

# Variable protection of $\beta 3$ -integrin-deficient mice from thrombosis initiated by different mechanisms

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Platelet integrin  $\alpha \text{IIb}\beta 3$  (GPIIb/IIIa) plays a central role in the initiation of arterial thrombosis, but its contribution to disseminated microvascular thrombosis is less well defined. Therefore, wild-type mice ( $\beta 3^{+/+}$ ),  $\beta 3$ -integrin-deficient mice ( $\beta 3^{-/-}$ ), and wild-type mice treated with a hamster monoclonal antibody (1B5) that blocks murine  $\alpha \text{IIb}\beta 3$  function were tested in models of large-vessel and microvascular thrombosis. In the large-vessel model, ferric chloride was used to injure the carotid artery, and the time to thrombosis was measured. In  $\beta 3^{+/+}$  mice, the median time to occlusion was 6.7 minutes,

whereas occlusion did not occur in any of the  $\beta 3^{-/-}$  mice tested ( $P < .001$ ). Fab and  $\text{F(ab')}_2$  fragments of 1B5 increased the median time to occlusion. To initiate systemic intravascular thrombosis, prothrombotic agents were administered intravenously, and platelet thrombus formation was monitored by the decrease in circulating platelet count. Three minutes after the injection of adenosine diphosphate (ADP), collagen + epinephrine, or tissue factor, the platelet counts in  $\beta 3^{+/+}$  mice decreased by 289, 424, and  $429 \times 10^3/\mu\text{L}$ , respectively.  $\beta 3^{-/-}$  mice and wild-type mice pretreated with 1B5 Fab (1

mg/kg, IP) were nearly completely protected from the effects of ADP. In contrast,  $\beta 3^{-/-}$  mice were only partially protected from the effects of collagen + epinephrine and minimally protected from the effects of tissue factor. In all cases, less fibrin became deposited in the lungs of  $\beta 3^{-/-}$  mice than in wild-type mice. These results suggest that though  $\alpha \text{IIb}\beta 3$  plays a dominant role in large-vessel thrombosis, it plays a variable role in systemic intravascular thrombosis. (Blood. 2001;98:1055-1062)

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## Introduction

Thrombosis, the leading cause of death worldwide, is marked by heterogeneity in inciting causes, contributing elements, locations, and pathologic consequences. As early as the mid-1800s, Virchow recognized that changes in the blood, the blood vessel wall, and blood flow could all contribute to initiating thrombosis. Many experimental and clinical observations have led to the conclusion that blood platelets play a primary role in the initiation of arterial thrombosis,<sup>1,2</sup> but the contribution of platelets to systemic intravascular thrombosis is less well defined.<sup>3</sup> Advances in understanding platelet physiology have led to the identification of a number of surface receptors that contribute to platelet adhesion, aggregation, or both, and the contributions of these receptors to platelet-mediated thrombosis are being defined.<sup>4</sup> Inhibitors of the platelet  $\alpha \text{IIb}\beta 3$  (GPIIb/IIIa) receptor are among the most effective anti-thrombotic agents in animal models of large-vessel arterial thrombosis.<sup>5,6</sup> Three  $\alpha \text{IIb}\beta 3$  antagonists, the Fab fragment of the mouse/human chimeric antibody 7E3 (abciximab), the peptide eptifibatid, and the peptidomimetic tirofiban, have demonstrated efficacy in preventing and treating coronary artery thrombosis in humans.<sup>7</sup> Less is known, however, about the contribution of  $\alpha \text{IIb}\beta 3$  to systemic intravascular thrombosis.<sup>3</sup>

We recently reported that  $\beta 3$ -integrin-deficient mice, which lack both  $\alpha \text{IIb}\beta 3$  and  $\alpha \text{V}\beta 3$ , have bleeding diatheses and platelet function abnormalities essentially identical to those of patients with Glanzmann thrombasthenia, who lack  $\alpha \text{IIb}\beta 3$ , and sometimes

$\alpha \text{V}\beta 3$ , receptor function on an inherited basis.<sup>8</sup> In the present study, we tested these animals in a number of different models of large-vessel and systemic intravascular thrombosis initiated by different stimuli. Because animals deficient in cell receptors on an inherited basis sometimes display responses that differ from those observed by blocking the receptor with monoclonal antibodies (mAbs) or other antagonists,<sup>9,10</sup> we also analyzed the effect of hamster mAbs we previously developed to mouse  $\alpha \text{IIb}\beta 3$ <sup>11,12</sup> in some of the same systems.

## Materials and methods

### Antibodies

Rabbit anti-mouse thrombocyte polyclonal antibody was from Inter-Cell Technologies (Hopewell, NJ); rabbit anti-human fibrinogen polyclonal antisera was from Behring (Marburg, Germany); mAb 350 specific for the heptapeptide sequence exposed at the new N-terminus of the  $\beta$  chain of fibrin after thrombin cleavage of fibrinopeptide B was from American Diagnostica (Greenwich, CT). Hamster mAb 1B5 and 9C2 against mouse platelet  $\alpha \text{IIb}\beta 3$  were prepared as described.<sup>11</sup> Purification of the antibodies was performed by subjecting culture supernatant to 50%  $(\text{NH}_4)_2\text{SO}_4$  precipitation, followed by chromatography of the dialyzed precipitates on an nProtein A AvidGel F column (Unisyn, Tustin, CA). Bound proteins were eluted by lowering the pH in a stepwise fashion. Fab or  $\text{F(ab')}_2$  fragments were prepared by incubating mAb with immobilized papain or

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pepsin, respectively, and separating Fab and F(ab')<sub>2</sub> fragments from IgG and Fc fragments by chromatography on protein A using either an Fab or an F(ab')<sub>2</sub> ImmunoPure preparation kit (Pierce, Rockford, IL). Fab and F(ab')<sub>2</sub> fragments were dialyzed against sterile, endotoxin-free, 0.9% normal saline before injection into mice. 1B5 IgG was conjugated with Alexa 488 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

### Fibrinogen and fibrin formation

Mouse fibrinogen was from Sigma Chemical (St Louis, MO). Mouse fibrin was prepared by incubating 0.5 mL mouse fibrinogen (0.25 mg/mL) in 10 mM Tris/HCl, 150 mM NaCl, pH 7.4, with 2 U human  $\alpha$ -thrombin (ERL, South Bend, IN) at 37°C for 15 minutes, and then it was dissolved in sodium dodecyl sulfate (SDS)-sample buffer. In some experiments, CaCl<sub>2</sub> was included in the reaction to promote factor XIII-mediated cross-linking of fibrin, and the reaction was terminated after 1 hour.

### Mice

C57Bl/6 and 129Sv mice were purchased from Jackson Labs (Bar Harbor, ME). The generation of integrin  $\beta 3^{-/-}$  mice by homologous recombination in embryonic stem cells has been previously described.<sup>8</sup> Wild-type ( $\beta 3^{+/+}$ ), heterozygous ( $\beta 3^{+/-}$ ), and  $\beta 3$ -null ( $\beta 3^{-/-}$ ) mice were descendants of F2 intercrosses and were on a mixed C57Bl/6-129Sv background. Mice were weaned at 3 weeks, maintained on a 12-hour light-12-hour dark cycle, and fed water and standard rodent chow (5001; Purina Mills, Richmond, IN) ad libitum. All procedures conformed to the recommendations of the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication number NIH 78-23, 1996) and were approved by Mount Sinai's Institutional Animal Care and Use Committee.

