Connexin 37 Limits Thrombus Propensity by Downregulating Platelet Reactivity
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Background—Formation of platelet plug initiates hemostasis after vascular injury and triggers thrombosis in ischemic disease. However, the mechanisms leading to the formation of a stable thrombus are poorly understood. Connexins comprise a family of proteins that form gap junctions enabling intercellular coordination of tissue activity, a process termed gap junctional intercellular communication.

Methods and Results—In the present study, we show that megakaryocytes and platelets express connexin 37 (Cx37). Deletion of the Cx37 gene in mice shortens bleeding time and increases thrombus propensity. Aggregation is increased in murine Cx37−/− platelets or in murine Cx37+/− and human platelets treated with gap junction blockers. Intracellular microinjection of neurobiotin, a Cx37-permeant tracer, revealed gap junctional intercellular communication in platelet aggregates, which was impaired in Cx37−/− platelets and in human platelets exposed to gap junction blockers. Finally, healthy subjects homozygous for Cx37–1019C, a prognostic marker for atherosclerosis, display increased platelet responses compared with subjects carrying the Cx37–1019T allele. Expression of these polymorphic channels in communication-deficient cells revealed a decreased permeability of Cx37–1019C channels for neurobiotin.

Conclusions—We propose that the establishment of gap junctional communication between Cx37-expressing platelets provides a mechanism to limit thrombus propensity. To our knowledge, these data provide the first evidence incriminating gap junctions in the pathogenesis of thrombosis. (Circulation. 2011;124:930-939.)

Key Words: connexin 37  ■  gap junction  ■  platelets  ■  thrombosis

Formation of platelet plug initiates hemostasis at sites of vascular injury and triggers pathological thrombosis in ischemic tissue disease. Recruitment of additional platelets and the development of contacts between platelets are necessary for the establishment of a stable thrombus. Mechanisms regulating thrombus propensity are poorly understood.

Editorial see p 873
Clinical Perspective on p 939

Connexins comprise a large family of proteins that form gap junction channels enabling intercellular coordination of tissue activity. The gating, permeability, and regulatory properties of a gap junction channel vary depending on its constituting connexin isoform.1 There are 21 connexin isoforms in the human genome, and mutations in some of these genes have been implicated in human hereditary diseases such as X-linked Charcot-Marie-Tooth disease, sensorineural hearing loss, oculodentodigital dysplasia, cataracts, and idiopathic atrial fibrillation.2 The present study focuses on connexin 37 (Cx37), an isoform expressed in endothelial cells (ECs), vascular smooth muscle cells, monocytes, and macrophages.3 Studies on genetically modified mouse models showed the involvement of Cx37 in the pathogenesis of atherosclerosis.4 5 Studies in humans indicate that a polymorphism at position 1019 of the GJA4 gene, encoding for human Cx37, may be a prognostic marker for atherosclerosis (reviewed in Reference 3). This C1019T polymorphism codes for a proline-to-serine substitution (P191S) in the C-terminal domain of the protein. Two separate studies...
showed a correlation between the presence of the Cx37–319S isoform and an increased prevalence of myocardial infarction in the sampled population.6,7 The Cx37–319S genotype was also shown to predict survival after an acute coronary syndrome.8 In 3 additional studies, the alternative isoform (Cx37–319P) was associated with an increased prevalence of stenosis in the coronary9,10 or the carotid arteries.11 Studies at the cellular level recently showed that Cx37 expression inhibited monocyte adhesion to the endothelium; this effect was more pronounced in monocytes expressing Cx37–319P than in those expressing Cx37–319S.5

In this study, we demonstrate that megakaryocytes and platelets express Cx37 and that Cx37 plays a crucial role in the physiological and pathological signaling in platelets leading to hemostasis and thrombosis. We found that gap junctional intercellular communication (GJIC) between aggregating platelets limits thrombus propensity by downregulating platelet reactivity. In addition, we show increased platelet response in healthy subjects homozygous for Cx37–1019C compared with subjects carrying the Cx37–1019T allele. Our results suggest that Cx37 gap junction channels allow the propagation between platelets of antiaggregating signals, a mechanism that may be severely decreased in Cx37–319P platelets.

## Methods

### Mice

Cx37+/+ and Cx37−/− mice were progeny of the original colony, on a C57BL/6J background.5,12 Genotyping was performed as described.5,12 Housing and animal experiments were approved by the Swiss Federal Veterinary Office.

### Preparation of Murine and Human Washed Platelets

Blood was drawn from the inferior vena cava into acid-citrate-dextrose solution to obtain platelet-rich plasma (PRP), and washed platelets were prepared as described in the online-only Data Supplement. Washed human platelets were prepared as described.13

### Preparation of Platelet-Rich Plasma in 96 Healthy Subjects

Ninety-six unrelated healthy men aged 18 to 40 years were recruited.14 All subjects gave written informed consent, and the study protocol was approved by the ethics committee of University Hospitals of Geneva. PRP was prepared as described in the online-only Data Supplement.

### Thrombopoietin-Differentiated CD34+ Cells

Cord blood cells were collected after informed consent was obtained. CD34+ cells were purified with a CD34 microbeads kit (Miltenyi Biotec) as described by the manufacturer and cultured with polyethylene glycol/recombinant human megakaryocyte growth and development factor. After 12 days of culture, cells derived from these cultures were used for investigations.

### Isolation of Murine Bone Marrow Cells

Single-cell suspensions were obtained from bone marrow flushed from femur and tibia. Cells adherent to gelatin-coated coverslips were fixed as described below.

### Western Blotting

Western blotting was performed as described.15 Blotted proteins were then incubated overnight at 4°C with rabbit polyclonal (2 μg/mL; Alpha Diagnostic International) Cx37 antibodies or anti-β-actin (1:1000; Sigma), which was used as a loading control. Specificity of the Cx37 labeling was confirmed by preabsorption of the polyclonal antibodies for 15 minutes at room temperature with 20 μg/mL cognate immunogenic peptide (Alpha Diagnostic International).

