Fibrillar type I collagens enhance platelet-dependent thrombin generation via glycoprotein VI with direct support of α2β1 but not αIIbβ3 integrin

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Summary

The role of collagens and collagen receptors was investigated in stimulating platelet-dependent thrombin generation. Fibrillar type-I collagens, including collagen from human heart, were most potent in enhancing thrombin generation, in a way dependent on exposure of phosphatidylserine (PS) at the platelet surface. Soluble, non-fibrillar type-I collagen required pre-activation of integrin α2β1 with Mn^{2+} for enhancement of thrombin generation. With all preparations, blocking of glycoprotein VI (GPVI) with 9O12 antibody abrogated the collagen-enhanced thrombin generation, regardless of the α2β1 activation state. Blockade of α2β1 alone or antagonism of autocrine thromboxane A_2 and ADP were less effective. Blockade of αIIbβ3 with abciximab suppressed thrombin generation in platelet-rich plasma, but this did not abolish the enhancing effect of collagens. The high activity of type-I fibrillar collagens in stimulating GPVI-dependent procoagulant activity was confirmed in whole-blood flow studies, showing that these collagens induced relatively high expression of PS. Together, these results indicate that: i) fibrillar type-I collagen greatly enhances thrombin generation, ii) GPVI-induced platelet activation is principally responsible for the procoagulant activity of fibrillar and non-fibrillar collagens, iii) α2β1 and signaling via autocrine mediators facilitate and amplify this GPVI activity, and *iv)* αIIbβ3 is not directly involved in the collagen effect.

Keywords

Coagulation, collagen, glycoprotein VI, platelets, thrombin generation

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Introduction

Platelet binding to collagen exposed at a damaged vessel wall is considered an initial and key step in thrombus formation (1). Two receptors mediate the stable interaction of platelets with collagen fibers: the adhesive receptor, integrin α2β1, and the signaling receptor, glycoprotein VI (GPVI), which is a member of the immunoglobulin receptor family; the contribution of additional receptors for collagen is still disputed (2). Both receptors appear to cooperate in collagen-induced thrombus formation: the low affinity GPVI receptor plays an activating role, e.g. inducing higher-affinity binding sites of integrin α2β1 for collagen. The activated integrin, in turn, stabilizes the binding of GPVI and the signaling through this receptor (3–6). Particularly at high, arterial shear rates, also platelet binding via glycoprotein Ib-IX-V to Willebrand factor (VWF, which avidly binds to collagen) participates in the interplay of collagen receptors, both directly and indirectly by stabilization via integrin αIIbβ3 (6, 7). Additional factors contributing to the stabilization of platelet-collagen contact are the autocrine mediators, thromboxane A_2 (TxA_2) and ADP, which are both released from activated platelets (8, 9), e.g. by enhancing the α2β1 and αIIbβ3 activation (10). In addition, these secondary mediators trigger nearby, flowing platelets to assemble on the primary layer of collagen-adherent platelets with as a result formation of a platelet thrombus.

Whereas the function and interplay of these direct and indirect collagen receptors in platelet adhesion and aggregate formation is relatively well understood, much less is known of the importance of these receptors in collagen-induced thrombin generation and coagulation. Earlier data indicate that type-I collagen fibers stimulate Ca^{2+}-dependent shedding of microvesicles and scrambling of plasma membrane phospholipids, with as a re-

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sult expression of procoagulant phosphatidylserine (PS) at the surface of vesicles and remnant platelets (11). The exposed PS is necessary but perhaps not sufficient for platelets to mediate prothrombinase activation, thrombin generation and clot formation (12). By comparing the PS-exposing effect of collagen with that of more specific GPVI-activating agonists, such as convulxin and collagen-related peptide, it was concluded that GPVI plays a prominent role in the procoagulant reaction of collagen (13, 14). This agrees with the observation that variation in platelet GPVI content led to a corresponding change in GPVI-mediated prothrombinase activity (15).

Typically, in washed platelets, fibrillar collagen by itself is only a weak stimulus of the procoagulant platelet response. Co-activation of platelets with thrombin is required to obtain significant procoagulant activity with collagen (16, 17). This may imply that, in analogy to collagen-induced aggregate formation, next to GPVI additional receptors (and ligands) support PS expression and thrombin formation; candidate receptors are glycoprotein Ib-V-IX (VWF), integrins $\alpha2\beta1$ and $\alpha IIb\beta3$ (fibrinogen), and the autocrine mediators ADP and TxA_2. An indication that the adhesive receptors indeed contribute to collagen-induced procoagulant activity comes from the observation that adhesion per se enhances GPVI-induced PS expression (18). Yet, non-fibrillar, soluble collagens with limited GPVI binding sites and to which platelets adhere mainly via $\alpha2\beta1$ (19), are only weak stimulators of PS expression and thrombin formation (20), which questions a role of $\alpha2\beta1$ in this response. Further, in collagen-adherent platelets, the autocrine ADP and TxA_2 mediate integrin affinity changes and may thus influence collagen-induced PS expression (6, 10). Whether and how these adhesive receptors interact with collagen to stimulate procoagulant activity, and what the precise requirement herein is of the collagen (fibrillar) structure, are still unresolved issues.