### Blood collection, blood counts, and bleeding time assay

Mice were anesthetized with methoxyflurane (Mallinckrodt Veterinary, Hazelwood, MO), and blood was collected into 0.1 volume of 3.8% sodium citrate by puncture of the retrobulbar venous plexus with a 12- to 15-mm-long glass capillary. Citrated whole blood was analyzed in either a Sero Diagnostic System 9018cp Analyzer (Sero Diagnostics, Allentown, PA) or a Mascot 800 (CDC Technologies, Oxford, CT) set to measure mouse blood cells. The bleeding time assay was performed as described<sup>8,13</sup> by cutting the distal 2 mm from the tip of the tail; assays were terminated at 12 minutes if the tail was still bleeding.

### Platelet preparation and aggregation studies

Citrated whole blood (350  $\mu$ L) was centrifuged (800g for 8 minutes) at 22°C to obtain platelet-rich plasma (PRP). After the PRP was removed, 200  $\mu$ L modified Tyrode buffer (138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 12 mM NaHCO<sub>3</sub>, 10 mM HEPES, 5 mM glucose, 0.35% bovine serum albumin, pH 7.35) was added to the red cell pellet, and the suspension was mixed. Platelet-rich buffer (PRB) was obtained by centrifuging (800g for 4 minutes) the suspension at 22°C, and PRB was then combined with the PRP in a mixture of PRP/PRB. Aggregation of platelets in PRP/PRB (1-3  $\times 10^8$ /mL) was performed as previously described.<sup>8</sup> For flow cytometry, PRP/PRB was incubated with Alexa 488-labeled mAb 1B5 IgG (5  $\mu$ g/mL) for 30 minutes at room temperature. Samples were analyzed on an Epics flow cytometer (Coulter, Hialeah, FL).

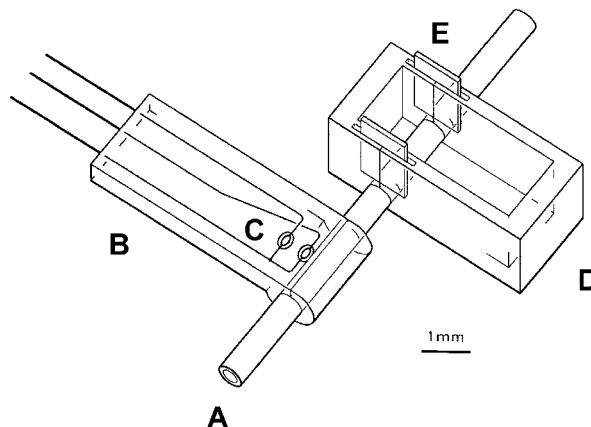
### Thrombosis models

**Large-vessel (carotid) artery thrombosis.** Male mice (10 to 12 weeks old) were anesthetized with methoxyflurane and maintained under anesthesia by inhaling through a nose cone a mixture of isoflurane-saturated air (3.2-5.2 mL/min) mixed with room air (100-130 mL/min). Body temperature was monitored with a rectal probe and maintained at 37°C  $\pm$  1°C by varying the output (from 0 to 12V AC) of an EPZ type halogen heat lamp (Sylvania, New York, NY) placed 16 cm from the mouse and aimed at the trunk. The left carotid artery was exposed, dissected free of surrounding tissue, and placed through slits into holes at the bottom of a rectangular

"boat" cast from 0.55-mm-thick Sylgard 184 silicone elastomer (Dow Corning, Midland, MI) (2 mm  $\times$  2 mm  $\times$  4.5 mm) (Figure 1). After insertion of the artery, the slits were sealed by inserting 2 mm  $\times$  1.5 mm gaskets of 0.13 mm polyester into slots in the walls at both ends of the boat (Figure 1). Changes in vessel temperature, an indicator of blood flow,<sup>14</sup> were monitored with a temperature probe placed distal to the boat. The body of the probe was made of silicone elastomer reinforced with silk cloth and contained a transverse cylindrical groove (0.55-mm diameter) at its distal end to hold the artery. Temperature differences between 2 thermistors, one touching the groove and a second approximately 1 mm from the groove, were continuously measured and recorded with Lab View software (National Instruments, Austin, TX). Heat fluctuations were minimized by surrounding the probe with Surgilube and placing a 0.13-mm-thick metal heat conductor on top of the probe. Once a stable signal was obtained, the proximal portion of the artery was clamped for 30 seconds with a microvascular clamp to establish the extent of temperature change with occlusion. To initiate thrombosis, at least 2 minutes after releasing the clamp, 5 to 10  $\mu$ L 20% (wt/wt) FeCl<sub>3</sub>  $\cdot$  6 H<sub>2</sub>O in Surgilube was added to the boat. Time to maximal temperature reduction was defined as the time from the application of FeCl<sub>3</sub> to the onset of the temperature deflection of a sustained reduction—that is, one in which there was a decrease in temperature equal to that observed after clamping the vessel and a duration longer than 30 seconds. Experiments were performed in a blinded manner.

**Models of systemic intravascular thrombosis.** Wild-type ( $\beta 3^{+/+}$ ) and  $\beta 3$ -deficient mice matched for sex and age (10-16 weeks) were anesthetized with inhaled methoxyflurane, and 125  $\mu$ L blood was collected into citrate for the determination of platelet count, hematocrit, and leukocyte count. The right jugular vein was exposed by a lateral neck incision, and a 27-gauge needle was used to inject over 10 seconds 0.1 mL normal saline, 5  $\mu$ g ADP (Sigma Chemical) in saline, 25  $\mu$ g collagen (equine tendon type I fibrillar collagen; Chronolog, Havertown, PA) plus 1  $\mu$ g epinephrine (Sigma), or undiluted recombinant tissue factor reconstituted in sterile water according to the manufacturer's instructions (Innovin; Dade, Miami, FL). One minute after the injection was complete, a second sample of blood was collected. Three minutes after the injection, mice were euthanized by cervical dislocation.

**Lung fibrin determination.** The sequential extraction procedure of Olman et al<sup>15</sup> was used to quantify fibrin deposition in lung tissue. Lungs were harvested and then rinsed at 4°C in extraction buffer composed of 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 U/mL aprotinin (Miles, West Haven, CT), 100 U/mL heparin (from porcine intestinal mucosa, grade I-A; Sigma), 0.1 M  $\epsilon$ -aminocaproic acid (Fluka, Milwaukee, WI), and 10 mM Tris/HCl, pH 7.4. Samples were frozen in cryovials on the surface of liquid nitrogen and stored at -20°C until further use. Lung tissue was thawed (by immersing the cryovial in a 37°C waterbath for 15 seconds), minced, homogenized in extraction buffer



**Figure 1. Schematic diagram of instrumented mouse carotid artery.** The proximal portion of the exposed carotid artery (A) is placed in the boat (D) through slits in the side walls, and the distal portion of the artery is placed in the groove of the temperature probe (B). Once the artery is positioned, FeCl<sub>3</sub> in Surgilube is placed in the boat to initiate thrombosis. A, artery; B, probe; C, thermistors; D, boat; E, gaskets.

