Comparative Studies of a Humanized Anti-glycoprotein IIb/IIIa Monoclonal Antibody, YM337, and Abciximab on *in Vitro* Antiplatelet Effect and Binding Properties

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The effects of YM337, the Fab fragment of a humanized anti-glycoprotein IIb/IIIa (GPIIb/IIIa) monoclonal antibody C4G1, on *in vitro* platelet function and binding properties were compared with those of abciximab, the Fab fragment of the human/murine chimeric anti-GPIIb/IIIa monoclonal antibody 7E3. Both agents completely inhibited platelet aggregation caused by all agonists tested except ristocetin. Further, both inhibited human platelet adhesion to von Willebrand factor, fibrinogen, fibronectin and subendothelial matrix with similar potency. Fibrinogen binding to washed platelets was dose-dependently inhibited by both agents. In binding assay using ^{125}I-YM337 and ^{125}I-abciximab, K_d values determined with platelet-rich plasma were 6.74±0.56 nM for YM337 and 6.65±1.45 nM for abciximab, and the number of binding sites were 42700±3000 for YM337 and 76000±5400 for abciximab. GPIIb/IIIa was precipitated from the solubilized fraction of platelets by both agents. In contrast, integrin $\alpha_v\beta_3$ was precipitated from the solubilized fraction of human umbilical vein endothelial cells by abciximab but not by YM337. Fibrinogen binding to purified GPIIb/IIIa was dose-dependently inhibited by both agents. In contrast, vitronectin binding to purified integrin $\alpha_v\beta_3$ was dose-dependently inhibited by abciximab but not by YM337, supporting the idea that abciximab reacts to integrin $\alpha_v\beta_3$. Therefore, YM337 was suggested to bind to a different epitope of GPIIb/IIIa from abciximab. These results suggest that YM337 specifically acts on platelet GPIIb/IIIa receptors and has similar inhibitory properties on platelet aggregation and platelet adhesion to abciximab.

Key words glycoprotein IIb/IIIa; monoclonal antibody; platelet aggregation; integrin; binding

Platelet aggregation is known to occur through the binding of fibrinogen to activated platelet glycoprotein IIb/IIIa (GPIIb/IIIa) and subsequent bridging of platelets *via* bound fibrinogen.[1,2] Therefore, GPIIb/IIIa antagonists, which inhibit fibrinogen binding to platelet GPIIb/IIIa, are considered to be potent inhibitors of platelet aggregation.

YM337 is the Fab fragment of a humanized anti-GPIIb/IIIa monoclonal antibody (hC4G1), produced by complementary-determining regions grafting of murine anti-GPIIb/IIIa monoclonal antibody C4G1.[3] This compound completely inhibited platelet aggregation induced by various agonists in monkeys and humans.[3,4] It also showed an antithrombotic effect in some animal models of thrombosis.[4—6]

Abciximab, developed by Centocor (Malvern, PA, U.S.A.), is also the Fab fragment of the human/murine chimeric anti-GPIIb/IIIa monoclonal antibody (c7E3), produced by chimerization of murine anti-GPIIb/IIIa monoclonal antibody 7E3, and inhibits human platelet aggregation.[7] Abciximab has been pleviously shown to bind to intgerin $\alpha_v\beta_3$ of human umbilical vein endothelial cells (HUVEC) and to inhibit the binding of ligands to HUVEC and purified integrin $\alpha_v\beta_3$.[8] Moreover, this antibody has been shown to bind to integrin $\alpha_M\beta_2$ (CD11b/CD18 or Mac-1) and to inhibit the binding of ligands.[9] It has been used for the prevention of cardiac ischemic complications of high-risk percutaneous transluminal coronary angioplasty (PTCA) patients in many countries.[10,11]

Previously, we compared the *ex vivo* antiplatelet effect of YM337 and abciximab in rhesus monkeys, and have shown that YM337 is an easy controllable GPIIb/IIIa receptor antagonist with a short half-life.[12] Further, YM337 inhibited platelet aggregation completely with little prolongation of template bleeding time, whereas abciximab prolonged template bleeding time.[12] However, the biochemical properties of YM337 have yet to be reported in detail.

In the present study, we examined the *in vitro* antiplatelet effects and binding properties of YM337 and directly compared with those of abciximab. We found that YM337 recognizes GPIIb/IIIa of platelets only but that abciximab does integrin $\alpha_v\beta_3$ of vascular endothelial cells as well, and that inhibitory properties of YM337 on platelet aggregation and platelet adhesion are similar to those of abciximab.

MATERIALS AND METHODS

Drugs The production and humanization of monoclonal antibody C4G1 have been described in detail elsewhere.[3,13] The Fab fragment of the hC4G1, YM337, was prepared using papain according to established methods.[14] Abciximab was purchased from Eli Lilly (Indianapolis, IN, U.S.A.) or Eli Lilly Netherland B.V. (Nienwegein, the Netherlands).

