Physical exercise reduces transplant arteriosclerosis in a mouse aorta transplantation model

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Background: Transplant arteriosclerosis limits long-term outcome after heart transplantation. The underlying mechanism of transplant arteriosclerosis remains alloreactivity, but it is also influenced by nonimmunologic cofactors. Physical exercise has well-established effects on the prevention of native arteriosclerosis. We hypothesized that physical exercise would reduce the development of transplant arteriosclerosis in an allogeneic transplantation setting.

Methods: Segments of the thoracic aorta from C57.Bl6 (H2b) or C3H.HeJ (H2k) mice were transplanted into the abdominal aortas of CBA.Ca mice (H2k), representing a major or minor alloantigen mismatch, respectively. Three days after surgery, recipient mice were assigned to either the control or physical exercise (consisting of 2 × 45 minutes of treadmill training per day) groups. Transplant arteriosclerosis was assessed and quantified by histology on day 28 after vessel transplantation. Endothelial cell integrity and function in histology sections and peripheral blood was assessed.

Results: All animals developed transplant arteriosclerosis with more severe luminal occlusion in the major alloantigen mismatch setting. Animals undergoing physical exercise developed significantly less severe transplant arteriosclerosis in both the major ($P < .0001$) and minor ($P < .0001$) antigen mismatches than their respective control groups without physical exercise. $CD31^+$ endothelial cells were present in significantly higher numbers in the grafts and circulating in peripheral blood in the exercise groups compared with their respective control. Above that, we found enhanced endothelial nitric oxide synthase-positive cells in both exercise groups compared with the untreated groups ($P = .01$ and $P = .02$, respectively).

Conclusions: Physical exercise has a protective effect against the development of transplant arteriosclerosis. This could be due to enhanced endothelial cell regeneration and function in the graft. (J Thorac Cardiovasc Surg 2015;149:330-7)

See related commentary on pages 337-9.

Cardiac transplantation is the gold standard therapy for end-stage heart failure. However, chronic rejection, which is known as cardiac allograft vasculopathy (CAV), in transplanted hearts, affects in excess of 50% of the recipients at 10 years.[1] CAV thus remains the most important cause of allograft loss in heart transplantation. The pathology comprises an occlusive disease of the coronary arteries, with similarities to atherosclerotic coronary artery disease (CAD) in patients who do not undergo transplant. CAV develops in both epicardial and intramural coronary vessels and may show concentric intima and media thickening.[2,3] Progression of CAV takes place in the absence of arteriosclerosis in other recipient arteries, indicating allogeneic immune responses as the underlying trigger. However several other factors have been identified to also contribute to progress of CAV. Kobashigawa and colleagues[4] described the effectiveness of early statin therapy in heart transplant recipients, leading to significantly lower serum cholesterol levels, better 1-year survival, and lower incidence of CAV. Also, cytomegalovirus serostatus positivity as well as episodes of cytomegalovirus infection influence development and progression of CAV.[5]

More recently, the endovascular endothelium and its microvascularization has been the focus of CAV research, indicating that pathologic coronary artery microcirculation is a negative prognostic factor for long-term outcome after cardiac transplantation. Therefore, impaired endovascular microcirculation possibly influences the onset of CAV in allografts.[6] The degree of microvascularization in vessels might also be influenced by physical exercise, given that for muscle, as well as Achilles tendon tissue, microvascular

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Abbreviations and Acronyms
CAD = coronary artery disease
CAV = cardiac allograft vasculopathy
cDNA = complementary DNA
eNOS = endothelial nitric oxide synthases
EPC = endothelial progenitor cell
PCR = polymerase chain reaction

filtration is enhanced under physical exercise.[7,8] Another beneficial effect of physical exercise in recipients of heart transplant was described by Hermann and colleagues,[9] who showed reduced systolic blood pressures and improved endothelial function as determined in duplex sonography of nitroglycerin-induced vasodilation. Direct experimental evidence supporting the observation of reduced transplant arteriosclerosis by physical exercise is currently missing.

Here, we hypothesize physical exercise directly counteracts the development of transplant arteriosclerosis in a murine allogeneic aorta transplantation model.

MATERIAL AND METHODS

Mice

C57.Bl6 mice (H2b), CBA.Ca (H2k) and C3H.HeJ (H2k) mice were obtained from Charles River or Jackson Laboratories (Wilmington, Mass) and housed under specific pathogen-free conditions. The mice were aged 8 to 12 weeks at the time of surgery. All animals received humane care in compliance with the German animal protection legislation, the Principles of Laboratory Animal Care, and the Guide for the Care and Use of Laboratory Animals.[10] The study was approved by the government board for animal welfare of Lower Saxony, Germany.

