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The extrinsic coagulation cascade and tissue factor pathway inhibitor in macrophages: A potential therapeutic opportunity for atherosclerotic thrombosis

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ABSTRACT

Objectives: The coagulation protease cascade plays the central requisite role in initiation of arterial atherothrombosis. However, the relative participation of the extrinsic as compared to the intrinsic pathway is incompletely resolved. We have investigated *in vivo* the relative importance of the extrinsic and intrinsic pathways to define which is more essential to atherothrombosis and therefore the preferable prophylactic therapeutic target. We further addressed which type of plaque associated macrophage population is associated with the thrombotic propensity of atherosclerotic plaques.
Methods: Both photochemical injury and ferric chloride vascular injury models demonstrated arterial thrombosis formation in ApoE deficient mice. We found that direct interference with the extrinsic pathway, but not the intrinsic pathway, markedly diminished the rate of thrombus formation and occlusion of atherosclerotic carotid arteries following experimental challenge. To explore which plaque macrophage subtype may participate in plaque thrombosis in regard to expression tissue factor pathway inhibitor (TFPI), bone marrow derived macrophages of both M and GM phenotypes expressed tissue factor (TF), but the level of TFPI was much greater in M- type macrophages, which exhibited diminished thrombogenic activity, compared to type GM-macrophages.
Results and conclusions: Our works support the hypothesis that the TF-initiated and direct extrinsic pathway provides the more significant contribution to arterial plaque thrombogenesis. Activation of the TF driven extrinsic pathway can be influenced by differing colony-stimulating factor influenced macrophage TFPI-1 expression. These results advance our understanding of atherothrombosis and identify potential therapeutic targets associated with the extrinsic pathway and with macrophages populating arterial atherosclerotic plaques.
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Introduction

Thrombotic occlusion of atherosclerotic arteries is well established as the proximal cause of myocardial infarction and stroke [1,2]. However, the molecular pathophysiology of this atherothrombotic syndrome is not only complex but incompletely elucidated [1,3,4]. Depending on arterial vessel size, type and host tissue bed, the mechanism may variously involve the vascular endothelium, platelets, leukocytes, coagulation proteins, numerous cytokines, and other issues [3,5]. The lack of a thorough understanding of atherothrombosis has impeded the development of more effective targeted therapeutics, and thus has limited the choices for improved long-term prophylactic therapy [6,7].

The two coagulation pathways conventionally implicated in atherothrombosis are the extrinsic and the intrinsic pathways. This division is useful for interpreting *in vitro* results, but in reality TF present in atherosclerotic lesions potently activates factor IX, an essential initiating requirement of the intrinsic pathway and then factor X, which is the locus of convergence of the extrinsic and intrinsic pathways [8,9]. Although both pathways could participate in atherothrombosis, a prevalent opinion is that the extrinsic pathway is most likely responsible for initiation of thrombosis while the intrinsic pathway most likely amplifies the incipient thrombus [7]. The relative potential merits of the extrinsic and intrinsic pathways as targets of therapeutic prophylaxis and their precise roles in atherothrombosis are incompletely resolved. Yet, clarification is essential for effectively directing efforts to discover and advance new pharmacological interventions offering prevention with a diminished bleeding risk [4,9].

The key molecule of the coagulation cascades is TF (Tissue factor or factor III) for initiation. TF is expressed by macrophages and macrophage foam cells within atherosclerotic lesions. Also found

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present is tissue factor pathway inhibitor (TFPI), which inhibits the TF initiated and driven coagulation cascade by neutralizing factors Xa, IIa, and VIIa [10,11]. Macrophages populating atherosclerotic lesions are of two differing and divergent phenotypes, namely M-macrophages and GM-macrophages. They differ in their surface markers, cholesterol uptake, and relative numbers populating plaques, with M-Macs predominating [12]. An unresolved issue is whether these two subpopulations differ in regard to TF and TFPI expression and their relative contributions to plaque associated thrombus generation *in vivo*.

To address the issues, we adopted a clinically relevant *in vivo* model with the question of whether the extrinsic or the intrinsic pathway represents a more important contributor to thrombosis *in vivo* and which presents as a more valid therapeutic target pathway. For these studies we further validated and focused on the physiologically authenticated $ApoE^{-/-}$ atherosclerotic mouse vessel photochemical injury model. First, we intravenously administered specific coagulation pathway inhibitors [6]. Second, we addressed the relative role of atherosclerotic plaque macrophages and subtypes by differentiating monocytes *ex vivo* into two divergent lineages macrophages in the presence of alternative macrophage development cytokines: macrophage colony-stimulating factor (M-CSF) to produce M-Mac and granulocyte-macrophage colony-stimulating factor to produce granulocyte-macrophage-CSF macrophages (GM-Mac). We compared their procoagulant activity, TF expression, and TFPI expression.

Methods

Animals

ApoE-deficient C57BL/6 J mice approximately 3 months of age were fed high fat chow for 90 + days after which 6–8 months old mice were subjected to the thrombosis-injury analysis. Normal ($ApoE^{+/+}$) C57BL/6 J mice with the same age and fed were used as control. Female mice 4–6 weeks old were used to isolate bone marrow cells. The Institute Committee on Use and Care of Animals approved all animal care and experimental procedures.