### Immunofluorescence

Cells were fixed in ice-cold methanol and immunolabeled with rabbit Cx37 antibodies. One antibody, used for the experiments shown in Figure 1C and 1D and Figure II in the online-only Data Supplement, was purchased from Alpha Diagnostic International. A second antibody together with its preimmune serum was kindly provided to us by Dr Daniel Gros (IBDM-LGPD, Marseille, France) and was used in the experiment in Figure I in the online-only Data Supplement.
Platelet Adhesion
Adhesion to immobilized fibrinogen or collagen was quantified as described.16

Bleeding Time
A stopwatch was started immediately on tail transection to determine time to cessation of bleeding.

Thrombosis Models
Intravital microscopy was performed on mouse mesenteric arteries. Platelets were labeled directly in vivo by the injection of rhodamine 6G. After selection of the studied field, vessel wall injury was generated by a filter paper saturated with a 20% FeCl3 solution. Time to vessel wall occlusion was measured, as determined by cessation of the blood cell flow. Thromboembolism was performed as described.16

Vasomotor Responses
Rings of descending thoracic aortas with intact endothelium were suspended in a multi-channel myograph. They were progressively stretched to a passive tension of 5 nN that gives the optimal length-tension relationship. Contractility with KCl and phenylephrine was measured. Then aortic rings were precontracted with phenylephrine to match the level of precontraction. Subsequent relaxations with acetylcholine or sodium nitroprusside were measured. Two aortic rings per mouse were used, and results were averaged.

Murine Platelet Aggregation
Platelet aggregation was performed as described.16 When indicated, Cx37+/−, washed platelets were preincubated with 200 μmol/L Cx37 mimetic peptides before stimulation (2 to 5 minutes). Platelet aggregation was measured turbidimetrically with the use of an optical Platelet Aggregation Profiler (model PAP-4, Bio/Data).

Human Platelet Aggregation
Washed Platelets
The platelet suspension was incubated with either vehicle or α-glycyrrhetinic acid (α-GA) before stimulation with the following agonists: arachidonic acid (AA), ADP, TRAP-6 peptide, or collagen.

Platelet-Rich Plasma
Platelet aggregation in PRP was performed with the use of the following agonists: AA, collagen, or ADP.

Fluorescein Isothiocyanate/Phalloidin Staining
Washed platelets were placed on coverslips coated with fibrinogen or collagen. Nonadherent platelets were washed away, and adherent cells were fixed and permeabilized in 0.1% Triton X-100 and incubated in fluorescein isothiocyanate/phalloidin for 15 minutes.

Evaluation of Gap Junctional Intercellular Communication
GJIC within platelet aggregates and in Cx37-expressing HeLa cells was evaluated by intracellular microinjection of neurobiotin. HeLa cells were transfected with a pIRE2-eGFP vector containing Cx37-J019C or Cx37-J019T cDNA.17 Neurobiotin was detected by incubating the fixed platelet aggregate or HeLa cells with streptavidin-rhodamine.

Statistical Analyses
Data are shown as mean±SEM. Standard t tests were used to calculate significance levels between groups. Results from intravital microscopy were compared with the Mann-Whitney test. Vasomotor dose responses were compared with a 2-way ANOVA for repeated measures. Aggregation data on human platelets are shown as median dose responses were compared with a 2-way ANOVA for repeated measures. Standard error of the mean. Statistical Analyses were performed with the STATA 7.0 software package (Stata Corp, College Station, TX) and Prism5 (Graphpad), and differences with P values <0.05 were considered significant.

Results
Cx37 Is Expressed by Platelets and Megakaryocytes
To test for the presence of connexins in platelets, we performed Western blots on total protein obtained from resting and activated human platelets. As shown in Figure 1A, both resting and activated human platelets showed a band at a molecular weight (≈37 kDa) similar to the one observed in mouse ECs, our positive control. This 37-kDa band disappeared on preincubation of the antibody with its immunogenic peptide, indicating the specificity of the reaction (Figure 1B). In contrast, we did not detect other vascular connexins (ie, Cx43 and Cx40) in human platelets (data not shown). Cx37 was also detected by immunofluorescence on the surface of isolated human (Figure I in the online-only Data Supplement) and murine (Figure 1C) adherent platelets. As expected, no staining was detected in human platelets with the use of preimmune serum (Figure I in the online-only Data Supplement) and in platelets obtained from Cx37−/− mice (Figure 1C). Finally, confocal microscopy of immunostained murine platelet aggregates revealed Cx37 staining at areas of cell-to-cell contact between platelets (Figure 1D and Figure II in the online-only Data Supplement).

We also studied Cx37 expression in megakaryocytes. Cx37 was detected in murine megakaryocytes with a recrudescence of Cx37 expression in forming pseudopods (Figure 2A). Finally, Cx37 expression was confirmed in thrombopoietin-differentiated human CD34+ cells by Western blot (Figure 2B). Thus, Cx37 is expressed by murine and human platelets and by murine megakaryocytes and thrombopoietin-differentiated human CD34+ cells.

Cx37 Deficiency in Mice Shortens Bleeding Time
Cx37−/− mice did not suffer spontaneous bleeding or thrombosis. Bleeding time was measured after tail transection of Cx37−/− and Cx37+/+ mice. We obtained significant differences in the cessation of bleeding: Bleeding time was on average shortened by >50% in Cx37−/− mice (n=20 per group; P<0.01) (Figure 3A). Thus, Cx37 deficiency in mice shortens bleeding time.

