Additive and Synergistic Effects of a Low-Molecular-Weight, Heparin-like Molecule and Low Doses of Cyclosporin in Preventing Arterial Graft Rejection in Rats

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Arteriosclerotic intimal proliferation is one of the main long-term complications of organ transplantation. Low-molecular-weight, heparin-like molecules prevent myointimal proliferation in arterial wall injury and limit rejection in skin allografts. Cyclosporin limits rejection but has no major effect on intimal proliferation. Therefore, an experimental protocol was designed to test whether heparin-like molecules interacted with low doses of cyclosporin to prevent arterial wall immune system injury and response in a model of arterial graft rejection in normotensive and hypertensive rats. Aortic allografts were performed in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) normotensive control rats. Four groups of 10 allografted (SHR and WKY) rats were used: one group was treated with placebo, one with low doses of cyclosporin (2 mg/kg body wt per day), one with low-molecular-weight, heparin-like molecule (1 mg/kg body wt per hour), and one with low doses of cyclosporin plus low-molecular-weight, heparin-like molecule. Ten SHRs and 10 WKYs were isografted and served as the control groups. All rats were killed 8 weeks after aortic grafting. Structural parameters of the grafted segment were measured by morphometric analysis on formalin-fixed sections with specific stains. The classical signs of immune system injury and response were present in the untreated allografts in SHRs and WKYs: inflammatory infiltration of the adventitia, medial injury, and intimal proliferative response. Low doses of cyclosporin had a significant beneficial effect on immune medial injury by increasing medial thickness and the number of remaining smooth muscle cells and decreasing the extracellular matrix injury. Cyclosporin had no protective effect on intimal proliferation. Low-molecular-weight, heparin-like molecules had a beneficial effect on both the medial injury and the intimal proliferative response that was independent of blood pressure. Heparin-like molecules increased medial thickness and partially prevented smooth muscle cell loss and extracellular matrix attack. They also significantly decreased the intimal thickness by acting more on the collagen content than on the smooth muscle cell density. Low doses of cyclosporin plus heparin-like molecules had a marked effect in preventing the arterial wall injury and response. This combination resulted in a normal medial appearance, increased medial thickness and smooth muscle cell number, and no intimal proliferation or adventitial inflammation. Thus, heparin-like molecules appear to act in concert with low doses of cyclosporin in preventing rejection-induced arterial wall remodeling in an experimental model of aortic allograft in rats, and their effects are independent of blood pressure.

KEY WORDS • heparin • cyclosporin • rats • graft rejection

Chronic arterial rejection is the major complication that arises from clinical organ transplants. In particular, posttransplant accelerated coronary arteriosclerosis is the main chronic complication after cardiac transplantation.1 Cyclosporin treatment has reduced the short-term rejection mortality rate, but posttransplant accelerated coronary arteriosclerosis appears to be the predominant pathology that limits the survival of long-term heart transplant patients,2 and this seems to be only slightly influenced by cyclosporin treatment.Billingham2 found that almost 40% of patients were suffering from this disease 3 years after transplantation. It is the sole reason for retransplantation after 1 year, and the third largest cause of mortality in cardiac transplantation. Bieber et al3 showed that posttransplant accelerated coronary arteriosclerosis involves the three layers of the arterial wall, with fibrointimal proliferation, medial necrosis, and adventitial inflammatory cell infiltration. The histopathological lesions of chronic posttransplant accelerated arteriosclerosis also occur after most other types of organ transplants, particularly those of the kidney.4

Posttransplant arteriosclerosis develops in response to immune injury of the arterial wall. This immunological injury to and response of the arterial wall can be experimentally reproduced by the aorto–aortic allograft model in rats.5–8 This experimental model is more
useful than experimental heart transplantation, as the former focuses on the pathological structure of the arterial wall. The longitudinal and regular morphology of the abdominal aorta is also suitable for quantitative analysis of arterial wall injury and response, whereas the irregular and complex anatomy of the coronary circulation is not. In this aortic allograft model, adventitial inflammatory cell infiltration provides evidence of the rejection process, injury is indicated by medial smooth muscle cell necrosis and breaks in the medial extracellular matrix, and intimal cellular proliferation and extracellular matrix accumulation are a measure of the vascular wall response. Hypertension increases the intimal proliferative response, and vasodilator treatment, which induces a fall in blood pressure, decreases the intimal thickness in this model in the same way that hypertension and its treatment modify the intimal response to balloon-induced deendothelialization and smooth muscle stretch.

