Fabry Disease in Mice Is Associated With Age-Dependent Susceptibility to Vascular Thrombosis

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Abstract. Fabry disease is an X-linked lysosomal storage disorder due to deficiency of α-galactosidase A (GLA) activity that results in the widespread accumulation of neutral glycosphingolipids. Renal failure, neuropathy, premature myocardial infarction, and stroke occur in patients with this condition primarily due to deposition of glycosphingolipids in vascular endothelial cells. The clinical consequences of Fabry disease suggest that vascular thrombosis may play a prominent role in the pathogenesis of this disease; however, the vasculopathy associated with Fabry disease has not been extensively studied. To determine if mice genetically deficient in *Gla* are susceptible to vascular thrombosis, a photochemical carotid injury model was used to induce occlusive thrombosis. In this model, *Gla−/0* mice displayed a progressive age-dependent shortening of the time to occlusive thrombosis after vascular injury that correlated with progressive accumulation of globotriasylceramide (Gb3) in the arterial wall. Bone marrow transplantation from *Gla−/0* to *Gla+/0* mice and from *Gla+/0* to *Gla−/0* mice did not change the thrombotic phenotype of the host. These studies reveal a potent vascular prothrombotic phenotype in *Gla*-deficient mice and suggest that antithrombotic therapies as well as therapies designed to reduce the vascular accumulation of Gb3 may have beneficial effects on thrombotic complications in patients with Fabry disease.

Fabry disease is an X-linked recessive disorder that results from deficiency of α-galactosidase A (GLA) enzymatic activity (1). This enzyme defect leads to widespread accumulation of neutral glycosphingolipids with α-galactosyl linkages consisting primarily of globotriaosylceramide (Gb3) (2). Clinical manifestations of Fabry disease include renal failure, painful neuropathies, angiokeratoma, myocardial infarction, and stroke, leading to premature mortality (2). Myocardial infarction and stroke are due to acute arterial thrombosis (3); therefore, the vascular accumulation of Gb3 may predispose affected individuals to these thrombotic complications. However, the thrombophilia associated with Fabry disease has not been extensively studied. Recently, a murine model of Fabry disease has been generated by targeted disruption of the *Gla* gene (4). These mice accumulate lipids in multiple organs similar to that observed in humans with Fabry disease. Thus, they provide a useful model to explore the pathogenesis of Fabry disease. A carotid injury model was used to elicit occlusive thrombosis in *Gla*-deficient mice and wild-type controls to determine whether *Gla*-deficient mice are more susceptible to arterial thrombosis.

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Materials and Methods

Mice

Gla-deficient mice (4) used in these experiments were bred from mice provided by Drs. Ashok Kulkarni and Roscoe Brady (National Institutes of Health, Bethesda, MD). These mice were back-crossed at least four generations to the C57BL6/J strain. Control C57BL6/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All mice were maintained on normal chow in specific pathogen-free facilities. All animal care and experimental procedures complied with the Principals of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

Carotid Arterial Thrombosis Protocol

Arterial thrombosis was performed using a carotid photochemical injury model as described previously (5). Briefly, the photochemical, rose bengal, was injected into the mouse tail vein while the right carotid artery was exposed to a green laser light. The rose bengal is activated by the green light, resulting in the liberation of toxic reactive oxygen species such as superoxide anions (6). This type of injury is clinically relevant, as endogenous superoxide anions appear to play an important role in the progression of vascular disease (7). Endothelial damage ensues at the site of the photochemical injury followed by the formation of a platelet/fibrin-rich occlusive thrombus (Figure 1). Flow in the vessel was monitored continuously, using a Doppler flow probe (model 0.5 VB; Transonics Systems, Ithaca, NY) connected to a flowmeter (Transonics model T106), throughout the protocol. The endpoint is occlusive thrombosis, defined as zero flow for at least 1 min.

