Reestablishment of the Endothelial Lining by Endothelial Cell Therapy

Stabilizes Experimental Abdominal Aortic Aneurysms

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Abstract:

Background—Loss of the endothelium and its replacement by a thick thrombus is a structural feature of human abdominal aortic aneurysms (AAAs). In AAAs, the relationship between aortic diameter expansion, presence of thrombus and lack of endothelial cells (ECs) remains unexplored. We hypothesized that reendothelialization by cell therapy would modulate aortic wall destruction and ultimately stabilize AAAs. We evaluated the impact of local seeding of rat aortic ECs (RAEC) or peripheral blood-derived outgrowth ECs (OECs) on AAA evolution.

Methods and Results—RAECs (n=30) or serum-free medium (controls, n=29) were seeded endovascularly immediately (D0) or 14 days (D14) after surgery in the rat xenograft model. RAEC seeding prevented AAA formation and stabilized formed AAAs at 28 days (diameter increase at D0+28: 51±6 vs 83±6%, D14+28: -1±4 vs 22±6% in RAECs and controls, respectively, p<0.01). This stabilizing effect was associated with the reestablishment of the endothelial lining, the suspension of proteolysis, and the reconstitution of new aortic wall rich in smooth muscle cells and extracellular matrix. Transplanted RAECs did not directly participate in aortic wall repair but exerted their healing properties through paracrine mechanisms, involving the upregulation of endothelial-derived stabilizing factors and the recruitment of resident vascular cells. In rats, the transplantation of OECs (n=7) significantly reduced by 30% the progression of AAA and restored the abluminal endothelium at 28 days, as compared with controls (n=9).

Conclusions—Our study demonstrates the potential of restoring the endothelial lining to control AAA dynamics and designates ECs as an efficient therapy to stop AAA expansion.

Key words: endothelium, cell transplantation, abdominal aortic aneurysm, endothelial progenitor cells, regeneration
Introduction

Abdominal aortic aneurysms (AAAs) are progressive enlargements of the aorta that represent the third cause of cardiovascular death, with a risk of rupture proportional to the external diameter. Current preventive treatments, e.g. prosthetic replacement (surgery) or strengthening (endoprosthesis) of the aorta are associated with a high mortality rate and a limited durability, respectively. For the past few years, our laboratory has studied the feasibility of therapeutic approaches based on the local modulation of aneurysmal wall biology through local gene or cell therapy to strengthen the diseased aorta.

AAAs are characterized by a thinning of the aortic media with rarefaction of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM) destruction triggered by inflammation, oxidative stress and proteolysis. Another structural feature of AAAs is the presence of a thick intraluminal thrombus with the loss of the abluminal endothelial monolayer. Although the association of luminal thrombus and AAA progression and rupture has been recognized, the contribution of endothelial loss has received little attention.

Endothelial cells (ECs) play a major role in the maintenance of vascular integrity, by controlling inflammation, thrombosis and, mural cell and matrix coverage. Alterations of the endothelial layer participate in the development of arterial lesions associated with atherosclerosis or restenosis. Regarding AAA pathogenesis, the causal relationship between aortic diameter expansion, presence of thrombus and lack of ECs remains unexplored. Nevertheless, there is evidence that induction of EC apoptosis promotes thrombin generation and platelet adhesion in vitro and initiates fibrin-rich thrombus formation in vivo. On the other hand, recent data suggest that the intraluminal thrombus is a major source of proteases in the aneurysmal wall that participate in arterial wall thinning and absence of luminal healing. These two observations...
suggested to us that loss of the endothelial lining may favor thrombus formation, wall atrophy and AAA expansion and that restoration of the integrity of the endothelial monolayer would modulate AAA degeneration.

Over the last decade, one of the therapeutic options to regenerate the damaged endothelium of injured arteries involves the transplantation of autologous cells of the endothelial lineage including \textit{ex vivo} expanded mature ECs or late endothelial progenitor cells (EPCs), also known as late outgrowth ECs (OECs)\textsuperscript{12,13}. Hence, the systemic delivery of ECs has been shown to enhance reendothelialization and prevent vessel remodeling following arterial injury\textsuperscript{14-16}.

Several lines of evidence suggest that vascular EC therapy provides a cellular source of endothelial-derived stabilizing factors that enhance arterial wall reprogramming and healing, rather than a direct participation in the restoration of endothelial lining\textsuperscript{12,14,17}. In the present study, we thought to evaluate the feasibility and the impact of local EC therapy on AAA expansion in the xenograft model in rats. This model offers the advantage to reproduce important features of human AAA lesions, including aortic wall atrophy, endothelial denudation and the presence of an intraluminal thrombus\textsuperscript{2,3}. Using this model, we show that endovascular delivery of mature aortic ECs or peripheral blood-derived OECs reduces the expansion of AAAs while reestablishing the integrity of the endothelial monolayer. The recovery of a luminal endothelium is associated with the reconstitution of new arterial wall replacing the thrombus. We further show that the healing properties of EC delivery rely mainly on paracrine mechanisms, involving the upregulation of endothelial-derived stabilizing factors and the recruitment of resident vascular cells into the renewed aortic wall.

\textbf{Material and Methods}

An expanded Methods section is available in the Online Data Supplement. The experiments were
performed in accordance with the European guidelines for animal care (86/609/EEC). Rats were anesthetized with pentobarbital (60 mg/kg IP). AAAs were generated in 8 week-old male Fischer 344 rats (Charles River Laboratories, France) by implanting an aortic xenograft from guinea pig, as previously described\textsuperscript{2,3}. The design of the in vivo experiments is illustrated in Figure 1.

Results

The endothelial phenotype of culture-expanded rat aortic ECs (RAECs) or peripheral blood-derived OECs was first confirmed in vitro prior to their use in cell therapy in vivo (Supplemental Results; Supplemental Figures 1 and 2).

Local mature ECs delivery prevents AAA formation and stabilizes formed AAA.

The impact of local seeding of mature ECs on AAA formation was evaluated in the xenograft model of AAA in rats (Figures 1A and 2A-C). Control AAAs expanded constantly between day 0 to day 28 (Figure 2B, C) and acquired an intraluminal thrombus, mimicking important features of human atherosclerotic AAAs\textsuperscript{1,18}. Local RAEC seeding at the same time as surgery significantly retarded AAA development till 7 days (D0+7) and up to 28 days (D0+28) after transplantation (Figure 2B, C).

To test whether mature ECs in formed AAA would stop further AAA expansion, RAECs were seeded into the aortic lumen, 14 days after the xenograft implantation on formed AAA (>50% diameter increase) (Figures 1B and 2D-F). The external aortic diameter had significantly increased 14 days after the xenograft but was not different between RAEC-treated and control rats at the time of cell seeding initiation (3.0±0.1 vs 2.7±0.1mm, respectively, NS). Whereas AAA aortic diameter continued to expand in control rats, RAEC seeding significantly suspended AAA expansion as early as 7 days and up to 28 days after cell therapy (Figure 2E, F). Thus,
mature EC therapy stabilizes established experimental AAAs.