Cx37 Deficiency in Mice Increases Thrombus Propensity
We used different thrombosis models to determine the effects of Cx37 deficiency. In the first model, thrombus formation was recorded by intravital microscopy, and we measured the time to form an occlusive thrombus in FeCl3-injured mesenteric arteries. In Cx37+/+ mice, thrombi grew to occlusive size in 50 minutes, and half of the vessels did not occlude by 60 minutes after injury, when observation was terminated. In Cx37−/− mice, thrombi grew to occlusive size in 20 minutes, and all injured arteries were occluded (Figure 3B). Although emboli generated during thrombus formation were rare events in Cx37+/+ mice, they were never observed in Cx37−/− mice.
Bleeding time and thrombus formation after arterial injury depend on multiple factors, including vasoconstriction and platelet response. Because Cx37 is highly expressed in ECs and in some types of vascular smooth muscle cells, we compared vasomotor responses in aortic rings of Cx37+/+ and Cx37−/− mice. Although maximal tension in response to 1 μmol/L phenylephrine was increased in Cx37−/− mice (Cx37+/+: 11.4±0.7 mN and Cx37+/+: 6.0±1.2 mN; n=4 per group; P<0.0075), no difference in sensitivity to the compound was observed between Cx37+/+ and Cx37−/− mice (Figure 4A). This suggests that a variance in intrinsic contractile properties between Cx37+/+ and Cx37−/− mice might result in a stronger vasoconstriction in Cx37−/− mice. We did not observe, however, a difference in maximal tension with 100 mmol/L KCl (Cx37+/+: 11.0±1.2 mN and Cx37+/+: 7.6±1.7 mN; n=4 per group; P=0.1965). Importantly, endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) relaxations were not different between the 2 groups of mice (Figure 4B and 4C). To exclude a contribution of vasoconstriction in thrombus formation, we next induced thromboembolism by intravenous injection of collagen and epinephrine in anesthetized mice. In this model, thrombus formation is recognized to be platelet dependent. Cx37+/+ mice survived 4.2±0.4 minutes compared with Cx37−/− mice that died at 1.9±0.3 minutes (mean±SEM; n=8 per group; P<0.001; Figure 5). These data indicate that thrombus forms more rapidly and extensively in Cx37−/− than in Cx37+/+ mice. Altogether, these experiments point to a major role of platelet Cx37 in thrombosis formation, although Cx37 in vascular wall smooth muscle cells may play an additional role in specific cases in which vasoconstriction occurs.

**Cx37 Deficiency in Mice Increases Platelet Aggregation**

The prothrombotic mechanisms of Cx37 deficiency were further studied by analyzing platelet function in Cx37−/− mice. Cx37−/− mice had a normal platelet count and morphology (data not shown). However, platelet aggregation studies revealed significant functional differences between Cx37+/+ and Cx37−/− mice. Platelet aggregation was increased in Cx37−/− mice compared with Cx37+/+ in response to AA (Figure 6A), ADP (Figure 6B), thrombin (Figure 6C), and collagen (Figure 6D). At a suboptimal concentration of ADP (2 μmol/L), the extent of aggregation was increased by ~50% in the Cx37−/− platelets (representative tracing in

**Figure 2.** Megakaryocytes express connexin 37 (Cx37). A, Cx37 is expressed in murine megakaryocytes with recrudescence of expression at the extremity of forming pseudopods. Photographs are combinations of fluorescent signal with transmitted light to localize cells, and nucleus is counterstained with DAPI (multiple slices of 0.32 μm; bar=20 μm). B, Cx37 expression by Western blotting of total proteins (50 μg) from thrombopoietin (TPO)-differentiated human CD34+ cells (TPO-CD34+ cells). CD34+ cells were cultured for 12 days in Iscove’s modified Dulbecco medium supplemented with polyethylene glycol/ recombinant human megakaryocyte growth and development factor at a final concentration of 10 ng/mL. Ten micrograms of total proteins from untransfected HeLa cells (HeLa wild-type [WT]) or transfected HeLa cells expressing Cx37−319S (HeLa Cx37-S) or Cx37−319P (HeLa Cx37-P) was used as negative and positive control, respectively. β-Actin was used as loading control.

**Figure 3.** Effect of lack of connexin 37 (Cx37) on hemostasis and thrombus propensity. A, After 2-mm tail-tip transaction, a stopwatch was started immediately to determine time to cessation of bleeding (n=20 per group; *P<0.05). B, Thrombus formation in FeCl3-injured mesenteric arteries recorded by intravital microscopy. Time to form an occlusive thrombus, *P<0.05.
Figure 4. Effect of lack of connexin 37 (Cx37) on vasomotor responses. A, Contraction of aortic rings from Cx37\(^{+/+}\) and Cx37\(^{-/-}\) mice in response to phenylephrine. Values are expressed as percentage of phenylephrine-induced maximal (max) contraction; n=4 per group. B and C, Endothelium-dependent relaxations in response to acetylcholine (ACh) and endothelium-independent relaxations in response to the NO donor sodium nitroprusside (SNP) in aortic rings of from Cx37\(^{+/+}\) and Cx37\(^{-/-}\) mice; n=4 per group.

Figure 6B; Cx37\(^{+/+}\) mice: 16.2±0.2% and Cx37\(^{-/-}\) mice: 36.0±0.3%; mean±SEM; n=4 per group; P<0.05). Aggregation in response to higher concentrations (5 and 10 \(\mu\)mol/L) was comparable in Cx37\(^{+/+}\) and Cx37\(^{-/-}\) mice (data not shown). Aggregation in response to low (1 and 2 \(\mu\)g/mL) and high (10 \(\mu\)g/mL) concentrations of collagen did not differ between Cx37\(^{+/+}\) and Cx37\(^{-/-}\) platelets (data not shown). However, the extent of aggregation in response to 5 \(\mu\)g/mL collagen was increased in average by ≈50% in the Cx37\(^{-/-}\) platelets (representative tracing in Figure 6D; Cx37\(^{+/+}\) mice: 23.5±5.3% and Cx37\(^{-/-}\) mice: 48.7±2.7%; mean±SEM; n=4 per group; P<0.05).

Increase in the extent of platelet aggregation was reproduced when murine Cx37\(^{+/+}\) platelets were pretreated with 200 \(\mu\)mol/L Cx37 mimetic peptides\(^5\) and then stimulated by either 5 \(\mu\)mol/L ADP (Figure 6E) or 5 \(\mu\)g/mL collagen (Figure 6F). Platelet adhesion to either immobilized fibrinogen (200 \(\mu\)g/mL) or collagen (50 \(\mu\)g/mL) was comparable in both genotypes when platelets were plated at a concentration of 120 G/L (Figure 6G). However, when Cx37\(^{-/-}\) platelets were plated at a higher concentration (250 G/L), they formed stable aggregates after 1 hour of incubation that were not washed out by 2 thorough washings with buffer. In contrast, Cx37\(^{+/+}\) platelet aggregates, when plated at the same concentration, were washed out, and only a dense platelet monolayer remained on immobilized fibrinogen or collagen (Figure 6H). These data indicate that Cx37 deficiency in mice increases platelet aggregation but not platelet adhesion to either immobilized fibrinogen or collagen under static conditions.

