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MELAGATRAN PREVENTS TISSUE FACTOR EXPRESSION IN HUMAN PLATELET-MONOCYTE HETEROTYPIC COMPLEXES

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Abstract – Platelets form heterotypic complexes with circulating monocytes, inducing the expression of the procoagulant Tissue Factor (TF) that leads to thrombin generation. We investigated the potential preventive effect of melagatran, a direct anti-thrombin drug, on TF expression by platelet/monocyte heterotypic complexes (PMHC) from healthy human donors. Flow cytometry and western blot analysis were performed to characterize surface and total TF protein expression in PMHC in venous blood samples drawn in the presence of heparin or heparin and melagatran (4µM). Addition of melagatran significantly lowered the percentage of TF positive PMHC (2.6±0.3 *vs.* 5.9±0.7 %, *p<0.01*). This was not due to a melagatran-induced decrease in activation of the platelets associated with monocytes in PMHC. Indeed, melagatran effect on TF expression was accompanied by an increase in cell surface P-selectin expression in PMHC (95.6±1.9 *vs*. 48±18 %, *p<0.001*), suggesting that platelet were actually more activated in PMHC from the melagatran-treated samples. Western blot analysis of PBMC extracts suggested that melagatran specifically targeted a (54kD) form of TF in monocytes. Although further investigation is warranted, these data suggest that melagatran decreases TF expression in PMHC.

Key words: Melagatran, Tissue factor, platelet/monocyte heterotypic complexes, peripheral blood mononuclear cells, P-selectin

INTRODUCTION

Tissue factor (TF, CD142), a single chain transmembrane glycoprotein, is the main trigger of the coagulation proteolytic cascade. TF acts as a receptor for coagulation factors VIIa and X, leading to FXa formation and generation of active thrombin from prothrombin proteolysis. In the vessel, this activation of the coagulation cascade by TF plays a major role in developing inflammatory lesions after myocardial infarction or renal ischemia-reperfusion injury (IRI) (8, 14). Under specific pathophysiological conditions such as myocardial ischemia, coronary atherosclerosis or sepsis, active TF becomes detectable at the surface of monocytes and endothelial cells (7, 11, 29). Although the precise sequence of events leading to the expression of active TF is not fully understood, cellular interactions play a central role in the expression of TF activity in circulating monocytes (2, 22).

Highly relevant in the clinical setting of ischemia-reperfusion injury is the induction of TF expression in monocytes triggered by activated platelets (3, 6, 13). Indeed, platelet/monocyte heterotypic complexes (PMHC) typically form in the blood stream during cardiovascular ischemic events (1, 16), which could lead to increased TF expression. Hence, the development of new strategies aiming at preventing TF expression is of the up most interest in ischemic diseases and transplantations.

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; **FACS,** Fluorescence-activated cell sorting; **FITC,** Fluorescein isothiocyanate; **mAb,** Monoclonal antibody; **PAR-1, 4,** Protease activated thrombin receptor-1, 4; **PBMC,** Peripheral blood mononuclear cells; **PBS,** Phosphate buffer saline; **PE,** Phycoerythrin; **PerCP-Cy5.5**, Peridinin chlorophyll protein-cyanine dye Cy™5.5; **PMHC,** Platelet/monocyte heterotypic complexes; **PRP,** Platelet rich plasma; **SDS-PAGE,** Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **TF,** Tissue factor.

Melagatran is the active molecule of the pro-drug Ximelagatran (Astra Zeneca). Derived from the site of fibrinogen that binds the active site of thrombin, melagatran selectively and reversibly inhibits free and clot-immobilized active thrombin (15). There have been reports of indirect effect of melagatran as well, such as a prevention of *in-vivo* and *ex-vivo* PAR-1 and PAR-4 -dependent thrombin-induced platelet activation (21, 24). To our knowledge, the potential effect of melagatran on TF expression in human PMHC has not been investigated.

Thus, the aim of the present study was to assess the potential preventive effects of melagatran on TF total and surface expression in freshly prepared human PMHC. Using flow cytometry, we found that melagatran decreased TF surface expression in PMHC. Western blot analysis suggested that melagatran exposure depleted a 54kD form of TF in PMHC.

