Early and Transient Release of Leukocyte Pentraxin 3 during Acute Myocardial Infarction

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Pentraxin 3 (PTX3) plays cardioprotective and anti-atherogenic roles in murine models. PTX3 blood levels raise during early acute myocardial infarction (AMI). Neutrophils from healthy subjects physiologically contain PTX3 in secondary (also called specific) granules. In this study, we report that circulating neutrophils release preformed PTX3 in the early phase of AMI (within 6 h from the onset of clinical symptoms). Depletion of intracellular PTX3 correlates with increased plasma levels and with platelet–neutrophil heterotypic aggregates. Neutrophil PTX3 returns to normal values 48 h after the onset of symptoms; concentration does not vary in matched healthy controls or in patients with chronic stable angina. In vitro, recognition of activated P-selectin+ platelets causes the formation of neutrophil–platelet heteroaggregates and the release of neutrophil PTX3. Purified or membrane-bound P-selectin triggers PTX3 release from resting neutrophils. Released PTX3 binds to activated platelets in vitro. Moreover, PTX3 binds to a substantial fraction of platelets from patients in the circulating blood. PTX3–bound activated platelets have a reduced ability to 1) form heterotypic aggregates with neutrophils and monocytes; 2) activate neutrophils, as evaluated assessing the upregulation of PTX3 in AMI patients not only is a solid biomarker, but it possibly represents the outcome of a homeostatic response to tissue injury (1, 8). The protein has a well-characterized cardioprotective action (19, 20). Concentrations of PTX3 raise in the circulating blood of wild-type mice after heart ischemia/reperfusion, as also occurs in AMI patients. PTX3 reduces the size of murine infarcts and the clinical severity of coronary artery occlusion (19). PTX3 in atherosclerosis-prone, apolipoprotein E-deficient mice attenuates the inflammatory response associated to the vessel lesions. Several molecular mechanisms are involved: PTX3 regulates in damaged cells and tissues the activation of the complement system (19) and attenuates P-selectin–dependent neutrophil recruitment at sites of inflammation (21, 22). The latter result is particularly intriguing, since P-selectin expressed by endothelial cells and activated platelets is a key signal regulating the activation and the function of circulating leukocytes via its counterreceptor P-selectin glycoprotein ligand 1 (23–28).
Table I. Main characteristics of patients with AMI or CSA

<table>
<thead>
<tr>
<th></th>
<th>AMI (n = 24)</th>
<th>CSA (n = 20)</th>
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<tbody>
<tr>
<td>ST segment elevation</td>
<td>14</td>
<td>–</td>
</tr>
<tr>
<td>Time after symptom onset, h (median, range)</td>
<td>3 (1–6)</td>
<td>–</td>
</tr>
<tr>
<td>Age, y (median, range)</td>
<td>66 (39–74)</td>
<td>66 (39–72)</td>
</tr>
<tr>
<td>Sex (M, F)</td>
<td>17, 7</td>
<td>14, 6</td>
</tr>
<tr>
<td>Troponin I concentration, ng/ml</td>
<td>0.72 (0.04-4.22)</td>
<td>–</td>
</tr>
<tr>
<td>Peak (median, range)</td>
<td>20.4 (6.5–202.0)</td>
<td>–</td>
</tr>
<tr>
<td>Previous AMI</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Previous PCI (%)</td>
<td>0</td>
<td>6 (30)</td>
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F: female; M: male; PCI: percutaneous coronary intervention; –, negative.

Materials and Methods

Study population

We studied blood cells from 64 subjects, including 24 consecutive patients with AMI, with elevation of the ST segment in the electrocardiogram. Each patient was studied twice: at the moment of enrollment, that is, within 6 h from the onset of symptoms, and at a later time point, that is, 48 h from the onset of symptoms. Table I depicts some characteristics of the patients, from the onset of symptoms, and at a later time point, that is, 48 h from the onset of symptoms.

