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ORIGINAL ARTICLE

Histaminylation of fibrinogen by tissue transglutaminase-2 (TGM-2): potential role in modulating inflammation

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Abstract Plasma fibrinogen plays an important role in hemostasis and inflammation. Fibrinogen is converted to fibrin to impede blood loss and serves as the provisional matrix that aids wound healing. Fibrinogen also binds to cytokine activated endothelial cells and promotes the binding and migration of leukocytes into tissues during inflammation. Tissue transglutaminase (TGM-2) released from injured cells could cross-link fibrinogen to form multivalent complexes that could promote adhesion of platelets and vascular cells to endothelium. Histamine released by mast cells is a potent biogenic amine that promotes inflammation. The covalent attachment of histamine to proteins (histaminylation) by TGM-2 could modify local inflammatory reactions. We investigated TGM-2 crosslinking of several biogenic amines (serotonin, histamine, dopamine and noradrenaline) to fibrinogen. We identified histaminylation of fibrinogen by TGM-2 as a preferred reaction in solid and solution phase transglutaminase assays. Histamine caused a concentration-dependent inhibition of fibrinogen cross-linking by TGM-2. Fibrinogen that was not TGM-2 crosslinked bound to un-activated endothelial cells with low affinity. However, the binding was increased by sevenfold when fibrinogen was cross-linked by TGM-2. Histaminylation of fibrinogen also inhibited TGM-2 crosslinking of fibrinogen and the binding to un-activated HUVEC cells by 75–90 %. In summary, the histaminylation of fibrinogen by TGM-2 could play a role in modifying inflammation by sequestering free histamine and by inhibiting TGM-2 crosslinking of fibrinogen.

Keywords Tissue transglutaminase · Histaminylation · Endothelial cell · Histamine · Fibrinogen · Inflammation

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Background

Fibrinogen plays an important role in hemostasis, wound healing and inflammation (Davalos and Akassoglou 2012). The fibrin gel serves as a provisional matrix to prevent blood loss and aid tissue repair (Greenberg et al. 1991). Thrombin-cleaved plasma factor XIII (factor XIIIa), a transglutaminase, catalyzes fibrin ten times faster than fibrinogen (Lorand et al. 1972). In contrast, tissue transglutaminase (TGM-2), an enzyme present in erythrocytes, endothelial cells, and extracellular matrix is efficient at catalyzing the cross-linking of soluble fibrinogen (Murthy and Lorand 1990; Shainoff et al. 1991). Purified TGM-2 catalyzes inter- and intra-molecular crosslinking of fibrinogens forming high molecular weight polymers, respectively (Murthy and Lorand 1990; Murthy et al. 1991; Shainoff et al. 1991). TGM-2 cross-linked fibrinogen may also promote endothelial cell signaling and adhesion (Belkin et al. 2005). In addition, there is growing interest in the effect of "aminylation" on protein structure and their role in cell biology (Walther et al. 2011). High concentrations of biogenic amines such as serotonin, histamine, and dopamine exist in a wide variety of tissues (Walther et al. 2011). Several biogenic amines are potential transglutaminase substrates (see latest review (Walther et al. 2011). In this study, we report histaminylation of fibrinogen by TGM-2 covalently couples free histamine to

fibrinogen and inhibits the formation of crosslinked fibrinogen multimers that bind to endothelial cells.

TGM-2 is a sulfhydryl rich calcium-dependent enzyme that is regulated by nitric oxide, redox state and GTP/ATP levels (Greenberg et al. 1991). It is synthesized and secreted by endothelial cells and is bound to fibronectin (FN) in the ECM and to the endothelial cell surface, where it functions as a co-receptor for FN (Greenberg et al. 1991; Lorand and Graham 2003). TGM-2 can assist the FN receptor in binding FN and other molecules (Lorand and Graham 2003). In addition, TGM-2 is active and can crosslink glutamine-containing proteins to either primary amines or other lysine bearing proteins (Greenberg et al. 1991; Lorand and Graham 2003). Endothelial cells contain a high level TGM-2 compared to other cell types (Greenberg et al. 1991; Lai et al. 2007). Significant amounts of TGM-2 can also be released from the injured erythrocytes and tissues at sites of vascular injury (Lorand and Graham 2003). Previous studies have shown that TGM-2 on hepatocytes and endothelial cells were able to crosslink fibrinogen (Martinez et al. 1989; Barsigian et al. 1991) although the function of this reaction was never defined. TGM-2 also catalyze the crosslinking of fibrinogen on the surface of B16/F10 melanoma cells (Cardinali et al. 1990). In this study, we investigated the effect of TGM-2 crosslinking of fibrinogen on the binding of the multivalent fibrinogen molecule to un-stimulated endothelial cells.

