Endothelial Seeding for Abdominal Aortic Aneurysms: Lessons Learned from the Past and Present

Running title: Mayr et al.; Endothelial seeding of aortic xenografts

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In this edition of Circulation, Gervais and colleagues\(^1\) report a novel approach to modulate the growth of aortic aneurysms in an animal model by using endothelial cells (EC) or outgrowth EC grown in culture which were then delivered directly into an aortic xenograft prior to implantation. The authors report that this therapeutic approach resulted in modulation of the inflammatory process in the aortic wall and decreased the rate of growth of the xenograft aneurysms. They propose that endothelial seeding may be a promising technique for managing the growth of aneurysms in humans. This paper brings back memories of the herculean efforts that were initiated by a number of laboratories to seed EC on vascular grafts with the hope that this technique would improve the patency of the grafts, decrease perianastomotic neointimal hyperplasia and loss of ischemic limbs\(^2,\,3\). In vitro assessment of the growth potential of adult human sources of endothelium suggested that there was adequate in vitro growth of cells to theoretically cover the surface of commonly used grafts\(^4\). In vitro studies also demonstrated that human EC proliferation and morphology was modulated by both extracellular matrix and growth factors\(^5\). Human trials of EC seeding gave variable results, none convincing\(^6,\,7\) enough to try to bring the technique to wide spread use. Because of concerns that the surface of prosthetic grafts would never provide a hospitable environment for endothelial cell growth and development, the focus of these efforts changed to first lining the grafts matrix proteins such as fibronectin\(^8\) or with growth factors that would potentiate endothelial cell growth\(^9\) along the luminal surface of grafts. Eventually as research into endothelial and smooth muscle cell biology progressed, this then led to attempts at ex vivo construction of complete living cellular vessels\(^10\).

With the previous efforts of endothelial seeding of grafts in mind, it is useful to consider the similarities and differences of the efforts of Gervais et al to seed EC onto aortic xenografts. First we should discuss the similarities.
During efforts to seed prosthetic grafts and aortic xenografts, great care was taken to characterize the endothelial identity of the cells used for seeding. Specific immunohistochemical markers of EC identity were used in early studies of EC seeding, and in these studies of aortic xenograft seeding, flow cytometry and mRNA analysis were also used to confirm the cellular identity of cells being seeded. Although five million rat ECs were injected, only few were detected in the intima 7 days after cell therapy. Quantitation of the initial and subsequent loss of ECs during the first few days after seeding would have been essential. In studies of cell therapy, it is customary to perform titration experiments to characterize the temporal aspects of engraftment. From the description of the experiments, it is possible that the number of cells engrafted at day one and day seven were similar. If that was so, the interpretation of these results might be different.

In *ex vivo* experiments to seed prosthetic vascular grafts, Fibroblast Growth Factor-1 was previously found to promote EC adherence to grafts. In the aortic xenograft seeding experiments in this manuscript, the authors attribute the benefits by cell therapy to paracrine effects and were able to confirm that basic Fibroblast Growth Factor (bFGF) produced by rat ECs could potentiate growth of human EC *in vitro*; however in seeded grafts, mRNA for bFGF was only present transiently in the wall up to seven days after seeding. While it is well known that EC can deposit bFGF into the extracellular matrix (ECM) where it can influence metastasis and angiogenesis, the cellular origin of the local bFGF production in seeded xenografts needs to be confirmed. The early bFGF expression did co-localize with GFP signal in the neointima of GFP+/+ rat EC-treated aortas, but given the poor engraftment, is it likely that a few seeded ECs account for a greater than 3-fold increase in vascular bFGF expression?

The relationship these authors draw between the ability of soluble rat derived bFGF in
culture medium to promote human EC growth in vivo, and the ability of bFGF to modulate rat 
EC growth on the lumen of experimental xenografts is unfounded based on the data presented in 
the paper. There were no experiments done in vivo to block the effect of bFGF or to detect bFGF 
protein in the matrix of the seeded aortic xenografts. These results would be more compelling if 
the authors show that bFGF protein is deposited in the matrix of the seeded xenografts. Then, it 
may be plausible that transient seeding of the aortic xenografts with EC could modulate the 
matrix characteristics of the xenografts to facilitate and sustain ingrowth of native endothelium.

