C1q-TNF Related Protein-1 (CTRP-1), a Vascular Wall Protein that Inhibits Collagen-Induced Platelet Aggregation by Blocking vWF Binding to Collagen

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EDITORIAL NOTE
G Lasser, J Ellsworth, P Bishop, J Fruebis designed and/or performed research and analyzed data. J Fruebis wrote the paper.
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P Guchhait, M Cruz and J Lopez designed and performed all studies done under flow conditions, in addition studies involving $\alpha_2\beta_1$ and selected studies under static conditions. In addition, J Lopez provided valuable expertise to the general design.

ABBREVIATIONS
CTRP-1, C1qTNF-related protein-1; CRP, collagen-related peptide; vWF, von Willebrand’s Factor; ECM, Extracellular Matrix; PRP, Platelet-rich plasma; CVF, cyclic flow variations; RU, Relative Units; ACT, activated clotting time;
ABSTRACT

CTRP-1 is a novel member of the C1q-TNF Related Protein family containing family characteristic collagen and TNF-like domains and shows marked expression in vascular wall tissue. We observed that recombinant human CTRP-1 specifically bound to fibrillar collagen and blocked collagen-induced platelet aggregation. CTRP-1 completely or partially prevented vWF and GPVI-Fc4 binding to collagen, respectively. However, GPVI-Fc4 failed to compete for the binding of CTRP-1 to collagen. CTRP-1 had no effects on α2β1 integrin I-domain binding to collagen. Using whole human blood under flow at low and high shear rates, CTRP-1 prevented platelets from accumulating on a collagen-coated surface but had no effects on ‘platelet-rolling‘ on a surface coated with vWF. These data suggest that CTRP-1 prevents collagen-induced platelet aggregation by specific blockade of vWF binding to collagen. By using the Folts vascular injury model in non-human primates (Macaca fascicularis), we were able to demonstrate that CTRP-1 can prevent platelet thrombosis in vivo. This effect was achieved in the absence of changes in activated-clotting time (ACT) and template cut bleeding times, suggesting CTRP-1 has promising anti-platelet thrombotic activity and most likely acts by pacifying the thrombogenic site of vascular injury.
INTRODUCTION

C1qTNF related protein-1 (CTRP-1) belongs to a family of proteins characterized by a common TNF alpha-like globular domain. A second, less conserved structural element in this family is the N-terminal collagen-like region with typical Glycine-X-Y repeats. The basic structure of proteins in the CTRP family appears to be a trimer that in turn can form higher order structures. While structurally related, members of this protein family are functionally diverse and include the plasma protein C1q that is involved in immune functions and possibly platelet hemostasis, Adiponectin and the hibernation proteins 20, 25, 27, which are thought to be regulators of metabolism. Other CTRP family members appear to have more structural or extracellular matrix-related functions, e.g. collagen types VIII and X, and CTRP-5.

Since C1q may play a role in collagen-induced platelet activation, we investigated whether CTRP-1 might exhibit similar properties. Initial studies indicated that CTRP-1 had affinity for collagen and blocked collagen-induced platelet activation. This raises the potential significance of CTRP-1 for human disease because of the central role collagen-induced platelet activation and thrombus formation plays in acute cardio- and cerebrovascular events. Spontaneous rupture of atherosclerotic plaque or medical interventions can cause an injury to the arterial endothelial lining thus exposing the subendothelial extracellular matrix to circulating blood. Collagens are a major component of the extracellular matrix and can directly and indirectly activate platelets. Three platelet receptors are thought to mediate these interactions. Integrin α2β1 is thought to have a major role in adhesion and platelet anchoring; GPVI, a member of the Ig superfamily is responsible for collagen binding, signalling and platelet activation, and GP Ib-V-IX (GPIb), through its indirect binding to collagen-bound ‘von Willebrand’s factor’ (vWF), enables the initial ‘rolling’ of platelets on a collagen-containing surface under high shear conditions.

In the present work, the mechanism by which CTRP-1 interferes with activation and aggregation of platelets by collagen was investigated and the efficacy to prevent platelet thrombosis in vivo was tested.
MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (University of Washington Regional Primate Center).

Protein expression, purification and characterization. Full-length recombinant human CTRP-1 was produced in CHO-DG44 cells transformed with a pZMP18-CTRP-1 expression vector. CTRP-1 was purified to homogeneity from the cell culture medium by sequential chromatographic steps and was characterized by various methods including amino acid analysis, N-terminal sequencing and peptide mapping. CTRP-1 was dissolved in PBS and stored at -80°C.

Expression and purification of soluble GPVI-Fc4 fusion proteins. Expression constructs encoding two versions of soluble human GPVI-Ig fusion proteins were generated by overlap PCR and homologous recombination in yeast. The first construct encoding GPVI-wtγ1Fc was modeled after the version described. The second version encoding GPVI-Fc4 contained the same mouse κ signal sequence and GPVI extracellular domain sequences, but included a modified human IgG1 Fc containing amino acid substitutions which prevent FcγRI binding and complement fixation.