Biogenic or naturally occurring amines are involved in many different physiological processes related to neurotransmission and cell signaling (Kimura et al. 2004; Sasaguri and Tanimoto 2004; Walther et al. 2011). Biogenic amines are primary amines that serve as substrates for TGM-2-mediated cross-linking reactions (Greenberg et al. 1991; Lorand and Graham 2003; Walther et al. 2011). They can be covalently attached to protein-bound glutamine residues by TGM-2 and function as competitive inhibitors of the cross-linking reaction (Fesus et al. 1985; Greenberg et al. 1991; Lorand and Graham 2003). Interest in TGM-2 and biogenic amines increased when the biogenic amine serotonin was reportedly cross-linked to fibrinogen by TGM-2 at the platelet surface and the crosslinked products bound to platelets (Szasz and Dale 2002; Walther et al. 2011). Histamine has also been shown to be incorporated into proteins including heterotrimeric G proteins Cdc42, $G_{\alpha 01}$, and $G_{\alpha q}$ by TGM-2 and might be involved in regulating cell signaling events (Vowinckel et al. 2012).

In this study, we investigated the interaction between several biogenic amines and fibrinogen using TGM-2. Histamine was the preferred substrates for TGM-2 crosslinking to fibrinogen. Histamine was able to inhibit formation of TGM-2 mediated crosslinking of fibrinogen thereby inhibiting binding of these complexes to endothelial cells. The implications of TGM-2 dependent histaminylation of fibrinogen on endothelial cell biology and inflammation will be discussed.

Materials and methods

Materials

[^{125}I]-fibrinogen (100 μCi/ml) was purchased from Amersham/Pharmacia biotech. [^{3}H]-histamine (23.2 Ci/mmol) and [^{3}H]-serotonin (23.7 Ci/mmol) were obtained from PerkinElmer life sciences (Boston, MA). Purified human plasma fibrinogen (depleted for coagulation factor XIII and plasminogen) was purchased from American Diagnostica Inc (Greenwich, CT). Human α-thrombin was a gift from Dr. J. W. Fenton, II (New York State department of Health, Albany, NY). 5-biotin-amido-pentylamine (BP) was obtained from ThermoScientific (Rockford, IL). Hirudin and all other reagents used in this study were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Expression and purification of recombinant TGM-2 and factor XIII A-chains

The recombinant TGM-2 and factor XIII A were expressed and purified from *E. coli* as a glutathione *S*-transferase fusion protein as described (Lai et al. 1994, 1998). Protein concentrations were quantified using the Bradford method (Bio-Rad, Hercules, CA). Purified proteins were stored in 50 mM Tris–acetate, pH 7.5, at −80 °C until ready to use.

Solid-phase microtiter plate transglutaminase assay

Transglutaminase activity of TGM-2 (or factor XIIIa) was determined by quantifying the incorporation of 5-biotin-amidopentylamine (BP) into *N*, *N*′-dimethylcasein (or fibrinogen) coated on a microtiter plate as described previously (Slaughter et al. 1992). The amount of BP incorporated into the casein was determined after a 45-min incubation at 37 °C and color developed using streptavidin-conjugated alkaline phosphatase and PNPP (Slaughter et al. 1992). Zymogen factor XIII A was converted to active factor XIIIa by incubating with 20 U/ml α-thrombin in a reaction mixture containing 0.1 M HEPES, pH 7.5, 10 mM Ca^{+2} and at 37 °C for 15 min and the reaction was stopped by adding 0.1 U/μl of hirudin. For IC_{50} determination, all assays were determined in the presence of 1 mM BP. The data represent the means of two triplicate experiments.

Solution phase transglutaminase assay

The assay was adapted from [^{3}H]-putrescine incorporation assay as described (Miraglia and Greenberg 1985). In brief,

transglutaminase activity of TGM-2 was quantified by measuring the incorporation of [^{3}H]-histamine (or [^{3}H]-serotonin) into fibrinogen at 37 °C for 45 min. The reaction mixtures contained 0.1 M HEPES, pH 7.5, 10 mM Ca^{+2}, 1 mM DTT, 1,000 μg/ml of fibrinogen, 0.862 μM [^{3}H]-histamine, 112.5 μM unlabelled histamine and various amount of recombinant TGM-2 (or factor XIIIa). After the reaction, reaction mixtures were precipitated with 25 % trichloroacetic acid (TCA). The precipitates were collected on Whatman GF/C filters and free unincorporated [^{3}H]-histamine/unlabelled histamine were washed out by 10 % TCA as described (Miraglia and Greenberg 1985). The incorporated radioactivity was quantified by liquid scintillation counting.