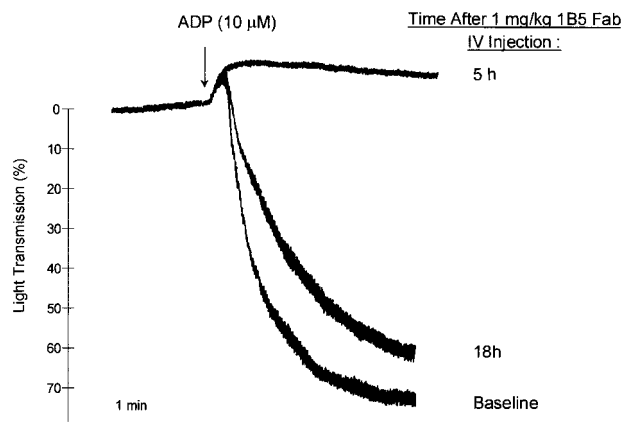
(0.5 mL buffer/100 mg tissue) for 10 minutes, and incubated on ice for 4 hours. The pellet, obtained after centrifugation at 16 000g for 30 minutes at 4°C, was washed twice, resuspended in 1 mL 8 M urea–4% SDS–2% dithiothreitol, incubated for 18 hours at 37°C, and recentrifuged at 16 000g for 30 minutes at room temperature. The supernatant containing the extracted fibrin was separated by SDS–polyacrylamide gel electrophoresis on a 7.5% gel and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) for immunoblotting. Fibrin  $\beta$  chain was detected by reacting membranes first with a 1:200 dilution of mAb 350, followed by horseradish peroxidase–conjugated antimouse antibody. Bands were visualized with enhanced chemiluminescence (ECL; Amersham, Little Chalfont, Buckinghamshire, United Kingdom). To quantify fibrin, the optical density of bands derived from samples containing equal amounts of lung protein were compared to standard curves of uncross-linked mouse fibrin by scanning densitometry.

**Histology, immunohistochemistry, and electron microscopy.** For light microscopy, specimens were fixed with 10% formalin in phosphate-buffered saline (PBS) overnight, embedded in paraffin, and stained with hematoxylin–eosin or modified elastic-tissue Masson trichrome. For immunohistochemical analysis, 5- $\mu$ m sections were deparaffinized, rinsed in xylene, rehydrated, and blocked first with 3% hydrogen peroxide and then with 2% ovalbumin in PBS. Sections were incubated at 37°C for 2 hours with either rabbit anti–mouse thrombocyte polyclonal antibody (1:20 000) or rabbit anti–human fibrinogen polyclonal antibody (1:2000). Sections were then incubated at room temperature for 30 minutes with biotin-conjugated antirabbit secondary antibody (Biogenics, Napa, CA). Bound antibody was detected by reaction with HRP-conjugated streptavidin and diaminobenzidine; specimens were counterstained with hematoxylin. To quantitate the accumulation of thrombi in the lung, the number of thrombi in half a dozen 40 $\times$  fields from 5 mice in each treatment group were averaged.

For electron microscopy of carotid arteries, mice were euthanized and then immediately perfused via a cannula in the left ventricle with 3 mL 4% paraformaldehyde in PBS. The injured area of the artery (or the corresponding area of the uninjured contralateral artery) was excised and fixed with 3% glutaraldehyde in 0.1 M cacodylate. Cross-sections of the artery were obtained and prepared for transmission electron microscopy. Blocks containing these sections were dehydrated in graded alcohol solutions and embedded in embed 812 (EMS; Fort Washington, PA.). Thin sections were stained with uranyl acetate and lead citrate and viewed with a JEM 100 CX microscope (JEOL, Tokyo, Japan). For scanning electron microscopy, the remaining artery was sectioned longitudinally, oriented with the lumen exposed, processed by critical point drying, mounted with silver paint, sputter coated with gold–palladium, and examined in a Hitachi S350 scanning electron microscope.

### Statistical analysis

Results are expressed as the mean  $\pm$  SD, unless otherwise indicated. In the thrombosis models, comparisons were made by Mann-Whitney *U* test.



**Figure 2. 1B5 Fab fragments.** Administration of 1B5 Fab fragments (1 mg/kg, IV) inhibits ex vivo ADP (10 mM)–induced platelet aggregation.

**Table 1. Mean fluorescence intensity of Alexa 488 1B5 IgG binding to mouse platelets isolated before or at various times after in vivo administration of 1B5 Fab**

Time after injection	Alexa 488 1B5 IgG mean fluorescence intensity	
	1B5 Fab 0.53 mg/kg IV	1B5 Fab 1 mg/kg IV
Baseline	3.30	3.10
7 minutes	0.68	ND
1 hour	0.78	0.02
5 hours	ND	0.04
18 hours	ND	0.60
24 hours	1.50	ND
5 days	3.10	ND

Results are from 2 individual mice. Similar results were obtained through the first 6 hours after injection in 2 additional mice receiving 1B5 Fab (1 mg/kg, IP) and 4 mice receiving 1B5 F(ab')<sub>2</sub> (1–2 mg/kg, IP).

ND, not determined.

Other results were analyzed by Student *t* test. *P* < .05 was considered significant.

## Results

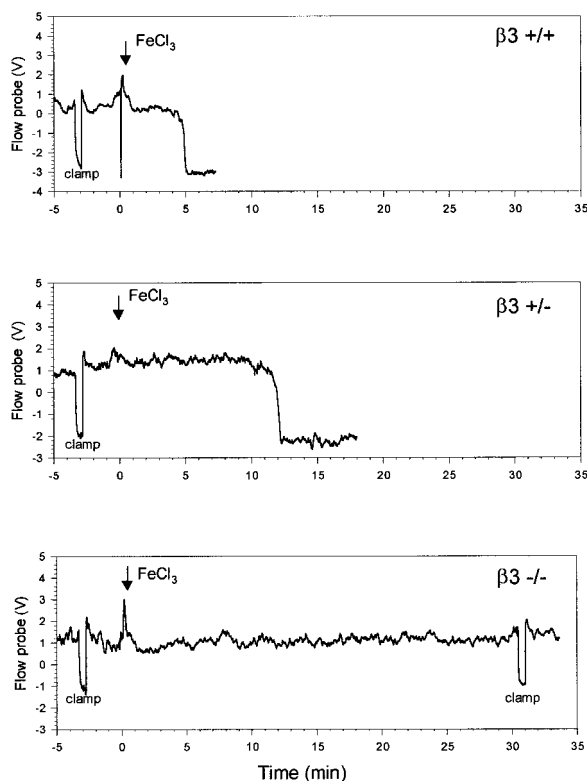
### Characterization of the in vivo effects of anti- $\alpha$ IIb $\beta$ 3 mAb 1B5

One hour after mice were injected with 1B5 Fab fragments (1 mg/kg, intraperitoneally [IP]) tail bleeding times were all greater than 12 minutes (*n* = 4), a result that was significantly different (*P* < .05) from the mean time of 1.5 minutes in untreated mice (*n* = 3). Neither 1B5 nor 1B5 F(ab')<sub>2</sub> had an effect on platelet count (data not shown). After a single intravenous bolus dose of 0.53 mg/kg 1B5 Fab, initial slopes of platelet aggregation induced by 10  $\mu$ M ADP were inhibited 85% at 10 minutes, 80% at 1 hour, 50% at 24 hours, and 15% at 5 days. A higher intravenous dose of 1B5 Fab fragments (1 mg/kg) abolished ADP-induced aggregation at 1 hour and 5 hours after injection; 60% inhibition remained at 18 hours (Figure 2), as determined by measuring differences in the initial slope of aggregation. In vivo administration of 1B5 Fab blocked ex vivo binding of 1B5 IgG as measured by flow cytometry, with a time-course consistent with the inhibition of platelet aggregation (Table 1).

