Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Late outgrowth endothelial progenitor cells engineered for improved survival and maintenance of function in transplant-related injury

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Keywords
A20, gene transfer, inflammation, ischaemia reperfusion injury, late outgrowth endothelial progenitor cells, transplant vasculopathy.

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Conflicts of Interest
The authors have declared no conflicts of interest.

Received: 21 July 2011
Revision requested: 21 August 2011
Accepted: 21 October 2011
Published online: 26 November 2011

doi:10.1111/j.1432-2277.2011.01387.x

Summary

Chronic allograft vasculopathy (CAV) is a major cause of organ transplant failure that responds poorly to treatment. Endothelial activation, dysfunction and apoptosis contribute to CAV, whereas strategies for protecting endothelium and maximizing endothelial repair may diminish it. Late outgrowth endothelial progenitor cells (LO-EPC) can home to areas of injury and integrate into damaged vessels, implying a role in vascular repair; however, in an allograft, LO-EPC would be exposed to the hazardous microenvironment associated with transplant-related ischaemia reperfusion (I/R) injury and persistent inflammation. We evaluated the *in vitro* effect of I/R injury and the proinflammatory cytokine tumour necrosis factor (TNF)-α on LO-EPC phenotype and function. We show that LO-EPC are intrinsically more tolerant than mature EC to I/R injury induced apoptosis, maintaining their proliferative, migratory and network formation capacity. Under inflammatory conditions, LO-EPC were activated and released higher levels of inflammatory cytokines, upregulated adhesion molecule expression, and were more susceptible to apoptosis. Lentiviral vector-mediated overexpression of the protective gene A20 in LO-EPC maintained their angiogenic phenotype and function, and protected them against TNF-α-mediated apoptosis, reducing ICAM-1 expression and inflammatory cytokine secretion. Administration of *ex vivo* modified LO-EPC overexpressing A20 might effect vascular repair of damaged allografts and protect from CAV.

Introduction

Organ transplantation is an effective treatment for end-stage organ failure. Current immunosuppressive drugs targeting T cell-dependent immune pathways have significantly reduced early graft loss from acute rejection, whereas chronic rejection remains refractory and is now the major cause of graft failure. There is a need for novel and effective approaches for targeting chronic rejection.

The pathogenesis of chronic allograft vasculopathy (CAV) is poorly understood but it is now generally accepted that proinflammatory cytokines, circulating alloantibodies, complement components and T lymphocytes all play important roles and that endothelial cells (EC) are not only a key target of these damaging stimuli, but also actively participate in the process through expression of a variety of ligands, receptors and secreted molecules [1]. EC activation and dysfunction cause chemotactic accumulation of mononuclear and polymorphonuclear leucocytes, some (together with EC themselves) expressing Fc receptors facilitating antibody-mediated damage [2]. Damage to and loss of the endothelial monolayer can progress, via smooth muscle cell proliferation, accumulation of transformed macrophage and cell debris,

to intimal thickening and ultimately luminal occlusion in small arteries throughout the transplanted organ. Restoring the endothelial monolayer, regenerating vascular networks and protecting them from transplant-related ischaemia reperfusion (I/R) injury and inflammation might be an effective strategy to control CAV.

Recent studies have suggested an important role for endothelial progenitor cells (EPC) in the homoeostasis of the vessel wall and an inverse correlation exists between circulating EPC numbers and risk factors for cardiovascular disease, such as atherosclerosis, diabetes, stroke, hypercholesterolaemia and hypertension [3–8]. It has been demonstrated experimentally and clinically that EPC contribute to both re-endothelialisation [9–11] and neovascularisation [10,12,13], which are functionally important in preventing CAV. Delivering *ex vivo* expanded autologous LO-EPC could be a novel approach for repair of damaged EC and lead to regeneration of vascular networks in the allograft. However, the role of EPC in allograft rejection is unclear. Some studies have demonstrated that an inverse correlation exists between circulating EPC numbers and renal transplant rejection [14–16]; others reported that EPC contribute to allograft rejection [17,18]. This controversy could be a result of transplant-related pathological influences on EPC and the strict definitions of EPC. Although autologous EPC would not be subject to adaptive immune responses, they would be exposed to the hostile environment of ischaemia and reperfusion, and the persistent transplantation-related inflammatory milieu associated with allografts might impair their reparative capacity or altering their behaviour.

Previous studies have raised concerns about the safety and efficacy of cell therapy using EPCs or stem/progenitor cells in cardiovascular disease [19–22]. We have investigated the influence of transplant-related pathological effects on late outgrowth EPC (LO-EPC) using *in vitro* I/R injury and the proinflammatory cytokine TNF-α. We have shown that LO-EPC can withstand simulated I/R damage, and maintain their proliferative, migratory and network forming capacity; however, they are not resistant to inflammatory signals. TNF-α activates LO-EPC and ultimately induces apoptosis when protein synthesis is impaired, suggesting that LO-EPC undergo a phenotypic change in response to inflammation, thus limiting their therapeutic potential. We attempted to enhance the therapeutic potential of LO-EPC by gene transfer of A20, a cytoplasmic zinc finger protein that has a unique role in preventing TNF-α-induced cellular activation and apoptosis [23,24]. Overexpression of A20 in LO-EPC reduced TNF-α-induced activation and apoptosis, prevented LO-EPC acquiring an inflammatory phenotype, and maintained their *in vitro* angiogenic capacity.

Materials and methods

Culture of late outgrowth EPC and mature EC

Late outgrowth EPC were purchased from Lonza. These cells are isolated from human umbilical cord blood and clonally expanded from endothelial progenitor cell populations. LO-EPC were cultured in endothelial basal medium EBM-2 supplemented with EGM-2 SingleQuots (Lonza) and additional 10% FBS. Cells from passage 2–8 were used. Mature endothelial cells isolated from umbilical veins, were purchased from Lonza. Human umbilical vein endothelial cells (HUVEC) were cultured in M199 medium (Sigma) supplemented with 20% FCS, endothelial supplement factors (Sigma) and heparin (Sigma). Passage 2–6 cells were used.

Vector construction and lentiviral vector production

A four-plasmid expression system was used to generate third generation lentiviral vectors (LVV) as previously described [25]. The vector plasmid HIV-CMV-A20 contains the human A20 (gene bank NM_006290) driven by the human cytomegalovirus promoter. An equivalent LVV expressing no transgenes (empty vector) was used as a control, while an LVV expressing enhanced green fluorescent protein (eGFP) was used to measure transduction efficiency. Transfections were done in 100-mm dishes using optimized ratios of constructs: 10 μg vector plasmid, 6.5 μg packaging plasmid, 2 μg Rev expressor and 3.5 μg VSV-G *env* plasmid DNA per 10 ml of medium. The medium was changed at 14–16 h and removed and filtered at 62 h (0.45 μm Millex-HA filter, Millipore). The vector titres used were 1×10^7 to 5×10^7 transduction units/ml (TU/ml) measured in 293 T cells.

In vitro transduction of LO-EPC and HUVEC

Late outgrowth endothelial progenitor cells or HUVEC were plated in 6- or 24-well plates at 50–60% confluence 24 h before transduction. Medium was replaced with a minimum of fresh growth medium containing the LVV at a multiplicity of infection (MOI) of 5 for 6 h at 37 °C in the presence of 8 μg/ml Polybrene (Sigma), after which additional fresh medium was added. The medium was changed after 24 h and cells were assayed 72 h after transduction.

Dil-Ac-LDL labelling

Late outgrowth endothelial progenitor cells were fluorescently labelled by incubation with 10 μg/ml DiI-labelled acetylated low density lipoprotein (DiI-Ac-LDL, Molecular Probes, Invitrogen, Paisley, UK) in complete EBM-2 medium for 4 h at 37 °C. After washing twice with PBS, the

cells were viewed and used in an *in vitro* Matrigel network formation assay.