Here, we set to study the contribution of collagen receptors and autocrine agents in thrombin generation with different collagens under physiologically relevant conditions, in platelet-rich plasma (PRP) that was triggered with a low dose of tissue factor. To investigate this, we compared type-I collagen preparations with different affinities for collagen receptors, i.e. Horm type-I collagen (used as reference) and fibrillar and non-fibrillar (soluble) collagens and, in addition, a collagen enriched in type-I fibers derived from human heart.

Material and methods

Materials

Abciximab (Reopro) was from Centocor (Leiden, The Netherlands); AR-C69931MX was kindly provided by Astra-Zeneca (Charnwood, UK). Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC) came from Bachem (Bubendorf, Switzerland), acetylsalicylic acid (aspirin) from Lorex Synthelabo (Maarssen, The Netherlands); unlabeled and Oregon Green-488 (OG488) labeled annexin A5 (annexin V) from Nexins Research (Hoeven, The Netherlands); recombinant human tissue factor from Dade (Miami, FL, USA). Human thrombin calibrator and thrombogram software were supplied by Synapse (Maastricht, The Netherlands). Other reagents came from Sigma (St. Louis, MO, USA). Fab fragments of monoclonal anti-human GPVI antibody 9O12 were produced and characterized as described (21). Anti-integrin $\alpha2\beta1$ mAb 6F1 was a kind gift from Dr. B. Coller (Mount Sinai Hospital, Boston, MA, USA).

Source and preparation of collagens

Fibrillar 'Horm' type I collagen, used as reference collagen preparation, was bought from Nycomed (Munich, Germany). Native fibrillar type-I collagen was prepared from bovine tendon, and pepsin-digested to obtain soluble (non-fibrillar) collagen, following a procedure described elsewhere (20). Tissue from healthy human heart, obtained by autopsy, was kindly provided by Dr. J. Cleutjens (Dept. of Pathology, Maastricht University). Permission was given by the local Medical Ethical Committee. Native total collagen fibrils were extracted from heart tissue, basically as described (22). Briefly, tissue was washed with phosphate-buffered saline containing protease inhibitors (10 mg ml^{-1} EDTA, 1 µg ml^{-1} pepstatin, 2 µg ml^{-1} aprotinin and 0.5 µg ml^{-1} leupeptin), homogenized, and extracted with 0.5 mol L^{-1} acetic acid. The mixture was vigorously shaken at 5°C for 24 h, then filtered and centrifuged at 48,400 g for 2 h. Collagen fibrils in the supernatant were precipitated by addition of 1.7 M NaCl and a 24-h incubation at 5°C. After 1 h of centrifugation at 35,000 g, the collagen-containing pellet was solubilized in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid. Fibrillar collagen content was determined by Sirius red staining.

Isolation of platelets and plasma

Blood was obtained from healthy volunteers after full informed consent according to the Helsinki declaration. Blood was drawn by venapuncture into 1/10 volume of 129 mM trisodium citrate. First 2.5 ml of blood were discarded. The blood was centrifuged at 260 g for 15 min to obtain PRP, and twice at 870 g for 10 min for platelet-free plasma (PFP). Platelet concentration was determined with a Beckman MicroDiff18 counter (Coulter Electronics, Luton, UK). PRP was normalized to 1.5×10^8 platelets ml^{-1} with autologous PFP.

Thrombin generation measurement

Thrombin generation was continuously measured in PRP using the thrombogram method essentially as described (23). Briefly, triplicate samples of 80 µl PRP (1.5×10^8 platelets ml^{-1}) were pipetted into wells of a 96-wells plate (Immulon 2HB, Dynex, Chantilly, VA, USA), containing 20 µl tissue factor (3 pmol L^{-1}) in buffer A (20 mM Hepes, 140 mM NaCl, 5 mg ml^{-1} BSA, pH 7.35). Plates were pre-warmed at 37°C in a well-plate reader (Molecular Devices), and pre-heated to 37°C for 5 min. Coagulation was started by automated addition of 20 µL of buffer B (20 mM Hepes, 0.1 M $CaCl_2$, 60 mg ml^{-1} BSA, pH 7.35) containing Z-GGR-AMC (2.5 mM). Final concentrations were 0.5 pmol L^{-1} tissue factor and 0.42 M Z-GGR-AMC. Fluorescence accumulation from cleaved AMC was continuously measured at excitation and emission wavelengths of 390 and 460 nm, respectively (37°C). First-derivative curves of accumulation of fluorescence were converted into curves of nanomolar thrombin using a human thrombin calibrator and by correction for the fluorescence due to α_2-macroglobulin-bound thrombin (24). PRP was pretreated with antibodies or inhibitors at maximally effective concentrations for 10 min (37°C). Thrombin generation

measurements were started at 10 min after collagen addition. All collagen samples were dialyzed against 10 mM acetic acid (overnight 4°C) before using for thrombin generation. Controls were always run with vehicle media.

The potency of collagen preparations to enhance platelet-dependent thrombin generation was evaluated from effects on the thrombin peak height. This thrombogram parameter is an indicator of the maximal rate of thrombin formation (23), and is sensitive to platelet activation (24).