High doses of cyclosporin prevent immune injury in this model of the aortic allograft in rats. Schmitz-Rixen et al not only reported that cyclosporin was beneficial in preventing medial injury in aortic allografts in rats but also showed that it had no major effect on intimal proliferation. These data have been recently confirmed by the study of Mennander and coworkers that describes the absence of any beneficial effect of cyclosporin on intimal proliferation in arterial allograft rejection. These experimental data fit well with the clinical data reporting the absence of any beneficial effect of cyclosporin treatment on accelerated posttransplant arteriosclerosis. Long-term treatment with cyclosporin is also frequently associated with hypertension.

Heparin and low-molecular-weight, heparin-like molecules have been reported to inhibit the intimal proliferative response to mechanical arterial wall injury in vivo. Lider et al have also recently reported that a low-molecular-weight, heparin-like molecule can partially prevent skin allograft rejection in mice. Experimental posttransplant arteriosclerosis appears to be a suitable target for heparin and heparin-like molecules because heparin can act on the two components of arterial graft rejection: immune injury and the intimal response. We have, therefore, examined the possibility that a low-molecular-weight, heparin-like molecule can act together with a low dose of cyclosporin to prevent chronic arterial graft rejection. This hypothesis was tested in an experimental design with our model of abdominal aortic allograft rejection in normotensive (Wistar-Kyoto [WKY]) and hypertensive (spontaneously hypertensive rat [SHR]) histoincompatible inbred rats.

**Methods**

**Experimental Design**

A total of 100 male SHRs (body weight, 190±30 g) and 100 male normotensive WKY control rats (body weight, 200±30 g), all 8 weeks old, were purchased from Ifa-Credo, Lyon, France. All animal care and use procedures were in accordance with the European Community Standards on the Care and Use of Laboratory Animals (No. 00577). Aortas were transplanted by using microsurgical techniques. Rats were anesthetized with pentobarbital (5 mg/100 g body wt i.p.). Two animals underwent operation simultaneously, one as the donor of the aortic graft and the other as the recipient. While it was viewed under the operating microscope, the infrarenal aorta was exposed from the left renal vein to the aortic bifurcation via a midline laparotomy. Any aortic branches in this segment were identified and ligated. Two microclips were placed, one below the renal arteries and the second above the aortic bifurcation. A 1-cm segment of aorta was removed from the donor animal and washed with saline. A similar resection of the infrarenal aorta was performed in the recipient rat. The donor segment was immediately inserted into the aorta of the recipient with end-to-end interrupted anastomoses using 9/0 nonabsorbable monofilament nylon sutures. Cold ischemic time was 30 minutes. No preservation solution other than saline was used. The patency of each graft was evaluated. No acute secondary graft thrombosis occurred during any of the experiments. The laparotomy was closed, and the rat was returned to its cage. All rats were fed a standard diet, and water was provided ad libitum.

