Tissue factor-bearing microparticles and thrombus formation

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Blood microparticles are vesicular structures with a diameter of 100 to 1000 nm that are present in the blood of normal subjects and in patients with various diseases. These microparticles are derived from cells that circulate in the blood and cells associated with the blood vessel wall. Microparticle membranes retain the protein receptors of their parent cells and may retain RNAs and other cytosolic content. Based on surface protein expression, microparticles are known to be derived from platelets, granulocytes, monocytes, endothelial cells, smooth muscle cells as well as tumor cells. Only a subpopulation of these microparticles express tissue factor.

Tissue factor is an integral membrane protein that is present on the plasma membrane of many cells not exposed to blood. This protein, a receptor for factor VII and factor VIIa, is required for the initiation of blood coagulation. Tissue factor, composed of 263 amino acid residues [1, 2], includes an N-terminal extracellular domain of 219 amino acids, a transmembrane domain of 23 amino acids and a 21-residue C-terminal cytoplasmic domain containing palmitate and stearate bound to a cysteine [3]. The extracellular domain is homologous to other members of the cytokine receptor superfamily. Tissue factor binds Factor VIIa to form the tissue factor/Factor VIIa complex on the cell surface that activates Factor IX, Factor X and Factor VII [4]. The Xray structure of tissue factor [5] and the complex of the active site-inhibited Factor VIIa and extracellular domain of tissue factor have been determined [6], and others have further refined this tissue structure [7].

The central dogma of blood coagulation has been that (1) blood clotting is initiated through the action of tissue factor; (2) tissue factor is normally not exposed to blood; (3) tissue factor is constitutively expressed on the surface of non-vascular cells including astrocytes, smooth muscle cells, epidermus, renal glomeruli, the vascular adventitia, and placenta; (4) upon vascular injury, tissue factor on non-vascular cells comes in contact with flowing blood. The interaction of extravascular tissue factor with plasma Factor VIIa initiates linked, enzymatic reactions that culminate in the generation of thrombin and the subsequent conversion of fibrinogen to fibrin. These events transpire on a time scale of seconds to minutes. In addition, some vascular cells can be induced to express tissue factor but the time scale for the appearance of tissue factor activity *in vitro* is measured in hours, and is inconsistent with

a primary role in hemostasis. For example, unstimulated monocytes and endothelial cells do not express tissue factor, but can be induced to express tissue factor on their surface under certain conditions [8–10]. Monocytes can be activated by endotoxin [11], immune complexes [12], certain cytokines [13], P-selectin [14] and platelets [15], leading to tissue factor expression whereas endothelial cells express tissue factor when exposed to certain cytokines or endotoxin [16–20].

Contradicting some of this dogma, tissue factor antigen can be detected in whole blood. The concentration of plasma tissue factor antigen has been reported to be about 150 pg/ml [21–23], although this value has been disputed [24]. Nemerson and colleagues discovered blood-borne tissue factor incorporation into experimental thrombi. This group examined thrombus formation on pig arterial media or collagen-coated glass slides exposed to flowing human blood in an *in vitro* perfusion system [25]. The thrombi that formed during a short perfusion stained intensely for tissue factor antigen and were primarily composed of fibrin. Immunoelectron microscopy revealed tissue factor-positive membrane vesicles in large clusters near the surface of platelets. By immunostaining, tissue factor-containing neutrophils and monocytes were identified in peripheral blood. These data challenged the existing paradigm that tissue factor is not exposed to blood prior to vascular injury, and raised the possibility that leukocyte-derived microparticles are a source of blood-borne tissue factor that is involved in thrombus propagation at the site of vascular injury. Although extravascular tissue factor is certainly important in initiating blood coagulation in some forms of host defense, circulating tissue factor represents an alternative mechanism for activation of blood coagulation.

