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Antithrombotic effect of a humanized anti-GPIIb/IIIa monoclonal antibody, YM207, in a photochemically induced thrombosis model in monkeys

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Abstract

We investigated the antithrombotic effects of a $F(ab')_2$ fragment of a humanized anti-platelet glycoprotein (GP) IIb/IIIa monoclonal antibody, YM207, in a platelet-rich thrombosis model. Thrombus was induced in a mesenteric venule of squirrel monkeys by irradiation with filtered light in combination with i.v. administration of a fluorescent dye (sodium fluorescein). Time to occlusive thrombus formation was significantly prolonged by i.v. bolus injection of 0.3 mg/kg of YM207. At doses of 1 mg/kg and 3 mg/kg, complete occlusion did not occur during 60 min of the observation. Platelet aggregation in platelet-rich plasma was inhibited by YM207 at doses 0.3 mg/kg or more. A good correlation was obtained between inhibition of platelet aggregation and the percentage of GPIIb/IIIa receptors blocked by YM207. Bleeding time was significantly prolonged at a dose of 3 mg/kg. Platelet counts showed no significant change. These results suggest that YM207 is effective in preventing platelet-rich thrombus formation without inducing the prolongation of bleeding time or a significant decrease in platelet count. YM207 may be a useful therapeutic agent for the treatment of thrombotic disorders.

Keywords: Humanized antibody; GPIIb/IIIa; Thrombosis, photochemically induced; Microcirculation; Bleeding time; (Squirrel monkey)

1. Introduction

Platelet thrombus formation is an important cause of arterial thrombotic disorders. The final common mechanism in the formation of a platelet aggregate is the linking of adjacent platelets by fibrinogen binding to the platelet GPIIb/IIIa receptor (Plow and Ginsberg, 1988; Phillips et al., 1988). This step is thought to represent an excellent target for the development of antiplatelet agents.

A number of anti-GPIIb/IIIa monoclonal antibodies that bind to GPIIb/IIIa and block its ability to bind fibrinogen have been developed (Coller et al., 1983; Coller, 1985; Pidard et al., 1983). In particular, treatment with the $F(ab')_2$ fragment of monoclonal antibody 7E3 has been found to abolish platelet thrombus formation in some experimental thrombosis models (Coller et al., 1986; Hanson et al., 1988). However, murine antibodies are highly immunogenic in humans. To reduce this immunogenicity, the monoclonal antibody 7E3 is now under extensive clinical investigation with chimeric Fab with a human constant region and a murine variable region (Topol and Plow, 1993). However, since chimeric antibodies are still immunogenic at the framework of their variable region (Bruggemann et al., 1989), it may be necessary for therapeutic purposes to fully humanize the antibody by altering both the

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variable and the constant domains. Recently, we achieved the humanization of the murine anti-GPIIb/IIIa monoclonal antibody C4G1 by CDR-grafting (Co et al., 1994). This antibody did not react with platelets from mice, rats, guinea pigs and dogs, but did react with those from humans, and cynomolgus, rhesus and squirrel monkeys. It has therefore been necessary to use monkeys to evaluate the antithrombotic effect of this antibody in vivo.

The present study evaluated the efficacy of $F(ab')_2$ fragments of humanized C4G1 (YM207) in the prevention of thrombus formation in a photochemically induced platelet-rich thrombosis model (Sato and Ohshima, 1984a). The rationale of this model was first described by Rosenblum and El-Sabban (1977). They reported the formation of platelet aggregates in arterioles on the cerebral surface of mice by exposure of the vessels to an appropriately filtered light from a mercury lamp in the presence of an intraluminal fluorescent dye. Sato and Ohshima (1984a) thereafter developed a simple model of experimental thrombosis with high reproducibility in the microvessels of the rat mesentery. An advantage of this thrombosis model in microvessels as compared with those in large vessels is that it allows continuous monitoring of the course of thrombus formation. Although this photochemically induced thrombosis model produces a platelet-rich thrombus, it has not been widely used in the investigation of the antithrombotic effects of anti-GPIIb/IIIa monoclonal antibodies.

