Coagulation Systems of Invertebrates and Vertebrates and Their Roles in Innate Immunity: The Same Side of Two Coins?

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Abstract

Bacterial infections represent a serious health care problem, and all multicellular organisms have developed defense mechanisms to eliminate pathogens that enter the host via different paths including wounds. Many invertebrates have an open circulatory system, and effective coagulation systems are in place to ensure fast and efficient closure of wounds. It was proposed early on that coagulation systems in invertebrates play a major role not only in sealing wounds but also in preventing systemic infections. More recent evidence suggests that vertebrates, too, rely on clotting as an immune effector mechanism. Here we discuss the evolution of clotting systems against the background of their versatile function in innate immunity.

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Coagulation Systems and Hemostasis

Upon injury, most, if not all, animals that contain body fluids, activate coagulation systems leading to the clotting of hemolymph (invertebrates) or blood (verte-

brates). The ultimate goal in this process is the formation of a stable clot, which is required to seal the wound and prevent the loss of body fluids. Key players in this process are blood cells and their products, as well as soluble factors that are secreted into body fluids by other organs such as the liver or the insects' equivalent of the liver, that is, the fat body. Soluble and cell-derived factors interact to varying degrees to form a stable clot and in many cases this involves activation of proteolytic cascades, for example during secondary hemostasis in vertebrates and hemolymph clotting in horseshoe crabs. This review aims to summarize the differences and similarities of invertebrate and vertebrate hemostasis with a focus on their roles in innate immunity.

Model Organisms to Study Hemostasis at the Molecular Level

Vertebrate Models

Bleeding disorders sparked a strong interest to understand the molecular basis of blood clotting in humans leading to a thorough characterization of the cells and proteins involved. Briefly, upon bleeding, primary hemostasis leads to the attachment and activation of platelets and to the formation of a primary, mostly cellular plug. During secondary hemostasis, proteolytic cascades are activated

Fig. 1. Clotting in insects and mammals: clotting in mammals involves the contact and the tissue factor (TF) system. For clarity only the final cleaved products are shown. There is increasing evidence for crosstalk between coagulation and the complement system [36]. In insects, coagulation involves the parallel activation of hemolymph proteins, such as Fondue and TG, as well as the prophenoloxidase-activating system. It is presently unknown whether a proteolytic cascade is involved in TG activation (arrows in light blue), although a protease is activated during clotting [39]. In both insects and vertebrates, clot formation leads to the TG/F XIIIa-dependent immobilization of bacteria limiting their further dissemination [32].

by two different pathways: the tissue factor-dependent extrinsic and the intrinsic pathway also known as the contact or kallikrein/kinin system (fig. 1). Both proteolytically driven paths ultimately lead to the activation of thrombin and factor XIII (F XIII) and the formation of a stable clot. Activated F XIII (F XIIIa) belongs to the family of transglutaminases (TG) and is required to stabilize the clot by crosslinking fibrin/fibrin complexes via covalent bonds between glutamines and lysines [1]. In humans, F XIII deficiency is rare. Patients suffer from serious hemorrhagic diathesis, characterized by lifelong severe bleeding and delayed wound healing, but they have a good prognosis, when prophylactically treated with F XIII-containing plasma products or human plasma-derived concentrates. It should be mentioned that dysregulation of F XIII is seen in patients suffering from bacteremia or sepsis [2, 3]. However, whether patients with F XIII deficiency are more prone to bacterial infections has not been reported yet. F XIII deficiency has been extensively studied using knock-out mice. To this end, animals deficient in F XIII show impaired wound healing [4], but it is not known whether these mice are more susceptible to invasive infections.

Clotting in Invertebrates

In this section we will briefly summarize how clotting occurs in some of the best-studied invertebrate model systems with emphasis on the comparative aspects. For details, the readers are referred to the other reviews in this issue. Despite their name, horseshoe crabs are ancient members of the chelicerates, which include spiders,

scorpions and mites. Clotting of horseshoe crab hemolymph is thoroughly characterized and involves the activation of either of two proteolytic cascades (with the central factors C and G; for more details, see the review by Cerenius and Söderhäll in this issue [56]). Ultimately, both cascades lead to the cleavage of coagulogen into coagulin, which polymerizes to form an insoluble polymer. Analogous to vertebrates this does not require TG. However, TG released from hemocytes increases clot stability through crosslinking different clot components including coagulin. Nevertheless, coagulogen shows no sequence or structural similarity to fibrinogen [5].

