Cyclosporin Suppresses Transplant Arteriosclerosis in the Aorta-Allografted, Cholesterol-Clamped Rabbit

Suppression Preceded by Decrease in Arterial Lipoprotein Permeability

Henrik Ørbaek Andersen, Gitte Madsen, Børge G. Nordestgaard, Birgit Fischer Hansen, Knud Kjeldsen, Steen Stender

Abstract The immunosuppressant cyclosporin has been suggested to aggravate as well as retard the development of transplant arteriosclerosis, the major long-term problem for patients with heart transplants. We examined the effect of human therapeutic levels of blood cyclosporin on the development of experimental transplant arteriosclerosis. The thoracic aorta from one rabbit was transplanted as an end-to-side bypass on the abdominal aorta of another rabbit, and plasma cholesterol was clamped at 5 to 7 mmol/L. Cyclosporin markedly suppressed the severity of transplant arteriosclerosis, judged both biochemically and histologically: cholesterol content in aortic transplants was reduced by 70% and 80% after 10 days and 20 days of cholesterol feeding, respectively (both comparisons, P<.01), and after 20 days of cholesterol feeding myointimal proliferation was totally inhibited in grafts from cyclosporin-treated animals, judged from maximal intimal thickness and intimal area on cross sections of grafts (both comparisons, P<.05). In another group of non-cholesterol-fed, aorta-transplanted rabbits, cyclosporin reduced by 90% (P<.01) an otherwise markedly increased permeability to low-density lipoprotein in transplanted aortas. These results suggest that cyclosporin causes a substantial decrease in the severity of transplant arteriosclerosis and that this effect is mediated at least partly via a large decrease in aortic lipoprotein permeability. (Arterioscler Thromb. 1994;14:944-950.)

Key Words • cyclosporin • transplant arteriosclerosis • aorta-allografted rabbit • arterial lipoprotein permeability • cholesterol feeding

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tort-term survival after heart transplantation improved markedly after the introduction of cyclosporin as the principal immunosuppressive drug. However, due to the development of an accelerated form of arteriosclerosis, a process limited exclusively to arteries of the transplanted heart, including the aorta up to but not beyond the suture line, late survival has not been significantly improved.1 The lesions of transplant arteriosclerosis resemble those of conventional atherosclerosis in that both consist of intimal cell masses. However, the two types of lesions differ in several important aspects, since transplant arteriosclerosis is characterized by diffuse and concentric rather than focal and asymmetric lesions. The lesions in transplanted arteries include intimal proliferation of smooth muscle cells, which may be triggered by an immunologic mechanism,2-6 whereas the development of foam cells in the vessel wall seems to be induced by elevated plasma cholesterol levels in humans5,7 and in cholesterol-fed rabbits with heart transplants.8-10 It should be stressed, however, that in human transplant arteriosclerosis cholesterol deposits may be less important than in human conventional atherosclerosis. No consensus exists on the nomenclature for this process in the arteries of transplanted organs; we use the term "transplant arteriosclerosis."

Studies on the effect of cyclosporin on transplant arteriosclerosis in humans conflict: cyclosporin has been suggested to inhibit,10 to aggravate,11 and to have no effect.3,12 In the rat model, in which plasma cholesterol is around 1 mmol/L, the effect of cyclosporin on transplant arteriosclerosis in both allografted hearts13-16 and aortas17-20 has also been ambiguous. An allograft is a tissue transplanted from a donor to a recipient of the same species but of a disparate genotype. In the rabbit model, in which human levels of plasma cholesterol are easily induced, the effect of cyclosporin per se on the development of transplant arteriosclerosis is unknown. In the present study we used the aorta-allografted, cholesterol-fed rabbit model,21 in which plasma cholesterol is clamped at human levels (5 to 7 mmol/L), to investigate the effect of human therapeutic levels of blood cyclosporin on the development of transplant arteriosclerosis. This model has the advantage that survival of the graft is not dependent on immunosuppressive treatment. To elucidate the mechanism of cyclosporin as a suppressor of experimental transplant arteriosclerosis, the effect of cyclosporin on the permeability of low-density lipoprotein (LDL) in the transplanted arterial wall was also investigated.

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Methods

Using outbred male white rabbits of the Danish Country Strain, the thoracic aorta from one rabbit was transplanted as an end-to-side bypass on the abdominal aorta of another rabbit. Anesthesia was induced and maintained with repeated small doses of intravenous pentobarbital. A total dose of about 50 mg/kg body wt was given on average to each rabbit. The experiments were approved by the Danish animal experiment inspectorate.

