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

Satsuki Fukushima, MD; Steven R. Coppen, PhD; Anabel Varela-Carver, PhD; Kenichi Yamahara, MD, PhD; Padmini Sarathchandra, PhD; Ryszard T. Smolenski, MD, PhD; Magdi H. Yacoub, FRS; Ken Suzuki, MD, PhD

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 I]:I-251–I-256.)

Key Words: antibody therapy ■ cell adhesion molecules ■ leukocytes ■ myocardial protection ■ reperfusion

Myocardial ischemia-reperfusion injury remains a major issue in clinical settings such as heart transplantation, heart surgery using cardiac arrest (particularly in cases with severely hypertrophied or deteriorated left ventricle [LV]), and primary angioplasty for acute myocardial infarction (MI). It is known that acute inflammation, which is initiated and augmented by a series of reactions between leukocytes and endothelial cells, is an important contributor to the injury. The leukocyte–endothelial interaction and the consequent transmigration of leukocytes into the myocardial interstitium are regulated by adhesion molecules such as P-selectin and intercellular adhesion molecule-1 (ICAM-1). Previous experimental studies have demonstrated that intravenous injection of either anti-P-selectin or anti-ICAM-1 antibody attenuates leukocyte-mediated reperfusion injury, suggesting the utility of antibody therapy for myocardial protection. However, further refinement is needed for the future success of this strategy in the clinical arena.

We hypothesized that co-inhibition of P-selectin and ICAM-1 may provide enhanced cardioprotection compared with inhibition of either single molecule, because these molecules are involved in different processes of the leukocyte–endothelial interaction. In addition, the use of retrograde intracoronary infusion as a delivery route for the antibodies may augment the therapeutic efficacy. This route will offer a more effective, direct access to the postcapillary venules,
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-selection and ICAM-1 by retrograde intracoronary injection to attenuate myocardial ischemia-reperfusion injury using our original model in rat.

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 released for reperfusion.

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 (Figure 1A to 1D). No arteries, arterioles, or capillaries were positively labeled. No antibody was detected on cardiomyocytes.

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-phenylindole (DAPI; DAPI; 1:1000). After observation and recording with a fluorescence microscope (Zeiss; Axioskop 40), 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.

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 antibodies were 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 were significantly higher than the control group. The LVEF was 56±5% and 64±6%, respectively.
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>LVOs (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
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 ($10^{178}/\text{mm}^2$) and ICAM-1 ($10^{241}/\text{mm}^2$) groups compared with the control group ($13,469/\text{mm}^2$, $P=0.027$ and $P=0.031$, respectively; Figure 6). The co-inhibition group ($6,825/\text{mm}^2$) 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 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.
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. Subsequent binding between β2-integrin on the leukocytes and its ligand, ICAM-1, on the endothelial cells strengthens the attachment of leukocytes to the endothelium. ICAM-1 also facilitates the transendothelial migration of leukocytes into the myocardial interstitium. In this study, we demonstrated that inhibition of either P-selectin or ICAM-1 reduced leukocyte infiltration into the ischemic myocardium, preserved regional blood flow, and reduced infarct size after ischemia-reperfusion. 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-strate of ICAM-1, or a CD11/18 leukocyte receptor blocker has failed to limit infarct size after thrombolysis or angioplasty in acute MI. Thus, further refinement will be needed for the future clinical success of antibody therapy for myocardial protection. In this article, we have demonstrated that co-inhibition of both P-selectin and ICAM-1 via the retrograde intracoronary route provided exceptionally powerful cardioprotection. It is noticeable that this treatment markedly reduced infarct size, from 56.8% (in the control group) to as small as 19.1%.

Our results support the concept that co-inhibition of P-selectin and ICAM-1 enhances the therapeutic efficacy against myocardial ischemia-reperfusion injury compared with inhibition of either single molecule alone. However, Lefer et al. reported that bolus intravenous infusion of both anti-P-selectin and anti-ICAM-1 antibodies did not add further protective effects against reperfusion injury to single antibody administration in a canine model. Briaud et al. reported that mice with a genetic deficiency of both P-selectin and ICAM-1 showed a significant reduction of leukocyte accumulation but without reduction in infarct size after ischemia-reperfusion, compared with the wild-type. Although reasons for these inconsistent results are uncertain, we would speculate that it could be because of the different experimental models used. The former study used intravenous injection for antibody delivery, by which the efficiency of injected antibodies to reach the targeted myocardium might not be consistent because of a varying degree of their degradation or entrapment in other organs and vessels. Results of the latter study employing knock out mice might potentially be confounded by the developmental effects of early loss of expression. Continuous total blockage of the P-selectin and ICAM-1 cascade since before birth might enhance alternative pathways of postreperfusion acute inflammation and myocardial damage.

Antegrade intracoronary infusion and intravenous injection are other feasible routes for the antibody therapy. Although different from these, the retrograde intracoronary route allows for direct access to the postcapillary venules where P-selectin and ICAM-1 are upregulated and play a role in the leukocyte–endothelial interaction. This unique ability could be a great advantage as a route for antibody therapy targeting these molecules. In addition, in the present study, the retrograde intracoronary route was successful in delivering antibodies into the ischemic myocardium before reperfusion with 30-second controlled incubation time. This process is believed to encourage the infused antibodies to effectively reach the target tissue, and firmly bind to their antigens, which are known to be upregulated even during the ischemic period.

It is true that this strategy requires additional procedures to the usual heart surgery or percutaneous coronary intervention before reperfusion, which will delay reperfusion by several minutes. However, we consider that this powerful treatment will provide greater benefits far overweighting the loss caused by the “several-minute delay” in reperfusion, thereby improving the overall outcome. We did not compare the effect of retrograde intracoronary infusion with that of antegrade intracoronary or intravenous infusion in this study, which may be a limitation of the study. However, the efficiency of intravenous injection in delivering antibody into the heart is much poorer compared with local (intracoronary) infusion methods, and there is lack of widely accepted, reproducible models of antegrade intracoronary injection in rats. This issue should be clarified using an appropriate large animal model. The lack of dose-dependent analysis may be another limitation of the study. However, we have carefully decided on the antibody dose by considering all the relevant previous reports and, using such the most promising dose of each antibody, we have provided proof of principal that the combined antibody therapy inhibiting both P-selectin and ICAM-1 via the retrograde intracoronary route provides effective myocardial protection.

In conclusion, the combined antibody therapy inhibiting both P-selectin and ICAM-1 via the retrograde intracoronary route could be a promising new strategy to attenuate myocardial ischemia-reperfusion injury.

Sources of Funding
We thank the Magdi Yacoub Institute (UK) and Great Britain Sasakawa Foundation (UK/Japan) for financial support. Ken Suzuki is a Senior Clinical Fellow of the Medical Research Council. Satsuki Fukushima is supported in part by a research fellowship from the Uehara Memorial Foundation (Japan). Sir Magdi Yacoub is a British Heart Foundation Professor of cardiothoracic surgery.

Disclosures
None.

References


A Novel Strategy for Myocardial Protection by Combined Antibody Therapy Inhibiting Both P-Selectin and Intercellular Adhesion Molecule-1 Via Retrograde Intracoronary Route
Satsuki Fukushima, Steven R. Coppen, Anabel Varela-Carver, Kenichi Yamahara, Padmini Sarathchandra, Ryszard T. Smolenski, Magdi H. Yacoub and Ken Suzuki

Circulation. 2006;114:I-251-I-256
doi: 10.1161/CIRCULATIONAHA.105.000794
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/1_suppl/I-251