In vitro model of I/R injury

Ischaemia reperfusion injury was simulated by anoxic (O_2 < 1% and CO_2 > 5%) and acidotic conditions with glucose and pyruvate deprivation in an otherwise physiological solution (Krebs' buffer). In addition, potassium chloride was added to the ischaemia solution to simulate the leakage of potassium ions into the extracellular environment; sodium lactate was added to simulate sodium overload associated with ischaemia, as described previously [26]. LO-EPC at 80% confluence were incubated with a minimal volume of ischaemia solution (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $NaH_2PO_4{\cdot}H_2O$, 2.5 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM $MgCl_2$, 0.5 mM sodium·EDTA·$2H_2O$, 20 mM sodium lactate, and 16 mM KCl, pH 6.2) under hypoxia in an anaerobic bag (BDH), at 37 °C for 4 h. Cells were then transferred to a 37 °C incubator with 5% CO_2 in air for 2 h with additional complete culture medium.

Proliferation assay

Proliferation was determined by BrdU incorporation during DNA synthesis using the Cell Proliferation ELISA kit (Roche) according to the manufacturer's protocol. Results were expressed as absorbance at wavelength 450 nm compared with a reference wavelength 690 nm.

Migration assay

Migration was evaluated using the ORIS^{TM} Cell Migration Assembly kit, according to the manufacturer's instructions. 100 μl of 5×10^4 cells was seeded into each well of a 96-well-plate using the ORIS^{TM} cell seeding stoppers, and incubated at 37 °C and 5% CO_2 for 16 h to allow cell attachment to the periphery of each well. The seeding stopper was then removed and unattached cells removed by gentle washing. Reincubation at 37 °C and 5% CO_2 permitted cell migration from the peripheral margin towards the centre of each well. Migration was examined microscopically throughout the incubation period. At the end of the incubation period, cells were stained with Hoechst for 15 min. Cells migrating to the detection zone were photographed and counted using Image J software. The value was normalized against untreated cells.

In vitro Matrigel network formation assay

Matrigel (BD Biosciences, Oxford, UK) of 50 μl was added to a precooled 96-well plate and allowed to solidify for 1 h at 37 °C. 5×10^3–1×10^4 cells in 150 μl complete growth medium were added to each well and incubated at 37 °C (5% CO_2). The formation of networks was observed by microscopy after overnight incubation.

Flow cytometry for ICAM-1 expression and apoptosis

Cells were detached by trypsin/EDTA and incubated with FITC-anti-CD54 (ICAM-1, R&D Systems, Abingdon, UK) or with an isotype control antibody, then analysed by flow cytometry (FACSCanto2, BD Biosciences). Apoptotic cells were identified using FITC-conjugated AnnexinV and Propidium Iodide (PI, Bender MedSystems, Vienna, Austria). In early apoptosis, membrane phosphatidylserine is externalized and can be detected by FITC-AnnexinV. Typically, viable cells were AnnexinV$^-$PI$^-$, early apoptotic cells AnnexinV$^+$PI$^-$, late apoptotic cells AnnexinV$^+$PI$^+$ and necrotic cells AnnexinV$^-$PI$^+$.

Cytokine array assay

The relative levels of multiple cytokines and chemokines in cell culture supernatants were measured simultaneously using a commercial Cytokine Antibody Array kit (R&D Systems) according to the manufacturer's instructions. Briefly, cell culture supernatants were diluted and mixed with a cocktail of biotinylated detection antibodies against a selection of cytokines and chemokines. The mixture was then incubated with a nitrocellulose membrane onto which duplicate cytokine/chemokine capture antibodies had been spotted. After incubation, membranes were washed and incubated with a horseradish peroxidase (HRP)-streptavidin complex. Signals were detected using a chemiluminescence system (ECL, GE Healthcare, Little Chalfont, UK). The array data were analysed by Image J software and expressed as densitometric units.

Western blot analysis

Western blot analyses were performed as described previously [26]. Specific proteins were identified using monoclonal antibodies to human A20 (R&D Systems), human phosphorylated IκBα (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Abcam Ltd, Cambridge, UK) followed by HRP-labelled secondary antibodies. Bands were developed with the ECL kit (GE Healthcare) and compared using Image J software. Protein expression was normalized to β-actin and expressed as relative densitometry units.

Caspase 3 activity assay

Caspase–3 activity in LO-EPC extracts was detected using the Caspase-Glo^{ry} 3/7 Assay kit (Promega), based on

cleavage of luminogenic substrate and generation of a 'glow-type' luminescence, according to the manufacturer's protocol. Activity of caspase-3 was expressed in relative light units (RLU) and normalized by total protein concentration.

Statistical analysis

All values are expressed as mean ± SE. The 'n' numbers in the results section relate to the number of experiments. Within each independent experiment, at least duplicate measurements were performed. The values from each experiment were pooled to allow statistical comparisons. Statistical comparisons between two groups were performed using Student's unpaired two tailed and unequal variance *t*-test. Statistical analyses for the normalized data were performed using one-sample *t*-test against a hypothetical mean value using GraphPad software. A probability value of $P < 0.05$ was considered statistically significant and expressed as * and one of $P < 0.01$ expressed as **.

Results

Properties of LVV-transduced LO-EPC

Late outgrowth EPC display a 'cobblestone' morphology in culture, typical of endothelial cells. The endothelial phenotype and angiogenic function of LO-EPC was further confirmed *in vitro* by their ability to take up Dil-Ac-LDL and to migrate into Matrigel and form a network.

Both LO-EPC and HUVEC were effectively transduced to express eGFP (transduction efficiency >89%) using an LVV at an MOI of 5; eGFP expression was stable for at least 1 month. LVV transduction did not impair LO-EPC morphology or their ability to take up DiI-Ac-LDL or to generate networks in matrigel (Fig. 1a–c). Co-culture of 5×10^3 nontransduced LO-EPC (labelled with Dil-Ac-LDL) and 1×10^4 HUVEC (transduced with an LVV expressing eGFP) in the *in vitro* Matrigel assay demonstrated that LO-EPC participated in and contributed to network formation by mature EC (Fig. 1d).

Response of LO-EPC to simulated I/R injury

Ischaemia reperfusion injury did not significantly affect LO-EPC morphology (Fig. 2a and c), whereas microscopy revealed that culture conditions simulating I/R caused significant deformation and detachment of HUVEC cultured under the same conditions (Fig. 2b and d). The capacity of HUVEC to form networks was disrupted following culture in simulated I/R conditions (Fig. 2f), but LO-EPC network formation was not affected (Fig. 2e).

The ability of LO-EPC to proliferate *in vitro* and to migrate was not influenced by exposure to simulated I/R culture conditions, whereas both proliferation and migration were impaired in HUVEC under the same conditions (Fig. 3a and b).