### Carotid artery thrombosis

Figure 3 contains typical temperature probe readings from a carotid artery after applying FeCl<sub>3</sub> to the mouse carotid artery. In  $\beta$ 3<sup>+/+</sup> mice (*n* = 7), whose genetic background is a mixture of C57Bl/6 and 129Sv strains, the median time to maximal temperature reduction was 6.7 minutes (mean  $\pm$  SD of 8.2  $\pm$  3.3 minutes). Time to maximal temperature reduction in pure C57Bl/6 and 129Sv mice was similar to that in the mixed  $\beta$ 3<sup>+/+</sup> mice, suggesting that there was little variation between the strains (Table 2). Median time to maximal temperature reduction in the heterozygous  $\beta$ 3<sup>+/-</sup> mice (*n* = 8) was 9.8 minutes (mean  $\pm$  SD of 9.8  $\pm$  1.8 minutes), which was longer than the median time in the C57/129  $\beta$ 3<sup>+/+</sup> mice, but the difference was not statistically significant (*P* = .4). In sharp contrast to the results in the  $\beta$ 3<sup>+/+</sup> and  $\beta$ 3<sup>+/-</sup> mice, however, no change in temperature occurred for up to 30 minutes in any of carotid arteries of the 5  $\beta$ 3<sup>-/-</sup> mice tested (*P* < .001 vs C57/129  $\beta$ 3<sup>+/+</sup> mice).

Microscopic evaluation of longitudinal sections of the arteries of wild-type mice (data not shown) revealed that the thrombus occluding the lumen was platelet- and fibrin-rich and, at the ends, contained trapped red cells. Electron microscopy (Figures 4, 5)



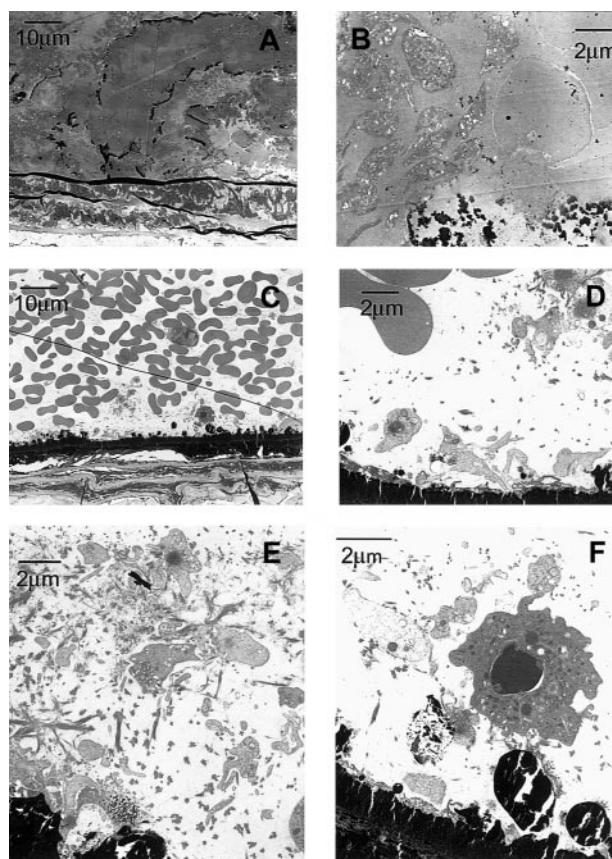
**Figure 3.  $\beta 3$ -null ( $\beta 3^{-/-}$ ) mice are protected from  $\text{FeCl}_3$ -induced thrombosis of the carotid artery.** To determine the extent of temperature change during the cessation of blood flow, the arteries were occluded for 30 seconds with a vascular clamp before the application of  $\text{FeCl}_3$  (arrow). Time to maximal temperature reduction occurred 5 minutes and 12 minutes after the initiation of injury in the  $\beta 3^{+/+}$  mouse and the  $\beta 3^{+/-}$  mouse, respectively. Up to 30 minutes after the addition of  $\text{FeCl}_3$ , no temperature reduction occurred in the  $\beta 3^{-/-}$  mouse, at which time the clamp was re-applied to document that the vessel was still patent.

confirmed these findings. In contrast, the lumens of arteries from  $\beta 3$ -null mice were lined by essentially a single layer of platelets with overlying red cells but no occlusive thrombus (Figures 4, 5).

The effect of antibody 1B5 on thrombotic occlusion in this model was tested by injecting 1B5 or control antibodies into wild-type mice. Control hamster mAb Fab (9C2, an anti-mouse  $\alpha \text{IIb}\beta 3$  mAb that does not inhibit receptor function) did not prolong the time to maximal temperature reduction when compared to untreated wild-type animals (Tables 2, 3; mean  $\pm$  SD time of  $7.0 \pm 1.5$  vs  $8.2 \pm 3.3$ , respectively). At 1 mg/kg, 1B5 Fab increased the median time to maximal temperature reduction from the control value range of 7.8 to 8.7 minutes to 12 minutes ( $P = .2$ ), and 1B5 Fab at 2 mg/kg increased the median time to 21.5 minutes ( $P = .006$ ) (Table 3, Figure 6). Control polyclonal hamster F(ab')<sub>2</sub> also had no effect on the time to maximal temperature reduction, whereas 1B5 F(ab')<sub>2</sub> at 2 mg/kg prolonged the median time to more than 30 minutes ( $P < .001$ ). Microscopic evaluation demonstrated more thrombus formation than in the  $\beta 3^{-/-}$  mice but less than in

**Table 2. Time to maximal temperature reduction of carotid arteries after application of  $\text{FeCl}_3$**

	Median time (min)	Mean time $\pm$ SD (min)	n	P (vs $\beta 3^{+/+}$ )
C57B1/6	7.3	$7.5 \pm 0.9$	4	NS
129Sv	7.3	$7.6 \pm 2.4$	4	NS
$\beta 3^{+/+}$ C57/129	6.7	$8.2 \pm 3.3$	7	NS
$\beta 3^{+/-}$ C57/129	9.8	$9.8 \pm 1.8$	8	NS
$\beta 3^{-/-}$ C57/129	> 30	> 30	5	< .001



**Figure 4. Transmission electron micrographs of a carotid artery from a  $\beta 3^{+/+}$  and a  $\beta 3^{-/-}$  mouse exposed to  $\text{FeCl}_3$  demonstrate platelet masses in the  $\beta 3^{+/+}$  mouse and a platelet monolayer in the  $\beta 3^{-/-}$  mouse.** The carotid artery of the  $\beta 3^{+/+}$  mouse (A-B) was fixed approximately 7 minutes after the application of  $\text{FeCl}_3$ , whereas the artery in the  $\beta 3^{-/-}$  mouse (C-F) was fixed after 30 minutes. Dark-staining  $\text{FeCl}_3$  accumulated along the internal elastic lamina and, in the wild-type mouse, within the thrombus. The vessel in the wild-type mouse was occluded by thrombus composed of platelets, fibrin, and erythrocytes (A-B). Primarily a single layer of platelets adhered to  $\text{FeCl}_3$ -treated vessel wall of the  $\beta 3^{-/-}$  mouse (C-D); fibrin and residual erythrocytes were also present. In addition to attaching directly to the damaged wall, platelets accumulated in areas rich in fibrin along the  $\beta 3^{-/-}$  artery (E). Along the damaged vessel in  $\beta 3^{-/-}$  mouse, adherent platelets recruited leukocytes, and additional platelets attached to the luminal surfaces of the leukocytes (F).

the arteries of wild-type mice. Treatment of wild-type mice ( $n = 6$ ) with aspirin at doses sufficient to inhibit ex vivo arachidonic acid-induced platelet aggregation (20 mg/kg, IP) had no effect on the time to maximal temperature reduction (data not shown). Moreover, aspirin did not prolong the time to maximal temperature reduction in mice receiving 2 mg/kg 1B5 Fab (data not shown).