In the present study, we optimized light intensity and dye concentration conditions of this model for use in squirrel monkeys, and used it to evaluate the antithrombotic effect of YM207 in platelet-rich thrombosis.

2. Materials and methods

2.1. Monoclonal antibody preparation

The production and humanization of monoclonal antibody C4G1 has been described in detail elsewhere (Co et al., 1994; Yano et al., 1994). $F(ab')_2$ fragments of humanized C4G1 were prepared using pepsin according to established methods (Harlow and Lane, 1988). Briefly, the whole molecule of the humanized C4G1 was digested by porcine pepsin (Sigma Chemical Co., St. Louis, MO) and the $F(ab')_2$ fragment was purified by combination of ion-exchange chromatography and hydrophobic chromatography. The purified $F(ab')_2$ showed as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The residual pepsin concentration in the $F(ab')_2$ preparation was measured by ELISA and found to be less than 50 ppm (lower limit of detection).

2.2. Photochemically induced thrombosis model

Twenty-one squirrel monkeys weighing 520–1000 g were anesthetized with sodium pentobarbital injected into the femoral muscle (20 mg/kg). The right femoral vein and artery were cannulated for the injection of dye and drug and for monitoring arterial blood pressure and heart rate, respectively. The small intestine was exteriorized via a midline incision in the abdominal wall into a bath and perfused with saline kept at 37° C. The mesentery around the ileum was spread out carefully on a small circular glass stage, and the other parts of the intestine were covered with moistened gauze. Microvessels in the mesentery were observed under transillumination with a halogen lamp. Venules of 42–77 μm in diameter were selected in which to produce microthrombi. Thrombus formation was induced in microvessels according to a method reported previously (Rosenblum and Sabban, 1977). Filtered light at a wavelength of 420–490 nm was passed through an epi-illumination system from a mercury lamp to irradiate the selected microvessel through an objective lens. Using a field stop, the area of irradiation around the microvessel was adjusted on the focal plane to a diameter of about 130 μm. The light intensity was controlled at 10.4 mW/mm^2. The experimental protocol was as follows: 1 min after the start of irradiation, YM207 was injected intravenously via the cannula. At 1 min after injection, a solution of sodium fluorescein (2.5% w/v, Kobayashi Seiyaku Kogyo Co., Tokyo, Japan) was injected through the femoral vein (1 ml/kg body weight). Irradiation with the filtered light was continued throughout the observation period. The course of thrombus formation was continuously monitored with a TV camera and recorded on videotape for 60 min after injection of sodium fluorescein. Time to occlusive thrombus formation, time course changes in thrombus area, and the luminal stenosis rate in diameter were used as indexes of antithrombotic activity. To analyze the thrombus area and luminal stenosis rate in diameter, the configuration of the thrombus was traced on a transparent sheet from the TV monitor frames at predetermined times. Thrombus area and the luminal stenosis rate in diameter were measured using a computer-controlled image-processing system (TVIP-2000; Nippon Avionics Co., Tokyo, Japan). Bleeding time was measured in the ear before and at 1 h after drug administration. A 23G needle was used to puncture a vessel on the surface of the ear and blood was blotted from the incision with filter paper every 30 s. Bleeding time was determined by measuring the time from incision until blood no longer stained the filter paper. Blood samples were collected from the left femoral vein before and after observation. At the end of the experiments the monkeys were killed with an overdose of pentobarbital.

2.3. Platelet aggregation assay

Platelet aggregation in whole blood was measured by the impedance method using a Chrono-Log whole blood aggregometer (C560, Chrono-Log Co., Havertown, PA). Aggregation to collagen 10 μg/ml was recorded for 7 min, and the maximum increase in resistance was calculated.