MATERIALS AND METHODS

Blood collection

Blood was collected by venopuncture from the antecubital vein of informed healthy volunteers (age range 23-55 years) who had not taken antiplatelet drugs within 14 days prior to donation. Whole blood was drawn with a 21-gauge needle into lithium heparin-containing tubes (BD® Vacutainer plasma tube, Becton Dickinson, France) in the presence or absence of 4µM melagatran (final concentration). Melagatran drug was provided by Astra-Zeneca.

Preparation of the Peripheral Blood Mononuclear Cell Suspension

PBMC were isolated from approximately 24 ml of freshly drawn blood. Blood was diluted in sterile phosphate buffer saline (PBS) and then carefully layered on top of a commercially available solution (Histopaque, 1.077g/ml, Sigma). After centrifugation for 25 min at 700g at 20°C, the interphase containing PBMC was collected and suspended in PBS/EDTA 1mM buffer. Cell suspension was pelleted by centrifugation at 350g for 5min and supernatant was replaced by fresh buffer and again centrifuged at 150g to remove loosely associated platelets (*ie*. those that were not engaged in complexes with PBMC).

Flow cytometry

To evaluate the percentage of platelet/monocyte complexes and platelet activation in PBMC suspensions, we used dual staining flow cytometry. Specifically, $CD14^+/CD41a^+$ (index of total PMHC) and $CD14^+/CD62P^+$ (index of activated platelets in PMHC) populations were analyzed and compared in suspensions from heparin or heparin/melagatran drawn blood. Approximately 400,000 cells were stained with saturating concentrations of the following fluorochrome-conjugated monoclonal antibodies (mAb): FITC-labeled mAb for the constitutive platelet marker CD41a (GPIIb glycoprotein of the platelet-specific complex GPIIb/IIIa), PE-labeled mAb for TF (CD142), PE-labeled mAb for the platelet activation marker P-selectin (CD62P) and PerCP- Cy5.5-labeled mAb for monocyte CD14 (endotoxin receptor) and corresponding isotype controls (all mAb were purchased at Becton Dickinson, France). After 20 minutes of incubation with antibodies in the dark at room temperature, the samples were fixed with a 5% formaldehyde/PBS solution. Flow cytometry was performed on a FACS Canto device (Becton Dickinson, USA). A minimum of 5,000 $CD14^+$ events were acquired for each analysis. The percentage of PMHC expressing TF was determined by the relative number of co-expression of the CD14 monocyte marker, the constitutive platelet marker CD41a, and TF/CD142 ($CD14^+/CD41a^+$/CD142 %). Activated platelets level in PMHC was characterized by the percentage of $CD62P^+$ in the $CD14^+$ population ($CD14^+/CD62P^+$, %). FITC and PE positive events percentage were obtained from Diva software, BD.

Electrophoresis and Immunoblotting

PBMC suspensions ($2.5\text{-}5.10^6$) were prepared in RIPA lysing solution (1mM EDTA, 150mM NaCl, 1% Sodium-deoxycholate, 0.1% SDS, 1% Triton, 50mM Tris-HCl, pH 7.4) with an anti-protease cocktail (phenylmethylsulfonylfluoride 170µg/ml, aprotinin 16µg/ml, benzamidin 16µg/ml, phenanthrolin 10µg/ml, pepstatin A 5µg/ml, leupeptin 4µg/ml, Sigma-Aldrich, France) and sodium orthovanadate (1mM). Protein concentration was measured using the bicinchoninic acid kit (Sigma-Aldrich, France). To control for platelet-TF protein expression, a platelet suspension from sodium citrate whole blood platelet rich plasma (PRP) was prepared as previously described (23), lyzed in RIPA solution as mentioned above. PBMC or isolated platelet lyzates containing 20µg or 10µg of protein, respectively, were boiled 5 min in Laemmli buffer (Sigma-Aldrich, France) and loaded on a 15% SDS-PAGE gel. Electrophoresis was performed with two different buffers (anode buffer: 0.2M Tris, pH 8.9 and cathode buffer: 0.1M Tris, 0.1M Tricine, 0.1% SDS, pH 8.25). Proteins were transferred onto an ECL nitrocellulose membrane (Amersham bioscience, France). The membrane was incubated with 1:1,000 diluted purified rabbit polyclonal anti-human brain TF antibody (2µg/ml, American Diagnostica, France). Specific protein bands were revealed following incubation with a peroxidase coupled anti-rabbit mouse IgG antibody (1:10,000 in TBS/0.5% Tween/5% Milk) for 1h at room temperature (Amersham Biosciences, France). After further washing, the peroxidase chemical signal was developed using ECL substrate (ECL Plus Western Blotting Detection Reagents, Amersham Pharmacia biotech, France) for 5 min.