Fibrinogen crosslinking

The cross-linking reactions were performed in a 20 μl reaction containing 100 mM HEPES, pH 7.5, 10 mM Ca^{+2}, 1,000 μg/ml of factor XIII free fibrinogen, and 200 μg/ml of TGM-2. To test the effects of histamine on crosslinking, various concentrations of histamine were included in the reaction (see figure legends). In some experiments, [^{3}H]-histamine (23.2 Ci/mmol) was included in the reaction. Control reactions in the absence of TGM-2 were included and also served as markers to identify positions of Aα, Bβ and γ chains of fibrinogen. After the reaction, SDS-PAGE loading buffer was added to stop the reaction. The reaction mixtures were loaded on a 5–15 % SDS-PAGE gel and bands were visualized by Coomassie blue staining. To visualize the incorporation of [^{3}H]-histamine, gel was dried on a piece of Whatman 3 MM paper and exposed to X-ray film.

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonenetics and maintained in EGM2 media (EBM2 plus serum) according to manufacturer's instruction (Clonenetics, Lonza, Walkersville, MD). For fibrinogen binding studies, cells were plated on to a 24-well plate 1 day before experiments.

Endothelial cells binding assay

[^{125}I]-fibrinogen and unlabelled fibrinogen (1: 10 = w/w) were mixed with 250 μl of binding buffer containing EBM2/1 % BSA and loaded onto confluent HUVEC cells. To test the effects of crosslinked fibrinogens on binding, [^{125}I]-fibrinogen and unlabeled fibrinogen were crosslinked by TGM-2 before mixing with binding buffer. For investigating the effects of histamine on TGM-2-mediated crosslinking, extensive dialysis was performed after crosslinking to remove free histamine before mixing with binding buffer. Binding was performed at 4 °C for 1 h, and cells were washed two times with EBM2/1 % BSA and one time with PBS. In all binding experiments, cells were carefully examined under light microscope and no significant detaching was observed. The bound materials were scraped off the plate with 250 μl of elution buffer containing 0.3 M NaOH, 1 % SDS and 2 % $NaHCO_3$. Radioactivity was determined by gamma-counter. To identify the bound species, cells were washed three times with PBS and bound fibrinogens were eluted 2× with 100 μl of buffer containing 10 mM HEPEs, 350 mM NaCl, and 5 mM EDTA and 0.1 % NP40. 25 μl of the eluted materials were loaded and separated on a 5–15 % SDS-PAGE gel. Bands were stained with Coomassie blue and the gel was dried on a piece of Whatman's 3 MM paper before exposed to X-ray film. The dried gel was stored at −80 °C for 3–5 days before it was developed.

SDS-polyacrylamide (SDS-PAGE) gel electrophoresis and X-ray autoradiograph

Samples were electrophoresed under reducing conditions with 3 % stacking and 5–15 % resolving gel (Bio-Rad, Hercules, CA). The molecular weight standards were purchased from Bio-Rad (Hercules, CA) and were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), Albumin (66 kDa) and ovalbumin (45 kDa). Gels were stained with Coomassie blue, and were dried on a piece of 3 MM filter paper. Autoradiography of the dried gels was performed by exposed the gels to X-ray film and stored at −80 °C for 3–5 days before developed.

Statistical analysis

Unless specified, all experiments were performed in triplicate and the results were presented as data ± standard deviation.

Results

In this study, we investigated whether biogenic amines including serotonin, histamine, dopamine, noradrenaline and putrescine could function as TGM-2 substrates and inhibit the incorporation of biotinylated pentylamine (BP) into *N, N′*-dimethylcasein coated microtiter plates (Table 1). Histamine was found to be the most effective inhibitor with an IC_{50} value of 160 μM followed by putrescine ($IC_{50} \sim 600$ μM), whereas up to 50 mM of dopamine and serotonin only inhibited ≤10 % of transglutaminase activity. Histamine was also the best inhibitor when dimethylcasein was replaced with fibrinogen or fibrin

Table 1 IC50 s of biogenic and primary amines in inhibiting transglutaminase activity of TGM-2

	Histamine	Serotonin	Dopamine	Noradrenaline	Putrescine
IC_{50}	160 ± 50 μM	>>10 mM	>>10 mM	>>10 mM	600 ± 100 μM

