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The blood compatibility challenge

Part 3: Material associated activation of blood cascades and cells

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Abstract

Following protein adsorption/activation which is the first step after the contact of material surfaces and whole blood (part 2), fibrinogen is converted to fibrin and platelets become activated and assembled in the form of a thrombus. This thrombus formation is the key feature that needs to be minimized in the creation of materials with low thrombogenicity. Further aspects of blood compatibility that are important on their own are complement and leukocyte activation which are also important drivers of thrombus formation. Hence this chapter summarizes the state of knowledge on all of these cascades and cells and their interactions. For each cascade or cell type, the chapter distinguishes that which is in widespread agreement from what there is less of a consensus.

Statement of significance

This paper is part 3 of a series of 4 reviews discussing the problem of biomaterial associated thrombogenicity. The objective was to highlight features of broad agreement and provide commentary on those aspects of the problem that were subject to dispute. We hope that future investigators will update these reviews as new scholarship resolves the uncertainties of today.

Introduction

Biomaterials at once activate several defense systems of the body, Figure 1. The humoral systems of coagulation and complement strongly interact with platelets and immune cells and recently yet
more interactions between thrombogenic and immunologic processes have been discovered. We discuss those systems separately and then refer to the manifold interactions at the relevant sections.

**Coagulation Cascade**

Biomaterials drive the generation of thrombin and the conversion of fibrinogen to fibrin necessitating the administration of heparin or other anticoagulants. The cascade of clotting factors beginning with Factor XII are amongst those in the protein adsorbate [1]. Activation of the coagulation pathway also activates the kinin-kallikrein system (via FXIIa), complement (via Factor XIIa, kallikrein, thrombin) and platelets (via thrombin). When endothelium is damaged, for example via biomaterial implantation, thrombomodulin levels decrease and tissue factor increases creating another source of thrombin [2] and the potential for activation of the intrinsic pathway via thrombin-mediated activation of FXI.

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In the absence of biomaterials, tissue factor is regarded as the key driver of coagulation following vascular injury. Biomaterial associated coagulation on the other hand can be related to the surface activity of contact phase molecules, which have a relatively strong abundance in the protein adsorbate, suggesting a prominent role for contact activation. Also polyphosphate nanoparticles on activated blood platelets were reported to activate coagulation via Factor XII [3]. However, it is interesting to note that under physiological conditions, the absence of Factor XII or other contact proteins (HMWK, kallikrein) has not been associated with abnormal bleeding [4]. Overall, evidence seems to suggest that FXII activation by biomaterial contact activates the intrinsic coagulation cascade, leading to formation of a clot. Yet there are other pathways that may augment or even replace the contact activation proteins in inducing biomaterial-induced coagulation.

The surface of activated blood platelets accelerate the coagulation cascade to produce thrombin, providing an amplification path that also links platelet and protein-mediated thrombosis [5].

There are multiple links between inflammatory and coagulant pathways. The role of blood born tissue factor in leukocytes and perhaps blood platelets [6, 7] in thrombosis and blood clot propagation has been highlighted [8, 9], and consequently some have argued that leukocyte activation and tissue factor release (and the extrinsic pathway) are key drivers also for biomaterial induced coagulation in blood [10-13]. On the other hand, the relevance of blood-born tissue factor for initiation of the coagulation (in distinction to propagation) is not generally accepted. A correlation between leukocyte inflammatory response and tissue factor expression has been demonstrated *in vitro* [11]. While the contribution of tissue factor (TF) involvement in the initiation of clotting in an ovine hemodialysis-model was shown [13], a correlation between leukocyte activation and blood clotting could not be demonstrated under defined *in vitro* conditions [11]. The rapid inactivation of tissue factor in blood has been regarded as the reason for this.

The impact of neutrophil elastase and cathepsin G on clot formation has also recently been further demonstrated in mice and highlights the complexity of the interactions taking place in clot formation and growth [14, 15].

Factor VII activating protease (FSAP or hyaluronic acid binding protein 2: HABP2) is a serine protease, supporting the extrinsic pathway of clotting activation by interfering with tissue factor
pathway inhibitor and by direct FVII activation. FSAP is activated by positively charged macromolecules [16] and surfaces [17] and may present an additional pathway of biomaterial induced coagulation activation.

Reliable analysis of blood coagulation has to reflect the complexity of the different activation pathways. Clotting times using whole blood or plasma using one of the established methods are crude measures of the ability of a material to activate the coagulation cascade. Whether plasma or whole blood is used to assess the properties of a test material further is a critical parameter. The pure initiation of the coagulation cascade does not necessarily lead to thrombus formation, but, as indicated above, the propagation from coagulation to the clot requires the presence of activated platelets [5].