Effect of Cyclosporin on Transplant Arteriosclerosis

Three successive experiments of individualized cholesterol feeding over 10, 20, and 20 days were performed. Ten, 10, and 8 transplanted rabbits were randomly assigned to intramuscular injections of cyclosporin or saline. Cyclosporin (Sandimmun, Sandoz) was given preoperatively (10 mg/kg) and subsequently in individualized doses once daily to obtain whole-blood trough levels in the human therapeutic range (0.08 to 0.33 μmol/L); blood cyclosporin concentrations were determined once a week (Emit Cyclosporine Assay, Syva Co). After a recovery period of 2 weeks, during which the rabbits were fed ordinary chow, each rabbit was fed cholesterol-enriched pellets (0 to 1 g cholesterol/rabbit per day) individually adjusted to achieve a mean plasma cholesterol concentration in the average human range of 5 to 7 mmol/L; plasma cholesterol concentrations were determined two to four times before cholesterol feeding, daily during cholesterol feeding in the 10-day experiment, and every second day in the two 20-day experiments. Lipoprotein cholesterol concentrations were determined at the start of cholesterol feeding, twice during cholesterol feeding in the 10-day experiment, and three times in the first 20-day experiment. Plasma and lipoprotein cholesterol concentrations were measured with an enzymatic kit (Boehringer Mannheim). High-density lipoprotein cholesterol was measured in the supernatant after precipitation of other lipoproteins (Boehringer Mannheim), very-low-density lipoprotein and intermediate-density lipoprotein were isolated by ultracentrifugation, and LDL cholesterol was calculated by the difference from total plasma cholesterol. After cholesterol feeding, the transplanted thoracic aorta and the rabbit's own corresponding thoracic aorta were removed, and the luminal surface area was outlined. The tissue was divided into an intima-inner media and an outer media layer, and each part was weighed. The tissues were stored at −20°C until further processing. Total, free, and esterified cholesterol contents were determined in the intima-inner media layers in rabbits from the 10-day and the first 20-day experiment.

In the second 20-day experiment, a 3- to 5-mm-long specimen of unopened aorta was taken from the central part of the graft. After fixation in neutral buffered formalin, this specimen was embedded in paraffin, and four serial sections for histomorphometric studies were prepared. The cross-sectional intimal area (expressed as number of points) and the ratio of area of the intima to area of the media were determined. Using the Leitz Microvic Computer Microscopy Module System, the maximal and minimal intimal thicknesses were likewise determined on each of the four cross sections of the same tissue, and the mean of the four measurements was recorded. Finally, the cross sections were evaluated independently and blindly for qualitative morphological features by two examiners (Dr Andersen and Dr Hansen, a pathologist).

Effect of Cyclosporin on Aortic Permeability

Eleven more rabbits received aorta transplants; 6 were given cyclosporin and 5 saline as described. After a recovery period of 2 weeks, during which the rabbits were fed ordinary chow, the 11 rabbits were injected intravenously with iodinated human LDL 1 hour before death. Blood samples were drawn at 10, 20, 40, and 60 minutes after injection. Immediately thereafter the animals were anesthetized (50 to 100 mg pentobarbital/kg), and the vascular system was perfused with 1 L saline via the left ventricle of the heart after the inferior vena cava was severed. Subsequently, the native thoracic aorta and the transplanted thoracic aorta were excised, the luminal surface area was outlined, and the tissue was separated into the intima-inner media and outer media layers. Trichloroacetic acid–precipitable radioactivity in plasma and in the intima-inner media was used to calculate aortic permeability by the "sink" method, in which aortic radioactivity is divided by the area under the plasma radioactivity curve.

Statistics

All results are given as mean±SEM. For comparison between the two groups, Wilcoxon's test for unpaired samples was used; P<.05 was chosen as the level of significance.

Results

Animals

Average body weight decreased after surgery and increased again during cholesterol feeding but did not reach preoperative levels in any of the groups except for the second 20-day experiment (Table 1). Cyclosporin-treated animals appeared less active than control rabbits, but otherwise the animals thrived.

