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Inhibition of Fibrinogen Binding to Platelets by MK-852, a New GPIIb/IIIa Antagonist

Anders Larsson[1] and Tomas L. Lindahl[2]

[1]*Department of Clinical Chemistry, University Hospital, Uppsala and*
[2]*Department of Clinical Chemistry, University Hospital, Linköping, Sweden*

ABSTRACT

MK-852 is a newly developed low molecular weight inhibitor of fibrinogen binding to platelets. Platelet aggregation and adhesion of platelets to damaged vessel walls are critical events in haemostasis, and uncontrolled aggregation may cause arterial thrombus formation. Depending on the location of the occluded vessel, this may result in unstable angina, myocardial infarction or stroke. Platelet aggregation requires binding of fibrinogen to the GPIIb/IIIa receptor on the platelet surface. Thus, inhibitors of fibrinogen binding to the receptor may constitute an efficient way of preventing thrombus formation.

We have used flow cytometry and FITC-labelled chicken anti-human fibrinogen antibodies to study the in vitro inhibitory effects of MK-852 on fibrinogen binding to platelets. We show that MK-852 is a very efficient fibrinogen receptor antagonist in vitro. Flow cytometry is well suited for clinical use and may be used to monitor treatment with MK-852 or other fibrinogen receptor antagonists.

INTRODUCTION

The use of anti-platelet drugs has transformed the treatment of ischemic heart disease (2). Today, aspirin is routinely given to patients with acute myocardial infarction and unstable angina. However, aspirin does not completely inhibit platelet aggregation. During the last few years several research groups have instead developed antagonists to the GPIIb/IIIa receptor. Fibrinogen binding to the platelet receptor GPIIb/IIIa has a very important role in platelet aggregation, and an inhibitor to this receptor may thus inhibit platelet aggregation and thromb formation. The first antagonist tested in patients was a murine monoclonal antibody known as 7E3 (3). This antibody has later been modified several times to reduce antigenicity (6). The results obtained with this antibody in ischemic heart disease and during coronary angioplasty has been very promising (19). The results

have lead to the development of several low molecular weight fibrinogen receptor blockers (1). Some of these antagonists are currently being evaluated in clinical studies.

In the present work we have used fluorescence-activated flow cytometry (FACS) and chicken anti-human fibrinogen-FITC to study the inhibitory effects of a new fibrinogen receptor antagonist (MK-852) (20) on ADP and immune complex induced platelet activation. Flow cytometry gives single-cell data, allowing the detection of a few activated platelets (15). Chicken antibodies are superior to mammalian antibodies for the measurement of platelet bound plasma proteins as they do not induce complement activation (10) or platelet activation (11).

MATERIALS AND METHODS

Reagents. MK-852 was generously provided by Merck, Sharp & Dohme (West Point, PA, USA). Adenosine-5′-diphosphate was obtained from Boehringer-Mannheim (Mannheim, Germany). All other chemicals were of reagent grade, most purchased from Merck (Darmstadt, Germany).

Antibodies. Fluorescein (FITC) labelled chicken anti-human fibrinogen was obtained from Biopool AB (Umeå, Sweden). Rabbit anti-human IgA was purchased from Dakopatts AS (Glostrup, Denmark).

Blood sampling. Venous blood was obtained from healthy volunteers who had taken no medication for at least 10 days. All volunteers gave informed consent before blood sampling. Blood was obtained from an antecubital vein without a tourniquet and the blood was collected in 5 mL sodium citrate tubes (367704, Becton Dickinson, Rutherford, NJ). Platelet-rich plasma was isolated by centrifugation at 140 x g for 10 minutes at room temperature. The concentration of platelets, size and purity were analyzed by a Coulter STKS cell counter (Coulter Diagnostics, Hialeah, FL).

Gelfiltration of platelets. 1 mL of platelet-rich plasma was separated on a Sepharose S-1000-column (1.5x9 cm) equilibrated with HEPES buffer. The first peak contained the platelets. The number of platelets, size and purity were analyzed by a cell counter.