In our first study, we demonstrated that the isografts did not significantly differ from the aortas of sham-operated control animals. Therefore, 10 syngeneic isografts were performed for each strain (SHR and WKY), which served as controls. Forty SHRs were allografted with aortas from WKYs, and 40 WKYs were treated similarly with aortas from SHRs. Ten animals of each recipient strain were treated with cyclosporin, 10 were treated with heparin, 10 were treated with heparin plus cyclosporin, and 10 allografted, vehicle-treated rats served as the allografted control group. This experimental protocol was designed to use a two-way analysis of variance (ANOVA) to test the effect of each drug alone and of their combination on the different parameters. In a two-factor ANOVA, a statistically significant positive interaction reflects a synergistic effect of the two treatments, whereas the absence of a significant interaction reflects only an additive effect of the two treatments. All treatments were begun 1 week before surgery and continued daily for 8 weeks. Cyclosporin A (Sandoz, Basel, Switzerland), dissolved in oil, was given by subcutaneous injection (2 mg/kg body wt daily). The low-molecular-weight, heparin-like molecule was given by continuous infusion (Lab Choay France IC 86 1772) with an osmotic infusion pump (Alzet, Alza Corp., Palo Alto, Calif.) that was placed in the subcutaneous tissue in the back. Active pumping began approximately 4 hours after implantation. The pumps contained 2 ml of solution that was pumped at a rate of 5 μl/hr. Heparin was infused for 8 weeks. Infusion pumps were replaced every 2 weeks while the rats were under ether anesthesia. Vehicle-treated animals received only subcutaneous injections of oil. Blood cyclosporin concentration was measured on total hemolyzed blood samples by a fluorescence polarization immunoassay (Abbott TDX analyzer) using a monoclonal antibody.

**Measurement of Aortic Graft Volumes**

Eight weeks after graft microsurgery, the rats were anesthetized with inactin (0.1 mg/100 g body wt i.p.), and the aortic graft volumes were determined by in vivo catheterization and isolation of the grafted segments. The catheter was filled with Evans blue dye and connected to a pressure transducer for simultaneous measurement of pressure and volume. The volume of the
TABLE 1. Effects of Rat Strain, Graft Type, and Treatment on Blood Pressure and of Treatment on Blood Cyclosporin Concentrations

<table>
<thead>
<tr>
<th>Blood pressure (mm Hg)</th>
<th>Strain effect</th>
<th>Treatment effect</th>
<th>Interaction of strain x treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (n=40)</td>
<td>F=68</td>
<td>F=1.63</td>
<td>F=1</td>
</tr>
<tr>
<td>SHR (n=40)</td>
<td>p&lt;10⁻³</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Blood cyclosporin (ng/ml)</td>
<td>p&lt;10⁻³</td>
<td>NS</td>
<td></td>
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</tbody>
</table>

Values are mean±SD. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. ANOVA, analysis of variance.

Morphological Studies

At the end of the pressure measurements the 1 cm of grafted abdominal aorta was fixed, in situ under anesthesia by infusion of 10% buffered formalin solution at mean arterial pressure. The graft was removed, dehydrated, and embedded in paraffin for light microscopy. Three successive sagittal sections (5 μm) were cut and stained, one for each of the structures in the aortic wall that was studied. Collagen fibers were stained with Sirius red and elastin/orcein, and the nuclei were stained with hematoxylin after periodic acid oxidation. Slides were analyzed in an automatic image processor (NS 1500, Nacchet-Vision, Paris, France).[17] An additional section was stained with Masson's trichrome for standard histological examination. Algorithms were developed to analyze the structures that were stained on each of the three successive sections. The first algorithm analyzed the mean medial thickness by measuring the distance between the internal and external elastic laminae (20 measurements on each section). The medial elastin network was analyzed in terms of the number of elastic laminae by measuring 10 fields in each section. The second algorithm analyzed the collagen matrix by measuring the relative area, density, and mean thickness of collagen fibers in 20 contiguous fields in each Sirius red-stained section. The third algorithm counted the number of nuclei within 20 fields in each section and measured the mean area of each nucleus. Repetitive measurements were performed, pooled, and averaged for each animal. Similar measurements of nuclear density and collagen were performed for determination of intimal proliferation.

