The maternal immune response to fetal platelet GPIbα causes frequent miscarriage in mice that can be prevented by intravenous IgG and anti-FcRn therapies

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Fetal and neonatal immune thrombocytopenia (FNIT) is a severe bleeding disorder caused by maternal antibody–mediated destruction of fetal/neonatal platelets. It is the most common cause of severe thrombocytopenia in neonates, but the frequency of FNIT-related miscarriage is unknown, and the mechanism(s) underlying fetal mortality have not been explored. Furthermore, although platelet αIIbβ3 integrin and GPIbα are the major antibody targets in immune thrombocytopenia, the reported incidence of anti-GPIbα–mediated FNIT is rare. Here, we developed mouse models of FNIT mediated by antibodies specific for GPIbα and β3 integrin and compared their pathogenesis. We found, unexpectedly, that miscarriage occurred in the majority of pregnancies in our model of anti-GPIbα–mediated FNIT, which was far more frequent than in anti-β3–mediated FNIT. Dams with anti-GPIbα antibodies exhibited extensive fibrin deposition and apoptosis/necrosis in their placentas, which severely impaired placental function. Furthermore, anti-GPIbα (but not anti-β3) antisera activated platelets and enhanced fibrin formation in vitro and thrombus formation in vivo. Importantly, treatment with either intravenous IgG or a monoclonal antibody specific for the neonatal Fc receptor efficiently prevented anti-GPIbα–mediated FNIT. Thus, the maternal immune response to fetal GPIbα causes what we believe to be a previously unidentified, nonclassical FNIT (i.e., spontaneous miscarriage but not neonatal bleeding) in mice. These results suggest that a similar pathology may have masked the severity and frequency of human anti-GPIbα–mediated FNIT, but also point to possible therapeutic interventions.

Introduction

Fetal and neonatal immune thrombocytopenia (FNIT) is a severe alloimmune disorder that results from fetal/neonatal platelet destruction by maternal antibodies generated during pregnancy (1–4). FNIT is the most common type of severe thrombocytopenia in live-born neonates and carries a major risk of intracranial hemorrhage, which can lead to neurological impairment or death (5–8). The incidence of FNIT has been estimated at 0.5–1.5 per 1,000 live-born neonates (1–4). This number, however, does not include miscarriages caused by the disease, since the rate of fetal mortality in affected pregnant women has not been adequately studied, although miscarriage has been reported by several groups (9–13). Currently, the mechanisms leading to miscarriage in these women and the therapies to prevent this devastating consequence are unknown.

Platelets play a critical role in hemostasis and thrombosis. Platelet adhesion, activation, and aggregation at the site of vascular injury lead to the formation of a platelet plug and the subsequent arrest of bleeding. However, accumulation of activated platelets at inappropriate sites (e.g., atherosclerotic lesions) may lead to thrombus formation and vessel obstruction (14–16). In addition, activated platelets may generate negatively charged phospholipids (e.g., phosphatidylserine [PS]) on their surfaces, which promote thrombin generation and fibrin formation (17–19). This procoagulant activity facilitates hemostasis but may also enhance the severity of thrombotic disorders. To date, there is no report regarding whether thrombosis in the placenta may be involved in the pathogenesis of FNIT and contribute to the miscarriage observed in this disease.

Integrin αIIbβ3 (GPIIb/IIIa) and the GPIbα complex are major glycoproteins on the platelet surface and are critically required for platelet adhesion and aggregation. In FNIT, most reported cases (75%–95%) have been characterized by maternal alloantibodies to fetal β3 integrin (20, 21), with few reported cases of FNIT associated with anti-GPIbα antibodies (22–27). This is in stark contrast to the 20%–40% prevalence of anti-GPIbα complex antibodies in patients with immune thrombocytopenia (ITP) (28–30), an analogous bleeding disorder in which patients have autoimmune responses to the same platelet antigens as in FNIT (β3 integrin and GPIbα). The underlying reason for the surprisingly low incidence of FNIT mediated by anti-GPIbα antibodies has not been explored, and the maternal immune responses to fetal platelet antigens remain to be elucidated.
In the current study, we developed two murine models of FNIT in syngeneic GPIbα-deficient (GPIbα−/−) and β3 integrin-deficient (β3−/−) mice. We found that anti-GPIbα caused miscarriage (complete lack of parturition) in most affected mothers and markedly enhanced fibrin deposition in their placentas, leading to impairment in placental function. This is different from FNIT as it is traditionally conceived, as a disorder primarily characterized by bleeding symptoms in neonates. The high incidence of miscarriage likely contributes to the rarity of case reports of anti-GPIbα–mediated FNIT. We further demonstrated that intravenous IgG (IVIG) and an mAb against the neonatal Fc receptor (FcRn) can prevent this devastating consequence.

