The Positive Effect of Immunosuppression on Adaptation of Venous Allografts to Arterialisation in Rats

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Abstract  Objectives and Design: We investigated whether immunosuppression was necessary for transplanted allogeneic veins to adapt to arterialisation. We used a transplant rat model with or without immunosuppression.

Material and Methods: Iliolumbar veins from Lewis (LEW) or Brown–Norway (BN) rats were transplanted into the abdominal aorta of isogeneic (LEW to LEW; group A) or allogeneic (BN to LEW; groups B and C) rats. Group C had daily intramuscular injections of 0.2 mg kg⁻¹ FK506. Light microscope evaluations of grafts were performed at 30 days following transplantation. We determined the presence of endothelial cells, the intensity of intimal proliferation and the degree of infiltration by Lewis major histocompatibility complex (MHC) class II positive, CD4-positive and CD8-positive cells into the adventitia.

Results: Groups A and C displayed similar results in intimal thickness (12.7 ± 7.0 μm vs. 15.0 ± 8.4 μm, respectively) and degree of adventitial infiltration by MHC class II positive, CD8-positive (0.8 ± 1.7 vs. 1.8 ± 2.6, respectively) and CD4-positive (12.5 ± 7.7 vs. 5.8 ± 4.6, respectively) cells. In contrast, allogeneic rats without immunosuppression (group B) showed infiltration of host immunocompetent cells and destruction of the venous wall with no histological signs of arterialisation.

Conclusion: Immunosuppressive therapy is necessary for venous allograft adaptation to arterialisation in rats.

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Vascular peripheral reconstructive surgery involves the interposition of an autologous vein into the arterial circulatory tree. This results in an adaptation of the venous wall to the new biomechanical conditions. Vein remodelling is a complex biological process that requires alterations in cell proliferation, cell death, cell migration and degradation and/or production of extracellular matrix components. These adaptive interactions amongst vascular cells, their substrates and the local environment lead to the typical anatomical changes associated with venous arterialisation: intimal hyperplasia, circumferential medial thickening with well-organised concentric layers of smooth muscle cells and adventitial neo-vascularisation.

In specific indications, allogeneic veins are used for peripheral vascular reconstruction in humans. Immunological studies have shown that venous allografts that had been cold-stored or cryopreserved were antigenic and elicited an immune response in the recipient. Allograft rejection could play an important role in the low patency rates of venous allografts. Currently, low patency is the primary limitation of venous allografts for clinical use.

Different types of immunosuppressive drugs have been examined as possible adjuncts for reducing antigenicity and improving the patency rates of venous allografts, but these studies have shown different results in animals and humans. Currently, immunosuppressive therapy following allovenous reconstruction is generally not accepted in clinical practice.

FK506 (tacrolimus) is a modern immunosuppressive drug that belongs to the group of calcineurin inhibitors. One of its most important immunosuppressant effects is the inhibition of interleukin-2 (IL-2)-dependent T-cell activation. FK506 is routinely used clinically, in combination with other types of immunosuppressive drugs, to inhibit immune responses following renal and liver transplantations. In previous studies, we confirmed the positive effect of FK506 administration on arterial transplantation. FK506 inhibited wall destruction in the abdominal aorta caused by acute rejection, assessed 30 days following transplantation into the rat arterial system.

This study aimed to investigate the process of allovenous arterialisation under conditions of FK506 immunosuppression. Because medial smooth muscle cell proliferation is a dominant feature of the venous arterialisation process, we hypothesised that immunosuppression would, to some degree, preserve smooth muscle cell content in allografted veins, and that smooth muscle cell preservation would be necessary for successful allovenous arterialisation.

Material and Methods

Principles of laboratory animal care were followed, and applicable national laws observed during the study. Ethical approval by local ethical committee was obtained for this study.

Animals

Male Lewis rats (LEW) (RT1<sup>a</sup>) in the weight range of 200–340 g (N = 26) were used as recipients of allogeneic or syngeneic iliolumbar vein grafts and male Brown–Norway rats (BN) (RT1<sup>k</sup>) in the weight range of 220–300 g (N = 9) were used as donors for the allogeneic group, and LEW (RT1<sup>a</sup>) in the weight range of 280–300 g (N = 5) were used as donors for the syngeneic group. Animals were obtained from Charles River, Germany. All the rats were maintained according to the National Institute of Health guidelines. Each transplanted animal was housed in a separate cage during the entire 30-day follow-up period.