The source of circulating PTX3 in AMI patients remains to be established. Several immune and tissue cells produce it in physiologic conditions in response to primary proinflammatory signals, including endothelial cells, monocytes–macrophages, and dendritic cells (2). However, the extent and the time kinetics of the enhancement of PTX3 systemic concentration in AMI better agree with the prompt release of a pre-existing PTX3 reservoir than with a de novo synthesis and secretion by tissue cells in response to local stimuli. Neutrophils represent an attractive candidate: they store PTX3 in secondary (also called specific) granules and release it in response to the recognition of both microbial and sterile inflammatory signals (29). Neutrophils in heart tissues derived from patients with advanced AMI appear depleted of the protein, possibly as a consequence of previous degranulation. Neutrophils are also known to undergo activation across the coronary vascular bed in patients with acute coronary syndromes, an event that is associated with the release of other biologically active molecules contained in primary granules, such as myeloperoxidase (MPO) (30, 31). In this study we examined whether in patients with AMI neutrophils release PTX3, and whether PTX3 modulates the acti-vatory cross-talk between activated platelets and leukocytes, which contributes to coronary thrombosis (32–37).

Table II. Markers of leukocyte and platelet activation/interaction in patients with AMI or CSA and in matched healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Early AMI (0–6 h)</th>
<th>Late AMI (48 h)</th>
<th>CSA</th>
<th>Healthy Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>24</td>
<td>24</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>4.3 ± 0.7a</td>
<td>6.4 ± 1.0a</td>
<td>&lt;2.5</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Average MPO content (MFI arbitrary units, within CD66b) cells</td>
<td>59.9 ± 4.11* #</td>
<td>126.2 ± 12.3</td>
<td>134.5 ± 5.6</td>
<td>154.4 ± 5.7</td>
</tr>
<tr>
<td>Neutrophil–platelet heteroaggregates (% of CD66b cells)</td>
<td>17.4 ± 2.3* #</td>
<td>7.9 ± 1.1</td>
<td>7.6 ± 0.7</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>Monocyte–platelet heteroaggregates (% of CD14+ cells)</td>
<td>11.2 ± 1.7</td>
<td>9.3 ± 1.9</td>
<td>4.1 ± 0.8</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>Neutrophil CD18 expression (MFI arbitrary units, within CD66b cells)</td>
<td>579.4 ± 18.6* #</td>
<td>415.7 ± 20.2</td>
<td>409.2 ± 22.2</td>
<td>413.8 ± 29.2</td>
</tr>
<tr>
<td>Platelets expressing P-selectin (% of CD61+ cells)</td>
<td>25.9 ± 1.9* #</td>
<td>9.1 ± 0.9</td>
<td>7.4 ± 0.9</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>Platelets with bound PTX3 (% of CD61+ cells)</td>
<td>5.7 ± 0.3* #</td>
<td>16.6 ± 1.6</td>
<td>2.8 ± 0.3</td>
<td>2.2 ± 0.2</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM. Isotopic control MFI values for average MPO content, 2.1 ± 0.1; for neutrophil–platelet heteroaggregates, 0.5 ± 0.2%; for monocyte–platelet heteroaggregates, 0.3 ± 0.1%; for neutrophil CD18 expression, 158.7 ± 18.3; for platelet P-selectin expression, 0.7 ± 0.1%; for platelets with bound PTX3, 0.3 ± 0.2%. *p < 0.001 versus late AMI, #p < 0.001 versus both CSA patients and matched healthy subjects.
**Blood sampling and processing**

Venous blood was obtained from patients and controls using a 19-gauge butterfly needle. The first 6 ml was used for routine laboratory determinations. Blood was collected in an EDTA-containing Vacutainer to obtain plasma for PTX3 determination. For studies of neutrophil and platelet interaction, blood was carefully collected in tubes containing 0.2 ml sodium citrate (109 mM) and a mixture containing Na2EDTA (50 mM), N-ethylmaleimide (60 mM), and aprotinin (2000 kIU/ml) to limit in vitro cellular activation as much as possible. Aliquots were either immediately fixed with an equal volume of ThromboFix or treated with FACS lysis solution and processed for flow cytometry, immunofluorescence, and confocal microscopy (23, 41).

