Early Plus Delayed Hirudin Reduces Restenosis in the Atherosclerotic Rabbit More Than Early Administration Alone

Potential Implications for Dosing of Antithrombin Agents

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Background—A 2-hour infusion of r-hirudin at the time of balloon angioplasty limits restenosis in atherosclerotic rabbits. Because thrombin activity in the vessel wall after angioplasty remains high for 48 to 72 hours, we hypothesized that a second infusion of hirudin at 24 hours would reduce restenosis more than early treatment alone.

Methods and Results—Femoral atherosclerosis was induced in 35 rabbits by air desiccation injury and a high-cholesterol diet. At the time of angioplasty, rabbits were randomly assigned to 1 of 4 groups: controls: heparin bolus, saline infusion at 24 hours; early hirudin: hirudin bolus 1 hour infusion, saline infusion at 24 hours; delayed hirudin: heparin bolus, hirudin infusion bolus at 24 hours; and early+delayed hirudin: hirudin bolus 1 hour infusion, hirudin infusion bolus at 24 hours. Rabbits were euthanized after 28 days. The early+delayed hirudin treatment group had less loss of minimal lumen diameter by angiography at 28 days. By histomorphometry, cross-sectional area narrowing by plaque was least in the early+delayed treatment group compared with controls (P=0.0001), early hirudin (P=0.01), or delayed hirudin (P=0.001). The early+delayed hirudin group also had a significant reduction in absolute plaque area and an improvement in lumen area compared with the other groups. No differences were observed between treatment groups with respect to the cross-sectional area encompassed by the internal or external elastic laminae.

Conclusions—Combined early+delayed administration of hirudin significantly reduces angiographic restenosis and cross-sectional area narrowing by plaque compared with early or late treatment alone. These results suggest that restenosis after balloon angioplasty is markedly influenced by thrombin-mediated events not only occurring early but also extending beyond the first 24 hours in this model. (Circulation. 1998;98:2301-2306.)

Key Words: restenosis ■ atherosclerosis ■ hirudin

Thrombin is generated in large amounts at sites of arterial injury and is a potent stimulus for platelet aggregation and thrombus formation.1 In vitro, thrombin is a potent smooth muscle cell (SMC) mitogen, and SMC proliferation is one of the proposed mechanisms of restenosis.2 We have previously shown that a bolus plus short-term (2-hour) infusion of recombinant desulfatohirudin (r-hirudin) or the specific factor Xa inhibitor antistasin reduces angiographic restenosis and luminal cross-sectional area narrowing by atherosclerotic plaque (CSAN-P) 28 days after angioplasty.3,4 In vitro, we have demonstrated that thrombin receptor mRNA expression is upregulated within 6 hours of exposure of quiescent vascular smooth muscle cells (VSMCs) to thrombin.5 However, prolonged exposure to thrombin (10 to 12 hours) is required to promote maximal VSMC mitogenesis. Hirudin blocks this proliferative response even when hirudin is administered as late as 32 hours after thrombin exposure.5 In the rabbit model, we have shown that thrombin activity associated with the vessel wall peaks at 48 hours after balloon angioplasty (BA). A short (2-hour) infusion of hirudin at the time of angioplasty significantly reduced vessel wall-associated thrombin activity for up to 24 hours after BA.6 By 48 hours, however, thrombin activity was equally elevated in control and hirudin-treated animals, returned to near-baseline levels by 72 hours, and remained low at 7 days. The present study was designed to determine the effect of early plus delayed administration of hirudin, compared with early or late administration alone, on restenosis after BA in the double-injury hypercholesterolemic rabbit model.