The IC_{50} values were determined using biotinylated pentylamine incorporation assay as described under "Materials and methods"

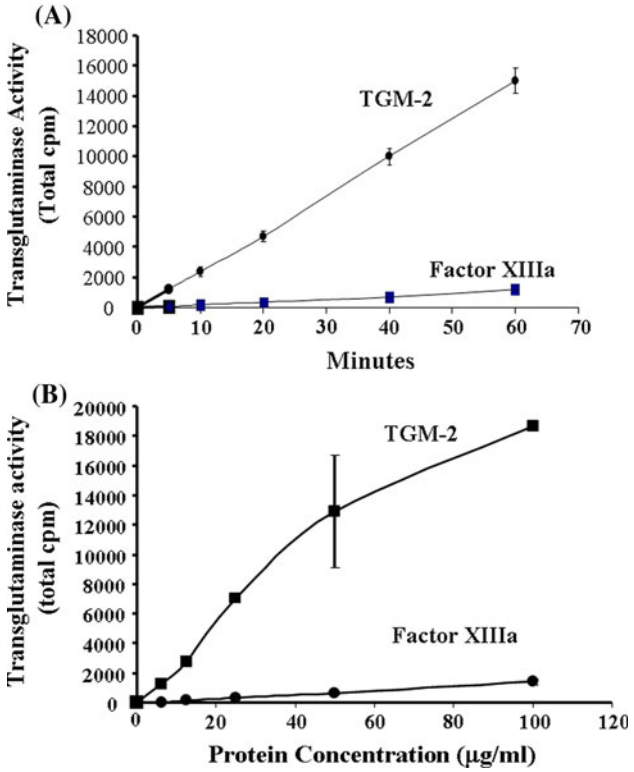


Fig. 1 Incorporation of histamine into fibrinogen by TGM-2 and factor XIIIa. **a** The solution phase transglutaminase assay was performed in a 100 μl reaction containing 0.1 M HEPES, pH 7.5, 10 mM calcium, 2 mM DTT, 1,000 μg/ml fibrinogen (factor XIII free), 0.8 μM of [^{3}H]-histamine (23.2 Ci/mmol) and 112 μM histamine, and 50 μg/ml of TGM-2 (or factor XIIIa) at 37 °C for 0–60 min. Incorporated [^{3}H]-histamine was measured by scintillation counting as described under "Materials and methods". **b** The same reaction conditions as in **a** except the reaction was performed at 37 °C with 0–100 μg/ml of TGM-2 (or factor XIIIa). All experiments were performed in triplicate and data were presented binding, the bound material was highly cross-linked fibrinogen as the average ± standard deviation

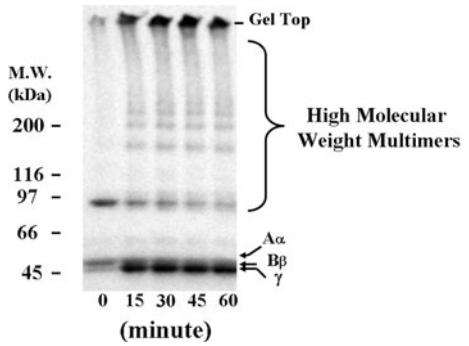
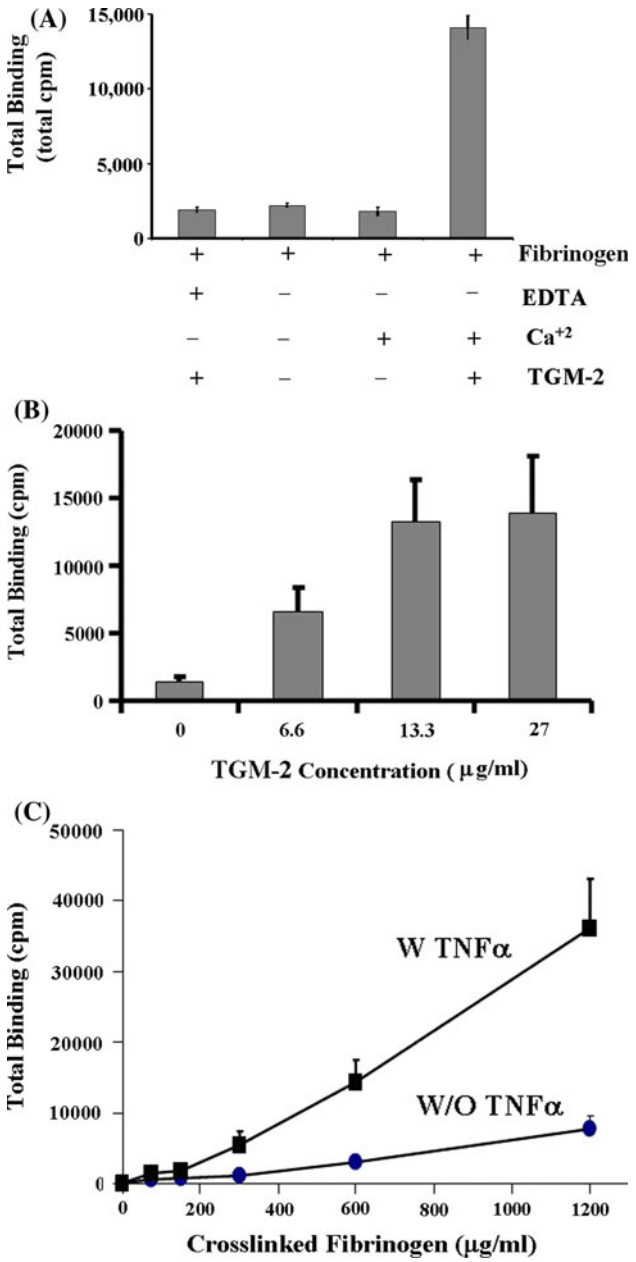


