Comparison of Differential Expression of $P2Y_{12}$ Receptor in Culprit Coronary Plaques in Patients With Acute Myocardial Infarction Versus Stable Angina Pectoris

Cheol Whan Lee, MD[a,†], Ilseon Hwang, MD, PhD[d,†], Chan-Sik Park, MD, PhD[b], Hyangsin Lee, MS[c], Duk-Woo Park, MD[a], Su-Jin Kang, MD[a], Seung-Whan Lee, MD[a], Young-Hak Kim, MD[a], Seong-Wook Park, MD, PhD[a], and Seung-Jung Park, MD, PhD[a,*]

$P2Y_{12}$ receptor antagonists may have pleiotropic benefits. Little is known, however, about the expression of $P2Y_{12}$ receptors in coronary atherosclerotic plaques. We investigated the expression of $P2Y_{12}$ receptor in coronary atherectomy tissues retrieved from patients with acute myocardial infarction (AMI) or stable angina pectoris (SAP). Tissue specimens were collected from 35 patients with AMI and 19 with SAP who underwent directional coronary atherectomy. Specimens were analyzed immunohistochemically using antibodies specific to $P2Y_{12}$ receptor and to markers of endothelial cells, macrophages, and smooth muscle cells. The 2 groups had similar baseline clinical characteristics. Plaque types were more likely to be cellular in the AMI group. The proportion of areas immunopositive for α-smooth muscle actin was smaller but those positive for CD31 and CD68 were larger in the AMI than in the SAP group. In addition, the relative area immunopositive for $P2Y_{12}$ receptor was significantly larger for AMI than SAP ($1.1 \pm 0.9\%$ vs $0.5 \pm 0.4\%$, respectively, $p < 0.001$). $P2Y_{12}$ receptor positivity coincided with areas positive for CD31 and α-smooth muscle actin. In conclusion, $P2Y_{12}$ receptor is present in coronary atherosclerotic plaques and is increased in culprit plaques of patients with AMI. $P2Y_{12}$ receptor may play a role in plaque destabilization. **(Am J Cardiol 2011;108:799–803)**

$P2Y_{12}$ receptors are expressed on endothelial cells, vascular smooth muscle cells, and platelets.[1,2] Clopidogrel can decrease markers of vascular inflammation and improve endothelial function.[3–7] Recent results from the Platelet Inhibition and Patient Outcomes (PLATO) trial showed that the cardiovascular benefits of ticagrelor outweighed those of clopidogrel despite being associated with more spontaneous bleeding events.[8,9] The vascular benefits of ticagrelor could not be explained by its faster and more potent platelet inhibition alone, suggesting that ticagrelor has platelet-independent pleiotropic effects coupled with $P2Y_{12}$ receptor antagonism. To date, however, little is known about the expression of $P2Y_{12}$ receptors in coronary atherosclerotic plaques and its relation to plaque instability. We therefore compared the expression of $P2Y_{12}$ receptors in coronary atherectomy tissues retrieved from patients with acute myocardial infarction (AMI) to those from patients with stable angina pectoris (SAP).

Methods

Atherectomy specimens were obtained from a biobank at our institution, which had collected tissues from 35 consecutive patients with AMI and 19 with SAP, defined as typical exertional angina with no change in symptoms within 1 month before the procedure. Patients were suitable for directional coronary atherectomy if they had a significant stenotic lesion with a large plaque burden but lacked heavy thrombi in a nontortuous epicardial coronary artery >3 mm in diameter.[10,11] Each specimen corresponded to the de novo lesion responsible for the clinical presentation in a single patient. Directional coronary atherectomy was performed using a Flexi-Cut catheter (Abbott Laboratories/Guidant Vascular Interventions, Santa Clara, California) under intravascular ultrasound guidance. The study protocol was approved by our institutional review committee, and all patients provided written informed consent.

Tissue specimens were fixed in formalin and embedded in donor paraffin blocks. Tissue microarrays were produced by re-embedding tissues from these paraffin blocks into arrays on recipient paraffin blocks. Sections from the master block were cut using a microtome, mounted on a microscope slide, and used for subsequent staining.