Results

GPIbα−/− mice were immunoresponsive to the GPIbα antigen on transfused WT platelets. The reported incidence of human anti-GPIbα–mediated FNIT is rare. Little information is available regarding how the maternal immune response to the GPIbα antigen occurs and whether GPIbα−/− mice are immunoresponsive to the GPIbα antigen after these antigen-positive platelets enter the blood circulation. Since there was no animal model to address this question, we first tested whether specific anti-GPIbα antibodies can be generated in GPIbα−/− mice and whether these antibodies can induce thrombocytopenia.

To develop a new FNIT model with GPIbα−/− mice, in order to compare the pathogenesis with that of anti–β3 integrin–mediated FNIT, we first backcrossed GPIbα−/− and β3−/− mice to a BALB/c background 10 times. The BALB/c GPIbα−/− and β3−/− strains were further crossed to each other to generate syngeneic gene-deficient mice. To mimic the human preconception exposure to the GPIbα antigen, or human mothers who had previous pregnancies that exposed them to fetal platelet GPIbα (e.g., human platelet antigen 2 [HPA-2]), we transfused GPIbα−/− females with WT platelets. We found that GPIbα−/− mice mounted a significant immune response to GPIbα after two transfusions of 10⁸ gel-filtered WT platelets, and both IgG1 and IgG2a isoantibodies were detected (Figure 1A), indicative of both Th1- and Th2-like immune responses. The antibodies from immunized GPIbα−/− mice were specific to GPIbα, as they did not recognize either GPIbα−/− platelets or WT red blood cells. The
specificity of anti-GPIbα polyclonal antibodies was also confirmed via Western blot analysis using a rat anti–mouse GPIbα mAb (p0p5) (31) as a control. Using murine platelets expressing the IL-4Rα/GPIb-IX complex, in which most of the extracellular domain of GPIbα is replaced by the α-subunit of the human IL-4 receptor (IL-4Rα) (32), we found that the anti-GPIbα antibodies specifically bound to platelets expressing WT GPIbα, but not IL-4Rα/GPIbα (Figure 1B). Since the expression levels of αIIbβ3 integrin, GPV, and GPIX are normal on the surface of IL-4Rα/GPIbα transgenic platelets (33), these anti-GPIbα antibodies do not cross-react with other proteins on platelets, including β3 integrins and glycoproteins in the GPIb-IX complex. Thrombocytopenia was efficiently induced in adult WT mice after injection of the anti-GPIbα antiserum (Figure 1C). These data indicate that GPIbα−/− mice are immunoresponsive to WT platelets and thus can be utilized in a model of FNIT, as we previously described in β3−/− mice (34).

**Miscarriage occurred in most of the mice with anti-GPIbα–mediated FNIT.** To compare the pathogenesis of FNIT mediated by anti-GPIbα and anti-β3 integrin (anti-β3) antibodies in these two strains of syngeneic mice, we determined antibody titers in GPIbα−/− and β3−/− females after 2 transfusions of 10^8 WT platelets (34, 35). There was no significant difference (data not shown) in the mean titers of anti-GPIbα and anti-β3 antibodies.

The immunized GPIbα−/− and β3−/− females and naive GPIbα−/− and β3−/− females (negative controls) were bred with WT males to mimic human FNIT (34, 35). We found, unexpectedly, that it was very difficult to obtain thrombocytopenic GPIbα−/− pups, and miscarriage occurred in most of the mice with anti-GPIbα–mediated FNIT (25 of 30, 83.3%), which was far more frequent than that mediated by anti-β3 (10 of 26, 37%; P < 0.0005) (Figure 2A) and compared with naive controls (0 of 17, 0%; P < 0.0001). Miscarriage was detected around day 16.5 to 18.5 of pregnancy. When the females were dissected after miscarriage, the conceptuses were much smaller than those of naive controls (Figure 2B), which was likely due to the resorption of conceptuses when miscarriage occurred. For the immunized females with a successful pregnancy, thrombocytopenia was observed in live GPIbα−/− pups (209.8 ± 13.9 × 10^9/l versus 286.7 ± 12.4 × 10^9/l in naive control mice, P < 0.001) and β3−/− FNIT pups (151.9 ± 12 × 10^9/l versus 341.5 ± 3.7 × 10^9/l, P < 0.001). Furthermore, we observed that the average number of pups per litter in the immunized GPIbα−/− group was lower than in the β3−/− group (0.4 ± 0.2 pups versus 3.3 ± 0.8 pups, P < 0.0005); naive GPIbα−/− and β3−/− mice generated 8.8 ± 0.6 and 8.2 ± 1.3 pups per litter, respectively (Figure 2C). These data suggest that the maternal immune response against the GPIb α antigen caused frequent fetal mortality, which is significantly more severe than that in anti-β3–mediated FNIT.