Fig. 2 Incorporation of [^{3}H]-histamine into fibrinogen by TGM-2 The cross-linking was performed in a 20 μl reaction containing 100 mM HEPES, pH 7.5, containing 7 μM of [^{3}H]-histamine (23.2 Ci/mmol, Perkin Elmer), 10 mM Ca^{+2}, 1,000 μg/ml of fibrinogen (factor XIII A free, American Diagnostica, CA), 200 μg/ml of TGM-2. The reaction was performed at 37 °C for 0, 15, 30, 45 and 60 min. At each time point, SDS-PAGE loading buffer was used to stop the reaction and ½ of the reaction mixtures were loaded onto a 5–15 % denaturing SDS-PAGE under reducing condition. On a separate lane, 10 μg of purified fibrinogen was also loaded. After electrophoresis, gels were stained with coomassie blue stain. The gels were dried on a piece of 3 MM filter paper and positions of fibrinogen Aα, Bβ and γ chains were labeled. The dried gel was then exposed to X-ray film at −80 °C for 3–5 days

to coat the plates. Histamine was selected for further investigation in this study as it was a potent biogenic amine that promotes inflammation. We used a solution phase transglutaminase assay to confirm this reaction would also occur in solution phase, and the results were the same as using immobilized protein on plate. In addition, TGM-2 was found to be a least 20 times more active than factor XIIIa (factor XIIIa) in the incorporation of [^{3}H]-histamine into fibrinogen in the solution phase assay (Fig. 1). Similarly, TGM-2 was more active than factor XIIIa in incorporating [^{3}H]-serotonin to fibrinogen (data not shown).

Time course studies demonstrated that TGM-2 initially catalyzed [^{3}H]-histamine incorporation into α–γ chains of fibrinogen, followed by the formation of high molecular weight crosslinked products, including high molecular weight multimers that failed to enter the gel (Fig. 2). The TGM-2 dependent reaction rate was rapid, with disappearance of monomeric alpha chains and formation of α–γ dimers and other higher molecular weight bands visible even at the 0 min. These higher molecular weight crosslinked products most likely were α–γ and Aα polymers as characterized by Shainoff et al. (1991) (Fig. 2). A ~97 kDa band was an intermediate crosslinked product and there was slowing disappearance of this band followed by the appearance of higher molecular weight products after 15 min of reaction. In contrast, the incorporation of [^{3}H]-serotonin into fibrinogen was only barely detectable even after many days exposure (data not shown). Histamine inhibited these crosslinking reactions in a concentration-dependent manner (data not shown).