Cyclosporin Treatment

Fig 1 depicts 24-hour blood cyclosporin in rabbits with aortic transplants after cyclosporin 8 mg/kg IM injection. Trough values did not increase during the study period; mean trough levels of cyclosporin were within the human therapeutic level of 0.08 to 0.33 μmol/L (Table 1).

Cholesterol Clamping

During the recovery period (after the operation but before cholesterol feeding), plasma cholesterol levels were higher in the cyclosporin-treated than in the saline-treated groups (Fig 2 and Table 1). During the individualized cholesterol-feeding period, however, the two treatment groups were clamped at similar mean plasma cholesterol levels. To achieve the desired plasma cholesterol level, cyclosporin-treated animals needed significantly less dietary cholesterol than did saline-treated animals.

Effect of Cyclosporin on Transplant Arteriosclerosis

Cyclosporin markedly suppressed accumulation of aortic cholesterol after both 10 and 20 days of individualized cholesterol feeding (Fig 3). At the levels of cyclosporin given, however, aortic cholesterol accumulation was not totally inhibited (Fig 3; compare cyclosporin-treated normal and aortic transplant animals). When aortic total cholesterol was separated into free
### TABLE 1. Body Weight, Cyclosporin Injections, Mean Trough Blood Concentrations, Mean Dietary Cholesterol, and Mean Plasma and Lipoprotein Cholesterol in Aorta-Transplanted Rabbits

<table>
<thead>
<tr>
<th></th>
<th>10-d Cholesterol Feeding</th>
<th>20-d Cholesterol Feeding</th>
<th>20-d Cholesterol Feeding</th>
<th>Aortic LDL Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline (n=5)</td>
<td>Cyclosporin (n=5)</td>
<td>Saline (n=5)</td>
<td>Cyclosporin (n=3)</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before operation</td>
<td>3.6±0.1</td>
<td>3.6±0.1</td>
<td>3.9±0.1</td>
<td>3.7±0.1</td>
</tr>
<tr>
<td>End study</td>
<td>3.5±0.2</td>
<td>3.3±0.1</td>
<td>3.7±0.1</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Cyclosporin, mg/kg per d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trough blood cyclosporin, μmol/L</td>
<td>9.8±0.3</td>
<td>6.8±0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet. cholesterol, g/kg per d</td>
<td>0.14±0.01</td>
<td>0.07±0.02*</td>
<td>0.14±0.02</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Plasm cholesterol, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During 2-wk recovery</td>
<td>0.76±0.04</td>
<td>1.86±0.12*</td>
<td>0.84±0.17</td>
<td>1.85±0.41</td>
</tr>
<tr>
<td>During cholesterol feeding</td>
<td>5.13±0.40</td>
<td>5.85±0.75</td>
<td>6.41±0.42</td>
<td>7.43±0.50</td>
</tr>
<tr>
<td>Lipoprotein cholesterol, mmol/L, during cholesterol feeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>1.14±0.54</td>
<td>1.40±0.60</td>
<td>1.24±0.41</td>
<td>1.53±0.42</td>
</tr>
<tr>
<td>IDL</td>
<td>0.77±0.37</td>
<td>0.99±0.33</td>
<td>1.10±0.37</td>
<td>1.34±0.34</td>
</tr>
<tr>
<td>LDL</td>
<td>1.94±0.85</td>
<td>2.30±0.85</td>
<td>2.05±0.67</td>
<td>2.96±0.66</td>
</tr>
<tr>
<td>HDL</td>
<td>0.60±0.04</td>
<td>0.95±0.06†</td>
<td>0.50±0.07</td>
<td>0.83±0.07‡</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; and HDL, high-density lipoprotein. Results are mean±SEM; for dietary cholesterol the range of cholesterol given to individual rabbits is in parentheses.

*P<.01 vs saline-treated group.
†P<.05 vs saline-treated group.
‡HDL cholesterol was higher (P<.05) in cyclosporin-treated groups compared with saline-treated groups when based on measurement in the supernatant after precipitation of apolipoprotein B-containing lipoproteins. However, in the 20-day experiment, HDL cholesterol was also measured in the d>1.063 g/mL fraction by using ultracentrifugation; by this measurement, HDL cholesterol was similar in both groups (data not shown).

and esterified cholesterol, the results were similar to those shown in Fig 3 (data not shown).