Bone Marrow Transplantation

Bone marrow transplantation experiments were performed similarly to previously described methods (8). Briefly, 78-d-old *Gla−/0*

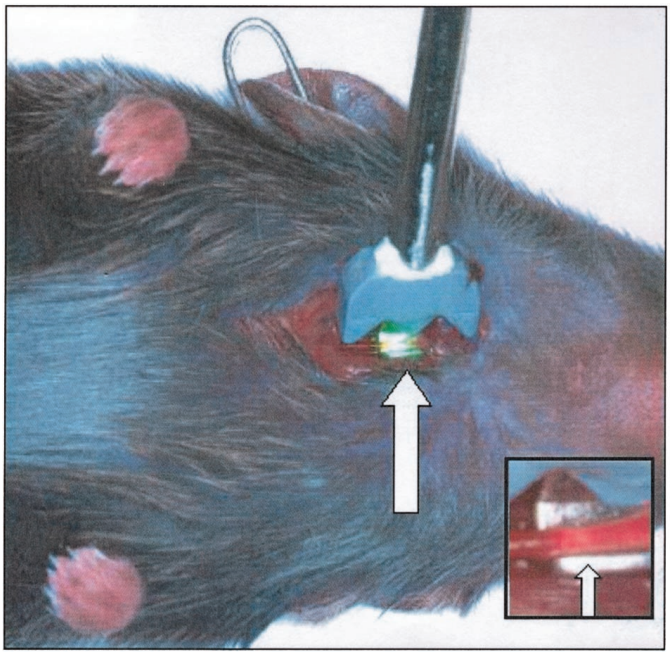


Figure 1. Carotid photochemical injury model. Doppler flow probe monitoring blood flow in right carotid artery, which is being exposed to green light (large arrow). Right lower inset shows gross appearance of clot within carotid artery (small arrow), at which time no flow is apparent by Doppler.

and *Gla+/0* mice were irradiated (2 × 650 rad) and then injected through the tail vein with 4×10^6 bone marrow cells harvested from the femurs of either *Gla−/0* or *Gla+/0* mice. Six weeks after transplantation, blood was drawn from the retro-orbital sinus, and platelet counts were determined from platelet-rich plasma (Coulter Counter Z2; Beckman Coulter, Miami, FL). Engraftment was confirmed by PCR analysis of whole blood using a 3-primer system designed to recognize the wild-type and mutant alleles (5'-3' wt –TCCACAG-CAAAGGATTGAAG and TTCTCCAAGGATACTACACTGTCA; 5'-3' mutant - TCCATCTGCACGAGACTAGT). The arterial thrombosis protocol was performed 3 to 5 d after retro-orbital bleeding. Six weeks after transplantation, the arterial thrombosis protocol was performed.

Histology

For analysis of vascular Gb3, the arterial vasculature was perfused with phosphate-buffered saline, and mid-carotid sections were excised and frozen in liquid nitrogen. The sections were stained for Gb3 with recombinant verotoxin B subunit provided by Diane Copeland at the Genzyme Corporation (Cambridge, MA). Frozen carotid artery sections were incubated with 1% normal goat serum for 30 min, rinsed with phosphate-buffered saline, and then incubated with biotinylated verotoxin B subunit (1 μg/ml diluted in 1% normal goat serum) for 60 min. After rinsing, the verotoxin binding was detected using Histostain SP for AEC kit per manufacturer's instructions (Zymed). Injured carotid artery sites were stained with Masson trichrome and Carstairs stains. Sections were viewed with a Nikon Eclipse E400 microscope.

Statistical Analyses

The statistical significance of differences in time to occlusion was determined using *t* test. $P < 0.05$ was considered significant.