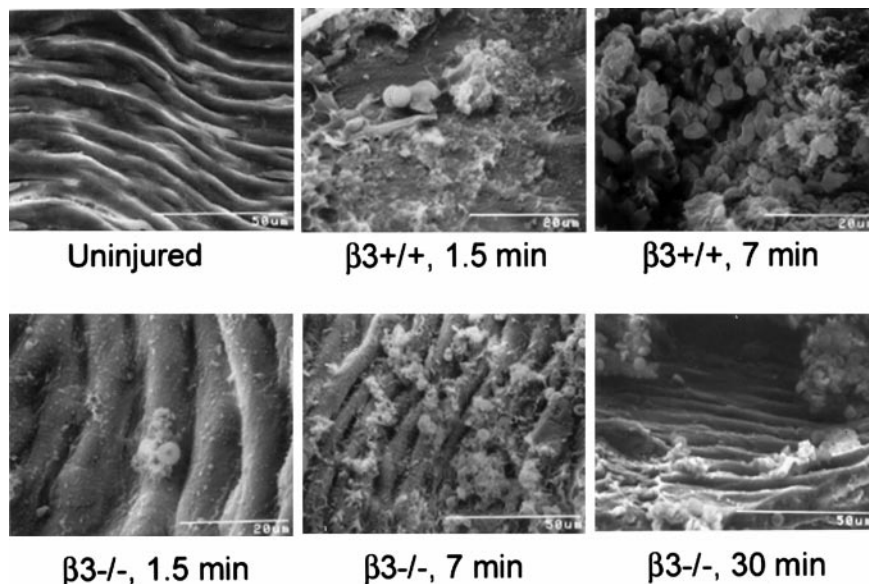
#### Models of systemic intravascular thrombosis

To initiate systemic intravascular thrombosis, prothrombotic agents (collagen + epinephrine, tissue factor, or ADP) were administered intravenously, and the incorporation of platelets into thrombi was monitored by the decrease in circulating platelet count after 1 minute.

Baseline platelet counts were similar in  $\beta 3^{-/-}$  and  $\beta 3^{+/+}$  mice ( $540 \pm 150$  and  $530 \pm 110 \times 10^3/\mu\text{L}$ ), as were basal white blood cell counts ( $4.5 \pm 2.3$  vs  $5.9 \pm 2.3 \times 10^3/\mu\text{L}$ ), but, as previously described,<sup>8</sup> the baseline hematocrit levels were lower in the  $\beta 3^{-/-}$  mice ( $30\% \pm 8\%$  vs  $40\% \pm 7\%$ ).

Injecting saline produced median decreases of  $62 \times 10^3$  and  $92 \times 10^3$  platelets per microliter in wild-type and  $\beta 3$ -null mice.

**Figure 5.** Scanning electron micrographs of carotid arteries of  $\beta$ 3<sup>+/+</sup> and  $\beta$ 3<sup>-/-</sup> mice exposed to FeCl<sub>3</sub> for differing time intervals demonstrate an occlusive platelet- and erythrocyte-rich thrombus in the  $\beta$ 3<sup>+/+</sup> mice and a platelet monolayer with small erythrocyte accumulations in the  $\beta$ 3<sup>-/-</sup> mice.



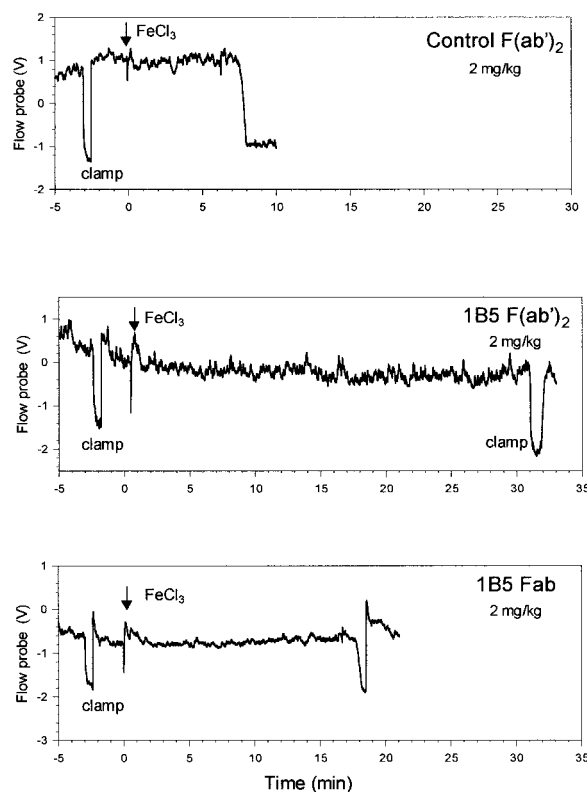
Three minutes after injection of ADP, collagen + epinephrine, or tissue factor, median platelet counts in the wild-type mice decreased by  $289 \times 10^3/\mu\text{L}$ ,  $424 \times 10^3/\mu\text{L}$ , and  $429 \times 10^3/\mu\text{L}$  (Table 4), indicating a gradation in prothrombotic effects. Essentially,  $\beta$ 3<sup>-/-</sup> mice were completely protected from the effects of injecting ADP because the median platelet count decrease ( $90 \times 10^3/\mu\text{L}$ ) was no different from that produced by saline ( $92 \times 10^3/\mu\text{L}$ ). Wild-type mice pretreated with 1B5 Fab (2 mg/kg, IP) were also substantially protected from the effects of ADP, exhibiting minimal changes in median platelet count. In addition,  $\beta$ 3<sup>-/-</sup> mice were partially, but significantly, protected from the effects of injecting collagen + epinephrine (Table 4;  $P < .01$ ), whereas the  $\beta$ 3<sup>-/-</sup> demonstrated minimal protection from the prothrombotic effects of tissue factor, sustaining a platelet count decrease almost as great as that of the control mice (Table 4).

Because thrombi are composed of varying mixtures of platelets and fibrin, we analyzed the microvascular thrombi in the lung for both elements by immunohistochemistry (Figure 7), and we analyzed the pulmonary vasculature for fibrin by immunoblotting (Figure 8). In all cases, more thrombi were present in the lungs of the wild-type than the  $\beta$ 3<sup>-/-</sup> mice. Tissue factor treatment resulted in a mean of  $3 \pm 0.4$  and  $1.7 \pm 0.3$  thrombi per high power field (hpf) in wild-type and  $\beta$ 3<sup>-/-</sup> mice, respectively ( $P = .08$ ); collagen + epinephrine resulted in  $1.8 \pm 0.5$  and  $0.5 \pm 0.4$  thrombi per hpf, respectively ( $P < .01$ ); and ADP resulted in  $0.5 \pm 0.2$  and  $0.03 \pm 0.2$  thrombi per hpf, respectively ( $P < .01$ ). The amount of fibrin extracted from the lungs of wild-type mice varied

depending on the agent injected, with ADP resulting in the least and tissue factor resulting in the most. In all cases, less fibrin accumulated in the lungs of  $\beta$ 3-null mice than of wild-type mice (Figure 9, Table 5).

