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Histamine Reduces GPIIb/IIIa-mediated Adhesion of Platelets to
TNF- α -activated Vascular Endothelium

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1 **Abstract**

2 Histamine and tumor necrosis factor- α (TNF- α) are critical mediators of acute and chronic
3 inflammation that are generated by mast cells and macrophages in atherosclerotic lesions or
4 systemically during allergic attacks. Both of them induce activation of vascular endothelium and
5 thus may play a role in thrombosis. Here we studied the interplay between histamine and TNF- α
6 in glycoprotein (GP) Iba-mediated platelet adhesion to cultured human vascular endothelial cells
7 under static and shear flow conditions. The stimulation of endothelial cells with histamine or
8 TNF- α increased the number of adherent or slow rolling GP Iba-coated microbeads or washed
9 human platelets. However, the application of histamine to endothelium pre-activated by TNF- α
10 inhibited GP Iba-mediated platelet adhesion. These effects were found to be associated with
11 changes in the concentration of ultra large von Willebrand factor (ULVWF) strings anchored to
12 endothelium. The results of this study indicate that histamine released during mast cell
13 degranulation may cause or inhibit thrombosis, depending on whether it acts on resting
14 endothelial cells or on cells pre-activated by other inflammatory stimuli.

15

16 **Keywords**

17 GP Iba; vWF; TNF- α ; histamine; platelet adhesion; endothelium

18 **Abbreviations**

19 GP, glycoprotein; vWF, von Willebrand factor; ULVWF, ultra large von Willebrand factor;
20 TNF- α , tumor necrosis factor- α , ADAMTS13, a disintegrin and metalloproteinase with a
21 thrombospondin type 1 motif - member 13; HUVEC, human umbilical vein endothelial cells;
22 BSA, bovine serum albumin; LSGS, low serum growth supplement; EDTA,
23 ethylenediaminetetraacetic acid; CCD, charge-coupled device.

24

1 The adhesion and accumulation of platelets at sites of vascular wall damage or inflammation is
2 the primary event in both arterial and vein thrombosis [1-5]. It is well established [6-8] that
3 initial adhesive interactions between platelets and vascular endothelial cells are mediated via
4 binding of the platelet membrane glycoprotein (GP) Ib-IX-V complex to endothelial von
5 Willebrand Factor (vWF). Endothelial cells store vWF in its highly-reactive, ultra large form
6 (ULVWF) within Weibel-Palade bodies [9-11] from which it is secreted constitutively or in
7 response to thrombogenic stimuli [12-14]. A portion of secreted ULVWF strings are anchored to
8 the endothelial cell membrane [15, 16]. Platelets adhere to these strings because of high-affinity
9 binding between the vWF A1 domain and the GP Ib α subunit of the GP Ib-IX-V complex [17,
10 18]. This process is, however, tightly regulated by hemostatic mediators such as the plasma
11 protease ADAMTS-13, which promptly cleaves ULVWF into smaller, less reactive dimers, thus
12 preventing the formation of occlusive thrombi [19-21].

13 Tumor necrosis factor- α (TNF- α) and histamine are important mediators of chronic and
14 acute inflammation. TNF- α produced by tissue resident macrophages activate endothelial cells
15 [22] and thus increases the flux of leukocytes to the inflamed tissue [23]. Histamine causes
16 inflammation when it is released from mast cells and basophils in response to allergen [24-26].
17 It can also be secreted by activated platelets [27, 28]. Bernardo et al. [14] demonstrated that
18 TNF- α stimulates ULVWF secretion and platelet aggregation to ULVWF strings. Numerous
19 evidence indicates that histamine can induce the secretion of proteins from Weibel-Palade
20 bodies, including P-selectin and ULVWF [2, 29-32]. Interestingly, ULVWF generated through
21 endothelium activation by histamine alone remains associated with the endothelial cell
22 membrane, likely due to the involvement of P-selectin in the ULVWF anchorage [16]. This leads
23 to rapid accumulation of platelets at histamine-activated endothelium [16, 27, 33].

1 Both TNF- α and histamine can simultaneously reach a high concentration in specific
2 regions of the body. For instance, the histamine level is high during allergic attacks [24], and
3 patients with allergy may have chronic inflammatory conditions such as atherosclerosis at which
4 TNF- α is extensively released [34]. Histamine secretion from mast cells abundantly found in the
5 atherosclerotic plaques [35] contributes to atherosclerosis even in the absence of the allergic
6 response [36, 37]. Meanwhile, platelets play a crucial role in this pathological process by either
7 recruiting leukocytes [38, 39] or by causing acute thrombotic events after rupture of a vulnerable
8 plaque [40]. The interplay between TNF- α and histamine in platelet-endothelium adhesion and
9 thrombus formation is not yet studied. Here, we investigate adhesive interactions of GP Ib α -
10 coated microbeads and human platelets with cultured human endothelial cells exposed to both
11 TNF- α and histamine under static and shear flow conditions.

12

1 **Materials and Methods**

2 *Reagents*

3 Histamine, TNF- α , bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic
4 (MES) acid hydrate, and 2% gelatin were obtained from Sigma-Aldrich (St. Louis, MO). Human
5 recombinant glycoprotein Iba (GP Iba) was purchased from R&D systems (Minneapolis, MN).
6 2.0 μ m diameter carboxylate-modified fluorescent latex microspheres (505/515 nm,
7 FluoSpheres®) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased
8 from Invitrogen (Carlsbad, CA). GTI-V3P monoclonal mouse anti-human-vWF (GTI
9 Diagnostics, Waukesha, WI) was used to block the A1 domain of vWF.