**Local mature ECs delivery promotes aortic wall reconstruction**

The stabilizing effect of RAEC transplantation on AAA formation was accompanied by the development of a CD31-positive endothelial monolayer together with the absence of thrombus and the formation of a thick neointima rich in αSMA-positive cells (**Figure 3A**). In control AAAs, the absence of endothelial lining with thrombus resulted in an increased vascular permeability for Evan’s Blue, as assessed by the increased fluorescence within the elastic network of the residual AAA wall (**Figure 3B**). In contrast, endothelial restoration after RAEC therapy abrogated the increased vascular extravasation of Evan’s Blue (**Figure 3B**). We further showed that local EC transplantation enhanced the aortic expression of the endogenous inhibitor of the extrinsic coagulation cascade, the tissue factor pathway inhibitor (TFPI) (fold induction: 3.7±0.7 in RAEC-treated relative to that of control AAAs, n=5, p<0.05).

Orcein and Sirius red staining demonstrated that local seeding of RAECs preserved the aortic medial elastic and collagen network structure and density and increased the elastin and collagen deposit in the neointimal wall, as compared to control AAAs (**Figure 3C, D**).

The ultrastructural changes in the reconstructed wall after RAEC therapy was examined by transmission electron microscopy at day 28. A thrombus mainly composed of platelets with few erythrocytes was evidenced in the lumen of control AAAs (**Figure 4A-C**). In contrast, two areas could be structurally differentiated based on cell phenotype and ECM organization in RAEC-seeded neointima (**Figure 4D**). The luminal part of the intima showed disorganized architecture, mainly composed of cells displaying characteristics of synthetic VSMCs, including rough endoplasmic reticulum and abundant collagen fibril embedment, reflecting the active remodelling process occurring in this area (**Figure 4E, F**). The deeper part of the neointima
showed an organized assembly of alternating and closely apposed bands of elongated mature contractile VSMCs and elastic lamella with collagen fibers in between, reproducing the classical microstructure of medial arterial lamellar units19 (Figure 4G, H). These data suggest a lumen-to-wall gradient of the reconstructed vascular wall in RAEC-treated AAAs.

**Local mature ECs therapy inhibits wall inflammation and destruction**

In human AAAs, destruction of ECM components and induction of VSMC apoptosis is triggered by inflammation, oxidative stress and MMP-dependent proteolysis4,18. Infiltration of the aneurysmal wall by CD68-positive macrophages was significantly inhibited 28 days after RAEC cell seeding (Figure 5A, B). This inhibition coincided with the parallel significant decrease in the number of adventitial isolectin B4-positive neovessels in RAEC-treated AAAs at day 28 (Figure 5A, B). Besides, although oxidative stress was abundant in AAA wall and thrombus of control rats, ROS production was decreased in the residual wall and almost undetectable in the neointimal wall of RAEC-treated aortas (Figure 5C). Concerning proteolysis, we showed that whereas the mRNA level of MMP-2,9,14 and their endogenous inhibitors, TIMP-1,3 remained unchanged at day 7 in the AAA wall of RAEC-seeded rats, MMP-12 gene expression was significantly down-regulated as compared to controls (Figure 5D). Concerning their activity, we showed that the active forms of MMP-2 and to lesser extent MMP-9 were increased at 7 days in the AAA wall of RAEC-seeded rats, but decreased at 28 days as compared to control rats (Figure 5E). Using *in situ* zymography, we showed that the early activation of MMP-2 in RAEC-seeded AAA wall was exclusively confined in the reconstructed neointimal wall (Figure 5E). Besides, we confirmed at a protein level that the total and active forms of MMP-12 were significantly decreased in the AAA wall of RAEC-treated rats as compared to controls, respectively (Figure 5F).
Neointimal cells after EC therapy originate from resident vascular cells but not from bone marrow-derived circulating progenitors

We next examined the origin of mural cells and ECs that composed the reconstructed vascular wall in RAEC-treated AAAs. When syngenic GFP+/+ RAECs were seeded endoluminally in rats immediately after the xenograft implantation, only few GFP cells were detected in the deepest part of the intima 7 days after cell therapy (n=4), whereas no GFP signal was evidenced at a later stage, i.e. 28 days post-infusion (n=4) (Figure 6A). These results demonstrate that although some transplanted ECs survived at an early stage, they do not directly participate in the long term cellular reconstruction of the new wall. In WT rats irradiated and rescued with bone marrow (BM) from GFP+/+ syngenic rats, the fraction of fluorescent peripheral blood leukocytes 3 weeks after BM transplantation (BMT) was 89±8%. At this delay, AAAs were generated in GFP+/+ BMT rats and WT RAECs were locally infused immediately after surgery (n=4). Twenty-eight days later, BM-derived GFP+/+ cells were only detected in the residual AAA wall itself but were totally absent from the newly-formed intima (Figure 6B), excluding the possibility that neointimal mural cells and ECs originate from BM-derived circulating progenitor cells. Finally, we transplanted GFP+/+ aortic isografts on both sides of the decellularized guinea pig xenograft before endovascular seeding of WT RAECs (n=3; Figure 6C). In those rats, the reconstructed arterial neointima consisted almost exclusively in GFP-positive cells at day 7 (Figure 6C), demonstrating that neointimal mural cells and ECs come from the neighboring aortic wall itself.

The healing properties of EC therapy involve the upregulation of endothelial stabilizing genes

Since transplanted ECs do not participate directly in the AAA wall reconstruction, we hypothesized that RAEC engraftment might stimulate endothelial and/or muscular wall repair
through the release of angiogenic/arteriogenic stabilizing factors. Quantitative real-time PCR experiments revealed that 7 days after endovascular operation, RAEC seeding significantly increased the mRNA aortic expression of the angiogenic stabilizing factors bFGF, eNOS, VE-cadherin and Robo4 (Figure 7A). Since bFGF plays an important role in the initiation of arteriogenic process, we analyzed its temporal and spatial expression in RAEC-treated aortas. bFGF protein was only expressed in RAEC-treated aortas at an early stage (D0+7) and was no longer detectable in the long term (D0+28; Figure 7B). Of interest, early bFGF expression co-localized with GFP signal in the deepest part of the neointima of GFP+/+ RAEC-treated aortas (Figure 7C). We confirmed that RAECs express high amount of bFGF proteins in vitro (Figure 7D), demonstrating that transplanted ECs act as a cellular source of bFGF. We then addressed whether bFGF secreted by donor RAECs might modulate the phenotype of vascular cells to promote aortic wall healing. We showed that the conditioned media from RAECs (RAEC-CM) stimulated the migration and proliferation of human ECs (HUVECs) and human AAA wall derived VSMCs in vitro (Supplemental Figure 3A, B). These effects were mediated through bFGF, as an anti-bFGF neutralizing antibody abrogated the migratory and proliferative effects of RAEC-CM on both vascular cells (Supplemental Figure 3A, B). As a positive control, we confirmed that recombinant rat bFGF promote the migration and proliferation of both human cells (Supplemental Figure 3A, B).

