Hirudin Reduces Tissue Factor Expression and Attenuates Graft Arteriosclerosis in Rat Cardiac Allografts

Hans Hölschermann, MD; Rainer M. Bohle, MD; Heiko Schmidt, MD; Hagen Zeller, MD; Ludger Fink, MD; Ulrich Stahl, MD; Helmut Grimm, MD; Harald Tillmanns, MD; Werner Haberbosch, MD

Background—Intravascular clotting has been implicated in the pathogenesis of cardiac allograft vasculopathy (CAV). We previously identified the expression of tissue factor (TF), the primary cellular initiator of blood coagulation, within the coronary intima, which was associated with neointimal thickening. In the present study, the effect of recombinant hirudin on CAV was assessed in Lewis to Fisher rat heterotopic cardiac allografts.

Methods and Results—Transplant recipients were randomized to a control group (n = 10) and a hirudin-treated group (n = 12; 2 mg·kg⁻¹·d⁻¹ SC). Histological evaluations of rejection, CAV, and TF staining were performed 120 days after transplantation. No significant differences were observed between the 2 groups with respect to the degree of rejection. Hirudin significantly (P < 0.05) suppressed the development of CAV in the graft microvessels, but it was less effective in large coronary arteries. Graft intimal cells, isolated by laser-assisted cell picking, showed a marked upregulation of TF gene transcription, which was prevented by hirudin (P < 0.01). As demonstrated by immunohistochemistry and quantitative analyses of TF mRNA levels by real-time polymerase chain reaction, hirudin treatment resulted in a significant reduction of TF protein and mRNA expression (P < 0.001).

Conclusions—Treatment with hirudin in this rat cardiac transplant model inhibited TF expression and decreased neointimal hyperplasia. These results suggest that TF inhibition by hirudin, in addition to its direct effect on thrombin, may attenuate the hypercoagulable state and prevent the development of CAV at least in restricted sites of the graft coronary vasculature. (Circulation. 2000;102:357-363.)

Key Words: transplantation | hirudin | arteriosclerosis

Cardiac allograft vasculopathy (CAV), the leading cause of morbidity and mortality among long-term heart transplant recipients,1 is characterized by a diffuse process of concentric narrowing of large epicardial arteries and small penetrating intramyocardial branches.2 Its heterogeneous presentation in large- and small-vessel compartments suggests that the rate of progression or the pathobiology of large- and small-artery disease may be discordant.3 CAV is believed to be the result of immune system– and non–immune system–dependent mechanisms,4 whereby an initial coronary endothelial injury leads to a deleterious disturbance of the local homeostatic vessel wall equilibrium. One change in endothelial cells that occurs apparently independently of immunological damage is predisposing the artery to fibrin deposits,5 which are not seen in stable grafts but are present in patients who subsequently develop a poor clinical outcome.6 Histological examinations characterizing intraluminal thrombus and fibrin formation along the graft vessel intima7 suggest that the intravascular activation of the coagulation system observed in cardiac transplant recipients8–11 might be closely involved in lesion formation. We previously demonstrated that tissue factor (TF) is aberrantly expressed within the coronary intima in rat cardiac allografts.12 Given its role as primary initiator of the coagulation cascade,13 intravascular TF expression might represent the predominant stimulus for fibrin deposition observed in allograft vessels.13

Therapeutic strategies have only partially fulfilled the expectation that they would retard neointima formation after transplantation.1 Pharmacological agents that equilibrate the hemostatic imbalance observed in the graft vessel system might favorably affect the development of CAV. Hirudin, a specific and potent inhibitor of thrombin,14 has been shown to attenuate cell proliferation in other models of vessel injury.15,16 In the present study, we examined the influence of recombinant hirudin on TF expression and CAV developing in long-term surviving rat cardiac allograft recipients.
Methods

Heterotopic Heart Transplantation
Adult male (250 to 300 g) Lewis (RT 1) and F-344 (RT 1) rats (Charles River Laboratories, Kingston, NY) were used as donors and recipients, respectively. Heterotopic heart transplantations were performed as described. Allografts were followed up for 120 days after transplantation. Graft function was assessed daily by palpation and rated on a scale of 0 to 4 (0=no mechanical activity, 4=vigorous normal beats).