Blocking Cx37 Channels Increases Aggregation of Human Platelets

The role of Cx37 in platelet function was further studied in human platelets. Washed platelets from 6 healthy volunteers were incubated 5 minutes with either 20 or 40 \(\mu\)mol/L of the gap junction blocker \(\alpha\)-GA or vehicle before a suboptimal concentration of AA (37.5 \(\mu\)mol/L), ADP (5 \(\mu\)mol/L), TRAP-6 (0.25 \(\mu\)mol/L), or collagen (0.5 \(\mu\)g/mL) was added. Maximal aggregation responses were recorded for each agonist used, and results are depicted in Figure 7. There was a graded amplification of the aggregation profile along with \(\alpha\)-GA concentrations. Interestingly, the aggregation response in platelets from 2 to 3 volunteers showed a stronger reaction to connexin inhibition. Moreover, the collagen lag time (ie, the interval between collagen addition and the onset of aggregation) decreased from a median value of >200 seconds after incubation with vehicle to 76 and 60 seconds after incubation with 20 and 40 \(\mu\)mol/L \(\alpha\)-GA, respectively (P<0.01). These observations in humans corroborate data in mice, confirming the involvement of Cx37 channels in limiting platelet aggregation.

Platelets Are Functionally Coupled During Aggregation

We next studied whether platelets formed functional gap junction channels during aggregation. To this end, platelets activated with thrombin (1 IU/mL) in a plate coated with immobilized fibrinogen (200 \(\mu\)g/mL) were microinjected with the Cx37-permeable tracer neurobiotin within an aggregate (encircled in Figure 8A, top left). The tracer was allowed to diffuse to neighboring platelets for 3 minutes, and then aggregates were fixed and subjected to immunolabeling to
detect neurobiotin. As illustrated in Figure 8A (top), we observed extensive diffusion of neurobiotin in 3 of 4 injections in human platelet aggregates. The diffusion of neurobiotin was inhibited in platelets exposed to a gap junction blocker (Figure 8A, top right: presence of gap junction blocker, carbenoxolone 50/µM; n=10053). To confirm these results, we repeated the experiments in aggregating platelets from Cx37−/− and Cx37+/+ mice. We detected spreading of the tracer into neighboring Cx37−/− platelets in 4 of 6 aggregates (Figure 8A, bottom left). In contrast, neurobiotin transfer was not observed between Cx37−/− platelets (Figure 8A, bottom right; n=4). These results support the view that Cx37 forms functional gap junction channels during platelet aggregation.

Cx37–319S–Expressing Cells Display Larger Channel Permeability

The human Cx37 gene exhibits a single nucleotide polymorphism resulting in a nonconservative amino acid change in the regulatory C-terminus of the Cx37 protein (P319S). To evaluate the consequence of this polymorphism on GJIC, we transfected Cx37–1019C or Cx37–1019T cDNA into communication-deficient HeLa cells. Clones with equal Cx37 expression level at the cell membrane were selected17 and injected with neurobiotin. As illustrated in Figure 8B and 8C, we observed extensive diffusion of neurobiotin in HeLa cells expressing Cx37–319S (n=9). In contrast, the diffusion of the tracer was markedly decreased (P<0.01) in HeLa cells expressing Cx37–319P (n=9). These results indicate a significant difference in the permeability of Cx37 polymorphic channels.

Platelet Aggregation Response in Humans Is Associated With the Cx37–C1019T Polymorphism

To investigate whether the Cx37 single nucleotide polymorphism may also be associated with platelet reactivity, we genotyped 96 healthy male subjects of Caucasian descent participating in a platelet function study.14 The respective frequencies of the 1019C and 1019T alleles were 66% and 34% and were in Hardy-Weinberg equilibrium. Platelet aggregation was evaluated in citrated PRP. As shown in the Table, there was a graded and significant increase in aggregation response with various agonists, except with AA and 20/µM ADP, according to the number of 1019C alleles.
There was, however, an obvious trend with the use of AA. Similar to platelet aggregation response in Cx37+/+ and Cx37−/− mice, aggregation of human platelet with a high concentration of ADP (20 μmol/L) did not differ across the genotype. Of note, we did not adjust for multiple tests on these correlated aggregation responses, and the error rate may be inflated as a consequence. Multivariable logistic regression analysis with independent variable coded 0 and 1 for association of the C1019T polymorphism with platelet aggregation size, our study indicates that the Cx37 single nucleotide polymorphism may constitute a marker for platelet reactivity.16,22 Soluble CD40L23 or Sema4D24 is shed from the platelet membrane surface on activation and can selectively activate platelets through Cx37–319P gap junction channels, which in turn results in decreased platelet reactivity.25

**Discussion**

Platelets are brought into close proximity at the site of vascular injury. Contacts that develop between nearby platelets allow interactions to occur among molecules on the surface of adjacent platelets. The various molecules involved in this process (reviewed in Brass et al19) include either receptors (αIIbβ3, Tyro3 family members, Eph kinases, SLAM), adhesion molecules (platelet/endothelial cell adhesion molecule-1 and thymocyte marker of the *Xenopus* or CTX family members such as junctional adhesion molecule-A, EC-selective adhesion molecule, CD226), or soluble molecules populating the gaps between platelets (thromboxane A2, ADP, Gas6, soluble CD40L, Sema4D). Signaling through SLAM-SLAM or ephrin-Eph kinases, interactions are dependent on or facilitated by contacts occurring between platelets during aggregation.19–21 Gas6 is secreted by activated platelets and can bind to its receptors Tyro3, Axl, and Mer to modulate αIIbβ3 outside-in signaling.16,22 Soluble CD40L23 or Sema4D24 is shed from the platelet membrane surface on activation and can selectively activate platelets through αIIbβ3 outside-in signaling.21,24 The importance of such signaling molecules in the perpetuation phase of platelet aggregation has been well documented with the use of in vitro studies or in vivo models of thrombus formation in genetically engineered mice.
We report here for the first time that human and murine platelets as well as megakaryocytes express the gap junction protein Cx37. Several proteins of tight junctions were previously identified on platelets such as junctional adhesion molecule-A, junctional adhesion molecule-C, and EC-selective adhesion molecule (reviewed in Brass et al). Interestingly, we show that mice deficient in Cx37 exhibit a shorter bleeding time and an increase in thrombus propensity and stability in 2 different thrombosis models. In addition, exposure of murine and human platelets to gap junction blockers increased their aggregation in response to multiple agonists. However, platelet adhesion to either immobilized fibrinogen or collagen was not influenced by Cx37 deficiency at low platelet concentration, indicating that contact between platelets is necessary for the expression of the phenotype; the participation of contacts between platelets and ECs and/or leukocytes was not excluded. Altogether, our data suggest that Cx37, like platelet/endothelial cell adhesion molecule-1 or EC-selective adhesion molecule, for example, plays a restraining role on thrombus propensity.