Preparation of 125I-CTRP-1 and 125I-GPVI-Fc4. Labeling of CTRP-1 and GPVI-Fc4 with 125I was accomplished by a modified version of the indirect Iodogen® labeling procedure as described by the manufacturer (Pierce Chemical, Rockford, IL). CTRP-1 and GPVI-Fc4 were labeled to specific activities of 4300 and 4900 cpm/ng, respectively. More than 95% of the cpm in the final preparations were precipitated in 10% TCA.

Synthesis and cross-linking of collagen-related peptide (CRP). CRP was synthesized (GKPGPPGPPGPPGPPGPPGPPGPPGPPGPPGPPGPPGPPGPPGPKGV) and cross-linked with glutaraldehyde as described. Valine was added to the sequence to improve recovery during resin cleavage, a molar ratio of CRP:glutaraldehyde of 1:290 was used. The majority of the cross-linked product was 30 kDa or larger.

Preparation of collagen, BSA, or CRP coated plates. MaxiSorp microtiter plates (Nunc #473768) were coated overnight at 37°C with equine fibrillar collagen I (Chrono-Par #385,
Chrono-Log, Havertown, PA) or bovine collagen VI (Chemicon, CC086, Temecula, CA) using collagen dissolved in 285 mM glucose, pH 2.7-2.9 to a final concentration of 0.01 mg/mL. Control plates were coated with an identical concentration of BSA dissolved in PBS. Dry, coated plates were stored at 4°C and were blocked for 1 h at room temperature (0.05 M Na phosphate, 0.109 M NaCl, 0.1% BSA, pH 7.5) prior to use. Reacti-Bind Maleic Anhydride clear strip plates (Pierce #15100) were coated with cross-linked CRP dissolved in PBS to a final concentration of 5 µg/mL. Prior to use, peptide-coated plates were blocked with Super Block (Pierce Chemical) in TBS for 1 h at room temperature.

**Binding of ¹²⁵I-CTRP-1 and ¹²⁵I-GPVI-Fc4 to collagen or CRP-coated plates.** The indicated concentration of ¹²⁵I-CTRP-1 or ¹²⁵I-GPVI-Fc4 was incubated in the absence (total binding) and presence (non-specific binding) of 250 µg/mL unlabeled CTRP-1 or GPVI-Fc4 in a final volume of 0.2 mL blocking buffer (0.05 M Na phosphate, 0.109 M NaCl, 0.1% BSA, pH 7.5). For competition of ¹²⁵I-GPVI-Fc4 binding to cross-linked CRP, each well was incubated with 0.05 µg/mL ¹²⁵I-GPVI-Fc4 in the absence (total binding) or presence (non-specific binding) of the indicated fold-excess of unlabeled GPVI-Fc4. Blocking buffer for CRP binding also contained 0.025% Tween. Plates were gently agitated for 3 h at room temperature before being placed on ice. Media were removed; each well was washed three times with 0.2 mL of ice cold blocking buffer, and radioactivity was measured. Binding data were analyzed by non-linear regression using Prism (GraphPad, San Diego, CA).

**Competition for collagen binding under static conditions.** 96-well plates were coated with 1 µg Type I (Sigma #885-1) or Type III (Sigma #C4407) collagen dissolved in 0.1 mL Tris-buffered saline (TBS) overnight at room temperature. Plates were subsequently blocked with 2% BSA in TBS for 1 h at 37°C. A mixture of either purified A1 (0.75 µM) or A3 (0.75 µM) domains of vWF, full length vWF (0.75 µM), or purified I-domain (0.75 µM) of α2β1 integrin were incubated in the presence and absence of CTRP-1 in a final volume of 0.2 mL TBS. After 1 h at 37°C, incubation mixtures were removed and the wells were washed with TBS. Collagen-bound A1 and A3 were detected by the addition of CR-1 (0.3 µg/mL) and anti-His HRP (1/2500, Sigma) antibodies, respectively. The α2-I domain was detected by the addition of anti-His HRP antibody (1:2500) and vWF was detected by the addition of anti-vWF-HRP antibody (1:500, DAKO, Carpinteria, CA) and visualized using secondary anti-mouse HRP (1:2500, Pierce) and OPD reaction solution.
Collagen-induced platelet aggregation. Collagen-induced platelet aggregation was performed using human platelet-rich-plasma (PRP). CTRP-1 was mixed with PRP by gentle rocking in a 96-well flat bottom plate. Collagen-I was added at a final concentration of 1.25 µg/mL and the plate was agitated at 37ºC on the microplate reader, turbidity was monitored as percent light transmitted at 632 nm after 21.5 min as described previously23. Platelet aggregation was determined in an aggregometer and platelet activation by measuring ATP release using a Chrono-Lum® luciferase reagent (Chrono-Log Col) following the manufacturer’s instructions.