10 *Endothelial culture*

11 Primary human umbilical vein endothelial cells (HUVEC) were purchased from
12 Invitrogen. Endothelial cells were grown out in T-75 flasks in low serum growth medium
13 (M200-LSGS; Invitrogen) supplemented with antibiotics. When 80% or greater confluence was
14 reached, the cells were harvested by treatment with 0.1% trypsin in EDTA (Sigma-Aldrich),
15 yielding approximately 2.0×10^6 cells per flask. HUVEC were reseeded, at passage 4-6, in 0.1%
16 gelatin-coated 24-well plates for static adhesion assays. Experiments were conducted within 24
17 hours after HUVEC reached more than 90% confluence in the 24-well plates. All the cells were
18 maintained at 37°C in a 5% CO₂ humidified incubator.

19 *Preparation of GP Iba-coated microbeads*

20 Covalent coupling of the amino-terminal end of GP Iba to the carboxylate group on the
21 surface of latex microspheres (microbeads) was accomplished through carbodiimide chemistry,
22 catalyzed by EDAC [41], using the protocol by Liu *et al.* [42]. Briefly, 400 μ L of MES buffer

1 was added to a 100 μL aliquot of microbeads and mixed by aspiration and sonication for five
2 minutes. The resulting suspension was then centrifuged at $6,708\times g$ for 10 minutes. Following
3 centrifugation, the microbead pellet was isolated and re-suspended in 500 μL of MES buffer.
4 This wash procedure was repeated three times, with the microbead pellet re-suspended in 100 μL
5 of MES in the final stage. 20 μL of EDAC was then added and mixed by aspiration, followed by
6 sonication. After a 20 minute incubation period with EDAC at room temperature, 2.5 μL of
7 recombinant human GP Iba (50 $\mu\text{g}/\text{mL}$), calculated to yield the surface density of 300 sites/ μm^2 ,
8 was added to the microbead-EDAC suspension. The final suspension was incubated at room
9 temperature for at least 12 hours before an adhesion assay, with continuous gentle vortexing.

10 Following the covalent attachment of GP Iba to the microbead surface, the microbeads
11 were re-suspended in 400 μL of phosphate-buffered saline (PBS), sonicated to break apart any
12 clumps that may have formed during the incubation period, and subsequently separated by
13 centrifugation at $6,708\times g$ for 10 minutes. After two additional wash steps conducted in 500 μL
14 PBS, protein-coated microbeads were re-suspended in 3 mL of 1.5% BSA, which served to block
15 non-specific binding, finally yielding a concentration of 400,000 particles/ μL .

16 The control group of microbeads was washed as described above. However, following
17 the third wash step the microbeads were re-suspended in 2% BSA and incubated overnight with
18 gentle vortexing. The suspension of control microbeads were washed twice in PBS and re-
19 suspended in 1.5% BSA immediately before an adhesion assay was performed.

20 *Preparation of human platelets*

21 Human venous blood was collected from healthy consenting volunteers into 4 mL
22 Vacutainer tubes (K_2EDTA , BD, Franklin Lakes, NJ) and used within 4 hours of donation.
23 Platelet rich plasma was prepared by centrifugation of whole blood samples ($400\times g$, 4 min).

1 Whole blood and platelet rich plasma were analyzed for platelet and red blood cell content using
2 a hemocytometer (BD, Franklin Lakes, NJ). Platelet rich plasma was then diluted with PBS to
3 the desired platelet concentration, and then used immediately in the adhesion experiments.

4 *Cell-free model to study GP Iba-mediated platelet adhesion*

5 We developed a well-controlled system consisting of carboxylate-modified fluorescent
6 microbeads to quantitatively describe GP Iba-mediated platelet adhesion to stimulated
7 endothelial cells. The microbead diameter (2.0 μm) was selected based on measured diameters of
8 human and mouse platelets (0.5 to 3.0 μm [43, 44]). Each platelet has 15,000 to 25,000 copies of
9 GP Iba [45, 46]. Based on this estimation and using the literature value of 50 μm^2 [47] for the
10 surface area of a washed human platelet, the lower bound for the GP Iba density is about 300
11 sites/ μm^2 . Assuming spherical geometry, our microbeads were coated with GP Iba at this value
12 of the receptor density. It is anticipated that a higher density of GP Iba will make the effects we
13 observed more pronounced. The concentration of microbeads in the BSA solution (400,000
14 particles / μL) was carefully controlled in adhesion assays to mimic platelet-endothelial
15 interactions in vivo. The range of platelet concentrations in human blood is between 150,000 and
16 450,000 platelets/ μL [48].

17

18 *Static Adhesion Experiments*

19 Confluent groups of HUVEC in a 24-well tissue culture plate were left untreated
20 (control) or incubated with TNF- α (50 ng/mL), histamine (10^{-6} mol/L), or their combination for
21 one hour at 37°C. In the combined activation group, histamine was introduced into the growth
22 medium 30 minutes into the TNF- α activation period. The supernatant from each well was then
23 removed and GP Iba-coated microbeads were applied to HUVEC for five minutes. All wells

1 were then washed three times with PBS to remove any microbeads that had not firmly attached
2 to the endothelium. After the wash procedure had ended, the wells were imaged under an
3 inverted microscope (Nikon Eclipse TiS) using 40X objective. Four randomly selected images
4 from each well were captured with a CCD camera (QImaging Retiga EXi), and saved in the
5 TIFF format at a resolution of 1392×1040 pixels.