Gap junction channels are made by the docking of 2 halves, each being located at the membrane of apposing cells. They consist of connexin hexamers, a structure referred to as connexons or hemichannels, which by themselves may promote interactions between cells, with or without the formation of a complete connexon. Among these connexins, 3 are known to be expressed in the platelets, thereby limiting exaggerated accumulation of platelets, thereby limiting exaggerated accumulation of platelets, and propensity of the aggregate. The identity of the gap junction channels made of Cx40. However, it cannot be ruled out that functional gap junctions between aggregating Cx37+/+ but not Cx37−/− platelets. In addition, this GJIC was abolished in murine and human platelets exposed to a gap junction inhibitor. Thus, the data presented here, in which not only Cx37-deficient mice but also pharmacological blockers and blocking peptides were used, strongly indicate that functional gap junctions rather than signaling through hemichannels or interaction of Cx37 with extracellular substances are directly involved in the antiaggregating effect of Cx37.

The importance of functional gap junction channels between platelets is further supported by the association of a known polymorphism in the human Cx37 gene and platelet aggregation responses. We found that healthy subjects homozygous for Cx37−/−1019C, coding for Cx37−319P, display an increased platelet response compared with subjects carrying the Cx37−/1019T allele, coding for Cx37−319S. Interestingly, we report here that Cx37−/319S gap junction channels are more permeable to neurobiotin than Cx37−319P channels. These observations led us to propose that antiaggregating signals diffuse more efficiently through Cx37−319S gap junction channels to decrease platelet reactivity.

cAMP inhibits platelets by activating protein kinase A, which in turn phosphorylates several intracellular substrates, resulting in the inhibition of multiple platelet functions, such as cytoskeletal reorganization, integrin activation, and secretion. In vitro studies performed with the use of flow chambers show that elevation of cAMP inhibits platelet adhesion to immobilized von Willebrand factor and collagen. In vivo, it has been demonstrated that initial accumulation of platelets during thrombus formation is inhibited by elevation of basal cAMP levels. The movement of cAMP through gap junction channels was demonstrated nearly 30 years ago. It is also interesting to note that cell-to-cell transfer of cAMP through Cx37 gap junction channels is thought to regulate several physiological processes. For example, female Cx37−/− mice display infertility. These mice lack mature Graaf follicles, fail to ovulate, and develop numerous inappropriate corpora lutea. Interestingly, Cx37 participates in the gap junctions between the oocyte and the granulosa cells of the follicle. Selective assembly of Cx37 into heterosexual gap junctions at the oocyte/granulosa cell interface and follicular gap junctions are known to play a crucial role in the maintenance of meiotic arrest of the oocyte in a follicle via low tonic amounts of cAMP signaling from the granulosa to the oocyte. Similar to the follicle, it is possible that Cx37 gap junction channels support the intracellular spread of antiaggregating signals like cAMP between platelets, thereby limiting exaggerated accumulation of platelets and propensity of the aggregate. The identity of the antiaggregating signals in platelets remains, however, to be determined. Recently, an antiadhesive signal for leukocytes has been reported to diffuse through endothelial gap junction channels made of Cx40. However, it cannot be ruled out that loss of connectivity between Cx37-deficient platelets may enhance the intracellular concentration of proaggregating signals in the newly recruited platelets at the edge of the...
aggregate (for example, induced by activation of P2Y12/ADP receptor).

To date, several gene polymorphism association studies have detected a link between the C1019T Cx37 polymorphism and coronary artery disease as well as myocardial infarction in various populations. Surprisingly, the published association studies appear contradictory. Whereas stenosis in coronary arteries seems to be associated with 1019C, coding for Cx37–319P, increased risk for myocardial infarction appeared to be associated with 1019T coding for Cx37–319S.6,7 In a previous study, we demonstrated that Cx37–319S–expressing monocytes display increased adherence to the endothelium, likely resulting in excessive monocyte recruitment and vulnerable atherosclerotic lesions.5 Because the endothelium, likely resulting in excessive monocyte adherence to 319S–expressing monocytes,6,7 in a previous study, we demonstrated that Cx37–319S polymorph may help to resolve this issue. It is noteworthy that platelets have been shown to be involved in restenosis after stent implantation,50 and 2 haplotypes of the platelet ADP receptor P2Y12 gene were shown recently to be associated with restenosis.51 Altogether, our data show that Cx37, by establishing gap junctional communication between aggregating platelets, limits thrombus propensity. We propose that transfer between platelets of an antiaggregating factor occurs after thrombus formation has begun following agonist stimulation. It is likely that the putative antiaggregating factor interacts with intracellular signaling pathways rather than by changing the sensitivity of the agonists’ receptors. In this respect, gap junctional communication between aggregating platelets should thus be considered a fine-tuner of the normal response. Although Cx37 in vascular smooth muscle cells may play an additional role in the in vivo experiments in which vascular constriction occurs, data obtained on platelet aggregation ex vivo evidenced differences according to the genotype in both murine and human platelets. These findings should open new avenues for the development of antiplatelet drugs that target thrombus propensity.

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Disclosures
None.