Operative procedures

The donor animals were anaesthetised with an intramuscular injection of ketamine (Narkamon<sup><sup>b</sup></sup>, Spofa a.s., Prague, Czech Republic) at a dose of 100 mg kg<sup>–1</sup> and xylazine (Rometar<sup><sup>c</sup></sup>, Spofa a.s., Prague, Czech Republic) at 10 mg kg<sup>–1</sup>. Two 1–1.5-cm long segments of the iliolumbar veins were excised and these excised veins were stored in saline solution at room temperature until transplantation. The mean ischaemic time for iliolumbar venous grafts during the entire experiment was 201 ± 103 min, with no significant statistical differences amongst the experimental groups (Table 1).

Animals that were used as recipients of venous grafts with planned follow-up were given less invasive anaesthesia to ensure more natural awakening. The recipients were anaesthetised with an intramuscular injection of sufentanil (Sufenta<sup><sup>d</sup></sup>, Janssen Pharmaceutica Inc., Beerse, Belgium) at 20 μg kg<sup>–1</sup> and azaperone (Stresnil<sup><sup>e</sup></sup>, Janssen Pharmaceutica Inc., Beerse, Belgium) at 1 mg kg<sup>–1</sup>. Following a midline laparotomy into the recipient’s infrarenal aorta, the venous grafts were transplanted with a running 10/0 mono-filament suture (Ethicon Inc., Sommerville, NJ, USA).

Neither anticoagulants nor anti-platelet drugs were administrated during the harvest, operative time or in the postoperative period.

Animal groups

The recipient animals were divided into three groups. Groups A and B comprised rats that received isogeneric (LEW to LEW) and allogeneic (BN to LEW) venous transplantations, respectively. Neither group was given immunosuppression. Group C comprised rats that received allogeneic venous transplantations (BN to LEW) and were given immunosuppression (Table 1).

Immunosuppressive therapy

FK506 (Prograf<sup><sup>f</sup></sup>, Astellas Pharma Inc., Tokyo, Japan) was the immunosuppressive drug given to the recipient animals in group C. FK506 was suspended in normal saline solution and administered intramuscularly in daily doses of 0.2 mg kg<sup>–1</sup>. On day 30, following transplantation, blood levels of FK506 were evaluated with an enzyme-enhanced immunoassay technique (Emit<sup><sup>®</sup></sup> 2000 Tacrolimus assay, Dade Behring Inc., Deerfield, IL, USA). The blood was drawn from infrarenal vena cava punctured prior to euthanasia.

On day 30, anaesthetised, heparinised animals in all experimental groups received a midline re-laparotomy to remove the venous grafts. The animals were then euthanised by intracaval administration of a lethal dose of...
The mean cold ischaemic time (CIT) of iliolumbar venous grafts was with no statistical difference between all the experimental groups (Group A vs. Group B p = 0.46, Group A vs. Group C p = 0.59, Group B vs. Group C p = 0.42).

Histology and immunohistochemistry

Histological and immunohistochemical analyses of venous grafts were performed according to methods described previously for our arterial transplantation model. Briefly, the venous grafts were embedded in Sakura Finetek Tissue Tek® Cryomold holders (Sakura Finetek, Tokyo, Japan) and Sakura Finetek Tissue Tek® O.C.T. compound (Sakura Finetek, Tokyo, Japan). The samples were subsequently frozen in 2-methylbutane (Fluka Chemika, Buchs, Switzerland) cooled by liquid nitrogen and then stored at −80 °C.

Histological analysis

The samples obtained were cut into 5-μm sections and stained with Haematoxylin–Eosin and a Van Gieson with elastica stain. The sections were taken from the mid-portion of the graft to avoid tissue that may have reacted to the suture material. Each section was photographed with a Olympus BX51 microscope at a magnification of ×1000. The images were digitally captured in TIFF format with no compression and analysed with Olympus DP-Soft software Version 3.2. Intimal thickness was measured from the endothelial surface to the inner border of the tunica media. Measurements were made in up to 10 locations in each section. The mean value and standard deviation (mean ± SD) were calculated for each vein and each animal group.

Immunohistochemical analysis

Information on the antibodies used for all immunohistochemical analyses is shown in Table 2.