Thrombin as a parameter of blood clotting is difficult to measure directly, because it is inactivated within seconds to the thrombin-antithrombin (TAT) complex, especially in presence of heparin. Prothrombin F1+2 fragment as byproduct of thrombin formation, the TAT-complex or fibrinopeptide A as byproduct of fibrin activation present more useful integral parameters of coagulation activation. More sophisticated measures such as thrombin generation rate may be more useful since one can incorporate such measures in mathematical models that may enable predictions under a wider range of experimental and perhaps even real-world conditions. A critical thrombin generation rate constant was one such parameter that was defined [18].

Ultimately though, all materials will drive plasma to coagulate following material contact unless anticoagulants are used. These levels of coagulation may not be clinically significant in many applications, but under in vitro conditions, placing blood in contact with a synthetic material will ultimately result in formation of a clot [19]. The lack of clinically significant levels of coagulation may arise from the dilutional effects of blood flow or from the continued presence of natural anticoagulants.

The need to use anticoagulants during blood collection argues against the above noted key role of tissue factor, although it is possible that clot initiation happens prior to the blood hitting the test tube. In that case though, one could not produce a blood sample that would not clot, effectively muting the ability to even study the problem anywhere other than in a person implanted with a material, and post-surgical recovery.

Hemolysis can influence thrombosis [20-22] through an effect on coagulation and other processes, although this is not well studied in the context of biomaterials. It has been thought that hemolysis is a consequence of shear stress in a device such as in a left ventricular assist device and not an effect of the material per se.
Platelet Activation

Platelet activation through biomaterials occurs via adhesion to adsorbed proteins (mainly fibrinogen) and indirectly through biomaterials-induced activation of coagulation and other systems. Platelet activation leads to spreading, a change in shape [23] and adhesion to surfaces as well as to platelets or to other cells via the GPIIb/IIIa receptor and P-selectin. It boosts the coagulation cascade by expression of phosphatidylserine, leads to the formation of microparticles, thromboxane formation and release of granule content [5, 24, 25]. These activated platelets create a nidus for thrombin generation and ultimately, occlusive thrombus formation or embolization. Such activation by biomaterials has been noted in experimental animals in several clinical scenarios [26-28]. In other situations, platelet activation is a useful diagnostic of potentially adverse interactions [27, 29].

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Material surface properties will affect the exposure of platelet binding regions in adsorbed proteins, and therefore affect the adhesion/activation of platelets. There are good correlations between the exposure of the γ-chain dodecapeptide in fibrinogen and platelet adhesion [30-32]. Platelet activation can occur with but also without adhesion to a material surface [26, 27]. Frequently, the activation level of adherent blood platelets is used as a measure of thrombogenicity and scored according to the platelet morphology. Small non-spreading cells are regarded as least activated while a pancake-like appearance show the highest activation level with several levels of pseudopod formation as intermediate stages [23, 33]. However, the platelet density on a surface and their morphology do not always correlate with thrombogenicity: various hydrogels are not thrombo-adhesive, but they support platelet aggregation and embolization. Furthermore, highly spreading blood platelets on hydrophobic materials are found which show clinically low thrombogenicity. A passivating effect of these blood platelets is assumed [34-36].

Activated platelets upregulate surface receptors like GPIIb/IIIa and P-selectin (CD62P), which is an important marker of platelet activation in vivo. The tendency of activated platelets to form platelet-leukocyte aggregates and stick to surfaces may suppress the level of detected P-selectin expression and raise the need for alternative parameters [37]. Release products of platelets, such as PF4 or β-TG release are established parameters [38, 39]. The platelet-leukocyte aggregates as such correlate with thrombotic events with cardiovascular devices and are suggested as a marker of platelet activation [40]. Material-induced aggregate formation has not been consistently characterized in vivo, making it difficult to correlate results from in vitro studies as well as testing the validity of the in vitro models used.