Platelet Aggregation Under Flow Conditions. The parallel plate flow chamber24 was composed of a polycarbonate slab, a silicon gasket, and a glass coverslip coated with type I collagen (Helena Labs, Beaumont, TX). Coverslips were incubated with a solution of 100 µg collagen or vWF/mL for 1 h at 37ºC. Citrated whole blood alone or with 20 µg CTRP-1/mL added was incubated for 5 min at room temperature, the platelets were labeled by incubating the blood sample with 0.2M mepacrine for 10 min in the dark, and the blood was perfused over the slide containing the immobilized collagen. A syringe pump was connected to the outlet port that drew blood through the chamber at defined flow rates (13 and 50 dynes/cm²) to generate a wall shear. The flow chamber was mounted onto an inverted stage microscope equipped with a high speed digital camera. Images were analyzed offline using MetaMorph Imaging System.

Folts vascular injury and bleeding models. The anti-thrombotic efficacy of CTRP-1 was tested in non-human primates (Macaca fascicularis) using the Folts model of vascular injury25. The right carotid artery was surgically exposed by blunt dissection and a flow probe (Transonic Systems, Ithaca, NY) was placed around the artery. After determining baseline blood flow, a partial stenosis (85-90% flow) was established by placing a nylon ring around the vessel proximal to the flow probe. A 2-4 mm wide crush-type injury was performed on the exposed artery, a stenotic ring was then positioned over the crush area, and blood flow was monitored. Each time blood flow approached zero (<0.7 mL/min), the vessel was lightly tapped to dislodge occluding thrombi, thereby creating cyclic flow variations (CFV). Once CFV were confirmed, animals were treated with the indicated amount of protein. Tapping the occluding blood vessel was ceased 30 min following treatment. Bleeding effects were examined by measuring activated clotting time (ACT) using the SCA2000 Coagulation
Analyzer (Synbiotics, San Diego, CA). In addition, template bleeding cuts were made on the forearm and time to hemostasis was determined (Surgicutt, ITC, Edison, N.J.).

RESULTS

CTRP-1 prevents collagen-induced platelet activation and aggregation. Incubation of PRP with fibrillar collagen type I produced a rapid activation and aggregation of platelets (Fig.1). Both processes were inhibited by CTRP-1 with virtually complete inhibition at CTRP-1 concentrations ≥ 10 µg/mL (Fig.1). CTRP-1 had no significant effects on activation of platelets by ADP, ristocetin or thrombin (data not shown).

Binding of $^{125}$I-CTRP-1 to collagen type I under static conditions. To evaluate whether CTRP-1 inhibition of collagen-induced platelet aggregation was due to CTRP-1 binding to collagen, the binding of $^{125}$I-CTRP-1 to immobilized fibrillar collagen type I was investigated. Binding of $^{125}$I-CTRP-1 to collagen I was saturable and specific (Fig.2). In contrast, little or no binding of $^{125}$I-CTRP-1 was observed to wells coated with an equivalent concentration of BSA (data not shown). Scatchard plots of the specific binding data were linear (Fig.2, inset), consistent with $^{125}$I-CTRP-1 binding to a single class of sites. $^{125}$I-CTRP-1 bound to fibrillar collagen type I with a $K_d$ and $B_{max}$ of $8.78 \pm 1.56 \mu g/mL$ and $493.3 \pm 150.5$ ng bound/well, respectively (mean ± SD, $n=4$ separate experiments). Thus, $^{125}$I-CTRP-1 bound to immobilized collagen type I with a $K_d$ of approximately 50 nM. The stoichiometry of $^{125}$I-CTRP-1 binding to collagen could not be readily determined since the amount of collagen bound to each well was not measured.

