Reperfusion Injury Intensiﬁes the Adaptive Human T Cell Alloresponse in a Human-Mouse Chimeric Artery Model


Objective—Perioperative nonimmune injuries to an allograft can decrease graft survival. We have developed a model for studying this process using human materials.

Methods and Results—Human artery segments were transplanted as infrarenal aortic interposition grafts into an immunodeﬁcient mouse host, allowed to “heal in” for 30 days, and then retransplanted into a second mouse host. To induce a reperfusion injury, the healed-in artery segments were incubated for 3 hours under hypoxic conditions ex vivo before retransplantation. To induce immunologic rejection, the animals receiving the retransplanted artery segment were adoptively transferred with human peripheral blood mononuclear cells or puriﬁed T cells from a donor allologeneic to the artery 1 week before surgery. To compare rejection of injured versus healthy tissues, these manipulations were combined. Results were analyzed ex vivo by histology, morphometry, immunohistochemistry, and mRNA quantitation or in vivo by ultrasound. Our results showed that reperfusion injury, which otherwise heals with minimal sequelae, intensiﬁes the degree of allologeneic T cell–mediated injury to human artery segments.

Conclusion—We developed a new human-mouse chimeric model demonstrating interactions of reperfusion injury and alloimmunity using human cells and tissues that may be adapted to study other forms of nonimmune injury and other types of adaptive immune responses. (Arterioscler Thromb Vasc Biol. 2012;32:353-360.)

Key Words: hypoxia • reperfusion injury • transplantation • graft arteriosclerosis • humanized arterial mouse model

Analyses of outcomes in clinical transplantation have established that nonimmune injuries to an allograft in the perioperative period can inﬂuence graft survival.1 For example, in human kidney transplantation, cadaver allografts generally fail at a faster rate than living donor grafts, often despite better human leukocyte antigen (HLA) matching,2 a difference attributed to the greater degree of injury incurred by cadaver grafts due to incubation in a hemodynamically unstable donor, longer cold ischemic times, and, in the case of non-beating heart donors, additional warm ischemic time.3 Furthermore, cadaver renal allografts with delayed graft function, a condition that correlates strongly with prolonged ischemic time and is believed to be caused by ischemia/reperfusion (IR) injury to the graft vasculature, have a signiﬁcantly shorter half-life than those without delayed function.1 Nonimmune factors are also thought to contribute to the development of graft arteriosclerosis (GA) of the coronary arteries in cardiac allografts, also referred to as cardiac allograft vasculopathy.4 GA is the major cause of clinical graft failure after the ﬁrst year posttransplantation and is characterized by a diffuse narrowing of the conduit arteries of the graft caused by intimal expansion and inadequate compensatory outward remodeling.5,6 GA-like vascular changes are also observed in other solid organ graft types, and both delayed graft function and episodes of acute rejection predispose renal allografts to GA.7 GA is restricted to the vessels of the graft, stopping at the suture lines, and is thought to be primarily a form of chronic rejection.4 A key question posed by the pathogenesis of GA is how nonimmune injuries interact with the adaptive immune-mediated alloimmune rejection response that underlies these processes. The “burden of injury” hypothesis proposes (1) that perioperative graft injuries are one of several types of injury that may be experienced by an allograft, including acute rejection...
episodes, chronic rejection, and preexisting comorbidities; (2) that grafts expand their intima in a similar manner as a response to all forms of injury; and (3) that as effects of injury add up, grafts that had suffered from perioperative injury simply reach clinically significant stenoses at an earlier time.8,9 Alternatively, the “immune modulation” hypothesis posits that perioperative graft injury changes the graft such that it interacts with the host adaptive immune system differently, eliciting an accelerated rejection response.4 In human specimens of cardiac allografts with GA, T cells are found within the arterial wall, primarily in a subendothelial location within the vessel intima and surrounding the vessel within the adventitia.10 These infiltrating T cell populations are biased toward production of interferon (IFN)-γ.11 Using human arterial segments implanted into immunodeficient mouse hosts, we have shown exogenous provision of IFN-γ in the absence of leukocytes causes vascular smooth muscle cell (VSMC) proliferation, intimal expansion, and eventual loss of lumen in the absence of leukocytic infiltrates or overt vascular injury.12 Thus changes in a graft vessel induced by nonimmune injury that favor T cell production of IFN-γ, such as release of inflammatory cytokines, could represent a form of immune modulation that could predispose to GA.