6 Anti-body blocking experiments were also conducted to evaluate the influence of GP
7 Iba-vWF binding under the inflammatory conditions studied. The vWF blocking assay was
8 identical to the static adhesion assay with the exception that the cells in the blocking antibody
9 groups were incubated with 6.7 μL of V3P in 200 μL of 2% BSA in PBS for 30 minutes at 37°C
10 immediately following TNF-α and/or histamine incubation, while other groups received just 2%
11 BSA in PBS. GP Iba-coated microbeads were then applied and imaged as described above.

12 Static adhesion of washed human platelets to histamine-activated endothelium was also
13 analyzed to validate the cell-free platelet model. In this experiment, HUVEC were stimulated
14 with 0, 10⁻⁴, or 10⁻⁶ mol/L of histamine for five minutes before applying GP Iba-coated
15 microbeads and/or platelets. The platelet and microbead adhesion to HUVEC was quantified
16 through image analysis using a custom MATLAB script (Fig. 2). It should be noted that the
17 systematic histamine concentration in blood can increase to 10⁻⁷ mol/L during allergic attack
18 [49]. The local concentration of serum histamine several minutes to hours after allergen exposure
19 may far exceed its measured systematic value. Spikes in the histamine concentration to much
20 higher than 10⁻⁷ mol/L may occur locally in the tissue containing a large number of activated
21 mast cells. To model this situation, we considered the histamine concentration of 10⁻⁶ mol/L in
22 the majority of experiments. 10⁻⁴ mol/L histamine was used only for short-term (5 minutes)
23 activation of HUVEC for the model validation purpose. It should be noted that histamine with

1 this concentration was used in numerous in vitro experiments [50-54]. 10^{-4} mol/L is a threshold
2 concentration for the histamine effect on leukocyte rolling [29]. This level is shown to saturate
3 the vWF response [16, 30].

5 *Flow Chamber Experiments*

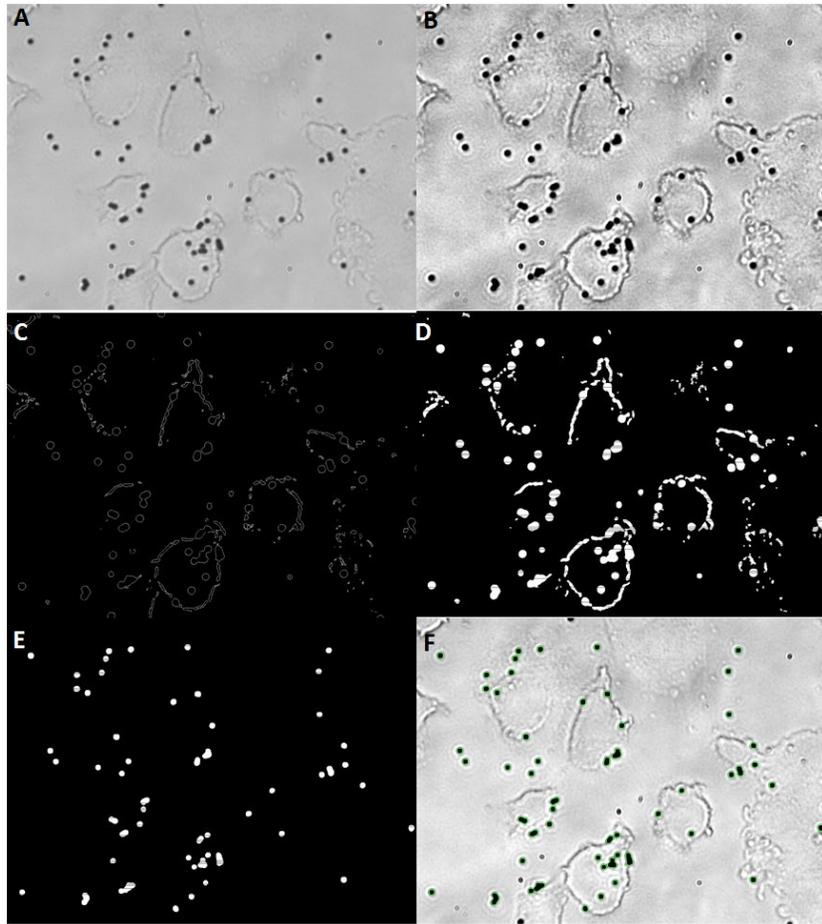
6 GP Iba α -coated microbeads were perfused over confluent HUVEC monolayers cultured in
7 a commercially available microfluidic flow chamber (Bioflux 200, Fluxion Biosciences, San
8 Francisco, CA). Briefly, passage 3-5 HUVEC were seeded in gelatin-coated micro-channels of a
9 48-well Bioflux plate at a density of 5×10^6 cells/mL. The width and height of these micro-
10 channels were 350 and 70 μ m, respectively. Media changes were performed twice daily for 2-3
11 days until confluence was reached. Confluent HUVEC monolayer in micro-channels were left
12 untreated (control) or exposed to selected inflammatory mediators (50 ng/mL of TNF- α , 10^{-6}
13 mol/L of histamine, or their combination) for one hour. Following the activation period,
14 HUVEC-lined micro-channels were washed by perfusion with the growth medium (M200).
15 Fluorescent GP Iba α -coated microbeads were then perfused over each micro-channel for a period
16 of 2 minutes under physiologic shear flow conditions (wall shear stress of 0.6 dyn/cm 2 , wall
17 shear rate of ~ 70 s $^{-1}$). Fluorescent images of the flowing beads were visualized using a 10X
18 objective in an inverted microscope (Nikon Eclipse TiS), and a total of 2400 images per micro-channel
19 were captured by a digital CCD camera (Qimaging Retiga EXi) at 20 frames/s. The number of
20 microbeads slowly rolling along the stimulated endothelium was quantified frame-by-frame
21 using methods similar to the analysis of static adhesion (see below) and normalized to the
22 control.