Crustaceans including crayfish and shrimps rely upon the crosslinking of soluble proteins, which show sequence similarity to storage proteins (vitellogenins). Individual vitellogenin-like molecules are crosslinked by a TG leading to polymers, which form the clot matrix [6, 7]. There is no evidence that this highly effective process involves any proteolytic cascades [7, 8].

Clotting in insects was originally studied in larger biochemically amenable species and shown to involve another kind of transport proteins, namely lipophorins, which are considered as the insect LDL and HDL equivalents (summarized in Bohn [9] and in the article by Eleftherianos and Revenis in this issue [57]). More recent studies on smaller model insects, such as mosquitoes and *Drosophila melanogaster*, confirmed the earlier results from other insects [10, 11], and added hexamerin (also called larval serum protein: a class of storage proteins) and its receptor to the list of storage proteins involved in

clotting [12]. The last work also identified additional more specialized clotting factors, including hemolectin [13, 14] and the *Drosophila* protein Fondue (fig. 1). Just like in vertebrates, hardening of the clot relies on a TG [15], but also on a second enzyme, namely phenoloxidase [7] (see also the review by Eleftherianos and Revenis in this issue [57]). While proteolytic processing is required for the activation of prophenoloxidase, the molecular mechanisms that lead to TG activation remain to be identified in insects.

The dual crosslinking in insects is in contrast to vertebrates, where only F XIII in its active form (F XIIIa) is responsible for clot hardening (fig. 1). It has been speculated that the presence of an open circulatory system, which, at least in soft-bodied animals is also used as a hydroskeleton [16], makes them more susceptible to the loss of body fluids [11]. Therefore, insect larvae may depend to a greater extent on efficient and fast crosslinking to seal wounds leading to the evolution of two parallel modes to achieve this goal.

Evolution of Clotting Systems

There are few proteins of clotting systems that show conservation across different major groups of animals. TG appears to contribute to clotting in all invertebrates and is homologous to human F XIIIa. TGs have diversified more in vertebrates than in invertebrates. For instance, 8 members of this family are present in the human genome, while only 1 or few copies are usually identified in that of insects [17]. Despite the conserved usage of TGs during clotting, their relative position in clotting cascades varies. In both humans and horseshoe crabs, TG is required for the final hardening of the clot, while in crustaceans the same enzymatic activity is necessary for the formation of the polymers that make up the clot matrix itself [7, 8]. Likely explanations for these discrepancies include different lifestyles and ecological situations, which vary widely across arthropods [18]. Some gene families have also expanded by one or several rounds of duplications. One example that is relevant for clotting systems is a large protein superfamily that includes insect hexamerins, phenoloxidase (both of which are described above) and hemocyanin, an oxygen transport molecule in chelicerates and crustaceans [19]. During their evolution proteins may also acquire novel functions, a process, which has been named recruitment, co-opting or moonlighting. Examples include: (1) hexamerins, which can act both as storage proteins and clotting factors, (2) a salivary

gland mucin, which has been recruited as a clotting factor [20], and (3) hemocyanin, which can acquire phenoloxidase activity [21, 22].

An additional important mechanism that has shaped the evolution of clotting factors more than conservation of proteins is the recombination (shuffling) of conserved domains into new proteins [23]. One example here is *Drosophila* hemolectin, which is exclusively released from hemocytes and is a significant constituent of *Drosophila* clotting fibers. Interestingly, hemolectin consists of domains (discoidin domain) that share homology with domains found in human proteins such as factor V and VIII, complement regulatory domains and domains with similarity to von Willebrand factor [14]. The same holds true for several proteins in the horseshoe crab clotting cascade including factors C and G and tachylectins where related domains as in insects, as well as domains with similarity to fibrinogens and sugar-degrading enzymes (xylanases, which have lost their active sites and act as lectins) have been identified [5]. The horseshoe crab clotting cascade also provides an interesting example of how similar modules in biochemical pathways with a possibly common evolutionary origin are used in completely different contexts. This process resembles the recruitment of single proteins, but here parts of whole biochemical pathways are recruited for a different function. In this case, some horseshoe crab clotting factors show structural similarity with proteins required for the establishment of dorsal-ventral polarity, in developing *Drosophila* embryos (see the review by Cerenius and Söderhäll in this issue [56]). Surprisingly, *Drosophila* itself uses a different part of the developmental pathway including Spätzle and its receptor (Toll) in an immune context. Thus, clotting systems appear to have been constructed relying both on conserved components as well as faster evolving and more divergent proteins, which may nevertheless converge onto similar solutions.

