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What have snakes taught us about integrins?

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Abstract. Snake venoms contain unique components that affect cell-matrix interactions. Disintegrins represent a class of low molecular weight, Arg-Gly-Asp (RGD)-containing, cysteine-rich peptides purified from the venom of various snakes among the Viperidae and Crotalidae. They bind with various degrees of specificity to integrins $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ expressed on cells. Snake venom metalloproteases (high molecular mass haemorrhagins) also contain disintegrin-like domains, in addition to zinc-chelating sequences. Membrane-anchored ADAMs (*A D*isintegrin *A*nd *M*etalloprotease domain), multidomain molecules consisting of metalloprotease, disintegrin-like, cysteine-rich, and epidermal growth factor domains, a transmembrane domain and a cytoplasmic tail, are a new family of proteins. In the light of the large number and wide distribution of ADAMs, they may participate in cell-cell fusion events, including sperm-egg binding and fusion, myoblast fusion and other cell-cell and cell-matrix interactions. The structure-function relationship of these molecules is discussed.

Key words. Snake venom disintegrin; metalloprotease; ADAMs; integrin $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_V\beta_3$; platelet aggregation; haemorrhage; cell-matrix interaction; cell fusion.

Introduction

Snake venoms contain many bioactive proteins with various biological activities, such as neurotoxins, haemorrhagins, coagulants, anticoagulants, phospholipase A and cardiotoxins [1–4]. Extensive studies of these venom proteins have helped us to understand the symptoms of snakebite victims and have contributed to rational treatments. Venom proteins of the Elapidae and Hydrophidae families are highly neurotoxic, blocking neuromuscular transmission with a risk of respiratory failure [5]. On the other hand, venom proteins of the Viperidae and Crotalidae families cause shock, intravascular clotting, systemic and local haemorrhage, oedema and necrosis [6, 7]. Fatality is usually caused by the synergistic effects of several components in the venom. Some snake venom components are unique in terms of their mode of action and specificity, such as the neurotoxin α-bungarotoxin, which blocks neuromuscular transmission by acting on the nicotinic acetylcholine (ACh) receptor of skeletal muscle, and disintegrins, antagonists of integrin $\alpha_{IIb}\beta_3$, which block platelet aggregation via binding to platelet membrane glycoprotein IIb/IIIa complex (i.e. $\alpha_{IIb}\beta_3$, fibrinogen receptor). Further elucidation of their mode of action and exploratory analysis of these unique molecules on the basis of their molecular structure-activity relationship has not only shed light on their detailed mechanism of action but has also opened avenues for development of new classes of drugs. For example, a thrombin-like enzyme purified from snake venom has been used to prevent venous thrombosis [8], and the discovery of disintegrins has led to the development of antithrombotic drugs which are under clinical trials [9–11].

Snake venoms affect blood coagulation and platelet aggregation in various ways [4, 12, 13]. Some venom components induce platelet aggregation, whereas other components inhibit platelet aggregation. Several recent reviews have dealt with disintegrins purified from haemorrhagic snake venoms (Crotalidae and Viperidae) [14–16]. Integrins, one of the adhesion receptor superfamilies, play important roles in mediating cell-cell and cell-matrix interactions. The widely distributed in-

tegrins have a heterodimeric structure consisting of transmembranar α and β subunits. So far, 15 α and 8 β subunits have been found. It has become apparent that integrins play important roles in platelet aggregation, inflammatory reactions, tissue remodelling, cell adhesion, migration, angiogenesis and other biological processes [17, 18]. Following the discovery of trigramin (the first disintegrin) in snake venom, many laboratories enthusiastically searched for other disintegrins in snake venoms and explored their possible clinical applications. The snake venom haemorrhagins – including HR1B, Ht-a and jararhagin – were found to contain a disintegrin-like domain [19–21]. Venom metalloproteinases affecting the interaction of collagen and its receptor (integrin $\alpha_2\beta_1$) were characterized. And a new family of membrane-anchored ADAMs was identified after a sperm protein fertilin β of rodents was found to contain a disintegrin and metalloproteinase domain [22]. These ADAMs may play important roles in cell-cell fusion, cell-matrix interaction and other potential normal cell functions [23–25].

Role of platelet integrins in thrombosis and haemostasis

Circulating platelets respond very rapidly to vascular injury. Coverage of the exposed subendothelium by platelets depends on the recognition of adhesive proteins [e.g. fibrinogen, collagen, von Willebrand factor (vWF), fibronectin and laminin] by specific platelet membrane glycoproteins, many of which are integrins (i.e. $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_V\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$). Among them, GPIa/IIa ($\alpha_2\beta_1$) and GPIIb/IIIa ($\alpha_{IIb}\beta_3$) are known to play important roles in mediating adhesion and aggregation of platelets [26]. Through membrane GPIa/IIa, platelets adhere to collagen in the exposed subendothelial layer in a Mg^{2+}-dependent manner [27] and become activated. The activation of platelets is associated with stimulation of several metabolic pathways, shape change, activation of the latent GPIIb/IIIa, enhancement of platelet coagulant activity and adenosine diphosphate (ADP) release reaction as well as thromboxane A_2 formation in reinforcing platelet aggregation. Subsequently, the binding of plasma fibrinogen to GPIIb/IIIa of the activated platelets leads to platelet aggregation, either forming a haemostatic plug to arrest bleeding under normal conditions, or forming a thrombus under pathological conditions such as chronic endothelial injury due to an atherosclerotic lesion [28, 29]. Patients with a deficiency or dysfunction of platelet membrane GPIa/IIa or GPIIb/IIIa are often detected because they have bleeding disorders [30–32], indicating that these two integrins expressed on the platelet membrane play essential roles in platelet function in haemostasis.