Similarly, LO-EPC were more resistant to I/R injury-induced apoptosis than were mature endothelial cells, as

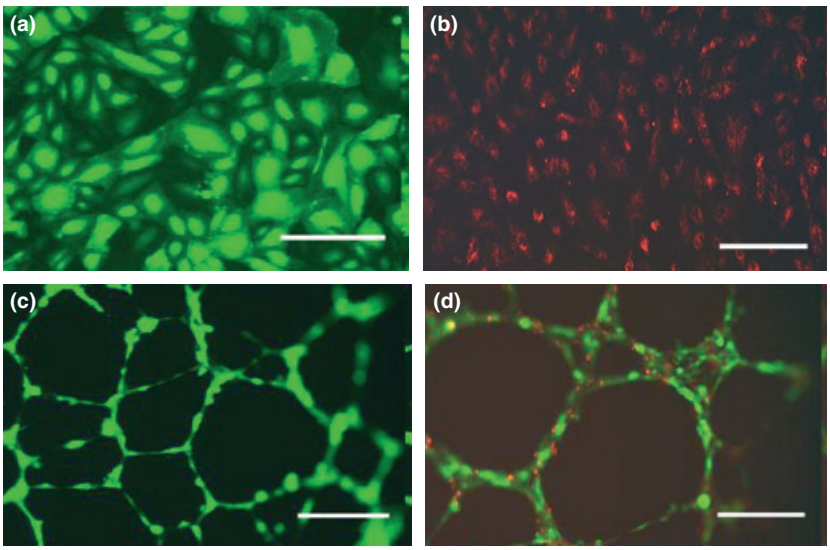

Figure 1 Properties of lentiviral vector (LVV)-transduced late outgrowth endothelial progenitor cells (LO-EPC). (a) Lentiviral vector encoding green fluorescent protein (eGFP) transduced LO-EPC efficiently at multiplicity of infection (MOI) 5 in the presence of 8 mg/ml polybrene; transduction efficiency was greater than 89% as assessed by flow cytometry (not shown). (b, c) LVV-transduced LO-EPC were able to take up DiI-Ac-LDL (b) and to form networks in Matrigel (c). (d) Coculture of 5×10^3 LO-EPC and 1×10^4 human umbilical vein endothelial cells (HUVEC) demonstrates that LO-EPC participate and contribute to HUVEC network formation *in vitro*; LO-EPC were labelled with DiI-Ac-LDL (red) and HUVEC were transduced with LVV expressing eGFP (green). Scale bar 200 μm.

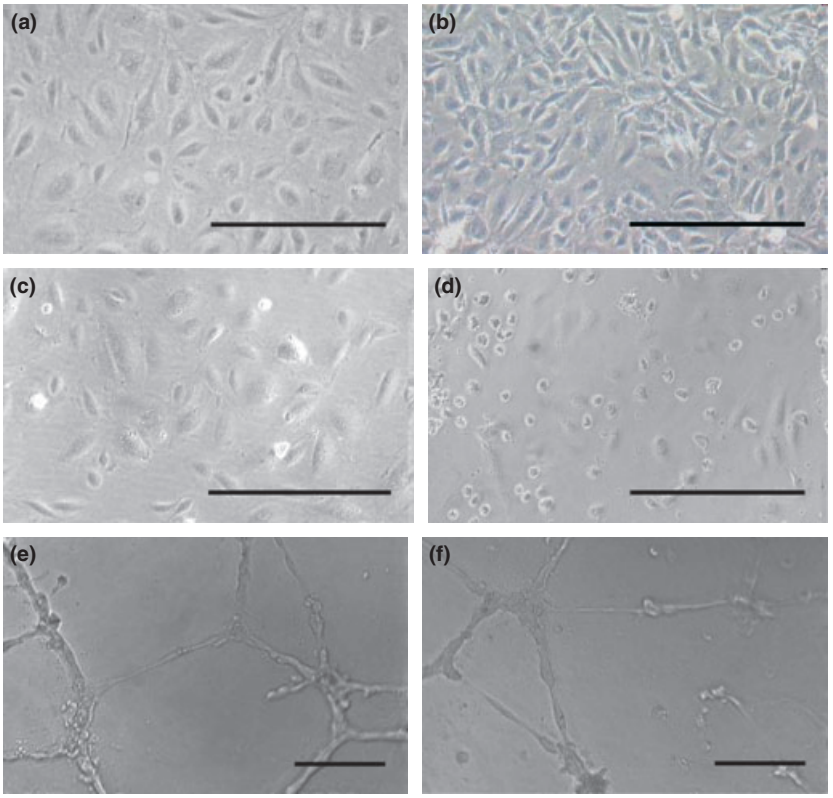

Figure 2 Resistance of late outgrowth endothelial progenitor cells (LO-EPC) to simulated ischaemia reperfusion (I/R) injury (I). (a, b) Representative phase contrast image of untreated LO-EPC (a) and human umbilical vein endothelial cells (HUVEC) (b). (c, d) I/R culture conditions did not significantly change the morphology of LO-EPC (c), but caused cell deformation and detachment in HUVEC (d). (e, f) LO-EPC developed normal, closed network units after being subjected to I/R culture conditions (e), while networks were disrupted in HUVEC under the same conditions (f). Scale bar 200 μm.

indicated by the percentage of cells positive for Annexin V and propidium iodide labelling (Fig. 3c). However, prolonged exposure to simulated I/R culture conditions (>16 h) led to apoptotic death in both HUVEC and LO-EPC (data not shown).

Ischaemia reperfusion injury did not cause significant activation in either LO-EPC or HUVEC as assessed by ICAM-1 expression or upregulation of proinflammatory cytokines/chemokines.

Response of LO-EPC to inflammatory stimulus

Both LO-EPC and HUVEC have detectable basal expression of ICAM-1, which is upregulated to a comparable level in both by recombinant TNF-α (10 ng/ml) (Fig. 4a).

Late outgrowth endothelial progenitor cells had a low basal expression level of most proinflammatory cytokines/chemokines, except for CXCL-1, IL-8, MIF and PAI which were relatively strongly expressed. TNF-α markedly upregulated the release of GM-CSF, MCP-1, IL-6 and soluble ICAM-1 (Fig. 4b) and moderately increased CXCL-1, IL-8 expression levels, but there was no significant change in secretion of G-CSF, MIF or PAI or other proinflammatory cytokines/chemokines. TNF-α-induced activation profiles of LO-EPC and mature endothelial cells (HUVEC) were similar (Fig. 4b). Both LO-EPC and HUVEC had low basal expression of proinflammatory cytokines of IL-2 family including IL-2, IL-4 and IL-15, which was not altered by TNF-α stimulation.

Tumour necrosis factor-α induced increase in adhesion molecule expression and chemokine and cytokine release likely resulted from activation of the NFκB pathway, which requires the phosphorylation, ubiquitination and degradation of the NFκB inhibitor IκBα [27,28]. Western blot analysis of LO-EPC and HUVEC subjected to TNF-α stimulation revealed that by 30 min, IκBα was phosphorylated, confirming the contribution of the NFκB pathway to the inflammatory cellular phenotype (Fig. 4c). LO-EPC and mature EC responded to TNF-α with comparable kinetics suggesting that both cell types have a similar sensitivity to inflammatory stimuli.

NFκB activation also upregulates 'protective' proteins such as A20. A very low level of endogenous A20 protein expression was detected in LO-EPC, which was upregulated on exposure to TNF-α (Fig. 4d), possibly protecting them against apoptosis (data not shown). Consistent with this, inhibition of protein synthesis by cycloheximide (CHX), which would inhibit *de novo* expression of endogenous A20, resulted in TNF-α induced apoptosis in LO-EPC, as shown by their changed morphology (Fig. 5a) and an increased level of both early and late apoptosis (Fig. 5b). Both LO-EPC and mature EC showed a comparable apoptotic response to TNF-α/CHX (Fig. 5c and d). Despite the upregulation of endogenous A20, no decrease in expression