Local delivery of ECs derived from circulating OECs reduces AAA formation, promotes aortic wall reconstruction and reestablishes the endothelial lining

Since the isolation of autologous mature ECs might not be practically feasible in humans, we tested whether peripheral blood-derived OECs have a significant promise to control AAA expansion. Endovascular OEC seeding at the same time as surgery significantly retarded AAA
development 28 days after transplantation (Figure 8A, B). As for mature ECs, the stabilizing effect of OECs was accompanied with the reconstruction of a VSMC-rich neointima and the reestablishment of the endothelial lining, as assessed by the SMA- and CD31-positive signals in OEC-treated AAA, respectively (Figure 8C). Moreover, OEC transplantation preserved the integrity of the medial elastic network as compared with control rats (Figure 8D).

Discussion

There is no EC in the lumen of AAAs, usually covered by thrombus. Moreover, the potential of correcting endothelial loss by cell therapy to stabilize AAAs has never been explored. The rational of using such a therapeutic approach stems from previous reports showing that systemic infusion of autologous expanded ECs, i.e., mature ECs or OECs, promotes re-endothelization and vascular repair in large diseased arteries in contexts such as arteriosclerosis or restenosis. Due to the restricted localization of AAAs, we have chosen the endovascular route for EC delivery rather than systemic delivery in order to avoid proangiogenic systemic effects and because local endovascular treatments of AAAs are associated with a low morbidity in patients. We show that the local seeding of mature ECs or peripheral blood-derived OECs reestablishes the endothelial lining and limits AAA development/expansion, supporting the notion that re-endothelization allows for functional repair of the aortic wall. This functional healing is associated with the blockade of the destructive process occurring in AAA wall and the regeneration of elastic and VSMC units below the reestablished endothelium.

EC transplantation limits aortic wall destruction

Destruction of the aortic ECM components by proteases is an early step in human atherosclerotic AAAs and is reproduced in the xenograft aneurysm model. MMP-9 and -12, two potent
elastin-degrading enzymes are upregulated in AAA and required for matrix injury and aortic
dilatation\textsuperscript{22,23} and their inactivation limits AAA in mice\textsuperscript{24,25}. The extent of MMP release and
ECM injury is spatially linked to the development of neovessels in the adventitia/media\textsuperscript{26} and
increased adventitial neovascularisation is associated with AAA degeneration or rupture\textsuperscript{26,27}. Our
present data show that the endovascular seeding of ECs decreases adventitial neovascularisation
and macrophage infiltrates, as well as the activity of MMP-9 and -12 in the residual aortic wall.
In addition, EC therapy limits the generation of ROS in the AAA wall thereby contributing to
decreasing MMP-dependent proteolysis\textsuperscript{18}. As a marker of a decrease in proteolytic burden,
elastin network is preserved upon EC therapy. Hence, EC addition leads to the disappearance of
the mural thrombus in the lumen of AAAs in concordance with the increase in expression of
TFPI, a regulator of tissue factor activity. In EC-treated aortas, the disappearance of the
thrombus, a structure recently identified as a promoter of AAA expansion likely contributes to
decrease proteolysis and aortic diameter increase\textsuperscript{5,11}.

**EC transplantation promotes aortic wall reconstruction**

In adult, maturation and stability of abnormal vasculature imply both the normalization of the
endothelial lining and, the coverage of ECs by organized layers of mural cells and the production
of ECM components\textsuperscript{28}. In our study, we show that EC therapy involves both mechanisms. First,
the endovascular seeding of culture-expanded mature ECs restores a continuous endothelial
lining in the lumen of treated AAA, replacing the intraluminal thrombus. This neo-endothelium
appears functional, as assessed by the correction of excessive vascular permeability towards
Evan's Blue. Secondly, EC therapy is associated with the construction of a thick elastic and
muscular arterial tissue between the internal elastic laminae and the newly-formed endothelium.
These results strengthen the association between the re-establishment of a functional endothelial
monolayer, the reconstruction of a new VSMC- and ECM-rich wall and the long term stabilisation/normalization of aneurysmal diameter.

We show that EC therapy promotes a lumen-to-wall gradient of the neointimal maturation in AAAs with two areas: the luminal aspect of the neointima consisting of synthetic VSMCs embedded in disorganized collagen network and the intermediate and abluminal aspect characterized by orderly arrangement of apposed bands of mature contractile VSMCs and elastic lamellae in between. Accordingly, the consolidation of AAA wall after EC therapy, through VSMC phenotypic changes and ECM deposits is reminiscent to the outward remodelling observed in arteriogenesis, i.e., maturation of small arterioles into much larger conductance arteries\textsuperscript{20}. Another similarity with arteriogenesis is the early increase in MMP-2 activity observed in the neointima of RAEC-treated aortas contemporary of extracellular scaffolding reorganization and VSMC mobilization during wall reconstruction\textsuperscript{29}. In addition, in contrast to pathological intimal hyperplasia, the neointima generated after EC transplantation did not grow until lumen obliteration. Indeed, the restoration of an endothelium and accumulation of elastin scaffold may have both contributed to stabilizing VSMC growth in our study. In favour of this hypothesis, ECs and/or elastin has been shown to negatively regulate VSMC proliferation\textsuperscript{30,31}. Altogether, our results demonstrate that the stabilization of AAA after EC therapy relies on the restoration of the endothelial integrity and the reconstruction of new arterial tissue, reproducing most of the remodelling events leading to vascular maturation/arteriogenesis.