Platelet adhesion and activation are shear dependent processes. The platelet itself is shear sensitive [41, 42] and von Willebrand factor (vWF), the ligand for the platelet adhesion receptor GPIb, undergoes shear dependent exposure of the adhesion motifs [43]. Therefore, the response of platelets in arterial and venous settings may be different and hemocompatibility tests need to consider and report the shear conditions. Levels and types of shear stress highly differ depending
on the device and thus improvement in in vitro blood compatibility due to changes in material chemistry may have little clinical impact, because the influence of flow predominates [44]. As an extreme case, modern continuous flow LVADs can lead to bleeding by destruction of the high molecular weight vWF multimers that are critical for platelet adhesion in capillaries [45]. Activated platelets release 0.1 – 1 µm sized microparticles (MPs) budding off from the cell membrane. Several animal and clinical studies have demonstrated that these MPs contribute to a pro-thrombotic state through the expression of phosphatidylserine and TF. This is true not just for platelets MPs but also for endothelial cells, monocytes and neutrophils MPs [46]. Cardiovascular materials have demonstrated to induce MP formation with significant impact on thrombosis and inflammation in animal experiments as well as clinical studies, highlighting the need of the biomaterial community to assess these as a form of material-induced activation and a biocompatibility marker. The presence of microparticles generated by LVAD in patients is also believed to contribute to endothelial dysfunction inducing a prothrombotic and pro-inflammatory state in patients, which can also alter the ability of endothelial cells to release vasodilatory mediators [47, 48]. The direct link between MPs and adverse events (such as stroke, organ failure, myocardial infarction), however, remains difficult to identify due to the limited number of clinical studies and the small number of patients involved.

Sefton believes that complement activation (particularly at the level of C1) is important for both adhesion and activation of blood platelets [49]. Recent research shows that C1q binds to platelets and is activated, leading to further complement activation through an increase in C3a [50-52]. The binding of C1q occurs either through a specific C1q receptor or through P-selectin after platelet activation. Complement activation may occur earlier than platelet activation, although it is likely that there is substantive crosstalk precluding such delineation. Given the crosstalk among these systems, platelet and leukocyte activation also occur in an interdependent fashion, albeit to different extents at different times.

In vitro platelet analysis in the frame of hemocompatibility assessment has several limitations. It is difficult to determine when platelet activation by a biomaterial, observed in in vitro assays, becomes clinically relevant; the same is generally true for any hemocompatibility parameter. Consequently, use of the term “highly activating” is of limited value without quantitative definition. Despite evidence that platelet functions and reactivity from patients with cardiovascular disease differ from healthy individuals [53-55], pre-clinical in vitro studies continue to be performed with the blood of healthy donors. This estimation of hemocompatibility therefore does not necessarily reflect the clinical performance and more tests with blood from patients should be performed.
Complement

The complement system, made up from more than 30 proteins is evolutionary related to the blood coagulation cascade and contributes to host defense. Biomaterials activation of the complement cascade leads to the release of reactive fragments C3a, C5a and SC5b-9 to plasma while complement proteins including C1q and C3b are often found in the protein adsorbate [56, 57]. The alternative pathway is particularly relevant for surfaces bearing a nucleophile group (OH, NH₂) capable of covalent binding of C3b [58, 59]. Complement inhibitors such as Factor H or C1q inhibitor may also bind to biomaterial surfaces or be incorporated into the protein adsorbate, such that complement activation is downregulated [60].

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Different pathways may be involved in biomaterials induced complement activation [61]. Surface induced activation of the alternative pathway was reported to be a consequence of nucleophilic surface groups – but several reports propose that this effect is conferred via adsorbed proteins and not via the chemical moiety itself [62, 63]. Some suggest [59] that mostly hydroxyl groups are responsible for strong complement activation and activation through amino groups seems to be less relevant. The classical pathway may be activated by C1q binding to non-specifically adsorbed immunoglobins (IgG, IgM) on a foreign surface as well as to non-self patterns on surfaces and gets activated to initiate the complement cascade [64]. Only few reports propose a mannose pathway involvement through adsorbed, glycosylated proteins [65] or nanoparticles [66].

Besides the activation of the complement cascade, biomaterials may affect also the course of the cascade. Complement inhibitors (Factor H, C1q inhibitor) interacting with surfaces crucially modulate the resulting complement activation [57]. Surface topography on a nanoscale range, like the high surface curvature of nanoparticles can sterically prevent the interaction of adsorbed complement factors and thus block the propagation of the complement cascade [67].

Activation of the complement system mainly has inflammatory consequences. Complement fragment C5a is a strong activator of myeloid leukocytes via their complement receptor C5aR1 (CD88). The adhesion of these cells to a complement-activating surface is mainly triggered by the presence of surface-bound complement fragment C3b [58].

Complement activation induced by the biomaterial at the site of implantation may also lead to deposition of complement products on endothelial cells and production of endothelial microparticles which can contribute to the pro-inflammatory and thrombotic state [68]. C1q has also been shown to modulate endothelial cells behavior and to play a role in angiogenesis [69]. While these processes have been studied in vitro and in thrombotic diseases, they have been poorly characterized with cardiovascular devices [68].