Preparation of Platelets Blood was withdrawn from healthy volunteers in the presence of a 1/10th volume of 3.8% sodium citrate, added as an anticoagulant, and centrifuged at 150×***g*** for 10 min at room temperature to obtain platelet-rich plasma (PRP). This residue was centrifuged further at 2000×***g*** for 10 min to obtain platelet-poor plasma (PPP). The number of platelets in PRP was counted with an automatic blood cell counter (MEK-5158, Nihon Kohden, Tokyo, Japan) and adjusted to the appropriate platelet count by adding PPP. Washed platelets were prepared as follows. PRP was mixed with 10 mM citrate and centrifuged at 800×***g*** for 15 min. The precipitate was suspended in 1 ml of Tyrode's-Hepes (3.8 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 2.9 mM

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NaH_2PO_4, 5.6 mM dextrose) pH 6.7 and 2 U/ml of potato apyrase (Sigma, St. Louis, MO, U.S.A.) was added. The mixture was allowed to stand at 37 °C for 10 min and, after addition of Tyrode's-Hepes pH 6.7, was centrifuged at 800×***g*** for 15 min. The platelets were resuspended in Tyrode's-Hepes pH 7.35 and the number was counted with an automatic blood cell counter. The suspension was diluted with Tyrode's-Hepes pH 7.35 to adjust to appropriate platelet count.

Platelet Aggregation Assay Platelet aggregation was induced by ADP (20 μM, Sigma), collagen (200 μg/ml, Sigma), epinephrine (10 μM, Sigma), ristocetin (1.5 mg/ml, Sigma) and U46619 (1 μM, Cayman Chemicals, Ann Arbor, MI, U.S.A.) and platelet aggregation was measured with an aggregometer (HEMA TRACER 801, MC Medical, Tokyo, Japan). Eighty microliter of PRP, previously adjusted to a concentration of 300000 platelets/μl, and 10 μl of either YM337 or abciximab were placed into a cuvette for measurement. After incubation at 37 °C for 1 min, 10 μl of each agonist was added to induce aggregation. The data (%) were expressed as the inhibition ratio of maximum platelet aggregation in the presence of the drug to that in the absence of the drug. The IC_{50} values were calculated from each inhibition curve.

Platelet Adhesion Assay Human von Willebrand factor (vWF) was purified from human plasma by the methods of Thorell *et al*.[15] Subendothelial matrix was prepared by the methods of Boomgaard *et al*.[16] HUVEC (Kurabo Industries, Osaka, Japan) were cultured to subconfluent, and then re-cultured for 2 d in 96 well plates (Nunc, Roskilde, Denmark) for tissue culture. After treatment of the plate with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 20 mM NH_4OH for 3 min at room temperature, the plate was washed 4 times with PBS to obtain a preparation of subendothelial matrix.

vWF, fibrinogen (Sigma), fibronectin (Novel Industries, Sweden) and bovine serum albumin (BSA, Sigma) diluted to 10 μg/ml with PBS were added to 96 well plates (Sumitomo Bakelite, Tokyo, Japan), and incubated at 4 °C overnight. The plate was then blocked at 37 °C for 1 h using 20 mM Tris–HCl pH 8.0 and 150 mM NaCl containing 3% BSA. YM337 and abciximab, diluted with 20 mM Tris–HCl pH 8.0, 150 mM NaCl, and 200000 platelets/μl of PRP were added to the blocked plates and the plates by which subendothelial matrix was prepared, and incubated at 37 °C for 1 h. The number of adhesive platelets was assessed by measuring endogenous acid phosphatase by the methods of Lu *et al*.[17] After washing twice with 20 mM Tris–HCl pH 8.0 and 150 mM NaCl containing 0.05% Tween 20, 50 mM citrate buffer pH 5.5 containing 4 mg/ml *p*-nitrophenylphosphate and 0.1% Triton X-100 was added, and incubation was continued at 37 °C for 1 h. After cessation of the reaction by adding 1 M NaOH, A405 was measured with a microplate reader (Thermo Max, Molecular Devices, Sunnyvale, CA, U.S.A.).

^{125}I-Labelling of Proteins Fibrinogen was dissolved in 0.1 M sodium phosphate buffer pH 7.3 at a concentration of 20 mg/ml. For the preparation of ^{125}I-YM337 and ^{125}I-abciximab, Iodo-beads (Pierce, Rockford, IL, U.S.A.) were added to 60 μl of 0.1 M Na-phosphate buffer pH 7.3 and 40 μl of 370 MBq/ml $Na^{125}I$ (ICN Biomedicals, Costa Mesa, CA, U.S.A.), and for the preparation of ^{125}I-fibrinogen Iodo-beads were added to 50 μl of 0.1 M Na-phosphate buffer pH 7.3 and 50 μl of 370 MBq/ml $Na^{125}I$. After 5 min, 100 μl of either 2 mg/ml YM337 or 2 mg/ml abciximab, or 200 μl of 20 mg/ml fibrinogen was added and the mixture was allowed to stand at room temperature for 10 min. The reaction mixture was applied to PD10 (Amersham Pharmacia, Uppsala, Sweden) equilibrated with PBS and fractions of 500 μl were collected. The radioactivity of each fraction was measured with a γ-counter (Aloka, Tokyo, Japan) and fractions in which labeled protein was eluted were collected. The protein in each fraction was determined by the Bradford method (Bio-Rad, Richmond, CA, U.S.A.).

