Structural and Functional Plasticity of Collagen Fibrils

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Collagen is a major component of the subendothelial matrix and participates in bleeding arrest by activating and aggregating platelets at the site of vascular injury. The most common type I collagen exists in both soluble and fibrillar forms, but structural exchangeability between the two forms is currently unknown. Using atomic force microscopy, we show that type I collagen switches between soluble and fibrillar forms in a pH-dependent and ion-independent manner. Fibrillar collagen is rope like with characteristic “D-bands.” The collagen fibrils can be disrupted with 0.1 M acetic acid and will reform when the pH is adjusted to 7.4. This structural plasticity leads to drastically different activities, with fibrillar collagen being significantly more active for platelets under static and flow conditions. More important, by probing with noncontact hopping probe ion-conductance microscopy, we find that platelets adherent to fibrillar collagen present primarily as high-density bubble shapes that have undergone rapid microvesiculation.

Keywords: collagen, platelets, microparticles

Introduction

BLEEDING ARREST is initiated by tethering platelets to the site of vascular injury (Sixma *et al.*, 1991). The tethered platelets adhere to and are activated on collagen, the most abundant component of the subendothelial matrix (Murata *et al.*, 1986). The human subendothelial matrix is composed mainly of fibrillar collagen types I and III (Murata *et al.*, 1986, 1987; Shekhonin *et al.*, 1987), which are also highly concentrated in fibrous atherosclerotic plaque (McCullagh and Ehrhart, 1974; McCullagh, 1983; Murata *et al.*, 1986, 1987; Murata and Motoyama, 1990). Two platelet receptors mediate the platelet/collagen interaction: glycoprotein VI and the integrin $\alpha 2\beta 1$. Platelets adherent to collagen are able to withstand the significant pulling force of the blood flow by forming multivalent ligand/receptor bonds to increase the binding avidity (Santoro and Cunningham, 1977).

Consistent with the notion, fibrillary collagen is significantly more active than soluble collagen in activating and aggregating platelets under static and flowing conditions (Brass and Bensusan, 1974; Bernardo *et al.*, 2004). We have previously shown that commercial fibrillar collagen reagents for platelet aggregation contain von Willebrand factor (VWF), which is, in part, responsible for more active col-

lagen fibrils (Bernardo *et al.*, 2004), as it synergizes with collagen to tether platelets to the collagen matrix (Lopez, 1994). However, highly purified collagen also presents with different activities in its soluble and fibrillary forms, suggesting an intrinsic structural base for the differential activity. The orderly structured collagen fibrils contain characteristic periodic “D-bandings” composed of ~ 67 nm repeats formed of quarter-staggered, side-by-side alignments of five triple helices (Cross and Templeton, 2004).

This rope-like structure (Bozec *et al.*, 2007) has been visualized with electron microscopy (EM) (Trelstad *et al.*, 1976), atomic force microscopy (AFM) (Lin and Goh, 2002; Friedrichs *et al.*, 2007), and time-lapse confocal reflection microscopy (Brightman *et al.*, 2000). AFM was also used to measure the elastic and viscous responses of type I collagen to mechanical forces (Grant *et al.*, 2012). However, these techniques are limited in the study of dynamic changes of collagen structure and their impacts on platelets. For example, EM requires sample fixation or freezing, whereas the repeated light exposures necessary for confocal microscopy generate heat that damages collagen structure and platelet viability. Although traditional AFM is capable of monitoring dynamic changes of collagen structure under force, which is known to influence the alignment of collagen fibrils

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(Saeidi *et al.*, 2009), its cantilever makes direct contact with, and thus can damage, the surfaces of adherent platelets.

There is also no report on the structural plasticity that changes collagen's ability to support platelet adhesion and activation. We have previously used hopping probe ion-conductance microscopy (HPICM) to monitor morphological changes of platelets adherent to fibrinogen and the rate of platelet microvesiculation induced by adenosine diphosphate (Zhang *et al.*, 2013; Liu *et al.*, 2015). HPICM uses ion-conductance as a probe to monitor dynamic changes of platelets adherent to collagen in real time and without physical contact with the adherent platelets, and chemical fixation. Using AFM and HPICM, we tested the hypothesis that collagen is structurally exchangeable between soluble and fibrillar forms and that such structural plasticity is associated with collagen's ability to activate platelets and to induce platelets to produce microparticles under static and flow conditions.