Discovery of disintegrins

Our previous studies revealed that the venoms of *Trimeresurus gramineus* [33, 34], *Echis carinatus* [35, 36], *Calloselasma rhodostoma* [37] and *Agkistrodon halys* (38, 39) contain peptides that are potent inhibitors of platelet aggregation. These components inhibit platelet aggregation elicited by a variety of agonists, including ADP, epinephrine, sodium arachidonate, collagen, thrombin and Ca^{2+} ionophore A23187, with a similar IC_{50}. As a working hypothesis, it was proposed that these inhibitors interfere with a common pathway of platelet aggregation [33, 34]. Subsequent studies of these venom inhibitors led to the discovery of disintegrins, a group of low molecular mass snake venom peptides (4–10 kDa) that bind with high affinities (K_d, $10^{-7} \sim 10^{-8}$ M) to both resting and activated platelet GPIIb/IIIa in a divalent-cation dependent manner [14–16]. The first disintegrin, trigramin, was purified from venom of the Taiwan viper *Trimeresurus gramineus* by Huang et al. in 1987 [40]. Its whole sequence was established and found to contain Arg-Gly-Asp (RGD) near its carboxyl terminus [41]. The tripeptide RGD within the molecule plays an essential role in mediating the binding of GPIIb/IIIa, and the specific conformation maintained by six disulphide bridges is also critical for its biological activity [41, 42]. After this finding, more than 40 peptides and their isoforms have been isolated and characterized, and were called disintegrins, based on their inhibitory activity on platelet membrane GPIIb/IIIa, a member of the integrin family [15] (fig. 1). Among the disintegrins shown in figure 1, only barbourin purified from the venom of *Sistrurus m. barbouri* contains KGD instead of RGD, and has a higher selectivity toward $\alpha_{IIb}\beta_3$ than $\alpha_V\beta_3$ or $\alpha_5\beta_1$ [43]. On the other hand, a number of $\alpha_{IIb}\beta_3$ antagonists do not belong to the disintegrin family, although they contain an RGD loop. These proteins include mambin, ornatin and decorsin. They show little amino acid homology with disintegrins (fig. 1). Mambin is a non-disintegrin (6.7 kD peptide) isolated from the venom of an Elapidae snake; its structure resembles that of neurotoxin [44]. But unlike neurotoxin, mambin is a potent inhibitor of platelet aggregation. Ornatin (4.4 kD) and decorsin (5.7 kD), purified from leeches, resemble the disintegrins in being cysteine-rich, RGD-containing molecules, capable of inhibiting ADP-induced platelet aggregation at nanomolar concentrations [45, 46]. However, the alignment of cysteines is very different from that of the disintegrins (fig. 1).

Possible applications of disintegrins

As potent inhibitors of platelet aggregation in vitro and efficient antithrombotic agents in several in vivo animal

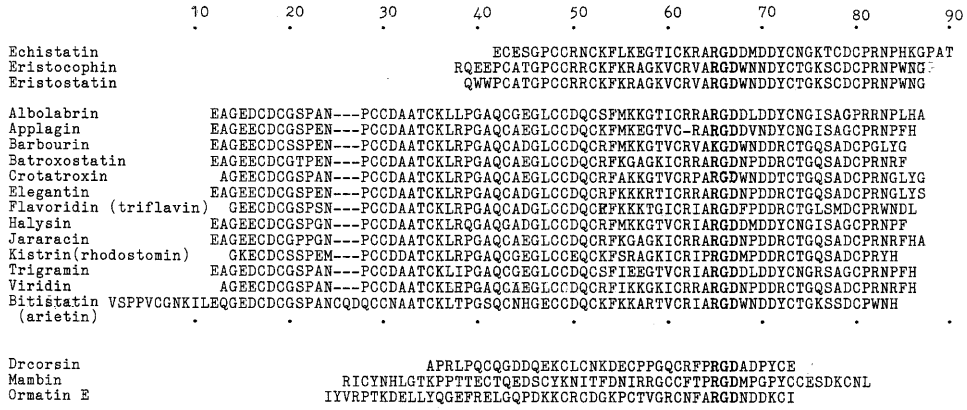

Figure 1. Amino acid sequences of disintegrins and other naturally occurring platelet $\alpha_{IIb}\beta_3$ antagonists in single-letter code. The spaces are inserted into amino acid sequences of medium disintegrins for better alignment with bitistatin (arietin).

models [43, 47–57], disintegrins were once considered to be highly potential antiplatelet agents, especially for use in arterial thrombosis. However, thrombocytopenia as a side effect was observed with echistatin, a disintegrin, when it was infused into baboons [58]. In addition, possible antigenicity and only a brief duration of the effect limit the development of intact disintegrins as therapeutic agents [51, 55, 56]. However, many extensive studies of disintegrins have revealed some potential uses of these peptides in the design of antithrombotic agents, the diagnosis of cardiovascular diseases and as novel tools for the study of cell-adhesion, cell migration, angiogenesis and other integrin-related disorders.

Design of antithrombotic agents

Platelet aggregation contributes significantly to the formation of arterial thrombi in coronary diseases and embolic stroke, leading to occlusion of arterial vessels, and subsequently the deficient oxygen supply of vital organs such as heart and brain [28, 29]. Thus reduction of platelet hyperactivity is an important therapeutic approach. However, a large number of patients suffering from ischemic complications cannot be controlled satisfactorily by currently available drugs such as aspirin and ticlopidine [59]. A more effective antithrombotic agent is urgently needed.