^{125}I-Fibrinogen Binding to Platelet The washed platelet suspension (300000 platelets/μl) were added to 200 μg/ml ^{125}I-fibrinogen, 1 mM $CaCl_2$ and either YM337 or abciximab. After further addition of 20 μM thrombin receptor agonist peptide (TRAP, Calbiochem, San Diego, CA, U.S.A.), the mixture was allowed to stand at room temperature for 1 h. For determination of non-specific binding, 10 mM EDTA was added. After the reaction, 100 μl of the mixture was layered over 200 μl of 20% sucrose and the tube was centrifuged at 10500×***g*** for 3 min. The tip of the tube was cut off and its radioactivity was measured with a γ-counter. The data (%) were expressed as the percentage of binding in the presence of the drug relative to that in the absence of the drug, and the IC_{50} values were calculated for each drug.

^{125}I-YM337 and ^{125}I-Abciximab Binding to Platelet ^{125}I-YM337 or ^{125}I-abciximab was added to PRP (100000 platelets/μl) or washed platelets (100000 platelets/μl), and the mixture was allowed to stand at room temperature for 1 h. For determination of non-specific binding, unlabeled YM337 or abciximab was added in an amount 100 times that of the labeled compound. For examination in the activated state, 20 μM of TRAP was added before addition of ^{125}I-YM337 or ^{125}I-abciximab. After the reaction, 100 μl of the mixture was layered over 200 μl of 20% sucrose and the tube was centrifuged at 10500×***g*** for 3 min. The tip of the tube was cut off and its radioactivity was measured with a γ-counter. K_d values and the number of binding sites per platelet were calculated from each saturation curve.

Immunoprecipitation from Platelets PRP was centrifuged at 800×***g*** for 15 min at room temperature and the sediment was suspended in Tyrode's-Hepes pH 6.7. This was centrifuged again and the sediment was resuspended in 1 ml of Tyrode's-Hepes pH 6.7. The membrane proteins of platelets in the suspension were labeled with ^{125}I by the lactoperoxidase method. The labeled platelets were washed with Tyrode's-Hepes pH 6.7 and solubilized with 1 ml of 20 mM Tris–HCl pH 7.4, 150 mM NaCl including 1% Nonidet P-40 (NP-40), 1 mM phenymethylsulfonyl fluoride (PMSF), 1 mM N-ethylmaleimide (NEM), 1 μM pepstatin. After addition of 10 μg/ml human γ-globulin fraction and 10 μg/ml anti-human immunoglobulin framework antibody (Jackson ImmunoResearch Laboratories, Weat Grove, PA, U.S.A.) to this lysate, the mixture was added to protein A-Sepharose (Amersham Pharmacia, Uppsala, Sweden) and incubated at 4 °C for 3 h to remove proteins which were adsorbed non-specifically to the protein A-Sepharose. To the lysate thus treated was added 10 μg/ml of YM337, abciximab or human γ-globulin fraction. After addition of anti-human immunoglobulin framework antibody and protein A-Sepharose, the mixture was incubated at 4 °C for 24 h. After the protein A-Sepharose

was washed, the proteins adsorbed were eluted with Tris/SDS sample buffer. The eluate was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4—20% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and the proteins were transferred to a polyvinylidene fluoride membrane and visualized by autoradiography with X-ray film.

Immunoprecipitation from HUVEC HUVEC (Kurabo Industries, Osaka, Japan) were cultured with a medium containing M199 (Gibco, Rockville, MD, U.S.A.), 15% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 90 μg/ml heparin and 1% Endothelial Cell Growth Supplement (Becton Dickinson; Franklin Lakes, NJ, U.S.A.). The cells (1×10^7) were washed with methionine- and cysteine-free Dulbecco's modified Eagle's medium and 15% dialysis FCS and metabolically labeled overnight with 3.7 MBq/ml of ^{35}S-methionine and cysteine (EXPRE ^{35}S^{35}S Protein Labeling Mix, NEN, Boston, MA, U.S.A.). The cells were then solubilized with 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM NEM, 1 μM pepstatin, 10 units/ml aprotinin, and 100 μg/ml DNase I. Human γ-globulin fraction and anti-human immunoglobulin framework antibody were added to the supernatant. This mixture was added to protein A-Sepharose and incubated at 4 °C for 3 h to remove proteins which were non-specifically adsorbed to the protein A-Sepharose. To the 200 μl of lysate thus treated was added 50 μg of YM337, abciximab or human γ-globulin fraction. After addition of anti-human framework antibody and protein A-Sepharose, the mixture was incubated at 4 °C for 30 h. After the protein A-Sepharose was washed, the proteins adsorbed were eluted with Tris/SDS sample buffer. The eluate was applied to SDS-PAGE by use of 6.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, dried with a gel drier and analyzed by a BAS-2000 image analyzer (Fujifilm, Tokyo, Japan).