Preparation of samples for flow cytometry. 2.5 µL platelet-rich plasma or 10 µL gel-filtered platelets were added to polystyrene tubes containing 50 µL HEPES-buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 1 g/L bovine serum albumin, and 20 mmol/L HEPES, pH 7.40) containing varying amounts of MK-852. In some experiments ADP was added

to the tubes to cause platelet activation and in some experiments 5 µL rabbit anti-human IgA was added to the tubes to form immune complexes. Samples were incubated for 10 min at room temperature followed by addition of 50 µL chicken anti-human fibrinogen-FITC, diluted 1:10 in HEPES-buffer. Samples were incubated for another 10 min at room temperature. The samples were then diluted and fixed with 500 µL ice-cold PBS containing 1% p-formaldehyde. Washing was tried but caused losses of cells without improving the analysis. Therefore washing steps were not used. The anti-fibrinogen antibody was tested at different dilutions and subsequently used at optimal concentrations to achieve maximal binding.

Flow cytometry. Labelled platelets were resuspended for analysis by Epics Profile II cytometer (Coulter Electronics, Hialeah, FL). The Epics Profile II cytometer was equipped with a 15 mW air-cooled 488 nm argon laser. Forward and side scatter and green (FITC) and red (phycoerythrin) signals were acquired with logarithmic amplification, with a 525 nm and 575 nm bandpass filter for collection of FITC and PE signals, respectively. Acquisition and processing of data from 10,000 cells were carried out with the Epics Elite Flow Cytometry software (Coulter Electronics, Hialeah, FL).

Based on light scattering properties, each cell is represented by a point in a rectangular coordinate system. The instrument was calibrated for fluorescence and light scatter using "Immunocheck" beads and "Standard Brite" beads (Coulter Electronics, Hialeah, Fl., USA). Based on light scattering properties, each cell is represented by a point in a rectangular coordinate system. A discrimination frame is placed around the platelet cluster utilising forward and side scatter. Analytical markers were set in the fluorescence channel to divide the negative control sample into two fractions containing 95-97% of the platelets and the brightest 3-5% of the platelets. Those platelets with fluorescence greater than the marker were identified as positive events.

RESULTS

Inhibition of ADP induced fibrinogen binding to platelets by MK-852. The platelets were activated with varying concentrations of ADP (0, 0.1 µmol/L, 1 µmol/L, 10 µmol/L, 100 µmol/L) in the presence of 1 mg/L of MK-852 or only HEPES-buffer. MK-852 was very efficient in inhibiting the ADP induced fibrinogen binding (Fig. 1). The fibrinogen binding in the presence of MK-852 was not significantly different from samples containing 10 mmol/L of EDTA. The binding of fibrinogen to the receptor is Ca^{2+} dependent. EDTA was used as a negative control as it inhibits the binding of fibrinogen to GPIIb/IIIa (17).

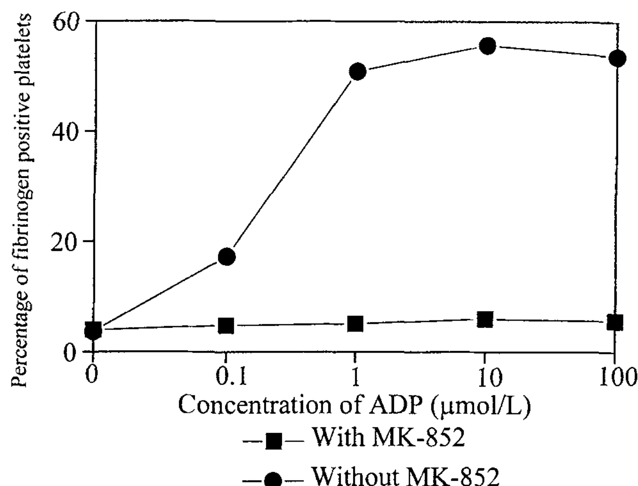

Figure 1. Inhibition of ADP induced fibrinogen binding to platelets by MK-852. The results are expressed as percentage of fibrinogen positive platelets in the presence of varying concentrations of ADP. The plasma fibrinogen concentration was 2.4 g/L.