Platelet-rich plasma and platelet-poor plasma was prepared by centrifugation of citrate-anticoagulated blood. Platelet counts in platelet-rich plasma were determined with an automatic cell counter (MEK-5158, Nihon Koden Co., Tokyo, Japan), and were adjusted to a count of 3×10^8 platelets/ml with platelet-poor plasma. Platelet aggregation in platelet-rich plasma was measured using an aggregometer (801, Niko Bioscience, Tokyo, Japan) by recording the increase in light transmission through a stirred suspension of platelet-rich plasma maintained at 37° C for 5 min. Aggregation was induced by ADP at 2 or 3 μmol/l in human platelet-rich plasma and at 20 μmol/l in monkey platelet-rich plasma. Collagen was used at 2 μg/ml in human platelet-rich plasma and at 10 μg/ml in monkey platelet-rich plasma. From in vitro studies, IC_{50} values were determined from the dose-response curve of mean values.

2.4. GPIIb / IIIa receptor sites blocked by YM207

Binding of ^{125}I-labeled YM207 to platelets was measured by incubating platelet-rich plasma at 10^8 platelets/ml with ^{125}I-YM207 at a final concentration of 1 μg/ml, a near-saturation dose. After 30 min incubation at 22° C, aliquots of the samples were layered over 20% sucrose in microcentrifuge tubes and centrifuged at 12000 g for 5 min at 22° C. Radioactivity in the platelet pellet was then determined. The decrease in GPIIb/IIIa receptor sites available for binding of the radiolabeled antibody after injection of YM207 was taken as the number of sites blocked by the in vivo treatment of unlabeled YM207.

2.5. Plasma concentrations of unbound YM207

Concentrations of unbound YM207 in plasma were measured by ELISA with anti-idiotype antibodies specific for YM207. Briefly, 96-well microtiterplates were coated with anti-YM207 antibody at 1 μg/ml. The plates were then blocked with 1% bovine serum albumin. Plasma samples were poured into the wells and incubated at room temperature for 1 h. After washing several times, the plates were incubated with 1 μg/ml of biotinylated anti-idiotype antibody at room temperature for 1 h and subsequently washed again several times. 100 μl of 1000-fold diluted horseradish peroxidase-conjugated streptavidin (Amersham Japan Co., Tokyo, Japan) were poured into each well. The amounts of the bound enzyme were finally quantified using of 2,2'-azido-bis(3-ethylbenzthazoline-6-sulfonic acid) (ABTS, Bio-Rad, Richmond, CA) and measurement of color absorbance at 414 nm.

2.6. Coagulation studies

The prothrombin time and the activated partial thromboplastin time were measured at 37° C using a coagulometer (KC4A; Amelung Co., Lehbrinksweg, Germany) and appropriate reagents (Ortho Diagnostic Systems Co., Tokyo, Japan).

2.7. Statistical analysis

The experiments were performed on groups of four or five monkeys each. Data are expressed as the means ± S.E.M. The groups were compared using one-way analysis of variance (ANOVA), with a P value of less than 0.05 considered significant.

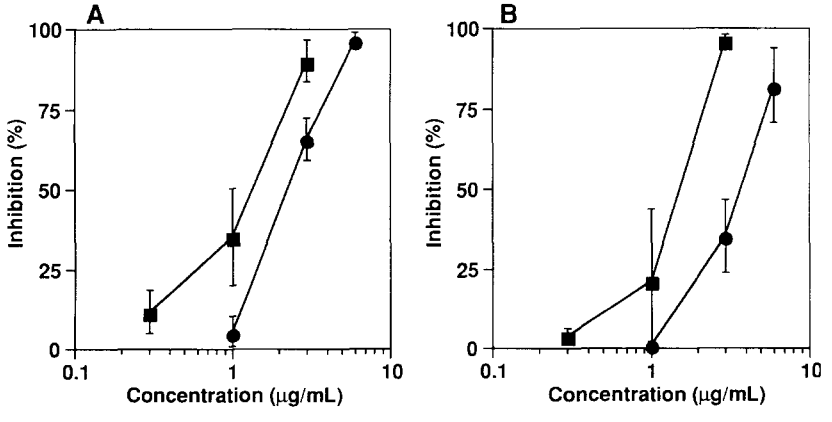

Fig. 1. Inhibitory effect of YM207 on ADP-induced (A) and collagen-induced (B) platelet aggregation in platelet-rich plasma from humans (■) and squirrel monkeys (●). Values are the means ± S.E.M. of at least three separate experiments.

