Stent-induced neutrophil activation is associated with an oxidative burst in the inflammatory process, leading to neointimal thickening

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Summary
Activation of leukocytes plays an essential role in the mechanism of restenosis. Prior research has focused on monocytes and little is known about the role of neutrophils in this process. Neutrophils are known to contribute to tissue injury through oxygen-derived free radicals that nitrate tyrosine. This study was designed to elucidate clinically the role of neutrophil-mediated oxidative burst in the regulation of the post-stent inflammatory process. In 36 patients undergoing coronary stenting, we serially measured serum levels of glycosyl-phosphatidil-inositol-anchored protein (GPI)-80, a modulator of Mac-1 on the surface of neutrophils, in samples of coronary sinus as well as peripheral blood. We also simultaneously measured the serum 3-nitrotyrosine/tyrosine ratio as an index of oxidative stress. The GPI-80 level and the 3-nitrotyrosine/tyrosine ratio increased in the coronary sinus after coronary stenting in a time-dependent manner; with the maximum increase of GPI-80 level (3.1±2.9 to 8.6±4.3 ng/ml, P<0.01) at 48 hours, and 3-nitrotyrosine/tyrosine ratio at 24 hrs (5.2±4.8 to 28.4±13.2×10⁻⁴, P<0.01), more strikingly than in the peripheral blood. In the coronary sinus blood, the 3-nitrotyrosine/tyrosine ratio was correlated with GPI-80 levels at 24 hr (R=0.58, P<0.001) and at 48 hr (R=0.41, P<0.01). Multiple regressions analysis showed that the maximum responses of GPI-80 level and 3-nitrotyrosine/tyrosine ratio were independent predictors of angiographic late lumen loss. Our results may support a hypothesis that Mac-1-dependent activation of neutrophils causes oxidative burst in the post-stent inflammatory process, possibly leading to restenosis.

Keywords
Neutrophil, Mac-1, GPI-80, nitrotyrosine, restenosis

Introduction
Activation of leukocytes, neutrophils as well as monocytes/macrophages, plays an essential role in the mechanism of restenosis after percutaneous coronary intervention (PCI) (1). Prior research has focused on monocytes as the preeminent inflammatory cell type involved in restenosis (2, 3). In contrast, little is known about the role of neutrophils in restenosis. Activation of β2 integrin, Mac-1 (CD11b/CD18) is responsible for firm neutrophil adhesion to the vessel surface (4, 5) and a Mac-1 modulator, glycosyl-phosphatidil-inositol-anchored protein (GPI)-80, may be involved in regulation of Mac-1-dependent neutrophil adhesion to the vessel surface and transmigration into the vessel wall (6, 7). Furthermore, neutrophils are known to contribute to oxidative tissue injury through peroxynitrite (8) or through myeloperoxidase (9) that potentiates nitration of tyrosine. This study was designed to clinically investigate activation of neutrophils and neutrophil-mediated oxidative burst in the inflammatory process of PCI-induced vessel injury and the development of restenosis. We serially measured serum levels of GPI-80 and nitrotyrosine in patients undergoing coronary stent implantation.

Methods
Sample preparation
The subjects included 36 patients with isolated atherosclerotic coronary artery disease in the proximal left anterior descending artery that underwent single elective coronary stent implantation. The stented lesions of each patient were only type A or type B lesions and the patients who underwent PCI for the com-
plex lesions or small vessel lesions (<2.5 mm of reference diameter) were excluded. All of the patients were receiving standard daily oral medications for angina, including 81 mg of aspirin, and none of these medications was discontinued or exchanged during PCI or the post-PCI follow-up period. The patients started receiving 200 mg of oral ticlopidine (n=26) or cilostazol (n=10) 2 days before PCI as a specific post-stent anti-platelet medication and therapy was continued for one month after PCI. Coronary stent implantation was performed from a femoral approach using the standard Judkins technique. Intravenous heparin was administered to maintain an adequate activated clotting time during the procedure and for 48 hrs after PCI. Follow-up angiography was recommended for all patients at 6 months after angioplasty, and was performed earlier if necessary based on clinical indications. Coronary lesions were assessed by quantitative coronary angiography (QCA). Lesion length, reference diameter and minimal lumen diameter were measured; the acute gain was calculated as the difference between minimal lumen diameter before and after PCI, and late lumen loss (minimal lumen diameter after PCI minus minimal lumen diameter at follow-up angiography) was calculated as an index of neointimal thickening. Prior to PCI, a coronary sinus catheter was positioned in the coronary sinus and left for 48 hr after the procedure for coronary sinus blood sampling. Coronary sinus blood as well as peripheral blood was collected before PCI and at 15 min, 24 hr, and 48 hr after coronary stenting. Whole blood was immediately collected into tubes containing ethylene diaminetetraacetate (EDTA). The EDTA-treated blood was centrifuged at 1,500 × g for 15 min. Follow-up angiography (QCA) was calculated as an index of neointimal thickening.