**FIGURE 1.** Transient depletion of neutrophil PTX3 in patients with AMI. Intracellular and plasmatic PTX3 were evaluated in samples from patients with AMI within the first 6 h (early AMI) and at 48 h after (late AMI) onset of symptoms in CSA patients and in healthy donors. A and B, Data are presented as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars). A, Significantly higher levels of plasmatic PTX3 were observed in early AMI compared with late AMI to CSA patients and to healthy subjects (all \( p < 0.0001 \)). B, Neutrophil PTX3 content was evaluated by flow cytometry in whole blood of AMI patients and controls. Significantly lower values were observed in early AMI compared with late AMI to CSA patients and healthy subjects (all \( p < 0.0001 \)). C and D, Representative flow cytometry profiles from an early AMI patient (C) and a CSA patient (D). Open profiles represent the fluorescence associated to the relevant isotypic control; filled profiles represent the fluorescence associated to PTX3. E and F, Representative confocal microscopy from an early AMI patient (E) and a CSA patient (F). PTX3 neutrophil content (Alexa Fluor 488, green) is lower in AMI patients. Nuclei were stained with Hoechst 33258 (blue). Original magnification \( \times 63 \), zoom 3.45.
PTX3 assay

PTX3 was assayed by a noncommercial sandwich ELISA (detection limit, 0.1 ng/ml) based on a rat mAb and a biotin-conjugated rabbit anti-serum as previously described (15, 16) ELISA for PTX3 does not cross-react with the short pentraxins CRP and serum amyloid P CRP was measured in the serum by nephelometry (42).