The effect of the histamine on the interaction between TGM-2 and endothelial cells complexes was investigated using confluent human umbilical vascular endothelial cell (HUVEC) culture as a model. [^{125}I]-fibrinogen and



◀ **Fig. 3** Fibrinogen binding to HUVEC cells is TGM-2-dependent. **a** Binding to HUVEC cells is dependent on TGM-2 in the presence of Ca^{+2}. [^{125}I]-labeled fibrinogen (25 µg/ml) and un-labelled fibrinogen (250 µg/ml) were incubated in a 100 µl reaction containing either 5 mM EDTA, 10 mM Ca^{+2} or TGM-2 (25 µg/ml) at 37 °C for 15 min before mixing with binding buffer (EBM2/1 % BSA) and loaded onto endothelial cell cultures as described under "Materials and methods". The results were the average of a triplicate experiment. **b** Binding is increased upon increasing TGM-2 concentration. [^{125}I]-labeled fibrinogen (25 µg/ml) and 250 µg/ml of unlabelled fibrinogen were mixed in a 100 µl reaction containing different amount of TGM-2 (0, 6.6, 13.3, 26.7 µg/ml) at 37 °C for 15 min. The reaction mixtures were mixed with EBM2/1 % BSA and loaded onto HUVEC cells. Cells were washed three times with EBM2/1 % BSA before bound materials were eluted as described under "Materials and methods". The data were subtracted by the control experiments with the same TGM-2 concentrations in the absence of calcium. The results were the average of a triplicate experiment. **c** [^{125}I]-labeled fibrinogen (240 µg/ml) and unlabelled fibrinogen (2,400 µg/ml) were mixed in a 100 µl reaction containing 120 µg/ml of TGM-2 at 37 °C for 15 min. After the reaction, crosslinked fibrinogens were diluted on ice in a series of dilutions and were mixed with EBM2/1 % BSA binding buffer before loading onto HUVEC cells previously treated with or without 10 ng/ml of TNFα. The results were the average of a triplicate experiment

unlabelled fibrinogen ($W/W = 1{:}10$) were cross-linked by human recombinant TGM-2. The cross-linked radiolabelled materials were then incubated with HUVEC cultured on a 24-well microtiter plate. Binding was performed at 4 °C for 1 h to prevent internalization. After washing, the materials bound was eluted and quantified with a gamma scintillation counter. [^{125}I]-fibrinogen bound to endothelial cells with low affinity and the binding was increased approximately sevenfold when fibrinogen was cross-linked by TGM-2 (15,000 cpm/well) (Fig. 3). The increase in fibrinogen binding was dependent on TGM-2 concentration and Ca^{+2} (Fig. 3a, b). We found that unlabelled crosslinked fibrinogens inhibited the binding by more than 85 %, while unlabeled fibrinogen actually enhanced the binding several fold suggesting that fibrinogen and cross-linked fibrinogens bind to different sites and that fibrinogen helps to form multivalent complexes with cross-linked fibrinogens on the endothelial cell surface (data not shown). The TGM-2 cross-linked fibrinogen binding was also increased in HUVEC cells pretreated with 10 ng/ml of TNF-α Fig. 3c).

When the cell bound materials were eluted from HUVEC cells after binding, the bound material was highly cross-linked fibrinogen and migrated at the top of the gel (Fig. 4, lane 1). No fibrinogen bands were detected when non-crosslinked fibrinogen was eluted from the cells (Fig. 4, lane 2).

Since histamine could rapidly be crosslinked to fibrinogen and competitively inhibit fibrinogen crosslinking by TGM-2, we tested whether it could inhibit the TGM-2 and calcium dependent binding of fibrinogen to un-activated endothelial cells. When fibrinogen TGM-2 and calcium were incubated with un-activated endothelial cells histamine inhibited the TGM-2 and calcium dependent fibrinogen binding to the HUVEC cells by 75–90 % (Fig. 5).

Discussion

The role of fibrinogen in coagulation, hemostasis and inflammation is well-established (Davalos and Akassoglou 2012). Biogenic amines have been the subject of research, particularly in the neurosciences for many years (Walther et al. 2011). However, it is clear now that these amines are also involved in many other physiological processes

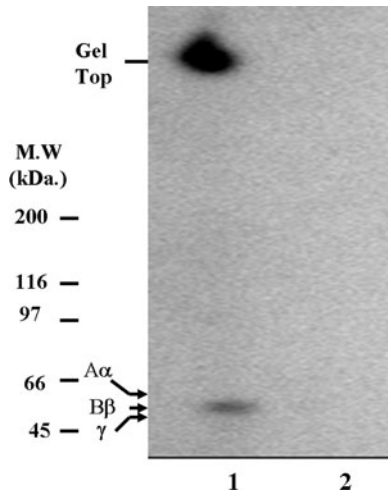


Fig. 4 Elution of surface bound fibrinogens from HUVEC cells 50 μg/ml of [^{125}I]-fibrinogen were incubated in a 100 μl reaction with (lane 1) and without (lane 2) 10 μg/ml of TGM-2 at 37 °C for 30 min. After reactions, mixtures were added to 24-well confluent HUVEC cell plate at 4 °C for 1 h. Cells were washed and surface bound proteins were eluted as described under "Materials and methods". 1/8 of the eluted materials were mixed with SDS-PAGE reducing buffer, separated by 5–15 % SDS-PAGE and exposed to X-ray film as describe under "Materials and methods"