Measurement of thrombus formation and PS exposure under flow

Flow experiments over collagen were performed at room temperature using blood anticoagulated with 40 μM PPACK (6, 25). Collagen preparations were spread on cleaned glass coverslips at an optimal density of 35 μg cm^{-2}. After coating for 1 h in a moist chamber, the coverslips were rinsed with saline, blocked during 30 min with Hepes buffer pH 7.45 (in mM: NaCl 136, glucose 10, Hepes 5, KCl 2.7, $MgCl_2$ 2, $CaCl_2$, 2), supplemented with 1% BSA, and washed again with saline. After staining with Sirius red, this resulted in about 40% area coverage with red colour. Whole blood was perfused for 4 min over the collagen surface at a moderately high wall-shear rate of 1000 s^{-1}. High-resolution transmission and fluorescent images were recorded in real-time with a Visitech digital imaging system (Sunderland, UK). PS exposure was detected by post-perfusion with rinsing Hepes buffer pH 7.45 containing 1 U ml^{-1} heparin and 1 μg ml^{-1} OG488-annexin A5.

Phase-contrast and fluorescent images were obtained from at least ten different collagen-containing microscopic fields, which were arbitrarily chosen. Area coverage from phase-contrast and fluorescent images was analyzed off-line, as described (6).

Statistics

Experiments were performed with PRP or blood from at least three different donors. Statistical indications were made using the Kruskal-Wallis test, followed by a Dunn test for comparison between treatments. Data are means±SE.

Results

Fibrillar but not soluble type I collagen enhances thrombin generation in platelet-rich plasma

Thrombin generation in PRP with normalized platelet count, triggered with a low concentration of tissue factor (0.5 pmol L^{-1}), relies on platelet activation and PS exposure (24). We determined the effects of several type-I collagen preparations on platelet-dependent generation of thrombin. Fibrillar Horm type-I collagen, which is mostly used as a reference collagen preparation to aggregate platelets (with fibers stabilized in an unpublished way), greatly enhanced the thrombin-generating process (Fig. 1A). Addition of 5 μg ml^{-1} Horm collagen to PRP resulted in a 1.70±0.06 fold (n=25) increase in thrombin peak height, which was accompanied by a shortening of the time-to-peak from 22.1±1.0 to 9.4±0.4 min in comparison to the control condition. Addition of 10 μg ml^{-1} annexin A5, a protein that specifically chelates PS (26), completely abolished thrombin generation in the presence of collagen (Fig. 1A). Platelet inhibition with cAMP-elevating PGE_1 also abolished the collagen effect, confirming it relied on activation of the platelets.

In comparison to Horm collagen, native type-I collagen fibrils (10 μM) were somewhat less effective in enhancing thrombin generation. Also with this purified fibrillar collagen, the time-to-peak was reduced and the peak height increased when compared to the control condition (Fig. 1B). On the other hand, a soluble type-I collagen – obtained by controlled proteolytic digestion of native type-I collagen fibers, lacking the telopeptides and the 'banded' quaternary structure of fibrillar collagen (20) -, was of little effect in stimulating thrombin generation. Dose-response curves showed that soluble collagen concentrations >20 μg ml^{-1} were weakly stimulatory, whereas the Horm and native type-I collagen fibers were already active at about ten-fold lower concentrations (Fig. 1C). Collagen that was purified from human heart tissue, enriched in type-I fibers, was also active in enhancing thrombin generation (Fig. 1D).

Principally GPVI determines the enhancement of thrombin generation by fibrillar collagen

To evaluate the importance of GPVI for the effect of collagen fibers, blocking Fab fragments were used of the mAb 9O12, directed against the collagen-binding site of human GPVI (21). Pre-incubation of PRP with 50 μg ml^{-1} 9O12 resulted in complete antagonism of the curve shift in thrombin generation induced by Horm type-I collagen (Fig. 2A). With both 9O12 Fab

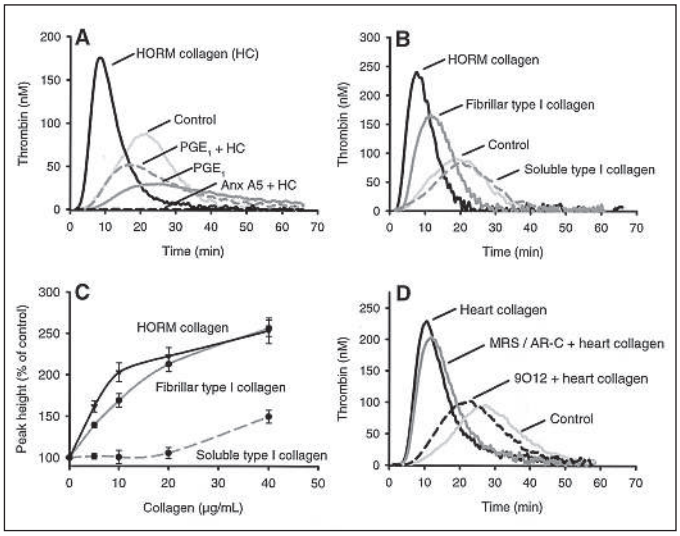

Figure 1: Effect of different collagen preparations on thrombin generation in PRP. Coagulation in PRP was initiated with 0.5 pmol L^{-1} tissue factor (f.c.) and 16.6 mM $CaCl_2$. (A) PRP was pre-incubated with vehicle (control) or activated with 5 μg ml^{-1} Horm type-I collagen (reference collagen, HC) for 10 min. Pretreatment before activation was with PGE_1 (10 μM) or annexin A5 (Anx A5, 10 μg ml^{-1}). (B) PRP was treated with vehicle (control) or was activated for 10 min with 10 μg ml^{-1} of either Horm type-I, fibrillar type-I collagen or soluble type-I collagen. (C) Dose-response of effect of collagens on thrombin peak height in thrombin generation (mean±SE, n=3–6). (D) Effect of collagen from heart tissue on thrombin generation. PRP was incubated with vehicle (control) or collagen from heart tissue (20 μg ml^{-1}); pretreatment was with anti-GPVI 9O12 (50 μg ml^{-1}) or MRS2179/AR-C69931MX (40 and 20 μM). Traces of thrombin generation are representative of three or more independent experiments.