Experiments using murine cardiac allograft transplantation have provided support for the immune modulation hypothesis, as grafts recently exposed to IR injury are more rapidly rejected by adoptively transferred effector T cell populations than are healed-in grafts.13 However, rodent models may not accurately reproduce human transplantation pathologies for several reasons. First, the consequences of ischemia and reperfusion to graft vessels may not be the same. Rodent endothelial cells (ECs) exposed to ischemia and reperfusion express xanthine oxidase, a major source of injurious reactive oxygen species; consequently, IR injury to rodent vessels precedes neutrophil extravasation.14 In contrast, human ECs do not express this enzyme,15 and infiltrating neutrophils are required as the major source of reactive oxygen species to produce IR injury in humans. Second, the immunologic characteristics of the cell populations that form the arterial wall differ between mice and humans. In particular, human ECs can activate resting CD4+ memory T cells to proliferate and produce effector cytokines,16 whereas mouse ECs cannot17 and may instead induce T-regulatory cells.18 Third, in the absence of specific immunization by a virus, most rodent transplant models involve rejection that is mediated by activating alloreactive naïve T cells,19 whereas adult humans, unlike mice, have a large population of alloreactive memory cells in the circulation whose frequency correlates with the frequency and severity of rejection.20 This difference may be particularly important for vascular rejection because human ECs selectively activate alloreactive memory T cells without the need for priming such T cells in secondary lymphoid organs.21 To address these differences, we and others have studied human alloimmune responses in chimeric mice receiving human artery grafts and allogeneic human T cells.22–26 To analyze the effects of human IR injury on the allogeneic T cell response to the artery wall, we have developed a new human/mouse chimeric model. This technical advance should be applicable to other studies of transplantation rejection and, if T cells autologous to the artery are used, to autoimmune diseases or diseases with an autoimmune component, such as atherosclerosis.

Methods

Arterial Transplantation

All human cells and tissues were collected under protocols approved by the Yale University Human Investigations Committee or the New England Organ Bank, and all experimental animal protocols were approved by the Yale University Institutional Animal Care and Use Committee. Human arterial transplantation was performed as previously described,22,23 and retransplantation into new recipients, with or without ex vivo hypoxia, is detailed in the Supplemental Methods, available online at http://atvb.ahajournals.org. No differences were noted between the responses of coronary and epigastric arteries, and experiments using both sources of human artery were pooled.

Human Peripheral Blood Mononuclear Cells Inoculation

 Peripheral blood mononuclear cell (PBMC) collection and inoculation into mouse hosts was performed as described previously22,23 and is detailed in the Supplemental Methods. Where indicated, T cells purified from PBMCs by magnetic bead immunoselection were inoculated instead of unfractonated PBMCs. The efficacy of adaptive transfers was assessed by analysis of mouse host peripheral blood by human T cells or immunoglobulins (see Supplemental Methods).

Analysis of Arterial Grafts

Harvested vessels were routinely assessed by conventional histology, morphometry, immunohistochemistry, and mRNA quantitation and electron microscopy as detailed in the Supplemental Methods. Cytokine production within the arterial wall was quantified by analyzing mRNA as described in the Supplemental Methods. Where indicated, changes in vessel morphology were monitored noninvasively by ultrasound examination using a VEVO 770 high-resolution imaging unit (Visual Sonics Inc., Toronto, Ontario, Canada) as described in the Supplemental Methods.

Statistical Analysis

All experiments involved comparisons between pairs of animals receiving human artery segments from adjacent portions of the same donor vessel subjected to distinct manipulations, and the data were analyzed by paired Student t tests. P<0.05 was considered significant.

Results

Induction of Reperfusion Injury in Human Artery Segments

Our overall objective was to develop a model to study the effects of perioperative injury on the subsequent development of human allograft rejection. To do so, we modified an established human/mouse chimeric system that we had developed and that has since been used by others to study human allograft vascular rejection.22–26 We began by developing a robust and reproducible model of IR injury of transplanted human artery segments in an immunodeficient mouse host. The conditions of the coronary vessels obtained from explanted recipient hearts of patients receiving human cardiac transplants or epigastric vessels from cadaver organ donors are highly variable and not subject to experimental control. We therefore first standardized and quiesced these vessels before the onset of the experiment by implanting them into a mouse host. To confirm that vessels had quiesced, we...
compared arteries harvested at 2 versus 30 days posttransplantation. At both time points, the implanted arteries showed a small intima, characteristic of healthy arteries, and a well-demarcated media defined in elastica van Giesson-stained sections by intact internal and external elastic lamina (Supplemental Figure IA and IB). Staining with anti-HLA-A, B antibody revealed that the artery segments remained entirely composed human cells (Supplemental Figure IC). Despite their similar appearance, human arteries at 2 days showed markedly higher expression of several inflammatory genes compared with pretransplant artery segments, the most robust of which was interleukin-6 (IL-6), and the level of IL-6 returned to pretransplant levels in the paired segment harvested 30 days posttransplantation (data not shown).