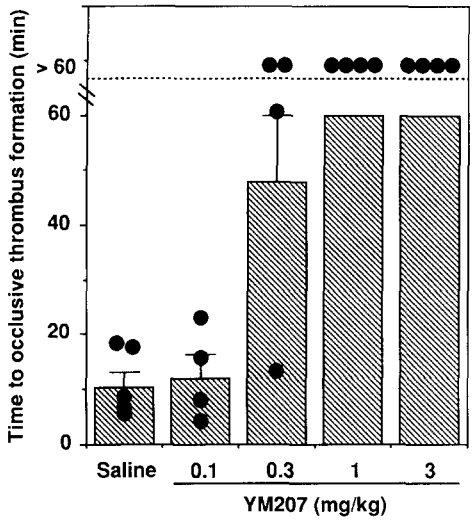

Fig. 2. Effects of YM207 on the formation of occlusive thrombus. Closed circles indicate the time to occlusive thrombus in each animal. Columns indicate the means ± S.E.M. for each group of four or five animals. Times greater than 60 min are given as 60 min for calculation of means ± S.E.M.

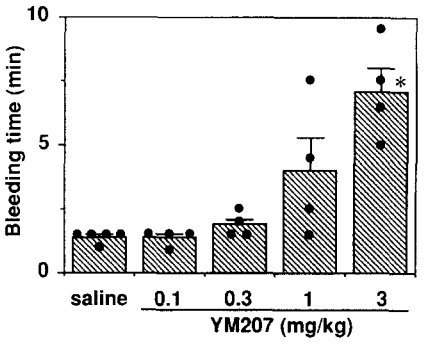

Fig. 3. Effect of YM207 on bleeding time. Closed circles indicate the bleeding time in individual animals. Columns indicate the means ± S.E.M. for each group of four or five animals. $^{*}P < 0.05$ versus the saline group by one-way ANOVA.

3. Results

3.1. Platelet aggregation in vitro

The inhibitory potency of YM207 on ADP- and collagen-induced platelet aggregation was measured using platelet-rich plasma from humans and squirrel monkeys (Fig. 1). YM207 concentration dependently inhibited ADP- and collagen-induced platelet aggregation in these plasmas. ADP- and collagen-induced platelet aggregation were inhibited with IC_{50} values of 1.1 and 1.3 μg/ml in human platelet-rich plasma and 2.4 and 3.7 μg/ml in squirrel monkey platelet-rich plasma. Inhibitory activity of YM207 in squirrel monkey platelet-rich plasma was 2- to 3-fold less than that in human platelet-rich plasma.

3.2. Thrombus formation and occlusion

The time to occlusive thrombus formation and the occlusion rate are shown in Fig. 2. Time to occlusive thrombus formation was calculated as the time at which blood flow in the microvessels was completely stopped by the developing thrombus. If blood flow did not stop within 60 min, this time was regarded as 60 min. In the saline group, the microvessels were occluded at 12.1 ± 4.2 min after initiation of the photochemical reaction in all animals. In the YM207 group, YM207 at a dose of 0.3 mg/kg prolonged the time to occlusive thrombus (48.0 ± 11.9 min) and decreased the occlusion rate (two of four animals). At doses of 1 and 3 mg/kg, occlusive thrombus was not observed in any animal.

Changes in the thrombus area and in the luminal stenosis rate in diameter are shown in Table 1. The data of thrombus area at 0.3 mg/kg were calculated from the values obtained from animals which showed no occlusion because it was difficult to measure accurately the thrombus area of occluded vessels on TV monitor. Increases in the thrombus area and in the luminal stenosis rate in diameter were observed only at the dose of 0.3 mg/kg, and were almost completely inhibited at doses of 1 and 3 mg/kg. The diameter of the venules showed no significant change (data not shown).