Since the recognition of the important role of tripeptide RGD, which is a part of the cell attachment domain of fibronectin, in constituting a recognition site [60], the pivotal role of the RGD peptide motif in integrin-mediated cell adhesive interaction with the extracellular matrix and plasma proteins has inspired many investigators to design RGD mimetics as antithrombotic agents [61]. Smaller linear peptides containing the RGD sequence have been shown to inhibit fibrinogen binding to activated platelets as well as platelet aggregation [42]. Nevertheless, these linear RGD-containing peptides exhibit low affinities towards platelet GPIIb/IIIa and weak antiplatelet activity. Surprisingly, the naturally occurring disintegrins are about 500–1000 times more potent than the short, linear RGD mimetics in inhibiting platelet aggregation. Their unique activity lies on the RGD sequence within a disulphide loop. Nuclear magnetic resonance (NMR) studies of the structures of two disintegrins, kistrin [62] and echistatin [63], have shed light on the reason for their high-affinity binding toward GPIIb/IIIa. The most important feature appears to be a hairpin loop that presents the binding sequence RGD at its tip. The RGD region was found to be highly flexible, and the side chains of Arg and Asp with positive and negative charges, respectively, are separated by glycine and turned in opposite directions. Based on this information on their steric structure, a series of RGD- and KGD-containing peptides, including cyclic peptides, were designed by computer modelling; some derivatives were found to be as potent as intact kistrin in a platelet aggregation assay [64–67]. To date, a variety of RGD- and KGD derivatives have been under clinical trials and have been demonstrated to be effective agents in reducing complications of ischemia, especially restenosis after percutaneous transluminal coronary angioplasty (PTCA) [68, 69].

Probe for analysis of platelet membrane GPIIb/IIIa

GPIIb/IIIa complex is the most abundant integrin (approximately 50,000 copies per platelet) expressed on the platelet surface [70]. This complex undergoes conformational changes when platelets are activated and subsequently associates with plasma fibrinogen, leading to platelet aggregation [71]. Binding assays performed us-

ing isotopically labelled disintegrins have demonstrated that unlike fibrinogen, which binds only to activated platelets, disintegrins bind with very high affinity to platelet membrane GPIIb/IIIa in both resting and stimulated platelets in a divalent-dependent manner [14]. Activation of platelets by ADP markedly enhances the binding affinity of some disintegrins (e.g. trigramin and halysin) towards platelet membrane GPIIb/IIIa [40, 47]. Based on their binding features, disintegrins can serve as a probe either for determining of GPIIb/IIIa content or for distinguishing the resting state from the activated state of platelet GPIIb/IIIa complex [72]. Although using radio labelled/ligand as a probe is the most popular technique for binding assays, the hazards of isotope disposal and isotopic labelling of the fibrinogen probe are major concerns. Recently, flow cytometry has become an extremely powerful technique for studying ligand-receptor interactions and for diagnosing platelet activation [73–77]. We successfully conjugated disintegrins with a fluorescent dye, fluorescein isothiocyanate (FITC), and performed a binding assay on them using flow cytometry [72]. These FITC-conjugated disintegrins have the same antiplatelet activity as intact disintegrins. After incubation with FITC-disintegrins, the fluorescence intensity of human platelets was dose-dependent and saturable, reflecting the specific binding of FITC-disintegrin to the platelet membrane GPIIb/IIIa complex. Activation of platelets with ADP markedly increased the binding of FITC-trigramin and -halysin, but not that of rhodostomin, consistent with data obtained from ^{125}I-disintegrin binding studies [40, 47]. Chelation of divalent cations with EDTA abolished FITC-disintegrin binding, indicating that binding of disintegrin to GPIIb/IIIa is divalent-cation dependent. Assuming a linear relationship between the fluorescence intensity and the amount of disintegrin bound on platelets measured by flow cytometry [78], the fluorescence intensity of platelets relative to the maximal binding fluorescence represents the extent of the GPIIb/IIIa level expressed on the platelet membrane. Using this method, we determined the platelet membrane GPIIb/IIIa levels of three Glanzman's thrombasthenia patients. The binding of FITC-disintegrin to platelets from these patients was strongly reduced and found to be less than 5% of normal platelets. They are thus type I thrombasthenia according to the classification of George et al. [30]. Owing to the simplicity and rapidity of the assay, FITC-disintegrins are ideal probes for determining the level of platelet membrane GPIIb/IIIa.

Measurement of GPIIb/IIIa blockade by $7E_3$

GPIIb/IIIa antagonists, including monoclonal antibodies against GPIIb/IIIa, RGD- or KGD-containing derivatives and especially the low molecular weight derivatives with cyclic structure, have recently been tested in clinical trials for treatment of coronary arterial thrombosis [61]. Among them, $7E_3$, a monoclonal antibody raised against GPIIb/IIIa and its chimeric fragment, $c7E_3$ Fab (Abciximab, or ReoPro), have been investigated as antithrombotic agents [9]. These GPIIb/IIIa antagonists block fibrinogen binding to the activated platelets, a common step in platelet aggregation. Thus they are expected to be the most ideal drugs for preventing platelet aggregation caused by variety of platelet agonists. Although $c7E_3$ Fab was shown to reduce ischemic complications in patients, e.g. restenosis after PTCA, this therapy led to an increased risk of bleeding if an overdose was infused [10, 11]. Since both antithrombotic activity and the adverse reaction of bleeding were highly related to the extent of platelet GPIIb/IIIa blockade, it will be very useful to establish a standard protocol for adjusting an optimal dose, in terms of both safety and efficacy, by monitoring the extent of GPIIb/IIIa blockade by $c7E_3$.