To exclude the potential effects of maternal GPIbα deficiency and maternal immune status on miscarriage incidence, we bred immunized GPIbα−/− females with heterozygous (GPIbα+/−) males. Assuming a Mendelian ratio, it was expected that half of the pups would be GPIbα−/− and half would be GPIbα+/−. Surprisingly, we did not find any GPIbα−/− pups, and all 11 pups delivered from these breeders were GPIbα+/− (P < 0.005). This suggests that maternal GPIbα deficiency and maternal immune status did not significantly affect pregnancy outcome in the anti-GPIbα–mediated FNIT model. Instead, it appears that the maternal immune response to fetal GPIbα caused severe pathology in the GPIbα antigen–positive fetuses (i.e., fetal death).

**Excessive fibrin deposition and apoptosis/necrosis in the placenta of immunized GPIbα−/− females.** Pregnancy is associated with an acquired hypercoagulable state that can cause gestational vascular complications, especially in the presence of other prothrombotic risk factors (36). Recently, it has been demonstrated that thrombus formation in the placenta can lead to spontaneous fetal loss (37). To test whether microthrombus formation was enhanced in the placenta of immunized GPIbα−/− mice, we used anti-fibrin antibody to stain the 14.5 days post coitum (d.p.c.) placentas (i.e., before miscarriage occurred) from immunized GPIbα−/− and β3−/− mice. Compared with the β3−/− group, immunized FNIT mothers with anti-GPIbα antibodies exhibited significantly more fibrin deposition in the placenta (P < 0.0005), suggesting that thrombus formation was enhanced in the placenta of GPIbα−/− females carrying GPIbα−/− fetuses (Figure 3, A and B). TUNEL staining revealed that significantly more apoptosis/necrosis occurred in placentas from immunized pregnant GPIbα−/− mice, compared with naive GPIbα−/− mice or immunized β3−/− mice (P < 0.0001) (Figure 4, A and B). By introducing FITC-dextran into the blood circulation of 15.5-d.p.c. pregnant mice (38), we found that compared
with naive pregnant GPIbα−/− mice, the amount of FITC-dextran reaching the fetal placenta circulation was significantly reduced in immunized pregnant GPIbα−/− mice (P < 0.0001), suggesting that fetal blood supply was markedly impaired in affected placentas (Figure 5, A and B). As a control, FITC-dextran infusion into other organs, such as liver, kidney, and spleen, was found to be comparable in naive and immunized pregnant GPIbα−/− mice (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI57850DS1). Interestingly, anti-thrombotic treatment of immunized pregnant GPIbα−/− mice with low-dose warfarin (5 μg/d) alleviated miscarriage in this FNIT model (8 of 15, 53.3% versus 25 of 30, 83.3%; P < 0.05). These data demonstrated, for the first time to our knowledge, that maternal antibodies to platelet GPIbα cause thrombus formation in the placenta and impair placental function, which likely accounts for the more frequent miscarriages observed in anti-GPIbα-mediated FNIT.

![Figure 3](image1)

**Figure 3**
Significantly more fibrin deposition was observed in placentas from immunized GPIbα−/− mice. (A) Representative pictures of 14.5-d.p.c. placenta from different pregnant females (as indicated), stained with anti-fibrin antibody. Scale bar: 100 μm. (B) Quantitative analysis detected significantly more fibrin deposition in placentas from immunized GPIbα−/− mice, compared with immunized β3−/− mice (n = 6–10 mice per group).