Methods

The study design is summarized in Figure 1. Bilateral focal femoral atherosclerosis was induced in 35 New Zealand White rabbits by air desiccation injury and a high-cholesterol diet. At the time of angioplasty, rabbits were randomly assigned to 1 of 4 groups: controls: heparin bolus, saline infusion at 24 hours; early hirudin: hirudin bolus 1 hour infusion, saline infusion at 24 hours; delayed hirudin: heparin bolus, hirudin infusion bolus at 24 hours; and early+delayed hirudin: hirudin bolus 1 hour infusion, hirudin infusion bolus at 24 hours. Rabbits were euthanized after 28 days. The early+delayed hirudin treatment group had less loss of minimal lumen diameter by angiography at 28 days. By histomorphometry, cross-sectional area narrowing by plaque was least in the early+delayed treatment group compared with controls (P=0.0001), early hirudin (P=0.01), or delayed hirudin (P=0.001). The early+delayed hirudin group also had a significant reduction in absolute plaque area and an improvement in lumen area compared with the other groups. No differences were observed between treatment groups with respect to the cross-sectional area encompassed by the internal or external elastic laminae.

Conclusions—Combined early+delayed administration of hirudin significantly reduces angiographic restenosis and cross-sectional area narrowing by plaque compared with early or late treatment alone. These results suggest that restenosis after balloon angioplasty is markedly influenced by thrombin-mediated events not only occurring early but also extending beyond the first 24 hours in this model. (Circulation. 1998;98:2301-2306.)

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desiccation endothelial injury followed by a 2% cholesterol/6% peanut oil diet for 28 days as described previously. The rabbits were randomly assigned to one of four treatment groups: control (CTL), early hirudin only (EH), delayed hirudin only (DH), or early plus delayed hirudin (EH+DH). BA was performed and rabbits were euthanized 28 days later for angiographic and histomorphometric analysis as previously described. The rabbits were treated according to the Animal Welfare Act specifications.

**Drug Administration**

CTL animals (n=6) received bolus heparin (150 U/kg, heparin sodium, 1000 USP units/mL, porcine intestinal mucosa, Solopak Laboratories) at the time of angioplasty and a 2-hour placebo infusion at 24 hours. Animals in the EH group (n=7) received an intravenous bolus of r-hirudin (1 mg/kg, CGP 39393, CIBA-Geigy Ltd) followed by an infusion of 1 mg kg\(^{-1}\) h\(^{-1}\) for 2 hours. These animals (CTL and EH) received a 2-hour saline infusion at 24 hours. Animals in the DH group (n=10) received bolus heparin (150 U/kg) at the time of BA and were treated at 24 hours with hirudin (1 mg kg\(^{-1}\) h\(^{-1}\) for 2 hours \pm 1 mg/kg IV bolus). Animals in the EH+DH group (n=12) received a 1-mg/kg IV bolus of hirudin followed by a constant infusion of 1 mg kg\(^{-1}\) h\(^{-1}\) for 2 hours at the time of BA and were treated again at 24 hours with hirudin (1 mg kg\(^{-1}\) h\(^{-1}\) for 2 hours \pm 1 mg/kg IV bolus).

**Angioplasty and Analysis**

After baseline angiography, BA was performed with a 2.5-mm angioplasty balloon in each femoral artery (three 60-second inflations at 10 atm). The site of minimal luminal diameter (MLD) was measured by quantitative angiography. After 28 more days, final angiography was performed and the distal arterial tree was perfused at physiological pressure with 4% buffered paraformaldehyde. Each femoral artery segment was cut in cross section at 2-mm intervals, dehydrated in 70% ethanol and xylene, and embedded in paraffin. Sections (5 μm) from each 2-mm segment were stained with Verhoeff–van Gieson elastin stain, and quantitative histopathology was performed at the site of the most severe luminal narrowing by an observer blinded to treatment groups. The internal elastic lamina (IEL) and external elastic lamina (EEL) were identified. Luminal narrowing was assessed as percent CSAN-P=(IEL area-lumen area)/IEL area\times100. Overall vessel size was measured by the total area bounded by the EEL.

**Statistical Analysis**

Data are reported as the number of femoral arteries in each experimental group and expressed as the mean±SEM. Angiographic and histopathological differences between treatment groups were analyzed by 1-way ANOVA followed by unpaired Student’s t test to evaluate 2-tailed levels of significance. Quantitative histomorphometric data were fitted by linear regression, and the fitted lines were plotted to demonstrate relationships between arterial wall component areas. A value of P=0.0125 was considered significant to account for the Bonferroni correction when 3 treatment groups were compared with controls. Otherwise, P<0.05 was considered significant.