Platelets were also activated with 100 μmol/L of ADP in the presence of varying MK-852 concentrations. 100 μg/L of MK-852 was very efficient in inhibiting fibrinogen binding (Fig. 2). The binding was not significantly different from samples containing 10 mmol/L of EDTA. 10 μg/L of MK-852 gave only a limited reduction in the percentage of fibrinogen positive platelets in comparison with only HEPES-buffer. However, the reduction in the mean fluorescence intensity was more pronounced. This indicates that there was a reduction in the amount of fibrinogen bound to each platelet with 10 μg/L of MK-852.

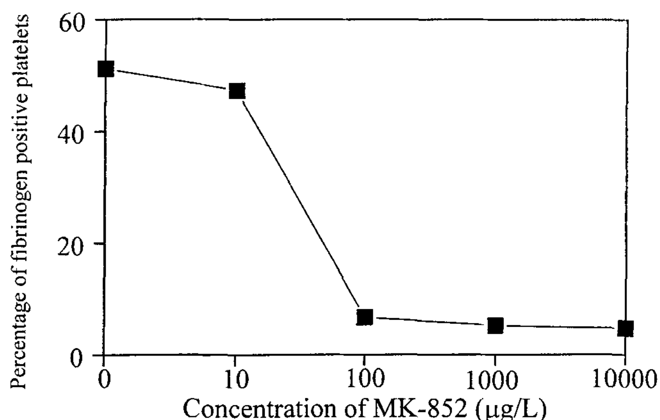

Fig. 2. Inhibition of ADP induced fibrinogen binding to platelets by varying concentrations of MK-852. The results are expressed as percentage of fibrinogen positive platelets in the presence of 100 μmol/L of ADP.

Displacement of bound fibrinogen by MK-852. Platelets were activated with 100 μmol/L of ADP. After 10 min, MK-852 was added in various concentrations to displace bound fibrinogen. Bound fibrinogen was detected with chicken anti-human fibrinogen-FITC. 100 μg/L of MK-852 was very efficient in inhibiting fibrinogen binding while 10 μg/L of MK-852 gave only a limited reduction in the percentage of fibrinogen positive platelets in comparison with only HEPES-buffer (results not shown). The results were not significantly different from the results obtained when MK-852 was added before ADP. Thus, MK-852 could displace platelet bound fibrinogen.

Effect of fibrinogen concentration on fibrinogen binding. Gelfiltered platelets were mixed with various volumes of platelet poor plasma. 20 or 100 μg/L of MK-852 (final concentration) were added and the platelets were activated with 100 μmol/L of ADP. With 20 μg/L of MK-852 there was a slightly increased percentage of fibrinogen positive platelets when the fibrinogen concentration was increased from 1.2 g/L to 10 g/L. With 100 μg/L of MK-852 there was no difference in the percentage of fibrinogen positive platelets (results not shown).

Inhibition of immune complex induced fibrinogen binding to platelets by MK-852. Rabbit anti-human IgA was added to PRP to form immune complexes and platelet activation was detected with antibodies against fibrinogen. The immune complexes increased the binding of anti-fibrinogen. This binding was inhibited by MK-852 in a dose dependent manner (Fig. 3). 10 μg/L of MK-852 gave only a limited reduction in the percentage of fibrinogen positive platelets while 100 μg/L of MK-852 was very efficient in inhibiting fibrinogen binding.

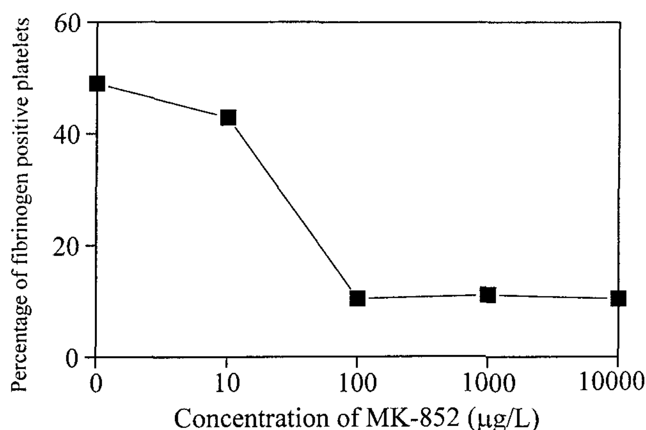

Fig. 3. Inhibition of CIC induced fibrinogen binding to platelets by varying concentrations of MK-852. The results are expressed as percentage of fibrinogen positive platelets. The plasma IgA concentration was 2.2 g/L.