![Figure 4](image2)

**Figure 4**
Significantly more apoptosis/necrosis was observed in placentas from immunized GPIbα−/− mice. (A) Representative pictures of the 14.5-d.p.c. placentas from different pregnant females with TUNEL staining. Scale bar: 200 μm. (B) Quantitative analysis revealed that significantly more apoptosis/necrosis occurred in placentas from immunized GPIbα−/− mice compared with naive GPIbα−/− mice or immunized β3−/− mice (n = 4–6 mice per group).
Anti-GPIbα antiserum activated antigen-positive platelets, enhanced fibrin formation in vitro, and accelerated thrombosis in vivo. Activated platelets can generate a procoagulant surface that promotes thrombin generation and fibrin formation (17–19). It has been reported that some anti-platelet autoantibodies in ITP patients stimulate platelets (39, 40), and anti-GPIbα antibodies may activate platelets under certain circumstances in a murine model (31). As described above, enhanced fibrin formation and apoptosis/necrosis were found in the placentas of mothers with anti-GPIbα antibodies. We suspected that anti-β3 and anti-GPIbα antibodies present in our murine model of FNIT might have differing effects on fetal platelet function, effecting the observed differences in fibrin formation and apoptosis/necrosis in the placenta.

Using aggregometry, we found that whereas anti-β3 serum inhibited aggregation, anti-GPIbα antiserum enhanced ADP-induced WT platelet aggregation in platelet-rich plasma (PRP) (Figure 6A). Similar effects were observed for antiserum in PRP from GPIbα−/− mice, which more appropriately reflects the in vivo platelet phenotype in GPIbα−/− fetuses from immunized pregnant GPIbα−/− mice. In addition, anti-GPIbα antiserum, but not anti-β3 serum, was able to induce mild platelet aggregation in WT PRP in the absence of agonist, and the platelet microaggregates were confirmed under light microscopy (Figure 6, B and C); this aggregation could be inhibited by RGD peptides (Supplemental Figure 2). We used WT platelets to specifically remove anti-GPIbα antibodies from antiserum and found that the preabsorbed anti-GPIbα serum failed to both enhance ADP-induced platelet aggregation and induce WT platelet aggregation in PRP (Supplemental Figure 3, A and B). Furthermore, the effects of murine anti-GPIbα antiserum on WT platelet aggregation (Figure 6, A and B) were not observed with GPIbα−/− platelets (Supplemental Figure 3, C and D). We also found that anti-GPIbα polyclonal serum induced WT platelet P-selectin expression and β3 integrin activation, which facilitated JON/A and fibrinogen binding (Figure 7, A–C). These effects seem to have been independent

![Figure 5](image_url) Significantly lower fetal blood supply was observed in placentas from immunized GPIbα−/− mice. (A) FITC-dextran was infused into 15.5-d.p.c. pregnant GPIbα−/− mice. Representative pictures of placentas are shown. ms, maternal side; fs, fetal side. Scale bar: 200 μm. (B) Quantitative analysis of the fluorescence-positive area at the fetal side of the placenta suggested that the fetal blood supply was severely impaired in immunized pregnant GPIbα−/− mice compared with naive GPIbα−/− mice (P < 0.0001, n = 4–6 mice per group).

![Figure 6](image_url) Anti-GPIbα antiserum cause antigen-positive platelet activation in vitro. (A) Polyclonal anti-β3, anti-GPIbα, or preimmune serum was incubated with WT PRP prior to induction of platelet aggregation with 2 μM ADP. Anti-GPIbα serum enhanced ADP-induced WT platelet aggregation, while anti-β3 serum inhibited aggregation. (B) Anti-β3 serum alone (blue curve), anti-GPIbα serum alone (red), or ADP (2 μM; black) was added to WT PRP at 2 minutes of reaction (arrow). Anti-GPIbα, but not anti-β3, serum was able to induce mild platelet aggregation in WT PRP in the absence of soluble agonist. (C) The platelets represented in B were visualized under light microscopy. Arrows indicate platelet microaggregates. Scale bar: 50 μm. Results in A–C are representative of 3 independent experiments.
of genetic background, since similar results were also observed in C57BL/6J mice (Supplemental Figure 4). The antiserum also induced PS surface exposure, a marker of early platelet activation (41), on WT platelets (Figure 7D). These data demonstrate that anti-GPIbα polyclonal antibodies are able to specifically induce GPIbα-positive platelet activation and aggregation.

