A Novel Strategy for Myocardial Protection by Combined Antibody Therapy Inhibiting Both P-Selectin and Intercellular Adhesion Molecule-1 Via Retrograde Intracoronary Route

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Background—Antibody therapy to inhibit either P-selectin or intercellular adhesion molecule-1 (ICAM-1) has been reported to provide myocardial protection against leukocyte-mediated reperfusion injury. Because these molecules play different roles in the leukocyte-endothelial interaction, co-inhibition of both may achieve further enhanced cardioprotection. In addition, the therapeutic efficacy of such antibody therapy may be affected by the delivery route used. Retrograde intracoronary infusion will offer an effective, direct access to the postcapillary venules, where the target event (leukocyte–endothelial interaction) takes place. We investigated the feasibility and efficiency of the combined antibody therapy targeting both P-selection and ICAM-1 via the retrograde intracoronary route to attenuate myocardial ischemia-reperfusion injury.

Methods and Results—Lewis rats underwent 30-minute left coronary artery occlusion. Just before reperfusion, anti-P-selectin monoclonal antibody (150 μg/kg), anti-ICAM-1 monoclonal antibody (200 μg/kg), both antibodies together, or control antibody were retrogradely infused into the left cardiac vein. At 24 hours after reperfusion, administration of either anti-P-selectin or anti-ICAM-1 antibody significantly (P<0.05) improved left ventricular ejection fraction and attenuated infarct size (40.6±3.2% and 34.8±3.5%, respectively) compared with the control (56.8±3.4%). This was associated with reduced leukocyte accumulation and improved regional blood flow in the ischemic area. Noticeably, co-administration of both antibodies achieved a much greater reduction in infarct size (19.1±3.6%), associated with greater attenuation in leukocyte infiltration, compared with administration of either single antibody.

Conclusions—Combined antibody therapy inhibiting both P-selectin and ICAM-1 via the retrograde intracoronary route could be a promising new strategy for myocardial protection against ischemia-reperfusion injury. (Circulation. 2006;114[suppl 1]:I-251–I-256.)

Key Words: antibody therapy ■ cell adhesion molecules ■ leukocytes ■ myocardial protection ■ reperfusion
where the target antigens are upregulated to facilitate leukocyte–endothelial interaction, as compared with any other infusion methods including intravenous and antegrade intracoronary infusion. In this article, we investigated the feasibility and efficiency of the combined antibody therapy targeting both P-selectin and ICAM-1 by retrograde intracoronary injection to attenuate myocardial ischemia–reperfusion injury using our original model in rat.9

Methods

Induction of Myocardial Ischemia-Reperfusion Injury

All studies were performed with the approval of the local ethical committee. The investigation conforms to the Principles of Laboratory Animal Care (National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (NIH Publication). Male Lewis rats (300 to 350 grams; Charles River, UK) underwent left thoracotomy under anesthesia with sodium pentobarbital (40 mg/kg, intraperitoneal) and mechanical ventilation. The left coronary artery (LCA) was occluded for 30 minutes and then coronary injection to attenuate myocardial ischemia-targeting both P-selection and ICAM-1 by retrograde intra-

Retrograde Intracoronary Infusion of Antibodies

Just before reperfusion, hearts were treated with retrograde intracoronary infusion of mouse monoclonal anti-P-selectin antibody (150 µg/kg, Santa Cruz; P-selectin group), mouse monoclonal anti-ICAM-1 antibody (200 µg/kg, Santa Cruz; ICAM-1 group), both antibodies (co-inhibition group), or mouse IgG (350 µg/kg, eBioscience; control group). The antibody dosages were decided based on the previous reports using intravenous infusion, because there were no reports in which antibodies to P-selectin or ICAM-1 were successfully infused into the coronary circulation. One-tenth of the reported dosages were used for retrograde intracoronary infusion, because the blood flow perfusing the heart is considered to be 5% to 10% of the systemic blood flow. Retrograde intracoronary infusion was performed as we have recently described. The perfusion area by this infusion method has been shown to fully encompass the whole ischemic area after LCA ligation. Through a 24-gauge catheter, which was inserted into the left cardiac vein, the appropriate antibody suspended in 0.5 mL phosphate-buffered saline was injected into the vein over a period of 5 seconds. Then, 25 seconds after the end of injection, occlusion of LCA was released for reperfusion. During this 30-second period, the stem of the cardiac vein was snared to prevent flush out of infused reagents.

Assessment of Cardiac Function and Dimension

At 24 hours after reperfusion, all the other rats were again anesthetized for echocardiography (n=14 for each group) using the Sequoia 512 system (Acuson, Siemens). LV diastolic dimension (LVDd) and LV systolic dimension (LVDs) were measured with M-mode. LV ejection fraction (LVEF) was calculated with 2-dimensional trace. After completion of echocardiography, the rats were used for one of the following assessments: measurement of infarct size (n=5 in each group), evaluation of regional myocardial blood flow (n=4), and histological analysis (n=5).