Examples of histological examinations of transplanted aortas in the two groups from the second-20 day experiment are shown in Fig 4. Neointimal proliferation was absent in grafts from cyclosporin-treated rabbits, and none of these grafts had ruptures of the elastic membranes. In contrast, all grafts from saline-treated rabbits showed neointimal proliferation including foam cells; there was edematous splitting of elastic membranes, and in three of five cases conspicuous rupturing of elastic membranes in the inner part of the media occurred. Adventitial accumulation of mononuclear cells was present in the grafts of four of five saline-treated rabbits, and in these grafts mononuclear cells were additionally located between elastic membranes. Such accumulation was never seen in the grafts from cyclosporin-treated rabbits. Maximal intimal thickness, intimal area, and the intimal area/media area ratio were all significantly lower in cyclosporin-treated aortic allograft rabbits than in saline-treated allograft rabbits (Table 2); the value of zero in cyclosporin-treated animals means that intimal tissue was not detected. Minimal intimal thickness (Table 2) as well as maximal and minimal thickness of media and medial area (data not shown) were not significantly different between the two groups.

![Fig 1](http://atvb.ahajournals.org/)  
Fig 1. Line graph showing blood cyclosporin concentrations (mean±SEM) during the 24-hour period after cyclosporin 8 mg/kg IM injection (n=3).
Discussion

The present study demonstrated that human therapeutic levels of blood cyclosporin markedly suppressed the development of transplant arteriosclerosis in the aorta-allografted rabbit clamped at a human level of plasma cholesterol.

The suppression of transplant arteriosclerosis by cyclosporin occurred in spite of a slightly higher plasma cholesterol level in cyclosporin-treated than in saline-treated rabbits during the 2-week period after surgery. The cholesterol-raising effect of cyclosporin in rabbits has been observed previously; however, the clamping of plasma cholesterol during the 10- or 20-day cholesterol-feeding periods eliminated this difference. The present study therefore examined the effect of cyclosporin on experimental transplant arteriosclerosis not mediated through differences in plasma cholesterol levels. Furthermore, because plasma cholesterol was clamped at human levels, the effects of both immunologic injury and the presence of elevated plasma cholesterol were studied. In this model myointimal cells proliferate and foam cells accumulate, which also occurs in the arteries of transplanted hearts in humans.

Experiments with heart transplantation in rabbits have depended on immunosuppression (cyclosporin) for the animals’ survival and have thus been unable to examine the effect of cyclosporin on transplant arteriosclerosis.

The cholesterol content in the transplanted aortas indicated that cyclosporin did not completely inhibit the development of transplant arteriosclerosis. Judged from the morphometric data, however, complete inhibition was obtained. Histological evaluation shows a significant development of transplant arteriosclerosis in transplanted hearts in cholesterol-fed rabbits despite treatment with cyclosporin, but in these studies rabbits were fed larger amounts of cholesterol than in the present study.

In the rat model, the findings on the effect of cyclosporin on transplant arteriosclerosis is equivocal. Using aorta-allografted rats, Mennander et al found a transient worsening of transplant arteriosclerosis by cyclosporin treatment; others found no effect or an inhibiting effect that disappeared after discontinuation of the treatment. One explanation for this discrepancy could be that studies with either a worsening effect or no effect used the lowest doses of cyclosporin (2 mg/kg SC, 5 mg/kg PO, or 5 mg/kg SC), whereas the one study with a reducing effect used a higher dose of cyclosporin (10 mg/kg SC). The latter dose is similar to the 8 to 10 mg/kg IM used in the present study. Studies with cardiac allograft in rats have shown that (1) discontinuation of cyclosporin treatment results in a worsening of transplant arteriosclerosis compared with continued treatment (15 mg/kg and 10 mg/kg IM, respectively); (2) an increase in the dose of cyclosporin from 1.5 mg/kg to 6 mg/kg SC reduces the development of transplant arteriosclerosis; and (3) cyclosporin in doses of 2.5 mg/kg SC and 10 mg/kg IM reduces the severity of transplant arteriosclerosis in rats with only weak immunohistological incompatibility. These results are not incompatible with our finding of cyclosporin as a suppressor of transplant arteriosclerosis.