Statistical Analysis

Results were obtained with blood samples from 8 different subjects. Data are expressed as mean ± SEM and were compared for statistical significance by unpaired t-test where a p value < 0.05 was considered significant.

RESULTS

To test whether melagatran reduces TF expression in PHMC, we prepared monocyte-enriched suspensions from blood collected in heparin- or heparin/melagatran- coated tubes (18). In this cell system, nearly all monocytes were recovered as PMHC for all conditions

tested, as shown by CD14/CD41a staining approaching 100% of the PBMC suspensions (data not shown).

Effect of melagatran on PMHC TF surface expression

TF expression in PMHC was evaluated using flow cytometry in samples prepared and labeled as described in Methods. As illustrated by the representative cytograms of figure 1A or the compiled data shown in figure 1B, TF expression was detectable in 5.9±0.7 % of PMHC in heparin-drawn blood. This percentage was significantly decreased to 2.6±0.3 % ($p<0.01$) when melagatran was added to heparin.

Effect of melagatran on PMHC P-selectin surface expression

To determine whether melagatran decreased TF expression by decreasing platelet activation, we compared the percentages of PMHC expressing P-selectin (a marker of platelet activation) in blood samples collected in the presence or absence of melagatran (Fig.2). As shown in figures 2A and 2B, melagatran treatment did not result in a decrease in platelet activation in PMHC. Rather, the proportion of P-selectin$^+$ PMHC was increased in the heparin/melagatran group compared to the heparin group (95.6±1.9 *vs*. 48±18 %, $p<0.001$).

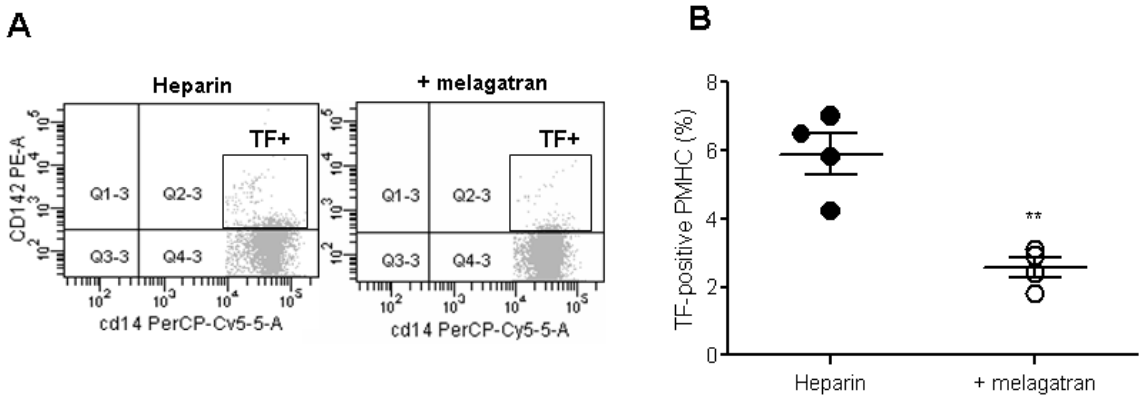

Figure 1. Melagatran-dependent modulation of TF surface expression in PMHC. Representative cytograms of PMHC isolated from heparin (left panel) and heparin+melagatran (4µM) (right panel) treated-blood samples (A). TF expression in PMHC present in PBMC isolated from heparin- or heparin + melagatran-drawn blood (B). Each individual value is represented as a dot. Bars represent means ± SEM (n = 4). * $p<0.05$ *and* ** $p<0.01$.