PS exposure on the platelet surface promotes the conversion of prothrombin into thrombin that mediates fibrin formation (17–19). In addition, the anti-GPIbα polyclonal antibodies generated in our anti-GPIbα model may block thrombin binding to platelet GPIbα (42, 43), which may increase free thrombin, further enhancing the conversion of fibrinogen to fibrin. To test this, we purified IgG from anti-GPIbα serum and preimmune GPIbα serum. We found that anti-GPIbα IgG significantly inhibited the binding of α-thrombin to murine WT platelets (Figure 8A). Thus, enhancement of PS exposure and thrombin generation as well as releasing thrombin from GPIbα may synergistically contribute to fibrin formation.

Using a perfusion chamber to investigate fibrin formation in vitro, we found that anti-GPIbα antiserum significantly accelerated fibrin network formation on platelets, compared with preimmune serum (Figure 8, B and C). In contrast, anti-β3 antiserum showed the trend of delaying fibrin formation (data not shown). In an intravital microscopy thrombosis model (44, 45), anti-GPIbα antiserum significantly accelerated thrombus formation (Figure 9, A and B), although anti-GPIbα antibodies inhibited the early stage of platelet–vessel wall interaction, which is mainly mediated by the engagement of GPIbα and von Willebrand factor (46); half of the BALB/c mice treated with anti-GPIbα serum, but none of those treated with preimmune serum, developed occlusive thrombi (5 of 10 versus 0 of 10, P < 0.01), despite thrombocytopenia in the anti-GPIbα antiserum–injected mice.

IVIG and anti-FcRn prevented miscarriage in anti-GPIbα–mediated FNIT. miscarriage is a devastating consequence, and thus, antenatal management is required. In previous work with the β3–/– model of FNIT, we found that maternal administration of IVIG ameliorated FNIT (34, 35), consistent with reported clinical data (1, 8), although the clinical data from different groups vary (1, 8, 47). Here, we investigated whether mice with anti-GPIbα–mediated FNIT similarly responded to IVIG treatment. This is intriguing, since we previously reported that in a murine model, IVIG did not effectively ameliorate anti-GPIbα–mediated ITP (48), which seems to be true in human ITP patients (49). Interestingly, we found that maternal administration of IVIG during pregnancy completely prevented miscarriage (0 of 5 versus 25 of 30, P < 0.0005) (Figure 10A), increased neonatal platelet count (265.3 ± 12.1 × 10^9/l versus 209.8 ± 13.9 × 10^9/l, P < 0.05), and increased the number of pups per litter (6.4 ± 1.2 versus 0.4 ± 0.2, P < 0.0001) in the anti-GPIbα–mediated FNIT (Figure 10, B and C).

FcRn plays an important role in extending IgG half-life in the circulation and mediating the transplacental transport of IgG from the mother to the fetus (50–52). We recently demonstrated that blockade of FcRn is an important mechanism of IVIG action and that anti-FcRn may be a potential new therapy for FNIT (35). However,
whether anti-FcRn treatment is able to prevent miscarriage has not been studied. This is particularly important, since it is completely unknown whether anti-FcRn will increase the local inflammatory response after it targets trophoblasts or endothelial cells in the placenta, which may enhance blood coagulation and exacerbate fibrin deposition. To test this, we used an anti-FcRn mAb to treat the immunized GPIbα−/− mice during pregnancy. Miscarriage was prevented (0 of 4 versus 25 of 30, P < 0.0005) (Figure 10A), and both neonatal platelet count (261.2 ± 18.4 × 10^9/l versus 209.8 ± 13.9 × 10^9/l, P < 0.05) and the number of pups per litter (4.5 ± 0.3 versus 0.4 ± 0.2, P < 0.0001) increased (Figure 10, B and C). Thus, in these animal models, we demonstrated that IVIG and anti-FcRn are both effective therapies for treating anti-GPIbα–mediated FNIT and preventing the prevalent fetal mortality observed in this devastating disease.