In vitro studies

Venous blood was collected after informed consent from healthy volunteers, who did not receive any medication for at least 2 wk. Blood was drawn with an 18-gauge needle, dripping freely into open tubes, containing 3.8% sodium citrate (1:9, v/v). Neutrophils and platelets were isolated as described (23, 38) and resuspended in HEPES-Tyrode buffer (pH 7.4) containing CaCl2 (1 mM). Neutrophils were incubated (5 min at 37˚C) either alone or with resting or activated autologous platelets (neutrophil/platelet ratio, 1:20); with CHO-P or with CHO (neutrophil/CHO ratio, 5:1); with MBL (0.5 μM) in the presence of cytochalasin B (2.5 μg/ml); or with purified P-selectin (5 μg/ml) (38). Reactions were stopped by the addition of an equal volume of ice-cold 10-fold fixing solution. The neutrophil MPO and PTX3 content and the CD18 neutrophil expression were then determined by flow cytometry and confocal microscopy (see below). To assess PTX3 binding to activated platelets, purified platelets (200,000/μl) were stimulated with TRAP-6 (15 μM) for 1–3 min at 25˚C or 37˚C in the presence of increasing concentrations (5–40 μg/ml) of biotinylated recombinant PTX3 or of human albumin. Reactions were stopped by addition of thrombofix. Samples were then labeled with Alexa Fluor 488-biotin and with the anti-CD61 mAb; the fraction of platelets with bound PTX3 was then determined by flow cytometry (see below). To assess platelet–neutrophil and platelet–monocyte heterotypic aggregates, purified platelets were loaded with the BCFE-AM intracellular dye as previously described (23, 38), resuspended at a final concentration of 200,000/μl, and stimulated with TRAP-6 (15 μM, 3 min at 37˚C) in the presence of increasing concentrations (5–40 μg/ml) of recombinant PTX3 or of human albumin. An equal volume of autologous whole blood was then added. After 3 min at 37˚C, reactions were stopped by the addition of ThromboFix. Samples were then labeled with mAbs against CD45, CD14, and CD66b and platelet–leukocyte heterotypic aggregates assessed by flow cytometry (see below). To assess fibrinogen binding to activated platelets, purified platelets (200,000/μl) were stimulated with TRAP-6 (15 μM for 3 min at 37˚C) in the presence of increasing concentrations (5–40 μg/ml) of recombinant PTX3 or of human albumin and 100 mg/ml human fibrinogen previously labeled with an Alexa Fluor 488-fluorescence protein labeling kit. Reactions were stopped with equal volumes of ThromboFix. Samples were then labeled with anti-CD61 mAbs against CD45, CD14, and CD66b and platelet–leukocyte heterotypic aggregates assessed by flow cytometry (see below). To assess PTX3 binding to activated platelets, purified platelets (200,000/μl) were stimulated with TRAP-6 (15 μM) for 1–3 min at 25˚C or 37˚C in the presence of increasing concentrations (5–40 μg/ml) of biotinylated recombinant PTX3 or of human albumin. An equal volume of autologous whole blood was then added. After 3 min at 37˚C, reactions were stopped by the addition of ThromboFix. Samples were then labeled with mAbs against CD45, CD14, and CD66b; and platelet–leukocyte heterotypic aggregates assessed by flow cytometry (see below). To assess fibrinogen binding to activated platelets, purified platelets (200,000/μl) were stimulated with TRAP-6 (15 μM for 3 min at 37˚C) in the presence of increasing concentrations (5–40 μg/ml) of recombinant PTX3 or of human albumin and 100 mg/ml human fibrinogen previously labeled with an Alexa Fluor 488-fluorescence protein labeling kit. Reactions were stopped with equal volumes of ThromboFix. Samples were then labeled with anti-CD61 mAbs to determine the fraction of platelets with bound fibrinogen, or with anti-CD61 and anti-CD62P mAbs to determine the fraction of platelets expressing P-selectin. Where indicated, platelets were labeled with anti-CD61 mAbs, permeabilized, and then labeled with anti-von Willebrand factor mAbs and the fraction of degranulated platelets was assessed by flow cytometry. To assess aggregation, purified platelets (200,000/μl) were stimulated into aggregometer cuvettes with TRAP-6 (15 μM) for 1–3 min at 25˚C or 37˚C in the presence of increasing concentrations (5–40 μg/ml) of recombinant PTX3 or of human albumin. An equal volume of autologous whole blood was then added. After 3 min at 37˚C, reactions were stopped by the addition of thrombofix. Samples were then labeled with Alexa Fluor 488-biotin and with the anti-CD61 and anti-PTX3 Abs (MNB4). PTX3-associated fluorescence was then determined within the CD61+ cell population. For in vitro determinations, neutrophil granular content of MPO or PTX3 was assessed in purified neutrophils fixed, permeabilized, and labeled as described above. Neutrophil CD18 expression was assessed in samples labeled with anti-CD45 and CD66b mAbs. For the determination of platelet–neutrophil and platelet–monocyte heterotypic aggregates, fluorescent BCFE-AM–loaded platelets were used and BCFE-AM fluorescence was traced within neutrophil (CD45+, CD66b+, CD14+) or monocyte (CD45+, CD66b+, CD14+) cell clusters (Supplemental Fig. 1). Samples were analyzed on a Gallios flow cytometer (Beckman Coulter, Miami, FL).

Confocal microscopy

Confocal microscopy was carried out as described previously (23). Briefly, whole blood samples were treated with FACS lysing solution, fixed, and labeled with the specific mAbs. Where indicated for intracellular Ags, samples were permeabilized with a Fix & Perm kit and then labeled. All mAbs were previously labeled with Zenon IgG labeling kits-Alexa Fluor. Hoechst 33258 (blue color) was used for counterstaining nuclei. Samples were then washed and plated to glass coverslips before analysis with a Leica TCS SP2 laser scanning confocal microscope (objective, ×63; numeric aperture, 1.4).