1 *Quantification of Microbead and Platelet Adhesion*

2 Digital images were analyzed using a custom program written in MATLAB®
3 (Mathworks, Natick, MA). Briefly, the original image (**Fig. 1A**) was imported into MATLAB®
4 as an array of intensity values. The image contrast was increased and further adjusted by the
5 Contrast Limited Adaptive Histogram Equalization (CLAHE) technique using the image
6 processing toolbox of MATLAB (**Fig. 1B**). Following contrast standardization, the grayscale
7 image was converted into a binary, black and white image. The edges of the objects (including
8 beads, cells, and debris) in this image were then detected using an edge-detection algorithm with
9 a Sobel filter (**Fig. 1C**). The image was then dilated, the holes within closed borders were filled
10 in, and any objects contacting the border of the image were removed. The filled image with clear
11 borders then underwent a two-stage erosion algorithm, which isolated partially connected and
12 small objects within the image. The debris field resulting from the erosion step consisted of small
13 amorphous objects representing cellular fragments (**Fig. 1D**), which were then removed from the
14 image through a morphological opening algorithm (**Fig. 1E**). Any remaining small objects were
15 finally removed using a filter targeting objects with an area less than 110 pixels, or 60% of the
16 calculated average microbead area. The perimeter of the remaining objects in the resulting black
17 and white image outlining only the microbeads of interest was determined. The original image
18 was then overlaid with the enhanced green-colored borders of the microbeads (**Fig. 1F**).

19 Morphologically opened images (**Fig. 1D**) depict adherent microbeads, i.e., the
20 microbeads left after the wash procedure, as white ‘blobs’ completely surrounded by the black
21 background. Since closely associated microbeads may be counted as one object by this method,
22 we first determined the average pixel-area of a microbead within the sequence of 36 to 48
23 images. 12 microbeads were selected from the images and their area was averaged, yielding the

1 value of 205 pixels per microbead. The number of microbeads within each blob was then
2 calculated by dividing the area of the blob by the pre-determined single microbead area. This
3 number was summed over all blobs to determine the total number of adherent microbeads in the
4 image.



5

6 **Figure 1:** Digital image analysis technique used to quantify adhesion of microbeads and
7 platelets to HUVEC. (A) Original image. (B) Image following contrast enhancement to
8 facilitate detection of the edges of microbeads or platelets (point to black spots). (C) Dark
9 objects were outlined based on their grayscale intensity relative to the background. (D)
10 These borders were used to generate a binary image with filled areas between closed borders. (E)
11 Cellular debris and other unwanted objects in the closed-border image were removed through
12 morphological opening algorithm, followed by a dilation filter to remove small objects,
13 producing a binary representation of adherent microbeads/platelets. (F) This binary image was
14 used to generate borders of microbeads/platelets which were then overlaid onto the original
15 image for visualization.

1 *Statistical Analysis*

2 In static adhesion experiments, the data from four images per well were averaged together for
3 three wells examined per experimental group. For flow experiments, the number of slow rolling
4 microbeads was calculated on a frame by frame basis, which was then averaged across the entire
5 image sequence. Reported is the mean flux of rolling microbeads averaged across each
6 individual microchannel, generating five individual data points from 12000 images that were
7 analyzed. All independent experiments were done in triplicate. Results are presented as the mean
8 \pm standard error of the mean (SEM). Statistical analysis was evaluated using a two-tailed,
9 unpaired Student's t-test. P-values less than 0.05 were considered statistically significant.