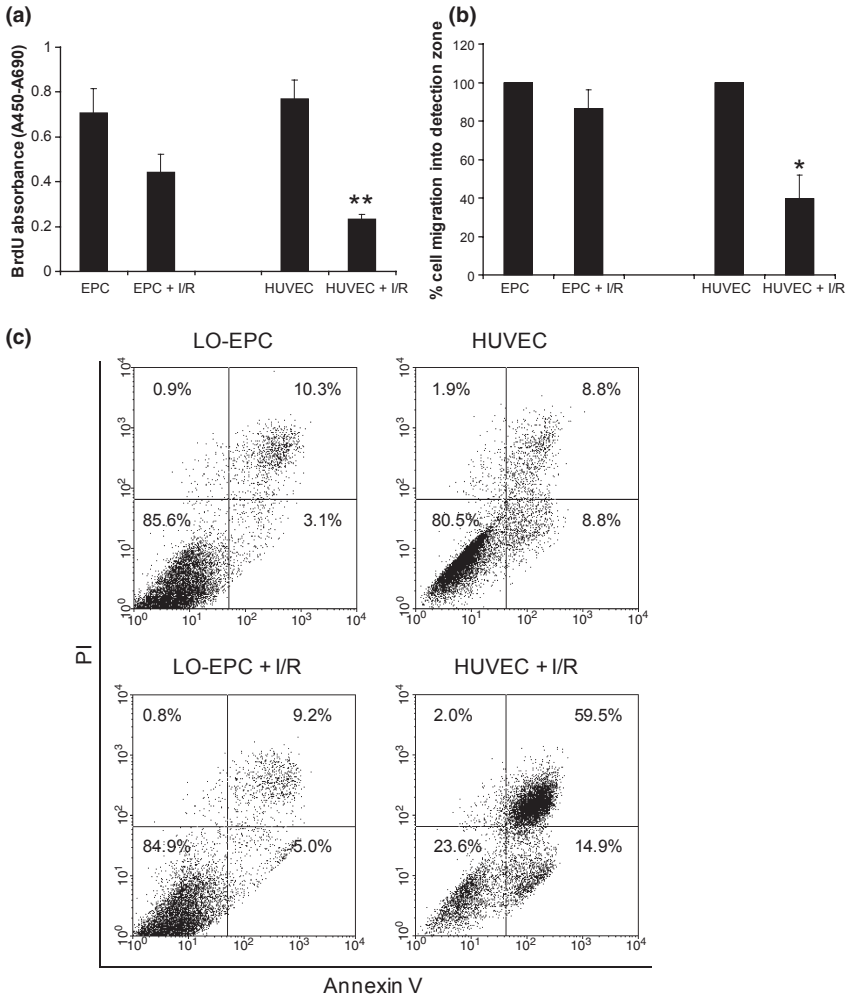

Figure 3 Resistance of late outgrowth endothelial progenitor cells (LO-EPC) to simulated ischaemia reperfusion (I/R) injury (II) (a) I/R culture conditions did not influence proliferation of LO-EPC, as determined by BrdU incorporation, but significantly inhibited the proliferation of human umbilical vein endothelial cells (HUVEC). $^{**}P < 0.01$ compared with untreated cells (data expressed as mean ± SE of three experiments). (b) I/R culture conditions did not influence cell migration in LO-EPC, as determined by counting cells migrating into the detection zone of ORIS^{TM} Cell Migration plates, but significantly reduced cell migration in HUVEC. The value was normalized against untreated cells. $^{*}P < 0.05$ compared with untreated cells (data expressed as mean ± SE of three experiments). (c) Representative FACS plots showed that LO-EPC are resistant to I/R-induced apoptosis, but I/R culture conditions increased both early and late apoptosis in HUVEC. Viable cells are identified as Annexin V^- PI^-; early apoptotic cells are Annexin V^+ PI^-; late apoptotic cells are Annexin V^+ PI^+.

of ICAM-1 was observed over the same time period (Fig. 4e). Both A20 and ICAM-1 were induced with similar kinetics. Apoptotic LO-EPC were less able to form networks, and had an impaired ability to migrate (Fig. 7d and e).

Overexpression of A20 protects LO-EPC from TNF-α-induced inflammation

As TNF-α-induced A20 upregulation appeared to protect LO-EPC from apoptosis, we hypothesized that overexpression of A20 may be an effective strategy for protecting autologous LO-EPC from the damaging effects of transplant-related inflammation by maintaining a nonactivated phenotype and resisting apoptosis. LVV-mediated A20 gene transfer led to stable A20 overexpression in LO-EPC as confirmed by Western blotting (Fig. 6a). A20 overexpression effectively inhibited both TNF-α-induced ICAM-1 upregulation (Fig. 6b) and TNF-α-induced secretion of cytokines and chemokines such as GM-CSF, MCP-1 and soluble ICAM-1, although the reduction of IL-6 secretion failed to reach statistical significance (Fig. 6c). Overexpression of A20 did not influence production of CXCL-1, IL-8, G-CSF, MIF and PAI (Fig. 6d), a range of cytokines and chemokines that were either only moderately increased or unaffected by TNF-α stimulation. The effect of A20 on TNF-α-induced activation in LO-EPC was initiated by inhibition of the NFκB pathway via inhibition of IκBα phosphorylation (data not shown).

It is believed that upregulation of NFκB leads to resistance to apoptosis [29,30]. In the present study, overexpression of A20 did not render LO-EPC susceptible to apoptosis, but protected cells from TNF-α-induced apoptosis (as measured by caspase 3 activity) in the presence of CHX (Fig. 7a).

Overexpression of A20 protected LO- EPC network formation *in vitro*

Tumour necrosis factor-α can upregulate angiogenic cytokines such as FGF-2 and VEGF by activating the NFκB