**Molecular and cellular mechanisms leading to aortic wall reconstruction after EC therapy**

The cellular mechanisms by which exogenous delivery of ECs enhances re-endothelialisation after vascular injury remains debated. Although delivered cells can engraft locally in the days following their injection, their long-term incorporation into the denuded endothelium is
controversial\textsuperscript{14,16}. In our study, we show that some transplanted ECs home locally and persist up to 7 days following injection. However, engrafted ECs were no longer detectable 28 days post-infusion in the AAA wall, demonstrating the lack of long-term residence of injected ECs in spite of potent regenerative potential. A plausible explanation is that injected cells release paracrine factors, including cytokines and growth factors into the surrounding tissue, that have the potential to modify the endogenous healing process\textsuperscript{32,33}. In favour of this hypothesis, we demonstrated that EC therapy at an early time point (when engrafted ECs were still detected) increased the aortic expression of endothelial-derived angiogenic/stabilizing factors, namely bFGF, eNOS, VE-cadherin and Robo4. It is assumed that these factors are involved in vessel maturation and stabilization via modulation of endothelial integrity, EC-mural cell interaction and/or VSMC recruitment\textsuperscript{34-36}. Given the role of bFGF in orchestrating early arteriogenic responses\textsuperscript{20}, we focused our attention on this cytokine. We show that cultured RAECs express high amounts of bFGF. \textit{In vivo}, bFGF production colocalizes with engrafted ECs, suggesting that transplanted ECs are the main cellular source of bFGF into the aortic wall. bFGF is a potent angiogenic factor that acts primarily on EC proliferation and survival\textsuperscript{37} and participates in endothelial regrowth after endothelial denudation\textsuperscript{38}. Furthermore, bFGF acts in synergism with other growth factors to promote vessel stability through the recruitment and growth of mural cells (pericytes/VSMCs) to the subendothelial space and is thus considered as an arteriogenic factor\textsuperscript{36,39}. In experimental AAAs, bFGF delivery into the aortic wall has been shown to limit aneurysmal formation by increasing medial VSMC density\textsuperscript{40}. These findings support our suggestion that one of the mechanisms by which transplanted ECs promotes AAA maturation/stability is the release of paracrine factors, such as bFGF that stimulate endothelial repair and VSMC growth. In favor of this hypothesis, we show that bFGF secreted by donor ECs
selectively promotes the migration and proliferation of human ECs and AAA wall-derived VSMCs.

The fact that injected ECs do not directly participate to AAA wall reconstruction questioned the origin of the endothelial and mural cells that constitute the regenerated neointima. Answering this question is facilitated by the fact that the aortic xenograft used in our study is decellularized and can thus be recolonized in situ only by cells from the recipient rat. Using BMT as well as double transplantation experiments, we demonstrated that all cells that form the new arterial wall have migrated from the flanking vasculature without any participation of BM-derived circulating progenitors. This result is in agreement with recent works showing that the majority of intimal cells after allograft transplantation or acute vascular injury in mice arise from vascular cells of the recipient adjacent arterial wall and not from circulating progenitor cells. In addition, compelling evidence suggests that during arteriogenesis, BM-derived cells neither incorporate into the wall of growing artery nor exhibit an endothelial or smooth muscle phenotype. Our study was not designed to discriminate between candidate source for ECs or VSMCs of the regenerated neointima after EC therapy. A plausible mechanism is that ECs that recolonize the AAA wall lumen after cell therapy derive from migrating and proliferating ECs from the adjacent healthy vasculature, as observed after mechanical arterial injury. Concerning intimal VSMC origin after EC therapy, several neighboring candidates can be proposed, including migrating medial VSMCs, vascular niche-derived progenitor cells or pericyte-like cells (pericytes/myofibroblastes) from the tunica adventitia.

Peripheral blood-derived OECs mimic the stabilizing effect of mature ECs on AAA development and remodelling

The use of mature ECs would involve invasive and complicated procedures, precluding clinical
transfer. An alternative option is the use of ex vivo expanded OECs that appear as a promising autologous cell source of ECs in vascular regenerative medicine. When cultured, a small subset of mononuclear cells isolated from peripheral blood expands in so-called late EPCs or OECs that possess high angiogenic and proliferative capacities, and a marked similarity with mature ECs. Along with this line, transfusion of OECs has been shown to promote the formation of functional vessels, to improve neovascularization in models of tissue ischemia and to promote reendothelialization of damaged vessels in vivo. In our study, we were able to obtain differentiated ECs (OECs) from rat peripheral blood mononuclear cells, with all cell markers and functional characteristics currently described. We show that transplantation of peripheral blood-derived OECs reproduces most of the stabilizing effects obtained with mature ECs in rat AAAs. These effects include reduction of AAA progression, recovery of the endothelial lining and reconstruction of new aortic wall. Our results represent the first demonstration that autologous OEC therapy reduces experimental AAA expansion. Since numerous studies have shown the safety and feasibility of EPC transplantation in humans, our study provide the first evidence that ex vivo expanded OECs may offer a valuable source of ECs in the future for AAA regeneration and repair.

Conclusion

We report that local transplantation of autologous ECs into rats limits AAA development and stabilizes formed AAAs. This stabilizing effect is paralleled by the reestablishment of a functional endothelial barrier at the interface with blood, demonstrating the importance of restoring the abluminal endothelium to control AAA dynamics. Moreover, we demonstrate that the addition of ECs is an effective therapy for AAA. Finally, our experiments highlight important
mechanisms involved in AAA repair after local EC-based therapy, including the paracrine release of endothelial stabilizing factors, especially bFGF by donor ECs that act on resident vessel wall cells to regenerate a new vascular wall. The results of this study are of particular relevance to future in vivo applications of EC-based therapy for regenerative medicine of large, diseased and dilated arteries.

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Conflict of Interest Disclosures: None.

References:


Figure Legends:

Figure 1. Experimental design of the endovascular EC therapy protocols. (A) Prevention study: endovascular infusion of ECs was performed immediately after the xenograft, i.e., before AAA formation (D0). (B) Stabilization study: endovascular infusion of ECs was performed 14 days after the xenograft, i.e., on already-formed AAA (>50% increase in the external aortic diameter, D14). Biological analysis was performed on dilated aortas harvested 7 days after the endovascular operation (D0+7 or D14+7), with the assumption that biologic changes at an intermediate delay provide mechanistic explanations for the impact of cell therapy on the remodeling observed 28 days after treatment (D0+28 or D14+28).
Figure 2. Endovascular mature EC seeding prevents AAA formation and stabilizes their expansion. (A, D) Endovascular infusion of RAECs or serum-free medium (control) in the grafted aorta was performed immediately (D0, prevention study) (A), or 14 days (D14, stabilization study) after the xenograft (D). (B, E) Macroscopic pictures of representative control or RAEC-seeded AAAs, 28 days after endovascular infusion in the prevention (D0+28) (B) or stabilization study (D14+28) (E). (C, F) External diameter increase at 7 (D0+7 or D14+7) or 28 (D0+28 or D14+28) days after endovascular infusion expressed as the percentage of the corresponding AAA diameter measured immediately after the endovascular infusion in the prevention (C) or stabilization study (F). Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05, **P<.01 vs control.

Figure 3. Endovascular mature EC therapy reestablishes the integrity of the endothelial monolayer and reconstructs a new vascular wall in the neointima. (A) VSMC or EC staining with anti-αSMA or anti-CD31 antibody, respectively, on cross sections of control (left) and RAEC-seeded (right) AAA at D0+28. CD31-positive ECs and elastin autofluorescence appear in yellow and green, respectively. Dashed line delimitates the internal elastic laminae. Arrowhead indicates the newly formed endothelium. (B) Aortic endothelial permeability to Evan’s blue dye (EBD) on cross sections of control (up) and RAEC-seeded (down) AAA at D0+28. Under Cy3 and FITC filters, EBD and elastin auto-fluorescence appears red and green, respectively. Co-staining of ECs with anti-CD31 antibody is shown for each cross section. (C, D) Representative staining (C) and computer-assisted quantification (D) of elastic (Orcein staining, top) or collagen (Sirius red staining, bottom) fiber network in the residual medial wall and neointima from cross sections of control (white column) and RAEC-seeded (black column) AAA at D0+28. Dashed line
delimitates the internal elastic laminae. Arrowhead shows disrupted elastic lamellae. Black arrow indicates newly synthesized elastin fibers. Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05 vs control. Scales bars =200μm.