Materials and Methods

pH-induced structural changes of type I collagen

Type I soluble and fibrillary collagen reagents were obtained from BioData Corp. (Cat. No. 101562, Horsham, PA) and Chrono-log Corp. (Cat. No. 385, Havertown, PA), respectively. The soluble collagen was induced to form fibrils at pH 7.4 with a 0.03 M bicarbonate/carbonate buffer (pH 10.8, 60 min at 37°C), and the fibrillar collagen was solubilized with 0.1 M acetic acid (pH 2.7, 60 min at 37°C) (Dong *et al.*, 1994). Both buffers did not contain calcium and magnesium. The soluble and fibrillar collagen was immobilized onto tissue culture dishes (200 µg/mL, 60 min at room temperature), washed with phosphate-buffered saline (PBS, pH 7.4), and scanned under NanoScope IV Multimode AFM (Digital Instruments Veeco Metrology Group) (Zhang *et al.*, 2013). The three-dimensional structure of the collagen was measured and analyzed with NanoScope software (Digital Instruments Veeco Metrology Group).

Platelet adhesion and microvesiculation

Blood samples were collected into acid-citrate dextrose (ACD: 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid, 10% v/v, pH 6.5) from healthy subjects (ages 26–54, 56% females) who had been medication free for at least 2 weeks. The Western Institutional Review (U.S.) and the Tianjin Medical University General Hospital Boards for Human Subject Research approved the study. Blood samples were centrifuged at 150 *g* for 15 min at 24°C to obtain platelet-rich plasma (PRP), which was further centrifuged at 900 *g* for 10 min to obtain platelets. The platelets were washed in citrate, glucose and sodium (CGS) buffer (13 mM sodium citrate, 30 mM glucose, and 120 mM sodium chloride, pH 6.5) and were suspended in Ca⁺⁺, Mg⁺⁺-free Tyrode's buffer (138 mM sodium chloride, 5.5 mM glucose, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, and 0.36 mM sodium phosphate dibasic, pH 7.4). Washed platelets adherent to immobilized collagen in 2 mL of Leibovitz L15 culture medium containing 1.26 mM of calcium (pH 7.4, Gibco 21083, Invitrogen, Carlsbad, CA) were scanned with HPICM every ~10 min (Zhang *et al.*, 2013).

The medium was collected after HPICM scans for measuring Annexin V⁺ and CD41a⁺ platelet microparticles using flow cytometry (Zhang *et al.*, 2013).

Platelet adhesion and aggregation on collagen and VWF under flow conditions

Blood samples were collected using sodium citrate as an anticoagulant (0.38% final concentration) and centrifuged at 150 *g* for 15 min at 24°C to collect PRP. PRP was perfused over immobilized collagen (100 µg/mL, 4°C, overnight) under either 2 or 15 dynes/cm² of shear stress (to mimic venous and arterial flows) for 1 min at room temperature in a microfluidic chamber system (Cellix Mirus Nanopump, Dublin, Ireland). After washing with PBS, platelet adhesion and aggregation (thrombus formation) were recorded under an Olympus IX-81 inverted stage microscope. For platelet adhesion, the data were quantified by measuring the areas covered by platelet thrombi. For platelet rolling, time-lapse images were continuously acquired at 1 frame/s for 30 s and were then overlaid to generate tracings of rolling platelets. The lengths of platelet-rolling traces were measured to calculate the rolling velocity of platelets (µm/s).

As control, the rolling of platelets on immobilized VWF under 15 dynes/cm² of shear stress was also quantified as previously described (Fredrickson *et al.*, 1998). The VWF substrate was prepared by incubating the flow chamber with 10 µg/mL of VWF, purified from human plasma cryoprecipitate, for overnight at 4°C.

Statistical analysis

The experimental data are presented as mean ± standard error of mean. The quantitative data were analyzed using either Student's *t*-test or ANOVA. A *p*-value of <0.05 was considered statistically significant.

Results

Structural plasticity of collagen

The type I collagen fibers were long axial rope-like structures with periodic and characteristic “D-banding” under AFM (Fig. 1A, B), consistent with previous observations (Lin and Goh, 2002; Friedrichs *et al.*, 2007). These collagen fibrils were ~0.15–0.2 µm in width. High-resolution images further showed that the D-bands did not cross the entire width of a collagen fiber, but formed orderly aligned small modules (Fig. 1C). Fibrillar collagen was efficiently dissolved in acetic acid (pH 2.7, 30 min, Fig. 1D) and it reformed its rope-like fibrils when the pH was adjusted to 7.4 (Fig. 1E) with similar “D-banding” (Fig. 1F).