The intact vessel wall has been thought to regulate the initiation of coagulation by partitioning extravascular tissue factor from circulating factor VII. The detection of circulating tissue factor in normal subjects requires additional hypotheses about the regulation of coagulation initiation. Tissue factor in blood may be encrypted so as not to cause thrombosis in the absence of specific stimuli. Alternatively, the concentration of tissue factor may be sufficiently low, below the threshold for activation of blood coagulation, until tissue factor is concentrated at a site of vascular injury. Also possible, the concentration of circulating tissue factor may be insufficient to overcome inhibition by tissue factor pathway inhibitor in the absence of endothelial injury. Nemerson suggested that 20% of tissue factor is available on the cell surface, 30% is intracellular and 50% is "latent or inactive" in non-vascular cells [26]. At present, it remains controversial whether constitutively circulating tissue factor-bearing microparticles express tissue factor activity. Several mechanisms of tissue factor activation are postulated, including phosphatidylserine exposure, dedimerization, decreased exposure to tissue factor pathway inhibitor and post-translational modifications including removal of glutathione groups and disulfide bond oxidation. Although protein disulfide isomerase is important for tissue factor activity and fibrin generation in vivo [27, 28], the mechanism by which this enzyme functions to yield activity is unknown. Furthermore, the role of protein disulfide isomerase in microparticles is unknown.

Bloodborne tissue factor-bearing microparticles and thrombus formation in vivo

The sole pathway to fibrin generation via the blood coagulation cascade requires initiation by tissue factor. Tissue factor is a major component of a host defense system that preserves the integrity of a high pressure circulatory system. This protein has now been shown to exist in three compartments: (1) in the adventitia and smooth muscle cells of the vessel wall; (2) inducible in cells in the endovascular compartment, including monocytes, granulocytes, and endothelial cells [11, 18]; (3) a subset of microparticles containing tissue factor that circulate constitutively in blood [25]. Each of these tissue factor compartments likely plays a different role in hemostasis and in thrombosis during pathological events.

Mindful that the traditional model of blood coagulation hinged on the exposure of subvascular tissue factor following vascular injury rather than circulating tissue factor, we have explored the contribution of circulating tissue factor-bearing microparticles to fibrin generation during thrombus formation in vivo. Intravital microscopy was utilized to monitor thrombus formation following laser injury to the cremaster arteriole of a live mouse [29]. This model system is well-suited to study circulating tissue without exposing sub-endothelial tissue factor, as laser injury causes minimal disruption of the endothelium. Following vessel wall injury, activated platelets accumulate at the site of injury and express P-selectin [30]. P-selectin, a leukocyte adhesion receptor, is expressed on activated platelets and stimulated endothelial cells [31, 32]. Fluorescently-labeled microparticles derived from mouse monocytes infused into a mouse prior to laser injury accumulate within the leading edge of the developing thrombus [33]. These monocyte-derived microparticles express tissue factor and fail to accumulate into thrombi when infused into P-selectin null mice, demonstrating that the accumulation of monocyte microparticles bearing tissue factor in the thrombus is dependent on the interaction of platelet P-selectin and its counterreceptor, P-selectin glycoprotein ligand 1 (PSGL-1), expressed on the surface of the monocyte microparticles.

To determine whether tissue factor derived from hematopoietic cells is delivered to the thrombus via tissue factor-bearing microparticles or circulating leukocytes expressing tissue factor on the plasma membrane, we examined the kinetics of tissue factor accumulation in the developing arteriolar thrombus with the kinetics of leukocyte-thrombus interaction and microparticle-thrombus interaction in the microcirculation of a living mouse [30]. Tissue factor rapidly accumulated in the developing thrombus, appearing immediately following vessel wall injury, peaking in about 60 seconds. In contrast, leukocyte-thrombus interaction was not observed until 2–3 minutes after vessel wall injury. Maximal leukocyte rolling and firm leukocyte adherence on thrombi in wild type mice were observed after approximately 8 minutes, and was dependent upon P-selectin and PSGL-1. In contrast, microparticle accumulation in the developing arteriolar thrombus was rapid, with peak accumulation within 50–60 seconds. The accumulation of hematopoietic cell-derived tissue factor in the developing thrombus correlates to the kinetics of microparticle accumulation and does not correlate temporally with leukocyte-thrombus interaction. These results indicate that tissue factor derived from hematopoietic cells is delivered by microparticles during the initial phase of thrombus development *in vivo*.