3.3. Bleeding time

The results of bleeding time at 60 min after initiation of the photochemical reaction are shown in Fig. 3. Bleeding times measured before injection of saline or YM207 ranged from 1 to 2 min. Bleeding time increased with YM207 in a dose-dependent manner, and was significantly prolonged at a dose of 3 mg/kg.

Table 1
Changes in thrombus area and luminal stenosis rate in diameter

	Thrombus area (μm^2)		Stenosis rate (%)	
	30 min	60 min	30 min	60 min
0.3 mg/kg	2662 ± 507 (3)	3963 ± 1252 (2)	64.3 ± 15 (4)	83.2 ± 11 (4)
1 mg/kg	750 ± 477 (4)	1413 ± 562 (4)	17.5 ± 7.7 (4)	27.2 ± 7.8 (4)
3 mg/kg	666 ± 284 (4)	822 ± 377 (4)	19.4 ± 4.3 (4)	23.2 ± 7.8 (4)

Values are the means ± S.E.M. for each group. () = number of animals.

3.4. Ex vivo platelet aggregation and GPIIb / IIIa receptor blockage

The results of ex vivo platelet aggregation are shown in Fig. 4. Platelet aggregation was slightly altered in animals treated with saline. ADP- and collagen-induced platelet aggregations in platelet-rich plasma were dose dependently inhibited by the injection of YM207 with a maximum effect at doses of 0.3 mg/kg and higher (Fig. 4A). Collagen-induced platelet aggregation in whole blood was inhibited by YM207 with a maximum effect at doses of 1 mg/kg and higher (Fig. 4B). GPIIb/IIIa receptors were dose dependently blocked after the injection of YM207. At a dose of 0.3 mg/kg, blockade of GPIIb/IIIa receptors was 83.3 ± 7.6% (Fig. 5). A good correlation was obtained between the inhibition of ADP-induced platelet aggregation and the percentage of GPIIb/IIIa receptors blocked (Fig. 5, inset). This indicates that the inhibition of ADP-induced platelet aggregation is primarily determined by the blockade of GPIIb/IIIa receptors. A similar correlation was obtained using collagen-induced platelet aggregation in both platelet-rich plasma and whole blood (data not shown).

3.5. Plasma concentrations of unbound YM207

Concentrations of unbound YM207 in plasma at the end of observation were measured with anti-idiotype antibodies. The YM207 concentrations increased dose dependently, being 2.8 ± 2.1, 10.1 ± 1.0 and 51.4 ± 10.4 μg/ml at doses of 0.3, 1 and 3 mg/kg, respectively.

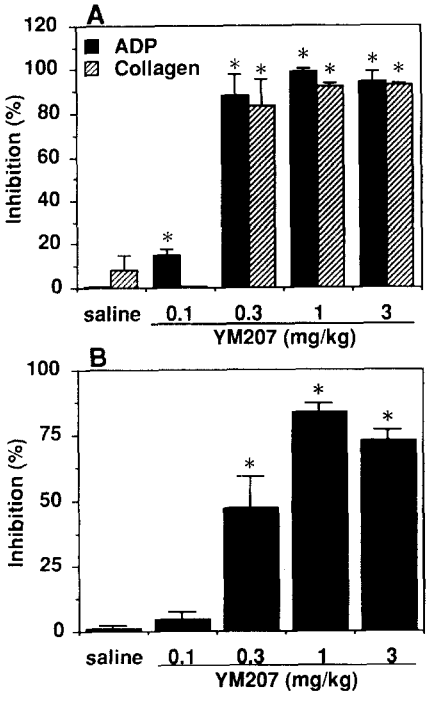

Fig. 4. (A) Inhibitory effect of YM207 on ADP- and collagen-induced platelet aggregation in PRP. (B) Inhibitory effect of YM207 on collagen-induced platelet aggregation in whole blood. Values are the means ± S.E.M. for each group of four or five animals. $^{*}P < 0.05$ versus the saline group by one-way ANOVA.