Samples were stained with hematoxylin and eosin to determine cellularity and general morphologic features. The area of each plaque was measured using a microscopic image analysis system (Motic Images Advanced 3.2, Motic, Xiamen, China). Plaques were classified as atheromatous (i.e., with necrotic cores and cholesterol clefts but without connective tissue matrix) or fibrocellular and graded as

Departments of [a]Medicine and [b]Pathology, Asan Medical Center and [c]Asan Institute of Life Science, University of Ulsan, Seoul, Korea; [d]Department of Pathology, School of Medicine, Keimyung University, Daegu, Korea. Manuscript received April 6, 2011; revised manuscript received and accepted May 6, 2011.

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*Corresponding author: Tel: 82-2-3010-3150; fax: 82-2-486-5918.
E-mail address: sjpark@amc.seoul.kr (S.-J. Park).

† Drs. Lee and Hwang contributed equally to this work.

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Table 1
Clinical characteristics of study subjects

Characteristics	AMI (n = 35)	SAP (n = 19)	*P* Value
Age (years)	56.4 ± 11.2	61.0 ± 6.9	0.107
Men/women	29/6	12/7	0.181
Current smoker	16 (45.7%)	6 (31.6%)	0.391
Diabetes mellitus	7 (20%)	7 (36.8%)	0.206
Hypertension	17 (48.6%)	11 (57.9%)	0.577
Total cholesterol (mg/dl)	192.9 ± 44.6	153.0 ± 30.1	0.001
Triglycerides (mg/dl)	179.4 ± 84.2	110.5 ± 45.5	0.002
High-density lipoprotein cholesterol (mg/dl)	34.8 ± 7.9	43.0 ± 14.4	0.031
High-sensitivity C-reactive protein (mg/L)	3.3 ± 3.3	1.7 ± 1.8	0.059
Multivessel coronary disease	17 (48.6%)	7 (36.8%)	0.567
Target coronary artery			0.795
Left anterior descending	19 (54.3%)	12 (63.2%)	
Left circumflex	3 (8.6%)	1 (5.3%)	
Right	13 (37.1%)	6 (31.6%)	
Medications at time of directional coronary atherectomy			
Aspirin	35 (100%)	19 (100%)	1.0
Clopidogrel	35 (100%)	19 (100%)	1.0
Angiotensin-converting enzyme inhibitor/angiotensin receptor blocker	2 (5.7%)	2 (10.5%)	0.607
β Blockers	7 (20%)	9 (4.7%)	0.060
Calcium channel antagonists	5 (14.3%)	11 (57.9%)	0.001
Statins	11 (31.4%)	13 (68.4%)	0.012

Table 2
Histologic characteristics of study subjects

Variables	AMI (n = 35)	SAP (n = 19)	*P* Value
Histology			
Atheroma	48.7 ± 26.1	31.1 ± 30.3	0.030
Fibrocellular area			
Sparsely cellular	35.5 ± 23.5	51.6 ± 30.4	0.035
Moderately cellular	7.6 ± 9.0	15.7 ± 24.6	0.179
Hypercellular	2.5 ± 5.1	0.5 ± 1.9	0.041
Thrombus	5.2 ± 16.2	0.0 ± 0.1	<0.001
Calcium	0.5 ± 1.9	1.0 ± 3.0	0.509
Total plaque area (mm^2)	704.4 ± 344.0	496.5 ± 314.9	0.034
Immunohistochemistry			
α-Smooth muscle actin	2.9 ± 2.7	12.3 ± 14.4	0.011
CD31	1.1 ± 1.6	0.2 ± 0.2	0.001
CD68	15.5 ± 13.6	7.0 ± 14.6	0.038
$P2Y_{12}$	1.1 ± 0.9	0.5 ± 0.4	<0.001

Data are expressed as percent positive areas (immunostained area/total plaque area × 100).

sparsely cellular (<30 spindle cells per high-power field), moderately cellular (30 to 100 spindle cells per field), or hypercellular (≥100 spindle cells per field). All slides were graded by 2 pathologists (C.-S.P. and I.H.) blinded to patients' clinical status. Any discrepancies between their findings were resolved by consensus.