Treatment
Animals were randomized to a control group (n=10) and a hirudin-treated group (n=12). All recipients were administered daily intraperitoneal cyclosporin A (Sandoz, Ltd), with the initial dose of 2 mg·kg body wt⁻¹·d⁻¹ reduced to 0.5 mg/kg on day 80. In addition, animals of the hirudin treatment group received the recombinant hirudin lepirudin (HBW 023, Hoechst Marion Roussel Germany GmbH) in 2 divided doses of 1 mg/kg body wt each, given every 12 hours subcutaneously. Animals treated with cyclosporine alone served as controls, receiving 2 injections of physiological saline subcutaneously daily.

Histological Examination
The heart allografts and the recipients’ own hearts were removed 120 days after transplantation. Native nontransplanted donor hearts served as controls. Hematoxylin and eosin–stained sections of paraffin-embedded samples were examined by standard light microscopy and scored for both the severity of rejection and CAV (Table 1, Figure 1), as recently described in detail. Large vessels were defined as arteries with >2 smooth muscle cell layers, and small

![Figure 1.](http://circ.ahajournals.org/)

**Figure 1.** Coronary arteries showing progressive CAV. Stage 0: normal small intramyocardial artery with intact internal elastic lamina and single-cell-layer endothelium (A). Stage 1 lesion: early coronary lesion with patchy endothelial protrusions and adherence of a few inflammatory cells (B). Stage 2 lesion: advanced lesion of CAV with concentric intimal proliferations and <50% obliteration of lumen (C). Stage 3 lesion: severe vasculopathy with high-grade intimal proliferations and inflammatory cell infiltrate (D). Magnification ×60; bar=20 μm; hematoxylin-eosin/elastica stained.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Rejection</th>
<th>Vasculopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No rejection</td>
<td>Vessel unaffected</td>
</tr>
<tr>
<td>1</td>
<td>Mild rejection with scanty mononuclear cell infiltrate, minimal or no fibrosis</td>
<td>Accumulation of inflammatory cells along intimal surfaces but with &lt;10% occlusion of the lumen</td>
</tr>
<tr>
<td>2</td>
<td>Moderate rejection with moderate mononuclear cell infiltrate</td>
<td>More advanced lesion, including definite intimal proliferation and thickening with &lt;50% occlusion of the lumen</td>
</tr>
<tr>
<td>3</td>
<td>Severe rejection with diffuse and severe mononuclear cell infiltrate, focal hemorrhage, and necrosis</td>
<td>High-grade occlusion of the vessel with &gt;50% occlusion of its lumen</td>
</tr>
</tbody>
</table>
vessels were defined as arteries/arterioles with ≤2 smooth muscle cell layers.

**Immunostaining**

Immunostaining was performed by the alkaline phosphatase/anti-alkaline phosphatase technique. Immunohistology for TF antigen was performed, as recently described in detail, with the murine monoclonal rabbit TF antibody AP-1 (0.375 μg/mL, kindly provided by Dr Michael D. Ezekowitz, Yale University, New Haven, Conn), the production, purification, and characterization of which are reported elsewhere. Negative controls were performed with mouse anti-rabbit immunoglobulin (clone MR 12/53, 0.425 μg/mL, DAKO). Sections were counterstained with hematoxylin and mounted in gelatin.