To evaluate whether the binding of $^{125}$I-CTRP-1 to collagen I was reversible, collagen I coated wells were first incubated for 3 h at 4°C with blocking buffer containing $^{125}$I-CTRP-1 alone and were then washed. Further incubation in the absence of unlabeled CTRP-1 resulted in little (14% of the total) of the previously bound $^{125}$I-CTRP-1 being released from the collagen I coated plates over a subsequent three-hour period. Incubation with increasing amounts of unlabeled CTRP-1, in contrast effectively displaced the collagen bound $^{125}$I-CTRP-1 with quantitative recovery of radiolabeled material in the media (data not shown). These data demonstrate that binding of $^{125}$I-CTRP-1 to fibrillar collagen type I was saturable, specific, reversible and of relatively high affinity.
**Inhibition of platelet adhesion on collagen I coated surfaces by CTRP-1 under high shear.** To evaluate whether CTRP-1 can inhibit collagen-induced platelet aggregation under biological conditions of high shear seen typically in small and large arterial vessels, vehicle alone or vehicle containing 20 µg/mL CTRP-1 was mixed with citrated whole blood from normal human donors and perfused through a collagen I coated flow chamber at 13 dynes/cm² (low shear) and 50 dynes/cm² (high shear). Flow of whole blood over the collagen-coated surface at high shear immediately produced adhesion and deposition of mepacrine-labeled platelets on the surface easily visualized under the microscope (Fig.3, upper left panel). Incubation of whole blood for 5 min at room temperature with 20 µg CTRP-1/mL prior to pumping through the flow chamber abolished the adhesion and deposition of platelets on the collagen coated slide (Fig.3, upper right panel). Similar results were seen with flow at 13 dynes/cm² (data not shown). Thus, CTRP-1 can inhibit collagen-induced platelet deposition under conditions of low and high rates of shear.

**Effect of CTRP-1 on the binding of vWF to collagen under static conditions.** 96-well microtiter plates were coated with collagen types I or III and binding of purified vWF to the plates was evaluated. In the absence of added vWF and in the presence or absence of 10 µg/mL CTRP-1, little or no vWF was detected on plates coated with either collagen I or III (Fig.4A). When vWF was included in the reaction mixtures in the absence of CTRP-1, binding of vWF was observed to both types of collagen. Binding of vWF to collagen I or III was reduced in a concentration dependent manner by CTRP-1 (Fig.4A). Addition of 25 µg/mL CTRP-1, the highest concentration tested, reduced vWF binding to background levels. Under these conditions, CTRP-1 appeared to be more potent at blocking vWF binding to type I versus type III collagen.

Two separate domains of vWF, the A1 and A3 domains appear to mediate collagen binding and their role was tested using purified A1 and A3. In the absence of added A1 domain, and in the absence or presence of 10 µg/mL CTRP-1, little or no A1 was detected on the collagen coated plates. Addition of A1 led to A1 binding to collagen I and III that was reduced in a dose-dependent manner by CTRP-1 (Fig. 4B). The binding of A1 domain to collagen type I was reduced to a greater extent by CTRP-1 than the binding to collagen type III. A1 binding to collagen type I was reduced to background levels by 25 µg/mL CTRP-1 (Fig.4B), similar results were observed for A3 domain binding (Fig. 4C).
CTRP-1 does not prevent platelet “rolling” on a vWF-coated surface under high shear.
Platelet adhesion to collagen under high shear requires the interaction of the platelet GPIb receptor with vWF bound to collagen through its A3 domain. The A1 domain of vWF, though able to bind collagen on its own and thought to also mediate the platelet-vWF interaction has a fast on/off rate producing the well-described “rolling” of platelets on a vWF-coated surface under high shear. To assess whether CTRP-1 could block the GPIb-vWF interaction, citrated whole blood was incubated for 5 min at room temperature with either vehicle alone or with vehicle containing 20 µg/mL CTRP-1 and flowed over a vWF coated surface at 50 dynes/cm². Platelet rolling over the vWF-coated surface was observed in the presence (Fig. 3 lower right panel) or absence (Fig. 3 lower left panel) of CTRP-1. Thus, CTRP-1 did not significantly affect the interaction of GPIb with vWF.

Effect of CTRP-1 on the binding of 125I-GPVI to collagen type I under static conditions.
To evaluate whether CTRP-1 could inhibit collagen-induced platelet aggregation by blocking the interaction of the platelet GPVI receptor with collagen, a soluble GPVI-Fc4 fusion protein was prepared. The GPVI-Fc4 was active as shown by its potent inhibition of collagen-induced aggregation of PRP (Supplement Fig. 1). In this assay, CTRP-1 was more effective at blocking platelet aggregation than GPVI-Fc4 (EC₅₀ 1.4 µg/mL vs. 7.0 µg/mL; Hill slope -3.3 and -1.3 for CTRP-1 and GPVI, respectively). These data demonstrate that the soluble GPVI-Fc fusion protein was active and could effectively compete with the platelet GPVI receptor for collagen-induced aggregation of platelets.

Binding of 125I-GPVI-Fc4 to immobilized type I collagen was saturable, specific, and of high affinity with non-specific binding less than 10% of the total binding (data not shown). Binding was concentration-dependent, approaching saturation at approximately 20 µg/mL with little or no non-specific binding. Scatchard plots were curvilinear suggesting the presence of two classes of binding sites. The high affinity site exhibited a K_d of 0.56 ± 0.18 µg/mL or 5.6nM and a B_max of 5.91 ± 2.67 ng bound/well (mean ± SD, whereas the low affinity site showed a K_d of 17.83 ± 3.12 µg/mL or 178 nM (mean ± SD) and a B_max of 27.22 ± 4.08 ng bound/well (mean ± SD). Thus, under static conditions, 125I-GPVI-Fc4 bound to a small number of high affinity sites and a larger number of low affinity sites on type I
collagen-coated plates. Thus, compared with the binding of $^{125}$I-CTRP-1 shown in Fig.2, $^{125}$I-GPVI-Fc4 bound to collagen type I with a higher affinity but lower capacity.