Estimation of the residual unoccupied GPIIb/IIIa using isotopically labelled $7E_3$ is the most popular technique for determining GPIIb/IIIa blockade [79–84]. Recently, Coller et al. have developed a rapid assay based on the ability of the activated platelets to induce clumping through the binding of available 'unoccupied' GPIIb/IIIa to fibrinogen precoated on beads. However, this assay only works if the occupied GPIIb/IIIa exceeds 80% [85]. On the other hand, our previous studies revealed that the binding sites of disintegrins appear to overlap with that of $7E_3$ [40, 47]. Furthermore, there is a good correlation between the inhibition of platelet aggregation and the blockade of FITC-crotavirin binding to platelets by $7E_3$. Crotavirin, a disintegrin, was isolated and purified from the venom of *Crotalus viridis* [86]. The amount of GPIIb/IIIa available for the subsequent binding of FITC-crotavirin to the $7E_3$-pretreated platelets inversely correlated to the extent of GPIIb/IIIa bound by $7E_3$, as shown by the observation that the sum of GPIIb/IIIa occupation by $7E_3$ and that by FITC-crotavirin approaches the total amount of GPIIb/IIIa expressed on the platelet membrane [87]. This indicates that percentage inhibition of FITC-crotavirin binding at the saturated dose reflects the extent of GPIIb/IIIa blockade by $7E_3$. Based on the characteristics of single-cell recording with flow cytometry where platelets exhibit a lower log light-scattering signal compared to leukocytes and erythrocytes, we can directly assess the binding of FITC-crotavirin to platelets even in whole blood preparations through proper gating by the light-scattering profile. This assay demands only a small volume (5 μl) of blood sample and is rapid, thus providing a feasible monitoring method for adjusting the therapeutic dose of $7E_3$ or its related derivatives. However, whether this technique can be applied to other GPIIb/IIIa antagonists awaits further study.

Disintegrin interaction with cells other than platelets

Since the disintegrins are RGD peptides, a number of studies have focused on their interaction with RGD-dependent integrins other than $\alpha_{IIb}\beta_3$. Both cultured cells and purified integrins have been used in these studies. Knudsen et al. [88] first showed that trigramin inhibited adhesion and spreading of C32 human melanoma cells on both fibrinogen and fibronectin. Applagin and echistatin inhibited the binding of vWF to platelets and adhesion of human umbilical vein endothelial cells (HUVEC) to vitronectin [89]. Rucinski et al. [90] and Soszka et al. [91] reported that batroxostatin and albolabrin, respectively, blocked the adhesion of $B_{16}F_{10}$ mouse melanoma cells to fibronectin. Triflavin (flavoridin) has been shown to inhibit $B_{16}F_{10}$ melanoma cell adhesion to the extracellular matrix (i.e. fibronectin, vitronectin and collagen type I) and lung colonization by $B_{16}F_{10}$ melanoma cell in vivo in a dose-dependent manner [92]. Adhesion of human hepatoma cells to extracellular matrix (ECM) components was also inhibited by this disintegrin [93]. Furthermore, the binding of FITC-conjugated triflavin to $B_{16}F_{10}$ melanoma cells was inhibited by Gly-Arg-Gly-Asp-Ser (GRGDS) and by monoclonal antibodies directed against $\alpha_5\beta_1$ and $\alpha_V\beta_3$, implying that this disintegrin binds via its RGD sequence to multiple integrin receptors on $B_{16}F_{10}$ melanoma cells [94, 95]. Scarborough et al. [43] investigated the effect of tergeminin, eristocophin (an isoform of enistostatin), echistatin and barbourin on the binding of fibronectin and vitronectin to their respective purified integrin receptor ($\alpha_5\beta_1$, $\alpha_V\beta_3$) in a solid-phase assay. Only echistatin inhibited fibronectin binding to the fibronectin receptor, and all peptides except barbourin blocked the binding of vitronectin to $\alpha_V\beta_3$. Barbourin specifically blocks fibrinogen binding to GPIIb/IIIa complex, but it does not affect binding of vitronectin to $\alpha_V\beta_3$. This specificity for the GPIIb/IIIa complex was related to substitution of the RGD sequence by KGD. Recently, Scarborough et al., reported that a group of disintegrins containing the RGDW or RGDN sequence blocked adhesion of M21 human melanoma cells to both vitronectin and fibronectin [96]. These data indicate that the amino acid sequence immediately adjacent to the RGD site of disintegrins can create an extended RGD locus which, coupled with a conformational effect on the RGD sequence, may be involved in determining disintegrin affinity and selectivity. In a number of studies, disintegrins have been shown to inhibit platelet aggregation induced by $B_{16}F_{10}$ melanoma cells, cervical carcinoma, hepatoma J-5, human prostate carcinoma, breast carcinoma and colon adenocarcinoma [97–99]. It is thought that the inhibitory effect of disintegrins on tumour cell adhesion to extracellular matrices and tumour cell-induced platelet aggregation may be responsible for the in vivo antimetastatic activity of the peptides. However, the exact mechanisms of action still remain to be solved. Echistatin appears to be a potent inhibitor of bone resorption in vitro [100] and in vivo [101]. Sato et al. showed that echistatin at nanomolar concentrations inhibits the resorption of bone by blocking the interaction of the vitronectin receptor ($\alpha_V\beta_3$) on the surface of osteoclasts with bone extracellular matrix [100].