**Results**

**Partial Thromboplastin Times**

The mean partial thromboplastin time increased by >2-fold after anticoagulation in all groups. The mean partial thromboplastin time at 1 hour among the groups treated with hirudin before angioplasty (CTL or EH) was not significantly different from those receiving hirudin (EH or EH+DH) treatment (131±14 versus 164±12 seconds, P=NS).

**Angiography**

The angiographic data are summarized in Table 1 and Figure 2. At baseline (before BA), there were no differences in MLD among the 4 treatment groups. The MLD increased significantly in all treatment groups at 30 minutes after BA, with no differences in the postangioplasty MLD between groups. All vessels were patent immediately after angioplasty in the CTL, the EH, and the EH+DH groups. Sixteen of 19 vessels were patent immediately after angioplasty in the DH group. All

**TABLE 1. Quantitative Angiographic Analysis**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Before BA</th>
<th>After BA</th>
<th>After BA</th>
<th>ΔMLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL (n=9)</td>
<td>0.91±0.08</td>
<td>1.32±0.08</td>
<td>0.87±0.12</td>
<td>-0.46±0.10</td>
</tr>
<tr>
<td>EH (n=13)</td>
<td>0.88±0.09</td>
<td>1.38±0.10</td>
<td>1.14±0.10</td>
<td>-0.24±0.07</td>
</tr>
<tr>
<td>DH (n=16)</td>
<td>0.84±0.06</td>
<td>1.29±0.04</td>
<td>0.91±0.08</td>
<td>-0.39±0.08</td>
</tr>
<tr>
<td>EH+DH (n=20)</td>
<td>0.85±0.06</td>
<td>1.36±0.09</td>
<td>1.24±0.06</td>
<td>-0.12±0.03</td>
</tr>
</tbody>
</table>

ΔMLD indicates change in MLD (mm) from after BA to 28 d after BA.

* Differences among treatment groups were not significant before or after BA.
† Differences among treatment groups were significant at 28 days by ANOVA (P<0.01).
§ EH+DH vs CTL (P=0.01).
|| ΔMLD was significantly different by ANOVA (P=0.001).
vessels that were occluded immediately after angioplasty were excluded from further analysis.

At 28 days, significant differences were observed between treatment groups with respect to luminal diameter ($P < 0.01$, ANOVA). Significantly larger luminal diameters were observed in the EH DH group compared with CTL (1.24 ± 0.08 versus 0.87 ± 0.12 mm, $P < 0.01$). There was a trend toward larger luminal diameters in the EH group compared with CTL (1.14 ± 0.10 versus 0.87 ± 0.12 mm, $P = 0.09$).

**Analysis of Angiographic Restenosis**

Angiographic restenosis was prospectively defined as the change in MLD from immediately after BA to 28 days after BA. The change in MLD for each group can be seen in Table 1. By ANOVA, significant differences between treatment groups were observed with respect to change in MLD ($P < 0.001$). As seen in Figure 2, the smallest change in MLD occurred in the EH DH group, which differed significantly from CTL ($P = 0.0003$), from EH ($P = 0.01$), and from DH ($P = 0.01$). As we have observed previously,$^3$ the EH group had significantly lower mean percent CSAN-P than CTL ($P = 0.01$). The DH group (which did not receive hirudin early after angioplasty) did not differ from CTL and was significantly more narrowed by plaque than the EH group ($P = 0.01$) and the EH DH group ($P = 0.001$). Thus, when treatment with hirudin at 24 hours is added to an initial treatment at the time of BA, significant additional reduction in CSAN-P is observed. This improvement is not observed when hirudin is administered at 24 hours without treatment at the time of BA.