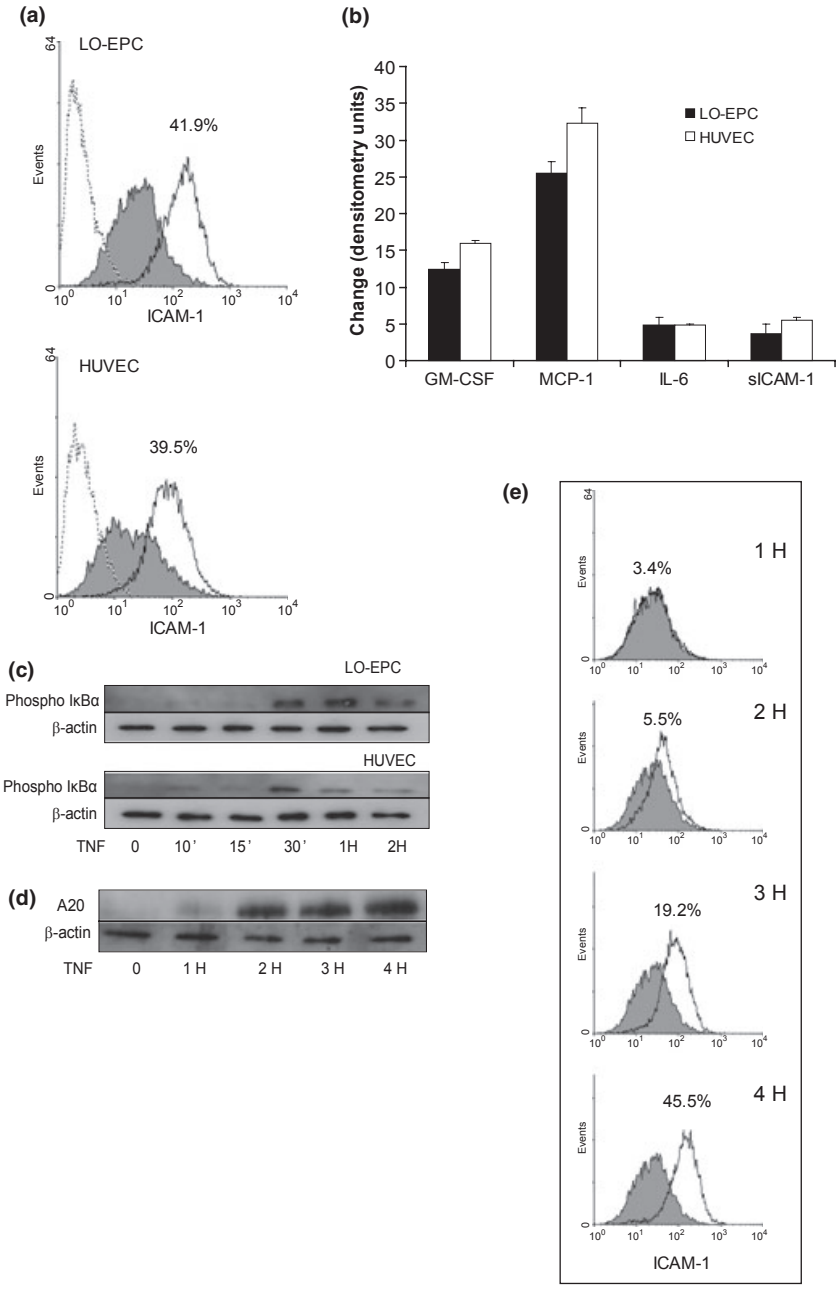

Figure 4 Effect of tumour necrosis factor (TNF)-α on late outgrowth endothelial progenitor cells (LO-EPC) activation. (a) Representative FACS plots of surface adhesion molecule (ICAM-1) expression in LO-EPC and human umbilical vein endothelial cells (HUVEC) in response to TNF-α stimulation. The empty histogram with dotted line represents untreated cells staining with isotype control antibody, the filled histogram represents untreated cells and the empty histogram with solid line represents cells treated with TNF-α (10 ng/ml) for 4 h. (b) TNF-α upregulates expression of inflammatory cytokines and chemokines in both LO-EPC and HUVEC. Relative expression levels in culture supernatants from LO-EPC and HUVEC following TNF-α stimulation for 4 h were measured using an R&D Cytokine Antibody Array kit and compared with supernatants from unstimulated cells. The data were analysed using Image J software and expressed as densitometric units (data represent mean ± SE of three experiments). (c) Phospho IκBα was used as indictor for NFκB activation and measured by Western blot of TNF-α treated cells. Time course of phospho IκBα expression measured by band intensity analysis of Western blots demonstrated a similar response to TNF-α stimulation in LO-EPC and HUVEC; β-actin protein expression is shown as a control. (d) Time course of endogenous A20 upregulation in response to TNF-α stimulation of LO-EPC as detected by Western blotting. (e) Time course of surface ICAM-1 expression in response to TNF-α stimulation of LO-EPC, as detected by flow cytometry. The filled histogram represents untreated cells and the empty histogram represent LO-EPC treated with TNF-α for the indicated time.

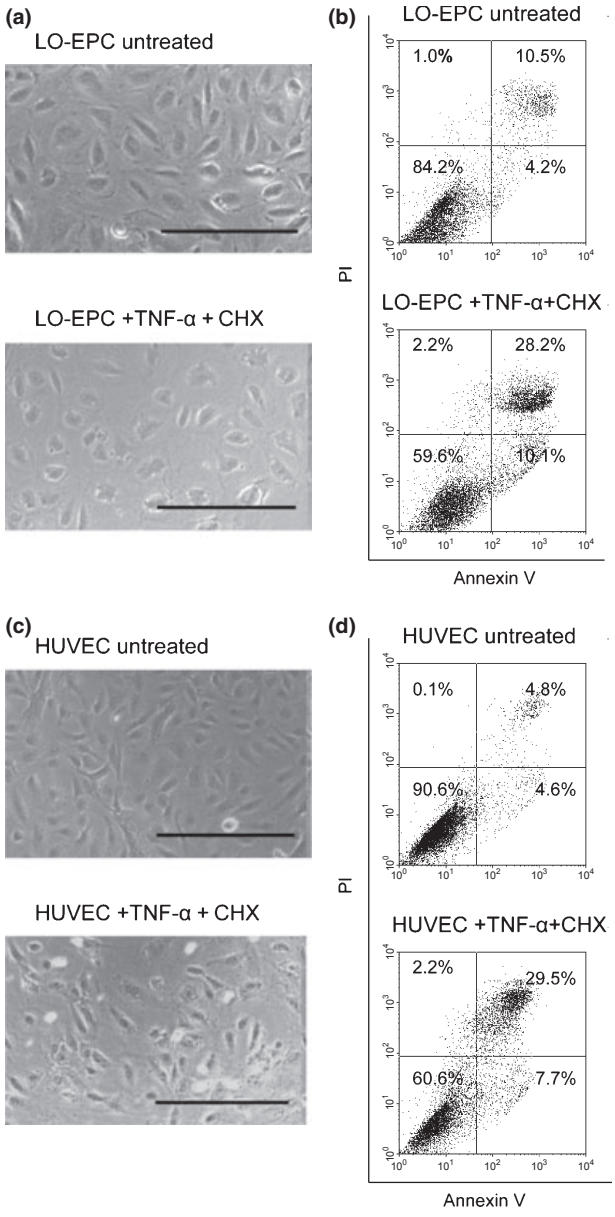

Figure 5 Tumour necrosis factor (TNF)-α induces apoptosis in late outgrowth endothelial progenitor cells (LO-EPC) and human umbilical vein endothelial cells (HUVEC) in the presence of the protein synthesis inhibitor, cycloheximide (CHX). (a, c) Representative phase-contrast images of LO-EPC (a) and HUVEC (c) show that TNF-α/CHX treatment caused cell deformation in both cell types. Scale bar 200 μm. (b, d) Representative FACS plots of LO-EPC (b) and HUVEC (d) show that TNF-α/CHX treatment induced apoptosis to a comparable degree in both cell types. Viable cells are identified as Annexin V^- PI^-; early apoptotic cells are Annexin V^+ PI^-; late apoptotic cells are Annexin V^+ PI^+.

pathway [20,31], whereas overexpression of A20 to inhibit NFκB activation could potentially compromise angiogenesis. LVV-induced overexpression of A20 in LO-EPC did not affect their capacity to form networks, compared with nontransduced LO-EPC or LO-EPC transduced with the empty vector (Fig. 7b).

A20-transduced LO-EPC were able to maintain network formation following treatment with TNF-α/CHX (Fig. 7c), whereas LO-EPC transduced with empty vector showed disrupted network units (Fig. 7d). In addition, overexpression of A20 was able to partially restore LO-EPC migration capacity after treatment with TNF-α/CHX compared with cells transduced with empty vector (Fig. 7e).

Discussion

Choosing the optimal endothelial progenitor cell to repair allograft endothelium damaged by transplant rejection

Endothelial cells play an active role in transplant vasculopathy, but have limited capacity for regeneration and repair. Transfer of endothelial progenitor cells might provide an attractive approach for repairing damaged endothelium within the graft as replacement and re-endothelialisation with expanded cells of recipient origin may abrogate allogeneic stimuli triggering chronic vasculopathy. EPC are cells of mesodermal origin found in the bone marrow, spleen, umbilical cord blood, and circulating in the peripheral blood [32]. Two recently identified types of EPC, early outgrowth (EO-EPC) and late outgrowth EPC (LO-EPC) have apparently distinct properties [33–38]. EO-EPC are short-lived cells (<2 weeks) that express markers for endothelium and for monocyte lineages [34,39]. EO-EPC do not differentiate into EC *in vivo*, but contribute to restoring endothelial function and enhance angiogenesis after tissue ischaemia, via a paracrine effect of secreted angiogenic growth factors [40]. Although EO-EPC have been shown to produce clinical benefit, these are a heterogeneous population of cells including monocyte-derived immune cells [40–42]; delivering large numbers of *ex vivo* expanded autologous early outgrowth EPC in transplantation might risk exacerbating immune rejection. Late outgrowth EPC appear after 2 weeks of culture in medium containing endothelial growth supplements. They are present in blood in very low numbers [43], are highly proliferative and are distinct from peripheral blood monocytes [43–45]. LO-EPC are a homogeneous endothelial-like progenitor cell population, which can integrate into damaged vascular beds and possess *de novo* vessel-forming ability, contributing directly to angiogenesis and secreting factors to promote EC repair [33,37,43–45]. Therefore, replenishing or supplementing with *ex vivo* expanded autologous LO-EPC could potentially repair allograft endothelium damaged by transplant rejection. In addition, *ex vivo* expansion provides the opportunity to genetically modify the cells