**Laser-Assisted Cell Picking and TF Reverse Transcriptase–Polymerase Chain Reaction**

Cryosections (5 μm) of the transplanted hearts (n=22), the recipients’ native hearts (n=22), and nontransplanted donor hearts (n=11) were prepared for mRNA extraction from complete heart slices. For cell picking, 3 sections of each heart were mounted on glass slides. Oligo cell samples of intimal cells (5 to 10 cells) of arteries from transplanted as well as from nontransplanted donor hearts were collected by cell picking after UV-laser microbeam dissection of cells (P.A.L.M.), as described in detail. cDNA synthesis and reverse transcriptase–polymerase chain reaction (RT-PCR) of cell-picking templates were performed as described. One half of the cDNA was used for porphobilinogen deaminase (PBGD) housekeeping-gene detection. The second half was used for TF PCR together with 10 pmol of reverse (5'-CTTTCTCGGCTTCCTTCTCCT-3') and forward (5'-AGTAAAAATTTAAGCCTTCCCTGGTA-3') primer sequence. PCR conditions for cell-picking templates were 95°C for 6 minutes 45 seconds, 55 cycles of (95°C for 20 seconds, 61°C for 30 seconds, and 73°C for 30 seconds), and 73°C for 5 minutes.

**Relative TF mRNA Quantification**

**mRNA Extraction**

Five to 10 cryosections (10 μm) of frozen rat heart tissue (left and right ventricle) were collected in a 1.5-mL reaction tube and lysed in 300 μL lysis buffer of the Dynabeads mRNA direct kit. On the basis of magnetic separation, mRNA is caught by attachment to oligo-dT fragments that are coupled to supermagnetic glass particles. Per sample, 100 μg of beads was applied. Isolated mRNA was finally dissolved in 20 μL DEPC-treated H₂O; 10 μL was reverse transcribed.

**Relative mRNA Quantification**

Relative mRNA quantification was performed by the Sequence Detection System 7700 (PE Applied Biosystems) and real-time PCR. On the basis of the following equation, we used comparative quantification (A_Ct) normalizing the target gene to an internal standard gene as recently described in detail:

\[ T/ R = K \times (1 + E)^{(C_T - C_T)} \]

where \( T_0 \) is the initial number of target gene mRNA copies; \( R_0 \) the initial number of standard gene mRNA copies; \( E \) efficiency of amplification; \( C_T \), the threshold cycle of the target gene; \( C_T \), the threshold cycle of standard the gene; and \( K \), a constant.

For internal calibration, we used mRNA transcribed from the PBGD gene, a ubiquitously as well as consistently expressed standard gene that is free of pseudogenes. In preliminary experiments we could show that amplification efficiency of PBGD and TF primer/probe sets was approximately equal and amounted to 0.9±0.02 (90±2%). \( K \) is assumed to be equal within a definite fluorogenic labeled primer/probe system and thus does not influence the comparison of calculated relative ratios.

**cDNA Synthesis and Real-Time PCR**

For cDNA synthesis, reagents and incubation steps were applied as described recently. Reagents for real-time PCR were also used as described there. Briefly, 2 μL cDNA was applied to each sample. Oligonucleotide primers were added to a final concentration of 300 nmol/L each, and hybridization probes (TF: 5'-AGTGGCTGCACAAACGGCCACC-3'; PBGD: 5'-CCAGCTACTTCCTCCC-3') to a final concentration of 200 nmol/L in a volume of 50 μL. Cycling conditions were adapted to 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 61°C for 60 seconds.

**Statistical Analysis**

Data are expressed as mean±SD. Differences were compared by the 2-tailed Mann-Whitney test. The Scheffé procedure was used to determine significance of TF mRNA levels between groups. The significance of differences of cell-picking samples from transplanted control and hirudin-treated hearts was determined by the 2-tailed Fisher’s exact test. Statistical significance was indicated by a value of \( P<0.05 \).

**Results**

**General Observations**

Neither general behavior nor body mass development was influenced by long-term administration of hirudin. Moreover, no hemorrhagic complications were observed with hirudin either in the immediate perioperative or in the late posttransplantation period. At the time the rats were euthanized, all the cardiac allografts were beating, with no statistically significant difference observed between groups with respect to the graft function score (3.30±0.82 versus 3.75±0.45; \( P=NS \)).