**Analysis of Arterial Remodeling**

By ANOVA, there were significant differences among the treatment groups with respect to plaque area ($P = 0.02$), lumen area ($P = 0.01$), intima/media ratio ($P = 0.004$), and CSAN-P ($P < 0.0001$). No differences were seen with respect to the area bounded by the IEL or the area bounded by the EEL. Figure 3 compares the individual components of the arterial wall between the CTL and the EH DH groups. Early plus delayed hirudin administration resulted in significant reduc-

**TABLE 2. Quantitative Histomorphometric Analysis**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>$P$, ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>EH (n=13)</td>
</tr>
<tr>
<td>CSAN-P, %</td>
<td>55±4</td>
</tr>
<tr>
<td>Plaque area, $\mu$m$^2$</td>
<td>0.72±0.1</td>
</tr>
<tr>
<td>Lumen area, $\mu$m$^2$</td>
<td>0.62±0.1</td>
</tr>
<tr>
<td>I/M ratio</td>
<td>2.27±0.67</td>
</tr>
<tr>
<td>IEL area, $\mu$m$^2$</td>
<td>1.35±0.18</td>
</tr>
<tr>
<td>EEL area, $\mu$m$^2$</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

I/M indicates intima/media ratio. See Figures 2 and 3 for individual statistical comparisons.
tion in absolute plaque area, improvement in absolute lumen area, and reduction in luminal CSAN-P. No differences were observed between these treatment groups with respect to overall arterial size (IEL or EEL area).

A positive correlation was seen across all treatment groups with respect to plaque area and elastic lamina areas as measured by either IEL ($R^2=0.44$, $P<0.0001$) or EEL ($R^2=0.43$, $P<0.0001$) areas. The 4 treatment groups did not differ, however, with respect to either IEL or EEL areas by quantitative histomorphometric analysis. As shown in Figure 4, the regression line comparing plaque area versus IEL area (panel A) is shifted significantly upward and to the left in the EH+DH group compared with CTL ($P<0.0001$). The same observation was made comparing the plaque area versus EEL area ($P<0.0001$) (panel B). As demonstrated in Figure 4, the smaller mean plaque area observed in the EH+DH group compared with CTL. Thus, although increasing plaque area is associated with increasing overall arterial area, the beneficial treatment effect observed with early plus delayed administration of hirudin resulted from reduced plaque area, increased lumen area, and reduced CSAN-P but not from changes in overall IEL or EEL areas.

**Discussion**

We have previously reported that thrombin inhibition with hirudin administered as a bolus and infusion during the first 2 hours after BA reduces angiographic restenosis and luminal CSAN-P at 28 days after BA in atherosclerotic rabbits. The present study confirms and extends these findings. We demonstrated that additional treatment with hirudin at 24 hours after BA resulted in significant further limitation of restenosis and further reduction of luminal CSAN-P at 28 days. We recently reported that the initial bolus and 2-hour infusion of hirudin inhibits thrombin activity in the arterial wall for up to 24 hours, long after its clearance from serum. At 48 hours, however, thrombin activity in the arterial wall increases and is not significantly less than in heparin-treated controls. The finding that additional dosing of hirudin at 24 hours further reduces restenosis suggests that the residual thrombin activity may be biologically relevant and/or that important thrombin-mediated events influencing the angioplastied artery may occur beyond the first 24 hours after injury in this model. The lack of effectiveness of delayed administration of hirudin again emphasizes the importance of early thrombin inhibition on outcome after BA.

The mechanism(s) of the beneficial effects of hirudin in the atherosclerotic rabbit model remains elusive. Thrombin is known to be a potent mitogen of VSMCs in culture. Although thrombin receptor mRNA expression is upregulated within 2 hours of exposure of quiescent cultured VSMCs to thrombin, we previously demonstrated that prolonged thrombin exposure (10 to 12 hours) was needed for maximal VSMC mitogenesis. Even if thrombin is blocked up to 4 hours after exposure to SMCs, the in vitro proliferative response is attenuated. In addition, progressive thrombin-induced recruitment of VSMCs into the growth fraction is significantly inhibited even when hirudin administration was