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Platelets play a key role in the pathogenesis and the acute complications of atherosclerosis such as myocardial infarction and stroke. Current antiplatelet drugs are the cornerstone of the treatment of this widespread disease, but their clinical benefit is relatively limited. Innovative research toward new targets in platelets for drug development is therefore mandatory. The present study reports that platelets and megakaryocytes express connexin 37, which belongs to the family of gap junction proteins. Gap junctions provide a pathway for direct communication between neighboring cells and thereby enable intercellular coordination of tissue activity. Deletion of the connexin 37 gene in mice shortens bleeding time and increases platelet aggregation and thrombus propensity. Aggregation of human platelets is also increased when treated with gap junction blockers, which effectively dampens the communication between platelets during the formation of an aggregate. The importance of functional gap junction channels between platelets is further supported by the association between a known polymorphism in the human connexin 37 gene (GJA4) and platelet aggregation responses. Connexin 37-built gap junctions between platelets therefore provide a mechanism to limit thrombus propensity by downregulating platelet aggregation. We propose that transfer between platelets of an antiaggregating factor occurs after the initiation of thrombus formation following platelet activation. Interestingly, the GJA4 polymorphism has been associated with atherosclerosis and myocardial infarction in previous studies. Our observations should open new avenues for the development of antiplatelet drugs that target thrombus propensity.
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Supplemental methods

Preparation of murine washed platelets

Whole blood was drawn from anesthetized mice from the inferior vena cava into acid-citrate-dextrose solution (ACD, 22 g trisodium citrate dihydrate, 8 g citric acid monohydrate, 25 g glucose dextrose in 1 L of H₂O), 1 volume anticoagulant/6 volumes blood and centrifuged at 100g (10 minutes) to obtain platelet-rich plasma (PRP). To prepare washed platelets, apyrase was added to PRP (final concentration, 1 IU/mL), and platelets were incubated for 10 minutes at 37°C, washed by adding 2 volumes ACD, and centrifuged at 2,000g (10 minutes). The platelet pellet was resuspended in Tyrode’s buffer (pH 7.3) containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, 5 mmol/L HEPES and 0.1% BSA. CaCl₂ (2 mmol/L final) and MgCl₂ (1 mmol/L final) were added immediately before platelet stimulation.

Preparation of human washed platelets

Six unrelated healthy volunteers were recruited. They denied taking any medication for at least 10 days before the start of the study. All subjects gave written informed consent, and the study protocol was approved by the ethics committee of University Hospitals of Geneva.

Venous blood was collected using a 19-gauge needle and no tourniquet after an overnight fast, in tubes containing ACD solution (25 g of trisodium citrate dehydrate, 14 g of citric acid monohydrate, and 20 g of anhydrous D(+)glucose in 1 L of H₂O, 1
volume anticoagulant/6 volumes blood). PRP was obtained after centrifugation at 530g during 15 minutes. Washed human platelets were prepared as described previously ¹ and resuspended at a density of 250 G/L in Tyrode's buffer (pH 7.3) containing albumin (0.35%), apyrase (0.02 U/mL), CaCl₂ (2 mmol/L) and MgCl₂ (1 mmol/L).

**Preparation of PRP in 96 healthy subjects**

Ninety-six unrelated healthy Caucasian men aged from 18 to 40 years were recruited ². They were all non-smokers and denied taking any medication for at least 10 days before the start of the study. All subjects gave written informed consent, and the study protocol was approved by the ethics committee of University Hospitals of Geneva.

Venous blood was collected using a 19-gauge needle and no tourniquet after an overnight fast, in tubes containing 0.105 mol/L sodium citrate (1 volume anticoagulant/9 volumes blood, BD Vacutainer®, Becton Dickinson). The first 3 mL of blood was discarded. PRP was adjusted to 250 G/L with autologous platelet poor plasma (PPP).

In order to test the purity of our human platelet rich plasma (PRP) preparations, PRP from 3 healthy volunteers were investigated by flow cytometry for T- and B-lymphocytes, monocytes and neutrophils contamination.

To obtain peripheral blood leukocytes, which served as positive controls, citrated whole blood was centrifuged at room temperature at 800g for 10 minutes without brake. The red blood cell/leukocyte solution was exposed to NH₄Cl 0.15 M for 30 minutes at 4°C to disrupt red blood cells, and centrifuged. The remaining pellet,
which contained the leukocytes was rinsed in PBS and adjusted to 500'000 cells per tube before staining.

Peripheral blood leukocytes or PRP samples from 3 healthy subjects were incubated with the following antibodies according to manufacturer’s instructions: FITC-conjugated anti-CD3 (clone UCHT1, DakoCytomation), RPE-conjugated anti-CD14 (clone TÜK4, DakoCytomation), PC5-conjugated anti-CD19 (clone J3.119, Beckman Coulter), PC7-conjugated anti-CD33 (clone P67.6, Becton Dickinson), PC5-conjugated anti-CD41 (clone P2, Beckman Coulter) or with isotype controls.

Flow cytometry was performed using a FACSARia (Becton Dickinson). Settings were made using leukocyte samples as fluorescence positive controls for CD3, CD14, CD19 and CD33. PRP was obtained from three healthy donors. Results were analyzed with Treestar FlowJo.

As shown in the Supplemental figure 3, contamination by T-lymphocytes (labelled by an anti-CD3 antibody) was 1.32 ± 0.7%, by B-lymphocytes (labelled by an anti-CD19 antibody) 0.17 ± 0.16%, by monocytes (labelled by an anti-CD14 antibody), 1.30 ± 0.81% and by neutrophils (labelled by an anti-CD33 antibody), 0.86 ± 0.05%. PRP contained 98.2 ± 0.91% platelets (labelled by an anti-CD41 antibody). Thus, PRP contamination by leukocytes was very low indicating that the Cx37 signal on Western blots was mainly coming from platelets.