Statistical Analysis

Results are expressed as mean±SD. The experimental design allowed a factorial two-way ANOVA to be used that would show the effects of rat strain and treatment and would compare the effects of the treatments. Linear regression curves and correlation coefficients were obtained by the least-squares method. Analysis of covariance was performed to test the influence of the various treatments on the relation between the density of adventitial inflammatory cells and the density of medial smooth muscle cells. In a pilot study, a nested ANOVA[18,19] was performed to determine the intraclass variance of the histomorphometric parameters in aortas from WKYs and SHRs that were given the two types of treatment for 3 months. Data were analyzed according to different levels of hierarchy: two strains, two treatments, 12 rats from each strain, and 20 fields from each section that were subjected to image analysis. The total number of measurements for each parameter was 960 (2x2x12x20). The results showed that for quantification of thickness, elastin, collagen, and nuclei, the observed variance was due to differences in strains and/or treatment but not to differences between rats from the same group or between fields from the same section.

Results

Blood Pressure and Cyclosporin Concentration

All SHR recipients had significantly higher blood pressures than did the WKYs. Allografts did not change blood pressure in SHRs or WKYs. The treatments did not alter the blood pressure in the two strains (Table 1).

A small "blank" of cyclosporin concentration was detected in the blood of control allograft and heparin-treated animals (Table 1). Cyclosporin alone increased the blood level of cyclosporin, whereas the combination of cyclosporin with heparin gave measurements that were higher than control values but lower than those with cyclosporin alone.

Aortic Volumes at 75 mm Hg

Allografted rats of both strains had a significantly increased aortic volume (F=44, p<0.001) than did isografted rats. There were also differences between the two recipient groups. The grafted aortic volumes in SHR recipients were greater than those in WKY recipients (F=10, p<0.001).

Cyclosporin or heparin alone did not change the allografted aortic volumes (F=0.6 for each treatment). In contrast, the allografted aortic volumes of rats treated with cyclosporin plus heparin were lower than those of untreated rats (F=6.2, p=0.01), rats given cyclosporin alone (F=4.5, p<0.05), or rats given heparin alone (F=5.4, p<0.05) and did not differ from those of isografted rats (F=2.7, NS). The cyclosporin and heparin interacted to statistically lower the volumes of allografted aortas (F=4.2, p<0.05) (Figure 1).

Morphological and Morphometric Results

Effect of the allograft. A monolayer of endothelial cells was present at the luminal pole of each arterial graft in all experimental groups. All untreated aortic allografts showed evidence of rejection under the light microscope (Masson's trichrome staining). The histological signs of arterial graft rejection were adventitial cellular infiltration, intimal thickening, and medial necrosis 2 months after transplantation. The lesions seen in this study were...
similar to those of other studies.\textsuperscript{5–8,12} Adventitial cellular infiltration indicated rejection, medial necrosis reflected the injury due to rejection, and intimal thickening reflected the arterial wall response (Figure 2).

The qualitative results were confirmed by the quantitative data. Medial thickness was lower in allografts than in isografts as a result of the disappearance of medial smooth muscle cells and of the decrease in collagen density and elastic laminae (Table 2). The intimal thickness of allografts was greater than that of the isografts (Table 3). There were also some differences between the two strains; these have been described in more detail in our first study.\textsuperscript{8} Allografted aortas were characterized by a significant increase in adventitial cell density (Table 4). The relation between rejection and arterial wall injury and response was indicated by the positive correlation between the intima/media thickness ratio and the density of adventitial inflammatory cells in untreated allografts ($r=0.53$, \( F=5.5, p<0.01 \)) and the positive correlation between adventitial inflammatory cell and medial smooth muscle cell densities ($r=0.7$, \( F=12.5, p<0.005 \)).

