hUCB progenitors have several distinct proliferative advantages over precursor cells derived from peripheral blood and BM, including the capacity to form a greater number of colonies, a higher cell-cycle rate, and longer telomeres (11, 12). These properties render hUCB cells an attractive source for EPCs.

In vein grafts, neointimal hyperplasia is diminished by interventions that accelerate re-endothelialization (4). Accordingly, we reasoned that infusion of exogenous EPCs could accelerate vein graft re-endothelialization and thereby diminish vein graft neointimal hyperplasia. To test this hypothesis, and the feasibility of using hUCB cells as a therapeutic agent, we performed interposition vein grafting in the carotid arteries of mice with severe combined immune deficiency (SCID), because they would not reject the infused hUCB cells. We examined the mechanisms underlying hUCB cell-mediated vascular repair, focusing on both direct differentiation (hardware theory) and paracrine secretion of factors (software theory) that may enhance EC regeneration/survival.

Methods

Additional methodological information is available in the Online Supplement.

Maintenance of SCID Mice

C57/B6/SCID mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Carotid Artery Interposition Grafting and hUCB injection

The operative procedures were performed as previously described (4).

Isolation of Lineage-Negative Human Umbilical Cord Blood (hUCB)

The lineage-negative subset of mononuclear cord blood cells was chosen for this study to avoid potential engraftment of donor lymphocytes and the resultant graft-versus-host disease.

Immunohistochemistry

Serial frozen sections of the vein graft were subjected to immunohistochemical staining with antibodies specific for human leukocyte antigen (HLA-ABC), human nuclear antibody (ANA), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2).

Vein Graft Morphometry

Morphometric analyses were performed on paraffin sections stained with Masson trichrome stain.

Data Analysis

One-way ANOVA was used to compare hUCB-injected and control grafts for neointima, media, and total wall area. A p -value <0.05 was considered significant.

Results

hUCB Cells Re-Populate Vein Graft Endothelium and Ameliorate Neointimal Hyperplasia

The BM of young animals contains more EPCs than that of old animals (13), and cells isolated from young BM are effective in suppressing native atherosclerosis (14). Since the umbilical cord blood is richer in precursor cells than the BM, we reasoned that the lineage-negative fraction of hUCB (enriched for EPCs) should accelerate vein graft re-endothelialization particularly well, and thereby diminish vein graft neointimal hyperplasia. To test this hypothesis, we infused hUCB cells into SCID mice subjected to interposition vein grafting. Vein grafts in the SCID mice developed neointimal hyperplasia comparable to that seen in immunocompetent mice (15). The average neointimal area was reduced by 41% when treated with hUCB ($p < 0.01$, Figure 1). Thus, infusion of hUCB cells attenuated neointimal hyperplasia.

To elucidate the mechanisms underlying hUCB-induced reduction of vein graft neointimal hyperplasia, we first examined whether hUCB cells could take up residence in the vein grafts and differentiate into mature ECs. To distinguish human from mouse cells in this model system, we performed immunohistochemistry for HLA-ABC, present on the surface of most nucleated human cells. We examined one section from each of the proximal, middle, and distal regions of each graft. With this survey, we found human cells in the luminal endothelial layer of the vein grafts in 5 of 8 hUCB cell-injected SCID mice (Figure 2A). The engrafted cells tended to be in clusters. The morphologic features of these cells were characteristic of ECs. There were distinct cellular borders with no evidence of cellular

fusion. Importantly, sample sections directly adjacent to those demonstrating HLA-ABC expression also expressed human PECAM-1, confirming that these cells were indeed ECs (Figure 2B). To quantitate the contribution of hUCB cells to the total vein graft endothelium, we stained serial vein graft sections with antibodies specific for mouse and human PECAM-1. By this analysis, donor-derived cells constituted $2.6 \pm 0.8\%$ in the middle of the graft, and $5 \pm 1\%$ in the proximal and distal regions, where turbulent blood flow and endothelial turnover is the greatest (15).