<table>
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<th>Table 1</th>
<th>Basic characteristics of each experimental group and operative procedures.</th>
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<td>Group</td>
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<tr>
<td>A</td>
<td>LEW to LEW</td>
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<td>B</td>
<td>BN to LEW</td>
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<td>C</td>
<td>BN to LEW FK506</td>
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LEV, male Lewis rats; BN, male Brown–Norway rats; CIT, cold ischaemic time; OP, operation duration.

Detection of CD4+ cells, CD8+ cells and Von Willebrand factor

Immunohistochemistry was performed on 8-μm-thick sections with a two-step indirect method. Briefly, the sections were fixed in cold acetone for 10 min. After rinsing in 0.2% Triton X100 and phosphate-buffered saline, the specimens were incubated with a primary antibody for 60 min. Endogenous peroxidase was blocked by incubating in 0.3% hydrogen peroxide (H2O2) and 70% methanol for 30 min. Next, the sections were incubated with a secondary antibody (Histofine® Simple Stain Rat MAX PO, Nichirei, Japan) for 30 min, then incubated with Dako Liquid DAB+ Substrate-Chromogen System (Dako Denmark A/S, Glostrup, Denmark) for 5 min. The specimens were counterstained and dipped in Entellan® (Merck KGaA, Darmstadt, Germany).

The slides were then scored in a blinded fashion. Cells were counted at five locations at ×1000 magnification. The cellularity was defined as the mean value of the cells counted.

Detection of Lewis MHC class II positive cells

Immunohistochemistry was performed on 8-μm-thick sections with a three-step indirect method. Briefly, the sections were fixed for 10 min in cold acetone. After sections were rinsed in 0.2% Triton X100 and phosphate-buffered saline, endogenous biotin was blocked with the biotin-blocking system (Dako Denmark A/S, Glostrup, Denmark). The tissues were then incubated in 10% horse serum to prevent non-specific binding, and then a primary antibody was applied for 60 min. Then, endogenous peroxidase was blocked in 0.3% H2O2 and 70% methanol for 30 min. The specimen was incubated with a secondary biotinylated horse anti-mouse antibody (Vector Lab, Burlingame, CA, USA), followed by an incubation with R.T.U. Vectastain Elite ABC Reagent (Vector Lab, Burlingame, CA, USA). Finally, specimens were incubated for 5 min with Dako Liquid DAB+ Substrate-Chromogen System (Dako Denmark A/S, Glostrup, Denmark), counterstained and dipped in Entellan® (Merck KGaA, Darmstadt, Germany).

Table 2 | The list of monoclonal and polyclonal antibodies used for immunohistochemical staining of iliolumbar venous grafts. |
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<td>Specificity</td>
<td>Origin</td>
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<tr>
<td>Von Willebrand factor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>CD4+ cells</td>
<td>Mouse</td>
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<tr>
<td>CD8+ cells</td>
<td>Mouse</td>
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<tr>
<td>RT1.8⁺ a</td>
<td>Mouse</td>
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a Major histocompatibility complex class II positive cells of host origin.
Statistical analysis

The values in the text and figures are expressed as the mean ± standard deviation (SD). Comparisons of intimal thicknesses and numbers of lymphocytes and Lewis MHC class II positive cells between the experimental groups were performed with the analysis of variance (ANOVA) method, followed by the Tukey HSD Multiple Comparisons test. The p values < 0.05 were considered significant.

Results

Animals

A total of 30 LEWs were intended for transplantations, but only 26 survived the procedure. Four animals died during the experiment. Three died due to perioperative bleeding (two from group A and one from group B) and one animal was overdosed with anaesthetics (group C). The increase in fatal bleeding in the group A, compared to the other groups, was due to technical failures, which arose initially (and this group was operated at the start of the experiment).

An increase from preoperative weight was observed in all groups at 30 days. The percentage weight increase was significantly higher (p < 0.01) in the syngeneic group A compared to both allogeneic groups. This was probably due to the lower weight of animals in syngeneic group A on day 0 when compared to both allogeneic groups (Table 1).

Immunosuppression

The mean blood level of FK506, measured on day 30 after transplantation, in group C was 5.57 ± 0.96 ng ml⁻¹. Diarrhoea was an adverse effect of FK506 administration, and it was observed in five out of seven animals in the immunosuppressed group C.

Graft patency

No venous graft thromboses or occlusions were observed in any of the animals on day 30 following transplantation.