including hemostasis and inflammation (Walther et al. 2011). The best studied biogenic amines include dopamine, serotonin (5-hydroxytryptamine; 5-HT) and histamine and they are primary amines that could be TGM-2 substrates (Walther et al. 2011). Biogenic amines are conjugated to glutamyl residues of polypeptides in vivo, through a transamidation reaction catalyzed by transglutaminases, in a reaction designated such as "serotonylation", or "histaminylation" (Dale et al. 2002; Szasz and Dale 2002; Prodan et al. 2007; Walther et al. 2011). Due to the limits of detection, the physiological significance of this post-translational protein modification has been minimally investigated. Protein-bound γ-glutamyl-histamine complexes were detected in liver and murine brain tissues but the physiological role was not defined (Walther et al. 2011). Recently, histaminylation of glutamine residues were found in several small and heterotrimeric G proteins Cdc42, $G_{\alpha o1}$, and $G_{\alpha q}$ indicating histaminylation could be involved in intracellular signaling (Vowinckel et al. 2012).

In this study, we found histamine was the preferred primary amine substrate for the covalent attachment to fibrinogen catalyzed by TGM-2. The data are in agreement with previous studies using carbobenzoxy-L-alanyl-L-glutamyl-valine ethyl ester and gliadin as glutamyl donor substrates (Pincus and Waelsch 1968; Qiao et al. 2005). We first showed that, in a solid phase transglutaminase assay, histamine was 4-fold and 300-fold better than putrescine and serotonin as a TGM-2 substrate. This may be an important reaction in vivo where fibrinogen is bound to fibrin, extracellular matrices or other cell surfaces. These findings were extended to fibrinogen in solution. Fibrinogen is the most prevalent coagulation protein in human plasma. There appears to be substantial variation in the preference of TGM-2 and factor XIIIa for primary amine incorporation into individual proteins. Serotonin was not a preferred TGM-2 substrate with fibrinogen, although it was previously found to be incorporated into gliadin, small GTPase and the surface of coat platelets in vivo (Dale et al. 2002; Walther et al. 2003).

The binding of fibrinogen to un-activated endothelial cells was a TGM-2 and calcium-dependent event suggesting that multivalent crosslinked fibrinogen complexes had higher tendency to bind to un-activated endothelial cells. It is unlikely that TGM-2 was mediating the binding as the binding was only minimum in the absence of calcium (Fig. 3). Although there was a TGM-2 dose-dependent increase in the binding in the presence of calcium, control experiments with TGM-2 in the absence of calcium (see Fig. 3b legend) did not show any increase in binding suggesting that TGM-2 was not mediating the binding to fibrinogen. In addition, fibrinogen was present in excess compared to TGM-2 in the binding experiments. The addition of un-crosslinked fibrinogens enhanced the binding suggesting that fibrinogen was promoting the binding of the multivalent crosslinked complexes (see "Results"). Previous investigation on the binding of TGM-2 crosslinked fibrinogen to HUVEC demonstrated that the binding was a calcium-dependent event (Martinez et al. 1989). In their studies, the binding of fibrinogen to HUVEC was not inhibited by RGDS peptide suggesting that integrins were

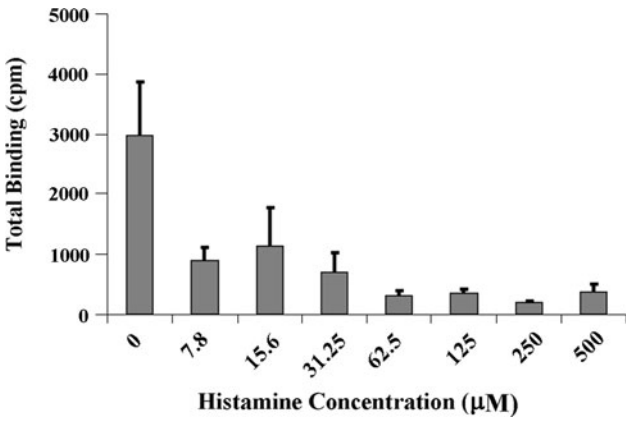


Fig. 5 Histamine inhibits TGM-2-dependent fibrinogen binding. The cross-linking of fibrinogen (1,000 μg/ml of unlabeled fibrinogens plus 50 μg/ml of [^{125}I]-fibrinogen) by TGM-2 in the presence of calcium was performed in a 100 μl reaction containing 0–500 μM of histamine. The reactions were performed at 37 °C for 30 min followed by extensive dialysis to remove free and unincorporated histamine. The dialyzed samples were mixed with binding buffer and added to confluent monolayer of HUVEC as described under "Materials and methods"

not directly involved in this TGM-2 dependent process (Martinez et al. 1989).