**Table 3.** Time to maximal temperature reduction of carotid arteries in wild-type mice pretreated with 1B5 Fab, 1B5 F(ab')<sub>2</sub>, or control antibodies

Treatment	Median time (min)	Mean time $\pm$ SD (min)	n	P
Control 9C2 Fab (1 mg/kg)	7.8	7.0 $\pm$ 1.5	3	—
1B5 Fab (1 mg/kg)	12.0	12 $\pm$ 0.5	3	.2 vs control Fab
1B5 Fab (2 mg/kg)	21.5	19.8 $\pm$ 5	3	.006 vs control Fab
Control polyclonal F(ab') <sub>2</sub> (1 mg/kg)	8.7	8.8 $\pm$ 1.1	3	
1B5 F(ab') <sub>2</sub> (1 mg/kg)	> 30	> 30	3	< .001 vs polyclonal F(ab') <sub>2</sub>



**Figure 6.** Antibody 1B5 prolongs the time to occlusive thrombus formation in carotid arteries of wild-type mice. Antibodies (2 mg/kg) were administered by intraperitoneal injection within 1 hour before arterial injury. Time to maximal temperature reduction occurred 8 minutes and 18.5 minutes after the initiation of injury in mice treated with control F(ab')<sub>2</sub> and 1B5 Fab, respectively. No temperature reduction occurred in the mouse treated with 1B5 F(ab')<sub>2</sub> for up to 30 minutes after the application of FeCl<sub>3</sub>.

**Table 4. Median decrease in platelet count ( $\times 10^{-3}/\mu\text{L}$ ) 3 minutes after indicated agents were injected**

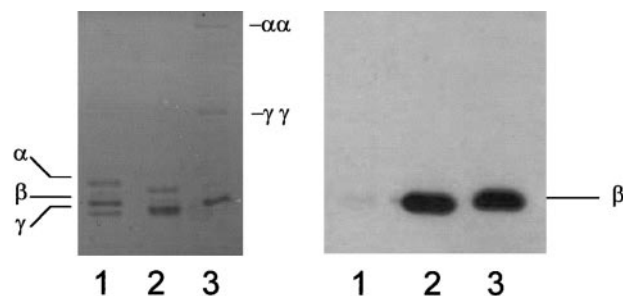
	Tissue factor	Collagen + epinephrine	ADP	Saline
WT ( $\beta 3^{+/+}$ )	429	424	289	62
	n = 11	n = 11	n = 6	n = 3
NULL ( $\beta 3^{-/-}$ )	334	268*	90*	92
	n = 8	n = 11	n = 5	n = 5
WT + 1B5 Fab	ND	ND	114*	ND
			n = 8	

WT indicates wild type; ND, not determined.

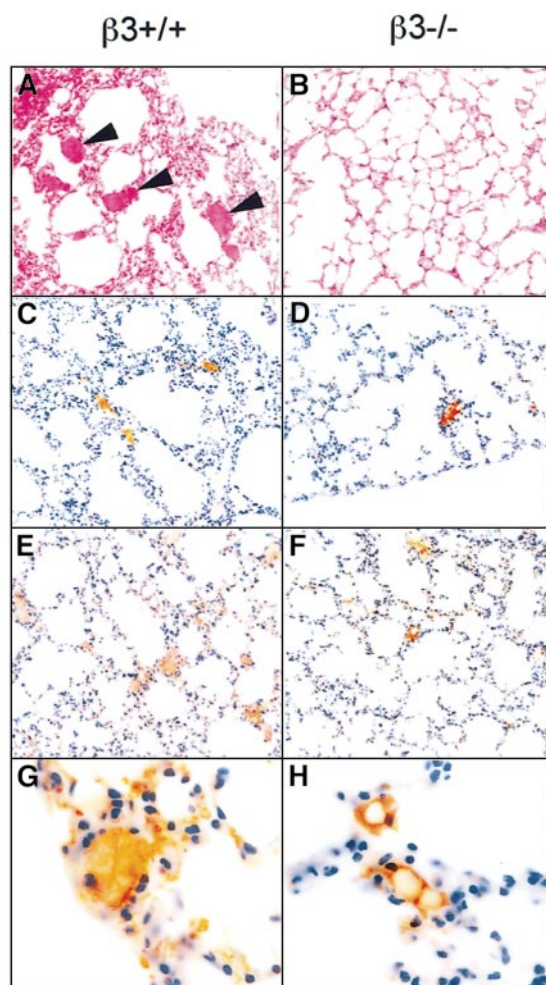
\* $P < .01$  (vs WT  $\beta 3^{+/+}$ ).

## Discussion

Mice lacking or overexpressing specific genes are uniquely powerful and attractive animals for developing models of human thrombotic disease. However, mouse platelets differ from human



**Figure 8. mAb 350 recognizes the  $\beta$ -chain of mouse fibrin but does not react with fibrinogen.** Mouse fibrinogen (lane 1), mouse fibrin prepared in the absence of calcium to limit endogenous factor XIII-mediated cross-linking (lane 2), or mouse fibrin prepared in the presence of calcium to promote endogenous factor XIII-mediated cross-linking (lane 3) was separated by polyacrylamide gel electrophoresis and visualized with Coomassie staining (left) or subjected to immunoblot analysis with mAb 350 (right).  $\gamma\gamma$ ,  $\gamma$ -dimer;  $\alpha\alpha$ ,  $\alpha$  polymer.

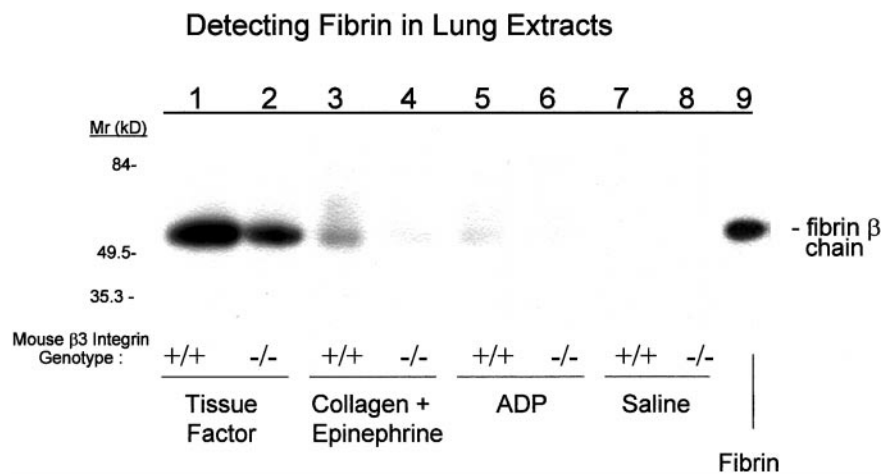


**Figure 7. Histology and immunohistochemistry with antibodies to platelets and fibrin(ogen) reveal increased platelet- and fibrin(ogen)-rich thrombi in the lungs of  $\beta 3^{+/+}$  mice injected with collagen + epinephrine as compared to  $\beta 3^{-/-}$  mice.** Mice were injected with collagen + epinephrine, and 3 minutes later sections of lung tissue were stained with hematoxylin and eosin (panels A-B, 40  $\times$ ). Arrowheads point to thrombi in the pulmonary vasculature of a  $\beta 3^{+/+}$  mouse; thrombi were present in the central and peripheral fields of the lung. Fewer thrombi were present in  $\beta 3^{-/-}$  mice, and, in the  $\beta 3^{-/-}$  mice, the thrombi (more than 70%) tended to be confined to the periphery of the lung tissue. Immunohistochemistry demonstrated that the thrombi were composed of fibrin(ogen) (C-D) and platelets (brown staining; E-F, 40  $\times$ ; G-H, 100  $\times$ ).