**Effect of cyclosporin alone.** Long-term treatment with cyclosporin significantly reduced medial allograft injury (Table 2). Medial thickness was significantly greater in cyclosporin-treated allografts than in untreated allografts but remained lower than in isografts ($F=6.6, p<0.01$). The number of remaining living cells in the media and medial collagen density were also higher. Last, the number of elastic laminae increased to a value similar to that of isografts. The intimal proliferation thickness of cyclosporin-treated rats was similar to that of untreated allografts (Table 3). The number of myointimal cells was significantly increased, but there was no significant change in collagen density from that of untreated allografts. Low doses of cyclosporin alone significantly

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**Figure 1.** Bar graphs showing effect of rat strain and treatment on allografted aortic volumes at 75 mm Hg perfusion pressure (values are mean±SEM expressed in cubic milliliters, \( n=10 \) per group). WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; cyclo, cyclosporin treatment; hep, heparin treatment.

**Figure 2.** Photomicrographs showing morphology of the aortic wall in isografted, and allografted Wistar-Kyoto rats (controls) treated with cyclosporin alone, heparin alone, or combined cyclosporin and heparin 2 months after grafting. Masson's trichrome stain, \( \times35 \).
TABLE 2. Effects of Rat Strain, Graft Type, and Treatment on Medial Injury

<table>
<thead>
<tr>
<th></th>
<th>Allograft (SHR and WKY)</th>
<th>ANOVA</th>
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<tr>
<td></td>
<td>Isograft (n=20)</td>
<td>Control (n=20)</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin (n=20)</td>
<td>Heparin (n=20)</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin plus heparin (n=20)</td>
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</tr>
<tr>
<td>Thickness (μm)</td>
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<td></td>
</tr>
<tr>
<td>WKY (n=50)</td>
<td>80±10</td>
<td>44±4</td>
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<tr>
<td></td>
<td>61±11</td>
<td>62±13</td>
</tr>
<tr>
<td></td>
<td>61±7</td>
<td>83±8</td>
</tr>
<tr>
<td>SHR (n=50)</td>
<td>88±7</td>
<td>54±9</td>
</tr>
<tr>
<td>Cell density (No./field)</td>
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<td></td>
</tr>
<tr>
<td>WKY (n=50)</td>
<td>20±3</td>
<td>0</td>
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<tr>
<td></td>
<td>9±4</td>
<td>9±4</td>
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<tr>
<td></td>
<td>21±4</td>
<td>13±2</td>
</tr>
<tr>
<td>SHR (n=50)</td>
<td>19±3</td>
<td>2±2</td>
</tr>
<tr>
<td>Collagen (%)</td>
<td></td>
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<tr>
<td>WKY (n=50)</td>
<td>15±5</td>
<td>7±3</td>
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<tr>
<td></td>
<td>9±4</td>
<td>16±6</td>
</tr>
<tr>
<td></td>
<td>16±8</td>
<td>16±4</td>
</tr>
<tr>
<td>SHR (n=50)</td>
<td>14±1</td>
<td>6.5±3</td>
</tr>
<tr>
<td>Elastin (No. of laminae)</td>
<td></td>
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<tr>
<td>WKY (n=50)</td>
<td>5.2±0.5</td>
<td>3.3±0.5</td>
</tr>
<tr>
<td></td>
<td>4.1±0.4</td>
<td>4.5±0.7</td>
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<tr>
<td></td>
<td>5.3±0.5</td>
<td>5.3±0.6</td>
</tr>
<tr>
<td>SHR (n=50)</td>
<td>5.4±0.5</td>
<td>3.8±0.4</td>
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</table>
| Values of morphometric parameters are mean±SD. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. ANOVA, analysis of variance.

Increased the number of adventitial inflammatory cells 2 months after the induction of the model (Table 4). As in untreated allografts, there was a positive correlation between intima/media thickness ratio and the density of inflammatory adventitial cells (r=0.5, F=5.3, p<0.05). In this group, there was also a positive correlation (r=0.46, F=4.6, p<0.05) between medial smooth muscle cell and adventitial inflammatory cell densities.