Discussion

The severity and prevalence of anti-GPIbα–mediated FNIT have not been adequately studied. In the current study, we established two new murine models of FNIT using syngeneic GPIbα−/− and β3−/− mice. Surprisingly, we found that with anti-GPIbα–mediated FNIT, most fetuses died and more than 83% of pregnant GPIbα−/− mothers did not deliver any pups. This miscarriage rate was far more severe than with anti-β3–mediated FNIT and revealed non-classical FNIT (i.e., no bleeding manifestations). Anti-fibrin and TUNEL staining demonstrated that fibrin deposition and apoptosis/necrosis were markedly enhanced in the placenta from immunized GPIbα−/− FNIT mice, compared with immunized β3−/− FNIT mice. We also observed that anti-GPlbα antibodies induced antigen-positive platelet activation, enhanced fibrin formation in vitro, and accelerated thrombus formation in vivo. Our data suggest that frequent miscarriage caused by anti-GPlbα antibodies may mask the severity and the reported incidence of this life-threatening disease. Importantly, we demonstrated that the devastating fetal loss can be efficiently prevented by IVIG and anti-FcRn therapies.

Anti-GPlbα–mediated FNIT has not been the subject of intensive study, since the reported incidence is rare. There is no report that the maternal immune response to fetal GPIbα may cause miscarriage in FNIT, although a genetic study suggested that the HPA-2 polymorphism (on GPIbα), but not the HPA-1 polymorphism (on β3 integrin), is associated with patients with recurrent spontaneous abortion (53). There are also case reports suggesting that anti-GPlbα complex antibodies were present in patients with habitual abortion (54). Anti-GPlbα antibodies in pregnant women with GPIb-IX deficiency are also associated with a few cases of intrauterine death (55–57). These observations in humans are consistent with our findings in the murine FNIT models in that miscarriages may occur in patients, accounting for the paucity of reported cases of anti-GPlbα–mediated FNIT.

Other clinical indices and symptoms of human FNIT are also well reproduced in our murine models (34, 35). The GPIbα−/− female mice immunized with WT platelets in this model may mimic the human mothers who have had previous pregnancies in which the maternal immune system is exposed to fetal/neonatal antigen-positive platelets due to uterine injury during delivery (58, 59) and...
thus may generate anti-GPIbα antibodies. Maternal immunization against fetal platelet GPIbα may also occur during human pregnancy when fetal GPIbα antigen–positive platelets leak into the maternal circulation via fetomaternal hemorrhage (58, 59). Since (a) β3 integrin (GPIIIa) is expressed on human placental syncytiotrophoblast microvilli that are in direct contact with maternal blood and may induce the immune response against HPA-1a on β3 integrin in primiparous mothers (60, 61) and (b) GPIbα has been reported to be expressed on human endothelial cells under certain conditions (62–64), we cannot exclude the possibility that GPIbα, like β3 integrin, may also be expressed on placental syncytiotrophoblasts and endothelial cells, thus contributing to maternal anti-GPIbα antibody generation during pregnancy.

Miscarriage or pregnancy loss affects approximately 10%–30% of human pregnancies (65, 66). In the current study, we observed enhanced placental fibrin deposition in immunized GPIbα–/− mice, compared with immunized β3/− mice. Our in vitro data demonstrated that anti-GPIbα, but not anti-β3, antiserum specifically induced the activation and aggregation of antigen-positive platelets (Figure 6 and Figure 7, A–C). We also observed that some anti-GPIbα serum, but not anti-β3 serum, from ITP patients induced platelet calcium mobilization, another indicator of platelet activation (C.M. Spring, G. Zhu, and H. Ni, unpublished observations). Furthermore, we demonstrated that anti-GPIbα antiserum enhanced PS expression (Figure 7D) and fibrin formation on platelets in vitro (Figure 8, B and C) and facilitated thrombus formation in vivo (Figure 9, A and B). The association between anti-GPIbα antibodies and enhanced thrombosis is in accordance with a human study in which anti-GPIbα antibodies were detected in approximately 18% of lupus anticoagulant patients and the majority developed thromboembolic events, such as abortion and arterial and venous thrombosis (67). The possibility that thrombus formation in the placenta likely accounts for miscarriage is further supported by our findings that anti-thrombotic treatment of immunized pregnant GPIbα–/− mice with low-dose warfarin (5 μg/d), which is able to traverse the placenta, alleviated miscarriage in this FNTI model.