The complement system also has several links to the coagulation system, both of which proceed in parallel in the protein adsorbate: coagulation factor XIIa can activate complement component C1 and thrombin can function as a C5 convertase [70]. In addition, the fibrinolytic plasmin has activity as a C5 convertase [71]. The complement system can modify coagulation by activating leukocytes to express and activate blood-born tissue factor [11, 70, 72, 73] or plasminogen...
activator inhibitor (PAI-1) [74]. As mentioned above, activated platelets can support complement activation by binding complement fragments to P-selectin and the C1q receptor [49, 75].

Heparin acts as inhibitor in both systems by association with either antithrombin (coagulation) or factor H (complement).

Complement activation in vitro is typically measured in serum, since calcium and magnesium are required cofactors (precluding the use of citrate or EDTA) and heparin is an inhibitor of some reactions but not all, complicating result interpretation. If whole blood or blood plasma has to be used, then hirudin can be used as anticoagulant, as it does not interact with the complement system [76]. The soluble fragment complement C5a is a possible analyte for the common, terminal complement pathway. Fast binding of C5a to its receptor on granulocytes may quench the concentration of the free peptide [77] and quantification of the soluble terminal complement complex (sC5b-9) has been suggested as a more reliable parameter to determine the activation of the complement cascade [78]. If differentiation between the classical or alternative pathway is desired, then quantification of Bb (alternative pathway) and C4d (classical pathway) or the use of specific inhibitors can help, however in advanced stages usually the whole complement cascade is activated.

The critical threshold for complement related incompatibility is undefined, yet it is presumed that higher amounts indicate greater incompatibility. While 5 mg/mL SC5b-9 is readily detectable, it is unclear whether this level (~ 1/3 of positive controls such as zymosan) is biologically significant.
Leukocyte Activation

Like platelets, myeloid leukocytes (neutrophils and monocytes) may adhere or be activated on a surface. Their adhesion to biomaterials is mediated via different factors with adsorbed proteins (fibrinogen, fibronectin and iC3b) being of primary importance. Upregulation of CD11b, loss of L-selectin, release of reactive oxygen species (oxidative burst), neutrophil elastase, cathepsin G or IL-8 are typical markers of activation [79, 80]. These release products lead to local tissue damage and recruitment and activation of more inflammatory cells.

Follow-on comments

Triggers for leukocyte activation on materials mainly are adsorbed proteins and adherent platelets. Additionally, mechanical factors and contamination with endotoxins may induce leukocyte activation.

Adsorbed and soluble complement products are the most prominent triggers of granulocyte and monocyte activation. Above all the anaphylatoxic peptides C3a and C5a induce cell activation [12] while adherence to the surface is mediated through surface-bound iC3b that binds to CD11b/CD18 [81]. Other pathways, such as kallikrein-induced activation, also contribute to the activation [82] and adherence can occur to already adsorbed and activated platelets [83, 84]. Little is known about how eosinophils react to bare materials, yet there is evidence that they may become activated with drug-coated materials (such as stents) [85, 86].

Physical factors of biomedical devices, such as disturbed flow in LVAD and cardiopulmonary bypass (CPB), also present a source of leukocyte activation and contribute to a pro-thrombotic and pro-inflammatory state of leukocytes [80].

An additional “external” factor that may also affect leukocyte activation are lipopolysaccharides (LPS) from Gram negative bacteria, which are also commonly known as endotoxin. They can easily contaminate the surface of cardiovascular devices and biomaterials synthesized in the lab [87]. A contaminated surface can activate several components of the inflammatory and thrombotic response in blood. Therefore all materials should be tested for endotoxin contamination prior to in vitro and in vivo studies [88, 89].

The first innate inflammatory response against foreign materials in blood includes neutrophils and monocytes. When activated, internal cell reorganization will lead to the presentation of CD11b and other adhesion ligands on the cell surface. This enables the cells to adhere to the surface and to interact with other neutrophils, monocytes and platelets. Additionally, they release soluble factors (elastase, myeloperoxidase) and microparticle and can form neutrophil extracellular traps (NETs).