**Figure 4.** Ultrastructural composition of the reconstructed wall after endovascular mature EC therapy. Left column (A, D): histology: thrombus (Thr) and disrupted internal elastic laminae in control rats (A) versus thick neointima (Ni) in RAEC-seeded animals (D); boxes indicate zones sampled for transmission electronic microscopy. Middle column (B, E, G): low-magnification overview of indicated zones: (B) partially or completely degranulated platelets (Pl) and red blood cells (Er) in controls; (E) abluminal Ni in RAEC-seeded animals showing somewhat disorderly deposition of extracellular collagen (Col) and a synthetic VSMCs; (G) orderly alternating and closely apposed bands of mature contractile VSMCs (section sign symbol) and elastic lamellae (El) deeper in the Ni; these two images suggest a periphery to lumen gradient of the Ni structural maturation. Right column (C, F, H): some details at higher magnification of corresponding areas: (C) two types of granules progressively disappearing from the platelets’ cytoplasm; (F) collagen bundles (Col) cut in different planes; (H) contractile filament bundles and focal densities (arrows) in the cytoplasm of a VSMC, collagen (Col) and elastin (El) fibers are also highlighted. Asterisk and section sign symbols indicate synthetic and contractile VSMCs, respectively.

**Figure 5.** Endovascular mature EC therapy decreases inflammation, adventitial angiogenesis, oxidative stress and proteolysis in the residual AAA wall. (A) EC and macrophage staining with isolectin B4 and anti-CD68 antibody, respectively, on cross sections of control (left) and RAEC-
seeded (right) AAA at D0+28. Dashed line delimitates the internal elastic laminae. Scale bars =200 μm. (B) Computer-assisted quantification of isolectin B4-positive microvessels and CD68-positive macrophages in the residual wall of control and RAEC-seeded AAA at D0+28. Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05 vs control. (C) In situ DHE staining (red signal) of control and RAEC-seeded AAA at D0+28. Dashed line delimitates the thrombus/neointimal layer. (D) Fold induction of MMP-2, -9, -12, -14 and TIMP-1, -3 mRNA levels (QRT-PCR) in RAEC-seeded relative to that in control aortas (n=5). *P<.05 vs control. (E) In situ zymography using DQ-gelatin (green fluorescence) or gelatin zymography to evaluate MMP-2 and -9 activity on cross-sections or protein extracts, respectively, obtained from RAEC-seeded and control AAA at D0+7 or D0+28. (F) Casein zymography and densitometric analysis of MMP-12 activity (left) and representative immunostaining to detect total MMP-12 (right) in RAEC-seeded relative to that in control AAA at D0+7. Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05 vs control.

Figure 6. Mature EC therapy indirectly participates to the reconstitution of a new aortic wall through the recruitment of local vascular cells. (A) Tracking of seeded RAECs in AAA wall using GFP+/+ RAECs. Representative double immunostaining to detect GFP (brown signal) and CD31 (blue signal) in GFP+/+ RAEC-seeded aortas at D0+7 or D0+28. The boxed areas represent higher magnification images of the deeper (dashed line box) or inner (straight line box) part of the RAEC-reconstituted neointima. Scale bars =250μm. (B) Implication of BM cells in RAEC-induced aortic wall reconstruction, using GFP+/+ BMT experiments. GFP+/+ BM cells were transplanted into irradiated WT rats and 3 weeks later, AAA were generated in GFP+/+ BMT rats.
and WT RAECs were endovascularly infused. Representative GFP immunostaining (brown signal) of WT RAEC-seeded aorta in GFP\(^{+/+}\) BMT rats at D0+28 is shown. The boxed areas represent higher magnification images of the residual wall (straight line box) or neointimal layer (dashed line box) from RAEC-seeded aortas. Scale bars =250μm. (C) Implication of local vascular cells in RAEC-induced aortic wall reconstruction, using GFP\(^{+/+}\) arterial transplants. GFP\(^{+/+}\) aortic donor isografts were implanted between the xenograft and the recipient abdominal aorta of WT rats, on both sides. Then, WT RAECs were endovascularly infused into the xenograft at D0 and rats were sacrificed at D0+7. Schematic representation of the transplant experiments is shown (left). Representative GFP staining and higher magnification images of the neointimal layer (straight line box) of WT RAEC-seeded AAA are shown (right). Ni; Neointima.

**Figure 7.** Mature EC therapy increases endothelial-derived stabilizing factor expression in the AAA wall. (A) Fold induction of vascular stabilization factor mRNA levels (QRT-PCR) in RAEC-seeded relative to that in control AAAs at D0+7 (n=5). *P<.05 vs control. (B) Representative immunostaining of bFGF on cross-sections of RAEC-seeded and control AAAs at D0+7 or D0+28. Dashed line delimitates the internal elastic laminae. Scale bars =100μm. (C) Triple immunostaining of bFGF (yellow), GFP (red) and nuclei (DAPI, blue) and elastin autofluorescence (green) on cross-sections of GFP\(^{+/+}\) RAEC-seeded neointima at D0+7. Scale bars =25μm. (D) Western blot analysis of bFGF and β-actin protein levels performed on RAEC cell lysates.

**Figure 8.** Peripheral blood-derived OEC therapy reduces AAA formation and reestablishes the abluminal endothelium in rats. Three million culture-expanded OECs or serum-free medium
(control) were endovascularly infused immediately after the xenograft implantation in rats. (A) Macroscopic pictures of representative control and OEC-treated AAAs, 28 days after endovascular infusion (D0+28). (B) External aortic diameter increase measured 7 (D0+7) or 28 (D0+28) days after endovascular infusion expressed as the percentage of the corresponding aortic diameter measured at D0. Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05 vs control. (C) Representative immunostaining of VSMCs and ECs using anti-αSMA or CD31 antibody, respectively, on cross sections of control and OEC-seeded AAAs, 28 days after endovascular infusion (D0+28). Dashed line delimitates the internal elastic laminae. (D) Representative staining and computer-assisted quantification of elastic (orcein staining) fiber network in the residual medial wall from cross sections of control and OEC-seeded AAAs, 28 days after endovascular infusion (D0+28). Scales bars =100 μm. Open circles represent individual values and the red line represents the mean value of the corresponding group. *P<.05 vs control.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 7
Figure 8
Reestablishment of the Endothelial Lining by Endothelial Cell Therapy Stabilizes Experimental Abdominal Aortic Aneurysms
Grégory Franck, Jianping Dai, Alexandre Fifre, Saravuth Ngo, Claire Justine, Stéphanie Michineau, Eric Allaire and Marianne Gervais