Sections of each tissue specimen were stained with rabbit polyclonal antibodies against $P2Y_{12}$ receptor (1:100; Novus Biologicals, Littleton, Colorado) and monoclonal antibodies against α-smooth muscle actin (1:200; mouse antihuman macrophage antibody clone 1A4; DAKO, Carpinteria, California), CD31 (1:100; mouse antihuman CD31 [platelet endothelial cell adhesion molecule-1] antibody clone 1A10; Leica, Newcastle, United Kingdom), and CD68 (1:200; mouse antihuman macrophage antibody clone KP-1; DAKO) using an Envisionplus immunostaining kit and 3,3-diaminobenzidine or 3-amino-9-ethylcarbazole as the chromogen as described by the manufacturer (DAKO). Briefly, samples were incubated with primary antibodies for 1 hour, washed 2 times for 5 minutes each with Tris buffered saline/Tween-20, incubated with secondary antibodies conjugated with horseradish peroxidase–labeled polymer (DAKO) for 1 hour, and washed again. As negative controls, adjacent sections were stained with species- and isotype-matched irrelevant antibodies including normal rabbit immunoglobulin G (Abcam, Cambridge, United Kingdom). A sample of platelets was used as a positive control for anti-$P2Y_{12}$ receptor antibodies. Cell types positive for $P2Y_{12}$ receptor were identified by immunostaining of serial sections with anti-$P2Y_{12}$ receptor antibodies. The immunopositive area was calculated as the ratio of positively stained regions to total plaque area.

For immunofluorescent staining, fixed sections were hydrated in phosphate buffered saline (PBS) for 10 minutes at room temperature, incubated with DakoCytomation Protein Block (DakoCytomation, Carpinteria, California) for 5 minutes at room temperature, and washed 3 times in PBS/Tween-20. Sections were subsequently incubated with mouse monoclonal antibody to human CD31 (Leica), CD68 (DakoCytomation), or α-smooth muscle actin (DakoCytomation) or rabbit polyclonal antibody to $P2Y_{12}$ receptor for 60 minutes at room temperature. After 3 additional washes in PBS/Tween-20, sections were incubated with fluorescein isothiocyanate–conjugated antirabbit immunoglobulin G or allophycocyanin-conjugated antimouse immunoglobulin G for 60 minutes at room temperature and washed 3 times with PBS/Tween-20. Coverslips were mounted onto glass slides using DAKO fluorescent mounting medium (DakoCytomation). Fluorescein isothiocyanate was excited using an argon laser at 488 nm and allophycocyanin was excited by a helium–neon laser at 633 nm. Detector slits were configured to minimize any crosstalk between channels. Images were collected on a Leica TCS-NT/SP confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a 40× objective (model NA 0.75) and a Zoom 1-4 × and processed using Leica TCS-NT/SP software (version LCS) and Adobe Photoshop 7.0 (San Jose, California).

Continuous variables are expressed as mean ± SD or median with interquartile range, whereas categorical variables are expressed as frequency. Continuous variables were compared using Student's *t* test or Mann–Whitney U test, and categorical variables were analyzed using chi-square test. Linear regression analysis was used to correlate areas positive for $P2Y_{12}$ receptor with those positive for markers for endothelial cells, macrophages, and smooth muscle markers. Statistical significance was defined as a 2-sided p value <0.05.