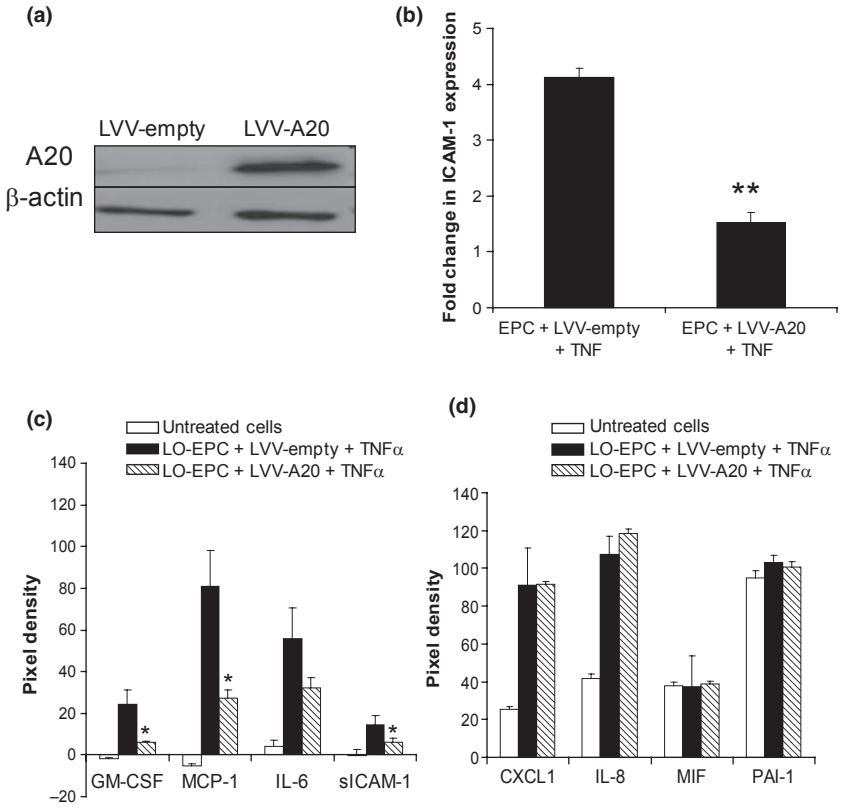

Figure 6 Effect of overexpression of A20 on tumour necrosis factor (TNF)-α-induced activation in late outgrowth endothelial progenitor cells (LO-EPC). (a) Western blot analysis of LO-EPC transduced with LVV-A20 demonstrates stable overexpression of A20. (b) Overexpression of A20 in LO-EPC significantly inhibited TNF-α-induced surface ICAM-1 expression as determined by flow cytometry. $^{**}P < 0.01$ compared with LO-EPC transduced with empty vector (data represent mean ± SE of three independent experiments). (c, d) Overexpression of A20 in LO-EPC inhibited GM-CSF, MCP-1, IL-6 and soluble ICAM-1 release in response to TNF-α stimulation, as determined by cytokine/chemokine array assay (c). $^{*}P < 0.05$ compared with LO-EPC transduced with empty vector (data represent mean ± SE of three independent experiments). Overexpression of A20 in LO-EPC did not influence CXCL-1, IL-8, MIF and PAI-1 release in response to TNF-α stimulation as determined by cytokine/chemokine array assay (d, $P > 0.05$). The open bar represents untreated cells, solid bar represents LO-EPC transduced with empty vector and cultured with TNF-α, and shaded bar represents LO-EPC transduced with LVV-A20 and cultured with TNF-α.

before reintroducing them into patients. By utilizing their natural homing ability, LO-EPC transfer could be an efficient way to deliver therapeutic genes to allografts.

LO-EPC resist ischaemia reperfusion injury

Transplanted EPCs have previously been found to exhibit a potent capacity *in vivo* to improve the neovascularisation of ischaemic tissue [46–48], but conflicting reports exist regarding their sensitivity to oxidative stress, which may be attributed in part to the diversity of cells and methods used. Ischaemia reperfusion injury damages cells in a biphasic process. Ischaemia initiates the injury by depriving cells of the energy needed to maintain ionic gradients and homoeostasis. Reperfusion exacerbates this damage by an overproduction of oxygen free radicals toxic to cells [49]. In this study, we used a previously established *in vitro* I/R model [26], in which ischaemic damage to LO-EPC was simulated by energy source deprivation combined with anoxia, and additional potassium chloride and sodium lactate; disrupting ionic gradients and Ca^{2+} homoeostasis as typically seen in severe ischaemia *in vivo* [50,51]. The pH was adjusted to 6.2 as severe ischaemia *in vivo* causes an extracellular acidification with pH values as low as 6.0 [52]. We have shown that *ex vivo* expanded LO-EPC can withstand simulated I/R injury-induced apoptosis, and maintain their capacity to proliferate, migrate and form vascular networks to a much greater degree than mature EC, which under the same *in vitro* conditions have impaired proliferation and migration, abrogated network forming capacity and undergo apoptosis. This may explain why EPC proved more beneficial than mature EC in animal models of hind limb ischaemia [53].