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SUPPLEMENTAL MATERIAL

Supplemental methods

Rat endothelial cell isolation and culture

Rat aortic endothelial cells (RAECs) were isolated from thoracic descending aortas of 8 week-old Fischer 344 rats by the primary explant technique, as previously described\(^1\). Briefly, the full length aorta was removed from the rat, cut into small pieces and placed with the endothelial layer side down on Matrigel-coated 35 mm dishes (BD Pharmingen, France) in complete ECGM-2 culture medium supplemented with 2% FCS, 2 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml IGF, 0.5 ng/ml VEGF165, 1 µg/ml ascorbic acid, 22.5 µg/ml heparin, 0.2 µg/ml hydrocortisone (PromoCell, Germany), and, 100 µg/ml primocin (InvivoGen, France). After 48h of incubation at 37°C in a 5%CO\(_2\) atmosphere, the explants were removed and the attached RAEC were grown to confluence. RAEC were recovered from the Matrigel by digestion using 100 U dispase (BD Pharmingen) and cultured on gelatin coated plastic in complete ECGM-2 medium without primocin.

Outgrowth ECs (OECs) were isolated from rat peripheral blood, as described previously with minor modifications\(^3\). In brief, mononuclear cells were isolated from 15 mL of rat venous blood by Ficoll separation and the mononuclear fraction without further purification step were resuspended in complete ECGM-MV2 culture medium containing 5% FCS, 5 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml IGF, 0.5 ng/ml VEGF165, 1 µg/ml ascorbic acid, 0.2 µg/ml hydrocortisone (PromoCell), and, 100 µg/ml primocin (InvivoGen) and seeded on rat tail collagen type I (BD Pharmingen)-coated well plates. A total of 20.10\(^6\) cells/well were seeded on a 12 culture-well plate and incubated in a 5% CO\(_2\) atmosphere at 37°C. Under daily observation, first media changes were performed 4 days after plating. Thereafter, the media was changed every 3 days.
Colonies of OECs with endothelial morphology appearing between 2-3 weeks (Supplemental Figure 2A) were trypsinized and expanded until several passages in a splitting ratio of 1:3 in complete ECGM-MV2 medium without primocin.

Expanded ECs (RAECs or OECs) from passages three to five were used for in vitro and in vivo studies. Conditioned medium was generated from RAEC cultures in Opti-MEM (Invitrogen) when cultures were approximately 70% confluent, then removed after 24h and all cellular debris were removed by centrifugation.

**Rat endothelial cell characterization**

The purity and the phenotype of all primary cultures were assessed by flow cytometry and immunofluorescence techniques using the following antibodies: PE-conjugated anti-KDR, PE-conjugated anti-CD31, FITC-conjugated anti-CD14, FITC-conjugated anti-CD45 and FITC-conjugated anti-CD34, purified anti-eNOS (all from BD Pharmingen), APC-conjugated anti-CD133 (Miltenyi Biotec, France), purified anti-vWF (Dako), anti-VE-cadherin (Santacruz) or anti-αSMA (clone 1A4, Sigma, France). Functional endothelial characterization of the primary culture was assessed by monitoring their ability to take up FITC-conjugated acetylated LDL (AcLDL, Molecular Probes, France) and DyLight® 594-conjugated Lycopersicon Esculentum (Tomato) Lectin (Vector) and to form tubular structures on Matrigel (BD Pharmingen).

**Human endothelial and vascular smooth muscle cell culture**

Primary human ECs, *i.e.* HUVECs were obtained from Laurent Muller and Catherine Monnot, Collège de France, Paris, France. HUVECs were cultured on rat tail collagen type I-coated plates in complete ECGM-2 culture medium (Promocell). Human vascular smooth muscle cells (VSMCs) were isolated from small pieces of human AAA wall by explants of the medial layer⁵. Fragments
from the maximum dilation zone of human atherosclerotic AAAs (asymptomatic; diameter >50 mm) were collected during elective surgery in the Department of Vascular Surgery of the Henri Mondor hospital in agreement with the local ethics committee. VSMCs were cultured on gelatin-coated plates in complete SMC medium 2 (Promocell, Heidelberg, Germany) containing 10% FCS, insulin (2.5 µg/ml), bFGF (1 ng/ml), and EGF (0.25 ng/ml). Cells were used at passage 4.

**Migration assays**

Cell migration was investigated using a 'scratch wound' method. Cells were grown until confluence in 24-well culture plates and starved overnight in Opti-MEM. The confluent monolayers were then scored with a sterile 200 µl-pipette tip to leave a scratch. Culture medium was then immediately removed (along with any dislodged cells) and replaced with fresh Opti-MEM medium, or with 24H-conditioned medium of RAEC culture supplemented or not with an anti-bFGF blocking antibody (5 µg/ml). Recombinant rat bFGF (10 ng/ml) diluted in Opti-MEM was used as a positive control. All scratch assays were performed in quintuplicate. Cells were incubated at 37°C for 6h. The progress of migration was monitored by collecting digitized images immediately and again 6h after wounding at the same location with an inverted microscope (Zeiss) and a digital camera (QICAM, Q imaging). The surface of the scratch was then analyzed using Image-J software. The extent of wound closure was defined as the percentage by which the original wound area at 0h has decreased at 6h.

**BrdU proliferation assays**

Cells (4000 cells/well) were seeded in 96-well plates. A day after, cells were starved overnight with Opti-MEM medium and cultured for 24h with fresh Opti-MEM medium, or with 24H-conditioned medium of RAEC culture, supplemented or not with an anti-bFGF blocking antibody
(5 µg/ml). Cells stimulated with recombinant rat bFGF (10-50 ng/ml) diluted in Opti-MEM were used as a positive control. BrdU labeling solution (10 µL) was added to each 100 µL of culture medium for the last 12 hr of culture and then BrdU incorporation ELISA (Roche Applied Science) was carried out according to the manufacturer’s instructions. Results are presented as percentage of BrdU incorporation into the DNA in Opti-MEM-cultured cells (set as 100%).

**Xenograft model of expanding AAA in rat**

AAAs were generated in 8 week-old male Fischer 344 rats (Charles River Laboratories, France) by implanting an aortic xenograft from guinea pig\(^7\). Briefly, guinea pig infrarenal aortas were decellularized using 0.1% sodium dodecyl sulfate to obtain intact tubes of aortic ECM which were grafted orthotopically into rats with 10-0 sutures. Fourteen days later, a chimeric AAA (>50% diameter increase) had developed from the degraded guinea pig ECM, colonized with cells and thrombus of rat origin\(^7\).