Disintegrins in angiogenesis

$\alpha_{IIb}\beta_3$ (platelet membrane GPIIb/IIIa) and $\alpha_V\beta_3$ are members of the β_3 subfamily of integrin heterodimeric adhesion receptors [17, 102]. $\alpha_{IIb}\beta_3$ is essential for platelet aggregation and controls platelet function in thrombosis and haemostasis. $\alpha_V\beta_3$ is expressed on many cells, where it influences cell migration and has an impact on angiogenesis, restenosis, tumour cell invasion and atheroclerosis [103]. These two integrins share the same β subunit, β_3, but have distinct α subunits, α_{IIb} and α_V. Their common macromolecular ligands include fibrinogen, fibronectin, thrombospondin, von Willebrand factor and vitronectin [17]. These common ligands contain RGD sequences, and RGD-containing peptides inhibit binding of these ligands to both receptors. Angiogenesis plays an important modulatory role in normal physiological processes, such as embryonic development, tissue repair and luteal formation [104, 105]. On the other hand, angiogenesis is also involved in some pathological phenomena, such as promoting growth of tumours, eliciting diabetic retinopathy and inflammatory diseases, e.g. rheumatoid arthritis [106, 107]. The importance of $\alpha_V\beta_3$ integrins in angiogenesis was first elucidated by Cheresh et al. in a series of experiments [108–110]. In the chick chorioallantoic membrane (CAM) assay, an in vivo assay for angiogenesis, LM609, an anti-$\alpha_V\beta_3$ monoclonal antibody, inhibited blood vessel growth induced by implanting an $\alpha_V\beta_3$-negative melanoma or by implanting a basic fibroblast growth factor (bFGF)-containing pellet on the CAMs of 10-day-old embryos. The cyclic RGD peptide inhibits tumour-induced angiogenesis in CAM [108] and hypoxia-induced neovascularization in the murine retina [111].

Disintegrins such as trigramin and rhodostomin inhibit adhesion between tumour cells and ECMs through blockade of $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins [93, 95, 112, 113]. These disintegrins as well as the synthetic RGD-containing peptides have been shown to inhibit the experimental metastasis of melanoma cells [90, 91, 114]. We also found that rhodostomin inhibited prostaglandin I_2 (PGI_2) formation by human umbilical vein endothelial cells (HUVECs) caused by ancrod-generated fibrin through the blockade of $\alpha_V\beta_3$ integrin [115–117]. Recently, we examined the effect of disintegrins, namely α- and β-crotroxin, purified from the venom of *Crotalus*

atrox on the processes related to angiogenesis (unpublished observations). α- and β-crotroxin inhibited both the adhesion of endothelial cells and $B_{16}F_{10}$ melanoma cells towards ECM, and migration of $B_{16}F_{10}$ towards chemoattractants, including fibronectin, and bFGF, through the blockade of integrins $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ and $\alpha_5\beta_1$ expressed on $B_{16}F_{10}$ and HUVECs. Both α- and β-crotroxin inhibited capillary-tube formation by bovine aortic endothelial cells. Their inhibitory effect on cell proliferation and angiogenesis in the CAM model may be primarily through the blockade of the $\alpha_V\beta_3$ integrin, which is crucial in angiogenesis processes, although we cannot exclude the possible involvement of $\alpha_5\beta_1$. However, Brooks et al. [109] showed that $\alpha_5\beta_1$ monoclonal antibody had little effect on angiogenesis in the CAM model. Thus, integrin $\alpha_V\beta_3$ is the primary target for disintegrins in antagonizing angiogenesis. Whether the ligation of $\alpha_V\beta_3$ in endothelial cells by disintegrins would lead to programmed death (apoptosis) and how the cell cycle is affected, as well as any changes in the Bcl 2: Bax ratio, warrants further study. Whether disintegrins could be developed as a therapeutic adjuvant in suppressing metastasis in certain tumours is currently under active investigation.

Venom metalloproteases

Bleeding is one of the most striking symptoms in victims bitten by snakes of the Viperidae and Crotalidae families. The most extensively studied snake venom proteins causing haemorrhaging are disintegrins and haemorrhagins. Haemorrhagins belong to a unique subfamily of metalloproteases that contain a conserved Zn^{2+}-chelating sequence, H-E-X-X-H, but which have no other similarities to other metalloproteinases [118]. It is believed that haemorrhage is caused by proteolysis of components of the basal lamina of the microcirculature, e.g. collagen and laminin [119]. Haemorrhagins can be classified into two groups based on their molecular size. Small haemorrhagins have a molecular mass of about 25 kDa, such as Ht-2, H2-protease Ht-e [120–122]. The molecular masses of the large haemorrhagins range from 50 to 90 kDa. HR1B from *T. flavoviridis* venom [19], jararhagin from *B. jararaca* venom [21] and Ht-a from *C. atrox* [20] are high molecular mass haemorrhagins that have been cloned and sequenced. They are mosaic proteins with a metalloproteinase domain similar to that of the small venom proteinase at the N-terminus, a disintegrin-like domain in the middle of the molecule and a cysteine-rich domain at the C-terminus (fig. 2). However, the conserved RGD sequence is replaced in this disintegrin-like domain by an Ser-Glu-Cys-Asp (SECD) sequence (HR-1B). Au et al. [123, 124] showed that rhodostomin (or kistrin) may share a common precursor with a putative haemorrhagin protein and suggested that disintegrin and the haemorrhagin protein may share a common gene sequence. Furthermore, the predicted amino-terminal sequence of jararhagin is preceded by 150 amino acids of a putative pro-protein sequence with a striking homology to pro-protein sequences found in the coding genes of the disintegrins trigramin [125] and rhodostomin [123, 124]. In a snakebite victim, snake venom metalloproteases and disintegrins promote haemorrhage in a synergistic manner, as metalloproteases degrade capillary basement membrane [126] and soluble disintegrins bind to platelet integrins, inhibiting platelet aggregation [16, 123]. Interest in the function of these haemorrhagic metalloproteinase has been generated due to the elucidation of the primary structure of different members of the venom haemorrhagic metalloproteinase-disintegrin family (fig. 2).