Platelet adhesion and microvesiculation on soluble and fibrillar collagen

Under HPICM, adherent platelets on soluble collagen mostly spread, with a few undergoing morphological transformation to high-density bubble shapes (HDBSs, Fig. 2A). Fully spread platelets on soluble collagen measured 7–15 µm and had a maximum height of ~1 µm (Fig. 2C), similar to those adherent to immobilized fibrinogen (Fig. 2D). By contrast, 91.6% ± 11.2% of platelets on fibrillar collagen were HDBSs (Fig. 2B), a significantly higher percentage than

FIG. 1. Structural plasticity of type I collagen: Fibrillar type I collagen detected by AFM (A, bar=1 μm) and its local enlargement (B, bar=0.2 μm), showing a rope-like structure that is composed of orderly arranged “D-bands.” Further enlargement shows that the D-bands do not run the entire width of the collagen fiber. While mostly orderly, these D-bands contain gaps on the surface (C, bar=0.1 μm). Type I collagen solubilized in acetic acid (D, pH 2.7) reformed fibrillar structures after the pH is adjusted to 7.4 (E, bar=1 μm). The reformed collagen fibrils contain similar “D-banding” structures (F, bar=0.2 μm). The images are representative of six to nine separate experiments. AFM, atomic force microscopy.

FIG. 2. Platelet adhesion to soluble and fibrillar collagen: Human platelets adherent to soluble (A; arrows indicate the morphology changes of an adherent platelet over the time of incubation) or fibrillar (B) collagen were repeatedly scanned with HPICM at intervals of ~ 12 –15 min. 3D images were constructed at $50 \times 50 \mu\text{m}$ (the bars at the lower right corner indicate the height of the platelets). (C) Shows a single platelet fully spread on soluble collagen, compared with platelets adherent to fibrinogen (D). Platelets spread on fibrillar collagen (E) rapidly change their morphologies to HDBSs (the first image was set at time 0), (F) when fibrillar collagen is added to the medium. All images are representative of 3 to 12 separate experiments. 3D, three dimensional; HDBS, high-density bubble shape; HPICM, hopping probe ion-conductance microscopy.

FIG. 3. Platelet microvesiculation through membrane shedding: HDBS platelets adherent to fibrillar collagen underwent rapid membrane shedding. (A–D) Arrows indicate platelet shedding [membrane fragmentation] and arrowheads indicate collagen fibrils. The images are representative of 20 separate experiments. (E) Levels of CD41a⁺ and Annexin V⁺ platelet microparticles from platelets adherent to soluble and fibrillar collagen (Student's *t*-test, *n* = 26). Platelets adherent to and spread on soluble collagen (F) were stimulated with fibrillar type I collagen and underwent microvesiculation [images taken 12 min (G) and 24 min (H) after stimulation, as shown by the disintegration of platelets, especially along the collagen fibrils (arrowhead). All images are representatives of three to nine independent experiments.

those on soluble collagen ($21.6\% \pm 5.1\%$, Student's *t*-test, *n* = 56, *p* < 0.001). Platelets spread on soluble collagen (Fig. 2E) rapidly changed to HDBS when fibrillar collagen was added to the medium (Fig. 2F). HDBS platelets adherent to fibrillar collagen underwent rapid membrane shedding (Fig. 3A–D), producing a significant number of microparticles (Fig. 3E). Similarly, incubating platelets adherent to soluble collagen (Fig. 3F) with fibrillar collagen also resulted in steady microvesiculation (Fig. 3G–H).

Platelet adhesion on collagen was also measured under both venous (2 dynes/cm²) and arterial (15 dynes/cm²) shear stresses. The platelets adhered mostly as singlets on soluble collagen under the venous shear stress of 2 dynes/cm² (Fig. 4A, the area covered by platelet thrombi: $11.2\% \pm 8.9\%$, *n* = 12), whereas the majority of them rolled under an arterial shear stress of 15 dynes/cm² (Fig. 4B). The rolling velocity of platelets under 15 dynes/cm² was $86 \pm 16 \mu\text{m/s}$, significantly slower than platelets rolling on VWF under similar shear stress (Fig. 4C) (Fredrickson *et al.*, 1998). By contrast, platelets quickly become adherent on fibrillar collagen under both venous and arterial shear stresses (Fig. 4D, E, and Supplementary Video S1; the areas covered by platelet thrombi: [Fig. 4D] $17.31\% \pm 1.6\%$ and [Fig. 4E] $8.6\% \pm 5.2\%$, *n* = 12). Figure 4F shows a large platelet aggregate formed on the collagen matrix under 2 dynes/cm² of shear stress.