Flow cytometry

All samples were analyzed as described (23). Briefly, for ex vivo determinations, to assess neutrophil–platelet heterotypic aggregates, whole samples were fixed with Thrombofix, labeled with mAbs against CD45, CD14, CD66b, and CD61; neutrophils were identified within the CD45+ CD66b+ and CD14+ populations and by their orthogonal light scatter. Neutrophils with adherent platelets were identified by the expression of the platelet Ag CD61 in the CD66b+ CD14+ cell cluster, whereas monocytes with adherent platelets were identified by the expression of CD61 in the CD66b+ CD14+ cell cluster. To study neutrophil granule content, whole blood samples were treated with FACs lysing solution, labeled with mAbs against CD45, CD14, and CD66b, fixed and permeabilized with a Fix & Perm kit, and labeled with mAbs against MPO or against PTX3. To assess platelet P-selectin expression, whole blood samples fixed with ThromboFix were labeled with mAbs against CD61 and P-CD62P. Platelets were identified within the CD61+ population and by their logarithmic light and forward scatter (Supplemental Fig. 1). To assess PTX3 binding to platelet–monocyte heterotypic aggregates, samples fixed with ThromboFix were labeled with anti-CD61 and anti-PTX3 Abs (MNB4). PTX3–associated fluorescence was then determined within the CD61+ cell population. For in vitro determinations, neutrophil granular content of MPO or PTX3 was assessed in purified neutrophils fixed, permeabilized, and labeled as described above. Neutrophil CD18 expression was assessed in samples labeled with anti-CD45 and CD66b mAbs. For the determination of platelet–neutrophil and platelet–monocyte heterotypic aggregates, fluorescent BCFE-AM–loaded platelets were used and BCFE-AM fluorescence was traced within neutrophil (CD45+, CD66b+, CD14+) or monocyte (CD45+, CD66b+, CD14+) cell clusters (Supplemental Fig. 1). Samples were analyzed on a Gallios flow cytometer (Beckman Coulter, Miami, FL).
**Statistical analysis**

Numerical values are represented by means ± SEM. Comparisons of markers of platelet and leukocyte activation between patients with AMI, patients with CSA, and healthy controls were made using ANOVA and Tukey’s test. Markers of cellular activation within 6 h and 48 h after onset of symptoms were compared by a paired t test. Associations between cellular markers of activation were assessed by Spearman correlation coefficients. All tests were two-sided and p values <0.05 were considered as statistically significant.

**Results**

**Neutrophils acutely and transiently release PTX3 in the early phase of AMI**

PTX3 blood concentrations are known to increase in patients undergoing AMI (15, 16). We confirmed these observations in patients with early AMI (within 6 h from the symptoms onset) (Tables I and II): plasma concentration of the molecule was indeed significantly higher than in patients with CSA (10.4 ± 1.1 versus 4.2 ± 0.3 ng/ml [mean ± SEM]; p < 0.001) or in matched healthy controls (10.4 ± 1.1 versus 3.8 ± 0.3 ng/ml [mean ± SEM]; p < 0.001). Plasma levels of PTX3 were substantially lower in the same patients at later time points (48 h after onset of symptoms) (10.4 ± 1.1 versus 5.7 ± 0.3 ng/ml [mean ± SEM]; p < 0.001) (Fig. 1).

Circulating neutrophils contain preformed PTX3 (29) and represent a candidate for early release of the protein in the circulating blood. Indeed, PTX3 content, determined by four-color flow cytometry, abated at early times of AMI: neutrophil PTX3-associated fluorescence was significantly lower in AMI patients than in either patients with CSA or in healthy controls [mean fluorescence index (MFI), 288.4 ± 25.7 versus 565.9 ± 11.7 and 513.7 ± 24.2, respectively; p < 0.001 for both comparisons] (Fig. 1). Conversely, neutrophil PTX3 content returned to normal levels in patients undergoing AMI at later time points as assessed by

![FIGURE 3. P-selectin–dependent release of neutrophil PTX3.](http://www.jimmunol.org/)