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Arterial wall components. Relative areas of arterial wall components in control (Ctls) compared with EH+DH treatment groups. CSAN-P is expressed as percent.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Relationship between plaque area vs IEL or EEL areas. Linear regression fit lines comparing plaque area with IEL area (A) and with EEL area (B). In each case, early hirudin+delayed hirudin fit line is significantly shifted upward and to left vs control ($P<0.0001$). Mean plaque area and corresponding mean elastic lamina area are depicted with respective regression lines for controls (solid line) and for EH+DH group (dashed line).
delayed for as long as 32 hours after thrombin exposure. Results of the present study, however, do not support a similar temporal profile with respect to thrombin inhibition and restenosis in vivo. For example, when hirudin administration was delayed for 24 hours after BA in vivo, neither angiographic restenosis nor luminal CSAN-P was significantly different from those in heparin-treated controls. Moreover, we have previously demonstrated that [H]thymidine labeling indices are increased by 4- to 10-fold at 72 hours after angioplasty in our model, but no detectable differences in [H]thymidine labeling indices were seen between hirudin-treated and control groups. Taken together, these studies suggest that hirudin limits restenosis by mechanisms other than simple inhibition of SMC proliferation.

Thrombin is a multifunctional serine protease with myriad known biological effects. These effects have complex interactions with numerous molecules and cell types known to be present in the arterial wall after injury. Thus, there are numerous potential mechanisms for the observed effects of hirudin on restenosis in the “double-injury” atherosclerotic rabbit model. Using cell-specific markers, we have previously characterized the cellular components of the atherosclerotic plaques in hirudin- versus heparin-treated rabbits. These studies, however, have failed to demonstrate obvious differences in the cellular composition of the plaques after hirudin treatment. Differences in cell migration might explain the effects of thrombin inhibition; however, this is difficult to study in a double-injury model, in which a baseline plaque is present at the time of angioplasty (as in human angioplasty). Differences in the composition and amount of extracellular matrix are another potential mechanism for the beneficial effects that we observed with hirudin.

Arterial remodeling to an overall smaller vessel size has been reported to occur after BA in humans and animal models, and several investigators have suggested this phenomenon as a mechanism to explain restenosis. In the present study, we did observe a significant correlation between plaque area and overall arterial area (IEL area or EEL area) across all the treatment groups and within each treatment group. This correlation has been reported previously in humans on the basis of both pathological and intravascular ultrasound observations. Within the treatment groups in the present study, however, we observed that the regression line relating plaque area to the elastic lamina area (IEL or EEL area) was shifted upward and to the left for the EH group compared with CTL (Figure 4). Thus, the beneficial effects observed in this study were related to significant differences with respect to plaque area, lumen area, and luminal CSAN-P but not to differences in overall arterial size. This is consistent with our previous observations that arterial remodeling to an overall smaller size is not the predominant mechanism to explain the beneficial effect of hirudin in our model.

The effects of specific inhibition of thrombin in humans undergoing BA remain uncertain. In the Helvetica trial, hirudin-treated patients had fewer acute complications, but these beneficial effects did not persist at later time points. In addition, there was no apparent effect of hirudin on late restenosis or target-vessel revascularization. Similar results were reported with Hirulog, another direct thrombin inhibitor. These studies limited the overall dose of hirudin (or Hirulog) to minimize hemorrhagic complications. It is important to note that the doses used in these clinical studies were less than those previously shown to minimize platelet and fibrinogen deposition in experimental models of deep arterial injury after BA and less than the dose used in the present study. In the present study, we used an intermittent dosing regimen of hirudin based on our previous observation (in an ex vivo model) that a 2-hour intravenous hirudin bolus and infusion reduced thrombin activity in the arterial wall at 24 hours, long after its anticoagulant effect (as measured by activated partial thromboplastin time) had returned to normal. This introduced the concept of using intermittent higher doses of hirudin to maintain low levels of thrombin activity in the arterial wall at later time points. Whether this dosing strategy may have applications in humans remains unknown. Optimal dosing in humans will require further clinical trials that explore the unique pharmacology of the thrombin inhibitors in patients with complex atherosclerosis undergoing coronary interventions.

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