Adhesion of platelets to newly exposed subendothelial collagen is believed to be an early event in arterial thrombosis. The integrin $\alpha_2\beta_1$ (i.e. GPIa/IIa) is the adhesion receptor for collagen [27]. The venom metalloproteinases, such as jararhagin [21, 127], catrocollastatin [128, 129] and crovidisin [130] have been reported to specifically inhibit the adhesion of collagen to platelets and subsequent platelet aggregation. Jararhagin has been referred to as a collagen receptor (GPIa/IIa) antagonist, while the mechanism of action of catrocollastatin and crovidisin is attributed to their collagen-binding activity rather than binding of GPIa/IIa [129–132]. Not only platelet aggregation but also the release reaction, thromboxane formation and increase of intracellular Ca^{2+} level of platelets in response to collagen were completely abolished by crovidisin [130].

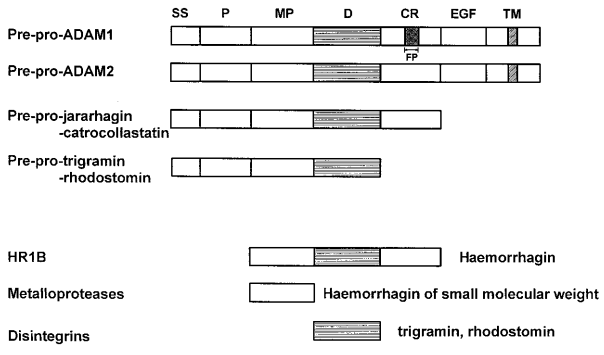

Figure 2. Domain composition of disintegrin-containing proteins. SS, signal sequence; P, prodomain; MP, metalloprotease; D, disintegrin; CR, cysteine-rich; EGF, epidermal growth factor repeat domain; TM, transmembrane domain. A short cytoplasmic tail follows in both ADAM 1 and 2 (PH-302α, and PH-30β) subunits. A putative fusion peptide (FP) is present in PH-30α.

Table 1. Protein sequences of the predicted binding loops of selected disintegrins, metalloproteases and membrane-anchored ADAMS.

Protein	Sequence
Disintegrin	
Kistrin (rhodostomin)	CRIP **RGD**-MPDDRC
Echistatin	CKRA**RGD**-DMDDYC
Barbourin	CRVA**KGD**-WNDDTC
Trigramin	CRIA **RGD**-DLDDYC
Metalloprotease	
HR-1B	CRES **TDEC**DLPEYC
Catrocollastatin	CRAS **MSEC**DPAEHC
Jararhagin	CRAS **MSEC**DPAEHC
ADAM	
Fertilin α	CRPA **EDVC**DLPEYC
Fertilin β	CRES **TDEC**DLPEYC
Meltrin α	CRGS **SNSC**DLPEFC

In the presence of crovidisin, the Mg^{2+}-dependent adhesion of platelets to collagen was diminished in a dose-dependent manner. In addition, collagen fibres were fluorescent after incubation with FITC-conjugated crovidisin, indicating that crovidisin binds directly to collagen fibres. Thus crovidisin blocks interaction between platelets and collagen fibres through its binding to collagen fibres, resulting in the blockade of collagen-mediated platelet function. Catrocollastatin also binds to collagen fibres, leading to a blockade of platelet aggregation [129]. Recently, the inhibition by jararhagin of the platelet response to collagen was thought to be mediated through the binding of jararhagin to platelet α_2-subunit (i.e. GPIa) via the disintegrin domain, followed by proteolysis of the β_1-subunit (e.g. GPIIa) with loss of the integrin structure necessary for the binding of macromolecular ligands [132]. However, both the action of crovidisin and catrocollastatin were independent of the proteolytic activity (or metalloproteinase domain), and they do not bind to integrin $\alpha_2\beta_1$ [129, 130]. A cyclic oligopeptide corresponding to the conserved fragment containing SECD, but not the linear SECD fragment, in the disintegrin-like domain of catrocollastatin (table 1) was shown to inhibit collagen-induced platelet aggregation and to bind to collagen [129]. Despite the sequence similarity among these metalloproteases, jararhagin and catrocollastatin/or crovidisin have different mechanisms. This striking discrepancy merits further investigation.

As well as $\alpha_2\beta_1$ antagonists, there are also venom $\alpha_2\beta_1$ agonists. Earlier studies showed that several noncoagulant, nonenzymatic snake venom components caused platelet aggregation and release reactions [12]. Of these proteins, the most extensively studied platelet aggregation inducers are trimucytin and aggretin, which have been purified from *Trimeresurus mucrosquamatus* and *Calloselasma rhodostoma* venoms, respectively. Both inducers cause platelet activation and aggregation through the activation of endogenous phospholipase C by acting as GPIa/IIa agonists [133, 134]. A monoclonal antibody (6F1) directed against GPIa/IIa, an integrin (i.e. $\alpha_2\beta_1$) expressed on platelet membrane, inhibited platelet shape change and aggregation induced by aggretin. ^{125}I-aggretin bound to platelets with a high affinity ($K_d = 4.0 \pm 1.1$ nM), and the number of binding sites was estimated to be 2119 ± 203 per platelet [134]. Furthermore, crovidisin blocked the aggregation of human washed platelets caused by trimucytin in a dose-dependent way but not that caused by aggretin (unpublished observations). Further study on the interaction of crovidisin-collagen and crovidisin-trimucytin at a molecular level may provide a new avenue for developing inhibitors to prevent detrimental effects of collagen. Such studies would also increase our knowledge of platelet-collagen interaction at a molecular level.