**Histopathological Findings**

The results of the morphological assessment of graft rejection and CAV are listed in Table 2 and depicted in Figure 2. There was no significant difference in the histological degree of rejection between the two groups, with most grafts having evidence of moderate rejection (mean rejection grade, 2.10±0.57 in the control group and 2.25±0.62; \( P=NS \) in the hirudin group). The nontransplanted native donor hearts generally demonstrated an entirely normal morphological structure.

Rat cardiac allografts in both groups developed coronary lesions indistinguishable in appearance from human CAV. Early lesions demonstrated patchy endothelial protrusions, endothelial swelling, and adherence of a few mononuclear cells along the vessel intima. More advanced lesions were characterized by a marked cellular expansion of the intima and an inflammatory infiltrate typically found within the internal elastica lamina and in a halo zone exterior to the adventitia. Occasionally, the internal elastic membrane was stretched and focally disrupted in advanced lesions. Diffuse fibrointimal thickening that markedly compromised the lumen resulted in a virtually complete occlusion of some coronary arteries. Representative photographs of affected vessels, in the stages as defined in Table 1, are depicted in Figure 1.

A total of 436 artery cross sections in 10 control and 12 hirudin rats were available for the study. Up to 90% of coronary vessels were affected by CAV in the control animals. Heart grafts from hirudin-treated recipients had significantly lower frequency and severity of small-vessel CAV than those from the control animals. As shown in Figure 2A, the overall incidence of intimal thickening of small coronary vessels (≤2 smooth muscle cell layers) was significantly lower in grafts from the hirudin group than in those
from controls (69±11% versus 89±8%; P<0.005). Similarly, the severity of intimal thickening of small vessels was significantly lower in hirudin-treated animals than controls (1.10±0.30 versus 1.47±0.37; P<0.05; Figure 2B). In the hirudin group, the distribution of vessels was shifted toward less severe luminal occlusion (Figure 2C). A nonsignificant reduction in the percentage of diseased large vessels (>2 smooth muscle cell layers), with a occlusive severity distribution similar to that of small vessels (Figure 2D), was found between the control and the hirudin groups (96±8% versus 85±14%; P=NS; Figure 2A). The average lesion grade of diseased large vessels also was reduced but did not differ significantly between groups (1.63±0.47 versus 1.46±0.82; P=NS).

**TABLE 2. Grading of Graft Rejection and Transplant Vasculopathy**

<table>
<thead>
<tr>
<th></th>
<th>Control Group (n=10)</th>
<th>Hirudin Group (n=12)</th>
<th>P (Hirudin vs Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft rejection</td>
<td>2.10±0.57</td>
<td>2.25±0.62</td>
<td>NS</td>
</tr>
<tr>
<td>Vessels graded, n</td>
<td>215</td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>Diseased vessels±SD, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large arteries</td>
<td>96±8</td>
<td>85±14</td>
<td>NS</td>
</tr>
<tr>
<td>Small arteries</td>
<td>89±8</td>
<td>69±11</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Mean disease grade±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large arteries</td>
<td>1.63±0.47</td>
<td>1.46±0.82</td>
<td>NS</td>
</tr>
<tr>
<td>Small arteries</td>
<td>1.48±0.37</td>
<td>1.10±0.30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Vessels positive for TF±SD, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large arteries</td>
<td>71±15</td>
<td>48±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Small arteries</td>
<td>52±9</td>
<td>28±6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Figure 2.** Effect of hirudin therapy on frequency and severity of CAV. A, Decrease in number of small vessels (≥2 smooth muscle cell layers) with intima proliferation is observed in hirudin group vs controls (P<0.005), whereas no significant difference occurred in large vessels. In total, 436 coronary arteries in control (n=10) and hirudin (n=12) groups were analyzed. B, Significant reduction in degree of intimal thickening is observed in small vessels in hirudin group vs control (P<0.05). C and D, In hirudin group, distribution of vessels is shifted toward less severe luminal occlusion.
TF Antigen Expression in Transplant Coronary Vessels

TF was detectable in rat coronary allograft vessels predominantly in the coronary intima, with moderate labeling of the adventitia and no or only faint focal staining of the media. A representative photograph of a transplant coronary artery section after immunostaining with antibodies to TF is shown in Figure 3. As reported earlier, intimal TF-positive cells were identified as endothelial cells lining the vascular lumen by colocalization of a rat panendothelial antigen in serial sections. TF expression on coronary endothelium was found in all stages of CAV but was detected most frequently within less severe arterial lesions.