Statistical analysis

Values were expressed as the mean ± SD. Serial changes in the variables were evaluated by repeated measures analysis of variance (ANOVA), for intra- and inter-group comparisons. Correlations between two parameters were assessed by simple linear regression. Simple and multiple regression analysis was performed for predicting angiographic late lumen loss. A P value of <0.05 was considered significant.

Results

Serial changes in GPI-80, and nitrotyrosine

Serial changes in plasma GPI-80 level and 3-nitrotyrosine/tyrosine ratio were shown in Figure 1. Although no change was evident 15 min after coronary stenting, an increase in the GPI-80 level was noted after 24 hr in both coronary sinus and peripheral blood. The maximal increase was seen at 48 hr (coronary sinus: 3.1±2.9 to 8.6±4.3 ng/ml, P<0.01; peripheral blood: 3.0±2.9 to 7.3±4.9 ng/ml, P<0.05). The changes were more striking in the coronary sinus than in the peripheral blood (Fig. 1, left). The 3-nitrotyrosine/tyrosine ratio did not also change at 15 min, but increased to a maximum at 24 hrs (coronary sinus: 5.2±4.8 to 28.4±13.2×10^-4, P<0.01; peripheral blood: 4.2±3.4 to 22.2±14.3×10^-4, P<0.01). The values decreased at 48 hr but remained higher than the baseline levels before PCI. These changes were also more striking in the coronary sinus than in the peripheral blood (Fig. 1, right).

Relationship between GPI-80 level and nitrotyrosine

In coronary sinus blood, the 3-nitrotyrosine/tyrosine ratio was correlated with GPI-80 level at 24 hr (R=0.58, P<0.001) and at 48 hr (R=0.41, P<0.01) (Fig. 2).

Prediction of late lumen loss

Prediction of angiographic late lumen loss was first assessed by simple regression analysis using various parameters including coronary risk factors, medications, lesion profiles and QCA variables possibly related to restenosis as well as values of...
GPI-80 and 3-nitrotyrosine/tyrosine ratio in the coronary sinus. The late lumen loss was predicted by diabetes (R=0.414, P<0.05), lesion length (R=0.416, P<0.05), reference diameter (R=0.384, P<0.05), GPI-80 level (R=0.469, P<0.05 at 24 hr; R=0.582, P<0.01 at 48 hr), and 3-nitrotyrosine/tyrosine ratio (R=0.536, P<0.01 at 24 hr; R=0.431, P<0.05 at 48 hr) (Table 1). Multiple regression analysis using these predictive parameters obtained from simple regression analysis data demonstrated that only GPI-80 level at 48 hr (r=0.522, P<0.05) and the 3-nitrotyrosine/tyrosine ratio at 24 hr (r=0.486, P<0.05) were independent predictors of late lumen loss (Table 2).