**FIGURE 3.** P-selectin–dependent release of neutrophil PTX3. The PTX3 content was assessed in purified human neutrophils exposed or not to resting or activated platelets, CHO cells, CHO cells stably expressing P-selectin, or to purified P-selectin. As a positive control of neutrophil activation, the fMLF peptide was used. A, Exposure to P-selectin–expressing cells, but not to resting platelets or parental CHO cells, causes neutrophil PTX3 depletion, as assessed verifying by flow cytometry the fraction of PTX3-expressing neutrophils and the amount of PTX3-associated fluorescence (MFI within CD66b⁺ cells). Expression of neutrophil PTX3 (green) in the presence of activated (B) but not of resting (C) platelets (glycoprotein Ib; Alexa Fluor 546, red) was also verified by confocal microscopy. Nuclei were counterstained with Hoechst 33258. D, Exposure to purified P selectin or to fMLF causes the depletion of both neutrophil PTX3 and MPO, as assessed verifying by flow cytometry the amount of PTX3-associated fluorescence (gray histograms) and of MPO-associated fluorescence (black histograms) (y-axis, MFI arbitrary units). Expression of neutrophil PTX3 (Alexa Fluor 488, green), which is conserved in untreated neutrophils (E), abates in the presence of fMLF (F) and of purified P-selectin (G), as verified by confocal microscopy. Nuclei were counterstained with Hoechst 33258 (blue). Original magnification ×63, zoom 3.45.
flow cytometry (MFI, 499.6 ± 12.8 at 48 h after the onset of the symptoms versus 288.4 ± 25.7 within 6 h; p < 0.001) (Fig. 1).

Figs. 1 and 2 depict the neutrophil PTX3 expression in patients with early AMI compared with patients with CSA or healthy controls, as assessed by confocal microscopy. Circulating neutrophils have a relatively short half-life: this result therefore suggests that the stimulus that elicits PTX3 release is effective in the earlier phases of AMI only.

**Activated platelets trigger neutrophil PTX3 release**

Neutrophils with low PTX3 content often physically interacted and formed aggregates in the circulating blood of early AMI patients with platelets (Fig. 2C). Indeed, the frequency of hetero-aggregates and the intracellular PTX3 content of circulating neutrophils were negatively correlated (r = −0.55, p < 0.005) (Fig. 2D). As expected, aggregates were rare in patients with CSA or in healthy controls, and neutrophil PTX3 content was unaffected (Fig. 2B, 2C). Platelet–leukocyte aggregates are a well-characterized hallmark of acute coronary syndromes and other systemic inflammatory diseases (11, 24, 36, 41, 43–45). Stable interaction between platelets and leukocytes depends on the activation of platelets, which, in turn, modulates the activation and adhesive properties of leukocytes. We therefore verified the expression of platelet P-selectin in the blood of patients and controls and observed that a substantial fraction of platelets from patients with early AMI are indeed, as expected (23, 43, 45), activated. Moreover, the fraction of platelets expressing P-selectin was negatively correlated with the neutrophil PTX3 content (r = −0.65, p < 0.001) and was positively correlated with the concentration of the molecule in the plasma (r = −0.67, p < 0.001; Fig. 2E, 2F).

We relied on three independent approaches to verify whether the recognition of activated platelets causes neutrophil PTX3 release and to identify the possible role of platelet P-selectin. First, neutrophils from healthy subjects were exposed to autologous activated or resting platelets. Second, neutrophils were incubated with CHO-P cells or with the parental CHO cells as negative control. Finally, neutrophils were challenged with purified human P-selectin. All stimuli effectively elicited the release of PTX3 (Fig. 3). Moreover, when neutrophils were challenged with activated...
(but not with resting) platelets, they both adhered to platelets, forming heteroaggregates, and released PTX3 (Fig. 3B, 3C). P-selectin recognition was almost as effective as the gold standard for neutrophil activation, the formylated fMLF peptide: it caused the release of molecules contained in the primary granules as well, such as MPO, thus resulting in virtually complete neutrophil degranulation (Fig. 3D–G).