Results

Effect of Age on Vascular Thrombosis in Gla-Deficient Mice

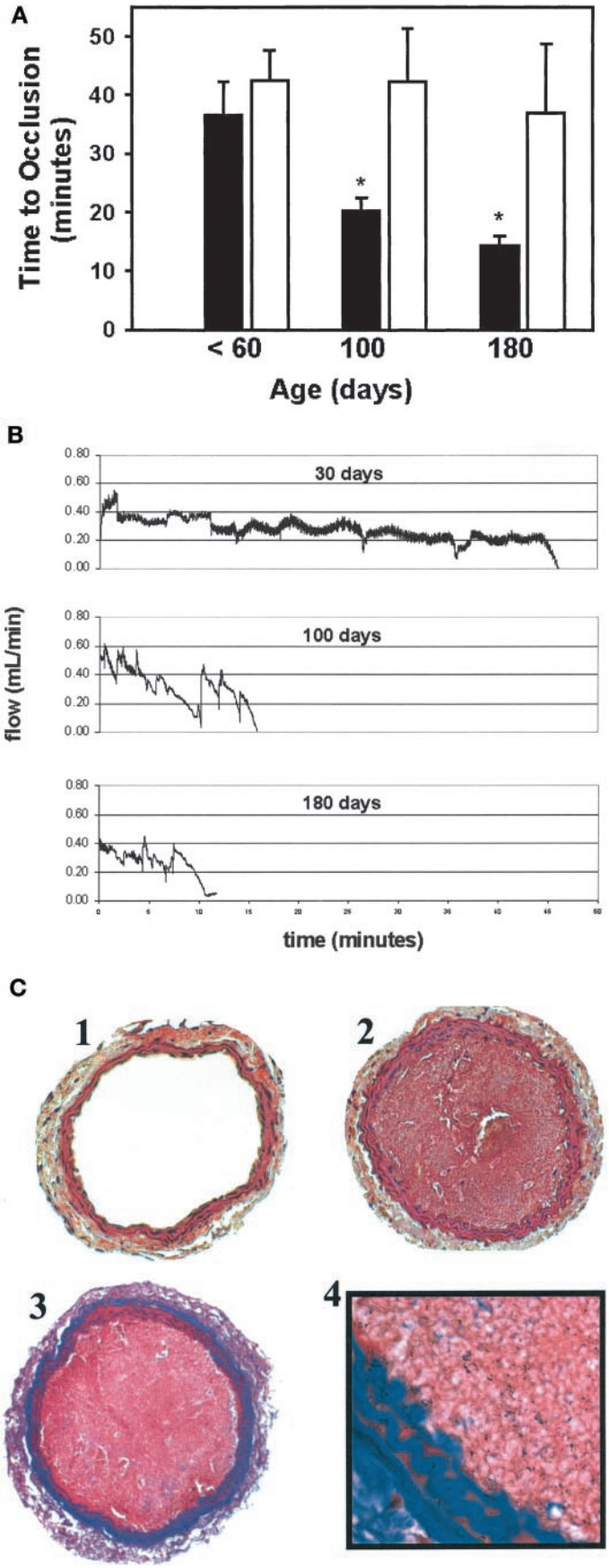
To determine the effects of *Gla* deficiency on vascular thrombosis, *Gla−/0* and *Gla+/0* mice at various ages were studied in the photochemical vascular thrombosis model. *Gla+/0* mice of different ages formed occlusive thrombosis at similar times (Figure 2A). In contrast, the time to occlusive thrombosis in *Gla−/0* mice became progressively shorter with age. By 100 d of age, the time to occlusive thrombosis in *Gla−/0* mice was significantly shorter than *Gla−/0* mice less than 60 d of age ($P < 0.04$) or 100-d-old *Gla+/0* mice (Figure 2A). This trend became more apparent with increasing age. Representative flow tracings are shown in Figure 2B. Figure 2C shows typical histology of an occlusive thrombus that corresponds to zero flow. At this stage of injury the wall of the carotid artery looks unremarkable by light microscopy. No differences in baseline carotid blood flow between 100-d-old wild-type and 100-d-old *Gla−/0* mice were observed (0.46 ± 0.1 and 0.43 ± 0.05 ml/min, respectively; P = NS). Thus, *Gla−/0* mice exhibited a progressive increase in vascular thrombotic tendency with age that was not observed in wild-type mice.

Vascular Gb3 Accumulation with Age in Gla−/0 Mice

Recent studies have documented progressive accumulation of Gb3 in various organs with age in *Gla−/0* mice (9). In this study, carotid sections were stained with verotoxin to examine the relationship between accumulation of Gb3 in carotid arteries and enhanced carotid thrombosis after injury. Gb3 staining of the carotid arterial wall was only faintly evident in 30-d-old *Gla−/0* mice, but the distribution and intensity increased markedly with age (Figure 3). Thus, the vascular accumulation of Gb3 correlated well with the increase in thrombotic tendency observed with age in the *Gla−/0* mice. No Gb3 staining was evident within the acute thrombus.

Effect of Bone Marrow Transplantation between Gla−/0 and Gla+/0 Mice on Vascular Thrombosis

Bone marrow transplantation was performed from *Gla−/0* to *Gla+/0* mice and from *Gla+/0* to *Gla−/0* mice to determine whether the source of the prothrombotic stimulus was due to a bone marrow–derived element, such as platelets. This is a potentially useful method to determine the *in vivo* consequences of altered platelet function (8). There were no differences in platelet counts between *Gla−/0* and *Gla+/0* mice before or after the transplantation. PCR analysis of whole blood from *Gla−/0* mice that received *Gla+/0* marrow revealed the presence of only the *Gla+* allele, whereas blood from *Gla+/0* mice that received *Gla−/0* marrow revealed only the *Gla−* allele, consistent with complete engraftment. As shown in Figure 4, the source of the bone marrow did not affect the thrombotic phenotype of the host. Although the times to occlusion were prolonged in the transplanted mice compared with non-irradiated mice, the time to thrombosis remained significantly shorter in the *Gla−/0* mice (45 ± 6 min; $n = 15$)



compared with the *Gla+/0* mice (67 ± 9 min; $n = 8$; $P < 0.02$).