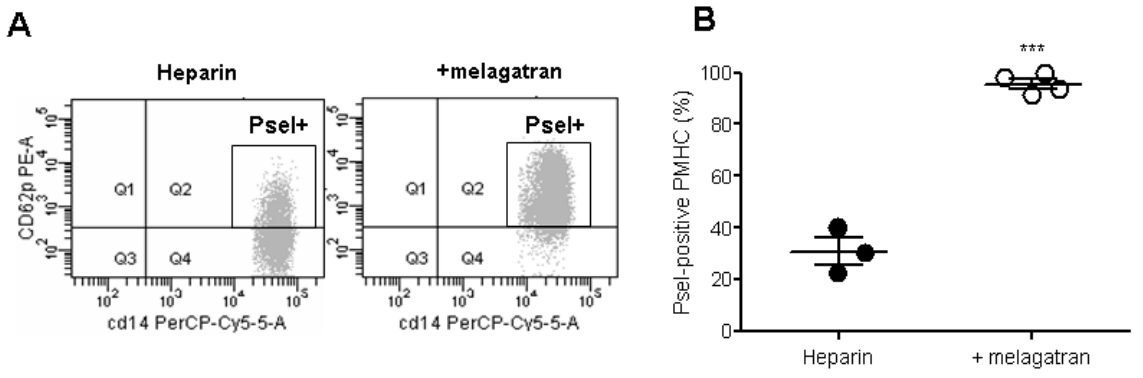

Figure 2. Melagatran-dependent modulation of P-selectin surface expression in PMHC. Representative cytograms of PMHC isolated from heparin (left panel) and heparin+melagatran (4µM) (right panel) treated-blood samples (A). P-selectin expression in PMHC present in PBMC isolated from heparin- or heparin + melagatran-drawn blood (B). Each individual value is represented as a dot. Bars represent means ± SEM (n = 3 or 4). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Melagatran depleted a 54 kD TF form in monocytes engaged in PMHC

To establish whether melagatran-induced decrease in TF expression in PMHC was due to a decreased in total expression, immunodetection studies were performed on PBMC whole cell lyzates using a purified polyclonal antibody against human TF. Beta actin was used as a loading control. As shown in immunoblots in figure 3B, a 54 kD band was detected in PBMC lyzates obtained from heparin-drawn blood, in addition to the 47 kD expected band for TF. The amount of the 54 kD TF detected was lowered in samples from heparin/melagatran treated blood samples (TF 53+54 kD/beta actin ratio: 0.74 *vs.* 0.05, $p<0.01$) (Fig. 3B, bar graph) whereas the 47kD TF was detected as a very minor band in both heparin and heparin/melagatran PBMC samples. Another, 53 kD TF band was weakly detected, but no detectable change in intensity occurred in the presence of melagatran (Fig. 3B). Two control experiments were performed to confirm the origin of the 53 and the 54 kD bands. First, a platelet extract was prepared and lyzed as described in Methods. As expected, only a 53 kD TF band was detected in absence of the 47kD TF form (Fig. 3A). The 53 kD TF band has been previously shown in resting platelet extracts, and identified as the platelet-associated human TF variant by Siddiqui *et al*. (27). However, the 54 kD TF protein band was not detected. In contrast, the 54 kD TF band was clearly detected in LPS stimulated PBMC, in addition to the expected 47 kD form known to be induced in monocytes under LPS stimulation (Fig.3A). Taken together, this data suggest that the 54 kD TF form detected in PMHC (that is regulated by melagatran) is of monocytic origin.

DISCUSSION

In the present study, we show for the first time that melagatran modulates TF expression on the surface of freshly isolated blood PMHC in human. Indeed, the addition of melagatran to heparin-drawn blood decreased TF expression in PMHC, as detected by flow cytometry. The decreased TF expression in PMHC induced by melagatran was not attributed to a decreased activation of the platelets engaged in PMHC interactions with monocytes. Instead, PMHC platelets expressed significantly more of the activation marker P-selectin in melagatran treated-blood samples. Western blot analysis on whole cell lyzates suggested that melagatran treatment specifically affected a 54kD form of TF expressed by PBMC.

The results of the flow cytometry analysis we conducted in human PMHC support the hypothesis that melagatran decreases TF expression while activating platelets as indicated by increased P-selectin. Although somewhat unexpected, this result is not in contradiction with earlier studies. First, the co-regulation of P-selectin and TF membrane expressions is still unclear. For example, the two proteins have been shown co-induced on platelet surface (19, 25), but they have also been found regulated independently of each other during epinephrine stimulation (5). Second, the effect of melagatran itself in platelet aggregation may explain this result. For example, although melagatran has shown potent, dose-dependent inhibitory effect on thrombin-induced platelet aggregation (21, 24), Soslau et al. have also reported a direct pro-platelet aggregation effect at low dose (28).