Coagulation and Immunity

Open wounds represent an ideal entry port for bacteria to invade an open (invertebrates) or closed circulatory system (vertebrates). These sites therefore bear a great risk to cause systemic infections. To prevent bacterial dissemination from the injured site, wound sealing has to occur quickly. Thus, remodeling of tissue integrity at the wounded site has to be considered an important part of the innate immune system. This is of particular importance for all animals with an open circulatory system, whereas a closed system might give more protection be-

cause it is restricted. Coagulation occurs within seconds and this has been shown to lead to sequestration of bacteria in invertebrates such as the horseshoe crab as well as insects (see the review by Cerenius and Söderhäll in this issue [56] and Lesch and Theopold [24]). Notably, antimicrobial peptides are contingently released, too, and it is therefore not surprising that for instance hemolymph clotting in horseshoe crabs is highly sensitive to endotoxin (LPS) and other microbial elicitors [5, 25, 26]. However, whether blood coagulation in closed circulatory systems may also prevent pathogen spreading and dissemination in a similar manner needs to be clarified, although new data point into this direction [27].

Invertebrate and Vertebrate Coagulation Systems and Their Roles in Immunity

As described above, hemolymph clotting in insects comprises coagulation of clotting factors as well as dual crosslinking by TG and phenoloxidase. Analysis of *Drosophila* mutants and knockdown lines in the corresponding genes reveal that both hemolectin and Fondue show only mild bleeding defects and are less relevant for wound closure, while mutants lacking phenoloxidase die from bleeding [14, 28]. Conversely, both larvae with reduced amounts of TG or Fondue, or mutants defective in prophenoloxidase activation are more sensitive to certain infections, supporting the notion that both activities also have an immune function in *Drosophila* [28–32].

The contribution of the vertebrate complement system to the innate immune defense against pathogens has been discovered decades ago, while the coagulation system has only recently been described to play a role in vertebrate innate immunity (see the review by Delvaeye and Conway [33]). In particular, activation of the intrinsic pathway of coagulation (contact system) has been found to occur after the exposure to pathogenic bacteria, such as Gram-positive *Streptococcus pyogenes* and *Staphylococcus aureus* as well as Gram-negative *Salmonella* and *Escherichia coli* [34]. Interestingly, the induction of the mammalian coagulation system is combined with the generation of antimicrobial activity. For instance, activation of the contact system leads to the release of antimicrobial peptides from high molecular weight kininogen, a contact system co-factor [35], and thrombin processing by neutrophil-derived proteinases results in the generation of broad-ranged host defense proteins [27]. It is striking that – similar to *Drosophila* lacking clotting factors – humans with defects in the contact system show only mild

bleeding defects. These findings indicate that certain parts of the coagulation system in invertebrates and vertebrates accomplish similar functions that are not in the first instance restricted to clotting, but rather part of the host defense system. Crosstalk between coagulation and complement further augments their respective contribution to innate immunity (fig. 1) [36].

TG – The Best Conserved Protein in Clotting Systems

Among the different clotting factors in invertebrate and vertebrate hemostasis, TG needs to be highlighted, as it is the only factor that has homologues in all species studied [37]. The multifunctional properties of TG were investigated in many species. However, TG has been especially intensively studied in different invertebrates, where its essential immune function has been described. For instance, in vivo gene silencing of shrimp clotting protein or TG made the animals more susceptible to bacterial (*Vibrio penaeicida*) or viral (white spot virus) infections [38]. Moreover, *Drosophila* larvae with reduced TG activity (TG-RNAi knockdown animals) were reported to have higher mortality rates than wild-type animals when infected with *Photorhabdus luminescens*, *S. aureus* or *Heterorhabditis bacteriophora* [32]. Subsequent studies on the molecular mechanisms employed by TG revealed that the enzyme targets bacterial surfaces and immobilizes bacteria within the hemolymph matrix. This involves the clotting factor fondue, which acts as a TG substrate [39] and as a consequence larvae with reduced fondue expression also show immune defects after infection with *H. bacteriophora* (see the article by Hyrs et al. in this issue [58]). Strikingly, similar results were observed in human plasma where bacterial crosslinking into the fibrin network was dependent on F XIII. These findings suggest a conserved role in early innate immune mechanisms for TG and F XIII [32].