Although the prevalence of hUCB cells was relatively low among all vein graft ECs, the effect of hUCB cells on overall vein graft re-endothelialization was substantial. Indeed, in 2-wk-old vein grafts from mice injected with hUCB cells, $80 \pm 17\%$ of the peri-luminal perimeter demonstrated mouse/human PECAM-1 expression by immunohistochemistry using an antibody that recognizes both human and mouse PECAM-1. In sham-injected mice, the corresponding endothelialization figure was only $22 \pm 8\%$ (Figure 3).

Because SMCs express low levels of human leukocyte antigens, HLA-ABC immunostaining may not be sufficiently sensitive to detect SMCs derived from injected hUCB cells. We stained specimens with an anti-human nuclear antibody (ANA), able to recognize all human cell types. Only rare (if any) neointimal and medial cells stained with ANA, even though there was abundant ANA staining in our positive control human aorta (data not shown).

Collectively, these findings indicate that hUCB cells can differentiate into ECs needed for vascular repair in the SCID mouse vein graft model, and thereby accord with previous observations showing that BM-derived EPCs can repopulate the damaged endothelium in vein grafts (16, 17) and in atherosclerotic vessels (14, 18). The contribution of hUCB cell direct differentiation to the vein graft endothelium obviously cannot account for the substantial difference in re-endothelialization between vein grafts from hUCB-treated and control mice. Moreover, the reduction in vein graft neointimal hyperplasia resulting from hUCB cell therapy is disproportionately greater than the contribution of hUCB cells to the total complement of vein graft ECs. For these reasons, we hypothesized that hUCB cells could exert paracrine effects that mitigate vein graft neointimal hyperplasia.

hUCB Cells Secrete Paracrine Factors

To determine the role of paracrine factors in hUCB-mediated reduction of neointimal hyperplasia, we asked whether hUCB cells in culture could secrete FGF-2 and VEGF—growth factors that are essential in stimulating EC proliferation and migration (19). The conditioned medium of cultured hUCB cells contained human VEGF and FGF-2 at levels similar to or even slightly higher than that obtained with cultured human umbilical vein endothelial cells (HUVECs, Figure 4A). We then tested whether infusion of hUCB cells resulted in increased expression of VEGF and/or FGF-2 *in vivo*. Indeed, vein grafts from hUCB-treated mice demonstrated enhanced expression of FGF-2 and VEGF (Figure 4B-E), primarily in the endothelial layer of the vein grafts. The extent of growth factor immunoreactivity was greater than hUCB engraftment in the endothelium. It is possible that the paracrine effects of hUCB cells promoted the survival and proliferation of endogenous vein graft ECs, which, together with injected cells, contributed to the substantial FGF-2 and

VEGF expression. Taken together, these data indicate that hUCB cell-mediated therapeutic effects on vein grafts include the paracrine mechanism.

hUCB Cells Reduce Inflammatory Response in the Vein Graft Recipients

The arterialization of vein grafts is characterized by acute and then chronic inflammation, which contributes to neointimal hyperplasia (20). To determine whether hUCB treatment could attenuate neointimal hyperplasia by reducing vein graft inflammation, we stained vein grafts for mouse Mac-1, to identify macrophages. Remarkably, the prevalence of macrophages was much less in 2-wk-old vein grafts from hUCB-treated mice than from sham-treated mice. In average, $17 \pm 5\%$ cells stained positive for Mac-1 in medium-treated vein grafts, whereas only $5 \pm 2\%$ cells expressed Mac-1 in hUCB-treated vessels ($p < 0.03$, Figure 5A-C). Thus, by facilitating vein graft re-endothelialization, hUCB cells may reduce vein graft monocyte recruitment into and macrophage proliferation within the vessel wall.

To test if reduced graft macrophage content induced by hUCB injection correlated with lessened systemic inflammation, we measured selected serum cytokines in our mice at 2 weeks postoperatively. While hUCB cell therapy had no effect on GM-CSF and IL-6, it reduced serum levels of TNF α and MCP-1 by 55.4% and 48.5%, respectively (Figure 5 B). These differences in serum cytokine levels likely resulted from hUCB cell effects both at and remote from the vein graft, such as in the reticuloendothelial system, where HLA-ABC staining also revealed evidence for hUCB cell engraftment (data not shown). These data collectively suggest that hUCB cell therapy may reduce vein graft inflammation through local and systemic anti-inflammatory effects.