10 **Results**

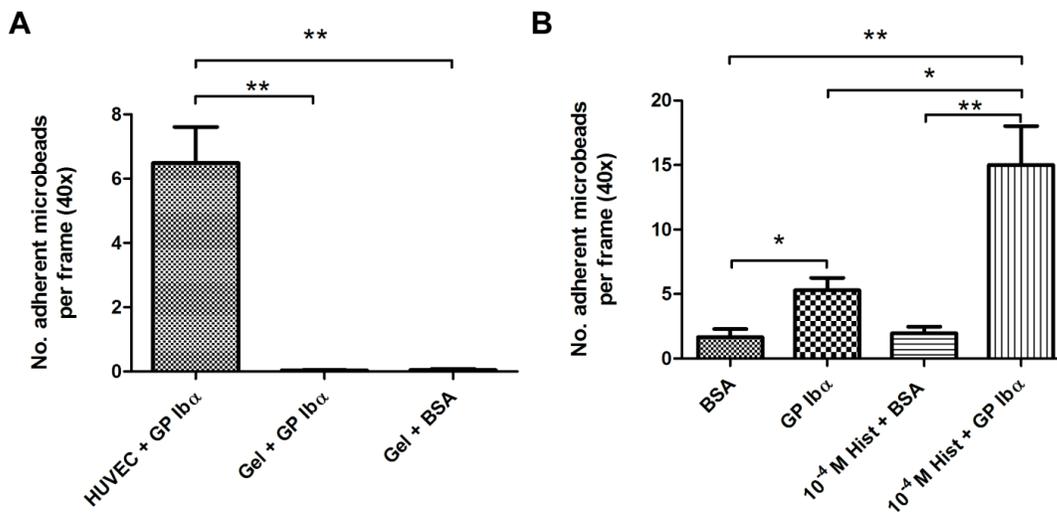
11 *Cell-Free Platelet Model Validation*

12 **GP Iba-coated microbeads** were evaluated against BSA-coated microbeads to assess non-
13 specific interactions to the tissue culture plate, gelatin coating, resting endothelium, and
14 histamine-stimulated endothelium. Both BSA and GP Iba-coated microbeads were applied to
15 **gelatin-coated** tissue culture plates in order to demonstrate the latex microbead interactions are
16 HUVEC-dependent (**Fig. 2A**). The specificity of GP Iba-coated microbeads for the HUVEC
17 monolayer was **then** evaluated on both resting HUVEC and HUVEC stimulated with 10^{-4} M
18 histamine in M200 for 5 minutes (**Fig. 2B**).

19 **Figure 3A** shows that GP Iba-coated microbeads (small bright circles) were minimally
20 adherent to the resting HUVEC monolayer. Following stimulation with 10^{-4} mol/L of histamine,
21 secreting ULVWF on HUVEC promoted increased microbead adhesion, and endothelial cells
22 retracted pseudopods and became round in shape with more gaps between cells (**Fig. 3B**). Only a

1 few human platelets (white amorphous dots) were seen attached to the resting endothelium (**Fig.**
 2 **3C**). As in studies using microbeads, stimulation with 10^{-4} mol/L of histamine **has been observed**
 3 **to induce** endothelial secretion of ULVWF which occurred primarily in a basolateral direction
 4 and created gaps in the previously confluent monolayer (**Fig. 3D**). Thus, both platelets and GP
 5 Iba-coated microbeads adhered primarily around the periphery of stimulated HUVEC, which **is**
 6 **consistent with** the localized presence of ULVWF in this region.

7

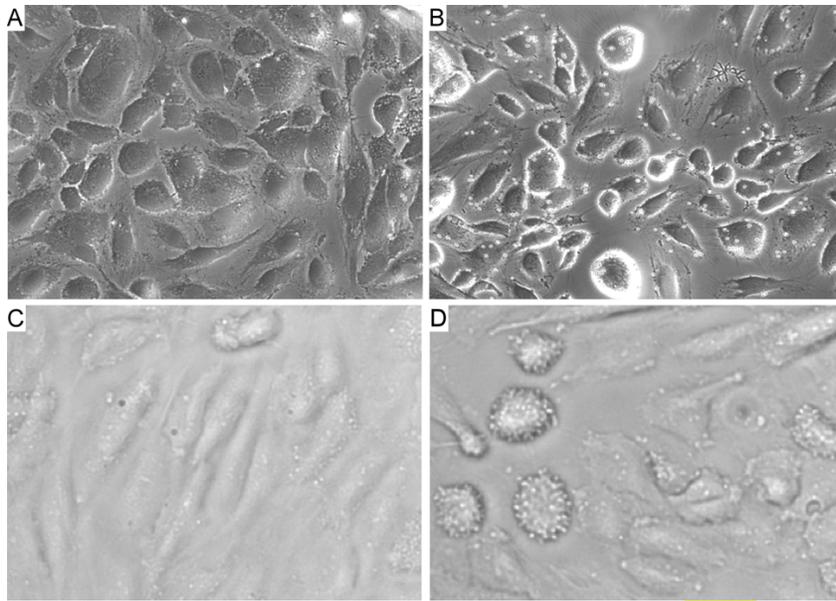


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9 **Figure 2:** Specificity of GP Iba-coated microbead adhesion to HUVEC. (A) Neither GP Iba- nor
 10 BSA-coated microbeads adhered in any significant manner to the 0.1% gelatin-in-PBS (gel)
 11 substrate, however, GP Iba-mediated adhesion increased significantly from 0.0355 ± 0.0175
 12 beads per frame (BPF) on gelatin to 6.49 ± 1.12 BPF on HUVEC. (B) GP Iba-coated
 13 microbeads adhere significantly stronger to HUVEC monolayer than do BSA-coated
 14 microbeads: 5.29 ± 0.966 GP Iba-coated microbeads were bound per frame on resting HUVEC,
 15 compared to 1.67 ± 0.631 BSA-coated microbeads. Histamine had no significant effect on BSA
 16 adhesion (1.67 ± 0.631 vs 1.96 ± 0.522). However, GP Iba adherence was significantly greater
 17 to histamine-stimulated HUVEC (15.0 ± 3.03 BPF) compared to resting HUVEC (5.29 ± 0.966
 18 BPF). Shown are Mean \pm SEM of six independent data points. * $p < 0.05$, ** $p < 0.01$.