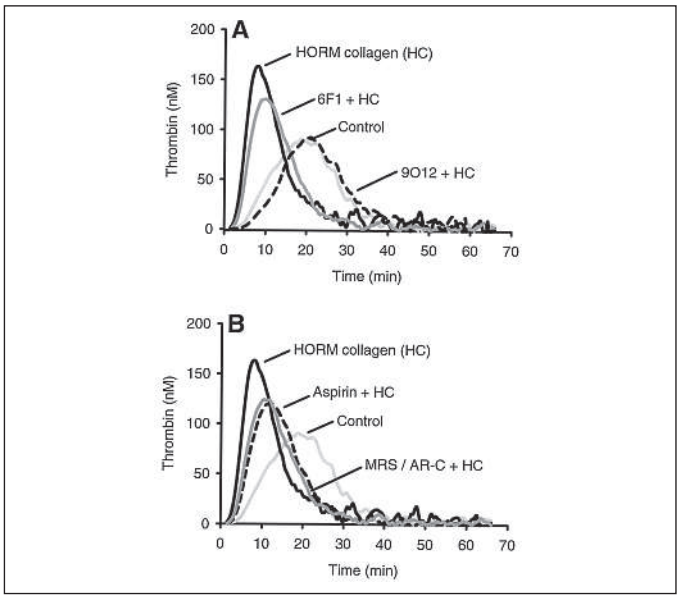

Figure 2: Contribution of GPVI, α2β1 and autocrine mediators to collagen-enhanced thrombin generation. PRP was incubated with vehicle (control) or activated with 5 μg ml^{-1} Horm type-I collagen (HC). Coagulation was initiated with tissue factor/$CaCl_2$ (see Fig. 1). (A) Pretreatment with anti-GPVI Fab 9O12 (50 μg ml^{-1}) or anti-α2β1 mAb 6F1 (10 μg ml^{-1}) before collagen activation. (B) Pretreatment with aspirin (100 μM) or ADP receptor blockers (40 μM MRS2179 and 20 μM AR-C69931MX) before activation. Curves are representative of at least three independent experiments.

and collagen present, thrombin generation was delayed and reduced to the level of untreated, control PRP (Table 1). The blocking anti-α2 mAb 6F1, active in plasma, was used to abolish the contribution of this integrin to collagen-induced activation (6). Differently from 9O12, pretreatment of PRP with saturating 6F1 resulted in an incomplete, 25% antagonism of the stimulation of thrombin generation by collagen (Fig. 2A and Table 1). Control experiments indicated that neither 9O12 nor 6F1 by itself altered thrombin generation in the absence of collagen.

Table 1: Effect of blocking agents on collagen-enhanced thrombin generation. PRP was untreated (control) or activated with 5 μg ml^{-1} Horm type-I collagen. Pretreatment was with anti-GPVI 9O12 Fab (50 μg ml^{-1}), anti-α2β1 6F1 antibody (10 μg ml^{-1}), aspirin (100 μM), MRS2179 (40 μM) and/or AR-C69931MX (AR-C, 20 μM), before activation with collagen. Thrombin generation was measured upon triggering with tissue factor/$CaCl_2$ (see Fig. 1). Data are compared to the control condition without collagen or antagonist (mean±SE, *n* experiments in triplicate).

	Time-to-peak (min)	Peak height (% of control)
Control	22.1±1.0	100 (25)
Horm type-I collagen		
+ vehicle	9.2±0.4	169.4±5.6 (20)
+ 9O12	16.7±1.2	95.2±6.6 (5)
+ 6F1	9.5±0.6	150.8±11.1 (4)
+ aspirin	10.5±1.0	144.8±12.8 (6)
+ AR-C69931MX	10.7±1.1	136.7±18.8 (4)
+ MRS2179	9.1±0.8	168.4±8.3 (6)
+ MRS + AR-C	10.9±0.7	133.8±12.3 (5)

The secondary mediators, TxA_2 and ADP contribute to the thrombin generation in tissue factor-triggered PRP (24, 27). To determine their involvement in the collagen effect, PRP was pretreated with aspirin and/or maximally effective doses of the $P2Y_1$ and $P2Y_{12}$ receptors antagonists, MRS2179 and AR-C69931MX, respectively. Aspirin pre-treatment resulted in partial reversal of the collagen effect on the thrombin peak (Fig. 2B); this is similar to the small inhibitory effect of aspirin, reported earlier in the absence of collagen (24). Also, pretreatment with AR-C69931MX (but not with MRS2179), caused a small, reducing effect on the thrombin peak (Table 1). This observation corresponds well with studies reporting that – in the absence of collagen – only $P2Y_{12}$ contributes to platelet procoagulant activity in man and mouse (27, 28). Together, these results indicate that the collagen-enhanced thrombin generation completely relies on GPVI activity, but can be reinforced by α2β1 binding and by release of autocrine TxA_2 and ADP.