**Thrombopoietin (TPO)-differentiated CD34⁺ cells**

Cord blood cells were collected after informed consent. Cord blood was diluted with phosphate buffer saline (PBS), mononuclear cells were separated on a Ficoll gradient (Lymphoprep; Nycomed Pharma) and washed twice in PBS. CD34+ cells were purified with a CD34 microbeads kit (Miltenyi Biotec) as described by manufacturer. The efficiency of purification was verified by flow cytometry
counterstaining with a CD34-PE antibody (Beckam Coulter) and analyzed with a Quanto II cytometer (Becton Dickinson). In the cells fraction containing purified cells, the percentage of CD34+ cells ranged from 90% to 98%. Then, CD34+ cells were cultured in Iscove’s modified Dulbecco medium (IDDM; GIBCO) with penicillin/streptomycin/glutamine and 11.5 μmol/L α-thioglycerol supplemented with polyethylene glycol (PEG)-rHuMGDF (gift of Amgen Corp., Thousand Oaks, CA) at a final concentration of 10 ng/mL. Cultures were performed in serum free conditions at 37°C in a fully-humidified atmosphere containing 5% CO₂ in air. After 12 days of culture, megakaryocytes derived from these cultures were used for investigations.

**Isolation of murine bone marrow cells**

Single-cell suspensions were obtained from bone marrow flushed from femur and tibia and diluted 10 times in DMEM 10% FCS. Cell suspension was incubated for 1 hour at 37°C on 0.2% gelatin coated coverslips. Non-adherent cells were then removed and the coverslips were washed twice with PBS. Adherent cells were fixed as described below.

**Western blotting**

Western blotting analysis of protein extracts from platelets, TPO-differentiated human CD34+ cells, transfected HeLa cells and bEnd3 cells was performed as described previously. Cells were harvested into an ice-cold solubilization buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 130 mmol/L NaCl, 0.2% sodium deoxycholate, 1% Nodinet-P40, 0.05% sodium dodecylsulfate, 2 mmol/L sodium orthovanadate, 125 mmol/L phenylarsine oxide and 2 mmol/L phenylmethyl sulfonyl fluoride, and stored at -80°C. After thawing, the samples were centrifuged for 20 minutes at 13,000g and
4°C. Supernatants containing solubilized material were recovered, and total amounts of protein were quantified using a bicinchoninic acid quantification assay (Pierce). Fifty μg protein was loaded on 10% SDS-polyacrylamide gels, electrophoresed, and electrotransferred onto nitrocellulose membranes (Millipore). Membranes were then soaked for 2 hours at room temperature in a 5% defatted milk saturation buffer (PBS/0.1% Tween20). Blotted proteins were then incubated overnight at 4°C with rabbit polyclonal (2 μg/mL; ADI) Cx37 antibodies or anti-β actin (1:1000; Sigma), which was used as a loading control. After rinsing, the membranes were incubated 1 hour with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to peroxidase (Jackson Laboratories), whichever appropriate. Specificity of the Cx37 labeling was confirmed by preabsorption of the polyclonal antibodies for 15 minutes at room temperature with 20 μg/mL cognate immunogenic peptide (ADI). Immunoreactivity was detected using the ECL chemiluminescent detection kit (Amersham), according to the manufacturer's instructions. The chemiluminescence reaction was visualized on high performance chemiluminescence film (Amersham, GE Healthcare).

**Immunofluorescence**

Adherent platelets, platelet aggregates and bone marrow cells were fixed in ice-cold methanol for 5 minutes at -20°C, and immunolabeled with rabbit Cx37 antibodies (Alpha Diagnostic International). Platelets were counterstained with Evans Blue in some experiments. Slides were mounted with Vectashield mounting medium (Vector laboratories) and examined with a confocal Zeiss LSM510 microscope. Images were captured using the software LSM510 Browser (Zeiss) and processed.
**Platelet adhesion**

Adhesion to immobilized fibrinogen or collagen was quantified as described. Human fibrinogen in PBS, pH 8.0, was coated onto microtiter plates at concentrations ranging from 1 ng to 2 μg/well and incubated overnight at 4°C. Plates were washed two times with PBS, pH 7.4, and blocked for 2 hours at room temperature with 20 mg/mL BSA in PBS. Fifty microliters of washed platelets (120 G/L) in Tyrode’s buffer 0.1% BSA were added per well. After 1 hour incubation, nonadherent platelets were removed and the wells were washed twice with 150 μL Tyrode’s buffer supplemented with 1 mmol/L MgCl₂. One hundred fifty microliters of pNpp buffer (0.1 mol/L citrate, pH 5.4, 0.1% Triton X-100, 5 mmol/L para-nitrophenylphosphate) were added for 1 hour at room temperature. Then, 100 μL of 2 mol/L NaOH was added and adherent platelets were quantified in a microplate reader at 405 nm. The percentage of platelets adhering was determined by calculating the ratio of bound/maximal signal at 405 nm, where maximal reading was obtained from a microtiter well containing 6 x 10⁶ platelets that was not subjected to washing procedures.

**Bleeding time**

For bleeding time measurements, 2-mm tail tips were transsected in mice anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg). A stopwatch was started immediately upon transsection to determine time to cessation of bleeding.

**Thrombosis models**

Intravital microscopy was performed on mouse mesenteric arteries. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg)
and xylazine (16 mg/kg). Platelets were directly labeled in vivo by the injection of 300 
μl rhodamine 6G (0.1%). After selection of the studied field, vessel wall injury was 
generated by a 1 mm large filter paper saturated with a 20% FeCl₃ solution for 4 
minutes. Fluorescent light was collected on an analog video-recorder mounted on an 
inverted microscope (Olympus BX50WI) equipped with a water immersion objective. 
Time to vessel wall occlusion was measured, as determined by a stop of the blood 
cells flow.

Thromboembolism was induced in mice anesthetized by intraperitoneal injection of 
sodium pentobarbital (60 mg/kg) by an intravenous administration of a mixture of 
collagen (0.5 mg/kg, equine collagen; Hormon Chemie, München, Germany) and 
epinephrine (60 μg/kg) into the jugular vein⁴.

**Vasomotor responses**

The descending thoracic aortas with intact endothelium were isolated, dissected free 
from perivascular tissues and cut into rings of 2 mm in length. Rings were suspended 
in a multichannel myograph (Multi Wire Myograph System 610M, Danish Myo 
Technology) filled with Krebs-Ringer bicarbonate buffer (in mmol/L: 118 NaCl, 4.7 
KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 0.026 EDTA and 11.1 glucose) 
at 37°C, aerated with 95% O₂ and 5% CO₂.