Effect of a heparin-like molecule. Long-term treatment with a heparin-like molecule also significantly reduced medial injury (Table 2). Medial thickness was significantly higher in heparin-treated allografts than in untreated allografts but was not different from that in the isografts (F=3.3, NS). The number of living cells remaining in the media, collagen density, and the number of elastic laminae were also increased. The effects of heparin treatment on medial protection against allograft injury were similar to those of low doses of cyclosporin alone. Heparin treatment greatly decreased intimal proliferative thickness compared with the untreated allograft (Table 3). Collagen density significantly decreased, but myointimal cell density increased. As with low doses of cyclosporin, the number of adventitial inflammatory cells was significantly higher in allografts that were treated with heparin alone than in untreated allografts (Table 4). There was no correlation between intima/media thickness ratio and the density of inflammatory adventitial cells (F=1, NS). As in the untreated and cyclosporin-treated allografts, there was a positive correlation (r=0.5, F=4.5, p=0.05) between medial smooth muscle cell and adventitial inflammatory cell densities.

Effect of heparin-like molecule plus cyclosporin. The effect of the combined treatment on injury and response in the allografted arterial wall was spectacular. Medial thickness was greater (F=104, p<0.001) than in the untreated allograft. The number of remaining living cells (F=381, p<0.001) and elastic laminae (F=88, p<0.001) and collagen density (F=17, p<0.001) in the media were also increased.

Except for medial thickness (F=0.40, NS), the effect of the combination of both treatments on medial smooth muscle cell (F=40, p<0.001), collagen (F=5.5, p<10-3), and inflammatory adventitial cells (F=1.5, p=0.05) was unchanged from that of the combination of both treatments on medial smooth muscle cell, collagen, and inflammatory adventitial cells. ANOVA, analysis of variance.
p<0.01). Nevertheless, there was no statistical interaction between cyclosporin and heparin (F=1.91, NS) did not significantly differ from those of isografted controls. Only the medial thickness was still less (F=8.18, p<0.01) than that of isografted aortas.

There was no intimal proliferation in the allografts that were treated with cyclosporin plus heparin (F=180, p<0.001). Nevertheless, there was no statistical interaction between heparin and cyclosporin in terms of a decrease in intimal thickness (Table 3). Therefore, the effect of cyclosporin plus heparin in preventing intimal thickening appears to be more than an additive effect. Cyclosporin and heparin interacted to reduce the number of smooth muscle cells and collagen density of the intimal proliferation.

The combination also interacted to prevent adventitial inflammation (Table 4). In contrast with cyclosporin and heparin alone, the density of inflammatory adventitial cells was significantly lower in allografts that were treated with cyclosporin plus heparin (F=15, p<0.001) than in untreated allografts. There was a tendency toward a negative correlation (r=−0.41, F=3.7, p=0.07) between medial smooth muscle cell and adventitial inflammatory cell densities in this group. Analysis of covariance showed the absence of any significant difference between isografts and the heparin plus cyclosporin–treated allografts (F=0.1, NS). Therefore, there was a negative correlation between adventitial and medial cell densities in isografts and heparin plus cyclosporin–treated allografts (Figure 3). In contrast, there was a general positive correlation between adventitial and medial cell densities in untreated, cyclosporin–treated, and heparin–treated allografts. Analysis of covariance showed that the heparin plus cyclosporin treatment had a significant influence on the relation between adventitial inflammatory cell and smooth muscle cell densities compared with cyclosporin or heparin alone.

**Discussion**

Experimental chronic arterial allograft rejection involves three types of lesions: medial necrosis, intimal proliferation, and inflammatory cell infiltration. The present study confirms our previous histological descriptions and those of Schmitz-Rixen et al., Mennander et al., and Häyry et al. Chronic arterial allograft rejection is a model of immune response–induced arterial wall injury. The medial necrosis is the consequence of the immune injury, whereas intimal proliferation represents the arterial wall response. The correlation between adventitial infiltration by inflammatory cells and the ratio of intimal proliferation to medial thickness in untreated allografts demonstrates one of the major features of the model, the importance of inflammation in the injury and response to chronic vascular immune aggression. Nevertheless, Mennander et al. reported that adventitial inflammation was predominant during the first month after arterial grafting in this model, a time when medial smooth muscle cells were still present. After this time, the adventitial inflammation decreased in parallel with the disappearance of medial smooth muscle cells. Our data confirm this result by showing a positive correlation between the number of medial smooth muscle cells and the adventitial inflammatory cell density in untreated allografts. These data show that not only endothelial cells but also medial smooth muscle cells participate in the chronic vascular rejection process.