Activated integrin α2β1 potentiates GPVI-mediated thrombin generation

In comparison to type-I collagen fibers, digested non-fibrillar collagen forms are more strongly dependent on α2β1 for platelet binding (13, 20). The divalent cation Mn^{2+} switches α2β1 and other integrins to a higher affinity state by exposing a cation- and ligand induced binding site (CLIBS) (29, 30). Others have shown that the affinity of platelet α2β1 for soluble collagen is significantly increased by Mn^{2+} treatment (31). We found that incubation of PRP with 2 mM $MnCl_2$ enhanced the effect of the soluble collagen (5 μg ml^{-1}) on thrombin generation; the Mn^{2+} ions caused a marked decrease in time-to-peak and a 1.12±0.07 fold increase in thrombin peak height (Fig. 3A). The stimulation by soluble collagen plus Mn^{2+} was partly reversed by the anti-α2 6F1 mAb and more than completely antagonized by anti-GPVI 9O12 fragments (Fig. 3B-C). In the latter case, the extra inhibition was not due to the simultaneous presence of Mn^{2+} and 9O12, as this combination alone did not influence thrombin generation. Possibly, the soluble collagen plus Mn^{2+} triggers a platelet-inhibiting pathway. Together, these results point to complete dependency of the stimulating collagen/Mn^{2+} effect on GPVI activity.

When Horm type-I collagen fibers were used instead of soluble collagen, pretreatment of PRP with Mn^{2+} resulted in a similar curve shift. Again, Mn^{2+} ions caused a 1.21±0.09 fold increase in thrombin peak height (Fig. 3C). The collagen/Mn^{2+} effect was largely inhibited by anti-α2 antibody and completely inhibited by anti-GPVI 9O12 Fab. To determine whether α2β1 contributes to the thrombin generation with GPVI-specific agonists, PRP was stimulated with convulxin (100 ng ml^{-1}), which is a snake peptide causing direct GPVI activation (32). In agreement with earlier data (24), convulxin treatment resulted in a 2.99±0.05 fold (*n*=4) increase in the thrombin peak. In the presence of anti-α2β1 mAb 6F1, the convulxin effect was hardly changed to a 2.89±0.04 fold increase, pointing to absence of integrin activity. Together, these experiments thus indicate that α2β1 contributes to the effects on thrombin generation of fibril-

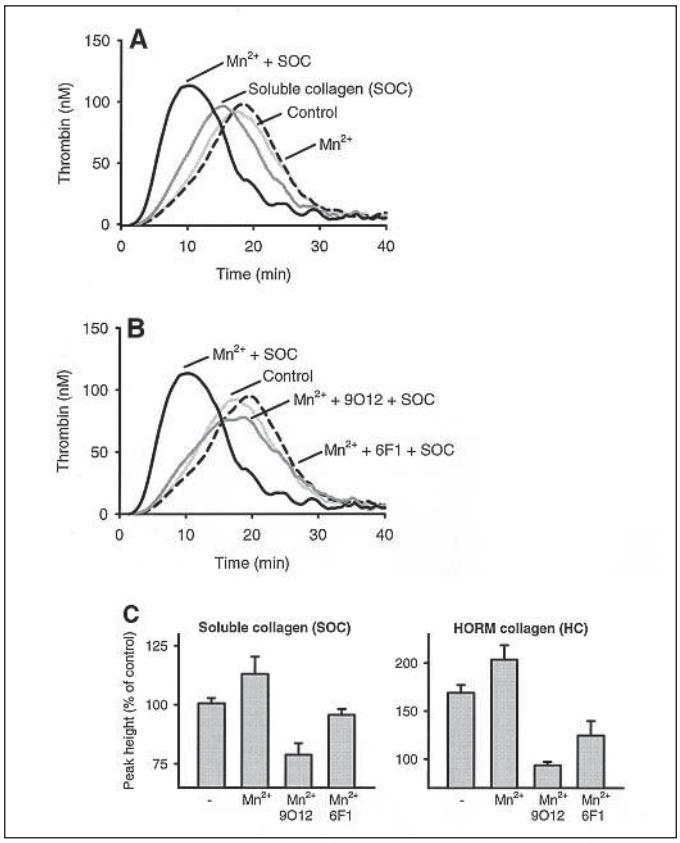

Figure 3: Potentiation of GPVI-mediated thrombin generation by activated integrin α2β1. (A) PRP was incubated with vehicle (control) or activated with 5 μg ml^{-1} soluble type-I collagen (SOC); where indicated, the PRP was pretreated with 2 mM $MnCl_2$ (Mn^{2+}) before activation. Thrombin generation was then measured upon triggering with tissue factor/$CaCl_2$, as in Fig. 1. (B) Conditions as for panel A, but PRP was pretreated with Mn^{2+} plus 10 μg ml^{-1} 6F1 or 50 μg ml^{-1} Fab 9O12 before activation with SOC. (C) Quantitative effect of treatments on thrombin generation with SOC or Horm type-I collagen (each 5 μg ml^{-1}). Data are percentages relative to the thrombin peak-height under control conditions taken as 100% (mean±SE, n=4–6). *P<0.05.