The cellular origin of these tissue factor-bearing microparticles was defined as hematopoietic through reciprocal bone marrow transplants between wild-type and low tissue factor mice. Low tissue factor mice express less than 1% of normal levels of tissue factor [34]. Following laser injury in a low tissue factor mouse, a platelet thrombus formed devoid of both tissue factor and fibrin [35]. Transplanting the bone marrow of a low tissue factor mouse into a lethally irradiated wild-type mouse recipient permitted the evaluation of hematopoietic-derived tissue factor in thrombus generation. There was a significant reduction in thrombus size in the low tissue factor/wild type mice chimera. By comparison, wild type bone marrow transplanted into low tissue factor mice restored tissue factor accumulation following a laser-induced vascular injury. Tissue factor appears immediately following vessel wall injury rather than the several minutes required for a leukocyte to roll and attach to a developing thrombus [30]. These data support a role for hematopoietic-derived tissue factor bearing microparticles in thrombus propagation in vivo.

P-selectin and PSGL-1 microparticle interaction was explored in genetically altered mice that over-express soluble P-selectin [36]. These mice have increased numbers of leukocyte-derived microparticles bearing tissue factor and have shortened clotting times. Labeled microparticles can be visualized in developing thrombi following vascular injury and infusion of inhibitory PSGL-1 antibodies into these mice prolongs plasma clotting times and decreases thrombus size [36, 37]. The P-selectin/PSGL-1 dependent accumulation of tissue factor-bearing microparticles within the developing thrombus leads to thrombin generation which in turn activates additional platelets through cleavage of the protease-activated receptor.

The relative contribution of vessel wall tissue factor either within the media or adventitia continues to be explored. In vascular injury models that cause disruption of the endothelium such as the ferric chloride and Rose Bengal models, the contribution of circulating microparticle tissue factor is not apparent [38]. For instance, Wang et al demonstrated in mice deficient of tissue factor within vascular smooth muscle cells that the size of a thrombus was significantly reduced following ferric chloride injury [39]. Nonetheless, the thrombi stained diffusely for tissue factor and fibrin consistent with a circulating source of tissue factor. In the mouse laser-induced vascular injury model, the endothelial layer appears intact thus limiting exposure to the subendothelial compartment of tissue factor. The extent of endothelial injury appears to dictate the relative contribution of vessel wall versus circulating tissue factor. The endothelium is activated following laser injury. Preliminary evidence in cell culture suggests that endothelial cells may serve as a source of tissue factor that initiates thrombin generation [10]. How these mechanistic insights apply to thromboembolic events in humans remains to be seen. In theory, the laser injury mimics thrombosis seen with intact endothelial activation akin to inflammation-induced thrombosis whereas the denudation models, including ferric chloride and Rose Bengal, approximate vascular trauma.

Limitations of current microparticle analysis technology

In line with the observation that tissue factor-bearing microparticles play a role in thrombus formation in vivo, pathologic alterations in circulating numbers or activity of microparticles have been explored as a potential mechanism for thrombosis in a large number of conditions

including malignancy, sepsis, coronary artery disease and arterial bypass surgery. The precise mechanism of microparticle accumulation and tissue factor activation in these pathologic conditions has yet to be established. The microparticle accumulation model in disease may be independent of P-selectin and PSGL-1 and may instead rely on an undefined receptor-ligand interaction. Alternatively, tissue factor may circulate on microparticles in an active form, leading to widespread activation of thrombin. Although an extensive literature exists on the perturbation of microparticle populations in various disease states, current analytical methods are inadequate for their accurate detection, identification and quantitation in plasma. Although there is agreement that microparticles exist and that various populations are altered in various disease states, analytical techniques used have failed to establish even the normal numbers and distribution of various microparticle species. For microparticles to be used as biomarkers for clinical laboratory medicine, new methods are required that will allow accurate, reproducible and robust measurements that can be applied in the routine hospital laboratory. Despite increasing interest in the field of microparticles and thrombotic disorders, the standardized measurement of microparticle populations remains a key limitation and compromises the existing literature on this subject.