Activated platelets as a target of neutrophil PTX3

A substantial fraction of platelets in patients with AMI (but not in CSA patients or healthy controls) circulated with PTX3 stably associated to the plasma membrane (Fig. 4A). This feature was clearly restricted to the later phases of AMI (Fig. 4A–C). Supporting evidence came from in vitro experiments: the recombinant purified molecule effectively bound to activated (but not to resting) platelets in a time- and temperature-dependent manner (Fig. 4D, 4E). Similar results were obtained when activated platelets were challenged with PTX3 released from fMLF-stimulated neutrophils (Fig. 4F).

PTX3 binding influenced some biological properties of platelets: in particular, PTX3 inhibited the platelet aggregation induced by various agonists, such as TRAP-6 and collagen (Fig. 5A, 5B), and interfered with the binding of fibrinogen to activated platelets (Fig. 5E), even if it did not influence α granule content upon activation, as assessed by evaluating the expression of P-selectin and the platelet von Willebrand factor content (Fig. 5D). Moreover, PTX3 influenced the inflammatory potential of activated platelets: the formation of heterotypic aggregates with autologous neutrophils (Fig. 6A) and monocyes (Fig. 6B) was prevented by activated platelets decorated with recombinant PTX3. The inhibitory effect of PTX3 on the formation of platelet–neutrophil heterotypic aggregates, as assessed by flow cytometry, was dose-dependent. Finally, in the presence of PTX3, activated platelets were significantly less effective at upregulating the expression of the CD11b/CD18 integrin, which is critical for the ability of leukocytes to adhere and to transmigrate within inflamed tissues and was similar in neutrophils challenged with resting or activated PTX3-bound platelets (Fig. 6C). The data raise the possibility that PTX3 decorates activated platelets and dampens their inflammatory potential, contributing to modulate the innate immune response and to limit collateral damages to bystander tissues.

**FIGURE 5.** PTX3 interferes with the ability of platelets to aggregate and to bind to fibrinogen. Increasing concentrations of PTX3 (open symbols) but not of albumin (filled symbols) (x-axis) inhibit platelet aggregation (y-axis) in response to TRAP-6 and to collagen in a dose-dependent manner (A–C). In contrast, P-selectin and von Willebrand factor expression after challenge with TRAP-6 were not altered (D). TRAP-6–activated platelets effectively bound to fibrinogen: this event was inhibited in a dose-dependent manner by PTX3 (E). Results in A are representative of seven independent experiments.
FIGURE 6. PTX3 interferes with the platelet–leukocyte cross-talk. Increasing concentrations of PTX3 (open symbols) but not of albumin (filled symbols) (x-axis) inhibit in a dose-dependent fashion the formation of heterotypic aggregates (y-axis) between activated platelets and monocytes (A) or neutrophils (B). C, Upregulation of neutrophil CD18 integrin. The effect of PTX3 on the cross-talk between activated platelets and neutrophils was assessed verifying by flow cytometry the expression of the CD18 molecule (MFI within CD66b+ cells, y-axis). Significant upregulation is observed in the presence of activated platelets. Increasing concentrations of PTX3 (open symbols) but not of albumin (filled symbols) (x-axis) inhibit in a dose-dependent fashion. Resting platelets do not influence CD18 expression, even in the presence of increasing concentrations of PTX3.

Discussion

The main objective of this study was to assess whether neutrophils represent a source of the long PTX3 in the blood of patients undergoing AMI and whether the protein regulates the interaction between neutrophils and activated platelets. We found that intracellular PTX3 is indeed depleted during AMI. This is an acute and transient event, which parallels the rise in plasma concentrations of the protein. Moreover, we found that PTX3, either as a recombinant moiety or released from degranulating neutrophils, binds to activated platelets and dampens their inflammatory potential. During late phases of AMI, characterized by the reduced activation of platelets and leukocytes, a substantial fraction of circulating platelets has PTX3 associated to membrane domains.