We next sought to introduce a reperfusion injury on grafts quiesced for 30 days. Reversibly clamping the segment in situ for up to 40 minutes led to reinduction of IL-6 but failed to result in neutrophil recruitment characteristic of IR injury at 6, 12, or 24 hours later (data not shown). In a second set of pilot experiments, human arterial grafts were harvested at 30 days along with a 1- to 2-mm cuff of mouse aorta on both ends. Grafts were either immediately transplanted into a second recipient animal or incubated ex vivo in medium at room temperature, either in room air or in a hypoxia chamber (less than 0.1% \( O_2 \)), for 3 hours before retransplantation. The duration of ex vivo hypoxia were selected, based on preliminary experiments, as conditions required to reproducibly induce positive staining with Hypoxyprobe (Figure 1A) but not cause extensive sloughing of ECs (Figure 1B) or loss of VSMC tone at 12 hours following retransplantation (data not shown). At 12 hours after retransplantation, the human artery segment subjected to ex vivo room air, which remained Hypoxyprobe negative, gave inconsistent results, but the artery segment made hypoxic for 3 hours ex vivo before retransplantation consistently showed a degree of mouse neutrophil infiltration (Figure 1C), a variable degree of focal endothelial disruption (Figure 1D), and morphological preservation of VSMCs (Figure 1E). Neither neutrophil recruitment nor EC disruption was observed in the artery segment that was immediately retransplanted. Ex vivo hypoxia also led to elevated IL-6 transcript levels 12 hours after retransplantation compared with the vessel that was immediately retransplanted (Figure 1F) or compared with the vessel exposed to ex vivo hypoxia before retransplantation (data not shown). At the ultrastructural level, ex vivo hypoxia followed by transplantation resulted in widespread mitochondrial swelling and clearing with disruption and loss of cristae (Supplemental Figure II), changes characteristic of IR injury,\(^{27}\) in both graft ECs and smooth muscle cells to a much greater extent than that seen in either quiesced human vessel segments before retransplantation (not shown) or to quiesced human vessel segments retransplanted without ex vivo hypoxia (Figure 1G and 1H). We concluded that we had successfully developed conditions for reproducibly producing reperfusion-dependent injury in a human artery segment that could be used as a model for human IR injury associated with transplantation.

Consequences of Reperfusion Injury

To test the consequence of reperfusion injury of human arterial grafts on subsequent allograft rejection, we initially examined the natural history of reperfusion injury by leaving the grafts in a second recipient mouse for 21 days after retransplantation and then analyzed the grafts by histology and immunohistochemistry. The human artery segments subjected to ex vivo hypoxia before retransplantation showed a small degree of intimal expansion (Supplemental Figure IIIA) that did not reach statistical significance compared with immediately retransplanted vessels. Even at this late time point, the graft was still composed of human cells (Supplemental Figure IIIB), and no evidence of active inflammation remained, assessed by anti-mouse GR1 staining (Supplemental Figure IIIC). In other words, reperfusion injury resulted in minimal permanent structural change following resolution of perioperative inflammation. Interestingly, the EC lining of the graft, despite focal disruptions at early times, appeared to have been completely repaired with human ECs. Importantly for the interpretation of subsequent experiments, overt rejection of the human artery segment in the immunodeficient mouse host did not occur in the absence of functional lymphocytes despite a nonimmune injury in the perioperative period.

We next used our reperfusion injury model to assess the effects of perioperative injury on a human T-cell allogeneic response to the transplanted artery segments. First, we compared vessels exposed to hypoxia ex vivo and then exposed or not to allogeneic human T cells by retransplanting 2 artery segments exposed to ex vivo hypoxia into paired recipients, 1 of which had been inoculated with human PBMCs 1 week before the second surgery and the other had not. At time of retransplantation, human T cells in the inoculated animal typically made up 1% to 5% of the circulating mouse mononuclear cell population (data not shown). The grafts were harvested 21 days after the second operation and analyzed by histology. Intimal expansion of the arterial grafts exposed to human T cells was markedly increased compared with paired human arterial grafts that were retransplanted in the recipients without human T cells (Supplemental Figure IVA). In both cases, the cells within the graft were overwhelmingly of human origin (Supplemental Figure IVB). Discrete foci of human CD45RO T cells were identified typically deep within the expanded intima, as well as within the adventitia (Supplemental Figure IVC). The majority of human ECs were lost from the lumen in specimens exposed to human T cells, but numerous microvessels formed by human ECs were noted within the intima (Supplemental Figure IVD). There was no obvious spatial relationship between the location of the human T cells and the location of the human EC-lined microvessels. Increased number of cells that stained with antibody to smooth muscle \( \alpha \)-actin, presumably VSMCs, were also noted (Supplemental Figure IVE), generally in clusters located superficially within the intima but separated from both the clusters of T cells and the human EC-lined microvessels.