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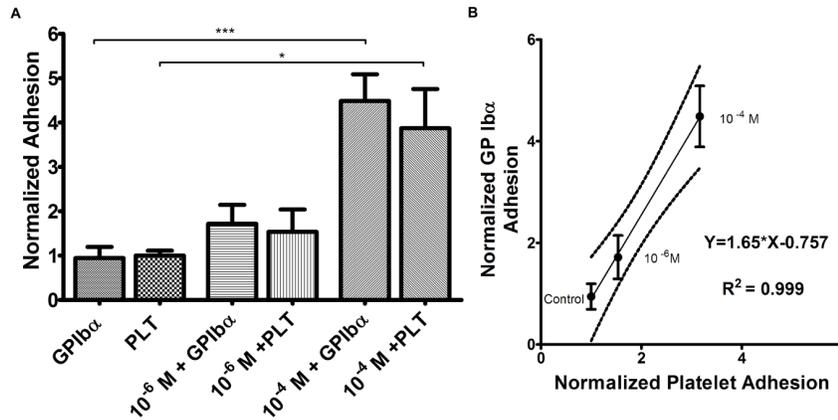


1
 2 **Figure 3:** Phase-contrast images showing adhesion of microbeads **(A,B)** and platelets **(C,D)** to
 3 resting and stimulated HUVEC. **(A)** GP Iba-coated microbeads (small bright circles) were
 4 minimally adherent to a resting HUVEC monolayer. **(B)** Following stimulation with 10^{-4} mol/L
 5 of histamine, endothelial cells retract pseudopods and become round in shape, forming gaps in
 6 the previously confluent monolayer. Adherent GP Iba-coated microbeads are found around the
 7 periphery of histamine-activated endothelial cells due to the localized presence of ULVWF
 8 strings in this region. **(C)** Human platelets (tiny white specs against grey background and cells)
 9 adhere minimally to a resting HUVEC monolayer. **(D)** HUVEC stimulation with 10^{-4} mol/L of
 10 histamine significantly increases platelet adhesion to HUVEC borders.
 11

12 **Figure 4** demonstrates that both platelets and GP Iba-coated microbeads adhere to
 13 histamine-stimulated endothelium in a dose-dependent fashion. As seen in **Fig. 4A**, stimulation
 14 with 10^{-4} mol/L of histamine significantly ($p < 0.0001$) increased the number of adherent
 15 microbeads to 4.49 ± 0.600 times that of the control. Similarly, the number of adherent platelets
 16 significantly ($p < 0.05$) increased to 3.16 ± 0.745 of the control value following stimulation with
 17 the same dose of histamine. **Figure 4B** depicts the data shown in **Fig. 4A** with normalized
 18 adherence of GP Iba-coated microbeads plotted against the normalized adhesion of washed
 19 human platelets. A linear regression gave us the equation of the best-fit line for our data (**Fig.**

1 **4B**), yielding an r^2 value of 0.999. This indicates that there exists a strong correlation between
 2 GP Iba α -coated microbead and platelet adhesion to activated endothelium.

3



4

5 **Figure 4:** Correlation in microbead and platelet adhesion to stimulated HUVEC under static
 6 conditions **(A)** Both human platelets (PLT) and GP Iba α -coated microbeads adhere to histamine-
 7 stimulated endothelium in a concentration dependent manner. Here, 10⁻⁴ M and 10⁻⁶ M indicate
 8 HUVEC incubation with 10⁻⁴ mol/L and 10⁻⁶ mol/L of histamine, respectively. **(B)** The
 9 correlation curve constructed from the GP Iba α and PLT data in **(A)**. Dotted lines show the 95%
 10 confidence interval. The correlation coefficient $r^2 = 0.999$. Mean \pm SEM of nine independent data
 11 points, **normalized to GP Iba α -coated microbead control with basis 1** * $p < 0.05$, *** $p < 0.0001$.
 12

13 Combined effect of TNF- α and histamine

14 As in the case of HUVEC exposed to histamine alone (**Fig. 4A**), the one hour incubation
 15 of HUVEC with 50 ng/mL of TNF- α significantly increased the number of adherent GP Iba α -
 16 coated microbeads (2.47 ± 0.392 of the control value, $p < 0.05$) (**Fig. 5A**). However, when TNF-
 17 α was co-incubated with 10⁻⁶ mol/L of histamine, a significant decrease ($p < 0.05$, 52.7%) in
 18 microbead adhesion was observed, when compared to the TNF- α alone activation. As seen in
 19 **Fig. 5A**, the number of microbeads adherent to TNF- α + histamine-activated endothelium was
 20 just 1.17 ± 0.187 of the control value. **Figure 5B** shows that the observed effects of TNF- α and
 21 histamine can be blocked by HUVEC incubation with a monoclonal antibody VP3 directed
 22 against the A1 domain of vWF. Specifically, adding this antibody to resting, TNF- α -activated,

1 and TNF- α + histamine-activated endothelium reduced the number of adherent microbeads to
2 0.714 ± 0.0714 , 0.929 ± 0.228 , and 0.696 ± 0.142 of the control value, respectively. Thus, both
3 TNF- α effect and the decrease in the number of adherent microbeads with histamine stimulation
4 of TNF- α -activated endothelium are associated with changes in the concentration of ULVWF
5 strings anchored to endothelium.