Surgical Technique

Transplantation of aorta grafts from C57.Bl6 (H2b), C3H.HeJ (H2k), or CBA.Ca (H2k) donor mice into CBA.Ca (H2k) recipient mice was performed using a technique described by Koulack and colleagues.[11] Briefly, the thoracic aorta of the donor mice was explanted, flushed with heparinized sodium chloride, and stored at 4°C. Grafts of 3 to 4 mm length were then transplanted into the abdominal aorta of the recipient animals (Figure 1, *A*).

Physical Exercise

Animals undergoing physical exercise were kept in regular cages, but were put into a cage with a treadmill twice daily for 45 minutes training per session. Training commenced as of postoperative day 3 after recovery from surgery. The physical exercise program was conducted until postoperative day 28 (Figure 1, *B* and *C*).

Experimental Groups

To resemble a full major alloantigen mismatch, C57.Bl6 (H2b) donor aortas were transplanted into CBA.Ca (H2k) recipient mice. A group of 10 recipients received no further treatment and constituted the control group, whereas another 10 recipients performed the treadmill training program described above. A second set of experiments was set up in a minor alloantigen mismatch, transplanting C3H.HeJ (H2k) vessels into CBA.Ca (H2k) mice. A group of 11 recipients received no further treatment and constituted the control group, whereas another 11 recipients performed the treadmill training program. Further, 2 control groups of CBA.Ca (H2k) mice undergoing sham surgery without aorta transplantation but performing treadmill training (CBA.Ca [H2k]; that is, the sham exercise group; n = 4) or not (CBA.Ca [H2k]; that is, the sham control group; n = 4), were analyzed and served as sham groups. Additionally, 3 CBA.Ca (H2k) animals (n = 3) underwent aorta transplantation receiving isogeneic grafts from CBA.Ca (H2k) mice. The experimental setup is shown in Figure 1.

Flow Cytometry

Circulating endothelial cells in peripheral blood of recipient mice were monitored by flow cytometry. Whole blood samples were collected into Eppendorf tubes and stained with antimurine CD31 antibody (Biolegend, San Diego, Calif). Analyses were performed on a FACSCanto flow cytometer (Becton, Dickinson and Co, Franklin Lakes, NJ). Obtained data were analyzed using FACSDiva 6.3.1 software (Becton, Dickinson, and Co).

Analyses of the Aorta Grafts

Transplanted aorta grafts were harvested on postoperative day 28. The grafts were then flushed with heparinized saline and imbedded into paraffin. Sections of 2- to 3-μm thickness were cut after complete removal of the anastomosis area and stained with hematoxylin and eosin or van Giesson elastin stain. Morphometric analysis of the graft was performed on 3 different van Giesson elastin–stained cuts, taken on 3 different levels being apart a minimum of 10 microtome cuts per aorta. Histologic photographs were taken at ×40 magnification using a light microscope connected to a digital camera (Olympus U-CMAD3, Olympus, Hamburg, Germany). Digitized images were analyzed using Photoshop CS4 software (version 11.0.2, Adobe, San Jose, Calif). Briefly, the endoluminal area, as well as the areas within the internal and external laminae elasticae, were circumscribed and the absolute pixel counts of each area was then recorded. From these measurements, the quotient for the thickness of the intima; that is, the intima to media ratio, was calculated. This ratio indicates relative thickness (%) of the intima (R = [area (intima)/area (media)] × 100%). For each individual experiment a mean value of the 3 sections analyzed was calculated.

Immunohistochemistry

Histologic sections of 2-μm thickness were deparaffinized and incubated with primary polyclonal rabbit against mouse endothelial nitric oxide synthases (eNOS) (Abcam, UK) or polyclonal rabbit CD31 (Santa Cruz Biotechnology, Heidelberg, Germany) antibodies over night at 4°C. Then, 1-step polymer antirabbit (Zytomed Systems GmbH, Berlin, Germany) was applied. To visualize the staining, the sections were incubated with dimethylaminoazobenzene (DAB-Komplex, Zytomed Systems GmbH, Berlin, Germany) and counterstained with hematoxylin (C. Roth GmbH, Karlsruhe, Germany). For further analyses, histologic sections were clustered into fields of identical size. Subsequently, the number of positive cells per field was counted.

Polymerase Chain Reaction (PCR)

For RNA extraction and complementary DNA (cDNA) preparation, formalin-fixed and paraffin-embedded tissue was cut. Tissue was suspended overnight in a proteinase K digestion solution. RNA was isolated using phenol-chloroform extraction followed by ethanol precipitation. Then, 10 μL RNA were transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) following the manufacturer's instructions. To increase the sensitivity of the subsequent real-time PCR analysis several thousand-fold, the cDNA was preamplified in 14 PCR cycles with nonrandom PCR primers (PreAmp Master Mix Kit, Applied Biosystems Darmstadt, Germany).[12] For analyzing the messenger RNA expression, the preamplified cDNA was evaluated by real-time PCR (TaqMAn 7500 Real-Time PCR System, Applied Biosystems, Carlsbad, Calif). Quantification was performed in