Endothelial cells must attempt to limit the propagation of thrombosis and have the thrombus adhere to the vessel wall to prevent embolization (MacCallum and Meade 1999). Endothelial, vascular smooth muscle cells and monocytes express TGM-2 and may interact with fibrinogen and fibrin during wound healing and inflammation. We postulated that there exists a mechanism that localizes adhesive proteins and blood cells crosslinked by TGM-2 to the vessel wall and that this reaction supports hemostasis, tissue repair and modulates inflammation. There is a well-established literature that links elevated fibrinogen to the risk of thrombosis (MacCallum and Meade 1999; Ariens et al. 2002). Several reports indicate that fibrinogen binds to ICAM-1 on endothelium that is activated by an inflammatory stimulus (Languino et al. 1993; Duperray et al. 1997). Fibrinogen serves as a bridge for leukocyte adhesion to activated endothelium as an initial step to the inflammation process that recruits leukocytes to inflamed tissues (Languino et al. 1993; Duperray et al. 1997). Fibrinogen binds to CD11b/CD18 on the leukocytes (Altieri et al. 1993). TGM-2 released from erythrocytes during tissue injury, present on endothelial and/or on monocyte surfaces can crosslink fibrinogen (Martinez et al. 1989; Barsigian et al. 1991). The cross-linked fibrinogen can also bind to endothelial cells that are either un-stimulated or activated with inflammatory cytokines TNFα. This previously un-recognized reaction could enhance inflammation and initiate blood cell adhesion and vascular obstruction. These complexes are potentially pro-inflammatory, prothrombotic and multivalent in nature. The binding of the TGM-2 mediated crosslinked fibrinogens to un-stimulated endothelial cells provides a site where leukocytes, platelets and/or fibrin could assemble, initiate, amplify and stabilize a microvascular obstruction and promote inflammation (MacCallum and Meade 1999; Ariens et al. 2002).

The effects of histamine are mediated through cell surface receptors including H1, H2, H3 and H4 (MacGlashan 2003; Walther et al. 2011). The allergic and inflammatory reaction induced by histamine is mediated in part by its binding to H1 on endothelial cell surface (Hao et al. 2008; Raveendran et al. 2011). There are three well-described sources of histamine in human: mast cells and basophils, gastric enterochromaffin-like cells, and histaminergic nerves in the brain (MacGlashan 2003). The wide distribution of receptors for histamine, as well as the wide distribution of mast cells and mobile basophils suggests that histamine is an important regulator of a variety of function. However, when histamine was conjugated to gliadin, it no longer exerted agonistic effects on histamine receptors, and therefore decreases its inflammatory potential (Qiao et al. 2005). The current studies suggest that fibrinogen may also serve to dampen histamine's pro-inflammatory effects in vivo. When fibrinogen was cross-linked in the presence of histamine, the binding to the HUVEC cells was reduced (Fig. 5). These results suggest that histamine can inhibit the TGM-2 and calcium-dependent binding of fibrinogen binding to un-activated endothelium. These results have three anti-inflammatory implications: (1) TGM2-mediated histamine incorporation into fibrinogen could reduce its agonistic effect on histamine receptors; (2) histamine inhibits TGM-2 and calcium dependent binding of fibrinogen to un-activated endothelial cells and thereby prevent further adhesive reactions mediated by fibrinogen on endothelium surface; (3) histamine released at sites of tissue injury antagonizes TGM-2's activity expressed on endothelial cells. The action of histamine may be as biologically important as spermidine, another primary amine substrate of TGM-2, which can delay lens opacification by inhibiting crosslinking of beta crystallin molecules (Lentini et al. 2011).

In summary, histaminylation of fibrinogen by TGM-2 has been demonstrated to occur with soluble and surface bound fibrinogen. This reaction could play a role in regulating inflammation at sites of tissue injury. TGM-2 on the cell surface or released in plasma could have multiple effects on acute and chronic inflammatory reactions mediated by histamine.

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Conflict of interest The authors declare that they have no conflicts of interest.

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