While TG and F XIII use similar modes of actions to eliminate an invading pathogen, their modes of activation may differ. F XIII is the last member of the vertebrate clotting cascade to be activated either by the induction of the intrinsic or extrinsic pathway. Once activated by thrombin, F XIII crosslinks newly generated fibrin networks to form a stable clot. Even though TG has been studied in several insects, its activation mechanism is yet unknown. Like F XIII, *Drosophila* TG uses clot components such as Fondue as major substrates [15]. It remains to be established, whether insect TG acts at a late stage of clotting like in humans or already during formation of the clot matrix such as in crustaceans (see above; fig. 1).

Clotting: At the Crossroads between Physiology and Immunity?

Taken together, these findings suggest that far beyond its physiological function in hemostasis, clotting plays a crucial role in the early immune response against invading microbes. This implies that the borders between both functions are not clearly defined, providing yet another example for recruitment of proteins (see above). Notably, this not only applies to TG. One additional example includes proteins such as hexamerins, which have been recruited to function both in clotting and housekeeping physiology. Another example are lipid transport proteins (lipophorins) in insects, which act as the insect's equivalent of LDL and HDL, but are also recruited as clotting factors and immune modulators [9, 10, 40]. Linking lipophorin to immunity, the composition of lipophorin particles has been found to vary depending on the immune status of the insect and includes pattern recognition molecules of the innate immune system [41, 42]. In addition, lipophorin particles are known to sequester LPS in the hemolymph and may therefore contribute immune stimulatory cargo both during incorporation into the clot and during uptake by hemocytes [41, 43]. Proteomic analysis of vertebrate HDL also reveals the presence of host defense proteins such as C-reactive protein, complement components, fibrinogen, kininogen and a secretory phospholipase A2 [44, 45], for which a regulatory function during immune responses has been suggested [46]. As part of the eicosanoids synthetic pathway, phospholipase A2 is a target for antiplatelet drugs. Notably, eicosanoids have also been implied in insect immunity [47] and more recently in the regulation of insect clotting (see the article by Hyrs et al. in this issue [58]). Finally, there is increasing evidence that the formation of atherosclerotic plaques, which involves clotting at the final stage of plaque formation and possibly platelets at an earlier stage, bears many similarities with an immune reaction and involves activation via Toll-like receptors, which bind endogenous ligands [48–50].

Cellular Contributions to the Clot

Hemostasis is to a great deal dependent on a cellular response, both in invertebrates and vertebrates, in particular at an early stage of wounding. Primary hemostasis in vertebrates leads to the attachment of platelets at the wound site and the formation of a cellular plug that

seals the wound. Insect cells that release clotting factors are usually called coagulocytes, but it is unclear whether these are specialized cells or normal hemocytes, which are activated by wound signals. In some insects this involves apoptosis-like mechanisms [51]. Similar to cells involved in blood clotting, insect hemocytes have been found to release membrane vesicles, which expose phosphatidylserine (PS), an early marker of apoptosis on their surface [51, 52]. In insects, PS modulates the activity of phenoloxidase, which is reminiscent of the activating role PS has on many vertebrate clotting factors [53].

Conclusions

Studies of clotting systems in both invertebrate and vertebrate model organisms have a long tradition. For obvious reasons, in many cases research on blood clotting was often initiated by studying disease-related phenomena such as the lack or decreased activity of clotting factors, the manipulation of clotting by certain pathogens and their virulence factors, or the aberrant endogenous activation of clotting for example during atherosclerosis. Research on invertebrate clotting has added a phylogenetic perspective, which shows that: (1) clotting is widespread amongst animals that contain body fluids, (2) some clotting factors are conserved but more often they have been assembled by recombining domains into new proteins or recruited from other branches of physiology, (3) despite these differences strikingly similar and phylogenetically ancient principles have emerged at the biochemical and cellular level, and (4) clotting is an integral part of the innate immune system. Further studies will show how clotting interacts with other immune reactions and whether sequestration of microbes by the clot is modified by previous exposure to the same antigen, in other words, whether clotting contributes to adaptive immunity [54, 55]. Today, there is a growing body of evidence that the vertebrate coagulation system also represents a part in the complex arsenal of the innate immune system [33]. This has led to a better appreciation of the clot's normal immune function and is expected to provide deeper insight into its pathophysiological function, thus, closing the gap between basic and clinical research.

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