Next, we compared the vessels exposed or not to ex vivo hypoxia before retransplantation and then exposed to allogeneic human T cells. To do so, we retransplanted paired artery
segments, one exposed and the other not exposed to ex vivo hypoxia, into paired recipients that had both been inoculated with human PBMCs from a blood donor (who was allogeneic to the artery donor) 1 week before the second surgery. The most striking finding is that human arterial grafts exposed to hypoxia ex vivo before being retransplanted in the mice with PBMCs had a significant increase (Table) of intimal expansion at 21 days compared with the control arteries (Figure 2A). Essentially all of the cells within the expanded intimas of both control arteries and arteries subjected to ex vivo hypoxia stained with a pan-HLA-A,B monoclonal antibody, establishing their human origin (Figure 2B). In the same
Table. Effects of Ex Vivo Hypoxia on T Cell–Dependent Intimal Changes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fold Change</th>
<th>SEM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>4.8</td>
<td>1.5</td>
<td>0.037</td>
</tr>
<tr>
<td>T Cell No.</td>
<td>3.4</td>
<td>2.1</td>
<td>0.27</td>
</tr>
<tr>
<td>Vessel No.</td>
<td>2.2</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>VSMC No.</td>
<td>4.5</td>
<td>1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>IFN-γ/CD3ε</td>
<td>0.95</td>
<td>0.27</td>
<td>0.53</td>
</tr>
<tr>
<td>IL-10/CD3ε</td>
<td>1.41</td>
<td>0.35</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Fold change was calculated as the mean the ratios of the measured parameter of paired artery segments quiesced for 30 d, harvested and subjected to ex vivo hypoxia vs not, and then retransplanted into paired mouse recipients and harvested 3 wk later. Six paired mouse recipients had been adoptively transferred with human PBMCs 7 d before the retransplant procedure. P values indicate the probability that the mean of the ratios is not different from 1.0. VSMC indicates vascular smooth muscle cell; IFN, interferon; IL, interleukin.

specimens, few if any mouse cells could be identified using a rat monoclonal antibody to mouse H-2D* (Figure 2B, inset), a mouse class I HLA allele readily detected on all artery wall cells of the C.B-17 mouse host (not shown). We identified infiltrating human T cells, mostly deep within the intima adjacent to the internal elastic lamina (Figure 2C). The numbers of T cells in the expanded intima showed considerable variation and did not reach statistical significance between the 2 groups (Table). There was a greater increase (Table) of human EC-lined microvessels in the intima (Figure 2D) of arteries that had been subjected to hypoxia, presumably reflecting an increased angiogenic response. Again, the location of microvessels did not closely correlate with that of the clustered T cells (Figure 2D) or VSMCs (Figure 2E). Finally, there was a clear increase (Table) in VSMCs within the intima of vessels that had been exposed to ex vivo hypoxia over control vessels. These cells occupied the more superficial regions of the intima, largely separated from the T cells (Figure 2E). We did not observe a significant change in transcripts encoding IFN-γ (Table), a pathogenic cytokine, or in IL-10 (Table), a protective cytokine. Levels of transcripts encoding IL-17A, another pathogenic cytokine, were below limits of detection. In other words, perioperative reperfusion injury dramatically increased alloimmune-mediated intimal expansion without causing a statistically significant effect on the intensity or the nature of the inflammatory infiltrate.

Although our histological analyses of human grafts retransplanted into mouse hosts that had been previously inoculated with human PBMCs allogeneic to the graft were most consistent with a T cell–mediated arteriopathy, other elements of the human innate or adaptive immune systems could contribute to the lesions that developed. In particular, some human B cells do engraft and host animals develop circulating levels of human immunoglobulins in the mg/mL range that could potentially recognize human autoantigens or alloantigens. To control for this possibility, we repeated the key experiment, comparing the response of retransplanted arteries exposed or not to ex vivo hypoxia into recipients that had been inoculated with purified T cells instead of whole human PBMCs. These animals developed similar lesions in their grafts to animals given whole PBMCs (Supplemental Figure VA and VB) but had 1000-fold lower levels of circulating human IgM and IgG (Supplemental Table I) and showed no detectable deposition of human IgG in the graft artery wall (Supplemental Figure VC). These data support our interpretation that the lesions produced in this model are mediated by T cells and that the T-cell response is exacerbated by nonimmune injury.

Having established a clear effect of reperfusion injury on the vessel caused by allogeneic T cells 21 days after retransplantation, we wished to examine the vessels at an earlier time to characterize the evolution of the lesions. To identify an early point at which lesions might be evident, we performed ultrasound examinations of living animals, measuring the thickness of the wall of the implanted artery segment. Analysis of implanted vessels 21 days posttransplantation had revealed an increase in wall thickness in the transplanted segments (Figure 3A) that correlated with changes measured by histology (Figure 3B). Ultrasound examination at earlier times revealed that a smaller degree of intimal expansion was detectable as early as 10 days after retransplantation in vessels subjected to ex vivo hypoxia compared in control retransplanted vessels (Figure 3C). The intima of vessels harvested at 10 days was larger in vessels subjected to ex vivo hypoxia than in control retransplanted vessels (Figure 3D), as detected by elastica van Giesson staining (Supplemental Figure VIA), but the total lesion size was smaller compared with 21 day specimens (Figure 3D), consistent with the ultrasound findings (Figure 3C). At these early times, there was human CD45RO+ T cell infiltration (Supplemental Figure VIB), human EC-lined microvessel formation (Supplemental Figure VIC), and the presence of VSMCs within the intima (Supplemental Figure VID), but these cells were not so obviously spatially separated as they were at 21 days. The ratio of VSMCs to T cells was higher at 10 days than at 21 days (6.2:1 versus 1.1:1), suggesting a different rate of change for these processes, although both populations continued to increase. We conclude that vessel infiltration by T cells and responses of intrinsic vessel wall cells, namely VSMCs and ECs, occur concomitantly.