### Table 1: Simple regression analysis for predicting angiographic late lumen loss.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Regression Coefficient</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>0.178</td>
<td>0.428</td>
</tr>
<tr>
<td>Male gender (yes/no)</td>
<td>0.092</td>
<td>0.746</td>
</tr>
<tr>
<td>Diabetes (yes/no)</td>
<td>0.414</td>
<td>0.042</td>
</tr>
<tr>
<td>Hypertension (yes/no)</td>
<td>0.189</td>
<td>0.386</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>0.113</td>
<td>0.461</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>0.126</td>
<td>0.413</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>-0.345</td>
<td>0.065</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>0.218</td>
<td>0.197</td>
</tr>
<tr>
<td>Statin (yes/no)</td>
<td>-0.316</td>
<td>0.078</td>
</tr>
<tr>
<td>ACEI (yes/no)</td>
<td>-0.244</td>
<td>0.181</td>
</tr>
<tr>
<td>ARB (yes/no)</td>
<td>-0.164</td>
<td>0.272</td>
</tr>
<tr>
<td>Lesion length (mm)</td>
<td>0.416</td>
<td>0.041</td>
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<tr>
<td>Reference diameter (mm)</td>
<td>0.364</td>
<td>0.049</td>
</tr>
<tr>
<td>Acute gain (mm)</td>
<td>0.149</td>
<td>0.444</td>
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<tr>
<td>GPI-80 24 hrs in CS (ng/ml)</td>
<td>0.469</td>
<td>0.018</td>
</tr>
<tr>
<td>GPI-80 48 hrs in CS (ng/ml)</td>
<td>0.582</td>
<td>0.004</td>
</tr>
<tr>
<td>3-nitrotyrosine/tyrosine 24 hrs in CS</td>
<td>0.536</td>
<td>0.008</td>
</tr>
<tr>
<td>3-nitrotyrosine/tyrosine 48 hrs in CS</td>
<td>0.431</td>
<td>0.035</td>
</tr>
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</table>

CS indicates coronary sinus

### Table 2: Multiple regression analysis for predicting angiographic late lumen loss.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Regression Coefficient</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes (yes/no)</td>
<td>0.136</td>
<td>0.542</td>
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<tr>
<td>Lesion length (mm)</td>
<td>0.165</td>
<td>0.391</td>
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<tr>
<td>Reference diameter (mm)</td>
<td>0.256</td>
<td>0.186</td>
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<tr>
<td>GPI-80 24 hrs in CS (ng/ml)</td>
<td>0.369</td>
<td>0.062</td>
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<tr>
<td>GPI-80 48 hrs in CS (ng/ml)</td>
<td>0.522</td>
<td>0.015</td>
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<tr>
<td>3-nitrotyrosine/tyrosine 24 hrs in CS</td>
<td>0.486</td>
<td>0.036</td>
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<tr>
<td>3-nitrotyrosine/tyrosine 48 hrs in CS</td>
<td>0.328</td>
<td>0.085</td>
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</table>

CS indicates coronary sinus

### Discussion

In the present study, we demonstrated that plasma level of GPI-80, a modulator of Mac-1 on the surface of neutrophils, and the 3-nitrotyrosine/tyrosine ratio, an oxidative stress marker, increased after coronary stenting in a time-dependent manner and that these changes were more striking in the coronary sinus than in the peripheral blood. The maximum response for the 3-nitrotyrosine/tyrosine ratio was 24 hr after PCI, while that for the level of GPI-80 was 48 hr. In addition, the 3-nitrotyrosine/tyrosine ratio was correlated with the GPI-80 level at 24 hr and at 48 hr after PCI. The maximum responses of these 2 parameters were independent predictors of angiographic late lumen loss (i.e., neointimal thickening) in the multiple regression analysis. These results suggest that neutrophils, activated through modulation of Mac-1 and inducing tyrosine nitration in the coronary circulation, possibly play a role in the post-PCI inflammatory process and restenosis.

### Mechanistic roles of neutrophils in neointimal hyperplasia

The monocytes/macrophages have long been considered to be the key players in the mechanism of atherosclerosis and resteno-
sis after PCI (2). In addition to monocytes/macrophages, several experimental studies have shown the early recruitment of neutrophils after vascular injury and the persistence of neutrophil products in the vessel wall (12, 13). Although it has been reported that rabbit vascular smooth muscle cells are stimulated to proliferate when they are cocultured with neutrophils or neutrophil-conditioned media (14), the contribution of neutrophils to intimal hyperplasia has not been well defined. Clinical studies also demonstrated the role of neutrophils and their association with restenosis (15–19). We previously demonstrated that Mac-1 was activated and up-regulated on the surface of neutrophils time dependently after PCI and the maximum response was shown at 48 hr (16–19). These changes were more strikingly observed in the samples from the coronary sinus than those from the peripheral blood, indicating the events locally occur in the coronary circulation. In addition, the activation and up-regulation of Mac-1 related to angiographic late lumen loss and restenosis (15–19). Mac-1 orchestrates the recruitment of leukocytes by promoting firm adhesion to, as well as transmigration across, fibrogenin and platelet ligands, such as glycoprotein Ibα (GPIbα) (20) and possibly intercellular adhesion molecule-2 (ICAM-2) (4), present at sites of vessel-wall injury. Monoclonal antibody blockade (21) and the absence of Mac-1 (22) reduces neointimal thickening after experimental angioplasty and stenting. Thus, Mac-1 is thought to be one of the important signaling proteins in the mechanism of restenosis. When neutrophils are activated and recruited to areas of injury, they release a number of proteolytic enzymes (e.g., elastase and collagenase), cytokines, free radicals and other products capable of promoting additional tissue injury (23). This process may lead to the progression of restenosis.