Preparation of Biotinylated Proteins Human fibrinogen (Sigma) and human vitronectin (Asahi Techno Glass, Tokyo, Japan) were commercially purchased, respectively. Adhesive proteins were biotinylated by adding sulfo-NHS-LC-biotin (Pierce) to adhesive protein solutions and allowing the mixture to stand for 2 h at room temperature. Excess biotin was removed by gel chromatography and the buffer was changed to TBS (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$).

Binding of Biotinylated Proteins to Purified Integrins To a 96 well microplate (MaxiSorp™, Nunc) was added either 100 μl of 2 μg/ml purified GPIIb/IIIa (Enzyme Research Laboratories, South Bend, IN, U.S.A.) or 50 μl of 2 μg/ml purified integrin $\alpha_v\beta_3$ (Chemicon, Temecula, CA, U.S.A.), both diluted with TBS, and the plate was left at room temperature overnight. After removing the solution, 200 μl of 3.5% BSA/TBS was added and the plate was incubated at 37 °C for 2 h. After washing with TBS once, 1 μg/ml of biotinylated-fibrinogen (b-Fg) or 0.4 μg/ml of biotinylated-vitronectin (b-Vn), diluted with TBS, was added in a volume of 50 μl. YM337 or abciximab was added in a volume of 50 μl and the reaction was allowed to proceed at 37 °C for 3 h. After washing 3 times with 0.05% Tween 20/TBS, 100 μl of Streptavidin biotinylated horseradish peroxidase complex (Amersham Pharmacia) diluted 1000-fold with TBS was added, and the plate was incubated at room temperature for 1 h. After washing 3 times with 0.05% Tween 20/TBS, 100 μl of Tetramethylbenzidine solution (Bio-Rad) was then added for color development. After cessation of the reaction by adding 50 μl of 1 M NaOH, A405 was measured with a microplate reader (Model 3350, Bio-Rad). The data were expressed as the binding (%) in the presence of the drug relative to that in the absence of the drug. The IC_{50} values were calculated from each inhibition curve.

RESULTS

Platelet Aggregation Assay The inhibitory effect of YM337 and abciximab against platelet aggregation caused by a variety of agonists (ADP 20 μM, collagen 200 μg/ml, U46619 1 μM, epinephrine 10 μM, ristocetin 1.5 mg/ml) is shown in Fig. 1. Both agents dose-dependently inhibited the platelet aggregation induced by all agents except ristocetin. The IC_{50} values of the two drugs for various agonists except ristocetin are shown in Table 1. Inhibitory concentrations for YM337 were lower than those for abciximab by a factor of 2.2 to 5.2.

Platelet Adhesion Assay Effects of YM337 and abciximab on human platelet adhesion to vWF, fibrinogen, fibronectin, subendothelial matrix and BSA are shown in Fig. 2. Both agents dose-dependently inhibited platelet adhesion to vWF, fibrinogen, fibronectin and subendothelial matrix up to the background level (BSA). Inhibitory potencies of YM337 on platelet adhesion to these adhesive proteins were similar to those of abciximab.

Fibrinogen Binding to Platelet The effect of YM337 and abciximab on the ^{125}I-fibrinogen binding to TRAP-stimulated washed platelets is shown in Fig. 3. Both agents inhibited fibrinogen binding to washed platelet in a dose-dependent manner. The IC_{50} values were 0.76 ± 0.11 μg/ml for YM337 and 1.04 ± 0.16 μg/ml for abciximab, indicating that inhibitory activities of the two compounds are closely similar.

YM337 and Abciximab Binding to Platelet Table 2 shows K_d values and number of binding sites per platelet for the two agents when PRP was used. K_d values for YM337 and abciximab were 6.74 ± 0.56 nM and 6.65 ± 1.45 nM, respectively, indicating that the two agents have similar affinity to platelets. The number of binding sites for YM337 was 42700 ± 3000, while that for abciximab was 76000 ± 5400. Binding site number for abciximab was about 1.8 times greater than that for YM337.

Table 3 shows the corresponding figures when human washed platelets were used. With unstimulated washed platelets, K_d values were 12.67 ± 1.00 nM for YM337 and 11.57 ± 0.70 nM for abciximab. The number of binding sites was 22500 ± 3200 for YM337 and 42800 ± 5900 for abciximab. With TRAP-stimulated washed platelets, K_d value for YM337 was 12.46 ± 0.83 nM, while that for abciximab was 12.62 ± 0.63 nM. Numbers were 27600 ± 3300 for YM337 and 53900 ± 3700 for abciximab. These findings indicate that the affinity of the two compounds to platelets is similar but that there are about twice as many binding sites for abciximab as for YM337. Activation of platelets did not affect K_d values or the number of binding sites for either agent.