Optical detection of microparticles using commercially available light scatter-based flow cytometry is the method most often employed to determine microparticle size and number. However, light scatter flow cytometry has limitations in resolving microparticles. The angle and amount of forward light scatter is dependent on several variables independent of particle size, including the wavelength of incident light, particle shape, presence of surface absorptive material, and relative refractive indices of particles and suspension medium. The standard method to identify a population of microparticles is performed by referencing the light scatter characteristics of a uniformly sized population of polystyrene beads even though the refractive index of beads is considerably greater than that of cellular membranes. Newer generation flow cytometers appear to offer improved resolution of microparticles and efforts are underway to formally evaluate their sensitivity in characterizing microparticle populations [40].

Several groups have explored alternative methods for microparticle characterization including atomic force microscopy [41] or dynamic light scatter [42], although neither of which has been explicitly adapted for the measurement of tissue factor bearing microparticles. Indirect methods of microparticle enumeration based on tissue factor activity have also been described [43–45]. Microparticles are concentrated either by centrifugation or antibody capture followed by incubation with factors VIIa and X [43, 46, 47]. The activity of tissue factor is then monitored using a chromogenic substrate for factor Xa. Alternatively, tissue factor antigen or activity can be measured in plasma or whole blood [44, 48–51]. Determination of microparticle size is not possible by such approaches.

We have taken an impedance-based approach to microparticle characterization which is based on Coulter-type resistive sensing, a classic methodology for detecting biological particles. Coulter counters are currently the primary devices used to size and count small biological particles for clinical and laboratory research. Detection via the Coulter principle relies on passage of a non-conducting particle through an electrolyte-filled aperture. The particle displaces conducting fluid from within the aperture and thus decreases the

conductance. This can be measured by a change in voltage or current, and the frequency and amplitude of the current or voltage pulse provides very accurate information on the number and size of the particles passing through the aperture. The volume of the aperture determines the size of the particle that can be measured. In general, particles with a diameter of 2 to 60% of the aperture diameter can be accurately sized in Coulter-type devices. A fluorescence detection system is required for antigen recognition.

Alternatively, particle size can be measured by tracking software that monitors Brownian movement [42]. Since particle movement is a function of particle size and shape, such measurements allow prediction of particle size, assuming microparticles are spherical. Fluorescence detection allows antigen identification. Although fluorescent antigen detection could be a surrogate measurement of microparticle number, no single antigen defines all microparticles of varying cellular origin and the number of antigens per microparticle are not uniform. This method shows promise in identifying very small particles (10–50 nm diameter), but only a very small percentage of identified particles are tracked long enough to be sized, potentially introducing a selection bias.

There are also a number of pre-analytic variables that have the potential to influence the measurement of tissue factor bearing microparticles. These include traumatic venipuncture, anticoagulant employed, time to centrifugation, speed of centrifugation, freeze-thaw cycles, and the sensitivity and/or specificity of different tissue factor antibodies. Some anti-human tissue factor antibodies used are derived from a panel of monoclonal antibodies with binding to distinct epitopes [52], and may be inhibitory to procoagulant function [7], Given the potential for microparticle measurement contributions to clinical diagnostics, the development, validation, and standardization of accurate microparticle measurement techniques is required.

Tissue factor-bearing microparticles and human disease

Cancer-associated thrombosis

The relationship between thrombosis and cancer dates back to the 1800's but the pathophysiologic basis for this association has been difficult to establish. Malignant cells are known to shed microparticles spontaneously that are highly concentrated with tissue factor [53–55]. Experimental models provide evidence that tumors generate tissue factor-bearing microparticles. In orthotopic murine models of human pancreatic cancer, tumor-derived tissue factor can similarly be measured in plasma. The concentration of tumor-derived tissue factor appears to correlate with total tumor burden [56]. Several weeks following subcutaneous injection of green fluorescent-labeled tumor cells into mice, tumor-derived microparticles can be visualized at the site of thrombus following a vascular injury [55].