platelets in a number of fundamental ways—including smaller size, greater number, alternative thrombin receptors, differences in signal transduction (reviewed in Tsakiris et al<sup>16</sup>)—and mouse vascular rheology is likely to differ from human as a result of the much higher pulse rate (approximately 600 beats per minute). Therefore, it is unclear whether there are fundamental differences in the thrombotic processes in mice and humans that would invalidate the use of mouse models. Moreover, it is important to recognize the major biologic differences between large-vessel thrombosis and systemic intravascular thrombosis. The current study was designed to address these issues by analysis of the role of the  $\alpha\text{IIb}\beta 3$  platelet receptor in a number of different mouse models of thrombosis. We found that  $\beta 3$ -null mice are variably protected from thrombosis based on the method used to initiate the thrombosis.

In the model of carotid artery thrombosis,  $\text{FeCl}_3$  penetrates the blood vessel wall and initiates the production of oxygen radicals, resulting in damage to the endothelium.<sup>17</sup> This led to occlusive thrombosis in the carotid arteries of wild-type but not  $\beta 3$ -null mice. To improve reproducibility of exposing the carotid artery to  $\text{FeCl}_3$ , we constructed a small boatlike structure to house the  $\text{FeCl}_3$  and localize its effects. Because the exposed carotid artery was too short to accommodate both the boat and a flow probe, we used a temperature probe to monitor blood flow in the artery. Previous studies by Kurz et al<sup>14</sup> demonstrated that reductions in blood flow as measured by a flow probe were highly correlated with changes in temperature. They also demonstrated that reductions in flow velocity did not occur until there was nearly an 80% reduction in the outer diameter of the carotid artery. Our unpublished observations correlating data from a flow probe and our temperature probe indicate that complete or near-complete cessation of blood flow is required to elicit a maximum temperature reduction as measured by our temperature probe. Thus, we conclude that  $\text{FeCl}_3$  induces occlusive or near-occlusive thrombus formation in the carotid arteries of wild-type but not  $\beta 3$ -null mice. Microscopic examination of the arteries supported our conclusions and revealed extensive platelet thrombi, fibrin, and red cells that obstructed the lumen of the carotid arteries in wild-type mice, whereas arteries from  $\beta 3$ -null mice, which lack both  $\alpha\text{IIb}\beta 3$  and  $\alpha\text{V}\beta 3$ , were patent and lined primarily with only a single layer of platelets. Studies using our mAb 1B5, which blocks the  $\alpha\text{IIb}\beta 3$  receptor but not the  $\alpha\text{V}\beta 3$  receptor, support the interpretation that the findings in  $\beta 3^{-/-}$  mice reflect the loss of the  $\alpha\text{IIb}\beta 3$

**Figure 9.** After the injection of prothrombotic agents, more fibrin deposits in the lungs of  $\beta$ 3<sup>+/+</sup> mice than in those of  $\beta$ 3<sup>-/-</sup> mice. Lung extracts from mice injected with the indicated agents were separated by polyacrylamide gel electrophoresis and subjected to immunoblot analysis with mAb 350 to detect fibrin.



receptor. They also make it unlikely that the results in the  $\beta$ 3<sup>-/-</sup> mice were due to anemia rather than to the  $\alpha$ IIb $\beta$ 3 defect. Thus, 1B5 prolonged the bleeding time, inhibited ex vivo platelet aggregation, and protected mice from the effects of thrombosis induced by FeCl<sub>3</sub>. Because the results of these experiments are similar to those obtained with other models of arterial injury<sup>5,6</sup> and with percutaneous coronary interventions in large arteries in humans,<sup>7</sup> and because there was concordance in the antithrombotic effects using  $\beta$ 3-null mice and the mAbs to  $\alpha$ IIb $\beta$ 3, we conclude that insights obtained on large-artery thrombosis with  $\beta$ 3-null mice are likely to be relevant to the inhibition of human  $\alpha$ IIb $\beta$ 3 with mAbs or other antagonists.

Many important clinical syndromes involve the entire systemic vasculature rather than just large arteries, among them diffuse intravascular coagulation, thrombotic thrombocytopenic purpura, and sepsis-related microvascular thrombosis. The relative contribution of  $\alpha$ IIb $\beta$ 3 receptors to these phenomena has not been systematically described, making it difficult to assess whether blockade of  $\alpha$ IIb $\beta$ 3 receptors is likely to be of therapeutic value. As a first step in addressing this question, we tested wild-type and  $\beta$ 3<sup>-/-</sup> mice in 3 different models of systemic intravascular thrombosis. These models lack the complexity of the human syndromes, which likely involve several agonists acting in concert. However, by choosing agonists that span the spectrum—from an effect primarily on platelet aggregation (ADP) to an effect primarily on thrombin generation (tissue factor)—they provide insight into the role of  $\alpha$ IIb $\beta$ 3-mediated platelet function in response to these prototypic agonists. Essentially,  $\beta$ 3<sup>-/-</sup> mice were completely protected from the effect of ADP injected intravenously. In contrast, they were only partially protected from the effects of collagen + epinephrine and only minimally protected from the effects of tissue factor. These differences probably reflect differences in the nature of the challenging agent. ADP probably exerts most of its effects by activating platelet  $\alpha$ IIb $\beta$ 3, thereby causing intravas-

cular platelet aggregation. In contrast, tissue factor injection most likely results more directly in fibrin formation, which may then trap platelets in an  $\alpha$ IIb $\beta$ 3-independent manner, perhaps in part through an interaction of platelet GPIb with von Willebrand factor adherent to fibrin.<sup>18-20</sup> The decrease in platelet count in wild-type mice after injecting collagen + epinephrine likely reflects 2 separate processes, platelet adhesion to collagen fibrils (through  $\alpha$ 2 $\beta$ 1, GPVI, and perhaps other receptors<sup>21,22</sup>) and  $\alpha$ IIb $\beta$ 3-mediated platelet-platelet interactions resulting from the release of ADP, thromboxane A<sub>2</sub>, and perhaps other agents from the adherent platelets. The partial protection against thrombocytopenia in the  $\beta$ 3-null mice is consistent with this interpretation given that their platelets are not deficient in collagen receptors, but they are unable to support  $\alpha$ IIb $\beta$ 3-mediated platelet-platelet interactions.

Data from the systemic intravascular thrombosis model initiated by injecting tissue factor are consistent with previous studies demonstrating that Glanzmann thrombasthenia platelets can interact with fibrin but not with fibrinogen.<sup>23</sup> They are also consistent with our previous studies in which administering antibody 7E3 F(ab')<sub>2</sub>, which inhibits  $\alpha$ IIb $\beta$ 3, to baboons had little impact on the thrombocytopenia and defibrination caused by injecting a sublethal dose of *Escherichia coli* along with C4b-binding protein (which lowers free protein S).<sup>3</sup> The 7E3 F(ab')<sub>2</sub>-treated animals did, however, show protection from developing microangiopathic hemolysis and renal insufficiency, suggesting that 7E3 F(ab')<sub>2</sub> prevented intravascular platelet aggregate deposition and resultant ischemic organ damage and the damage to erythrocytes traversing the partially obstructed blood vessels.<sup>3</sup> Our data are also in accord with the clinical observation that patients with Glanzmann thrombasthenia are not protected from developing disseminated intravascular coagulation. Seligsohn observed this syndrome in one patient after major blood loss after cesarean section and in another patient after a hemolytic transfusion reaction (U. Seligsohn, personal communication, 2000).