Immunoprecipitation from Platelet and HUVEC Figure 4 illustrates the results of immunoprecipitation from the

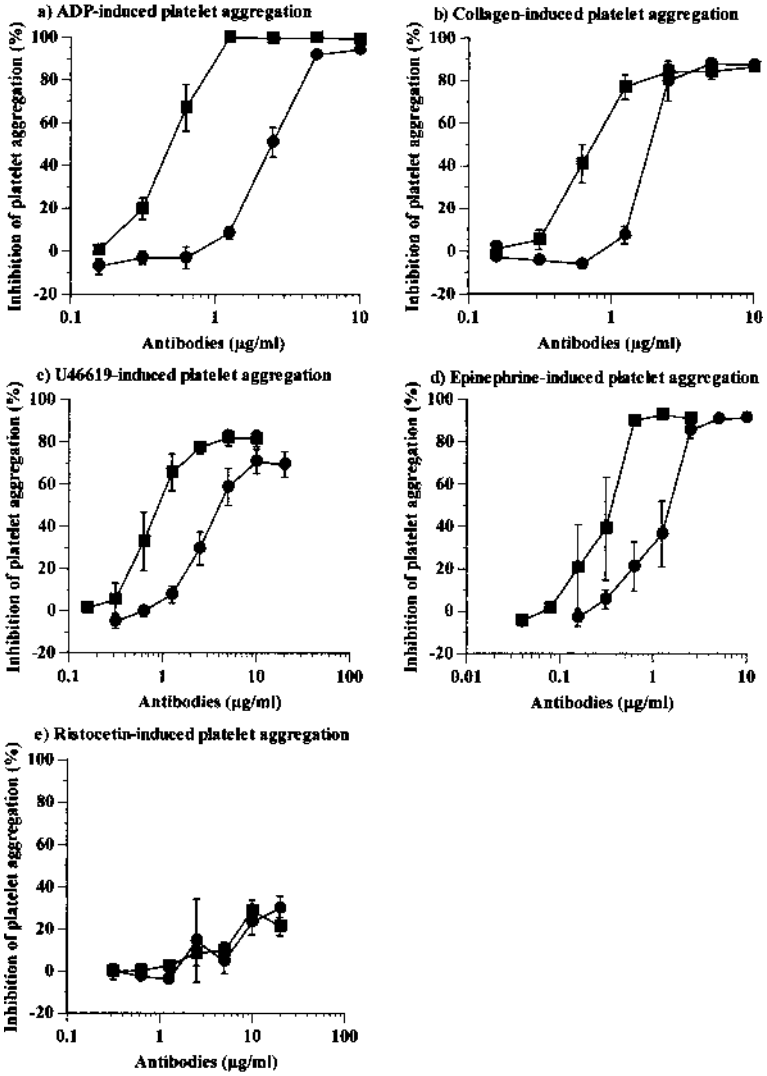

Fig. 1. Inhibitory Effects of YM337 (■) and Abciximab (●) on Platelet Aggregation Induced by Various Agonists

Platelet aggregation induced by 20 μM ADP (a), 200 μg/ml collagen (b), 1 μM U46619 (c), 10 μM epinephrine (d) and 1.5 mg/ml ristocetin (e) was measured using PRP in the presence of increasing concentrations of YM337 and abciximab. Data are presented as mean±S.E.M. (ADP: n=7 for YM337, n=8 for abciximab, collagen: n=8, U46619: n=5, epinephrine: n=4 for YM337, n=5 for abciximab, ristocetin: n=5).

Table 1. IC_{50} Values of YM337 and Abciximab on Platelet Aggregation Induced by Various Agonists

Drug	IC_{50} value (μg/ml)			
	ADP	Collagen	U46619	Epinephrine
YM337	0.507±0.053	0.937±0.215	1.189±0.238	0.383±0.109
Abciximab	2.518±0.160	2.072±0.217	6.191±1.108	1.463±0.303

IC_{50} values were calculated by nonlinear regression of data. Data are presented as mean±S.E.M.

solubilized fraction of platelets (a) and HUVEC (b) using YM337 and abciximab. From the solubilized fraction of platelets, both agents precipitated a protein having a molecular weight corresponding to GPIIb/IIIa. From the solubilized fraction of HUVEC, on the other hand, abciximab precipitated a protein with a molecular weight of integrin $\alpha_v\beta_3$, while no specific protein was precipitated by YM337 in comparison with the control.

Binding of Biotinylated Proteins to Purified Integrins Figure 5 illustrates the inhibitory effects of YM337 and abciximab on the binding of b-Fg to purified GPIIb/IIIa (a) and b-Vn to purified integrin $\alpha_v\beta_3$ (b). Both YM337 and abciximab inhibited b-Fg binding to purified GPIIb/IIIa in a dose-dependent manner. Abciximab showed dose-dependent inhibition of b-Vn binding to purified integrin $\alpha_v\beta_3$, but YM337 exerted no significant inhibition up to 100 μg/ml. IC_{50} value for b-Fg binding to purified GPIIb/IIIa was 0.324±0.042 μg/ml for YM337 and 0.037±0.002 μg/ml for abciximab, indicating that inhibition by abciximab was about 9 times greater than that by YM337. IC_{50} value for abciximab with respect to b-Vn binding to purified integrin $\alpha_v\beta_3$ was 0.297± 0.034 μg/ml.