Tunica intima

In all groups, at 30 days following transplantation, the luminal surface of the venous allograft was covered by a monolayer of endothelial cells. In immunosuppressed iliolumbar vein allografts, hyperplasia of the subendothelial neointima developed during that period. The neointima was formed primarily from elastic tissue (Fig. 1). The same feature was observed in syngeneic arterialised venous grafts of group A. The intima thicknesses did not differ between these two experimental groups (Table 3).

Figure 1  Representative light microscopic histological features of immunosuppressed (group C) and non-immunosuppressed (group B) rat venous allografts obtained at 30 days following transplantation into the infrarenal abdominal aorta. The process of arterialisation in allogeneic immunosuppressed venous grafts was represented by: (C1) (Van Gieson with elastica) intimal hyperplasia and formation of elastic tissue laminae in the neointima (layer of smooth muscle cells in the tunica media is marked with black arrows); (C2) (Von Willebrand factor positive cells) endothelialisation of the luminal surface (black arrows show positive monolayer of endothelial cells) and (C3) (Lewis MHC class II positive cells) low adventitial infiltration of immunocompetent cells (adventitial layer is marked with black arrows). In allogeneic non-immunosuppressed venous grafts, massive infiltration of the host immunocompetent cells led to tissue rejection. (B1, B3) (Van Gieson with elastica, Lewis MHC class II positive cells) the disorganisation of the allograft wall structure is evident with no signs of arterialisation (venous wall is marked by black arrows) and (B2) (Von Willebrand factor positive cells) the luminal surface of disorganised venous allografts was covered by endothelial cells (black arrows show the monolayer of endothelial cells). Magnification = ×100.
The absence of immunosuppression in allogeneic ilio-lumbar vein grafts (group B) resulted in the destruction of the intima and disorganisation of the whole venous wall structure. Minimal or no signs were observed of elastic tissue formation in this layer when compared to syngeneic or immunosuppressed venous grafts (Fig. 1). However, this elastic tissue formation was not quantified. The intimal thickness was statistically lower in venous allografts without immunosuppression than in venous allografts with immunosuppression ($p < 0.01$) (Fig. 2).

**Tunica media**

In immunosuppressed venous allografts, the process of adaptation to arterial pressure led to the formation of multiple smooth muscle layers at 30 days following transplantation. Minimal or no infiltration of host MHC class II, CD4-positive and CD8-positive cells was observed in the medial layer of immunosuppressed venous allografts. These histological signs of venous arterialisation were identical to those observed in syngeneic venous grafts (Fig. 1).

In contrast, no typical concentric smooth muscle layers were observed in non-immunosuppressed allogeneic venous grafts at 30 days following transplantation. Furthermore, the medial layer was infiltrated with all types of host immunocompetent cells in these allografts (Fig. 1).

**Tunica adventitia**

In immunosuppressed venous allografts on day 30 the adventitial layer was characterised by the infiltration by mononuclear cells (Fig. 1). Moreover, adventitial infiltration of CD4+ cells was significantly lower in immunosuppressed allogeneic animals ($p < 0.01$) compared to that observed in isogenic grafts. However, the degree of adventitial infiltration by CD8+ and Lewis MHC class II + cells was comparable in immunosuppressed allogeneic and syngeneic arterialised veins (Table 3).

In non-immunosuppressed venous allografts, tissue rejection led to massive wall infiltration by immunocompetent cells (Fig. 1). The total amounts of CD4+, CD8+ and host MHC class II positive cells that infiltrated the adventitial layer in the absence of immunosuppression were significantly higher ($p < 0.001$) than those observed in immunosuppressed allogeneic veins (Table 3).

**Discussion**

The present study examined the morphological changes of venous allografts implanted into the arterial system of rats. The results revealed that immunosuppression was necessary for the development of venous wall adaptation to arterial pressure within 30 days of transplantation. The suppression of the recipient immune response in the early post-transplantation phase led to the preservation of venous smooth muscle cells in transplanted veins. The presence and subsequent proliferation of these smooth muscle cells contributed to an increase in vascular wall thickness and mass, the typical features of venous arterialisation observed in syngeneic conditions.1
In the absence of immunosuppression, allogeneic venous grafts were unable to develop the typical signs of venous wall arterialisation. Allogeneic smooth muscle cells were destroyed by massive infiltration of immunocompetent cells and were not able to proliferate or adapt to the new biomechanical conditions in the arterial system.