**FIGURE 3.** Line plot of the relation between medial smooth muscle cell and adventitial inflammatory cell densities. There was a general positive correlation in untreated, cyclosporin–treated, or heparin–treated allografts (r=0.68, F=42, p<0.001) and a negative correlation in isografts and cyclosporin plus heparin–treated allografts (r=−0.4, F=4.75, p<0.05). Analysis of covariance demonstrated a significant influence of cyclosporin plus heparin (F=22, p<0.001) on this relation compared with heparin or cyclosporin alone. ○, Untreated allografts; □, cyclosporin treated; ●, heparin treated; ♦, cyclosporin plus heparin treated; Δ, isograft controls; open symbols, Wistar-Kyoto; filled symbols, spontaneously hypertensive rats. Values are mean±SD.
**Heparin Effects**

Low doses of cyclosporin protect the aortic media against destructive processes. An increase in medial smooth muscle cell density and partial protection of the extracellular matrix indicate the beneficial effect of cyclosporin in preventing arterial wall immune injury. These results are similar to those of Schmitz-Rixen et al. and Mennander et al., who used different doses of cyclosporin. Paradoxically, in our study low doses of cyclosporin apparently increased adventitial inflammation. This result is probably due to the chronology of the rejection process. The evidence of rejection probably decreases in untreated allografts in relation to the disappearance of smooth muscle cellular antigens 2 months after grafting. In contrast, low doses of cyclosporin could delay the appearance of rejection, so that the inflammation appeared greater in cyclosporin-treated animals than in untreated allografts 2 months after grafting. This inflammation could be related to the persistence of medial cellular antigens, as suggested by the positive correlation between medial smooth muscle cell and adventitial inflammatory cell densities. Nevertheless, the fibrointimal proliferative response was still present. This phenomenon may be analogous to the cyclosporin-resistant chronic obstructive arteriosclerosis that is seen in organ transplantation, particularly in heart transplantation.

Jonasson et al. have reported a decrease in intimal proliferative thickness in carotid artery deendothelialization in rats with high doses of cyclosporin. In contrast, Fens et al. did not find any change in intimal proliferation thickness after cyclosporin treatment in a carotid artery deendothelialization model in cholesterol-fed rabbits. In vitro, cyclosporin decreases the smooth muscle cell proliferation rate in a dose-dependent fashion. Therefore, clinical and experimental data tend to show that cyclosporin alone does not prevent the arteriosclerotic lesions of the arterial wall rejection process. Our data for low doses of cyclosporin confirm this general agreement.

**Heparin Effects**

Low-molecular-weight, nonanticoagulant, heparin-like molecules had two beneficial effects: one on the vascular wall injury and the other on the intimal proliferative response. Nevertheless, as with low doses of cyclosporin, heparin alone increased the density of adventitial cellular inflammation 2 months after grafting. The reason is probably similar to that suggested for cyclosporin: a delayed peak of rejection due to the persistence of medial cellular antigens.