In addition to platelet activation and PS expression induced by the anti-GPIbα antibodies, the interaction between α-thrombin and platelet GPIbα (42, 43) was also inhibited by these antibodies (Figure 8A). A similar effect may also occur in human FNTI patients with anti-GPIbα antibodies. Blocking thrombin binding to GPIbα would lead to increased free circulating thrombin, allow for increased cleavage of fibrinogen into fibrin, and activate platelets via protease-activated receptors 1 and 4 (PAR-1 and PAR-4), which may further enhance thrombosis. We indeed observed that platelets from these anti-GPIbα–injected mice exhibited significantly higher P-selectin expression and JON/A binding and tended to form microaggregates (Supplemental Figure 5, A–C). Thus, anti-GPIbα antibodies may activate platelets and enhance platelet aggregation, as well as generating procoagulant surfaces or more circulating thrombin, which would enhance thrombus formation in affected placentas. The observed extensive thrombi and fibrin deposition in the placenta may not only block fetal blood supply, as indicated by FITC-dextran placental perfusion (Figure 5), but also directly cause the death of trophoblast cells (68), thus synergistically contributing to the higher miscarriage rate in anti-GPIbα–mediated FNTI.

It is notable that our observations that anti-GPIbα antibodies activate platelets, enhance platelet aggregation, and generate a procoagulant status may be important in designing anti-thrombotic therapy. Anti-GPIbα–based therapies are presently under preclinical investigations (ref. 69 and G. Zhu and H. Ni, unpublished observations). Since our findings suggest that intact anti-GPIbα antibodies might aggravate the severity of thrombosis, manufacturers of this potential therapy may need to take this into consideration and avoid this adverse effect by using monovalent Fab or Fv antibody fragments. In addition, since thromboembolic events occur in approximately 5% of ITP patients (70–72), it may be worthwhile to address whether these patients have anti-GPIbα antibodies.

FNTI is a devastating disease, and effective therapeutic intervention is currently limited and unstandardized (73). We previously demonstrated that maternal IVIG infusion ameliorated neonatal thrombocytopenia in the anti-β3 FNTI model (34). However, we also reported that IVIG was not as effective in ameliorating thrombocytopenia caused by anti-GPIbα antibodies as compared with anti-β3 antibodies in a murine ITP model (48), which was supported by subsequent studies in human ITP patients (49). It was therefore of interest to determine whether IVIG was efficacious in ameliorating thrombocytopenia in the anti-GPIbα–mediated
FNI,T model. Here, we observed that maternal IVIG treatment significantly prevented miscarriage and ameliorated neonatal thrombocytopenia; anti-FcRn treatment was similarly effective, but the effective dose of anti-FcRn was at least 100-fold lower. The mechanisms of IVIG and anti-FcRn therapy are still not fully understood, but blocking transplacental transport of anti-GPIb antibodies may play an important role in the amelioration of FNI,T.

It is currently unknown whether the maternal immune response against fetal GPIbα is indeed a significant cause of miscarriage in humans. Screening the polymorphisms of GPIbα (e.g., HPA-2) in women experiencing miscarriage and habitual abortion and detecting anti-GPIbα antibodies during their pregnancies may provide important information to address this critical question. If anti-GPIbα antibodies are indeed a risk factor for miscarriage in humans, identifying these women and treating them during early pregnancy with IVIG or anti-FcRn therapies may lead to the prevention of this nonclassical but devastating FNI,T.

In summary, we found that the maternal immune response to fetal GPIbα caused an extremely high rate of miscarriage, which is different from the current definition of FNI,T: a disease that is primarily characterized by bleeding symptoms in neonates. We further demonstrated, for the first time to our knowledge, that anti-GPIbα antibodies cause platelet activation and generate a procoagulant status, which led to prominent placental fibrin deposition and impaired placental function. The frequent miscarriage in the anti-GPIbα FNI,T model may potentially explain why there are so few reported FNI,T cases mediated by anti-GPIbα antibodies. Finally, we demonstrated that the devastating fetal loss can be efficiently prevented by maternal treatment with IVIG and anti-FcRn therapy.

Methods

Mice. GPIbα−/− mice were described previously (74). β3−/− mice (34, 75) were provided by Richard O. Hynes (Massachusetts Institute of Technology, Boston, Massachusetts, USA). Both GPIbα−/− and β3−/− mice were backcrossed to a BALB/c background 10 times and bred to generate syngeneic genome-deficient mice. Experiments were performed when mice were 6–10 weeks of age. BALB/c WT mice were purchased from Charles River. All mice were housed in the St. Michael’s Hospital Research Vivarium.

Reagents. FITC-conjugated goat anti-mouse IgG was purchased from Sigma-Aldrich. IVIG (Gamunex) was obtained from Bayer Inc./Canadian Blood Services. The 1G3 hybridoma cell line producing anti-FcRn mAb (IgG1) was purchased from ATCC.