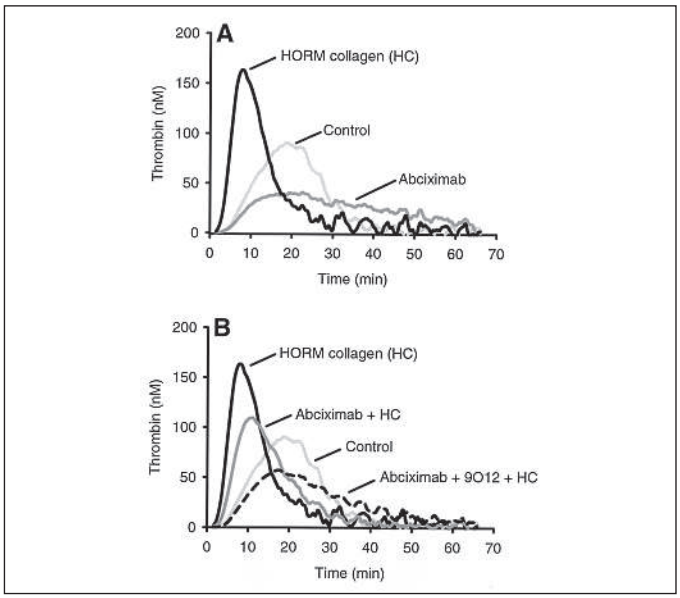

Figure 4: GPVI-enhanced thrombin generation is not mediated by integrin αIIbβ3. (A) PRP was incubated with vehicle (control), 5 μg ml^{-1} Horm type-I collagen (HC), or 40 μg ml^{-1} abciximab. (B) PRP was pretreated with abciximab alone or in combination with 50 μg ml^{-1} anti-GPVI Fab 9O12, before activation with Horm collagen. Thrombin generation was measured after triggering with tissue factor/$CaCl_2$. Curves are representative of three or more independent experiments.

lar and non-fibrillar collagens but not of convulxin, likely by enhancing the activating effect of platelet GPVI.

Integrin αIIbβ3 is not directly involved in collagen-enhanced thrombin generation

Platelets adhere to collagen-associated proteins like VWF via integrin αIIbβ3. Antagonists of αIIbβ3 such as abciximab along with VWF antagonists have been reported to suppress platelet-dependent thrombin generation (33–36). In the absence of collagen, we found that abciximab greatly delayed and diminished the formation of thrombin at a maximally effective dose of 40 μg ml^{-1} (Fig. 4A). In combination with Horm collagen (Fig. 4B), abciximab slightly prolonged the time-to-peak from 9.2±0.4 to 11.8±0.9 min (n=6); and it partially lowered the thrombin peak (from 169.4±5.6% to 121.3±6.9% of control level, P<0.01). Notably, in combination with abciximab, anti-GPVI 9O12 further diminished the thrombin generation to give a trace similar to that of abciximab alone (62.7±12.2% of control level). Thus, treatment with 9O12, but not abciximab, was needed to completely antagonize the collagen-enhancing effect on thrombin generation. These results indicate that the enhancing effect of GPVI is mostly not mediated by αIIbβ3 and, thus, that the effect of the integrin relies on a distinct mechanism.

Collagen enriched in type-I from human heart tissue enhances thrombin generation via GPVI

In human myocardium, collagen is abundantly present as a network of mostly type-I fibrils with fewer type-III fibrils (37). For comparison of the above results with bovine type-I collagen, we purified a collagen preparation from human heart tissue. Microscopic observation of this preparation, spread on glass, showed intense Sirius red staining with thin collagen fibers. At maximally effective concentration, this fibrillar collagen significantly enhanced thrombin generation in PRP: time-to-peak shortened with 15.3±2.1 min and peak height increased by 1.8±0.4 fold (Fig. 1D). The procoagulant effect of the heart collagen was almost completely inhibited with anti-GPVI mAb 9O12. Thus, GPVI appears to be a key receptor in the procoagulant activity of this collagen derived from the human cardiovascular system.

Type-I collagens differently stimulate PS exposure on adherent platelets under flow

Using the fibrillar Horm collagen, it has been described that GPVI is the major collagen receptor regulating thrombus formation of human and mouse platelets under flow conditions (6, 25). Using moderately high shear conditions (4 min at 1000 s^{-1}), checked to be sensitive to GPIb blockade, we performed a series of flow experiments where human blood was perfused