Validation of pathway coagulation factor inhibitors in vitro

Extrinsic pathway inhibition

In order to verify that the inhibitors and antibodies were competent to suppress coagulation on a biochemical level, *in vitro* activity was defined by the clotting time in single-stage clotting assays. The time to clot formation during gentle agitation was analyzed with a START4 analyzer (Diagnostica Stago, Parsippany, NJ). Inhibitors or antibodies were added to 50 μl of mouse brain acetone tissue standard solution (50 mg/ml), mixed and incubated at RT for 1 minute, then 50 μl murine plasma was added. After incubation at 37 °C for 1 minute, 50 μl of pre-warmed 25 mM $CaCl_2$ completed the reaction, and the clotting time was recorded. Pro-coagulant activity (PCA) was interpolated from a standard clotting time curve using human TF standard (Ortho Diagnostic Systems, Raritan, NJ) and expressed as a composite value of time and mUnit of TF. The extrinsic pathway inhibition was expressed as relative PCA compared with mouse brain acetone tissue standard without inhibitors. A series of 3 small molecule VIIa inhibitors were synthesized as described [13,14]. We tested 5 commercially available and one each of our anti-TF and anti-VII antibodies *in vitro*. We tested the best VIIa inhibitors at final concentrations of 20, 10 and 5 μM. Neutralizing anti-TF antibody (R&D Systems, Minneapolis, MN) was analyzed at 20,10 and 5 μg/ml. Corn trypsin inhibitor (CTI, Enzyme Research Laboratories, South Bend, IN) was tested at 100, 50, 25 μg/ml, and anti-fXI antibody (from Prof. Ruggeri) was tested at 5, 2.5 and 1.3 μg/ml *in vitro*. As a further validation of our factor VIIa small molecule inhibitor, 1 μg/g of inhibitor or PBS control was intravenously injected in C57BJ/6 L mice. The tail was incised 3–5 minutes after injection at the point along its length where its diameter was 2.0-2.5 mm was then immersed in 37 °C saline and the bleeding time was recorded. The tail was examined 12 hours later for signs of continued bleeding.

Intrinsic pathway inhibition

The activated partial thromboplastin time (aPTT) was used to assess intrinsic pathway inhibitor activity. An aPTT-XL kit (Pacific Hemostasis aPTT-XL, Thermo Fisher Scientific, Rockford, IL) was used. Briefly, 100 μl aPTT reagent was incubated with the antibody or inhibitors for 5 min, and 100 μl of normal murine plasma was added. Following incubation at 37 °C, 100 μl of pre-warmed 25 mM $CaCl_2$ completed the reaction, and the time to clotting was recorded. The inhibitors and antibodies were tested at the same concentrations as in the extrinsic pathway inhibition analyses.

Thrombosis models

Photochemical injury model

We adopted the $ApoE^{-/-}$ atherosclerosis photochemical injury model [6]. For induction of injury and for flow measurement normal or ApoE-deficient mice were anesthetized with Isoflurane (Phoenix Pharmaceutical Inc, Saint Joseph, MO). The left carotid artery was exposed and fitted with a miniature Doppler flow probe (model 0.7PSB; Transonic Systems, Ithaca, NY) connected to a transonic flow meter (model TS420 Perivascular Flowmeter Module; Transonic Systems Inc., Ithaca, NY). Flow data was recorded and analyzed using WinDaq software (DATAQ Instruments, Akron, OH). Rose Bengal solution was injected at 25 μg/g into the left external jugular vein. A 543 nm green laser (model LHGR-0200, Research Electro Optics, Inc., Boulder, CO) was directed at the right carotid bifurcation at the sight of an atherosclerotic plaque to induce injury. As controls, normal ($ApoE^{+/+}$) mice of the same age, strain and same high fat diet feeding were instrumented identically. Fig. 1(a) is a photograph of the experimental arrangement for photochemical injury and flow monitoring. Occlusion times were determined to be complete and stable when the flow rate dropped below 0.1 ml/min for at least 10 minutes. For mice in which no stable occlusive thrombus formed within 60 minutes, occlusion time was recorded as 60 minutes.

$FeCl_3$ injury model

This model was performed as described by Farrehi using C57BJ/6 J mice 6–12 months of age [15]. The left carotid artery of $ApoE^{-/-}$ and normal ($ApoE^{+/+}$) mice were exposed and instrumented for flow measurements as described. A small dot size of filter paper soaked with 6% $FeCl_3$ was placed on the artery for 3 minutes then removed. The site was washed with PBS and flow was measured until occlusion or for 30 minutes.

Extrinsic versus intrinsic pathway participation in thrombus formation

In order to distinguish between extrinsic and intrinsic pathways role in thrombus initiation; and to identify whether the extrinsic pathway could be as a target for intervention, the following factor specific inhibitors were injected with Rose Bengal solution into separate groups of mice prior to induced injury; (1) a small molecule factor VIIa inhibitor injected at 1 μg/g mouse weight [16], (2) goat anti-mouse TF antibody (0.2 μg/g), (3) corn trypsin inhibitor (CTI) (4 μg/g) against factor XIIa, and (4) anti-mouse fXI antibodies (0.25 μg/g for 4 mice and 0.75 μg/g for 2 mice). The inhibitors/antibodies doses were determined by their *in vitro* activity and calculated the dose were comparable after the injection of inhibitors/antibodies that were directly diluted in blood (total blood volume equals 60-80 ml/kg mouse weight). The VIIa inhibitor was administered at the same dose also tested and determined from bleeding time *in vivo*. CTI and fXI antibodies were tested in the C57Bl/6 J/$FeCl_3$ model and they each significantly delayed thrombus formation at

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