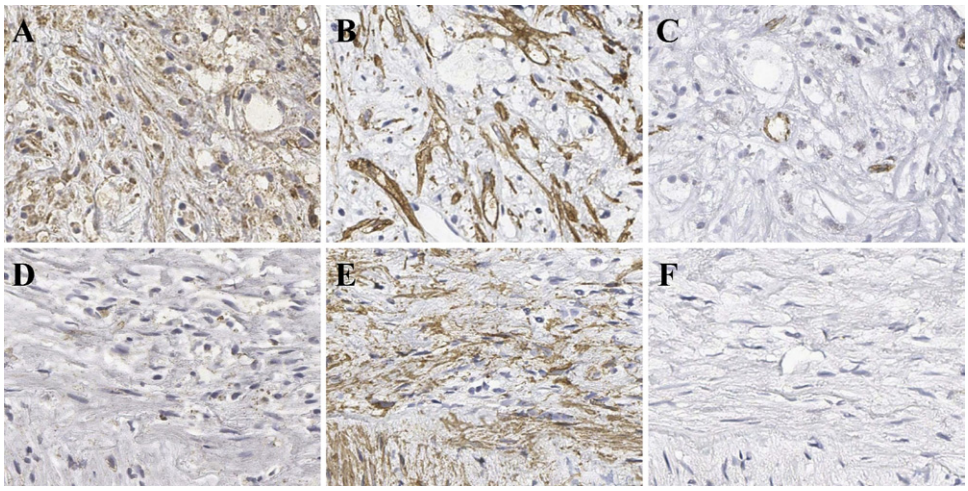

Figure 1. Representative images of immunohistochemical staining (magnification 400×) from patients with acute myocardial infarction *(A to C)* or stable angina pectoris *(D to F)*. *(A)* P2Y$_{12}$ receptor was strongly stained on plaque from a patient with acute myocardial infarction *(brown)*. Serial sections of immunostaining show strong positive areas of *(B)* α-smooth muscle actin *(brown)* and *(C)* focal positive areas of CD31 *(brown)*. *(D)* P2Y$_{12}$ receptor was weakly stained on plaque from a patient with stable angina pectoris. *(E)* Alpha-smooth muscle actin *(brown)* was strongly stained, *(F)* but CD31 was not stained in similar areas.

Figure 2. Immunofluorescence staining (magnification 800×) of coronary plaques of a patient with acute myocardial infarction using antibodies to *(A, D, G)* P2Y$_{12}$ receptor *(green)*, *(B)* α-smooth muscle actin *(red)*, *(E)* CD31 *(red)*, and *(H)* CD68 *(red)*. P2Y$_{12}$ receptor-immunopositive cells colocalized with cells positive for *(C)* α-smooth muscle actin *(arrows)* or *(F)* CD31 *(arrows)* but not with *(I)* CD68.

Results

Baseline clinical characteristics were similar between the 2 groups except for their lipid profiles and medications (Table 1). Median time from symptom onset to reperfusion was 5.5 hours (interquartile range 2.5 to 7.5) for ST-segment elevation AMI (n = 27) and 35.5 hours (interquartile range 24 to 90) for non–ST-segment elevation AMI (n = 8). At the time of directional coronary atherectomy, larger percentages of patients in the SAP group were taking calcium channel blockers and statins than those in the AMI group.

Total plaque area was smaller, whereas atheroma area was larger, in the AMI than in the SAP group (Table 2). Plaque types were more likely to be cellular in the AMI group. Thrombi were significantly more common in the AMI than in the SAP group (68.6% vs 1.1%, p <0.001). Relative plaque area immunopositive for α-smooth muscle actin was significantly smaller in the AMI group than in the SAP group, whereas areas immunopositive for CD31 and CD68 were significantly larger in the AMI group (Table 2). In addition, relative area immunopositive for P2Y$_{12}$ recep-

tor was significantly larger in plaques from patients with AMI than from those with SAP (1.1 ± 0.9% vs 0.5 ± 0.4%, $p < 0.001$). In multivariate analysis, areas immunopositive for $P2Y_{12}$ receptor ($r = 0.54$) were significantly correlated with those positive for CD31 (beta 0.30, $p = 0.022$) and CD68 (beta 0.36, $p = 0.007$).

Representative immunohistochemical staining of $P2Y_{12}$ receptor is shown in Figure 1. Immunoreactivity with the anti-$P2Y_{12}$ receptor antibody was stronger in the AMI than the SAP plaque. Staining of serial sections of these plaques demonstrated a similar distribution of $P2Y_{12}$ receptor- and α-smooth muscle actin-immunopositive areas in AMI, but not in SAP. Likewise, CD31 was focally stained on the similar area in AMI but not in SAP. To determine cellular localization of the $P2Y_{12}$ receptor, we performed double-immunofluorescence staining on coronary atherosclerotic plaques from a patient with AMI (Figure 2). Confocal immunofluorescence staining revealed that $P2Y_{12}$ receptor immunoreactivity colocalized with cells positive for CD31 and α-smooth muscle actin but not with those positive for CD68.