Discussion

Here we describe a new human/mouse chimeric animal model that allows for examination of the effects of a reperfusion injury on a human T cell–mediated adaptive alloimmune rejection response to an artery segment in vivo. The use of human materials is important because the mechanisms of reperfusion injury are significantly different in human and rodents.14,15 The injury we observed that was induced by ex vivo hypoxia followed by reperfusion is correlated with neutrophil recruitment, a characteristic feature of human IR injury, and can be further studied in its own right to better understand the details of IR injury to human ECs. Also, the adaptive immune response to allogeneic human ECs is strikingly different from that in mice and rats.16–18 Because VSMCs cannot activate allogeneic human T cells26,29 and because no human leukocytes can be detected in the grafts that are harvested after 30 days in the first mouse recipient, the human ECs of the graft are likely to be the only competent
Figure 2. Histological analyses of retransplanted human artery segments at 21 days: effects of ex vivo hypoxia on the effects of allogeneic T cells. Adjacent human artery segments were quiesced in pairs of mouse hosts for 30 days, subjected or not to ex vivo hypoxia, retransplanted into pairs of recipient mice that had been inoculated with human peripheral blood mononuclear cells (PBMCs) 1 week before retransplantation, and harvested 3 weeks later. Grafts were stained with elastica van Giesson (EVG) (A) or immunostained with anti-human leukocyte antigen (HLA)-A,B (B) or anti-mouse H-2Dd (B, inset), anti-human CD45RO (C), anti-human CD31 (D), or anti-smooth muscle actin (SMA) (E). Arrows point to internal elastic lamina. Results are representative of 6 independent experiments. Scale bars=100 μm.
antigen-presenting cells in our system. Finally, our system depends on alloreactive memory cells, which are present in significant numbers in clinical transplant recipients but are typically lacking in mice unless they are specifically infected with viruses before use. Translational studies using humanized mouse models, despite being limited by scarce materials and few tools for genetic manipulation, may more accurately predict potentially useful therapies.

Our initial findings do not completely support either the burden of injury hypothesis or the immune modulation hypothesis for the interplay between nonimmune injury and adaptive immunity. The finding that hypoxic human arterial grafts have severe injury with significant intimal expansion only in the presence of human PBMCs is consistent with immune modulation. However, the change in the adaptive T-cell response to the graft does not appear to closely correlate with either the magnitude of the T cell infiltrate or the pathogenic cytokines produced by the T cell infiltrate. Perhaps mediators other than those analyzed to date are responsible for the observed changes. Alternatively, perhaps IR injury sensitizes VSMCs and ECs to the actions of activating cytokines such that the same amount of IFN-γ produces a much more intense response.

The intimal lesions in our model raise several new questions for investigation. In our original human/mouse chimeric model of artery rejection, the rapidly expanding intima was composed almost entirely of infiltrating T cells with few if any VSMCs. In the new model, the expanded intimas contain clusters of T cells, clusters of smooth muscle-like cells, foci of angiogenic vessels, and an accumulation of extracellular matrix. The presence of VSMC-like cells may be linked to the reduced density of T cell infiltrate, resulting in less VSMC killing. The features of separated T cells and VSMC zones and the presence of an angiogenic response in the new model more closely resemble clinical GA, although the positions of the T cells and VSMC-like cells are inverted in relation to the lumen and the media observed in clinical specimens.

Of technical note, we introduced the use of noninvasive ultrasound imaging to serially follow changes in the vessel wall. We show that there is a good correlation between changes in vessel wall thickness assessed by ultrasound and by histology, even though the absolute magnitude of wall thickness does not exactly match. We cannot tell whether this is a systemic measurement error in the ultrasound technique or a post mortem change in the processing of specimens for histology. Nevertheless, although ultrasound does not provide the detailed information offered by histology, it can be very useful in assessing the time point at which vessels may be harvested to optimize information. This is particularly important when performing experiments with human tissues, where the number of individual specimens is often limited.

In summary, we describe a new model by which to study interactions of injury, immunity, and the intrinsic cells of the vessel wall using human cells and tissues. Our initial observations convincingly link perioperative injuries to pathological intimal expansion in the transplantation setting and have opened up several new questions for investigation. We also believe that this model is a significant technical advance that can be extended to settings other than transplantation, such as stroke or myocardial infarction, where initial ischemic and reperfusion injuries can lead to an autoimmune response that influences clinical outcome.

Acknowledgments
We thank Todd Jensen, Louise Benson, and Lisa Gras for assistance in surgical procedures.