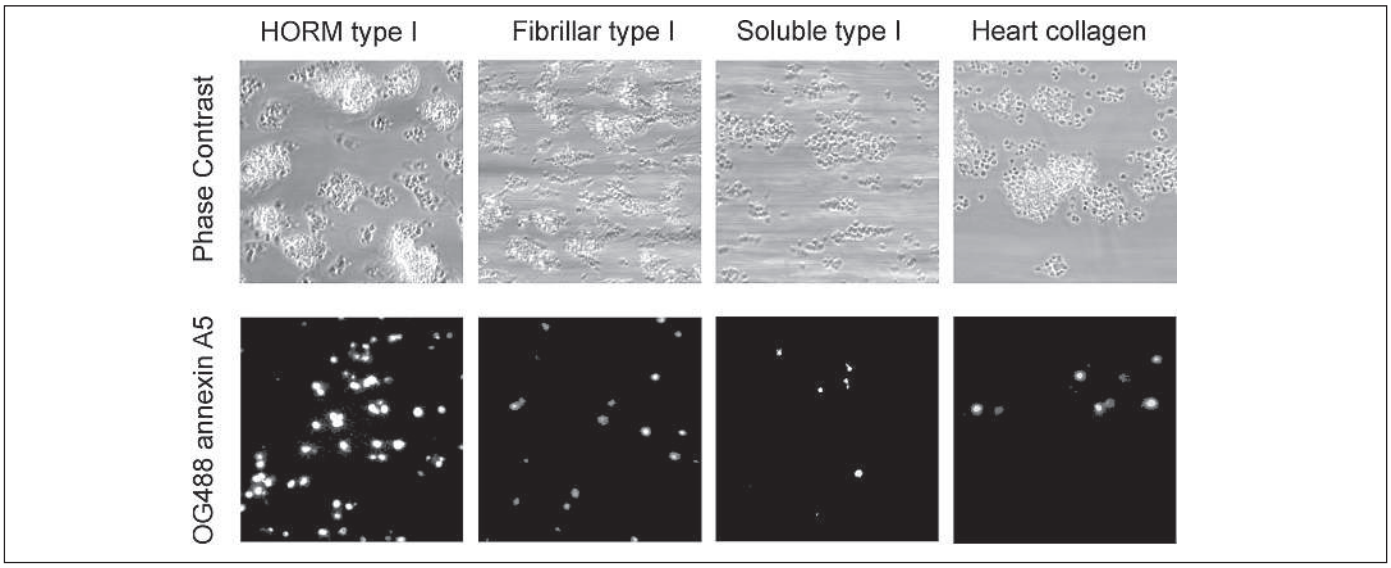

Figure 5: Effect of various collagens on platelet deposition and PS exposure under flow. Whole blood, anticoagulated with 40 μM PPACK, was perfused for 4 min at a shear rate of 1000 s^{-1} over Horm type-I collagen, fibrillar type-I fibers, soluble type-I collagen, or collagen from human heart tissue (each spread at 35 μg cm^{-2}). Adhered platelets were stained post-perfusion with OG488-annexin A5 (1 μg ml^{-1}). Shown are representative phase contrast (120×120 μm) and fluorescent (150×150 μm) images after perfusion.

over surfaces coated with the different collagen preparations. With all collagen forms, it appeared that platelet deposition increased with the spreading density up to a spreading concentration of 30–40 μg cm^{-2}, at which platelet deposition was maximal under this experimental condition (data not shown). At this density (35 μg cm^{-2}), staining of the collagen surfaces with Sirius red showed a dense, uniform distribution of red fibers (for fibrillar collagens) or of small red spots (soluble collagen). After 4-min perfusion of blood over the fibrillar Horm collagen and (bovine) type-I collagen surfaces, multi-layered platelet aggregates were detected (Fig. 5). Perfusion of the blood over soluble type-I collagen resulted in formation of smaller aggregates, while perfusion over human heart collagen gave aggregates of variable sizes. Total surface area covered by adherent platelets – earlier demonstrated to increase about linearly in time (10) – was similar for all (fibrillar and soluble) collagens at this time point (Table 2). Staining with OG488-annexin A5, to measure PS expression, was typically higher for Horm collagen (10.5% of surface area covered with fluorescence) than for the bovine fibrillar type-I collagen (3.8% coverage with fluorescence); annexin A5 staining was still lower for the heart (0.7%) and soluble type-I (0.25%) collagen (Fig. 5 and Table 2).

GPVI blockade by 9O12 Fab severely impaired aggregate formation (not shown) and PS exposure (Table 2) on all collagens. However, 9O12 did not reduce platelet adhesion, except for heart collagen (Table 2). Platelet adhesion to all collagens was abolished by combined blockade of GPVI with 9O12 and α2β1 with 6F1 mAb (not shown), such as was demonstrated earlier for Horm collagen (10). This suggested that the heart collagen was limited in adhesion sites for (unstimulated) α2β1, in a similar way as recently found for (type I) collagen in human plaque tissue (38). GPVI-dependent adhesion of platelets to the plaque collagen can be suppressed by blocking of the ADP receptors, e.g. causing α2β1 activation (38). To determine whether this was also the case for the GPVI-dependent adhesion to heart collagen, flow experiments were performed in the presence of MRS2179 (40 μM) and AR-C69931MX (20 μM). Indeed, ADP receptor blockade resulted in a greatly reduced platelet coverage with heart collagen (12±2% of control, mean±SE, n=3). In contrast, the blockers were much less inhibiting on platelet adhesion to Horm and fibrillar type-I collagens (131±16% and 89±9% of controls, respectively).

Table 2: Effect of anti-GPVI treatment on collagen-induced platelet adhesion and PS exposure under flow. Whole blood, anticoagulated with 40 μM PPACK, was perfused for 4 min at shear rate of 1000 s^{-1} over various collagens (see Fig. 5). The blood was pretreated for 10 min with anti-GPVI 9O12 (50 μg ml^{-1}). Adhered platelets were stained post-perfusion with OG488-annexin A5 (0.5 μg ml^{-1}). Data show surface area coverage of all platelets (phase contrast) and of fluorescent-labeled platelets (mean±SE, *n*=3–4 experiments with blood from different donors).