Our observations about the ability of tissue factor to overcome the deficiency of  $\alpha$ IIb $\beta$ 3 in initiating thrombus formation also provide a potential explanation for the reported success in using recombinant activated factor VII, which presumably augments the effects of tissue factor, to treat patients with Glanzmann thrombasthenia who have uncontrolled hemorrhage.<sup>24-27</sup> The reverse implication is that optimal therapy of systemic intravascular thrombosis may require inhibition of both tissue factor-mediated fibrin deposition and platelet  $\alpha$ IIb $\beta$ 3. This last consideration takes on particular

**Table 5.** Lung fibrin deposition 3 minutes after injection of prothrombotic agents in wild-type ( $\beta$ 3<sup>+/+</sup>) or  $\beta$ 3-null ( $\beta$ 3<sup>-/-</sup>) mice

	Fibrin (mg/mL)	
	WT ( $\beta$ 3 <sup>+/+</sup> )	Null ( $\beta$ 3 <sup>-/-</sup> )
Saline	2	2
ADP	3	2
Collagen + epinephrine	10	3
Tissue factor	150	100

WT indicates wild type.

importance in light of the recent announcement of the premature termination of a clinical trial evaluating the effect of activated protein C on sepsis-related mortality as a result of a beneficial effect of the treatment.<sup>28</sup> These findings suggest that systemic

intravascular thrombosis may be a major contributor to sepsis-related mortality, which raises the possibility that combined anticoagulant and anti- $\alpha$ IIb $\beta$ 3 therapy may be more efficacious than treatment with either alone.

## References

- Fuster V, Stein B, Ambrose JA, Badimon L, Badimon JJ, Chesebro JH. Atherosclerotic plaque rupture and thrombosis: evolving concepts. *Circulation*. 1990;82(suppl):II47-II59.
- Schafer AI, Kroll MH. Nonatheromatous arterial thrombosis. *Annu Rev Med*. 1993;44:155-170.
- Taylor FB, Collier BS, Chang AC, et al. 7E3 F(ab')<sub>2</sub>, a monoclonal antibody to the platelet GPIIb/IIIa receptor, protects against microangiopathic hemolytic anemia and microvascular thrombotic renal failure in baboons treated with C4b binding protein and a sublethal infusion of *Escherichia coli*. *Blood*. 1997;89:4078-4084.
- Ruggeri ZM. Mechanisms initiating platelet thrombus formation. *Thromb Haemost*. 1997;78:611-616.
- Collier BS, Folts JD, Smith SR, Scudder LE, Jordan R. 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*. 1989;80:1766-1774.
- Gold HK, Collier BS, Yasuda T, et al. Rapid and sustained coronary artery recanalization with combined bolus injection of recombinant tissue-type plasminogen activator and monoclonal anti-platelet GPIIb/IIIa antibody in a canine preparation. *Circulation*. 1988;77:670-677.
- Lincoff AM, Califf RM, Topol EJ. Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. *J Am Coll Cardiol*. 2000;35:1103-1115.
- Hodivala-Dilke KM, McHugh KP, Tsakiris DA, et al.  $\beta$ 3-Integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest*. 1999;103:229-238.
- Illera MJ, Cullinan E, Gui Y, Yuan L, Beyler SA, Lessey BA. Blockade of the  $\alpha$ V $\beta$ 3 integrin adversely affects implantation in the mouse. *Biol Reprod*. 2000;62:1285-1290.
- Bader BL, Rayburn H, Crowley D, Hynes RO. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all  $\alpha$ V integrins. *Cell*. 1998;95:507-519.
- Lengweiler S, Smyth SS, Jirouskova M, et al. Preparation of monoclonal antibodies to murine platelet glycoprotein IIb/IIIa ( $\alpha$ IIb $\beta$ 3) and other proteins from hamster-mouse interspecies hybridomas. *Biochem Biophys Res Commun*. 1999;262:167-173.
- Smyth SS, Tsakiris DA, Scudder LE, Collier BS. Structure and function of murine  $\alpha$ IIb $\beta$ 3 (GPIIb/IIIa): studies using monoclonal antibodies and  $\beta$ 3-null mice. *Thromb Haemost*. 2000;84:1103-1108.
- Dejana E, Callioni A, Quintana A, de Gaetano G. Bleeding time in laboratory animals. II: a comparison of different conditions in rats. *Thromb Res*. 1979;15:191-197.
- Kurz KD, Main BW, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res*. 1990;60:269-280.
- Olman MA, Simmons VL, Pollman DJ, et al. Polymerization of fibrinogen in murine bleomycin-induced lung injury. *Am J Physiol*. 1996;271:L519-L526.
- Tsakiris DA, Scudder L, Hodivala-Dilke K, Hynes RO, Collier BS. Hemostasis in the mouse (*Mus musculus*): a review. *Thromb Haemost*. 1999;81:177-188.
- Ni H, Denis CV, Subbarao S, et al. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest*. 2000;106:385-392.
- Loscalzo J, Inbal A, Handin RI, von Willebrand protein facilitates platelet incorporation into polymerizing fibrin. *J Clin Invest*. 1986;78:1112-1119.
- Parker RI, Gralnick HR. Fibrin monomer induces binding of endogenous platelet von Willebrand factor to the glyocalicin portion of platelet glycoprotein Ib. *Blood*. 1987;70:1589-1594.
- Béguin S, Kumar R, Keularts I, Seligsohn U, Collier BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. *Blood*. 1999;93:564-570.
- Clemetson KJ. Platelet collagen receptors: a new target for inhibition? *Haemostasis*. 1999;29:16-26.
- Sixma JJ, van Zanten GH, Huizinga EG, et al. Platelet adhesion to collagen: an update. *Thromb Haemost*. 1997;78:434-438.
- Niewiarowski S, Levy-Toledano S, Caen JP. Platelet interaction with polymerizing fibrin in Glanzmann's thrombasthenia. *Thromb Res*. 1981;23:457-463.
- Poon MC, Demers C, Jobin F, Wu JW. Recombinant factor VIIa is effective for bleeding and surgery in patients with Glanzmann thrombasthenia. *Blood*. 1999;94:3951-3953.
- Chuansumrit A, Sangkapreecha C, Hathirat P. Successful epistaxis control in a patient with Glanzmann thrombasthenia by increased bolus injection dose of recombinant factor VIIa [abstract]. *Thromb Haemost*. 1999;82:1778.
- Ancliff P, Liesner R, Khair K, Hann I. The use of recombinant factor VIIa in a patient with severe Glanzmann's thrombasthenia to facilitate insertion of a Port-a-Cath. *Blood Coagul Fibrinolysis*. 1999;10:447-448.
- Poon MC, d'Oiron R. Recombinant activated factor VII (NovoSeven) treatment of platelet-related bleeding disorders: International Registry on Recombinant Factor VIIa Congenital Platelet Disorders Group. *Blood Coagul Fibrinolysis*. 2000;11:S55-S68.
- Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med*. 2001;344:699-709.