To evaluate whether the binding of CTRP-1 and GPVI-Fc4 is to the same or different sites on the collagen matrix, competition studies were performed using the solid phase collagen-binding assay. Addition of increasing amounts of unlabeled GPVI Fc4 reduced the amount of $^{125}$I-GPVI-Fc4 bound to collagen (Fig. 5, top panel). A fifty-fold excess of unlabeled GPVI reduced $^{125}$I-GPVI-Fc4 binding by $>$80%, with higher concentrations completely blocking $^{125}$I-GPVI-Fc4 binding. Unlabeled CTRP-1, in contrast, was a less effective competitor for $^{125}$I-GPVI-Fc4 binding. The maximum competition achieved under these conditions was about 40% at a 500-fold excess of unlabeled CTRP-1 (Fig. 5). Addition of increasing amounts of unlabeled CTRP-1 reduced the amount of $^{125}$I-CTRP-1 bound to collagen I (Fig. 5, lower panel). Over an identical range of concentrations, unlabeled GPVI-Fc4, on the contrary, failed to compete for $^{125}$I-CTRP-1 binding (Fig. 5). Taken together, these data show that the binding sites for CTRP-1 on immobilized fibrillar type I collagen partially overlap with those of GPVI-Fc4.

Synthetic ‘collagen-related peptide’ (CRP), cross-linked with glutaraldehyde was used as functional agonist for GPVI\textsuperscript{16,26}. In preliminary studies, cross-linked CRP (CRPxl) was a potent inducer of platelet aggregation (EC\textsubscript{50} 0.26 ±0.07 µg/mL), which could be blocked by GPVI-Fc4. Saturation analysis revealed that binding of $^{125}$I-GPVI-Fc4 to CRPxl was saturable at 10-30 µg/mL, specific, and of high affinity (data not shown). Little or no specific binding of $^{125}$I-GPVI-Fc4 to an unrelated control peptide was observed. Scatchard plots of the specific binding data were curvilinear, indicating two classes of sites. The K\textsubscript{d} and B\textsubscript{max} for the high affinity site were 0.076 ± 0.016 µg/mL and 2.45 ± 0.19 ng bound/well and 2.43 ± 0.58 µg/mL and 15.4 ± 4.0 ng bound/well for the low affinity site. Binding of $^{125}$I-GPVI-Fc4 to CRPxl was reduced by unlabeled GPVI-Fc4 in a dose-dependent manner (Fig. 6). Unlabeled CTRP-1 also reduced the amount of $^{125}$I-GPVI-Fc4 bound, but compared with GPVI-Fc4 it was a less effective competitor (Fig.6). These data demonstrate that both CTRP-1 and GPVI-Fc4 recognize and bind to CRPxl. The relative affinity of GPVI-Fc4 binding to CRPxl is however more than 20-times higher than that observed for CTRP-1.
Effect of CTRP-1 on the binding of collagen to α2β1 integrin under static conditions.
Platelets also interact with collagen through the α2β1-platelet receptor, an interaction mediated by the I-domain of α2β1. To evaluate, whether CTRP-1 can inhibit the α2β1 integrin pathway, the ability of CTRP-1 to compete for I-domain binding to collagen was examined. In the absence of added I-domain, and in the presence or absence of 10 µg/mL CTRP-1, little or no I-domain was detected on plates coated with either collagen I or III. Exogenous I-domain bound to both types of collagen as detected by the anti-I domain specific antibodies. In contrast to the inhibition of intact vWF or vWF A1 and A3 domain binding to collagen, under identical conditions, CTRP-1 failed to compete for I-domain binding (Supplement Fig. 2). These data suggest that the collagen binding domains of α2β1 integrin and CTRP-1 are distinct.

In vivo anti-thrombotic effects of CTRP-1 in a Folts vascular injury model. A mechanical crush injury was made to the carotid artery of non-human primates (Macaca fascicularis) and blood flow through the injured, highly thrombogenic blood vessel was monitored. Reduced blood flow in this model indicates blockage of the artery by platelet-rich thrombus. A typical tracing of blood flow in a CTRP-1 treated animal is shown in Fig. 7A. The cyclic flow pattern recorded during the pre-treatment phase indicates rapid occlusive thrombus formation that requires mechanical manipulation (tapping) in order to dislodge the occluding platelet thrombus. This manipulation reestablishes blood flow only for a brief period before renewed thrombus forms. A single i.v. injection of CTRP-1 (0.5 mg/kg) reestablished blood flow, prevented thrombus formation, and maintained patency of the blood vessel for the complete duration of the 3-hour experiment. A control animal, injected with BSA (Fig. 7B) showed similar initial cyclic flow variation, however, the platelet thrombus became permanently occlusive once mechanical intervention stopped 30 min after treatment. The results of all nine animals studied summarized in Fig. 7C show permanent occlusion of the injured blood vessel developed in all control animals (n=3), whereas both doses of CTRP1 (0.5 and 1.0 mg/kg, n=3 each) prevented thrombus formation and maintained blood flow.