**Endovascular cell therapy of AAA in rats**

Five million RAEC (n=30) were resuspended in serum-free ECGM-2 medium and seeded into the aortic lumen either immediately after the xenograft implantation (D0, prevention study) or 14 days after, *i.e.* on formed AAA (D14, stabilization study) in rats, as previously described\(^8\). 200 µl serum-free ECGM-2 medium were infused as controls (n=29). Aortic diameter was measured *in situ* under a beating heart immediately after cell therapy and before euthanasia, at day 7 (prevention: D0+7; stabilization: D14+7) or day 28 (prevention: D0+28; stabilization D14+28). All rats were euthanized by an overdose of intravenous pentobarbital. The AAA lumen was gently rinsed with saline, and harvested. Half of AAA was fixed in 4% PFA and embedded in paraffin and the second half was snap-frozen in liquid nitrogen and kept at -80°C. In some experiments,
GFP+/+ RAEC isolated from syngenic homozygous GFP overexpressing male Fischer rats were used and AAA were embedded in OCT and kept at -80°C. Three million OECs (n=16) were resuspended in serum-free ECGM-MV2 medium and seeded into the aortic lumen immediately after the xenograft implantation (D0). 200 µl serum-free ECGM-MV2 medium were infused as controls (n=18). AAA samples were harvested at day 7 (D0+7) or day 28 (D0+28), as described for RAEC therapy.

**GFP+/+ Bone marrow reconstitution**

BMT was performed as described previously. GFP+/+ bone marrow cells were harvested from femurs of 8 week-old male GFP+/+ Fischer rats. Six week-old male WT Fischer rats were lethally X-irradiated with a total dose of 9.5 Gy (60Co Cobalt source, BioMep SARL). One day later, the recipient rats (n=6) received unfractionated GFP+/+ bone marrow cells (3. 10^7) in 0.9 mL saline by tail vein injection. Three weeks after BMT, rats were anesthetized, 50 µl of peripheral blood was collected by tail vein puncture on heparin tubes (BD) and analyzed by flow cytometry before AAA generation and WT RAEC seeding.

**Flanking GFP+/+ arterial transplantation**

The transplantation procedure consisted of interpositioning a GFP+/+ aortic isograft between the xenograft and the rat recipient abdominal aorta on both sides. The anesthetized GFP+/+ male Fischer rat was killed by exsanguination, and its infrarenal abdominal aorta was flushed, cut into four pieces and kept in saline at room temperature. The recipient WT Fischer male rat was anesthetized, and its abdominal aorta was mobilized from the left renal artery toward the iliac bifurcation and excised after clamping. The guinea pig xenograft flanked by two GFP+/+ donor isografts was implanted orthotopically in the recipient rat by 2 end-to-end anastomosis each consisting of 10 symmetrically placed 10-0 sutures (Ethicon, Johnson & Johnson). Immediately
after the arterial graft, WT RAEC were seeded into the aortic lumen (n=3, excluding one animal that died perioperatively).

**Endothelial permeability**

Endothelial permeability was investigated by monitoring extravasation of Evan's Blue dye (EBD). The red auto-fluorescence of EBD-albumin conjugate is evident in tissue sections examined by fluorescence microscopy\(^{10}\). Three rats from each group (control, RAEC) were injected 28 (D0+28) days after cell therapy, under anaesthesia through the jugular vein, with 0.15 mL of 3% EBD (Sigma) in 0.9% saline. After 30 min, rats were euthanized by transcardial perfusion using 100 ml cold saline and the AAA were harvested, embedded in OCT and kept at -80°C for histology.

**Histological, immuno-histochemical and -fluorescence analysis**

Five µm thick paraffin-embedded cross sections were stained with orcein and Sirius red mixture for visualization of elastic and collagen fibers, respectively. Immunostaining on paraffin-embedded cross sections was performed using the following antibodies: mouse monoclonal anti-αSMA (clone 1A4, Sigma, Lyon, France), mouse anti-rat CD68 (clone ED1, Serotec, Düsseldorf, Germany) and rabbit anti-bFGF (Abcam). After incubation with a biotin-conjugated anti-species antibody (Vector Laboratories), immunostaining was amplified using peroxidase-conjugated streptavidin complexes (Vector Laboratories) and peroxidase was detected using VIP or DAB (Vector Laboratories) substrate. Sections were counterstained with hematoxylin, mounted in Eukitt and examined with a bright field microscope (Zeiss, France). For double immunostaining study, 10 µm thick frozen cross sections were incubated with the mouse anti-rat CD31 antibody (Becton Dickinson) followed by the incubation with a biotin-conjugated anti-mouse antibody (Vector Laboratories), peroxidase-conjugated streptavidin complexes and Histogreen substrate (Vector Laboratories). Then, GFP signal was revealed with the rabbit anti-GFP antibody (Abcam),
followed by incubation with biotin-conjugated anti-rabbit antibody, peroxidase-conjugated streptavidin complexes and VIP substrate.

For the immunofluorescence studies, 10 µm thick frozen cross sections were incubated with mouse anti-GFP (Abcam), rabbit anti-bFGF (Abcam) or mouse anti-CD31 antibody (clone TLD-3A12, Becton Dickinson). These primary antibodies were revealed by incubation with an Alexa 555 or Alexa 647-conjugated anti-mouse or anti-rabbit antibody. Nuclei were stained with DAPI and sections were mounted in Mowiol. Fluorescence was examined with a fluorescence microscope (Axiolmage D1, Zeiss) in sequential scanning mode for triple detection of Alexa 555, Alexa 647, DAPI and elastin green-autofluorescence. To test endothelial permeability, extravasation of EBD-albumin was observed on 10 µm thick frozen cross sections by direct fluorescence microscopy (excitation wavelength 540 nm; emission wavelength 670 nm).

**Computer-assisted morphometric analysis**

*In situ* macroscopic and microscopic images were digitally captured using the Axiovision 4.8 Software (Zeiss). Customized programs were used to quantify the remodeling of the vessels, the elastic fiber content, the inflammatory infiltrate, VSMC density, and neovessel density, the observer being blinded to treatment allocation.

**Elastic and collagen fiber content**

The orcein- or Sirius red-stained surface was quantified using AxioVision and the corresponding elastin and collagen densities were expressed as a percentage of corresponding aortic surface.

**Aortic inflammatory infiltrate, medial VSMC density and adventitial neovascularization**

CD68-positive macrophages, αSMA-positive VSMCs and adventitial/medial isolectin B4-positive microvessels in aortic sections were quantified after immunostaining. The total aortic surface was determined in parallel and results were expressed as the number of cells or neovessels per
Oxidative stress analysis and MMP activities

Dihydroethidine hydrochloride (5 mM, Molecular Probes) was applied to freshly cut frozen aortic sections (10 µm thick) for 30 min at 37°C to reveal the presence of reactive oxygen species (ROS) as red fluorescence (585 nm, AxioImager A1, Zeiss)\(^{11}\).