Medial protection resulted in persistent medial smooth muscle cells and less destruction of the extracellular matrix. The effects of low-molecular-weight, heparin-like molecules on the rejection process were first described by Lider et al. in a mouse skin-allograft model. They reported that low doses of heparin-like molecules without anticoagulant effects altered T-lymphocyte functions in vitro and in vivo. Reports indicate that T lymphocytes have an endoglycosidase that specifically degrades the heparan sulfate side chains of the extracellular proteoglycans in vitro. Heparanase activity can generally be blocked in vitro by heparin. Inhibition of endogenous heparanase by a suitable dose of heparin limits tissue lymphocyte traffic. Others have shown anti-inflammatory effects of heparin or heparin-like molecules in several models. Bradfield and Born described the inhibition of lymphocyte emigration from blood into lymphoid tissue by heparin-like molecules in vivo. Heparin also modulates the complement system. Jaques reported a Clq-C1s junction inhibitory effect and a Cl esterase stimulatory effect in vitro. Other anticomplement effects have been attributed to heparin.

Heparin may also act on the two components of intimal proliferation, the smooth muscle cells and the extracellular matrix. Intimal thickness is clearly decreased by heparin. Clohesy and Tearney reported that heparin prevented intimal proliferation, and more recently Snow et al. obtained similar results in a model of carotid artery deendothelialization in rats. Nonanticoagulant, low-molecular-weight, heparin-like molecules were as effective as anticoagulant molecules in preventing intimal proliferation. In vitro, heparin inhibits smooth muscle cell migration and proliferation. Our present data also indicate that heparin-like molecules can limit intimal proliferation in response to arterial wall immune injury. The content of intimal proliferation was also modified by heparin. Collagen density decreased and cell density increased, indicating that heparin acts more on cell tropicinity than on the cell proliferative capacity. These results on intimal proliferation are in agreement with those of Snow et al., who reported a decrease in collagen density with heparin in a model of carotid artery deendothelialization in rats. In vitro, Tan et al. described a decrease in collagen synthesis by smooth muscle cells in culture after the addition of heparin. Castellot et al. reported that the production of a heparin-like molecule by bovine aortic endothelial cells in culture could inhibit smooth muscle cell proliferation in vitro. Heparan sulfates could be synthesized by normal quiescent endothelial cells and so prevent smooth muscle cell migration and proliferation. Endothelial injury may disrupt this physiological balance.

**Heparin Plus Cyclosporin**

The heparin plus cyclosporin combination dramatically protects the allografted arterial wall against rejection. Medial injury is prevented, and there is no intimal proliferation. The medial thickness is preserved, as are the elastin and collagen contents. Medial smooth muscle cell density remains similar to that of the isografted aortic wall. Intimal proliferation disappears. Despite the persistence of large amounts of medial cellular antigens, adventitial inflammatory infiltration is also lower than in untreated and cyclosporin- or heparin-treated allografts (covariance analysis).

Chronic arterial rejection is a major determining factor in the outcome of organ transplants. The immune-response remodeling of the arterial wall involves both medial injury and intimal proliferation. Cyclosporin alone, by its specific effect on T-helper lymphocytes, lowers immunological rejection and medial injury but has no major beneficial effect on intimal proliferative
response. Heparin, by its inhibitory effect on heparanase activity, acts nonspecifically on the tissue traffic of lymphocytes, macrophages, and smooth muscle cells. It partially inhibits medial injury and prevents the intimal proliferative response. The combination of heparin plus cyclosporin, by acting on different cell targets, prevents medial injury and the intimal proliferative response. The beneficial effect of heparin plus cyclosporin cannot be attributed to an increase in cyclosporin concentration because blood cyclosporin levels decreased in the presence of heparin. The positive statistical interaction between the effects of cyclosporin and heparin on two components of graft rejection, adventitial cellularity and persistence of medial smooth muscle cells, suggests that the two treatments act synergistically on rejection injury. In contrast, cyclosporin and heparin do not interact in the prevention of intimal thickening. Therefore, the beneficial effect of the combination on the intima predominantly involves an additive effect rather than a synergistic effect.

References

Additive and synergistic effects of a low-molecular-weight, heparin-like molecule and low doses of cyclosporin in preventing arterial graft rejection in rats.
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doi: 10.1161/01.ATV.13.1.112
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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