To evaluate effects of CTRP-1 treatment on bleeding, we measured the activated clotting time (ACT) and time-to-hemostasis (TTH) following template cuts to the forearm of the non-human primates used in this study between 5 and 60 min post treatment. ACT and TTH
values remained unchanged by treatment with CTRP-1. ACT values before treatment were 120s and remained at 116s and 126s in the low and high dose CTRP-1 group, respectively. Baseline TTH values were 171s, 215s, 162s, 144s in the BSA, 0.5mg CTRP-1, 1.0mg CTRP-1, Abciximab (0.25mg/kg) groups, respectively. TTH in the same groups after treatment were 154s, 208s, 158s and >1200s. (All SD were <10%)  

DISCUSSION

CTRP-1 is a novel member of the C1qTNF related protein family and similar to other family members, the basic structural unit of the recombinant protein is a homotrimer, which can dimerize to form a hexamer. Both forms are maintained by disulfide bonds and remain highly stable in vitro. The expression of CTRP-1 was examined in different species by Northern blot, in situ hybridization and immunohistochemical analysis and was found to be expressed in a number of tissues, most prominently in vascular wall tissue. The majority of CTRP-1 in the vasculature originated from smooth muscle cells with endothelial cells contributing a smaller proportion (manuscript in preparation).

Data from saturation- and competition binding studies presented show CTRP-1 specifically binds to collagen. Furthermore, we show that CTRP-1 inhibits collagen-induced platelet activation and aggregation. This activity might have clinical significance, because collagen is known to elicit platelet thrombosis following damage to the inner lining of the arterial wall - caused either by spontaneous rupture of atherosclerotic plaque or by medical intervention -, which can lead to life-threatening thrombogenic conditions including myocardial infarction (MI) and stroke. Interaction of platelets and collagen can occur along at least three different receptor-mediated pathways (GPIb, GPVI, Integrin α2β1). The ability of CTRP-1 to interfere with any of these three receptor pathways was investigated under static and under flow conditions simulating shear flow conditions seen typically in smaller human arteries. The inhibition of platelet activation in the static and flow studies occurred over a similar range of CTRP-1 concentrations suggesting similar mechanism in both assay paradigms. The data show that CTRP-1 binds to immobilized fibrillar collagen type I with virtually all the hallmarks of a receptor-mediated process. Importantly, the results from the competition
studies suggest that CTRP-1 binds to sites on collagen that are largely distinct from those that bind the soluble GPVI fusion protein. GPVI is thought to mediate collagen-induced platelet activation by binding to the CRP domain in fibrillar collagen\textsuperscript{16,18,30,31}. Indeed, the present results show that cross-linked CRP activates platelets, that GPVI-Fc4 inhibits collagen- and CRP-induced platelet activation, and that GPVI-Fc4 binds specifically to immobilized, cross-linked CRP or collagen. It is of interest that the apparent affinity and binding capacity of CTRP-1 for collagen I was different from that for GPVI-Fc4: the binding of CTRP-1 was of lower affinity but higher capacity. Since the amount of available collagen on the plates was not measured in these studies, the stoichiometry of CTRP-1 and GPVI-Fc4 binding could not be accurately assessed. On a mass basis, however, the number of binding sites on collagen I for CTRP-1 is approximately 80-times the number of high affinity sites for GPVI-Fc4 measured under identical conditions. This finding is consistent with our observation that GPVI-Fc4 did not compete for the binding of CTRP-1. Although CTRP-1 and GPVI appear to recognize different sites on fibrillar collagen I, CTRP-1 did exhibit a low affinity for a portion of GPVI-Fc4 collagen binding sites, suggesting a partial overlap of their collagen binding domains. The dose-dependent inhibition of GPVI-Fc4 binding to CRP observed with CTRP-1 further supports this notion. Thus, CTRP-1 appears to bind to collagen to at least two distinct sites, one that overlaps with and also recognizes GPVI, and a second site that is largely independent of this pathway.