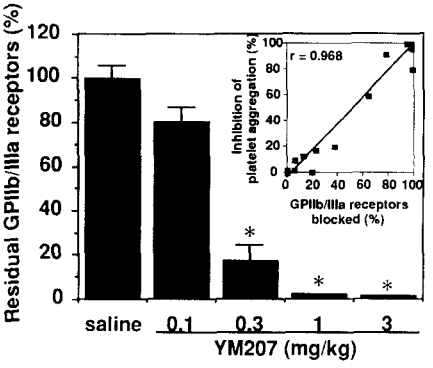

Fig. 5. Residual GPIIb/IIIa receptors after injection of YM207. Values are the means ± S.E.M. for each group of four or five animals. $^{*}P < 0.05$ versus the saline group by one-way ANOVA. (Inset) Correlation between the inhibition of ADP-induced platelet aggregation and the percentage of GPIIb/IIIa receptors blocked.

3.6. Platelet counts and coagulation times

Neither platelet counts nor coagulation times obtained before and after injection of YM207 showed any significant change (data not shown).

4. Discussion

Because YM207 has shown a high degree of species specificity, with slightly less activity in monkey platelets and little activity in other species as compared with humans, we applied the photochemically induced thrombosis model to monkeys. Species specificity has been noted in experimental studies with other GPIIb/IIIa receptor antagonists (Cox et al., 1992; Cook et al., 1993a,b).

In this previously reported method (Sato and Ohshima, 1984a; Rosenblum and El-Sabban, 1977), photochemical reaction causes the formation of active oxygens that damage the endothelium (Sato et al., 1987). Consequently, platelets are considered to adhere and aggregate on the damaged vessel, resulting in the formation of an occlusive platelet thrombus. The formation of an occlusive platelet thrombus at the site of irradiation was confirmed in the animals used in this study. This model has been previously used to evaluate antiplatelet agents (Sato and Ohshima, 1984b; Fukuda et al., 1985) and thrombolytic agents (Kawasaki et al., 1994).

YM207 at a dose of 0.3 mg/kg prolonged the time to occlusive thrombus formation. At doses higher than 0.3 mg/kg, complete occlusion did not occur and the increase in thrombus area and the luminal stenosis rate

in diameter was almost completely inhibited. Although YM207 almost completely inhibited the growth of the thrombus, it did not prevent the formation of the small mural thrombus at the early stage of thrombosis. These findings suggest that YM207 can inhibit platelet aggregation, but not platelet adhesion to the vessel wall.

The antithrombotic effect of YM207 was accompanied by the inhibition of ex vivo platelet aggregation and the blockade of GPIIb/IIIa receptors. Good correlation was obtained between the inhibition of platelet aggregation and the percentage of GPIIb/IIIa receptors blocked. Platelet aggregation in platelet-rich plasma was almost completely inhibited when more than 80% of GPIIb/IIIa receptors were blocked. YM207 significantly inhibited platelet aggregation and thrombus formation at a dose of 0.3 mg/kg. The inhibitory activity of YM207 on platelet aggregation in vitro was 2- to 3-fold less in the squirrel monkeys than in humans. These results suggest that YM207 may prevent thrombus formation at doses less than 0.15 mg/kg in humans.

YM207 significantly prolonged bleeding time only at the 3 mg/kg dose. Bleeding time was prolonged in two animals at the dose of 1 mg/kg, but mean value was not statistically significant versus saline group. The doses of 0.3 and 1 mg/kg resulted in inhibition of occlusive thrombus formation and ex vivo platelet aggregation, but no significant prolongation of bleeding time. The inhibition of platelet aggregation and prolongation of bleeding time following administration of GPIIb/IIIa receptor antagonists have been discussed in a number of reports. In experimental study, some GPIIb/IIIa receptor antagonists caused significant increases in bleeding time at the same dose at which they inhibited thrombus formation (Holahanm et al., 1991; Coller et al., 1989; Ramjit et al., 1993). Indeed, Ramjit et al. (1993) have suggested that increased bleeding time may constitute a marker of antiplatelet activity for this class of agents. However, some compounds show differences between doses at which they inhibit platelet aggregation and prolong bleeding time in guinea pigs (Cook et al., 1993a; Collen et al., 1994). The differences among results for these agents may be due to species differences or to the type of blood vessels in which bleeding time was measured. In clinical trials of the anti-GPIIb/IIIa antibody 7E3, the prolongation of bleeding time was not predictive of hemorrhagic events in patients (Bernardi et al., 1993). However, bleeding episodes and transfusion were more frequent in the group given the 7E3 Fab by bolus plus infusion than in the placebo group (The EPIC investigators, 1994). Clinical studies will be required to determine whether YM207 carries less of the potential risk of prolonged bleeding time associated with this class of agent.