**Modulation of Mac-1 on the surface of activated neutrophils**

We demonstrated in this study a time-dependent increase in serum levels of GPI-80, a Mac-1 modulator. GPI-anchored proteins, including urokinase-type plasminogen-activator receptor (uPAR, CD87), (24) FcyRIIib (CD16b) (25), and CD14 (26), have been recently recognized as important candidates for the regulation of Mac-1 function. A novel GPI-anchored protein, GPI-80, on human neutrophils regulates Mac-1-dependent neutrophil adherence and migration (6, 7). In the process of neutrophil adherence to and subsequent transendothelial migration into the PCI-injured vessel wall, it is thought that a large portion of neutrophils adhered to the endothelial surface can detach and recirculate in the blood stream. However, the molecular mechanisms responsible for leukocyte transendothelial migration or for detachment and subsequent recirculation are largely unknown. GPI-80 enhances Mac-1-dependent neutrophil adhesion at an early phase after vessel-wall injury while playing an inhibitory role at a later phase. Therefore, GPI-80 is possibly involved in the regulation of not only neutrophil attachment, but also detachment from the vessel surface that is essential for migration (6, 7). GPI-80 is expressed on the surface of neutrophils and partially sheds into the blood stream. It has been demonstrated that GPI-80, released from activated neutrophils into the blood stream, was absolutely dependent on adhesion via Mac-1 (27, 28). In our results, the change in serum GPI-80 level after PCI showed a similar time course to the change in the activation and up-regulation of Mac-1 on the surface of neutrophils that we previously demonstrated, and the maximum response of GPI-80 at 48 hr independently predicted late lumen loss. These results suggest a role for GPI-80 in the regulation of Mac-1-dependent neutrophil migration and restenosis after stenting. In addition, the ELISA measurement of serum GPI-80 levels is simple to perform, while assessment of Mac-1 on the surface of neutrophils requires a complex technique such as flow cytometry. Thus, GPI-80 levels measured by ELISA may be a very good surrogate marker for Mac-1.