Measurement of Infarct Size

The heart was then excised, cut into 5 sections in base–apex axis, and frozen in OCT compound. The embedded samples were cut into 10-µm cryosections, which were fixed in 4% paraformaldehyde. These sections were incubated with monoclonal anti-rat granulocyte antibody (1:20; Serotec) and then with R-phycocerythrin conjugated secondary antibody (1:200; Serotec). The sections were counterstained with 4',6-diamidino-2-phenyindole (DAPI; 1:1000). After observation and recording with a fluorescence microscope, the sections were stained with hematoxylin and eosin. The number of polymorphonuclear leukocytes (PMNLs) per high-power field was calculated to give an indication of the degree of acute myocardial inflammation. Nine different fields of the area at risk of infarction in each section were randomly selected and analyzed.

Detection of Antibody Injected Into the Heart Via Retrograde Intracoronary Route

In addition, monoclonal anti-ICAM-1 antibody was labeled with Alexa Fluor 546 (Invitrogen) and was infused in the same manner of retrograde intracoronary infusion (n=2). The heart was reperfused for 30 seconds and excised; 10-µm cryosections were counterstained with DAPI. After fluorescence recording, the sections were stained with hematoxylin and eosin.

Statistical Analysis

All values are expressed as means±SEM. Statistical comparison of the data were performed using 1-way ANOVA followed by Bonferroni test for individual significant difference. A value of P<0.05 was considered statistically significant.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript written.

Results

Distribution of Antibodies Injected Via Retrograde Intracoronary Route

The Alexa Fluor 546-labeled monoclonal anti-ICAM-1 antibody was exclusively attached to endothelial cells of veins, venules, and postcapillary venules (Figure 1A to 1D). No arteries, arterioles, or capillaries were positively labeled. No antibody was detected on cardiomyocytes.

Enhanced Cardiac Function After Antibody Therapy

Cardiac function was measured with echocardiography (Table 1). Baseline values (intact normal animals; n=6) of heart rate, LVDd, LVDs, and LVEF were 365±15 bpm, 6.7±0.3 mm, 4.0±0.2 mm, and 78.3±1.2%, respectively. After ischemia and reperfusion, all the groups demonstrated a reduction of LVEF compared with the baseline data (P<0.05). However, both the P-selectin and ICAM-1 groups...
demonstrated improved LVEF compared with the control group ($P<0.041$ and $P<0.001$, respectively). Furthermore, LVEF in the co-inhibition group was enhanced compared with the P-selectin group ($P<0.029$). Heart rate in every group tended to be higher (not significant) than the baseline, presumably caused by surgical stress.

**Reduced Infarct Size by Antibody Therapy**

The area at risk of infarction and the infarct area were determined by Evans Blue and TTC staining (Figure 2). The area at risk of infarction was similar among all groups (% to the whole LV; $51.2\pm2.6\%$ in the control, $54.1\pm2.1\%$ in the P-selectin, $54.0\pm0.8\%$ in the ICAM-1, and $53.8\pm1.4\%$ in the co-inhibition group). Both the P-selectin ($40.6\pm3.2\%$) and ICAM-1 ($34.8\pm3.5\%$) groups demonstrated a significant decrease in infarct size compared with the control group ($56.8\pm3.4\%, P=0.024$ and $P=0.002$, respectively; Figure 3). Noticeably, the co-inhibition group ($19.1\pm3.6\%$) demonstrated a much greater reduction of infarct size compared both to the P-selectin ($P=0.002$) and ICAM-1 ($P=0.021$) groups.

**Cardiac Function After Ischemia-Reperfusion**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>P-Selectin</th>
<th>ICAM-1</th>
<th>Co-Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>452±13</td>
<td>449±11</td>
<td>456±12</td>
<td>443±16</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>6.2±0.2</td>
<td>6.0±0.3</td>
<td>5.9±0.2</td>
<td>6.2±0.2</td>
</tr>
<tr>
<td>LVODs (mm)</td>
<td>4.8±0.2</td>
<td>4.2±0.3</td>
<td>4.2±0.2</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>52.3±2.0</td>
<td>60.1±2.2*</td>
<td>65.1±1.3*</td>
<td>68.4±2.0†</td>
</tr>
</tbody>
</table>

Cardiac function and dimensions were evaluated by echocardiography. $n=14$ in each group.

* $P<0.05$ vs the control group.
† $P=0.029$ vs the P-selectin group.