Sources of Funding
This work was supported by National Institutes of Health Grants P01-HL070295 and R01-HL109455. Drs Wang and Rao received support from training grant T32-GM07205, and Dr Fogal received support from training grant T32-HL007950.
Disclosures

None.

References


Reperfusion Injury Intensifies the Adaptive Human T Cell Alloresponse in a Human-Mouse Chimeric Artery Model

Arterioscler Thromb Vasc Biol. 2012;32:353-360; originally published online November 3, 2011;
doi: 10.1161/ATVBAHA.111.239285
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/2/353

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/11/03/ATVBAHA.111.239285.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Material

Supplemental Methods

Arterial transplantation

Adjacent segments from a donor human artery were transplanted into pairs of mice for each experiment to allow for pair-wise comparisons, and data from individual experiments were pooled to generate sufficient numbers for analysis. In experiments involving re-transplantation, pairs of human artery grafts were allowed to “heal in” for 30 days following transplantation into C.B-17 SCID/beige mice at which time the mice were anesthetized and the human arterial grafts, along with a 1-2 mm cuff of mouse aorta on each end, were harvested. One graft of each pair was immediately re-transplanted into a second recipient SCID/beige mouse while the other was incubated \textit{ex vivo} in RPMI 1640 medium either in room air or in a hypoxia chamber (BioSpherix Ltd, Redfield NY) at 0.1% O\textsubscript{2} for three hours at room temperature prior to re-transplantation. Survival rates for both the initial surgery and the re-transplant procedure exceeded 95%. To harvest transplanted arterial grafts, animals were anesthetized and arterial grafts were perfused with normal saline and excised before death. Arterial grafts were either snap frozen in optimum cutting temperature (OCT) compound or fixed in 10% formalin and then paraffin embedded. Serial 5-µm or 30-µm transverse sections were cut for morphometric and immunohistochemical, or RNA analyses respectively. Where indicated, human artery segments harvested at 30 days from the initial host were analyzed immediately after \textit{ex vivo} manipulation (i.e. without re-transplantation) to assess the extent of hypoxic changes by staining 5 µm sections with Hypoxyprobe™ Gemini Kit (NPI, In., Burlington, MA).

Human peripheral blood mononuclear cells (PBMC) inoculation
PBMC were collected by leukapheresis from adult volunteer donors, enriched by density centrifugation using Lymphocyte Separation Medium (MP Biomedicals LLC, Solon OH) and cryopreserved prior to adoptive transfer as described previously (1,2). Where indicated, the recipient animal was given an ip inoculation of $3 \times 10^8$ human PBMC from a volunteer donor allogeneic to the artery source seven days prior to artery re-transplantation. In some experiments, T cells were purified from total PBMC by negative immunoselection using magnetic beads (Dynabeads Untouched Human T Cell Kit, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. Such cells were 97% pure for CD3+ cells and animals were inoculated with $2 \times 10^8$ T cells. Successful adoptive transfer resulted in a distinct population of CD3+ human T cells (stained with anti-CD3 FITC, Beckman Coulter, Inc., Brea, CA) in the circulation detected by FACS analysis using (FAC Scan, CellQuest software, Becton, Dickinson and Company (BD), Franklin Lake, NY). Adoptive transfers were also tested in some instances by ELISA (Human IgM and IgG ELISA sets, Bethyl Laboratories, Inc, Montgomery, TX) for human IgM or IgG in the mouse sera according to the manufacturer’s directions.

**Analysis of Arterial Grafts**

Mice were sacrificed at indicated times post-transplantation and harvested vessels were routinely assessed by preparing either frozen or paraffin-embedded 5-μm cross sections and stained with either hematoxylin and eosin (H&E) or Elastica–van Gieson (EVG). Morphometric measurements were made on EVG-stained sections using ImageJ software (available at http://rsbweb.nih.gov/ij/). To study specific cell populations within the vessel wall, cryosections were stained with mouse mAbs against human CD31 for ECs or smooth muscle α-actin (both from Dako North America, Inc., Carpinteria, CA) for VSMC using the avidin–biotin peroxidase staining method (Vector Laboratories, Burlingame, CA). Graft-infiltrating human lymphocytes
were quantified by staining for human CD45RO (Ebioscience Inc., San Diego, CA). Mouse
neutrophils were quantified by staining 5-μm sections with rat anti–mouse Gr-1 (BD). Human
cells were identified by staining 5-μm sections with mouse anti human HLA-A, B (W6/32,
Dako). The anti-human CD31, anti-human CD45RO and anti-HLA-A, B mAbs used in this study
do not cross-react with mouse cells.