Membrane-anchored ADAMs

Families of membrane-anchored cell surface adhesion molecules include cadherin [135], immunoglobulin superfamily members [136], selectin [137] and integrins [17]. A recently discovered gene family encoding membrane proteins with a disintegrin and metalloprotease domain (ADAM) is unique among cell surface proteins in containing both a potential adhesion domain as well as a potential protease domain. mRNA coding for ADAMs has been found in a wide array of mammalian tissues as well as in lower eukaryotes [23, 24]. These molecules contain pro-, metalloprotease-like, disintegrin-like, cysteine-rich, endothelial growth factor (EGF)-like, transmembrane and cytoplasmic domains (fig. 2). They are related to domains found in a family of soluble snake venom proteins, the snake venom metalloproteases. The first ADAMs described – fertilin α and β – are expressed in spermatogenic cells [138, 139]. Some ADAM metalloprotease-like domains are thought to degrade ECM components, and some are involved in integrin-mediated interactions. The process of mammalian fertilization involves the binding of sperm to a large ECM (the zona pellucida) surrounding the egg, penetration across this matrix and finally sperm-egg binding and fusion. However, little is known about sperm-egg interactions at a molecular level [140]. Recently, a guinea-pig sperm protein, PH-30 (called fertilin), which has been implicated in sperm-egg membrane binding and fusion, was found to contain a membrane-anchored disintegrin-like domain that shares significant sequence homology with snake venom disintegrins [138]. The RGD sequence that appears in snake venom disintegrins was not found within the disinte-

Table 2. Potential functions of ADAMs.

ADAM	Synonyms	Metalloprotease	Adhesion	Fusion
ADAM 1	Fertilin α	+	+	+
ADAM 2	Fertilin β		++	
ADAM 3	Cyritesin		+	
ADAM 4	-		+	
ADAM 5	t MDC II		++	
ADAM 6	DMC IV		+	
ADAM 7	EAP 1		++	
ADAM 8	MS 2	+	+	
ADAM 9	MDC 9	+	++	+
ADAM 10	MPcDNA	+	+	
ADAM 11	MDC		++	+
ADAM 12	Meltin α	+	+	+
ADAM 13	-	+	+	
ADAM 14	adm-1			
ADAM 15	Metargidin; MDC 15 vascular MDC	+	++	

grin-like domain of PH-30 (table 1). Through a disintegrin-like domain, PH-30 recognizes $\alpha_6\beta_1$ integrin on the egg membrane, and this cell-cell interaction at the egg membrane plays an essential role in sperm-egg fusion [22].

The presence of a disintegrin-like domain is a feature of the mature fertilin β (fig. 2). The best-characterized disintegrins (e.g. trigramin and rhodostomin) are globular proteins with a projecting 13-amino acid loop with the sequences RGD or KGD at the tip of the loop. On the other hand, the disintegrin domains of venom metalloproteases (e.g. jararhagin and HR1B) contain a 14-amino-acid loop instead at the analogous position. There is a free cysteine in the middle of this loop which is thought to be important either for binding to its target cell molecule or for maintaining the proper configuration of the recognition site. GoH3, an anti-$\alpha_6\beta_1$ monoclonal antibody function blocking as well as a peptide analogue derived from the disintegrin loop of fertilin β (i.e. M14, CRLAQDEADVTEYC) abolished mouse sperm binding to the egg. However, RGD peptide had no effect [141]. All mammalian eggs analysed (mouse, hamster, human) express integrin $\alpha_6\beta_1$ at their surface. RGD-containing peptides inhibit binding of human and hamster sperm to golden hamster eggs. Whether different species of mammalian eggs use RGD-dependent (e.g. hamster) and RGD-independent (mouse) integrin as sperm receptors remains to be determined. The binding between sperm fertilins and egg plasma membrane integrin is a general mechanism for interaction between mammalian gametes. However, it remains to be determined for each mammalian species which sperm fertilin(s) binds to which egg integrin(s). Both fertilin α and β contain full disintegrin domains, and the mature fertilins α and β are the proteolytic processed forms of fertilins α and β lacking the pro- and metalloprotease domains as detected on fertilization-competent epididymal sperm [142]. This discovery indicates that a membrane-anchored protein with a disintegrin-like domain may function as a ligand for the integrin expressed on the target cell, and this kind of recognition could occur through an RGD-independent interaction. However a recent study found that the presence of an RGD sequence in metargidin (from an epithelial carcinoma cell line MDA, MDC 15) strongly suggests that metargidin could act as a membrane-anchored integrin ligand containing an RGD motif [143]. In addition, several of the ADAM tails (ADAMs 8, 9, 10, 13 and 15) are rich in proline, which suggests that they might contain binding sites for cytoskeleton-associated proteins or SH3 domain-containing proteins, a large group of signalling molecules [144]. A GST fusion protein containing two proline-rich regions of the tail region of ADAM 9 serves as a binding site for the SH3 domain of Src, a protein tyrosine kinase [145], and it is likely that some of these tails have signalling potential. Moreover, ADAMs may signal by binding to integrins (or other) receptors, so the possibility for bidirectional signalling exists [25].