A key component of the mechanism by which CTRP-1 inhibits collagen-dependent platelet functions appears to be blockade of vWF binding to collagen. The binding of vWF to collagens I and III is clearly inhibited by CTRP-1 under static as well as under flow conditions simulating arterial shear stress. The major binding site of vWF for collagen I is located in the A3 domain, while the A1 domain is thought to play a key role in binding to the platelet GPIb receptor, it also binds to collagen VI\textsuperscript{16,17,29,31-35}. Using isolated vWF A1 and A3 domains, we were able to show that under static conditions CTRP-1 blocked their binding to fibrillar collagen I. Conversely, platelets rolling on a vWF-coated surface, were not significantly inhibited by CTRP-1, suggesting CTRP-1 does not interfere with the interaction of vWF and platelets, which is mediated by the A1 domain of vWF and the platelet GPIb receptor. Furthermore, we were not able to show any direct interaction of platelets with CTRP-1.
Using the purified I-domain\textsuperscript{16} of the $\alpha2\beta1$ receptor, we observed that CTRP-1 had no measurable effects on I-domain binding to collagen, suggesting inhibition of collagen-mediated platelet functions by CTRP-1 is independent of the $\alpha2\beta1$ platelet receptor.

Taken together, these data suggest a dual mechanism for the inhibition of collagen-induced platelet function by CTRP-1; a major mode of action appears to be the blockade of vWF binding sites on collagen leading to the prevention of platelet interaction with collagen in particular under arterial, high-shear blood flow conditions. Blockade of GPVI binding sites on collagen might represent a secondary and additive mechanism for the action of CTRP-1. However, this does not sufficiently explain the mechanism by which CTRP-1 can prevent platelet aggregation under static conditions in vitro. Although unlikely, it is possible that CTRP-1 interacts directly with vWF, CRP and GPVI to prevent their interaction with collagen. Certainly, the partial blockade of the GPVI binding site on collagen could account for some of the effects of CTRP-1 under these conditions. In addition, a recent report by Bernado et.al.\textsuperscript{36} demonstrates that fibrillar collagen as used in the current studies is contaminated with small amounts of vWF. Although the role of vWF clearly is to mediate the initial interaction of platelets with injured vasculature and exposed collagen under high-shear conditions seen in arterial vasculature, a contribution to platelet aggregation under static in vitro conditions cannot be excluded. Lastly, static conditions might increase the inhibitory activity of CTRP-1 as compared to high-shear flow by allowing less-specific binding to interfere with platelet-collagen interaction.

While the exact mechanism contributing to the observed activity of CTRP-1 in vitro is not yet established, the data collected under dynamic flow conditions clearly show that CTRP-1 can prevent collagen-induced platelet adhesion on a collagen matrix by preventing vWF from binding to collagen. The anti-thrombotic effect of CTRP-1 seen in non-human primates show that CTRP-1 can act as a potent anti-thrombotic, preventing platelet thrombosis on an otherwise highly thrombogenic injured carotid artery. Interestingly, two measures of hemostasis indicated normal hemostasis in the treated animals. Adverse bleeding, seen when using current anti-thrombotic agents represents a small, but real clinical problem and the search for a ‘magic bullet’, i.e. anti-thrombotic therapy with no or only negligent effect on general hemostasis is a topic of ongoing investigations\textsuperscript{37,38}. 
Further investigations into efficacy and possible adverse bleeding events are needed to better understand the potential of CTRP-1 as a site-specific anti-thrombotic agent and to address whether this activity is related to its biological function.

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REFERENCES

FIGURE LEGENDS

FIGURE 1  CTRP-1 prevents collagen induced platelet activation and aggregation.
Collagen-induced platelet activation (5µg/mL collagen I) - measured by ATP-release (A) -
and platelet aggregation (B) are inhibited by CTRP-1 (5µg/mL). The inhibition of platelet
aggregation by CTRP-1 is dose-dependent (C), for this experiment PRP was incubated with
1.25 µg/mL collagen I.

FIGURE 2  Binding of 125I-CTRP-1 to fibrillar collagen type I. Plates were coated with
fibrillar collagen type I and incubated with the indicated concentration of 125I-CTRP-1 in the
absence (total binding, closed circles) and presence (nonspecific binding, open circles) of 250
µg/mL of unlabeled CTRP-1. Specific binding (solid triangles) was calculated as the
difference between the total and nonspecific values. Each point represents the mean of
duplicate wells and is representative of four separate experiments. Insert shows the
Scatchard plot of the specific binding data. B = bound 125I-CTRP-1; F = free 125I-CTRP-1.

FIGURE 3 CTRP-1 blocks adhesion of platelets on a collagen type I-coated surface
at high shear. CTRP-1 does not affect “rolling” of platelets on a vWF-coated surface.
Upper Panels: Citrated whole human blood was incubated with either vehicle alone (upper
left panel) or with 20 µg/mL CTRP-1 (upper right panel) and was perfused over collagen
type I coated coverslips through a flow chamber at 50 dynes/cm². Lower Panels: Citrated
whole human blood was incubated with either vehicle alone (lower left panel) or with 20
µg/mL CTRP-1 (lower right panel) and was perfused over vWF-coated coverslips through
the flow chamber at 50 dynes/cm². The pictures shown are the initial frames of brief movies
and are representative of repeated experiments.