The bitmap gating was set to exclude immune complexes (9). However some immune complexes had the same forward and side scatter as platelets and were thus analysed. The immune complexes bind some anti-fibrinogen antibody resulting in an increased percentage of positive events. This probably explains the higher percentage of fibrinogen positive particles in the presence of MK-852 in the experiment with immune complex activation than in the experiment with ADP.
Positive particles could also be detected in platelet poor plasma when immune complexes were present in the samples suggesting that the positive particles were anti-IgA-IgA complexes.

DISCUSSION

Platelets contribute to normal haemostasis by adhering to damaged endothelial surfaces and then aggregate at the site of damage. If the aggregation is not inhibited, a thrombus is formed that may occlude the vessel or cause embolization. Platelet adhesion is mediated by several platelet receptors that interact with adhesion molecules in the damaged area. In contrast to adhesion, platelet aggregation is mediated only by the platelet GPIIb/IIIa receptor, which is found only on platelets and megakaryocytes. The number of GPIIb/IIIa receptors in the platelet membrane are extremely large (approximately 30,000-50,000), making it one of the most dense receptors for adhesion and aggregation of any cell.
When the platelet is activated, there is a structural change in the GPIIb/IIIa receptor (16). Upon activation, the receptor will bind several different ligands, including fibrinogen, fibronectin, vitronectin and von Willebrand factor. At physiological protein concentrations it is mainly fibrinogen that is bound, which has a dimeric structure that allows the interaction with two platelets leading to platelet aggregation. It has been shown that the binding of fibrinogen to the GPIIb/IIIa receptor is essential for thrombus growth (13). Therefore, the blockade of GPIIb/IIIa might be a superior approach in preventing arterial thrombus formation (2). Like other members of the integrin family, GPIIb/IIIa contains a recognition site for the peptide sequence Arg-Gly-Asp (RGD) (3,4). Several substances that block this site have been tested, including monoclonal antibodies (2), polypeptides isolated from leeches (14) or snake venoms (5). Recently several peptides have been developed that block the RGD site. One of theses inhibitors is MK-852 that currently is being evaluated in a Phase II trial of unstable angina. Peptides that block the RGD site have theoretical advantages to monoclonal antibodies (19). The peptides are usually more rapidly cleared which allows for a finer titration and the rapid clearance is an advantage if bleeding problems occur as the inhibition will cease quickly once the infusion is stopped. The antigenicity of a peptide is less than for a monoclonal antibody which will eliminate the problems with human

anti-mouse IgG antibodies associated with in vivo use of monoclonal antibodies.

We used chicken antibodies to detect fibrinogen binding as these antibodies are superior to mammalian antibodies for the estimation of platelet bound plasma proteins. Chicken antibodies do not induce complement activation (10,12) or platelet activation (11,12). The results show that MK-852 is an effective blocker of ADP-induced fibrinogen binding to platelets. 10 µg/L of MK-852 or larger concentrations reduced the binding of fibrinogen to platelets.

We also wanted to see if MK-852 could inhibit immune complex mediated fibrinogen binding. Circulating immune complexes (CIC) can be found in many diseases such as autoimmune diseases, malignancies and infectious diseases (18) and CIC are known to play a pathogenic role in autoimmune diseases. There is also an increased risk of thrombosis and thrombocytopenia associated with these diseases (17). We have previously shown that immune complexes containing mammalian antibodies are efficient promoters of platelet activation measured as fibrinogen binding or microparticle formation (8,9). Activated platelets are rapidly cleared from the circulation, which may contribute to the thrombocytopenia in these patients. We show that MK-852 can block CIC mediated fibrinogen binding to platelets. Further studies have to be performed to see if MK-852 also can reduce the incidence of thrombosis and thrombocytopenia in patients with CIC.

In this study we show that MK-852 is a very efficient fibrinogen receptor antagonist in vitro and that flow cytometry can be used to monitor the inhibitory effects of MK-852 on fibrinogen binding to platelets.