Unbound YM207 concentrations in plasma remained relatively high at the end of the experiment, and showed a good correlation with the inhibition of platelet aggregation in vitro and ex vivo. Platelet counts did not change throughout the experiment.

In summary, YM207, the $F(ab')_2$ fragment of humanized anti-GPIIb/IIIa monoclonal antibody, showed potent antithrombotic activity in a monkey model without causing prolongation of bleeding time. These results suggest that YM207 may be useful in the treatment of patients with or at high risk of platelet-dependent thrombus formation.

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References

Bernardi, M.M., R.M. Califf, N. Kleiman, S.G. Ellis and E.J. Topol, 1993, Lack of usefulness of prolonged bleeding times in predicting hemorrhagic events in patients receiving the 7E3 glycoprotein IIb/IIIa platelet antibody, Am. J. Cardiol. 72, 1121.

Bruggemann, M., G. Winter, H. Waldmann and M.S. Neuberger, 1989, The immunogenicity of chimeric antibodies, J. Exp. Med. 170, 2153.

Co, M.S., S. Yano, R.K. Hsu, N.F. Landolfi, M. Vasquez, M. Cole, J.T. Tso, T. Bringman, W. Laird, D. Hudson, K. Kawamura, K. Suzuki, K. Furuichi, C. Queen and Y. Masuho, 1994, A humanized antibody specific for platelet integrin gpIIb/IIIa, J. Immunol. 152, 2968.

Collen, D., H.R Lu, J.-M. Stassen, I. Vreys, T. Yasuda, S. Burting and H.K. Gold, 1994, Antithrombotic effects and bleeding time prolongation with synthetic platelet GPIIb/IIIa inhibitors in animal models of platelet-mediated thrombosis, Thromb. Haemost. 71, 95.

Coller, B.S., 1985, A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex, J. Clin. Invest. 76, 101.

Coller, B.S., E.I. Peershke, L.E. Schudder and C.A. Sullivan, 1983, A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoprotein IIb and/or IIIa, J. Clin. Invest. 72, 325.

Coller, B.S., J.D. Folts, L.E. Scudder and S.R. Smith, 1986, Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model, Blood 68, 783.

Coller, B.S., J.D. Folts, S.R. Smith, L.E. Scudder and R. Jordan, 1989, Abolition of in vivo platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. Correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors, Circulation 80, 1766.

Cook, N.S., H.G. Zerwes, C. Tapparelli, M. Powling, J. Singh, R. Metternich and A. Hagenbach, 1993, Platelet aggregation and fibrinogen binding in human, rhesus monkey, guinea-pig, hamster and rat blood: activation by ADP and a thrombin receptor

peptide and inhibition by glycoprotein IIb/IIIa antagonists, Thromb. Haemost. 70, 531.

Cook, N.S., O. Bruttger, C. Pally and A. Hagenbach, 1993, The effects of two synthetic glycoprotein IIb/IIIa antagonists, Ro 43-8857 and L-700,462, on platelet aggregation and bleeding time in guinea pigs and dogs: evidence that Ro 43-8857 is orally active, Thromb. Haemost. 70, 838.

Cox, D., Y. Motoyama, J. Seki, T. Aoki, M. Dohi and K. Yoshida, 1992, Pentamidine: a non-peptide GPIIb/IIIa antagonist – in vitro studies on platelets from humans and other species, Thromb. Haemost. 68, 731.