**Neutrophil activation and oxidative burst**

Activated neutrophils produce reactive oxygen species such as oxygen free radicals. Reactive oxygen species production was directly induced by cross-linking of β2 integrins including Mac-1 with their surface-bound ligands through tyrosine kinase-dependent signaling (29). On the other hand, strongly activated neutrophils generate nitric oxide via inducible nitric oxide synthase (30). Oxygen free radicals react with nitric oxide to form peroxynitrite, a powerful oxidant that may directly oxidize proteins, lipids, and DNA through a nitronium-like intermediate with resultant carbonyl formation from side-chain and peptide-bond cleavage (31). Peroxynitrite has an affinity for tyrosine residues and reacts with tyrosine to produce nitrotyrosine (32). In addition, neutrophils also nitrate tyrosine through myeloperoxidase activity (9). Thus, nitrotyrosine is suggested to be one of the products of a neutrophil-induced oxidative burst mediated through Mac-1 signaling. Furthermore, nitrotyrosine is detected in the atheroma deposits of human coronary arteries by immunohistochemistry using an anti-nitrotyrosine antibody (33). Therefore, nitrotyrosine is possibly associated with atherogenesis or restenosis formation in human coronary arteries. In this study, we demonstrated that the 3-nitrotyrosine/tyrosine ratio increased in a time-dependent manner after coronary stenting with a maximum at 24 hr. However, it decreased at 48 hours whereas the GPI-80 level kept rising. It is puzzled why the serial change pattern of 3-nitrotyrosine/tyrosine ratio was discrepant from that of GPI-80 level. Neutrophil activation is associated with not only oxidative burst but also proteolysis, phagocytosis, etc. Prior to neutrophil activation reaches maximum, oxidative burst may occur as an event promoted by neutrophils in the early activation process. However, in our results, the correlation between 3-nitrotyrosine/tyrosine and GPI-80 level was observed at 48 hr as well as at 24 hr, independently of the maximum response discrepancy, suggesting a close linking of neutrophil activation to oxidative burst after PCI. Interestingly, the maximum response of 3-nitrotyrosine/tyrosine ratio at 24 hr could also independently predict late lumen loss. Recently, Chen et al. (34) demonstrated that vascular injury in wild type mice increased neointimal thickening in association with an increased nitrotyrosine/tyrosine ratio; but in leukocyte oxidase (Nos2) deficient mice, neointimal formation was reduced, suggesting a direct role of reactive oxygen species/reactive nitrogen species production by NADPH oxidase in vascular repair after injury. This experiment greatly supports the validity of our clinical results. Nitto et al. (30) demonstrated that the signal for the release of GPI-80 from neutrophils was associated with the redox state of neutrophils. Therefore, we hypothesize that shedding of GPI-80 into serum
indicates a link between Mac-1-dependent neutrophil adhesion and oxidative signals. Our finding of the correlation between 3-nitrotyrosine/tyrosine ratio and GPI-80 level supports this hypothesis and suggests that GPI-80 may serve as a clinical marker for neutrophil-induced nitrosative stress or further oxidative burst.

**Study limitations and clinical implications**

We cannot be certain that the increases in the level of GPI-80 and 3-nitrotyrosine/tyrosine ratio were caused by PCI-induced vascular injury rather than by the cardiac catheterization procedure including artery puncture, coronary artery catheterization, or coronary sinus catheterization for 48 hr. Although we did not specifically evaluate this issue in the present study, we have confirmed that diagnostic coronary angiography alone did not affect these parameters (data not shown). In our results, GPI-80 and 3-nitrotyrosine/tyrosine ratio was greater in the coronary sinus than in the peripheral blood in each sampling point, namely, the transcardiac gradients were present. The presence of transcardiac gradients suggests that the changes in parameters might less depend on the systemic effects of catheterization but rather locally caused by PCI procedure. Lesion length and reference diameter are accepted predictors of angiographic late lumen loss. In our results, both of these were predictors of the late lumen loss in simple regression analysis. However, neither could be independent predictors in multiple regression analysis. Since our study did not include patients with complex lesions or small vessel lesions, lesion profiles alone might not affect 6 months’ angiographic outcome. Our results of the relationship between the 3-nitrotyrosine/tyrosine ratio and serum GPI-80 levels linked to late lumen loss are merely observational results, and our data alone cannot be used to infer the pathophysiological mechanism of neo-intimal thickening or restenosis. In addition, nitrotyrosine is a final product derived from the actions of peroxynitrite or myeloperoxidase, and thus, represents an indirect marker of neutrophil-induced oxidative stress. Our conclusions would have been stronger if we simultaneously monitored peroxynitrite, myeloperoxidase, or directly measured the production of reactive oxygen species. Alternatively, chlorotyrosine measurement would be informative, since the neutrophil oxidative burst is accompanied by hypochlorite and thus, chlorotyrosine formation. In this study, we measured neither myeloperoxidase nor chlorotyrosine. However, we believe our study supports a hypothesis based on several experimental evidences that Mac-1-dependent neutrophil-induced oxidative burst may be modulated in part by GPI-80 and may play a role in neointimal thickening after vascular injury.

The introduction of coronary stents has markedly reduced the rate of restenosis and the use of drug-eluting stents has further reduced the restenosis rate to less than 10%. However, the issue of restenosis has not been completely resolved and we should continue trying approaches targeting restenosis. Welt et al. (35) suggested targeting neutrophils directly in addition to monocytes/macrophages as a rational therapeutic strategy for preventing restenosis. Since our study suggests that free radical production by activated neutrophils plays a clinically important role in the mechanism of restenosis, we can envision a significant clinical advantage of inhibiting the neutrophil-induced oxidative burst.

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**References**