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Vascular pentraxin 3 controls arterial thrombosis by targeting collagen and fibrinogen induced platelets aggregation

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ABSTRACT

Aim: The long pentraxin PTX3 plays a non-redundant role during acute myocardial infarction, atherosclerosis and in the orchestration of tissue repair and remodeling during vascular injury, clotting and fibrin deposition. The aim of this work is to investigate the molecular mechanisms underlying the protective role of PTX3 during arterial thrombosis.

Methods and results: PTX3 KO mice transplanted with bone marrow from WT or PTX3 KO mice presented a significant reduction in carotid artery blood flow following $FeCl_3$ induced arterial thrombosis ($-80.36 \pm 11.5\%$ and $-95.53 \pm 4.46\%$), while in WT mice transplanted with bone marrow from either WT or PTX3 KO mice, the reduction was less dramatic ($-45.55 \pm 1.37\%$ and $-53.39 \pm 9.8\%$), thus pointing to a protective effect independent of a hematopoietic cell's derived PTX3. By using P-selectin/PTX3 double KO mice, we further excluded a role for P-selectin, a target of PTX3 released by neutrophils, in vascular protection played by PTX3. In agreement with a minor role for hematopoietic cell-derived PTX3, platelet activation (assessed by flow cytometric expression of markers of platelet activation) was similar in PTX3 KO and WT mice as were haemostatic properties. Histological analysis indicated that PTX3 localizes within the thrombus and the vessel wall, and specific experiments with the N-terminal and the C-terminal PTX3 domain showed the ability of PTX3 to selectively dampen either fibrinogen or collagen induced platelet adhesion and aggregation.

Conclusion: PTX3 interacts with fibrinogen and collagen and, by dampening their pro-thrombotic effects, plays a protective role during arterial thrombosis.

1. Introduction

Pentraxin 3 (PTX3) is an essential component of the humoral arm of innate immunity and belongs to the pentraxin superfamily: soluble, multifunctional, and pattern recognition proteins [1]. Pentraxins share a common C-terminal pentraxin domain, which in the case of PTX3 is coupled to an unrelated long N-terminal domain [2]. PTX3 in humans, like CRP, correlates with other clinical markers of atherosclerosis [3] and is independently associated with the risk of developing vascular events [4–6]. PTX3 was detected in the myocardium and in the vasculature under different pathological conditions (i.e. in the context of angiogenesis, vascular restenosis, atherosclerosis) [7], a finding paralleled by the observation of increased PTX3 plasma levels in patients with cardiovascular disorders [8,9]. These data prompted the research toward the investigation on the role of PTX3 as biomarker, player or both in the context of cardiovascular disease.

The high degree of conservation of PTX3 between mouse and human [1] supports the investigation of its pathophysiological role in mice. PTX3 deficiency has been associated with increased fibrin deposition in wounded tissues [10], increased inflammation as a result of defective control of P-selectin mediated neutrophil recruitment [11], increased atherosclerosis [12], and cardiac damage [13]. These observations point to a cardiovascular protective effect of PTX3, potentially associated with the tuning of inflammation during cardiovascular diseases [7], and, as a consequence, the increased levels observed in humans might represent an extreme attempt of

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the body to limit an excessive inflammatory response. This conclusion is further supported by the finding that genetically determined high levels of PTX3 do not influence the risk of acute myocardial infarction (AMI), suggesting that PTX3 concentration itself is unlikely to be even a modest causal factor for AMI [14].

We have recently shown that, after tissue injury, the local acid micro-environment activates PTX3 which orchestrates tissue repair [15] and that PTX3 deficiency is associated with defective repair of wound healing and with increased thrombosis [15]. The aim of this work is to investigate the mechanisms underlying the protective role of PTX3 during arterial thrombosis. In details, we characterized in vivo arterial thrombosis in PTX3 chimera mice generated by bone marrow transplantation and investigated the molecular mechanisms responsible for the phenotype observed taking advantage of PTX3/P-selectin double KO mice. In vivo and in vitro studies were then carried out to selectively investigate the contribution of platelets, leukocytes and vascular wall in the increased thrombus formation observed in PTX3 deficient mice.

2. Materials and methods

2.1. Animals

The generation of PTX3 KO animals was described in detail before [16]. P-selectin KO mice were from the Jackson Laboratories. P-selectin/PTX3 KO mice were generated by crossing the two animal models. All mice were on a C57BL/6J background (from more than 10 generations). The investigation conforms to the European Commission Directive 2010/63/EU and was approved by the Ethical Committee (Progetto di Ricerca 2009/3 and Progetto di Ricerca 2012/02).

2.2. Bone marrow transplantation

Wild-type (WT) or PTX3 KO mice were lethally irradiated with a total dose of 900 cGy. Two hours later, mice were injected in the tail vein with 5×10^6 nucleated bone marrow (BM) cells obtained by flushing of the cavity of a freshly dissected femur from a WT or PTX3 KO donor. Recipient mice received gentalyn (0.4 mg/mL in drinking water) starting 10 days before irradiation and maintained thereafter [11]. At 8 weeks after BM transplantation, the $FeCl_3$ injury model was carried out as described below.