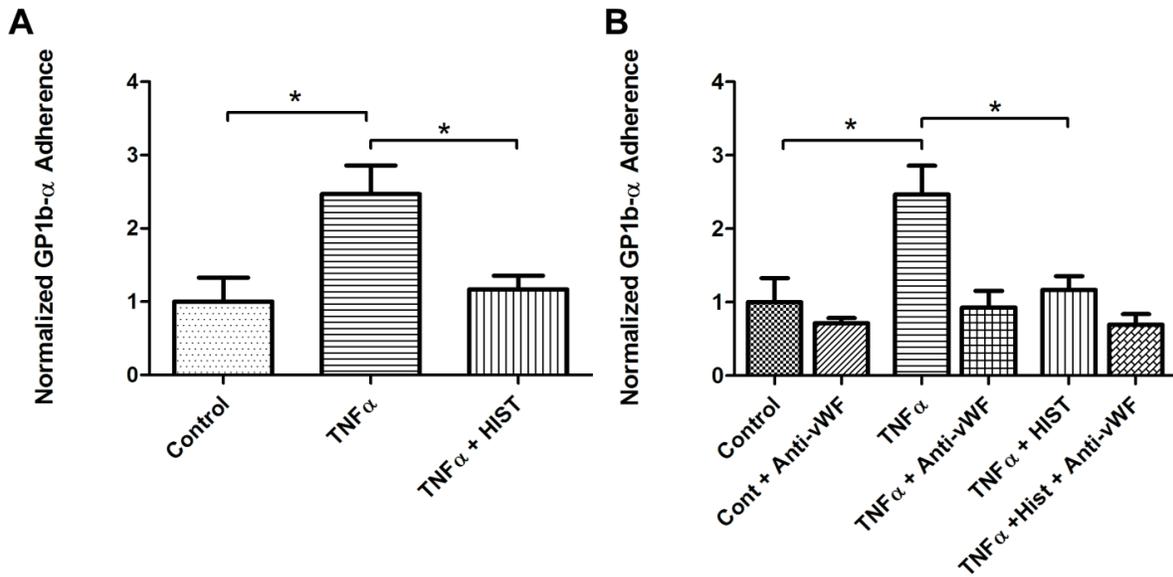
6 The GP Ib α -coated microbead adhesion to HUVEC exposed to single and multiple
7 inflammatory mediators was quantified under flowing conditions (**Fig. 6**). HUVEC monolayers
8 stimulated with 10^{-6} mol/L of histamine demonstrated a slight, but not significant, increase in the
9 number of slow rolling microbeads (measured as beads per frame (BPF) normalized against the
10 control) than did the unstimulated HUVEC monolayer (1.25 ± 0.181 vs. 1.00 ± 0.0944 , i.e., 25%
11 increase). Stimulation with 50 ng/mL of TNF- α produced a significantly ($p < 0.05$) greater effect
12 on GP Ib α -mediated adhesion, resulting in a 33% increase in the number of slow rolling
13 microbeads compared to the control (1.33 ± 0.0916). As in static adhesion experiments, the
14 number of microbeads interacting with TNF- α -activated HUVEC under flow was significantly (p
15 < 0.05) lower when HUVEC was additionally stimulated with histamine (1.01 ± 0.041 vs. $1.33 \pm$
16 0.0916). It is worth mentioning that GP Ib α -coated microbeads were observed to adhere to the
17 periphery of the endothelial cells under flow conditions, which is slightly different from the
18 behavior of platelets that adhere like “beads on a string” [19]. This discrepancy may be attributed
19 to the lack of secondary adhesion receptors such as GPIIb/IIIa ($\alpha_{IIb}\beta_3$), which would form
20 stronger bonds with vWF than GP Ib α .

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2
 3 **Figure 5:** Effect of TNF- α and histamine on adhesion of GP Iba-coated microbeads (GP1b- α) to
 4 HUVEC under static conditions. (A) HUVEC exposure to TNF- α at 50 ng/mL for 60 minutes
 5 significantly increases microbead adhesion to HUVEC. When 10^{-6} mol/L of histamine (HIST)
 6 was added alongside TNF- α , a significant decrease in microbead adhesion was observed. (B)
 7 For each inflammatory regime tested in (A), the same experiment was performed in the presence
 8 of a monoclonal antibody VP3 directed against the vWF A1 domain. A 30 minute incubation
 9 period with VP3 resulted in reduced microbead adhesion to near control values. Mean \pm SEM of
 10 nine independent data points, normalized to control with basis 1. * $p < 0.05$.

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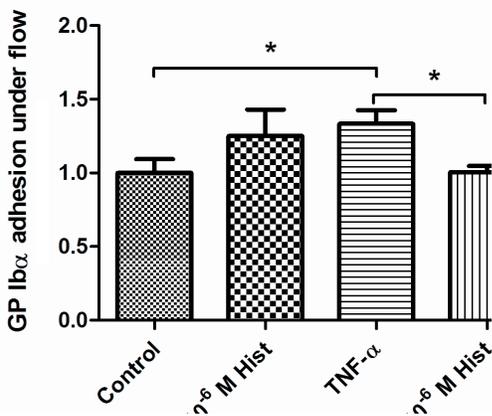


Figure 6: Effect of TNF- α and histamine (Hist) on adhesion of GP Iba-coated microbeads to HUVEC under flow conditions. The number of slow rolling microbeads increases with HUVEC exposure to 10^{-6} mol/L of histamine or 50 ng/mL of TNF- α . The combined exposure of HUVEC to TNF- α and histamine, however, results in a significant decrease in rolling adhesion of microbeads, compared to TNF- α alone stimulation. Mean \pm SEM of six independent data points, normalized to control with basis 1. * $p < 0.05$.