DISCUSSION

In the present study, YM337 and abciximab inhibited *in vitro* platelet functions including platelet aggregation, platelet adhesion and fibrinogen binding to platelets. Though both agents had similar affinity to platelets, the number of

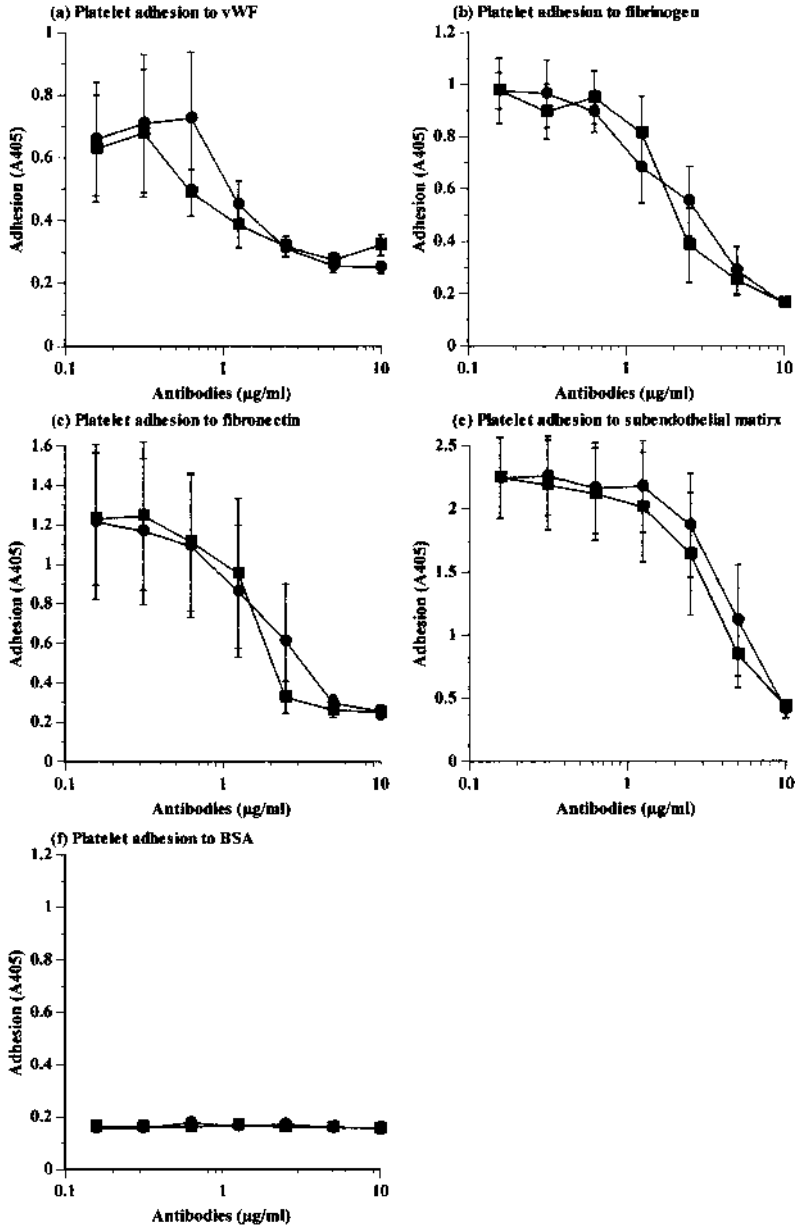

Fig. 2. Effects of YM337 (■) and Abciximab (●) on Platelet Adhesion to Various Proteins

Platelet adhesion to 10 μg/ml of vWF (a), 10 μg/ml of fibrinogen (b), 10 μg/ml of fibronectin (c), subendothelial matrix (d) and BSA (e) coated onto 96 well plates was measured using PRP in the presence of increasing concentrations of YM337 and abciximab. The number of adherent platelets in each well was determined by measurement of endogeneous acid phosphatase activity. Data are presented as mean±S.E.M. (vWF, fibronectin: $n=5$, fibrinogen, subendothelial matrix, BSA: $n=4$).

binding sites of abcixmab were about twice that of YM337. Further, YM337 immunoprecipitated GPIIb/IIIa of platelets only, and inhibit fibrinogen binding to purified GPIIb/IIIa. In contrast, abciximab immunoprecipitated integrin $\alpha_v\beta_3$ of HUVEC in addition of GPIIb/IIIa of platelets, and inhibit both fibrinogen binding to purified GPIIb/IIIa and vitronectin binding to integrin $\alpha_v\beta_3$.

Both YM337 and abciximab completely inhibited the platelet aggregation in PRP caused by all agonists used except ristocetin. Fibrinogen binding to platelet GPIIb/IIIa represents the final step in platelet aggregation, and GPIIb/IIIa antagonists are therefore considered to exhibit strong inhibition of platelet aggregation. The aggregation caused by ristocetin is known to be due to bridging of platelets through the binding of GPIb and vWF, and the involvement of GPIIb/IIIa is therefore considered small. This explains the weak inhibitory activity of two agents against ristocetin. These findings are consistent with those obtained with other GPIIb/IIIa antagonists.[18,19]

Platelet adhesion assay revealed that both agents inhibited platelet adhesion to vWF, fibrinogen, fibronectin and subendothelial matrix with similar potency. Both agents inhibited platelet adhesion to fibrinogen, fibronectin and subendothelial matrix up to the background level (BSA), suggesting that most of these adhesion phenomena involves platelet GPIIb/IIIa. In contrast, platelet adhesion to vWF was not completely inhibited by these agents, suggesting that this adhesion involves factors other than GPIIb/IIIa. Since it has been shown that vWF in the platelet can bind to platelet GPIb in addition to GPIIb/IIIa,[20] a possible relationship be-

tween platelet adhesion to vWF and GPIb in addition to GPIIb/IIIa could be considered. In a preliminary study with Aurin Tricarboxylic Acid (ATA), which inhibits the binding of GPIb/IX/V complex to vWF,[21] both agents completely inhibited platelet adhesion to vWF in the presence of ATA, suggesting that both GPIIb/IIIa and GPIb are involved in platelet adhesion to vWF.