Using an impedance-based flow cytometer, modified specifically for microparticle enumeration and characterization, we observed that some individuals with cancer have high levels of circulating tissue factor-bearing microparticles [57]. Individuals with pancreatic cancer, one of the most prothrombotic malignancies, have very high levels of circulating tissue factor-bearing microparticles and elevations in tissue factor-bearing microparticles are associated with a 4-fold increased risk of thrombosis in cancer patients. The microparticles

in pancreatic cancer patients are derived, in part, from the underlying tumor. Circulating microparticle levels normalized following surgical resection of the pancreatic cancer and microparticles in pancreatic cancer co-expressed the epithelial tumor antigen, MUC1. Other groups have similarly observed high levels of tissue factor activity in plasma samples from patients with cancer and its association with thrombotic events [43, 44, 58]. Prospective trials are underway to determine whether the presence of tissue factor-bearing microparticles predicts the increased incidence of subsequent venous thromboembolic events and whether primary thromboprophylaxis strategies are benefical in such patients [59].

Tissue factor-microparticles and sepsis

Inflammation, infection, endotoxemia and sepsis are each associated with increased thrombotic risk, including disseminated intravascular coagulation. Elevated microparticles [60] and tissue factor-bearing microparticles with tissue factor activity [47] have been described in trauma and septic patients. A major component of the resulting prothrombotic state may be disruption of endogenous anticoagulant function [61]. Mouse models of endotoxemia confirm activated coagulation due to increased microparticle tissue factor activity [62]. This endotoxin-mediated procoagulant state is suppressed in mice either exposed to an inhibitory anti-tissue factor antibody or genetically lacking either myeloid or total hematopoietic tissue factor [63].

Cardiac bypass surgery

Exposure of blood to abnormal flow and a synthetic circuit during cardiopulmonary bypass creates a hypercoagulable state. During cardiopulmonary bypass, patients generate procoagulant microparticles detected from pericardial blood, but not detected in the systemic circulation by flow cytometry [64]. When compared to microparticles from healthy donors, microparticles from pericardial blood obtained during cardiac surgery are prothrombotic in an *in vivo* venous stasis model in rats [65].

Hemolytic anemias

Several forms of hemolytic anemia associated with prothrombotic risk have been reported to produce elevated microparticle numbers and tissue factor activity. These procoagulant microparticles may be derived from erythrocytes themselves, but also from monocytes and endothelial cells in both sickle cell anemia [66] and paroxysmal nocturnal hemoglobinuria [67–69]. In sickle cell disease, vasoocclusive crisis leads to increased microparticle number, as well as overall procoagulant activity and thrombin generation, compared to sickle cell patients not in crisis [70]. Microparticle generation in paroxysmal nocturnal hemoglobinuria is postulated to results from complement-injured monocytes that become susceptible to injury due to deficiency of surface CD55 and CD59 [67]. Elevated procoagulant microparticles have also been described in non-transfused patients with beta-thalassemia intermedia, many of whom had undergone splenectomy [71].

Hemophilia corrected by procoagulant microparticles

Platelet-leukocyte interactions are mediated by activated platelet P-selectin expression and leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) [31]. The co-expression of PSGL-1 and tissue factor on circulating microparticles indicates their leukocyte origin. These

microparticles accumulate in a growing thrombus through binding to activated platelets [33]. Procoagulant microparticles are capable of correcting the bleeding phenotype in mice with severe hemophilia A and are generated through P-selectin-Ig binding to PSGL-1 [72]. In this study, a fusion protein comprised of the extracellular domain of P-selectin and the human Fc region of IgG immunoglobulin was used in PSGL-1 null and wild-type mice to study procoagulant microparticles with increased tissue factor activity. Addition of this chimeric protein to blood from patients with severe hemophilia A led to increased microparticle number and tissue factor activity.

Conclusions

Tissue factor-bearing microparticles potentially play an important role in pathologic processes that lead to thrombosis and likely a critical role in normal hemostasis. Microparticles that express tissue factor may be derived from a number of different vascular cells. Despite the expanding literature on this subject, difficulties with microparticle analyses strongly argue that many of the current ideas and conclusions may need significant revision once more accurate measurements of microparticles are established.

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