PTX3 is in normal conditions contained in neutrophil secondary (also called specific) granules (21, 29). In vitro, microbial and sterile inflammatory signals trigger its secretion (21, 29). To the best of our knowledge, this is the first demonstration of such an event taking place in human patients. Activation of neutrophils across the coronary vascular bed is a hallmark of acute coronary syndromes (30, 31, 37). Activated neutrophils release the content of their primary granules in the blood: MPO circulating levels in particular are associated with and may predict clinical outcomes in patients with AMI (30, 31, 37, 46). PTX3 levels also substantially change during AMI and represent predictors of clinical outcomes (46, 47). The early kinetics of PTX3 make it a particularly promising signal in the early phases of AMI after the onset of chest pain, when conventional cardiac biomarkers, such as troponins, have a relatively low concentration in the peripheral blood and thus lack precision and sensitivity (48). An ideal marker of ongoing myocardial infarction should rise and fall early and as such could be valuable for patient triage and for timely definition of appropriate intervention (48). Assessment of neutrophil PTX3 content may add to the assessment of the blood concentration of the protein, which has been shown to represent an independent predictor of the clinical outcomes of patients; further studies on large patient populations are required to address this issue.

Which signals trigger the release of PTX3 in AMI patients? In vitro, most stimuli that cause the release of MPO (23, 38) also result in the release of PTX3 (see Fig. 2). P-selectin, either as a soluble or as a cell-associated moiety, triggers PTX3 release from human neutrophils (Fig. 2), with an efficiency comparable to that of previously described stimuli, including microbes, such as Staphylococcus aureus and Escherichia coli, or cytokines, such as TNF-α (29). PTX3 released from activated leukocytes acts as a negative feedback loop on the inflammatory response in experimental models of inflammation in vivo (21).

PTX3 binds to endothelial P-selectin glycosidic moieties, thus interfering with neutrophil recruitment to activated endothelium and limiting the collateral damages associated to leukocyte activation in diseases characterized by systemic inflammation, including acute respiratory distress syndrome and sepsis (21, 22). PTX3 specifically enhances LPS-elicted tissue factor activity in vitro, possibly promoting activation of the coagulation cascade in the presence of microbial moieties (49, 50). The effect is specific for bacterial endotoxin, since PTX3 does not influence the action of primary proinflammatory cytokines, such as TNF-α and IL-1β. These results hint at a possible involvement of PTX3 in thrombotic events associated to sepsis. PTX3 per se does not influence tissue factor expression and activity in monocytes and endothelial cells (49, 50), making it unlikely that this is the case in sterile vascular injuries, such as those occurring in acute coronary syndromes.

Actually, our results suggest that a loop that negatively regulates the inflammatory response acts in AMI, thus limiting the noxious effects of neutrophils in the heart. PTX3 levels are known to be increased in patients with more severe cardiovascular disease and to independently predict mortality in patients with AMI. PTX3 levels most likely reflect a paroxystical inflammatory response to acute aterothrombosis, thus identifying patients at higher risk of a negative outcome (8, 20). The ability of PTX3 to modulate the hemostatic and inflammatory properties of platelets may however play a protective function. Platelets tightly interact with neutrophils and contribute to their activation and recruitment in inflamed tissues via multiple signals: P-selectin, together with β2 and β3 integrins, plays a key role (51, 52). In turn, the activation of neutrophils, which is effective at fighting invading microbes, is deleterious in conditions of sterile vascular injuries, such as the thrombosis of coronary atherosclerotic plaques (37). The release of the PTX3 prestored in neutrophil granules during AMI may contribute to interrupt this loop, via modulation of events down-
stream of P-selectin recognition, including β2 integrin upregulation and heteroaggregate formation. This model agrees well with the clinical data on PTX3 expression in AMI (8, 20).

Besides platelet activation, the recognition of formylated peptides and other sterile signals of tissue injuries, such as mitochondrial DNA, has been shown to trigger the paroxysmal activation of neutrophils (53, 54), an event that has severe clinical consequences. The release of PTX3 from neutrophils early after acute stimulation could provide a substantial advantage, given in particular its protective action on the heart and vessels. Further studies and appropriate animal models are necessary to verify this hypothesis.

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Disclosures

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References


Corrections


The second institution in the author affiliations was published incorrectly. In addition, the author list was published incorrectly. The corrected author list and affiliation list are shown below.

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