Discussion

It has been shown that soluble procollagen forms fibrils when its terminal propeptide is cleaved by the procollagen metalloproteinases (Kadler *et al.*, 1988). The elongated collagen fibrils are more flexible and extensible (Graham

et al., 2004), necessary for maintaining the integrity of vessels that undergo continuous constriction and relaxation. In this study, we further show that type I collagen undergoes rapid structural exchanges between soluble and fibrillar forms (Fig. 1). We have termed this the “structural plasticity of collagen.” Unlike the previously reported process of collagen polymerization (Evans and Drouven, 1983; Jiang *et al.*, 2004), this structural plasticity is pH dependent, but calcium independent, because it was detected at acidic and neutral pH in calcium- and magnesium-free buffers. More importantly, it significantly affects collagen's ability to support platelet adhesion and to activate and aggregate platelets under static (Fig. 2) and flowing (Fig. 4) conditions.

Fibrillar collagen is predominantly present in the sub-endothelial matrix, and its fibrillar structure may have helped platelets recognize the collagen substrate (Brass and Bensusan, 1974). In this study, we show that this structural plasticity is also important for collagen to interact with platelets in several ways (Figs. 2 and 3). First, fibrillar collagen enabled and accelerated platelet adhesion against the antagonist forces of blood flow (Fig. 4), potentially by clustering platelet receptors through multivalent collagen fibrils to achieve high avidity. This multivalent interaction has been demonstrated extensively for platelet binding to VWF multimers under flow conditions (Wijeratne *et al.*, 2013; Gogia and Neelamegham, 2015) and for the formation of fibrin, which crosslinks platelets to stabilize a hemostatic plug (Porter and Hawn, 1949; Kirchhofer *et al.*, 1995). The multivalent interaction may be a prerequisite for these ligands to effectively mediate platelet adhesion, activation, and aggregation under constant blood flow (Santoro and Cunningham, 1977; Kadler *et al.*, 1988; Dong *et al.*, 2002).

FIG. 4. Platelet adhesion to collagen under flow conditions: PRP was perfused over either soluble (A, B) or fibrillar (D, E) collagen under venous (2 dynes/cm², A, D) and arterial (15 dynes/cm², B, E) shear stress for 3 min. Time-lapse images (bar = 20 μm) were acquired at 1 frame/s and overlaid to generate platelet rolling trails. The velocity of platelet rolling was calculated from the average length of the platelet rolling trails (C, $n = 26$, Student's *t*-test). (F) Shows a large platelet aggregate formed on fibrillar collagen under 2 dynes/cm² of shear stress (bar = 5 μm, unlike other images in the figure, this image was captured with platelet suspension flowing through the chamber so that flowing marks are visible). The images are representatives of 12–36 independent experimental runs.

Second, platelets adherent to fibrillar collagen presented distinct HDBS morphology and produced significantly more microparticles than those on soluble collagen (Fig. 3), suggesting that this multivalent interaction between collagen fibrils and platelets also promotes microvesiculation. This possibility is consistent with the elevated levels of platelet microparticles found in patients with myocardial infarction, because of platelet interaction with fibrillar collagen exposed at the site of ruptured atherosclerotic plaques (McCullagh, 1983; Murata *et al.*, 1986, 1987). Furthermore, such HDBS platelet morphology changes (named as balloons or blebs by other groups) have also been observed *in vitro* in platelets adherent to immobilized collagen, which are also demonstrated to yield procoagulant surfaces and rapidly break up to form numerous procoagulant microvesicles (Heemskerk *et al.*, 1997; Agbani *et al.*, 2015). This finding also validates our previous report that HDBS platelets are sensitized to vesiculate (Zhang *et al.*, 2013).

Third, the structural and function plasticity of collagen may contribute to disease development. For example, non-enzymic crosslinking is a major cause of collagen dysfunction in the aging process (Anrather *et al.*, 1997). The crosslinking is accelerated in patients with diabetes (Anrather *et al.*, 1997), a well-known risk factor for atherosclerosis (Kalofoutis *et al.*, 2007). Oxidative stress, which contributes to various disease states, also regulates collagen crosslinking (Jelenska and Kopec, 1983; Lahav *et al.*, 2003). On the one hand, inter- and intrachain disulfide bonds

are involved in the crosslinking of type IV collagen (Weber *et al.*, 1988). On the other hand, reactive oxygen species promote the enzymatic degradation of type I collagen fibrils (Mukhopadhyay and Chatterjee, 1994).

Conclusions

In summary, we have shown that type I collagen is structurally plastic, undergoing exchanges between soluble and fibrillar forms. This plasticity influences the way collagen activates platelets under static and flow conditions. While the data reported here come from *in vitro* experiments, this collagen plasticity may have physiologic and pathologic implications because collagen is a key component of not only the subendothelial matrix but also other extracellular matrixes.

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Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Video S1

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