	Phase contrast	OG488-Annexin A5
	(% surface area covered)	
Horm type-I collagen	16.4±1.3	10.5±1.5
+ 9O12	15.6±1.2	1.6±0.4
Fibrillar type-I collagen	22.8±4.3	3.8±1.0
+ 9O12	19.0±3.2	0.3±0.04
Soluble type-I collagen	11.9±3.8	0.23±0.11
+ 9O12	15.9±0.9	0.04±0.02
Heart collagen	25.5±2.4	0.7±0.04
+ 9O12	2.2±0.6	0.1±0.02

Discussion

The present data show that especially fibrillar Horm and bovine type-I collagens are potent stimulators of platelet-dependent thrombin generation in PRP. Thus, under conditions of coagulation triggered with tissue factor, platelet interaction with fibrillar collagens greatly enhances the rate of thrombin generation (correlated with the thrombin peak-height). This potentiating effect of the fibrillar collagens is completely abolished by treatment of PRP with the blocking anti-GPVI mAb 9O12. Accordingly, platelet activation by the signaling collagen receptor GPVI plays a key role in this procoagulant collagen effect, which is in agreement with earlier observations that GPVI causes PS expression on platelets and stimulates prothrombinase activity (3, 13, 21, 25). We further show that the effect of fibrillar collagen on thrombin generation is antagonized by platelet inhibition with PGE_1 and by scavenging PS with annexin A5, indicating that it relies on platelet activation and subsequent PS expression.

We also find that a fibrillar collagen preparation from human heart, enriched in type I and fewer type III collagens, significantly stimulates thrombin generation in the presence of platelets. Also in this case the stimulating effect is blocked by anti-GPVI mAb and thus relies on GPVI. On the other hand, a soluble form of bovine type I collagen, lacking the telopeptides and the typical striated morphology of fibrillar collagen, requires prior activation of $\alpha2\beta1$ (with Mn^{2+}) for stimulation of thrombin generation. The stimulation also in this case ultimately relies on GPVI, as is concluded from the complete suppression by anti-GPVI mAb.

Soluble type I collagen expresses multiple $\alpha2\beta1$ binding sites (8, 20). It has been described that Mn^{2+} ions and weak agonists such as ADP increase the binding of this collagen to platelets by changing $\alpha2\beta1$ to an intermediate, higher affinity conformation (9). Our results indicate that, not only with soluble collagen but also with fibrillar type I collagen, pre-activation of the integrin with Mn^{2+} ions enhances thrombin generation. Because GPVI blockade completely reverses the effect of collagen/Mn^{2+}, GPVI must act as a principal platelet receptor in all collagen-enhanced procoagulant activity. In a different context, it has already been observed that integrin activation in interplay with human and mouse GPVI regulates platelet aggregation and thrombus formation under conditions where coagulation was absent (3–6). These present results significantly extend this work by showing that activated $\alpha2\beta1$, also under coagulant conditions, enhances the signaling function of GPVI likely by reinforcing platelet contact with collagen. In contrast, they do not provide evidence for a direct signaling effect via $\alpha2\beta1$ in the collagen-stimulated thrombin generation.

In the absence of collagen, the secondary mediators, TxA_2 and ADP, have a modest stimulatory effect on platelet procoagulant activity, notably via stimulation of $P2Y_{12}$ receptors (27, 28). This appears also to be true in thrombin generation measurements with collagen present (Table 1), possibly by enforcing platelet-collagen interaction. This modest effect differs from the regulation of platelet aggregation, where autocrine TxA_2 and ADP are considered to be relatively important (39).

Various studies have reported that integrin $\alpha IIb\beta3$ has a supporting role in platelet-dependent thrombin generation (11, 33). It is also proposed that the $\alpha IIb\beta3$ antagonist abciximab affects the procoagulant activity of platelets adhered to collagen (36). We note a clear lowering effect of abciximab on thrombin generation levels both in the absence and presence of collagen. In addition, we find that the enhancing effect of collagen on thrombin generation persists in the presence of $\alpha IIb\beta3$ antagonism with abciximab, which makes it unlikely that the integrin is directly involved in the platelet-collagen interactions implicated in procoagulant activity. Abciximab then may act by interfering in the interaction of platelets with fibrin(ogen) or coagulation factors.

In general, we find a correlation between collagen-stimulated, PS-dependent thrombin generation in PRP and collagen-induced PS exposure under flow (in the absence of coagulation). In either assay, fibrillar type I collagens are more active than the soluble collagen. Only the heart-derived collagen is less active in causing PS exposure (but not aggregate formation) under flow than is predicted from the enhancing effect on thrombin generation. On the other hand, with all collagens including the heart collagen, thrombin generation as well as platelet activation (PS exposure) and aggregate formation under flow are completely dependent on GPVI activity.

The differences in effects of the collagen preparations are likely to reside in the contribution of $\alpha2\beta1$. We find that only for heart collagen adhesion under flow relies on GPVI. Similarly to the situation with plaque-derived collagens (38), this may point to a low number of interaction sites for the (unactivated) $\alpha2\beta1$ integrin. Adhesion to the other collagens is not exclusively dependent on GPVI, such in agreement with previous findings with Horm collagen (6, 10). However, as described elsewhere, for human as well as murine platelets, GPVI activity is likely to play a role in the stable adhesion to collagens (40). Others have shown that soluble type-I collagen relies more strongly on $\alpha2\beta1$ for platelet adhesion and thrombus formation than 'banded', fibrillar collagen (8). The present results are compatible with the presence of a significant number of interaction sites for (unactivated) $\alpha2\beta1$ in both soluble and fibrillar collagen, which seem to variably allow GPVI-collagen interaction. Together, the results depict a physiological mechanism, in which GPVI interaction with collagen fibers, supported by $\alpha2\beta1$, plays a central, bridging role in not only primary thrombus formation, but also platelet-dependent coagulation.

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