To quantify mRNA levels, serial sections of artery or grafts were immersed in water, centrifuged
briefly, and then rapidly resuspended in RLT lysis buffer (QIAGEN Inc., Valencia, CA). RNA
was isolated using RNeasy mini kits (QIAGEN) according to the manufacturer's protocol. RT
with random hexamer and oligo-dT primers was performed according to the Multiscribe RT
system protocol (Applied Biosystems Inc., Carlsbad, CA). All RT-PCR reactions were prepared
with TaqMan 2× PCR Master Mix and predeveloped assay reagents from Applied Biosystems.
The specific primers used were purchased from Applied Biosystems: GAPDH
(Hs99999905_m1), CD3ε (Hs00167894_m1), IFNγ (Hs00174143_m1), IL-17A
(Hs00174383_m1), IL-10 (Hs 00961622_m1), and IL-6 (Hs00174131_m1). Samples were
analyzed on an iCycler or iQ5 (Bio-Rad Laboratories). Samples processed without the RT
enzyme were used as negative controls. The expression level of each target was normalized to
that of GAPDH unless otherwise indicated in the figures.

In some experiments quiesced human arterial grafts were analyzed by electron microscopy either
immediately following harvest from the initial mouse host or 12 h following re-transplant into a
second host, with or without a period of ex vivo hypoxia prior to re-transplant. Following
euthanasia, graft segments were cleared of blood in situ by perfusion with 0.01 sodium nitrite in
normal saline followed by fixation perfusion with 200 mls of 2% glutaraldehyde in 0.1M HEPES (pH 7.4) containing 2mM calcium chloride and then immersion in the same glutaraldehyde fixative at 4 degree for 4h. The fixed tissue was cut into pieces no larger than 3mm at any one length and was rinsed 5 times in 0.1M cacodylate buffer pH 7.5 with repeated agitation. After several buffer rinses, samples were incubated for 1 h at RT in 1% osmium tetroxide containing 1.5% potassium ferricyanide. After rinsing 4 times in de-ionized water, they were bulk stained in 2% uranyl acetate in 0.05 M maleate buffer at pH 5.5. After rinsing 3 times in deionised water to remove any buffer salts, samples were dehydrated by multiple exchanges of ethanol solutions, using progressively higher ethanol concentrations up to 100%, followed by two exchanges in dry 100% acetonitrile. Samples were then incubated in 50% acetonitrile 50% Quetol epoxy resin overnight. This was followed by five daily changes of absolute resin. The resin mixture contained: 9 g Quetol 651 (Agar, Stansted, Essex, UK), 11.6 g nonenyl succinic anhydride hardener, 5 g methyl-5-norbornene-2,3-dicarboxylic anhydride hardener and 0.5 g benzylidimethylamine catalyst. The samples were then cured at 60 °C overnight. Several 1μm thick were stained with 1% toluidine blue to guide subsequent trimming and, thin sections (60–90 nm) were cut using a Leica Ultracut UCT ultramicrotome and collected on bare 300 mesh copper grids. Grids were counterstained with saturated ethanolic uranyl acetate, followed by Reynolds lead citrate, each for 3 min. Grids were viewed using an FEI Tecnai G2 transmission electron microscopes operated at 120 keV and using a 10 μm objective aperture to improve contrast. Images were taken using an AMT XR60B digital camera running Deben software. Images were subjected to statistical evaluation of morphologically altered mitochondria per cell (SMCs and ECs) by unbiased estimation in 10 random fields of view at a magnification of x7K. Mitochondria were judged as injured by the following criteria: swollen and disrupted
mitochondria with electron-translucent or curvilinear alterations of the cristae, ghost mitochondria with focal and/or discontinuous outer membrane. Relative quantification for between-groups (hypoxic Vs non-hypoxic) was carried out and the absolute number of mitochondria was analyzed using GraphPad Prism version 5.02 with student t-test to determine differences between samples and a value of P<0.05 considered statistically significant.

Serial ultrasound monitoring

Ultrasound examinations were performed using a VEVO 770 high-resolution imaging unit (VisualSonics Inc. Toronto, Ontario, Canada) equipped with a 40-MHz high-frequency transducer (focus length 6mm, resolution 40x80μm). The mice were anesthetized with 1.5% isoflurane in oxygen, their body temperature was monitored by a rectal probe and maintained at 36–38°C using heating stage and lamp. All hair was removed from the abdomen using a chemical hair remover (Nair, Church & Dwight Co., Inc., Princeton, NJ, USA). Pre-warmed US gel was used as a coupling agent on the skin of the mice. All images were acquired in fundamental brightness mode B (B mode) with optimization of the gain setting, which were kept constant throughout the experiment.

References for Supplemental Methods


**Supplemental Figures**

Supplemental Figures are cited in the main text.
Supplemental Figure I. Histological analyses of transplanted human artery segments: effects of residence in mouse hosts. Human coronary artery segments were transplanted as
infra renal aortic interposition grafts in an immunodeficient mouse host as described in the Methods and recovered at 2 or 30 days post transplantation. (A) H&E staining; (B) EVG staining; (C) immunostaining with anti-HLA-A,B; and (D) immunostaining with mouse IgG control. Note that at both time periods, the artery appears healthy with minimal intima and is composed of human cells. Arrows point to internal elastic lamina. Bar equals 100 µm.