ADAMs might be involved in cell fusion events (table 2). ADAM 1 (fertilin α) and other ADAMs (e.g. ADAMs 9, 11, 12) contain a potential fusion peptide, a relatively hydrophobic stretch of $\sim$23 amino acids embedded in the cysteine-rich domain. Like viral fusion peptides, the candidate ADAM fusion peptides can be modelled as either an α-helix or β-sheet with one very hydrophobic face [146]. Although ADAMs 1 and 12 have been implicated in pathways leading to cell-cell fusion, no direct proof has formally been shown.

In addition to fertilization and myoblast fusion, other potential functions are under intensive investigation. ADAMs 1–5 are expressed on early spermatogenic cells

and could be involved in adhesive events between developing sperm and Sertoli cells [147]. ADAMs 1, 2 and 12 have been implicated in cell-cell fusion and myoblast fusion. The *kuz* gene that encodes a metalloprotease-disintegrin protein is essential for the partitioning of neural and nonneural cells during the development of both the central and peripheral nerve systems in *Drosophila*. The development of a neuron requires a *kuz*-mediated positive signal from neighbouring cells, raising the possibility the *kuz* homologues may act similarly during mammalian neurogenesis [148]. Using a polymerase chain reaction (PCR) approach, Herren et al. identified one member of the ADAM family (MDC15) which is expressed in umbilical vein endothelium and aortic smooth muscle. The sequence of MDC15, including the presence of an RGD sequence in the disintegrin-like domain, its expression in cultured vascular cells and its upregulation in atherosclerotic lesions, suggests that MDC15 may be a potential regulator of vascular function and may be involved in the development of atherosclerotic lesions [149]. Through their proteolytic, adhesive and/or fusogenic functions, ADAMs may be implicated in a variety of important physiological and pathological processes.

Other snake venom non-disintegrins affecting platelet function

Viper venoms also contain non-disintegrin proteins which affect platelet aggregation and function [15]. Binding of plasma von Willebrand factor (vWF) to platelet glycoprotein Ib is assumed to play a key role in the earliest phase of primary haemostasis. Recently, several investigators have shown that under high shear stress, platelet aggregation is initiated by interaction between multimeric vWF and platelet GPIb, a phenomenon assumed to occur in stenosed coronary arteries in vivo [150–153]. An impressive review deals with snake venom proteins modulating the interaction of vWF and platelet GPIb and describes the structure of the snake venom vWF-binding proteins and GPIb-binding proteins, and especially the characterization of snake venom GPIb-binding proteins which block the receptor for vWF [154]. The use of recombinant strategies for further investigation of the structure-function relationships of these proteins appears to hold promise in developing of antithrombotic agents.

Prospective research on disintegrin-related molecules

Further study on disintegrins will probably be focused on the application of disintegrins as tools for studying the mechanism of action of platelet aggregation, other integrin-related function and signal transduction involved in various kinds of cells, including platelets, endothelial cells, fibroblasts, smooth muscle cells and tumour cells. The integrins $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_2\beta_1$ (GPIa/IIa) and $\alpha_5\beta_1$ are targets for snake venom disintegrins. The specificity of these disintegrins should be fully characterized using purified integrins or specific integrin-bearing tumour cell lines. Disintegrins might be an ideal alternative to monoclonal antibodies for mapping ligand recognition sites on GPIIa/IIIa, GPIa/IIa, $\alpha_V\beta_3$ and other integrins. The elucidation of the steric structure of these disintegrins would be very helpful for drug design directed against integrin-related diseases. Some disintegrins appear to be relatively selective in recognizing specific integrins, and this selectivity can be further enhanced by producing recombinant proteins or synthetic peptides with optimal substitutions. Studies of disintegrins have the potential to contribute both academically and clinically to the important fields of thrombosis, cell adhesion, cell migration, angiogenesis and integrin-related diseases. The haemorrhagic metalloproteinases with a disintegrin-like domain (high molecular weight metalloproteinases) are responsible for local bleeding in envenoming, and they may play a role in systemic bleeding. Platelets and plasma proteins (e.g. fibrinogen, vWF) are targets for these enzymes. More work on the structure-function relationships of the metalloproteases needs to be carried out to understand fully how they interfere with haemostasis. For example, the role of the disintegrin-like domain in inhibition of platelet function and in recognizing integrin $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ or collagen requires further clarification. Venom metalloproteinases are useful tools for investigating mechanisms of ligand-receptor and cell-cell interactions in normal cell function. In addition to a metalloprotease domain and a disintegrin domain, ADAMs have a C-terminal cysteine-rich domain, an EGF domain and a cytosolic tail. Thus their roles in mediating heterotypic sperm-egg fusion and homotypic myoblast-myoblast fusion need to be clarified. A combination of biochemical and genetic studies is needed to elucidate the exact role of fertilin α, fertilin β and meltrin α in cell-fusion pathways in different species. In this process, we should advance our understanding of cell-cell and cell-matrix interactions. Further analysis of fertilin should enrich our knowledge about certain forms of infertility and may be useful in designing novel contraceptive agents. Overall, there are many mysteries about ADAMs that we have yet to explore. This tale of snake venom disintegrins, metalloproteinases and ADAMs reminds me that in the Bible it was a snake that aroused Adam and Eve's curiosity about the acquisition of knowledge, an event whose consequences we are still living with. Snakes have been evolving in parallel with other reptiles, birds and mammals for many millions of years and still have a lot to teach us about mammalian haemostasis and cell biology.

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