**Improved Regional Myocardial Reflow by Antibody Therapy**

The degree of regional reflow in the ischemic myocardium after reperfusion was evaluated with colored microspheres. At 24 hours after reperfusion, both the P-selectin ($73.3\pm4.5\%$) and ICAM-1 ($72.9\pm1.7\%$) groups demonstrated significant improvement in the degree of regional reflow compared with the control group ($56.1\pm2.9\%$, $P=0.021$ and $P=0.009$, respectively; Figure 4). The co-inhibition group ($81.7\pm4.3\%$) showed further improvement in reflow compared with the control group ($P<0.001$), although this was not significantly different from the control group ($P=0.021$).
P-selectin or ICAM-1 group ($P=0.742$ and $P=0.415$, respectively).

**Attenuated Leukocyte Infiltration by Antibody Therapy**

At 24 hours after reperfusion, the myocardium of ischemic areas had lost its regular structure and contained necrotic cardiomyocytes and cell infiltration into the disrupted tissue (Figure 5A to 5D). Immunofluorescence and hematoxylin/eosin staining on the same section demonstrated that the absolute majority of infiltrating cells were granulocyte antigen-positive cells having polymorphonuclei (PMNL) (Figure 5E and 5F). This myocardial damage and PMNL infiltration are typically seen in the Control group (Figure 5A). These changes indicating acute inflammation post ischemia-reperfusion appeared to be attenuated by antibody administration (Figure 5B to 5D), most clearly in the co-inhibition group (Figure 5D). The number of PMNL infiltrating into the ischemic myocardium was reduced in both the P-selectin ($10,178 \pm 729 /\text{mm}^2$) and ICAM-1 ($10,241 \pm 518 /\text{mm}^2$) groups compared with the control group ($13,469 \pm 433 /\text{mm}^2$, $P=0.027$ and $P=0.031$, respectively; Figure 6). The co-inhibition group ($6825 \pm 895 /\text{mm}^2$) showed further reduction in the PMNL number compared with all other groups ($P<0.001$ versus the control group, $P=0.024$ versus the P-selectin group and $P=0.021$ versus the ICAM-1 group).

**Discussion**

We demonstrated that either anti-P-selectin or anti-ICAM-1 antibody administration via the retrograde intracoronary route limited infarct size and improved cardiac function after ischemia-reperfusion injury. These effects were associated with reduced leukocyte infiltration into the ischemic myocardium and improved regional myocardial blood flow, suggesting the underlying mechanism of the therapeutic effects obtained. Noticeably, such cardioprotection against leukocyte-mediated reperfusion injury was further enhanced by co-inhibiting both molecules, highlighting the remarkable effectiveness of the combined antibody therapy targeting both

**Figure 4.** Degree of regional myocardial reflow. RMBF was measured with colored microspheres at 24 hours after reperfusion. The degree of regional reflow in the ischemic area was significantly improved in the ICAM-1, P-selectin, and co-inhibition groups as compared with the control group. $^*P<0.05$ vs the control group.

**Figure 5.** Histological findings. Myocardial damage and PMNL infiltration, which were typically seen in the control group (A), appeared to be attenuated by antibody administration (B to D), most clearly in the co-inhibition group (D, bar =100 μm). Immunofluorescence and hematoxylin/eosin staining on the same section (E and F, bar =20 μm) demonstrated that the majority of infiltrating cells were granulocyte-antigen positive (red). Nuclei are shown in blue (DAPI).

P-selectin and ICAM-1 by retrograde intracoronary injection to attenuate myocardial ischemia-reperfusion injury.

Myocardial damage after ischemia-reperfusion is accelerated and augmented by acute inflammation that initiates and progresses as a result of the leukocyte–endothelial interaction

**Figure 6.** Number of PMNL infiltrating into the ischemic myocardium. The number of PMNL infiltrating into the area at risk of infarction was calculated at 24 hours after reperfusion. Both the P-selectin and ICAM-1 groups demonstrated a significantly reduced number of PMNLs compared with the control group. The co-inhibition group showed the smallest number of PMNLs among the groups. $^*P<0.05$ vs the control group, $\dagger P=0.024$ vs the P-selectin group, $\ddagger P=0.021$ vs the ICAM-1 group ($n=5$ in each group).
at the postcapillary venules. Importantly, each step of the interaction is regulated by a distinct set of adhesion molecules. P-selectin, which is constitutively found in the endothelial cells and is mobilized to the cell surface rapidly after activation by inflammatory mediators, is known to mediate the initial entrapment of leukocytes. Thus, further refinement will be needed to determine the possible role of ICAM-1, or a CD11/18 leukocyte receptor blocker in the leukocyte-mediated reperfusion injury.

These results, which are consistent with previous reports, corroborate the important roles of both molecules in leukocyte-mediated reperfusion injury.

Although the efficiency of anti-P-selectin or anti-ICAM-1 antibodies for myocardial protection has not been tested in patients, multicenter clinical trials to block β2-integrin–ICAM-1 binding using an antibody to CD11/CD18, a sub-ABSTRACT

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**Disclosures**

None.

**References**


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