Representative of 6 independent experiments.
Supplemental Figure II. Mitochondrial morphology prior to and following re-transplantation of human artery segments. Adjacent human artery segments were quiesced in mouse recipients for 30 days and then either perfusion fixed in situ, re-transplanted immediately or subjected to ex vivo hypoxia for three hours prior to re-transplantation into new hosts. Re-
transplanted vessels were perfusion fixed 12 hours later. Mitochondrial morphology in graft endothelial cells and smooth muscle cells was analyzed by electron microscopy as described in the Supplemental Methods. Representative images displaying various morphological changes are presented here including (A) mitochondria with parallel stacks of cristae indicative of normal or minimal injury; (B) mitochondria with disruption of cristae, indicative of moderate injury; and (C) mitochondria with swollen electron-translucent matrix (open arrow) and outer membrane disruption (arrows), indicative of severe injury. Magnifications: A. x150k; B, x25k; c, x100k. Bar equals 100 nm. Representative of 3 independent experiments.
Supplemental Figure III. Histological analyses of re-transplanted human artery segments at 21 days: effects of ex vivo hypoxia. Adjacent human artery segments were quiesced in mouse recipients for 30 days and then either re-transplanted immediately or subjected to ex vivo hypoxia for three hours prior to re-transplantation into new hosts. Vessels were harvested 21 days later and analyzed by (A). EVG staining or immunostaining for (B) HLA-A, B or (C) mouse Gr-1.
Note that the inflammatory cells observed at 12 h in the artery subjected to *ex vivo* hypoxia are no longer present and that the artery remains composed of human cells in both instances. There is little evidence of permanent damage due to reperfusion injury as shown by the intact internal elastic lamina. The intima of the graft subjected to hypoxia appears slightly larger, but the difference is not statistically significant. Arrows point to internal elastic lamina. Bar equals 100 μm. Representative of 4 independent experiments.
Supplemental Figure IV. Histological analyses of re-transplanted human artery segments at 21 days: effects of allogeneic T cells. Adjacent human artery segments were treated as in Supplemental Figure II except that both artery segments were subjected to ex vivo hypoxia prior to re-transplantation and one segment was re-transplanted into a mouse host that had been inoculated with human PBMC one week prior to re-transplantation whereas the other host had
not received PBMC. Grafts were harvested 3 weeks later and stained with (A) EVG or immunostained with (B) anti-HLA-A,B; (C) anti-human CD45RO; (D) anti-human CD31 or (E) anti-SMA. Note that the presence of allogeneic T cells in the circulation of the recipient mouse causes a marked increase in the vessel intima which contains human T cells, human EC-lined microvessels and human VSMCs. Arrows point to internal elastic lamina. Bar equals 100 μm. Representative of 6 independent experiments.
Supplemental Figure V. Histological analyses of re-transplanted human artery segments at 21 days: effects of ex vivo hypoxia on the effects of purified allogeneic T cells. Adjacent human artery segments are quiesced in pairs of mouse hosts for 30 days, subjected or not to ex vivo hypoxia, re-transplanted into pairs of recipient mice that had been inoculated with purified T
cells from human PBMC 1 week prior to re-transplantation and harvested 3 weeks later. Grafts were stained with (A) EVG or immunostained with (B) anti-human CD45RO; (C) anti-human IgG. Panel (D) depicts staining of re-transplanted human artery segments with anti-human IgG of a human artery graft harvested at 21 days from a mouse host that had been inoculated 1 week prior to re-transplant with whole human PBMC instead of purified T cells for comparison to panel (C). Arrows point to internal elastic lamina. Representative of 3 independent experiments. Bar equals 100 μm.
Supplemental Figure VI. Histological analyses of re-transplanted human artery segments at day 10: effects of ex vivo hypoxia on the effects of allogeneic T cells. Grafts were stained with (A) EVG or immunostained with (B) anti-human CD45RO; (C) anti-human CD31; or (D) anti-SMA. Note that the augmenting effect of ex vivo hypoxia on the changes caused by allogeneic T cells is evident at 10 days and the changes are similar to but less pronounced than those observed at 21 days. Arrows point to internal elastic lamina. Bar equals 100 μm. Representative of four independent experiments.
**Supplemental Table I Human IgM and IgG level in mouse sera**

<table>
<thead>
<tr>
<th></th>
<th>IgM</th>
<th></th>
<th>IgG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>PBMC</td>
<td>154.700</td>
<td>95.033</td>
<td>1480.000</td>
<td>277.143</td>
</tr>
<tr>
<td>T cells</td>
<td>0.390</td>
<td>0.407</td>
<td>0.453</td>
<td>0.312</td>
</tr>
<tr>
<td>Control</td>
<td>0.073</td>
<td>0.019</td>
<td>0.028</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Table I**

Human IgM and IgG are measured as mean and standard deviation (SD) in sera of mouse recipients who get re-transplant of human artery segments quiesced for 30 days in previous mouse hosts. The re-transplant recipients had been adoptively transferred with human PBMCs, purified T cells from human PBMCs, or no human cells 7 days prior to the re-transplant procedure.