Genotyping. We designed the primers for GPIbα genotyping: (a) forward primer (5′-AGTCTGGGAGGTGTGGAAGC-3′) binds 5′ of the pgk-neo cassette; (b) reverse primer 1 (5′-CACGAGACTAGTGAGACGTG-3′) was neo (mutant) specific; (c) reverse primer 2 (GGTATCAGATCTCC CAGGC) was WT specific. PCR products were 288 bp (mutant) and 680 bp (WT). The primers and methods for β3 integrin genotyping were described previously (75).

Induction and treatment of fetal and neonatal immune thrombocytopenia. Gel-filtered WT platelets were prepared as previously described (45). GPIbα−/− and β3−/− females were immunized twice with 109 gel-filtered WT platelets (containing GPIbα and β3 integrin antigens) at weekly intervals (34, 35) and then bred with WT males. Naive GPIbα−/− and β3−/− females bred with WT males were used as controls. The antibody titration of the antigen by flow cytometry was performed as we previously described (34). Body weight of females was monitored during pregnancy; a decrease in body weight (i.e., ≥1 g) was indicative of miscarriage. For IVIG or anti-FcRn mAb treatment, IVIG (1 g/kg) or anti-FcRn (10 mg/kg) was intraperitoneally injected into the immunized knockout females every 5 days, from 1.5 d.p.c. to delivery.

Platelet P-selectin expression, JON/A, and fibrinogen binding. Gel-filtered WT platelets (2 × 107/ml) were incubated with polyclonal anti-β3, anti-GPIbα, or preimmune serum (1:10 serum dilution for P-selectin expression and fibrinogen binding: 1:50 dilution for JON/A binding). After 1 hour incubation, the samples were washed with PBS by centrifugation at 600 g for 10 minutes. FITC-labeled anti-P-selectin antibody (BD Biosciences), PE-conjugated JON/A antibody (Emfret Analytics), or Alexa Fluor 488–conjugated fibrinogen (Invitrogen) was then added to the platelets and incubated for 1 hour, followed by detection on a FACSCalibur II flow cytometer (BD Biosciences).

Anti-fibrin and TUNEL staining. The placenta samples were collected from 14.5-d.p.c. knockout females and fixed in formalin. The 4-μm tissue slides were deparaffinized and then dehydrated. After antigen unmasking, the slides were blocked with normal goat serum. Fibrin was stained with a mouse anti-human fibrin antibody (T2G1, Accurate Biochemicals), which cross-reacts with mouse fibrin, but not fibrinogen, coupled to Alexa Fluor 488 as per the manufacturer’s protocol (Invitrogen). The nuclei were counterstained with DAPI (Sigma-Aldrich). TUNEL staining of the placenta was...
performed according to the manufacturer’s instructions (Roche Applied Sciences). The fluorescence pictures were randomly taken under a Nikon E800 microscope. The fluorescence-positive areas were analyzed using ImageJ software (http://rsweb.nih.gov/ij/).

Placental perfusion studies. FITC-dextran (MW 2,000,000; Sigma-Aldrich) was dissolved in distilled water and infused into 15.5-d-p.c. naive or immunized GPIbα−/− mice (80 mg/kg mice) via the tail vein, and 15 minutes later, the placentas and control organs (i.e., liver, kidney, and spleen) were isolated and snap-frozen (38). Serial frozen sections were assayed and photographed under a Nikon E800 microscope. The fluorescence-positive area at the fetal side of the placenta was analyzed using ImageJ software.

Platelet aggregation assays. Platelet aggregation was performed as we previously described (45). 25 μl anti-f3, anti-GPIbα, or pre-immune serum was incubated with 250 μl WT or GPIbα−/− PRP (3 × 10^9 platelets/ml) for 10 minutes prior to induction of aggregation with ADP. To test the ability of the anti-serum to induce platelet aggregation, serum alone was added to PRP after 2 minutes of reaction. These platelets were then taken from the aggregometer and stained with FITC-labeled Annexin V (BD Biosciences), which binds phosphatidylserine on platelets (41). For anti-GPIbα serum to induce platelet aggregation, serum alone was added to PRP after 2 minutes of reaction. The experimental procedures on mice were approved by the Animal Care Committee of St. Michael’s Hospital, Toronto, Ontario, Canada.

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