Aortic rings were allowed to equilibrate for 45 minutes and were progressively 
stretched to a passive tension of 5 mN that gives the optimal length-tension 
relationship. Contractility with KCl and phenylephrine, were measured. Then, aortic 
rings were precontracted with phenylephrine (0.5 – 1 μmol/L) to match the level of 
precontraction. Subsequent relaxations with acetylcholine or sodium nitroprusside
were measured and calculated using PowerLab4/25 interface and PowerLab software (AD Instruments).

**Murine platelet aggregation**
Platelet aggregation was performed as described in 4. Briefly, whole blood, drawn from anesthetized mice from the inferior vena cava into 3.13% citrate (1 volume anticoagulant/9 volumes blood), was centrifuged at 100g (10 minutes) to obtain PRP and additionally at 2,000g (10 minutes) to obtain PPP. PRP and PPP were pooled from 4 to 6 mice. The platelet suspension was incubated at 37°C with the following agonists: ADP (Sigma) 1-10 μmol/L; collagen (Endotell) 1-10 μg/mL. Platelet response to thrombin 0.05-1 UI/mL (Endotell) and AA (Helena) 0.15-1.5 mM was investigated on washed platelets. When indicated, WT washed platelets were pre-incubated with 200 μM Cx37 mimetic peptides (2-5 minutes). Platelet aggregation was measured turbidimetrically using an optical Platelet Aggregation Profiler (model PAP-4, Bio/Data).

**Human platelet aggregation**
Platelet aggregation was measured turbidimetrically using an optical Platelet Aggregation Profiler (model PAP-4, Bio/Data). Aggregation responses were measured for each agonist used during 5 min and maximal aggregation value was recorded.

*Washed platelets*
The platelet suspension was incubated at 37°C either with vehicle or αGA at 20 or 40 μmol/L during 5 minutes before stimulation with the following agonists: AA (Bio/Data...
Corporation), 37.5 µmol/L; ADP (Sigma), 5 µmol/L; TRAP-6 peptide (Bachem), 0.25 µmol/L or collagen (Horm, Nycomed), 0.5 µg/mL.

**PRP**

Platelet aggregation in PRP was performed using the following agonists: arachidonic acid (AA) 1.5 mmol/L (Bio/Data), Horm collagen 1 µg/mL (Nycomed) or ADP 2, 5 and 20 µmol/L (Helena Biosciences Europe).

**FITC-phalloidin staining**

Coverslips were placed in 24-well plates and coated with 100 µg/ml fibrinogen. Washed platelets (250 G/L) in Tyrode’s buffer supplemented with CaCl$_2$ and MgCl$_2$ were added to each well for 15 to 60 minutes at 37°C. Non adherent platelets were washed away and adherent cells fixed in 1% paraformaldehyde in PBS for 15 minutes at room temperature. After three washes with PBS, excess aldehyde was quenched with 10 mmol/L ethanolamine in PBS (or 0.1 mol/L glycine in PBS) for 5 minutes. Platelets were permeabilized in 0.1% Triton-X 100 in PBS for 1 minutes and incubated in FITC-phalloidin (Sigma) diluted 1:100 in PBS for 15 minutes. The slide was removed from the well and then washed three times in PBS. Slides were then mounted in Vectashield (Vector Laboratories). Pictures were taken on an LSM 510 Meta inverted confocal microscope (Zeiss).

**Evaluation of gap junctional intercellular communication**

GJIC within platelet aggregates and in Cx37-expressing HeLa cells was evaluated by intracellular microinjection of neurobiotin. HeLa cells were transfected with a pIRES2-eGFP vector containing *Cx37-1019C* or *Cx37-1019T* cDNA$^5$. Neurobiotin (2%) was prepared in 150 mM LiCl and 10 mM Hepes (pH 7.2). Washed mouse or human
platelets (6 x 10^6) in Tyrode’s buffer containing 0.1% BSA were added to Petri dishes coated with fibrinogen. Thrombin (1 IU/mL) was added to activate platelets. The tracer was injected within the forming platelet aggregate using a thin-tip glass microelectrode and was allowed to fill platelets within the aggregate by simple diffusion for 3 minutes. Experiments were also performed in the presence of the gap junction blocker carbenoxolone (50 μmol/L). At the end of the injection, the electrode was removed and platelet aggregates were fixed with 4% PFA, rinsed with PBS and permeabilized with 0.3% Triton X-100 for 20 minutes. Neurobiotin was detected by incubating the fixed platelet aggregate with streptavidine-rhodamine for 30 minutes.

Using a similar methodology, one cell in a large cluster of HeLa cells expressing Cx37-319S or Cx37-319-P was micro-injected. Fluorescent cells were viewed on an inverted TMD-300 microscope (Nikon) equipped with a 40X phase 3 DM objective with a numerical aperture of 0.7 (Zeiss). Images were captured with a Visicam digital camera (Visitron Systems) connected to a personal computer running Metafluor 4.01 software (Universal Imaging).
Suppl. figure 1
Suppl. figure 2
Suppl. figure 3
**Legend to the supplemental figures**

**SUPPLEMENTAL FIGURE 1:**
Immunocytological staining of human platelets. Green fluorescence staining localized Cx37 on human platelets (scale bar, 2 μm). Platelets were counterstained with Evans Blue (red).

**SUPPLEMENTAL FIGURE 2:**
Cx37 immunostaining (in green) on platelet aggregates obtained from Cx37+/− mice. Multiple photographs (z slice interval of 0.76 μm) are combinations of fluorescent signal with transmitted light to localize cells (scale bar, 5 μm).

**SUPPLEMENTAL FIGURE 3:** Representative flow cytometry profiles of CD3, CD14, CD19, CD33 and CD41 expression in platelet-rich plasma (PRP). Cells were gated on PRP preparations according to size and granularity parameters (left column) so that any leukocyte could be detected within the same gate. Positive expression of CD3, CD14, CD19 and CD33 was determined using leukocyte preparation. Contamination with CD3+, CD14+, CD19+ or CD33+ cells in PRP was inferior to 1.35%. More than 98% of events were CD41+. Results were expressed as mean±SEM of positive cell percentage and are indicated in the upper right quadrant of dot plots (n=3).
References