FIGURE 4  CTRP-1 blocks intact vWF, vWF A1 domain, and vWF A3 domain
binding to fibrillar collagen types I and III under static conditions. Plates were coated
with collagen types I (gray bars) or III (black bars) and were incubated with vehicle control
or CTRP-1 alone, or the indicated mixtures of CTRP-1 with intact vWF (Panel A), vWF A1
domain (Panel B), or vWF A3 domain (Panel C). Each bar represents the mean ± SD of 6
separate measurements. Differences were significant comparing vehicle and vWF alone
(*p<0.0001), A1 domain alone (*p<0.0001), or A3 domain alone (*p<0.02) by an unpaired,
two-tailed Student’s t-test. Differences were significant (**p<0.0001) relative to incubation
with vehicle alone by ANOVA.

FIGURE 5  Cross competition for GPVI-Fc4 and CTRP-1 binding to fibrillar collagen.
Plates were coated with collagen type I as described under Methods. Top Panel: each well
was incubated with 0.3 µg 125I-GPVI-Fc4/mL in the presence or absence of the indicated
fold-excess of unlabeled GPVI-Fc4 (diamonds) or CTRP-1 (circles); Bottom Panel: each
well was incubated with 0.3 µg 125I-CTRP-1/mL in the presence or absence of the indicated
fold-excess of unlabeled CTRP-1 (circles) or GPVI-Fc4 (diamonds). The 100% values for
$^{125}\text{-GPVI-Fc}4$ and $^{125}\text{-CTRP-1}$ binding in the absence of competitors were $2.87 \pm 0.15$ and $16.38 \pm 2.11$ ng bound/well (mean ± SD, n = 8 separate measurements) respectively. Each point represents the mean ± SD of four separate measurements from two separate experiments.

**FIGURE 6** Competition for $^{125}\text{-GPVI-Fc}4$ binding to cross-linked CRP by unlabeled GPVI-Fc4 or by CTRP-1. Plates were coated with CRP and each well was incubated with 0.05 µg/mL $^{125}\text{-GPVI-Fc}4$ in the absence or presence of the indicated fold excess of unlabeled GPVI-Fc4 (closed circles) or unlabeled CTRP-1 (open circles). The 100% values for $^{125}\text{-GPVI-Fc}4$ binding in the absence of competitors were $0.94 \pm 0.01$ ng bound/well. Each point represents the mean of triplicate measurements and is representative of two separate experiments.

**FIGURE 7** CTRP-1 prevents platelet thrombosis in the Non-Human Primate Folts vascular injury model and reestablishes and maintains blood flow. Panel A and B show typical individual flow traces recorded. Treatment with CTRP-1 (0.5 mg/kg) prevents platelet thrombosis and reestablishes blood flow through the injured carotid artery (A). No treatment effect was seen in a BSA treated control (B). Arrows indicate the time of treatment. The result of the complete study is shown in panel C (n=3, each group, shown is the mean ± SEM). Pre-treatment stenotic blood flow was similar in all three groups. BSA-treated control animals showed permanent occlusion with no residual blood flow (dark bars), treatment with 0.5 mg/kg (open bars) and 1.0 mg/kg CTRP1 (gray bars) reestablished blood flow, which was maintained throughout the study. Average blood flow is shown over two time intervals, a 30 min period immediately following cessation of intervention and a subsequent 2 h time window indicating longer-term effects.
Figure 1  CTRP-1 prevents collagen-induced platelet activation and aggregation.
Figure 2  Saturation binding of $^{125}$I-CTRP-1 to collagen type I.
Figure 3  CTRP-1 Blocks adhesion of platelets on a collagen-coated surface at high shear. However, CTRP-1 has no effect on platelet “rolling” on a vWF-coated surface.
Figure 4  CTRP-1 blocks vWF binding to collagen types I and III under static conditions.
Figure 5  Competition for $^{125}$I-GPVI-Fc4 (panel a) and $^{125}$I-CTRP-1 (panel b) binding to collagen type I by unlabeled GPVI or CTRP-1.
Figure 6  Competition for $^{125}$I-GPVI-Fc4 binding to cross-linked CRP by unlabeled GPVI-Fc4 or CTRP-1.
Figure 7  CTRP1 prevents platelet thrombosis in the Folts vascular injury model, reestablishes and maintains blood flow.
C1q-TNF related protein-1 (CTRP-1), a vascular wall protein that inhibits collagen-induced platelet aggregation by blocking vWF binding to collagen

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