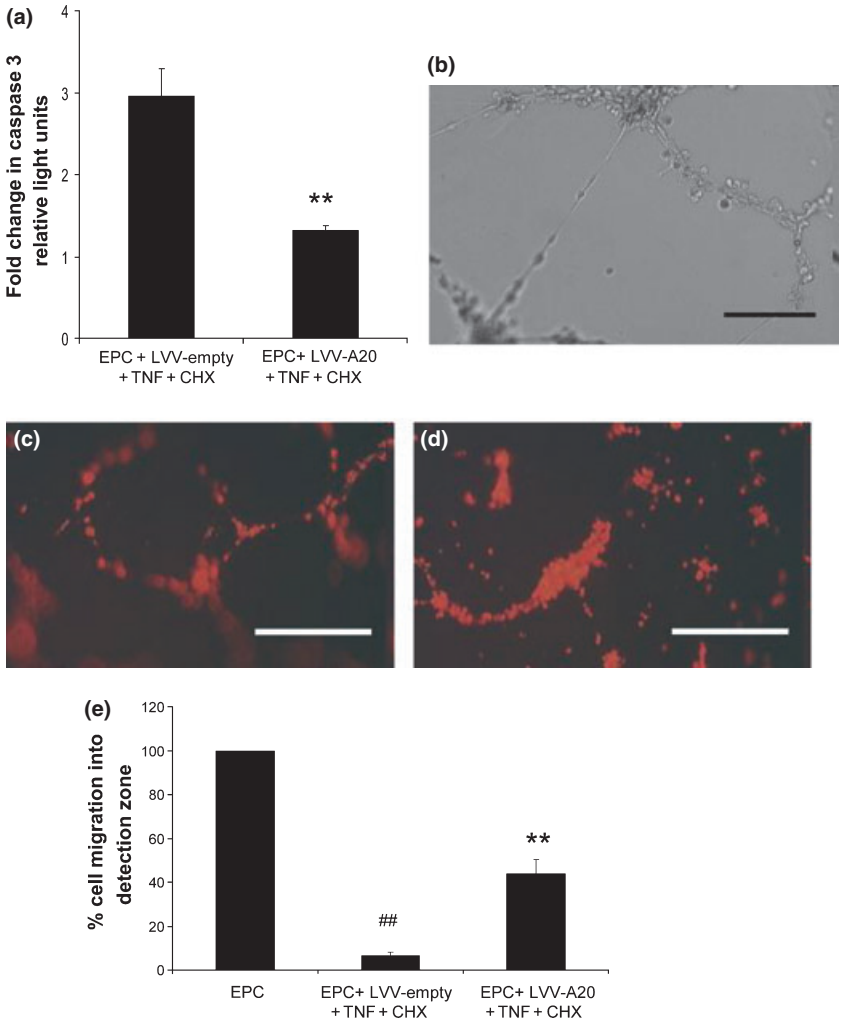

Figure 7 Effect of overexpression of A20 on tumour necrosis factor (TNF)-α-induced apoptosis, angiogenesis and migration. (a) Overexpression of A20 significantly inhibited TNF-α-induced apoptosis in late outgrowth endothelial progenitor cells (LO-EPC) stimulated with TNF-α in the presence of cycloheximide (CHX), as determined by caspase 3 activity (measured using a luminescent substrate and expressed as relative light units). $^{**}P < 0.01$ compared with LO-EPC transduced with empty vector (data represent mean ± SE of three independent experiments). (b) Overexpression of A20 in LO-EPC did not affect their networks formation. Scale bar 200 μm. (c, d) LO-EPC overexpressing A20 and treated with TNF-α/CHX were able to maintain network formation (c), whereas LO-EPC transduced with empty vector showed disrupted network units under the same conditions (d). Scale bar 200 μm. (e) LO-EPC treated with TNF-α/CHX significantly impaired their ability to migrate. $^{\#\#}P < 0.01$ compared with untreated LO-EPC. Overexpression of A20 in LO-EPC treated with TNF-α/CHX was able to restore their ability to migrate. $^{**}P < 0.01$ compared with LO-EPC transduced with empty vector. Migration was determined by counting cells in the detection zone. The value was normalized against untreated cells. Data represent mean ± SE from three independent experiments.

Some reports suggest that EPC are more resistant than mature EC to oxidative stress, correlating with high endogenous expression and activity of the anti-oxidant manganese superoxide dismutase (MnSOD) [54,55]. In contrast, Ingram *et al.* claimed that late outgrowth EPC derived from both cord blood and adult blood are *more* sensitive to oxidative stress compared with mature EC [56]. These conflicting studies used a superoxide generator (Ly83583) and/or increasing H_2O_2 concentration to provoke oxidative stress which induces ASK-1 pathway-dependent apoptosis in EPC, whereas the present study used I/R conditions simulating *in vitro* clinical transplantation, previously shown to induce mitochondrial-dependent apoptosis in mature EC [26].

The influence of inflammatory environment on LO-EPC

For endothelial repair, LO-EPC must migrate into the graft and resist a harsh environment resulting from acute and chronic transplant-related inflammatory processes.

Although more resistant than mature EC to I/R injury, LO-EPC and EC responses to inflammatory signals are similar. TNF-α activates LO-EPC generating a pro-inflammatory phenotype upregulating expression of the surface adhesion molecule ICAM-1, and secretion of cytokines and chemokines including GM-CSF, MCP-1, IL-6 and soluble ICAM-1. Although LO-EPC activation did not compromise their ability to migrate and form networks (data not shown), upregulation of cytokines and chemokines may convert LO-EPC from their initial proposed angiogenic function to a proatherogenic function potentially precipitating chronic rejection. These risks have been raised by others [20,21]. Although Liu *et al.* [20] demonstrated beneficial effects of A20 on activation, EC apoptosis is potentially more detrimental than activation in promoting vasculopathy as apoptotic EC would expose the subendothelial matrix leading to platelet binding and activation, and promoting coagulation and thrombosis [57]. TNF-α can induce both activation and apoptosis in LO-EPC depending on their cellular state. We show that LO-EPC are resistant to TNF-α-mediated apoptotic death correlating with upregulated endogenous A20 expression by TNF-α. Use of the protein synthesis inhibitor CHX to stop *de novo* synthesis of 'cytoprotective' genes resulted in TNF-α-induced apoptotic death in LO-EPC, suggesting that resistance to TNF-α-induced apoptosis depends on adequate protein synthesis. This mimics the acute and chronic cellular stresses in LO-EPC in the allograft microenvironment including ischaemia, low nutrient availability, and persistent and prolonged inflammatory stimuli, which would cause impaired *de novo* protein synthesis rendering LO-EPC highly susceptible to TNF-α induced apoptosis.

Role of overexpression of A20 in modulating the LO-EPC response to TNF-α

Tumor necrosis factor-α has a number of direct effects on LO-EPC causing release of an array of pro-inflammatory molecules, but also upregulation of A20 expression through activation of the NFκB pathway. Given that both ICAM-1 and endogenous A20 expression were upregulated by TNF-α in a synchronous fashion, simply inhibiting the NFκB pathway to prevent LO-EPC activation would fail as this would render LO-EPC susceptible to TNF-α-mediated apoptosis by blocking endogenous A20 expression. The fact that A20 inhibits TNF-α-induced activation and apoptosis in early signalling cascades [58] make it more likely that it would only affect NFκB activation triggered by certain stimuli. This combination of properties makes A20 an ideal candidate for modulating TNF-α-mediated inflammation.

We found that similar to mature EC, TNF-α stimulation of LO-EPC induced a proatherogenic phenotype. Although TNF-α induces 'protective' genes, such as A20, as part of the early response, we showed that upregulated endogenous A20 expression always correlated with expression of proinflammatory molecules such as ICAM-1 suggesting that an increased endogenous A20 would be insufficient to inhibit LO-EPC activation. Overexpression of A20, however, could inhibit surface ICAM-1 expression and that of several cytokines and chemokines, maintaining the LO-EPC in their unstimulated phenotype despite the presence of TNF-α. These findings are consistent with a recently published study that demonstrated the benefit of overexpression of A20 on LO-EPC in an inflammatory environment [20] but we also demonstrate that overexpression of A20 over endogenous (upregulated) A20 was able to inhibit NFκB in LO-EPC without sensitizing these cells to TNF-α-mediated apoptosis, preserving the capacity of LO-EPC to form networks and, importantly, retaining their ability to integrate with mature EC to form a chimeric network in the presence of TNF-α initiated apoptotic stimuli.

Our findings demonstrate that *ex vivo* expanded and gene-modified LO-EPC are functionally more robust than native LO-EPC, and suggest that they may provide a therapeutic option for repair and replacement of damaged EC in an allograft in order to avoid development of CAV.

Authorship

JZ: designed research study, performed research, collected and analysed data, and wrote manuscript. EMB: helped research design, analysed data, helped write and review the manuscript. MLO: contributed important reagents, read and contributed to writing the manuscript. JAB: helped research design, helped write and review the manuscript. NWM: contributed important reagents, read and approved manuscript. AML: helped design research study, analysed data, helped write and review the manuscript.

Funding

British Heart Foundation Grant PG/10/74/28483 and an NIHR Cambridge Biomedical Research Centre award RG52162.

References

1. Kwun J, Knechtle SJ. Overcoming chronic rejection-can it B? *Transplantation* 2009; **88**: 955.
2. Wehner J, Morrell CN, Reynolds T, Rodriguez ER, Baldwin 3rd WM. Antibody and complement in transplant vasculopathy. *Circ Res* 2007; **100**: 191.