Inflammatory cells in the vessel wall secrete cytokines that have been shown to stimulate medial SMC migration and proliferation. To determine if hUCB treatment reduced intimal cell proliferation, we stained the vein grafts for proliferating cell nuclear antigen (PCNA). In 2-wk-old vein grafts from mice treated with control medium, $28 \pm 4\%$ of intimal and medial cells expressed PCNA. By contrast, in vein grafts from mice treated with hUCB cells, only $12 \pm 3\%$ of intimal and medial cells expressed PCNA (Supplemental Figure 1). These data indicate that by enhancing re-endothelialization and preventing recruitment of inflammatory cells into the vessel wall, hUCB cells suppress vein graft cell proliferation, and thereby reduce neointimal hyperplasia.

Discussion

This study provides the first evidence that hUCB cells are capable of reducing neointimal hyperplasia in vein grafts, by accelerating vein graft endothelialization and reducing vein graft inflammation and cell proliferation. Our data with hUCB therapy for vein grafts accord with studies that use adult EPCs or EPC-eliciting cytokines to reduce neointimal hyperplasia after arterial injury (21, 22).

BM-derived cells have been shown to contribute to the endothelium of vein grafts (17, 20). Moreover, the severity of vein graft neointimal hyperplasia correlates inversely with the rapidity of vein graft re-endothelialization (4). It is conceivable that, by supplying excess

EPCs to re-endothelialize the vein graft, hUCB cell infusions may reduce the initial injury that triggers vein graft neointimal hyperplasia.

The mechanisms underlying the therapeutic efficacy exerted by the injected stem cells are postulated to be mediated by either differentiation into functional reparative cells that replace injured tissues, or secretion of paracrine factors that promote tissue repair. Experimental studies have shown that adult stem cells can transdifferentiate into ECs (23). We demonstrated using ROSA26 mice as donors that stem cells from the BM could engraft and differentiate into ECs in the aortas of apoE^{-/-} mice (14). In the current study, we found that hUCB cells contribute to the vein graft EC complement not only directly, by differentiating into mature ECs, but also indirectly, by up-regulating growth factor secretion that may promote vein graft re-endothelialization by endogenous EPCs and ECs, supporting the involvement of both hardware and software mechanisms in stem cell-mediated vascular repair.

Intimal hyperplasia and accelerated atherosclerosis in vein grafts is considered to be the result of an inflammatory process. Endothelial injury, resulting from mechanical vessel damage and hypoxia during surgery and altered shear stress after surgery, facilitates the adhesion and influx of inflammatory cells into the intima (24). MCP-1 and its receptor CCR2 are key mediators in vascular inflammation, acting as a potent chemotactic agent to monocytes (25). Vein graft neointimal hyperplasia can be reduced by eliminating TNF receptor-1-triggered activation of NFκB (4), inhibiting NFκB itself, or antagonizing the NFκB target gene product MCP-1 (26). In this context, the hUCB cell-mediated reductions in serum TNFα and MCP-1 levels may well contribute significantly to the efficacy of hUCB cell therapy for vein graft disease. Remarkably, inflammatory cell infiltration in the graft vessel wall was diminished in hUCB-treated grafts. These findings highlight the key role of hUCB cell-mediated re-endothelialization in limiting inflammatory cell recruitment to the vessel wall and systemic inflammation. IL-6 and GM-CSF are acute phase reactants involved in vascular graft remodeling. We did not, however, detect a reduction in serum IL-6 and GM-CSF levels after hUCB treatment. The precise mechanisms underlying the differential regulation of IL-6 and GM-CSF *versus* MCP-1 and TNFα in relation to hUCB treatment remain to be elucidated. It is possible that the rapid normalization of these acute phase reactants, presumably after the acute phase inflammation, might have contributed to this finding.