2.3. $FeCl_3$ injury: experimental arterial thrombosis model

Experimental arterial thrombosis was induced as previously described [10]. Briefly, mice (8–12 week old) were anesthetized with ketamine chlorhydrate (75 mg/kg) and medetomidine (1 mg/kg; Virbic). The left carotid artery was dissected free and placed in the probe (model 0.7V, Transonic System) connected to transonic flow meter (Transonic T106). After blood flow stabilization (baseline flow constant for 7 min), filter paper imbibed with $FeCl_3$ (10–20%) was applied downstream of the probe to the top of the exposed carotid. After 3 min, the filter paper was removed, the carotid artery was washed with PBS, and the flow was recorded for 30 min. In a group of experiments, human recombinant PTX3 (hPTX3, 5 mg/kg per mouse [11,17]) or PBS (as negative control) was injected iv before the arterial thrombosis experiment. Data are presented as the percentage, compared to the basal level, of carotid artery blood flow during the 30 min of observation and time to carotid occlusion (less than 20% of carotid blood flow).

2.4. Platelet count, tail bleeding time and fibrinogen, PT and aPTT measurement

Platelet was counted optically from whole blood collected by orbital sinus bleeding into Unopette System reservoirs. Bleeding time was measured on tails of anesthetized mice, and immersed in saline solution at 37 °C. After 3 min the last part of the tail, 3 mm from its end, was cut with a sharp scalpel blade, then immediately re-immersed in saline solution. Bleeding was followed visually, and time was determined as the interval (sec) from the tail transection to cessation of bleeding; 900 s was considered the cut off time for the purpose of statistical analysis [18]. Fibrinogen levels were measured as previously described [19], from plasma (PPP) obtained after centrifugation of blood [collected by cardiac venipuncture from anesthetized mice into 3.8% sodium citrate (1:10 vol:vol)] at 1000 *g* for 10 min. For prothrombin time (PT) test, PPP was mixed with Tissue Factor (TF) (containing phospholipid) at 37 °C and an excess of calcium chloride (25 mM) was added to initiate coagulation. Time was determined as the interval (sec) between calcium addition and clot formation. For activated partial thromboplastin time (APTT) test, PPP was incubated at 37 °C with phospholipid (cephalin) and a contact activator (e.g. Kaolin) was added followed by the calcium (all pre-warmed to 37 °C). Addition of calcium initiates clotting and time was determined (sec) as the interval taken for a fibrin clot to form.

2.5. Real time quantitative polymerase chain reaction

Total RNA was reverse transcripted as described [12,20,21]. 2 μL of cDNA was amplified by real time quantitative PCR with 1X Syber green universal PCR master mix (BioRad, Italy). The specificity of the Syber green fluorescence was tested as described [21]. PTX3 primers used have been described previously [12]. Each sample was analyzed in duplicate using the IQTM-Cycler (BioRad). The PCR amplification was related to a standard curve ranging from 10–11 mol/L to 10–14 mol/L and data were normalized for the housekeeping gene ribosomal protein L13a (RLP13a).

2.6. PTX3 plasma levels — ELISA assay

PTX3 plasma levels were measured with a sandwich ELISA using 2 anti-murine PTX3 mAb (2C3 and 6B11) as described [22]. The ELISA assay did not cross-react with the short pentraxins CRP and SAP.

2.7. P-selectin expression and integrin $\alpha 2\beta 3$ activation on platelet surface

Platelet rich plasma (PRP), isolated following centrifugation at $100 \times g$ with no break of citrated blood, from PTX3 KO and WT mice was stimulated with collagen (10 μg/mL, Mascia Brunelli, Italy) and U46619 (10 μM, Cayman Chemical, USA), an analogous of thromboxane for 1 or 10 min, for P-selectin or integrin $\alpha_2\beta_3$, respectively, and then stained with the anti-CD42c FITC and anti-CD62P PE or $\alpha_2\beta_3$ PE antibodies (Emfret, Germany). Samples were fixed with PFA 4% and the expression of the platelet activation markers was evaluated through flow cytometry (BD FACS Calibur).

2.8. Platelet–leukocyte aggregates analysis

Citrated blood from PTX3 KO and WT mice was stimulated with ADP (40 μM, Sigma-Aldrich, Italy) for 30 s or 1 min and fixed with BD lysis buffer; samples were stained with the anti-CD45 PE (BD) and anti-CD42c FITC antibodies (Emfret, Germany) and analyze through flow cytometry (BD FACS Calibur).

2.9. Histology and immunofluorescence

Mouse carotid arteries and lungs were excised, perfused with PBS, fixed in 10% neutral formalin, processed and then embedded in paraffin as described [23]. Section of 5 μm were stained with hematoxylin and eosin, or DAPI and fluorescent antibodies against PTX3 and fibrin and visualized with *Axiovision* software [23] at 10× magnification. PTX3 protein expression in carotid arteries was analyzed as described [12,13].