Reduction of Coronary TF Expression With Hirudin

For comparisons in terms of TF antigen labeling, a total of 221 arterial segments were analyzed in the control group and 215 segments in the hirudin group. Positive vascular TF staining was found in \( \approx 50\% \) of the arterial sections (small vessels, 52\%\%; large vessels, 71\%\%\%) in the control group. As shown in Figure 4, treatment with hirudin was associated with a significant decrease in the frequency of small vessels positive for intimal TF staining (control, 52\%\%\%; hirudin, 28\%\%\%; \( P<0.001 \)). Similarly, fewer large coronary vessels positive for TF antigen were found in hirudin-treated animals; however, half of the large vessels still stained for TF (48\%\%; \( P<0.001 \) versus control). The number of vessels positive for TF in the recipients’ native hearts was also found to be significantly reduced with hirudin versus controls (small vessels, 27\%\%\% control versus 12\%\%\% hirudin; large vessels, 22\%\%\% control versus 3\%\%\% hirudin; \( P<0.001 \)).

Figure 3. Presence of TF antigen in coronary lesions. A, Small intramyocardial artery from control heart with no endothelial TF staining. B, Endothelial TF expression in small coronary artery of transplanted heart. C and D, Endothelial TF detection at luminal border of intimal proliferation. Anti-TF immunohistochemistry; A and B, bar=20 \( \mu \)m; C and D, bar=40 \( \mu \)m.

Figure 4. Effect of hirudin therapy on intimal TF antigen expression. Decrease in number of vessels positive for intimal TF staining is observed in hirudin group vs control (\( P<0.001 \)) in both large and small vessels.
The upregulation of TF mRNA was absent in the nontransplanted donor hearts (P<0.001), while in the hirudin group (P=0.075). Animals treated with hirudin also showed reduced TF gene transcript levels in native recipient hearts (native hearts: P=0.06 vs control). K=constant.

TF protein expression observed in the allograft coronary endothelium was related to the induction of TF gene transcription in response to transplantation. Confirming our own previous data, qualitative RT-PCR of laser-dissected coronary endothelial cells detected TF mRNA transcripts in >80% of intima samples in the transplanted donor hearts, whereas TF mRNA was detectable in only ≈20% of intima samples in the nontransplanted donor hearts. Treatment with hirudin prevented the observed induction of TF gene transcription in the allograft coronary endothelium: whereas in the control group (without hirudin), TF mRNA transcripts were demonstrable in 7 of 8 cell samples (88%) of laser-dissected allograft coronary intima cells, in the hirudin-treated group, TF transcripts were detected in only 1 of 7 endothelial cell samples (14%) (P<0.01).

Quantification of TF mRNA by real-time PCR revealed a significant induction of TF gene transcripts in control cardiac allografts compared with the nontransplanted native donor hearts (P<0.001) or with the native recipient hearts (P<0.001). This upregulation of TF mRNA was absent in the hirudin group (Figure 5). TF transcript levels in transplanted hearts of hirudin-treated animals were even lower than in the nontransplanted donor hearts (P=0.075). A similar reduction of TF gene transcription with hirudin was observed in the recipients’ native hearts. Taken together, hirudin therapy reduces TF gene transcription in both the recipients’ native hearts as well as the transplanted hearts.

Discussion

We report here that recombinant hirudin significantly attenuates the development of microvessel transplant vasculopathy. The association with reduced TF expression in this vessel compartment suggests that this advantageous effect may be related to the suppression of aberrant TF synthesis occurring in graft coronary vessels.