Similarly to platelet microparticles, leukocyte microparticles play a role in hemostasis and inflammation and contribute to pathological conditions [90]. No in vitro study has yet characterized the mechanisms associated with material-induced leukocyte microparticles. There is however clinical evidence that leukocyte microparticles generated with cardiovascular devices such as CPB and LVAD also contribute to endothelial dysfunction and coagulation [47, 48].
Granulocyte activation also can lead to NETosis (neutrophil extracellular trap formation), which is characterized by the expulsion of nuclear DNA along with citrullinated histones and granular content (e.g. elastase) [91]. These components are highly thrombogenic [92] and have been identified in thrombi from coronary arteries [93] and from failed stents [86]. While these have become an area of active research in thrombosis and inflammation [94, 95], the biomaterials community has been slow to recognize the potential impact of NETs in biocompatibility and limited in vitro studies exist. Recently, Sperling and Maitz suggested that NETs may also contribute to biomaterial-induced thrombogenicity [96]. The formation of NETs was also observed on CoCr (a common biomaterial used in stents) in the presence of platelets in vitro [97].

It is generally considered that biomaterials do not induce an adaptive, lymphocyte dependent immune response [98] but this may deserve more attention in hemocompatibility research [99]. There are a few cases, like nickel or chromium allergy, where released ions transform self-proteins to foreign epitopes and induce a T-cell response. It is startling that in the absence of an antigen, T cells can be activated, but it was reported that cytokines at the inflammatory site around the implant may cause a non-clonal bystander activation of T-cells [100]. A recent paper [101] points to a pivotal role for B cells in the foreign body response.

The activation of immune responses sometimes also has distinct beneficial effects. Microbial infection is a persistent risk for implant materials in the body and antimicrobial defense is a leading task of neutrophil granulocytes. The antimicrobial capacity of biomaterials-adherent neutrophils however is still largely unexplored [102], which significantly affects our ability to design cardiovascular biomaterials that can resist infection. Whole blood incubation was shown to support anti-microbial activity against various gram-positive and gram-negative strains [103]. On the other hand, other studies indicate that neutrophils have been shown to promote bacterial growth and to create a protective environment for biofilms [104, 105].

More positive effects of material-induced inflammation were noted for endothelial regeneration following vascular prosthetic implants [106]. Activated neutrophils can support monocyte infiltration and subsequent phenotype switching may suggest a beneficial impact [107]. Since biomaterial implantation, inter alia, damages blood vessels, transmigration through activated endothelium may not be the primary mechanism for generating of monocyte-derived tissue macrophages. In addition, there are tissue resident macrophages that are different from the monocyte-derived phenotype [108]. Conversely, the inflammatory response in the surrounding tissue induced by cardiovascular implantation may also result in the release of macrophage wound healing factors that could promote endothelialization.

In the context of an inflammatory response, the variety of cytokines generated will dictate the consequences of that response. Pro-inflammatory properties (M1) of macrophages activated by Interferon γ and pro-regenerative properties (M2) after activation by IL4 or IL10, for example, are observed [109] in vitro. The relevance of these clear M1/M2 distinctions in vivo is less clear, although the notion of phenotypic differences among macrophages has been exploited in recent in vivo biomaterial studies [110, 111]. It has also been suggested that neutrophils can exhibit distinct phenotypes and may thus play different roles in inflammation, all the more so in the presence of biomaterials [112-114]; but this concept is not yet widely accepted.
Leukocyte activation manifests itself in diverse forms, and the mechanisms of crosstalk between platelets and leukocytes have been well demonstrated, blurring the line between inflammation and thrombosis [84]. Activated leukocytes can directly contribute to plasma coagulation on various pathways. They express tissue factor and contribute to coagulation in the process of thrombosis [12, 115-117]. The relevance of this pathway in biomaterial-associated thrombogenicity, however, is unclear [11].

In addition to local effects of cell adhesion / activation systemic clinical consequences that have been directly related to leukocyte activation include infection, myocardial infarction as well as organ injury and failure [80, 118]. Leukocyte removal filters have been introduced in cardiopulmonary bypass (CPB) [119] yet released inflammatory mediators from the trapped cells could contribute to the inflammatory response [120].

In an in vitro model, the determination of leukocyte activation is limited to local effects. Additionally, usually blood donation is done by healthy donors while clinical complications may result from the altered state of leukocytes in ill patients. Leukocytes are generally in a state of higher activation in cardiovascular diseases, such as in heart failure and left ventricular dysfunction [121, 122]. This can further contribute to complications following implantation of a medical device. Preclinical hemocompatibility testing will need to address these clinical conditions better in the future.

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Activation of enzymes and cells

- Coagulation cascade
- Platelet activation
- Complement activation
- Leukocyte activation

Biomaterial surfaces