MMP-2, -9 and MMP-12 activities were evaluated by gelatin or casein zymography, respectively\(^{12}\). Briefly, after AAA pulverization in liquid nitrogen, aortic lysates were prepared with ice-cold 0.2% Triton X-100 in 0.05 M Tris-HCl, pH 7.6 containing 0.01 M CaCl\(_2\). Protein content was determined by BCA quantification. 15µg per lane of extracted proteins were subjected to a 10% SDS-PAGE containing 0.1% gelatin or 0.1% casein. After electrophoresis, gels were washed in 2.5% Triton X-100 for 30 minutes, incubated for 24 hours at room temperature in 50 mM Tris, pH 8.2 containing 10 mM CaCl\(_2\), and finally stained with 0.008% Coomassie brilliant blue (Sigma Chemical Co). Quantitative analysis of MMP activity was performed using QuantityOne software (Bio-Rad, Hercules, CA).

For in situ zymography, we incubated freshly cut frozen AAA sections (10 µm thick) with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) according to the manufacturer’s protocol. We detected MMP-2 and -9 proteolytic activities as green fluorescence\(^{11}\).

Immunoblotting

RAEC lysates were prepared with ice-cold 1% Nonidet P-40 in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.1 mM Na\(_3\)VO\(_4\), and 0.5 mM phenylmethylsulfonyl fluoride. Lysates were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed overnight with anti-bFGF and anti-actin antibody at
4°C. After incubation with peroxidase-linked secondary antibodies, immunoreactive proteins were visualized by ECL reagent substrates (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Transmission Electron Microscopy**

AAA samples (RAEC-treated and control rats at D0+28, n=4) were excised and immediately placed in fixative solution (4% PFA, 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 1 hour. Samples were postfixed in 1% osmium tetraoxide, dehydrated, and embedded in Lowecryl resin. Sections of 70 to 80 nm were cut on a Reichert UltracutE, stained with 2% uranyl acetate plus Reynold’s lead citrate and viewed using a Phillips CM120 transmission electron microscope.

**Total RNA extraction, cDNA synthesis and Quantitative Real-time Polymerase Chain Reaction**

AAA walls were ground to a fine powder in liquid nitrogen and total RNA from each AAA sample was extracted in Trizol reagent using PureLink RNA Micro Kit according to manufacturer’s instructions (Invitrogen, Cergy Pontoise, France). The purity and concentration of total extracted RNA were evaluated using a spectrophotometer (NanoDrop Technologies, Montchanin, DE). The RNA was reverse transcribed using the high-capacity VILO SuperScript reverse transcriptase (Invitrogen, France). The resulting cDNA mixture was stored at -80°C until further use. The mRNA levels of 16 candidate genes (Supplemental Table) were determined by quantitative real-time–polymerase chain reaction (QRT-PCR) using Custom TaqMan Gene Expression Assays (Applied Biosystems, France). QRT-PCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems, France). Amounts of mRNA of interest were normalized to that of GAPDH (ΔCt = CT gene of interest – CT GAPDH). Fold induction of gene expression in RAEC-treated to that of control AAA samples is shown and represented as $2^{-\Delta\Delta Ct}$. 
Statistics

All data are expressed as means±SEM. Differences between groups were analyzed using the non-parametric Mann–Whitney \( U \) test. A value of \( P<0.05 \) was considered as statistically significant.
**Supplemental Results**

**Characterization of rat aortic ECs and peripheral blood-derived OECs**

Flow cytometry characterization of rat aortic endothelial cells (RAECs) showed positivity for the endothelial markers (KDR and CD31) and no expression of markers of monocytes (CD14) and hematopoietic cells (CD45) (Supplemental Figure 1A). By immunofluorescence, RAECs, similarly to HUVECs, expressed specific markers of mature ECs, such as VE-cadherin and von Willebrand factor (vWF) but were negative for the mesenchymal marker, αSMA (Supplemental Figure 1B). Expanded RAECs possessed functional characteristics of ECs, i.e., AcLDL internalization and lectin binding (Supplemental Figure 1C), and vascular structure formation in Matrigel® (Supplemental Figure 1D).

OECs appeared after 2-3 weeks as isolated colonies in cultures of mononuclear cells from rat peripheral blood and showed an EC-cobblestone morphology (Supplemental Figure 2A), as previously described. Large colonies were selected and expanded over several passages. Rat OECs at passage 3 presented all the features of ECs including cobblestone-like morphology, flow cytometric or immunofluorescence EC marker expression (KDR, CD31, eNOS, vWF), and capacity of AcLDL internalization and tube formation on Matrigel® (Supplemental Figure 2B-E). These cells were negative for CD14, CD34 and CD133 (Supplemental Figure 2C). These results confirmed the late EPC and differentiated EC phenotype of rat OECs.
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Supplemental Figure 1
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**Supplemental Figure Legends**

**Supplemental Figure 1. Characterization of rat aortic endothelial cells (RAECs).** (A) FACS analysis for KDR-PE, CD31-PE, CD14-FITC and CD14-FITC markers (dark line) and their corresponding isotype (red line) on RAEC cultures at P3. (B) VE-cadherin, vWF and αSMA immunostaining performed on RAEC and HUVEC primary cultures at P3. (C, D) Ability of RAECs to uptake DyLight 594-conjugated Tomato Lectin (red) and FITC-conjugated-AcLDL (green) (C), and to form tubular structures on Matrigel® (D). Nuclei are stained with DAPI (blue). Scale bars =100µm.

**Supplemental Figure 2: Characterization of outgrowth endothelial cells (OECs) derived from rat peripheral blood.** (A) OECs appear after 2-3 weeks as colonies in cultures of rat circulating mononuclear cells (left panel) and possess a cobblestone-like morphology (right panel). Dashed line represents the migration front of the cluster. (B) OECs maintain a cobblestone-like morphology throughout their amplification, e.g., at P3. (C) Flow cytometry analysis for KDR-PE, CD31-PE, CD34-FITC, CD133-APC and CD14-FITC markers (dark line) and their corresponding isotype (red line) on OEC cultures at P3. (D) eNOS and vWF immunostaining performed on OEC primary cultures at P3. (E) Ability of OECs to fix FITC-conjugated-AcLDL (green), and to form tubular structures on Matrigel®. Nuclei are stained with DAPI (blue).

**Supplemental Figure 3: bFGF secreted by RAECs selectively promotes the proliferation and migration of human ECs (HUVECs) and AAA wall-derived VSMCs in vitro.** Proliferation (A) and migration (B) assays were performed on HUVECs or human aortic VSMCs. Cells were stimulated with RAEC-conditioned media (RAEC-CM), supplemented or not with an anti-bFGF blocking antibody (5 µg/ml). Recombinant rat bFGF (10 and 50 ng/ml for HUVECs and VSMCs, respectively) was used as a positive control. Results are expressed as percentage of BrdU uptake.
at 24h or wound closure at 6h, n=5. Representative data of 3 independent experiments are shown. *P<.05; **P<.001 vs corresponding value.
Supplemental References