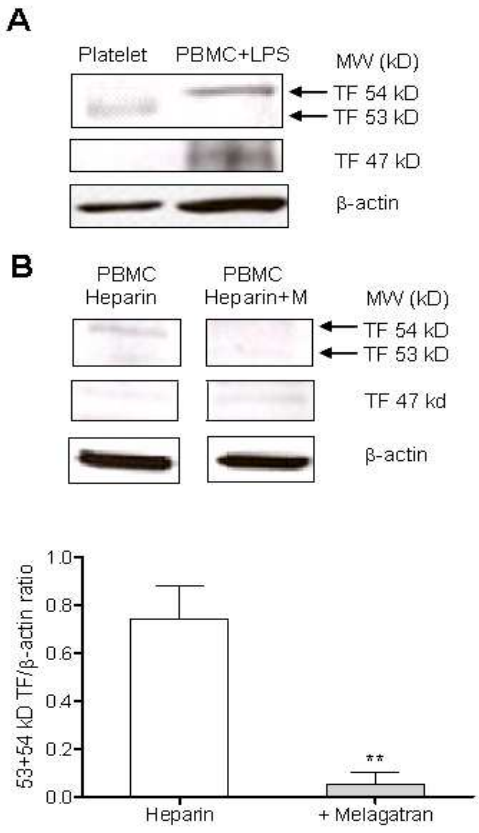

Figure 3. Melagatran-dependent modulation of 53/54 kD TF protein expression in PBMC. Representative immunoblots showing TF 53/54 kD bands as well as the 47kD expected band detected or not in fresh purified platelets extract and LPS-stimulated PBMC (4h with 5µg/ml) (A) or in PBMC isolated from heparin- or heparin+melagatran- drawn blood (B). Each picture is representative of 3-4 independent experiments. Bar graphs are means ± SEM of 4 experiments. ** $p<0.01$. *M: Melagatran*

Whereas we used high concentration of melagatran, residual low amounts of melagatran remaining on platelet surface may explain the induction of platelet degranulation we observed by PMHC P-selectin expression (Fig. 2). Third, studies have also suggested a direct interaction of free melagatran (i.e., not associated of thrombin) with GPIbα and PAR-4 platelet thrombin receptors. Finally, the role of activated platelets in triggering the expression of TF in PMHC is still poorly understood. GPIIbIIIa antagonists (such as abciximab, eptifibatide or clopidogrel) have shown differential effects on platelet-monocyte interaction and surface TF expression in the blood (26, 30). Hence, anti-GPIIbIIIa treatment in patients receiving coronary angioplasty or stenting showed reduced PMHC formation but increased platelet-induced TF expression (9, 10, 12, 20).

A 54 kD form of TF was detected in PMHC

The results presented in figure 3 suggest that melagatran regulates a 54 kD form of TF in PMHC. In LPS-stimulated PBMC, the anti-TF polyclonal antibody recognized two forms of TF: the 47 kD TF form, which is commonly described as the fully glycosylated TF (4, 7, 17), and a 54 kD form that has not been reported before (Fig. 3A). In platelets, we did not detect the 47 kD form but only one 53 kD TF form, which has been described previously (Fig. 3A) (27). We detected a very minor 47 kD TF form in PBMC extracts, irrespective of the treatment (Fig. 3B). In PMHC, we detected a trace amount of the 53 kD, and the level of expression was not modified by the addition of melagatran. Conversely, the 54 kD band detected in LPS-stimulated PBMC was clearly detected in PHMC, and addition on melagatran strongly decreased its level of expression. Taken together, these data suggest that the 54 kD TF detected in PMHC originated from the monocyte rather than the platelet, and that melagatran-induced regulation of TF expression in PMHC is through the regulation of the expression of this 54 kD form.

Taken together, this study suggests that melagatran has anti-TF properties in human PHMC. Further investigations are required to confirm the nature and the melagatran-induced regulation of the proposed 54 kD form of TF we detected in PMHC. If confirmed, this new property of melagatran may prove useful in the context of ischemia-reperfusion injury.

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