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REFERENCES

1. Alig L, Edenhofer A, Hadváry P, Hurzeler M, Knopp D, Muller M, Steiner B, Trzeciak A & Weller T. Low molecular weight, non-peptide fibrinogen receptor antagonists. J Med Chem 1992; 35: 4393-4407.
2. Coller BS. Platelets and thrombolytic therapy. N Engl J Med 1990; 322: 33-42.
3. Coller BS. 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 1985; 76: 101-108.

4. Ginsberg MH, Loftus JC & Plow EF. Cytoadhesins, integrins and platelets. Thromb Haemost 1988; 59: 1-6
5. Gould RJ, Polokoff MA, Friedman PA, Huang T, Holt JC, Cook JJ & Niewiarowski S. Disintegrins: A family of integrin inhibitory proteins from viper venoms. Proc Soc Exp Biol Med 1990; 195: 168-171
6. Jordan RE, Knight DM, Wagner C, McAleer MF, McDonough M, Mattis JA, Coller BS, Weisman HF & Ghrayeb J. A dramatic reduction of the immunogenicity of the anti-GPIIb/IIIa monoclonal antibody, 7E3 Fab, by humanization of the murine constant domains. Circulation 1992; 86: I-410.
7. Kekomaki R, Kauppinen H L, Penttinen K & Myllylä G. Interaction of immune complexes and platelets in rabbits immunized with hapten-carrier conjugates. Acta Pathol Microbiol Scand Sect C 1977; 86: 207-214.
8. Larsson A, Egberg N & Lindahl TL. Platelet activation and binding of complement components to platelets induced by immune complexes. Platelets 1994; 5: 149-155.
9. Larsson A, Nilsson B, Lindahl TL, Frödin L & Wahlberg J. Immune complex induced thrombocytopenia after in vivo treatment with horse anti-lymphocyte antibodies. Thromb Haemorr Dis 1994; 8: 53-56.
10. Larsson A, Wejåker PE, Forsberg PO & Lindahl TL. Chicken antibodies: A tool to avoid interference by complement activation in ELISA. J Immunol Methods 1992; 156: 79-83.
11. Lindahl T L, Festin R & Larsson A. Studies of fibrinogen binding to platelets by flow cytometry: An improved method for detection of platelet activation. Thromb Haemostas 1992; 68: 221-225.
12. Lindahl, TL & Larsson, A. C1q binding to platelets induced by monoclonal antibodies and immune complexes- A flow cytometric analysis. Platelets 1993; 4: 73-77.
13. Phillips DR, Charo IF, Parise LV & Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex Blood 1988; 71: 831-843.
14. Seymour JL, Henzel WJ, Nevins, B, Stults JT & Lazarus RA. Decorsin, a potent glycoprotein IIb-IIIa antagonist and platelet aggregation inhibitor from the leech *Macrobdella Decora.* J Biol Chem 1990; 265: 10143-10147
15. Shattil S J, Cunningham M & Hoxie J A. Detection of activated platelets in whole blood using activation dependent monoclonal antibodies and flow cytometry. Blood 1987; 70: 307-315.
16. Shattil S J, Hoxie J A, Cunningham M & Brass L F. Changes in platelet membrane glycoprotein IIb/IIIa complex during platelet activation. J Biol Chem 1985; 260: 11107-14.

17. Steiner B, Cousot D, Trzeciak A, Gillessen D & Hadváry P. Ca2+-dependent binding of a synthetic arg-gly-asp (RGD) peptide to a single site on the purified platelet glycoprotein IIb/IIIa complex. J Biol Chem 1989; 264: 13102-13108
18. Theofilopoulos A N & Dixon F J. Immune complexes in human diseases. Am J Pathol 1980; 100: 530-591.
19. Topol EJ & Plow EF. Clinical trials of platelet receptor inhibitors. Thromb Haemost 1993; 70: 94-98
20. Yuan AS, Hand EL, Hichens M, Olah TV, Barrish A, Fernandez-Metzler C & Gilbert JD. Determination of MK-852, a new fibrinogen receptor antagonist, in plasma and urine by radioimmunoassay. J Pharm Biomed Anal 1993; 11: 427-434

Offprint requests to: Anders Larsson
Department of Clinical Chemistry,
University Hospital,
S-751 85 Uppsala, Sweden