Figure 5. Effect of hirudin therapy on TF transcript levels. TF mRNA quantification in heart slices was performed by real-time RT-PCR. A significant upregulation of TF mRNA in transplanted hearts was seen in control group (P<0.001 vs nontransplanted). This upregulation was prevented with hirudin (P<0.001 vs control). In contrast, transplanted hearts from hirudin-treated animals even had fewer TF gene transcripts than nontransplanted hearts (P=0.075). Animals treated with hirudin also showed reduced TF gene transcription in native recipient hearts (native hearts: P=0.06 vs control). K=constant.

The results of the present study show a significant decrease in CAV with hirudin. Treatment with hirudin reduced the severity and frequency of graft intimal thickening and shifted the distribution of occlusive severity toward milder forms. A reduction of CAV with hirudin was observed in both vessel compartments of the transplanted hearts; however, its beneficial effect was restricted mainly to the microcirculation. The different responsiveness of the macrovascular and microvascular beds might reflect the well-known heterogeneous presentation of CAV in large and small coronary arteries. Alternatively, because the components of hemostasis are differentially regulated in large and small vessels, thrombin generation may vary in distinct coronary compartments. Further studies are needed to evaluate whether higher doses of hirudin are necessary to affect CAV also in the macrovascular coronary bed or whether the resistance of macrovascular vessels to hirudin might reflect local mechanisms that compensate or superimpose the antithrombotic potency of hirudin.

This possibility might be supported by a further finding of the present study. Blood coagulation is activated when blood is exposed to TF, a membrane-bound glycoprotein normally absent from circulating blood. We previously demonstrated that TF is aberrantly expressed within the intima of coronary arteries after rat cardiac transplantation. These data have meanwhile been confirmed in human transplant vasculopathy. The finding of the present study that treatment of heart transplant recipient rats with hirudin reduces both neointima formation and aberrant expression of TF within the graft coronary intima might suggest a direct relationship between intravascular TF expression and the intimal proliferative response. The advantageous effect of hirudin against CAV may rely at least in part on the ability of hirudin to inhibit endothelial TF expression and thereby cause a shift in the prothrombotic endothelial phenotype associated with atherogenic mechanisms. This hypothesis is supported on the one hand by reports that hirudin reduces the expression of TF, cell proliferation, and vascular lesion development in other animal models, and on the other hand by the fact that the blockade of TF, known to be a strong chemotactic factor for smooth muscle cell migration, suppresses neointima formation after artery injury. However, neointima formation in CAV probably involves a complex intercellular network of macrophages, T lymphocytes, endothelial cells, and smooth muscle cells, generating a vast number of stimulatory cytokines and growth factors. With this, although it is attractive, a causal association of TF expression with CAV remains to be proved. Further studies are necessary to elucidate whether reduced expression of TF or the blockade of the hemostatic and nonhemostatic effects of thrombin with hirudin (or both) is responsible for the prevention of graft neointima formation. As demonstrated by qualitative and quantitative TF mRNA analysis, the mechanism by which hirudin suppresses TF
expression apparently is transcriptional and seems not be restricted to the allografted heart, because a downregulation of TF transcript levels was also observed in the recipients’ native hearts. This effect of hirudin could result either from direct inhibition of TF gene transcription or from inactivation of thrombin, which is known to be an agonist itself for TF induction in endothelial cells.28

Although the prevention of CAV with hirudin was restricted primarily to the small vessels, our observations might still have important clinical implications. The fact that small intramyocardial branches, which are not amenable to mechanical interventions, are affected by CAV29 is probably the reason that the long-term results of coronary revascularization procedures are disappointing.30 Thus, an urgent need exists for seeking out pharmacological options that might be beneficial in retarding lesion formation in this particular coronary compartment. The promising data on the beneficial effect of hirudin might prepare the ground for initiating clinical trials testing the efficacy of hirudin to reduce vascular lesion development after heart transplantation.

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References
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