YM337 dose-dependently inhibited fibrinogen binding to platelets to the same extent as abciximab. Fibrinogen binding to GPIIb/IIIa is the common final step in platelet aggregation induced by a variety of stimuli. Both agents are considered to inhibit platelet aggregation by inhibiting the binding between fibrinogen and GPIIb/IIIa. In addition to GPIIb/IIIa, integrin $\alpha_v\beta_3$ and integrin $\alpha_5\beta_1$ are known as receptors of platelets binding with fibrinogen. However, the number of integrin $\alpha_v\beta_3$ per platelet is reported to be only 50—100[22] or 1500,[23] while that of GPIIb/IIIa is approx. 40000[24,25] and most fibrinogen binding to platelets is considered to be due to its binding to GPIIb/IIIa.

Binding study revealed that the two agents have similar affinity to platelets and that their affinity does not change on activation of platelets. Further, abciximab has about twice as many binding sites on platelets as YM337. The K_d value and

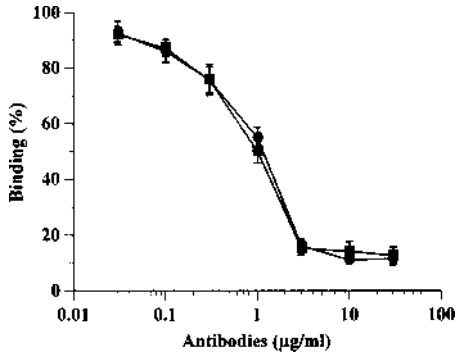

Fig. 3. Effects of YM337 (■) and Abciximab (●) on ^{125}I-Fibrinogen Binding to TRAP-Stimulated Washed Platelets

Binding of ^{125}I-fibrinogen was measured using washed platelets stimulated with 20 μM TRAP in the presence of increasing concentrations of YM337 and abciximab. Data are presented as mean±S.E.M. (n=6).

Table 2. K_d Values and Molecules Bound per Platelet of YM337 and Abciximab Binding to Human PRP

Drug	K_d (nM)	Molecules/platelet
YM337	6.74±0.56	42700±3000
Abciximab	6.65±1.45	76000±5400

Binding of ^{125}I-YM337 and ^{125}I-abciximab was measured using human PRP obtained from normal donors. K_d values of YM337 and abciximab and the number of YM337 and abciximab molecules bound per platelet were calculated from respective saturation curves. Data are presented as mean±S.E.M. (n=6).

Table 3. K_d Values and Molecules Bound per Platelet of YM337 and Abciximab Binding to Unstimulated and TRAP-Stimulated Human Washed Platelets

Drug	K_d (nM)		Molecules/platelet	
	Unstimulated	Stimulated	Unstimulated	Stimulated
YM337	12.67±1.00	12.46±0.83	22500±3200	27600±3300
Abciximab	11.57±0.70	12.62±0.63	42800±5900	53900±3700

Binding of ^{125}I-YM337 and ^{125}I-abciximab was measured using unstimulated or 20 μM TRAP-stimulated human washed platelets. K_d values of YM337 and abciximab and the number of YM337 and abciximab molecules bound per platelet were calculated from respective saturation curves. Data are presented as mean±S.E.M. (n=5).

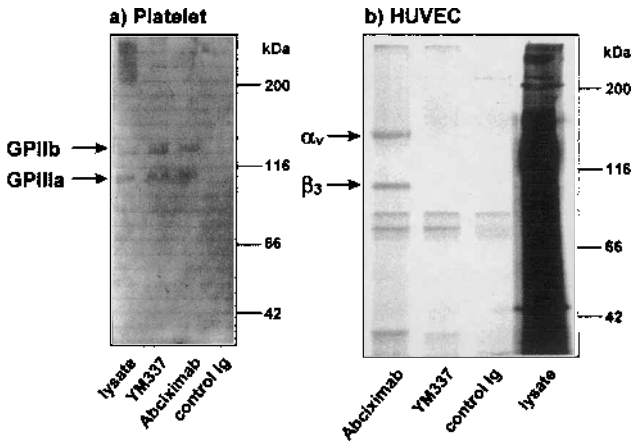

Fig. 4. Immunoprecipitation of YM337 and Abciximab from Platelets (a) and HUVEC (b)

(a): Platelets were ^{125}I-labeled and lysed with detergents. The ^{125}I-labeled platelets lysates were allowed to react with YM337, abciximab or human γ-globulin fraction (control Ig), and then these reaction mixtures were incubated with anti-human immunoglobulin framework antibody. (b) HUVEC were metabolically ^{35}S-labelled and lysed with detergents. The ^{35}S-labelled HUVEC lysates were allowed to react with YM337, abciximab or human γ-globulin fraction (control Ig), and then these reaction mixture were incubated with anti-human immunoglobulin framework antibody.