24

25

26 **Discussion**

1 The results presented in this report suggest that histamine may play a dual role in platelet
2 adhesion to vascular endothelium. When applied to resting endothelial cells, histamine increases
3 the number of ULVWF strings on the endothelial surface and thus induces platelet accumulation
4 on endothelial cells, in line with published data [16]. However, if histamine is released near
5 endothelium activated by inflammatory mediators such as TNF- α , it may have an inhibitory role
6 in GP Iba-mediated platelet-endothelial cell interactions. This is most likely caused by the
7 histamine-induced detachment of endothelium-anchored ULVWF strings secreted after TNF- α
8 exposure. This detachment may occur, for example, due to histamine-induced F-actin
9 redistribution in endothelial cells with disappearance of dense peripheral bands of F-actin and
10 stress fibers [55, 56]. The weakening and rearrangement of the actin cytoskeleton induced by
11 histamine may reduce the strength of ULVWF binding to the endothelial cell surface [57]. The
12 dual, paradoxical effect of histamine on platelet-endothelium adhesion may be one of the reasons
13 behind conflicting reports on the role of mast cells in thrombosis. Mast cell-deficient mice were
14 found to be more susceptible to lethal thromboembolism than wild-type mice, and evidence
15 shows that mast cells accumulate at sites of venous thrombosis, where they serve as repair cells
16 [58, 59]. On the other hand, mast cells activated by diesel exhaust particles were found to initiate
17 inflammation and thrombosis in the lungs of hamsters [60]. Thus, histamine released from mast
18 cells may cause or inhibit thrombosis, depending on whether it acts on resting endothelial cells or
19 on cells pre-activated by other inflammatory stimuli.

20 A large number of mast cells are colocalized with macrophages in atherosclerotic plaques
21 [35], where their activation leads to a significant increase in local histamine concentration [36,
22 37, 61]. Our data underlines the need for understanding whether mast cells are already present at
23 sites where atherosclerosis would be developed or they migrate to early atherosclerotic lesions in

1 response to stimuli released by activated cells in these regions. In the former case, platelet
2 adhesion to histamine-activated arterial endothelium could initiate atherosclerosis. Indeed,
3 adherent platelets can capture flowing monocytes via platelet P-selectin binding to monocyte
4 PSGL-1 [41, 62]. This leads to the accumulation of monocytes in the intimal layer of an artery,
5 where monocytes differentiate into macrophages and then foam cells. If the latter case is true,
6 histamine release from mast cells will have an anti-thrombotic effect on dysfunctional
7 endothelium at the plaque. However, activated mast cells will still cause monocyte adhesion and
8 transendothelial migration. Indeed, our recent investigation [63] shows that the number of slow
9 rolling and firmly adherent monocytes is higher on endothelium activated sequentially by TNF- α
10 and histamine than that on endothelium activated by TNF- α alone, with no slow rolling or firm
11 adhesion events observed when endothelial cells are exposed to histamine alone. This
12 synergistic effect indicates that histamine release from mast cell may lead to direct capture of
13 monocytes by endothelium (i.e., without involvement of platelets) provided endothelium is pre-
14 activated by TNF- α .

15 The methods used in this work are well suited to study functional characteristics of
16 receptor-ligand interactions involved in thrombosis. Fluorescent latex microbeads can be
17 covalently coated with platelet, leukocyte, or any other recombinant protein at physiologically
18 relevant concentrations, forming a model ideal for studying adhesion, translocation, and
19 aggregation under well controlled conditions free from the involvement of other proteins,
20 cytokines, and cellular elements present in previous studies using viable platelets or CHO cells
21 [16, 62]. Furthermore, the difficulty in working with human platelets has impeded their use in
22 quantitative studies. Platelets become activated extremely easily, for instance, centrifuging
23 platelets in PBS yields a platelet pellet which cannot be broken apart and re-suspended. For these

1 reasons, we propose the use of GP Ib α -coated microbeads as a model to evaluate platelet
2 adhesion to the vascular endothelium, the first step in thrombosis and related pathologies.

3 In summary, we developed a cell-free model of a human platelet by covalently coating
4 latex microbeads with human recombinant GP Ib α . Using this model, we were able to quantify
5 GP Ib α -mediated platelet adhesion to histamine- and/or TNF- α -activated endothelium under
6 static and flow conditions. The results of this work demonstrate that histamine may have both
7 stimulatory and inhibitory effects on platelet-endothelial adhesion: resting endothelium exposed
8 to histamine is more adhesive to platelets, while histamine exposure of TNF- α -activated
9 endothelium leads to reduced adhesion of platelets. Future studies will specifically examine the
10 role of mast cell activation in platelet-endothelial cell interactions.

11

12 **Conflict of Interest Statement**

13 The authors declare that they have no conflict of interest.

14

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