To prove their point about the utility of cell seeding, these authors also used late 
outgrowth ECs to seed aortic xenografts. Gervais et al harvested peripheral blood monocytes, 
cultured them, and then selected the colonies that had an “endothelial morphology” for 
subsequent passage and seeding experiments. The endothelial lining of blood vessels shows 
remarkable heterogeneity in structure and function.12 Surprisingly, rat EC isolated from the 
thoracic descending aorta or obtained as late outgrowth ECs from monocytes were equally 
effective for aneurysm repair. It is well known that macrophages can develop an endothelial 
phenotype and secrete growth factors such as bFGF and Vascular Endothelial Growth Factor13, 
14, which may explain their near equivalent capability to modulate the growth of the 
experimental aneurysms as compared to rat EC. A more important question is why the late 
outgrowth ECs did not outperform their mature EC counterparts? One of the promises of stem 
cell therapy is that stem cells offer a superior source of cells for tissue engineering due to their 
regenerative potential. However, just like ECs, there was no evidence for long-term residence of 
the seeded late outgrowth ECs. This is reminiscent of previous studies with early outgrowth 
endothelial progenitors (EPCs). The criteria for what defines an EPC in terms of surface markers 
remain highly controversial.15,16 Unlike late outgrowth ECs, EPCs do not give rise to
endothelial cells. Instead, the EPC definition has also been obscured by the uptake of platelet microparticles by monocytes/macrophages\textsuperscript{17,18} and the mechanisms for the \textit{in vivo} improvements observed in preclinical models after EPC administration remain elusive, with paracrine effects, rather than differentiation, being the most likely explanation. Similarly, the authors in this study speculate that injected cells secrete paracrine factors that have the potential to modify the endogenous healing process. This conclusion is not specific enough from a mechanistic standpoint.

A common practice in cell therapy experiments is to compare the cell treatment against a saline injection or a plain medium control. Cell culture media are supplemented with growth factors including bFGF and in the case of ECs additional supplements such as heparin. Although cells were cultured in serum-free media before injection to ensure that they contain no extraneous proteins, cells can selectively retain proteins from the bovine serum supplement\textsuperscript{19}. Endothelial cells, in particular, show a substantial carry-over of bovine proteins in the conditioned medium\textsuperscript{20}. Their glycocalyx, which is located on the apical surface of ECs, will bind growth factors such as FGF. Thus, the seeded cells could just act as a delivery vehicle for growth factor supplements from the \textit{in vitro} culture. To further demonstrate that the tissue repair is indeed dependent on the secretory activity of the seeded cells and not due to a cross-contamination of the cell preparations with growth factors from the serum supplement, conditioned medium from the EC culture as well as non-EC cells need to be used as controls\textsuperscript{19}. While the authors demonstrate that rat ECs contain bFGF proteins \textit{in vitro}, the protein data were not underpinned by mRNA expression levels comparing bFGF expression in rat EC’s and late outgrowth EC’s. There are experiments published which show that healing of experimental aneurysms in rats may be modulated by systemic\textsuperscript{21} and local administration of bFGF\textsuperscript{22,23}. Based
on the literature and the findings in this paper, it would be of great interest to know whether coating bFGF onto aortic xenografts provided the same protection against aortic dilation as did the process of cell seeding.

An important difference in the concept of endothelial seeding of prosthetic grafts in animals vs. humans that is relevant to these rodent experiments is the fact that given enough time, most dog, sheep and baboon models of arterial graft healing would develop an endothelial lining with all of the appropriate anti-inflammatory and thromboresistant properties as the native vessels. However, in human vascular grafts, a complete healing process has never been documented\textsuperscript{24, 25}. Substantial ongoing adherence of platelets to human vascular grafts has been detected years after graft implantation\textsuperscript{26}, suggesting that these grafts never develop an antithrombotic layer even though they develop a well-defined pseudointima\textsuperscript{27}. Detailed study of the differences between human and animal endothelial cell growth in vitro revealed the need to grow human endothelium on a collagen substrate that allowed for adherence of growth factors, heparin and matrix proteins such as fibronectin\textsuperscript{5, 28}. Canine, bovine and ovine endothelial cells have no absolute requirement for specific matrix proteins or growth factors to achieve serial growth in vitro.

The authors are to be commended for seeking a potential solution to halt the growth of aneurysms in humans. This is a significant clinical problem, and the potential complications associated with interventions for aneurysms are not trivial despite major advances with minimally invasive endovascular techniques. However, for the experiments by Gervais \textit{et al} to have significant clinical relevance, it would be important to show that the extracellular matrix lining their aneurysmal xenograft replicates the environment found in human abdominal aortic aneurysms. Proteomic methodology has previously been used for the first detailed analysis of
the ECM in human AAA and identified markers of pathological ECM remodeling related to Matrix Metalloproteinase-12 activity. This methodology could be used to assess the proteome of the aneurysmal xenograft for comparison with the proteome of human aneurysms. If the matrix of the rat xenograft aneurysm is capable of binding growth factors that the human aneurysm matrix cannot, these studies will remain relevant to rats, rather than humans.

**Conflict of Interest Disclosures:** None.

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