Discussion

Premature vascular thrombotic events such as stroke and myocardial infarction are devastating complications of Fabry disease (2). The disease is relatively rare (2); therefore, the true incidence of these thrombotic complications in Fabry patients is unknown. However, premature obstructive vascular disease due to coronary glycolipid deposition and premature myocardial infarction have been reported in Fabry patients (10). Similarly, cerebrovascular manifestations due to ischemic stroke have been reported in up to 24% of Fabry patients, with the majority of these events occurring before age 40 (11). Therefore, patients with Fabry disease are predisposed to premature arterial thrombotic complications. The underlying vascular change predisposing to thrombotic events is thought to be vascular accumulation of Gb3, which also plays a role in the other complications of Fabry disease (2). Recent studies focusing on vascular changes in Fabry patients have demonstrated evidence of endothelial and leukocyte activation (12) and paradoxically enhanced endothelium-dependent vasodilation (13,14). Gb3 also appears to be a critical mediator of the hemolytic uremic syndrome, which is characterized by widespread thrombosis (15). Verotoxin, which binds to Gb3 to initiate the hemolytic uremic syndrome, has been shown to render endothelial cells highly thrombogenic in cell culture (16). These studies suggest that Gb3-ligand interactions may be involved in signaling pathways, leading to a thrombogenic vasculature.

The phenotype of the *Gla*-deficient mouse model differs from the human condition in that the mice do not appear to develop renal failure up to 80 wk of age (9). However, from 10 to 20 wk of age, Gb3 has been shown to increase in the kidney and other tissues of these mice. Furthermore, lamellar bodies were noted within proximal and distal tubular cells, parietal and visceral glomerular epithelial cells, and peritubular capillary endothelial cells. Oshima *et al.* suggested that reduced

Figure 2. Effect of age on thrombosis in Fabry mice. (A) With increasing age, the time to occlusive carotid thrombosis progressively shortens in Fabry mice (filled bar); $n = 12$, 8, and 6 for ages <60, 100, and 180 d, respectively (*$P < 0.05$ compared with age-matched wild-type mice), whereas occlusion times in wild-type mice (open bar) are not affected by age; $n = 8$, 4, and 4. respectively. (B) Representative compressed carotid blood flow tracings from Fabry mice of various ages. (C) Histology of carotid artery. Sections harvested from perfusion fixed mouse carotid arteries 50 min after the onset of photochemical injury. Panel 1 is a Masson trichrome stain demonstrating the widely patent uninjured left carotid artery (×200). Panel 2 is a Masson trichrome stain demonstrating occlusive thrombosis in the injured right carotid artery (×200). Panels 3 and 4 are low-power (×200) and high-power (×1000) views of an injured section stained with Carstairs stain (arterial wall collagen and smooth muscles cells are blue and red, respectively; intraluminal thrombus stains red [fibrin] and faint blue [platelets]). No differences were noted in the appearance of the cross-sections between the groups.