3. Xiao Q, Kiechl S, Patel S, *et al.* Endothelial progenitor cells, cardiovascular risk factors, cytokine levels and atherosclerosis–results from a large population-based study. *PLoS ONE* 2007; **2**: e975.
4. Zampetaki A, Kirton JP, Xu Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res* 2008; **78**: 413.
5. Schmidt-Lucke C, Rossig L, Fichtlscherer S, *et al.* Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 2005; **111**: 2981.
6. Choi J-H, Kim KL, Huh W, *et al.* Decreased number and impaired angiogenic function of endothelial progenitor cells in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1246.
7. Urbich C, Dimmeler S. Risk factors for coronary artery disease, circulating endothelial progenitor cells, and the role of HMG-CoA reductase inhibitors. *Kidney Int* 2005; **67**: 1672.
8. Vasa M, Fichtlscherer S, Aicher A, *et al.* Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; **89**: e1.
9. Kong D, Melo LG, Gnecchi M, *et al.* Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries. *Circulation* 2004; **110**: 2039.
10. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, *et al.* Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation* 2003; **108**: 457.
11. Karra R, Vemullapalli S, Dong C, *et al.* Molecular evidence for arterial repair in atherosclerosis. *Proc Natl Acad Sci USA* 2005; **102**: 16789.
12. Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. *Circulation* 2003; **108**: 3122.
13. Xu Q, Zhang Z, Davison F, Hu Y. Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in ApoE-deficient mice. *Circ Res* 2003; **93**: e76.
14. de Groot K, Bahlmann FH, Bahlmann E, Menne J, Haller H, Fliser D. Kidney graft function determines endothelial progenitor cell number in renal transplant recipients. *Transplantation* 2005; **79**: 941.
15. Soler MJ, Martinez-Estrada OM, Puig-Mari JM, *et al.* Circulating endothelial progenitor cells after kidney transplantation. *Am J Transplant* 2005; **5**: 2154.
16. Steiner S, Winkelmayer WC, Kleinert J, *et al.* Endothelial progenitor cells in kidney transplant recipients. *Transplantation* 2006; **81**: 599.
17. Sathya CJ, Sheshgiri R, Prodger J, *et al.* Correlation between circulating endothelial progenitor cell function and allograft rejection in heart transplant patients. *Transpl Int* 2010; **23**: 641.
18. Woywodt A, Schroeder M, Gwinner W, *et al.* Elevated numbers of circulating endothelial cells in renal transplant recipients. *Transplantation* 2003; **76**: 1.
19. Rabelink TJ, de Boer HC, de Koning EJ, van Zonneveld AJ. Endothelial progenitor cells: more than an inflammatory response? *Arterioscler Thromb Vasc Biol* 2004; **24**: 834.
20. Liu JW, Dunoyer-Geindre S, Blot-Chabaud M, *et al.* Generation of human inflammation-resistant endothelial progenitor cells by A20 gene transfer. *J Vasc Res* 2010; **47**: 157.
21. Zhang Y, Ingram DA, Murphy MP, *et al.* Release of proinflammatory mediators and expression of proinflammatory adhesion molecules by endothelial progenitor cells. *Am J Physiol Heart Circ Physiol* 2009; **296**: H1675.
22. Breitbach M, Bostani T, Roell W, *et al.* Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* 2007; **110**: 1362.
23. Heyninck K, Beyaert R. A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. *Trends Biochem Sci* 2005; **30**: 1.
24. Daniel S, Arvelo MB, Patel VI, *et al.* A20 protects endothelial cells from TNF-, Fas-, and NK-mediated cell death by inhibiting caspase 8 activation. *Blood* 2004; **104**: 2376.
25. Zhao J, Pettigrew GJ, Bolton EM, *et al.* Lentivirus-mediated gene transfer of viral interleukin-10 delays but does not prevent cardiac allograft rejection. *Gene Ther* 2005; **12**: 1509.
26. Zhao J, Bolton EM, Bradley JA, Lever AM. Lentiviral-mediated overexpression of Bcl-xL protects primary endothelial cells from ischemia/reperfusion injury-induced apoptosis. *J Heart Lung Transplant* 2009; **28**: 936.
27. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO J* 1995; **14**: 2876.
28. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 1988; **242**: 540.
29. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 1996; **274**: 782.
30. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 1996; **274**: 787.
31. Ko Y, Totzke G, Gouni-Berthold I, Sachinidis A, Vetter H. Cytokine-inducible growth factor gene expression in human umbilical endothelial cells. *Mol Cell Probes* 1999; **13**: 203.
32. Marsboom G, Janssens S. Endothelial progenitor cells: new perspectives and applications in cardiovascular therapies. *Expert Rev Cardiovasc Ther* 2008; **6**: 687.
33. Yoder MC, Mead LE, Prater D, *et al.* Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 2007; **109**: 1801.

34. Asahara T, Murohara T, Sullivan A, *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964.
35. Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. *Circ Res* 2000; **87**: 434.
36. Hill JM, Zalos G, Halcox JP, *et al.* Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; **348**: 593.
37. Ingram DA, Mead LE, Tanaka H, *et al.* Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004; **104**: 2752.
38. Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. *J Am Coll Cardiol* 2008; **51**: 660.
39. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 2004; **95**: 343.
40. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003; **107**: 1164.
41. Zhang SJ, Zhang H, Wei YJ, *et al.* Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture. *Cell Res* 2006; **16**: 577.
42. Asakage M, Tsuno NH, Kitayama J, *et al.* Early-outgrowth of endothelial progenitor cells can function as antigen-presenting cells. *Cancer Immunol Immunother* 2006; **55**: 708.
43. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000; **105**: 71.
44. Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood* 2005; **106**: 1525.
45. Ingram DA, Mead LE, Moore DB, Woodard W, Fenoglio A, Yoder MC. Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. *Blood* 2005; **105**: 2783.
46. Kalka C, Masuda H, Takahashi T, *et al.* Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* 2000; **97**: 3422.
47. Assmus B, Schachinger V, Teupe C, *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 2002; **106**: 3009.
48. Tateishi-Yuyama E, Matsubara H, Murohara T, *et al.* Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002; **360**: 427.
49. Torras J, Cruzado JM, Grinyo JM. Ischemia and reperfusion injury in transplantation. *Transplant Proc* 1999; **31**: 2217.
50. Maxwell SR, Lip GY. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. *Int J Cardiol* 1997; **58**: 95.
51. Duchen MR. Roles of mitochondria in health and disease. *Diabetes* 2004; **53**(Suppl. 1): S96.
52. Ferrari R, Cargnoni A, Bernocchi P, *et al.* Metabolic adaptation during a sequence of no-flow and low-flow ischemia. A possible trigger for hibernation. *Circulation* 1996; **94**: 2587.
53. Kocher AA, Schuster MD, Szabolcs MJ, *et al.* Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001; **7**: 430.
54. He T, Peterson TE, Holmuhamedov EL, *et al.* Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. *Arterioscler Thromb Vasc Biol* 2004; **24**: 2021.
55. Dernbach E, Urbich C, Brandes RP, Hofmann WK, Zeiher AM, Dimmeler S. Antioxidative stress-associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress. *Blood* 2004; **104**: 3591.
56. Ingram DA, Krier TR, Mead LE, *et al.* Clonogenic endothelial progenitor cells are sensitive to oxidative stress. *Stem Cells* 2007; **25**: 297.
57. Cailhier JF, Laplante P, Hebert MJ. Endothelial apoptosis and chronic transplant vasculopathy: recent results, novel mechanisms. *Am J Transplant* 2006; **6**: 247.
58. Heyninck K, De Valck D, Vanden Berghe W, *et al.* The zinc finger protein A20 inhibits TNF-induced NF-kappaB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-kappaB-inhibiting protein ABIN. *J Cell Biol* 1999; **145**: 1471.