Exaggerated SMC proliferation, a critical process in intimal hyperplasia following vein grafting, is thought to be inflammation driven. Several studies have highlighted the importance of MCP-1, which stimulates the migration and proliferation of quiescent SMCs in the vessel wall (27). Inhibition of MCP-1 inhibits SMC proliferation and attenuates vein graft thickening both *in vitro* and *in vivo* (28). Using PCNA staining, we have demonstrated that cell proliferation in the intima and media is markedly reduced in the vein grafts from hUCB-treated mice relative to control grafts, and the reduction correlates with enhanced re-endothelialization, diminished inflammatory cell infiltration in the vessel wall, and decreased MCP-1 levels in the serum. These observations lend further support to the notion that endothelial injury, inflammation, and SMC proliferation and migration represent essential and consequential steps in vein graft intimal hyperplasia.

In conclusion, the present study establishes the important role of hUCB cells in limiting vascular remodeling, implicating the significance of cellular therapy in vessel injury. A broad implication of these findings includes cord blood banking for short-term supply of EPCs at times of vascular stress, for example following vein graft surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Infusion of hUCB cells inhibits vein graft neointimal hyperplasia. Vein grafts collected 4 weeks postoperatively were perfusion-fixed; paraffin sections were stained with Masson trichrome staining (MT). Cross-sections with MT stain are shown in (A) for control and in (B) for hUCB treated graft. Graft dimensions as shown in (C, original magnification $\times 400$) were measured by computerized morphometry as in Methods, and the means $\pm$ SE of 8 specimens from each group are displayed (D). Compared with control: *, $p < 0.05$.

Figure 2.

hUCB cells populate the vein graft endothelium and differentiate into ECs. Serial sections of hUCB-treated vein grafts were immunostained for human HLA-ABC (**A**, and **C**), and human PECAM-1, an EC marker (**B** and **D**), from hUCB- (**A** and **B**) and medium- (**C** and **D**) treated mice, respectively, and counterstained with hematoxylin.

Figure 3.

Therapy with hUCB cells enhances vein graft re-endothelialization. Vein grafts were subjected to immunohistochemistry using an antibody that recognizes both human and mouse PECAM-1, and counterstained with hematoxylin. Representative specimens from hUCB-treated (**A**) and control (**B**) mice sacrificed at 2 weeks after surgery are shown. Serial sections stained with non-immune IgG demonstrated no brown precipitate (not shown). The percent of each vein graft luminal perimeter occupied by PECAM-1-positive ECs was quantitated as in Methods, and the mean $\pm$ SE of 10 sections from 5 independent vein grafts from each group is plotted (**C**). Compared with control: * $p < 0.05$.

Figure 4.

hUCB cells secrete VEGF and FGF-2. **A**, HUVECs and hUCB cells were cultured in EGM medium for 5 days, and the medium was subsequently subjected to ELISA for VEGF or FGF-2. Shown are the means $\pm$ SE from 4 independent experiments with 4 distinct isolates of hUCB cells and HUVECs. Compared with HUVECs: *, $p < 0.05$. **B-E**, Sections of vein grafts harvest 2 weeks after surgery were subjected to immunoperoxidase staining for VEGF (**B**, **C**) or FGF-2 (**D**, **E**), and counterstained with hematoxylin, which shows strong staining for VEGF and FGF-2 in vein grafts treated with hUCB cells (**B**, **D**). In contrast, only faint staining for VEGF and FGF-2 is detected in vein grafts treated with medium alone (**C**, **E**). Serial sections stained with non-immune IgG demonstrated no brown precipitate (not shown).

Figure 5.

Infusion of hUCB cells diminishes vein graft macrophage infiltration and serum cytokine levels. Two week-old vein graft sections harvested from the indicated hUCB/medium treatment group were immunostained with anti-Mac-1 antibody (**A & B**). The percentage of Mac-1 positivity was quantitated as in Methods, and the mean $\pm$ SE of 10 sections of 5 vein grafts from each group is plotted (**C**). Serum was harvested from SCID mice immediately before vein graft harvest, and subsequently subjected to immunoassay for TNF α , MCP-1, GM-CSF, and IL-6, as in Methods. Shown are the mean $\pm$ SE of 5 serum samples from each group of mice collected at 2 week postoperatively, treated either with medium alone, or with hUCB cells (**D**). Compared with control: *, $p < 0.05$; **, $p < 0.01$.