Discussion

This study is the first to show that $P2Y_{12}$ receptor is present in human coronary atherosclerotic plaques, that its level of expression is higher in plaques from patients with AMI than from those with SAP, and that $P2Y_{12}$-immunopositive cells are endothelial cells and vascular smooth muscle cells. These findings support the hypothesis that $P2Y_{12}$ receptor inhibitors may have a dual antiischemic effect by inhibiting platelet activation and plaque destabilization.

Clinical benefits of $P2Y_{12}$ receptor inhibitors are largely driven by their antiplatelet activity. More recently, however, $P2Y_{12}$ receptor antagonists have been shown to have platelet-independent effects on the vasculature. For example, $P2Y_{12}$ receptor-mediated vasoconstriction of human internal mammary arteries, as demonstrated by 2-MeSADP–induced contraction of submaximally precontracted vessels, was blocked by a selective reversible $P2Y_{12}$ receptor antagonist but not by clopidogrel.[2] Clopidogrel does not inhibit adenosine diphosphate (ADP)-mediated vasoconstriction, probably because of pharmacokinetics.[12,13] In contrast, ticagrelor was shown to inhibit ADP-mediated vasoconstriction of isolated human arteries.[14] In addition, thrombin, which is generated during vessel injury, can increase expression of $P2Y_{12}$ receptor in vascular smooth muscle cells, resulting in increased vasoconstriction and a greater proinflammatory response to ADP.[15] In our study $P2Y_{12}$ receptor was more strongly expressed in culprit plaques of patients with AMI than in those with SAP. Areas immunopositive for $P2Y_{12}$ receptor were correlated with CD68 immunopositive areas ($r = 0.46$, $p = 0.001$), and $P2Y_{12}$ receptor was more frequently found in vascular smooth muscle cells around areas of inflammatory cell infiltrations, suggesting that vascular smooth muscle cells at sites of inflammation more actively produce $P2Y_{12}$ receptor. ADP released by a platelet thrombus may contribute to vasospasm in a culprit lesion, worsening the ischemia caused by the thrombus. Therefore, the additional benefits of ticagrelor compared to clopidogrel may be due, at least in part, to inhibition by ticagrelor of $P2Y_{12}$-mediated vasoconstriction.

$P2Y_{12}$ receptor inhibitors may improve endothelial dysfunction by direct interaction with the endothelial $P2Y_{12}$ receptor, which is, as yet, poorly characterized.[16,17] Nitric oxide–dependent coronary vasodilation by clopidogrel has been shown to be independent of its antiplatelet activity in an animal study.[16] Clopidogrel has been found to increase endothelial nitric oxide bioavailability and decrease biomarkers of oxidant stress and inflammation in patients with coronary artery disease.[5] Moreover, clopidogrel, but not aspirin, has been found to block plasma-promoted endothelial activation.[17] We found that CD31, an endothelial cell marker, was strongly expressed in culprit plaques of patients with AMI, that there was a significant correlation between areas positive for CD31 and $P2Y_{12}$ receptor, and that CD31-immunopositive cells colocalized with cells positive for $P2Y_{12}$ receptor. These findings suggest that endothelial cells express $P2Y_{12}$ receptor, especially in culprit plaques of AMI. Optimal $P2Y_{12}$ receptor blockade has been reported to decrease endothelial injury during percutaneous coronary intervention,[18] and high maintenance-dose clopidogrel compared to standard-dose clopidogrel has been shown to improve endothelial function and decrease inflammation,[19] indicating that $P2Y_{12}$ receptor antagonists have a protective effect on endothelial cells. Thus $P2Y_{12}$ receptor is likely involved in plaque destabilization by activating endothelial cells. At present, however, very little is known about the biological functions and signaling pathways of $P2Y_{12}$ receptors in endothelium.[1,20]

One limitation of this study is that atherectomy specimens were obtained from selected lesions in large vessels because calcified, tortuous, and small vessels are not suitable for directional coronary atherectomy. Another limitation is that $P2Y_{12}$ receptor expression could not be confirmed by western blot analysis.

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