The endothelial layer is a major barrier for lipoprotein penetration from plasma into the arterial wall.
Endothelial cells are the first foreign cells encountered by the host immune system in transplanted hearts or aortas. Significant endothelial denudation is observed in coronary arteries of transplanted rabbit hearts when cyclosporin is not administered, whereas in a similar animal model in which cyclosporin is used endothelial denudation is not observed. Such endothelial denudation would cause a large increase in arterial lipoprotein permeability with transplanted aortas without cyclosporin treatment (Reference 21 and present results). Alternatively, the large increase in arterial permeability could be related to leukocyte migration through intact endothelium, which would increase the incidental leakage of lipoproteins. In the present study this permeability increase was largely attenuated by cyclosporin.

A high arterial lipoprotein permeability would mean a large transport of lipoprotein cholesterol from plasma into the arterial wall; this may contribute to the accelerated development of transplant arteriosclerosis with foam cells. In normal cholesterol-fed rabbits, Nielsen et al report that a high aortic LDL permeability is a strong predictor for the severity of conventional atherosclerosis. Also, in cholesterol-fed rabbits in which the endothelial layer is removed with a balloon catheter, both the arterial lipoprotein permeability and the development of atherosclerosis are increased. The present findings seem compatible with the concept that cyclosporin protects the endothelial cells of transplanted arteries against immunologic injury, which then lowers the arterial lipoprotein permeability and thereby reduces the development of transplant arteriosclerosis.

A cytotoxic effect of cyclosporin on vascular endothelium in vitro can occur, but often the concentrations used are higher than those in the present study. However, in vivo studies using rat or rabbit carotid arteries, rat aortas, or coronary arteries from transplanted rabbit hearts have not shown any endothelial cytotoxic effect of cyclosporin. Yilmaz et al, in transplanted rat kidneys treated with cyclosporin, report a normal ultrastructure in the arteries but endothelial damage in the tubular compartments; this may be explained, however, by a higher concentration of cyclosporin in renal tissues compared with the blood compartment. Furthermore, balloon catheter-induced deendothelialization of arteries in vivo causes an increased lipoprotein permeability similar to the untreated

**Table 2. Histological Quantification of Severity of Transplant Arteriosclerosis in Thoracic Allografts**

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=5)</th>
<th>Cyclosporin (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimal minimal thickness, μm</td>
<td>43±25</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Intimal maximal thickness, μm</td>
<td>234±116</td>
<td>0.0±0.0*</td>
</tr>
<tr>
<td>Intimal area, No. of points</td>
<td>151±74</td>
<td>0.0±0.0*</td>
</tr>
<tr>
<td>Intimal area/medial area</td>
<td>0.34±0.14</td>
<td>0.0±0.0*</td>
</tr>
</tbody>
</table>

Allografted rabbits were treated with saline or cyclosporin and exposed to a 20-d cholesterol-feeding period. Results are mean±SEM.

*P<.05 vs saline-treated group.
grafted arteries in the present study. Therefore, since our present in vivo study demonstrated that cyclosporin markedly reduced the LDL permeability of grafted arteries, it seems likely that any cytotoxic effect of cyclosporin on the vascular endothelium either does not act at the blood concentration used in the present study or is less important than the protective effect of cyclosporin against immunologic injury.

Another mechanism for the reducing effect of cyclosporin on transplant arteriosclerosis is suggested from in vitro studies in which cyclosporin may inhibit smooth muscle cell proliferation, either directly or indirectly via an inhibition of endothelin, a compound liberated from endothelial cells with a stimulatory effect on smooth muscle cell proliferation. An indirect inhibition of smooth muscle cell proliferation by cyclosporin may also be achieved by the inhibition of induction of DNA synthesis by mutagens such as platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, or insulin-like growth factor. Since the introduction of cyclosporin as the principal immunosuppressant for patients with heart transplants, the 1-year survival rate has increased to at least 80%. However, cyclosporin may aggravate the major long-term problem of transplant arteriosclerosis. If the present findings apply to humans, it seems more likely that the use of cyclosporin has led to increased numbers of cardiac transplantations with improved short-term survival, thereby improving the clinical recognition of transplant arteriosclerosis.

In conclusion, the present study demonstrated that human therapeutic levels of cyclosporin reduce the development of transplant arteriosclerosis in the aortic allograft rabbit when plasma cholesterol is clamped at human levels. This enabled the study of the effect of cyclosporin in a transplant arteriosclerosis model with development of both myointimal proliferation and foam cells.

Acknowledgments

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References


Cyclosporin suppresses transplant arteriosclerosis in the aorta-allografted, cholesterol-clamped rabbit. Suppression preceded by decrease in arterial lipoprotein permeability.

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