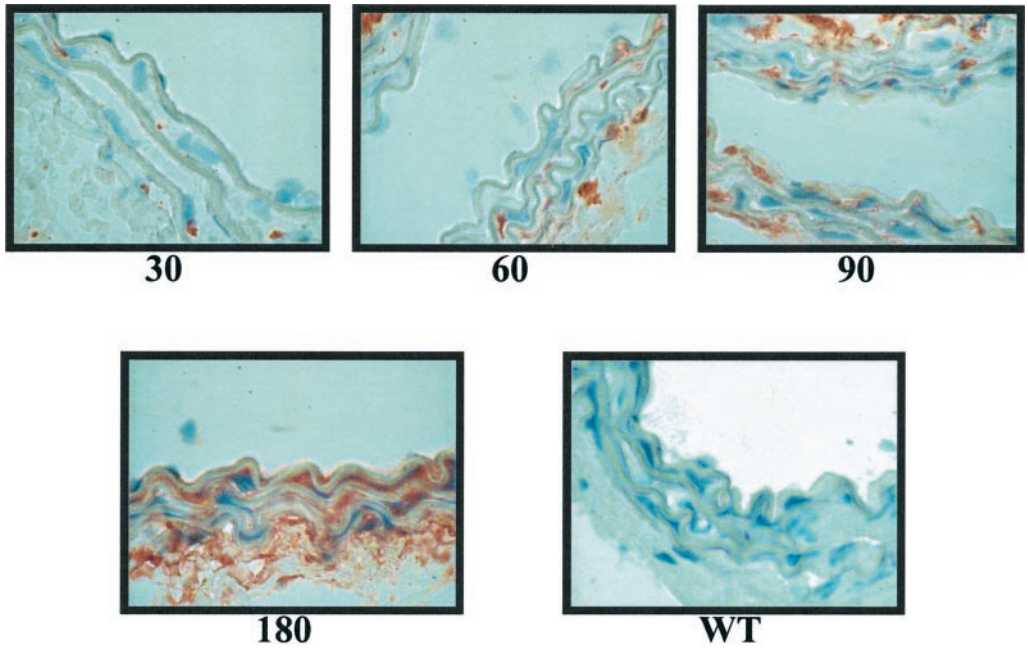


Figure 3. Effect of age on carotid Gb3 accumulation in Fabry mice. Frozen cross-sections of carotid arteries from Fabry mice of various ages stained with biotinylated verotoxin. The sections are from *Gla−/0* mice at 30, 60, 100, and 180 d of age. The wild-type (WT) section is representative of a 180-d-old mouse.

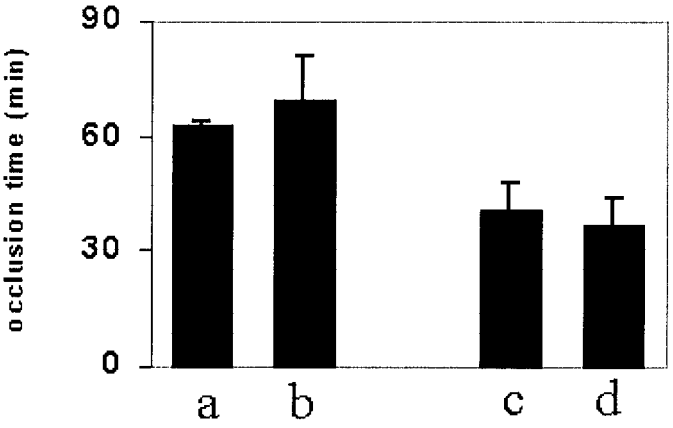


Figure 4. Effect of bone marrow transplantation on thrombosis. No significant differences in the time to occlusive thrombosis were noted between control *Gla+/0* mice receiving *Gla+/0* marrow (a; ($n = 2$) and *Gla+/0* mice receiving *Gla−/0* marrow (b; $n = 6$). Similarly, no differences were noted between control *Gla−/0* mice receiving *Gla−/0* marrow (c; $n = 7$) and *Gla−/0* mice receiving *Gla+/0* marrow (d; $n = 8$).

endothelial lesions in the Fabry mice compared with humans may affect expression of many of the phenotypic manifestations seen in patients with Fabry disease. Thus, although *Gla*-deficient mice do not completely mimic the disease manifestations of humans with this condition, they serve a useful tool for studying derangements of glycolipid metabolism. The findings in our study are particularly useful as they represent the first overt vascular phenotype in these mice.

In the current study, we demonstrate a potent thrombotic phenotype in *Gla*-deficient mice that becomes more severe with age. This phenotype correlates with progressive accumulation of Gb3 within the arterial wall and is not associated with differences in baseline carotid blood flow. The age dependence of this phenotype suggests that accumulation of Gb3 within the vascular wall may contribute to the enhanced thrombogenicity. Bone marrow transplantation from *Gla−/0* to *Gla+/0* mice and from *Gla+/0* to *Gla−/0* mice did not affect the host phenotype supporting a prominent contribution by the vascular wall toward the thrombotic phenotype. Further studies are required to identify the precise mechanisms responsible for this phenotype. However, these findings provide a useful model for the vasculopathy associated with Fabry disease and a preclinical endpoint for measuring the effectiveness of various therapeutic interventions (9,17–21) targeting Gb3 accumulation and thrombosis.

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