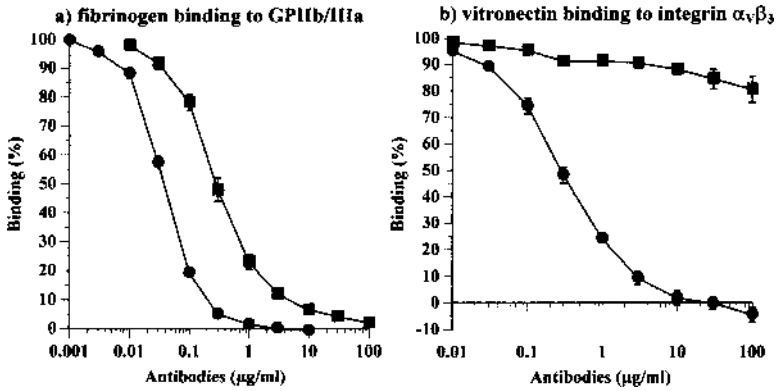

Fig. 5. Effects of YM337 (■) and Abciximab (●) on b-Fg Binding to Purified GPIIb/IIIa (a) and b-Vn Binding to Purified Integrin $\alpha_v\beta_3$ (b)

Binding of b-Fg to purified GPIIb/IIIa and b-Vn to purified integrin $\alpha_v\beta_3$ were measured in the presence of increasing concentrations of YM337 and abciximab. Data are presented as mean±S.E.M. (n=5).

binding site number of abciximab obtained in this study are consistent with those of Tam *et al.*[8] and Wagner *et al.*[26] It has been reported that approx. 40000 molecules of fibrinogen[8] and 35000 to 50000 molecules of anti-GPIIb/IIIa monoclonal antibodies are bound to one platelet.[27,28] This agrees closely with the number (42700±3000) of binding sites obtained for YM337 in this study. Although it is not understood why abciximab has more binding sites on platelets than YM337, our previous study demonstrated that the binding of an RGD peptide to platelets was inhibited by abciximab, but not by YM337.[13,29] Further, our preliminary data showed that YM337 did not compete with abciximab in binding to purified GPIIb/IIIa (data not shown). These results suggested that YM337 binds to a different epitope of GPIIb/IIIa from abciximab.

Both agents precipitate GPIIb/IIIa from the solubilized fraction of platelets and abciximab alone precipitates integrin $\alpha_v\beta_3$ from the solubilized fraction of HUVEC. The data concerning abciximab obtained in the present study are in agreement with those of Tam *et al.*[8] However, YM337 did not precipitate any specific protein from the solubilized fraction of HUVEC, indicating that YM337 does not recognize proteins expressed in HUVEC. Further, abciximab inhibited both fibrinogen binding to purified GPIIb/IIIa and vitronection binding to purified integrin $\alpha_v\beta_3$, whereas YM337 inhibited only fibrinogen binding to purified GPIIb/IIIa. These results also suggested that YM337 and abciximab recognized different epitopes of GPIIb/IIIa.

Both GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) and integrin $\alpha_v\beta_3$ are members of the integrin superfamily. Integrin, a heterodimer glycoprotein consisting of α- and β-chains, is present on the surface of many cells and is involved in various physiological phenomena.[30] While GPIIb/IIIa is expressed only on platelets and its precursor megakaryocytes, integrin $\alpha_v\beta_3$ is expressed on various cells including platelets, vascular endothelial cells, smooth muscle cells, osteoclasts and epithelial cells. GPIIb/IIIa and integrin $\alpha_v\beta_3$ bind through the RGD sequence of ligands. Homology among integrins recognizing the RGD sequence is relatively high.[30] Therefore, it is possible that abciximab recognizes integrin $\alpha_v\beta_3$ in addition of GPIIb/IIIa.

Though both agents inhibited the binding of fibrinogen to platelets with similar potency, the inhibitory potency of abciximab on the binding of b-Fg to purified GPIIb/IIIa was about 9 times greater than that of YM337. A reason for this difference may be that Triton X-100, contained in the purified GPIIb/IIIa preparation, altered the antigenicity of GPIIb/IIIa, resulting in lower affinity of YM337 towards GPIIb/IIIa. YM337 and abciximab may have different epitopes on GPIIb/IIIa with the result that the effects of Triton X-100 differ between the two agents.

In conclusion, these results suggested that YM337 specifically acts on platelet GPIIb/IIIa receptors in contrast to abciximab and binds to a different epitope of GPIIb/IIIa from abciximab, and has similar inhibitory potencies on platelet aggregation and platelet adhesion to abciximab. Like abciximab, YM337 may have therapeutic potential in the prevention of cardiac ischemic complications of PTCA patients.

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