Fukuda, T., M. Sato, Y. Nakamura, N. Ohshima and Y. Inagaki, 1985, Inhibitory effect of trapidil on platelet thrombus formation in the microvessels of rat mesentery, Blood Vessel 16, 573.

Hanson, S.R., F.F. Pareti, Z.M. Ruggeri, U.M. Marzec, T.J. Kunicki, R.R. Montgomery, T.S. Zimmerman and L.A. Harker, 1988, Effect of monoclonal antibodies against the platelet glycoprotein IIb/IIIa complex on thrombosis and hemostasis in the baboon, J. Clin. Invest. 81, 149.

Harlow, E. and D. Lane, 1988, Antibodies – a Laboratory Manual (Cold Spring Harbor Laboratory Press, New York) p. 630.

Holahanm, A., M.J. Mellott, M.A. Bednarek and S. Timmons, 1991, Prevention of reocclusion following tissue-type plasminogen activator-induced thrombolysis by the RGD-containing peptide, echistatin, in a canine model of coronary thrombosis, Pharmacology 42, 340.

Kawasaki, T., S. Kaku, T. Takenaka, K. Yanagi and N. Ohshima, 1994, Thrombolytic activity of a novel modified tissue-type plasminogen activator, YM866, in a photochemically induced platelet-rich thrombosis model. J. Cardiovasc. Pharmacol. 23, 884.

Phillips, D.R., I.F. Charo, L.V. Parise and L.A. Fitzgerald, 1988, The platelet membrane glycoprotein IIb-IIIa complex, Blood 71, 831.

Pidard, P., R.R. Montgomery, J.S. Bennett and T.J. Kunicki, 1983, Interaction of AP2, a monoclonal antibody specific for the human platelet glycoprotein IIb/IIIa complex, with intact platelets, J. Biol. Chem. 258, 12582.

Plow, E.F. and M.H. Ginsberg, 1988, Cellular adhesion: GPIIb/IIIa as a prototypic adhesion receptor, Prog. Hemost. Thromb. 9, 117.

Ramjit, D.R., J.J. Lynch, Jr., G.R. Sitko, M.J. Mellott, M.A. Holahan, I.I. Stabilito, M.T. Stranieri, G. Zhang, R.J. Lynch, P.D. Manno, C.T-C. Chang, R.F. Nutt, S.F. Brady, D.F. Veber, P.S. Anderson, R.J. Shebuski, P.A. Friedman and R.J. Gould, 1993, Antithrombotic effects of MK-0852, a platelet fibrinogen receptor antagonist, in canine models of thrombosis, J. Pharmacol. Exp. Ther. 266, 1501.

Rosenblum, W.I. and F. El-Sabban, 1977, Platelet aggregation in the cerebral microcirculation. Effect of aspirin and other agents, Circ. Res. 40, 320.

Sato, M. and N. Ohshima, 1984a, Platelet thrombus induced in vivo by filtered light and fluorescent dye in mesenteric microvessels of the rat, Thromb. Res. 35, 319.

Sato, M. and N. Ohshima, 1984b, A new experimental model for inducing platelet thrombus in the microvasculature: basic study on its feasibility, Blood Vessel 15, 36.

Sato, M., T. Natori and N. Ohshima, 1987, Effects of scavengers of active oxygens on the platelet thrombus formation in microvessels induced by photochemical reaction, in: Microcirc Ann, ed. M. Tsuchiya (Nihon-Igakukan, Tokyo) p. 135.

The EPIC investigators, 1994, Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty, New Engl. J. Med. 330, 956.

Topol, E.J. and E.F. Plow, 1993, Clinical trials of platelet receptor inhibitors, Thromb. Haemost. 70, 94.

Yano, S., K. Suzuki, M. Katoh, Y. Sugita, S. Kaku, K. Kawamura and Y. Masuho, 1994, Epitopes and biological activities of two monoclonal antibodies to platelet integrin $\alpha_{IIb}\beta_3$, J. Biochem. 116, 778.