There are two principal animal models described in the literature for studying the process of arterialisation in vivo. The first one, which was also used in this study, generates neointimal hyperplastic lesions and is performed by the dissection, removal and storage of vessels prior to insertion into the arterial circulatory system. The second one, first described by Kwei et al., connects the animal's external jugular vein to the carotid arterial circulation in situ (arterio-venous connection). Thus, the arterialised venous segment is never exteriorised from the body of the animal. The main difference between these models is the status of endothelial and smooth muscle cells at the beginning of the remodelling process. In the 'in situ model', at 7 days post-surgery, the intact endothelial layer promotes the proliferation of smooth muscle cells, the formation of smooth muscle cell layers and caused minimal apoptosis in the vascular wall; however, the 'ex situ model', at 7 days, showed significant cell loss and vessel degeneration. This resulted secondary to increased tensile stress following exposure to arterial blood pressure, vessel-wall ischaemia and free-radical oxidative injury associated with reperfusion. In the ex situ model, venous wall cell death occurred early, following implantation, both by necrosis and apoptosis. This was soon replaced by cellular proliferation in the adventitia and the tunica media. This resulted in increased microvessel density and smooth muscle cell regeneration during the first week. Neointimal formation took place thereafter.

Because ischaemic–reperfusion injury is inevitable in venous transplantation, all the venous grafts in our experiment were damaged at implantation. We assumed there were no differences in damage between the groups, because all groups had similar cold ischaemic times. However, smooth muscle cell regeneration and proliferation, followed by neointimal hyperplasia, were only possible in allogeneic grafts with immunosuppression, followed by neointimal hyperplasia, were only possible in allogeneic venous grafts with immunosuppression. Without immunosuppression, allogeneic venous grafts exhibited massive infiltration by immunocompetent cells and destruction of the venous wall.

In this study, we used FK506 for immunosuppression, which is more potent than cyclosporine A. In other studies, a combination of cyclosporine A and mycophenolate mofetil was used in dogs following allogeneic venous transplantation. They found a 100% patency rate at 20 weeks after implantation and immune reactions were markedly reduced. However, given alone, neither cyclosporine A at a 10 mg kg$^{-1}$ per day nor mycophenolate mofetil at 20 mg kg$^{-1}$ per day improved the overall patency of venous allografts. In the work of Mingoli et al., cyclosporine A at 20 mg kg$^{-1}$ day reduced aneurysmal dilatation and immunological responses at 30 days following allogeneic venous transplantsations in dogs. However, discontinuing cyclosporine A immunosuppression led to the occlusion of cold-stored grafts as well as cryopreserved venous allografts within 30 days.

Other studies have reported on the therapeutic potential of immunosuppression for the treatment of vein graft disease in syngeneic vein-to-artery implantations. Schachner et al. demonstrated reduced intimal thickness and decreased inflammatory cell infiltration following perivascular application of rapamycin in a mouse model. This reduction of vein graft disease was associated with a decreased amount of CD8$^+$ cell infiltration. In our model, we found no differences in the adventitial CD8$^+$ cellular infiltration between syngeneic grafts and allogeneic immunosuppressed grafts. Moreover, the extent of intimal proliferation in immunosuppressed allografts was comparable to that observed in syngeneic grafts.

Immunosuppressive therapy following allogeneic reconstructions is not generally accepted in clinical practice, despite experimental and clinical evidence of the potential benefits. We believe that inhibition of rejection and reduced destruction of the allogeneic venous wall with low systemic immunosuppression could improve the low patency rate of allogeneic transplantation. In our clinical practice, we have given this drug to patients with allogeneic alloarterial grafts at a mean daily dosage of 2 mg, which resulted in mean blood levels of 5 ng ml$^{-1}$. We found no or minimal adverse effects associated with this immunosuppressive regimen in a small, but high-risk, group of vascular patients. However, a controlled prospective clinical study is needed to investigate the impact of this immunosuppressive regimen on the patency rates of allovenous grafts.

In conclusion, the present study considered the positive effect of FK506 immunosuppression on the wall-remodeling process of venous allografts following implantation into the arterial system in rats. We suggest that the principal effects of FK506 under these conditions were the suppression of CD8$^+$ infiltration in the venous adventitial layer and the prevention of vascular smooth muscle cell depletion. The use of FK506 in clinical venous allotransplantation could be potentionally beneficial for increasing of the allovenous bypasses patency rates by the inhibition of venous wall destruction by the host’s immune system.

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References


