

Endothelial-derived microparticles: Biological conveyors at the crossroad of inflammation, thrombosis and angiogenesis

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Summary

Endothelial microparticles (EMP) are complex vesicular structures that can be shed by activated or apoptotic endothelial cells. EMP are composed of a phospholipid bilayer that exposes transmembrane proteins and receptors and encloses cytosolic components such as enzymes, transcription factors and mRNA derived from their parent cells. Thus, EMP behave as biological conveyors playing a key role in the tuning of vascular homeostasis. This review focuses on the multifaceted roles of EMP, notably in coagulation, inflammation and angiogenesis and also on the mechanisms that trigger their formation. In this con-

text, EMP could compromise vascular homeostasis and then represent key players in the pathogenesis of several inflammatory and thrombotic diseases. Consequently, elucidating their role and their mechanisms of formation will bring new insights into the understanding of endothelial-associated diseases. Moreover, in the future, it can open novel therapeutic perspectives based on the inhibition of EMP release.

Keywords

Microparticles, endothelium, coagulation, inflammation, thrombosis, vascular homeostasis, vesiculation

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Introduction

It is now well established that disruption of endothelial integrity represents a crucial event in the initiation and development of cardiovascular diseases. The endothelium is able to respond to physical and chemical signals by production of a wide range of factors that regulate vascular tone, thrombo-resistance, cell proliferation and vessel wall inflammation. In addition to these soluble molecules, the endothelium can shed microparticles (MP) as a result of cell activation or apoptosis. Since the first description of MP as circulating 'dust' in 1967 (1), numerous studies have reported the *in vitro* release of MP from different subtypes and their presence in human plasma. MP were defined as small vesicular structures with an heterogeneous diameter (from 0.1 to 1 micron), resulting from the remodelling of membrane phospholipids and expressing phosphatidylserine (PS) and antigens representative of their parent cells. MP harbouring endothelial-associated molecules were identified both *in vitro* and in human plasma and therefore, were called endothelial-derived MP (EMP). Moreover, the exposure of pro-coagulant phospholipids and specific receptors at their surface also indicate that EMP behave as bio-messengers linking inflam-

mation, thrombosis and angiogenesis. This brief review summarises the mechanisms leading to EMP formation, their structure and function relationship as well as their physiopathological involvement in vascular homeostasis.

Mechanisms of EMP formation

The current knowledge on EMP formation derives mainly from experiments on isolated or cultured endothelial cells. Indeed, *in vivo* mechanisms involved in EMP generation still remain mostly unclear.

Remodelling of membrane phospholipids

Under physiological conditions, cell membrane is defined by an asymmetric distribution of negative phospholipids being sequestered into the inner leaflet of the membrane. Disruption of

phospholipids asymmetry is a universal feature of cells undergoing activation or apoptosis, leading to exposure of phosphatidylserine (PS) on the outer leaflet as a consequence of the calcium-dependent activation of scramblase and floppase/ABC1 and the inhibition of translocase/flippase activities (2–4). Moreover, MP formation and shedding necessitate modifications in cell structural architecture involving disruption of cytoskeleton protein organisation. Altogether, these events lead to plasma membrane budding, formation of membrane blebs and MP release into the extracellular fluid.

Inducers of endothelial vesiculation

Cultured endothelial cells can release EMP after activation by a variety of prolonged stimuli, essentially inflammatory stimuli. Combes et al. first described the generation of EMP from human umbilical endothelial cells (HUVECs) stimulated by tumour necrosis factor- α (TNF- α) (5). Inflammatory cytokines (6), bacterial lipopolysaccharides, reactive oxygen species (6), plasminogen activator inhibitor (7), thrombin (8), camptothecin (9), C-reactive protein (10) and ureamic toxins (11) are also able to induce EMP generation *in vitro*.

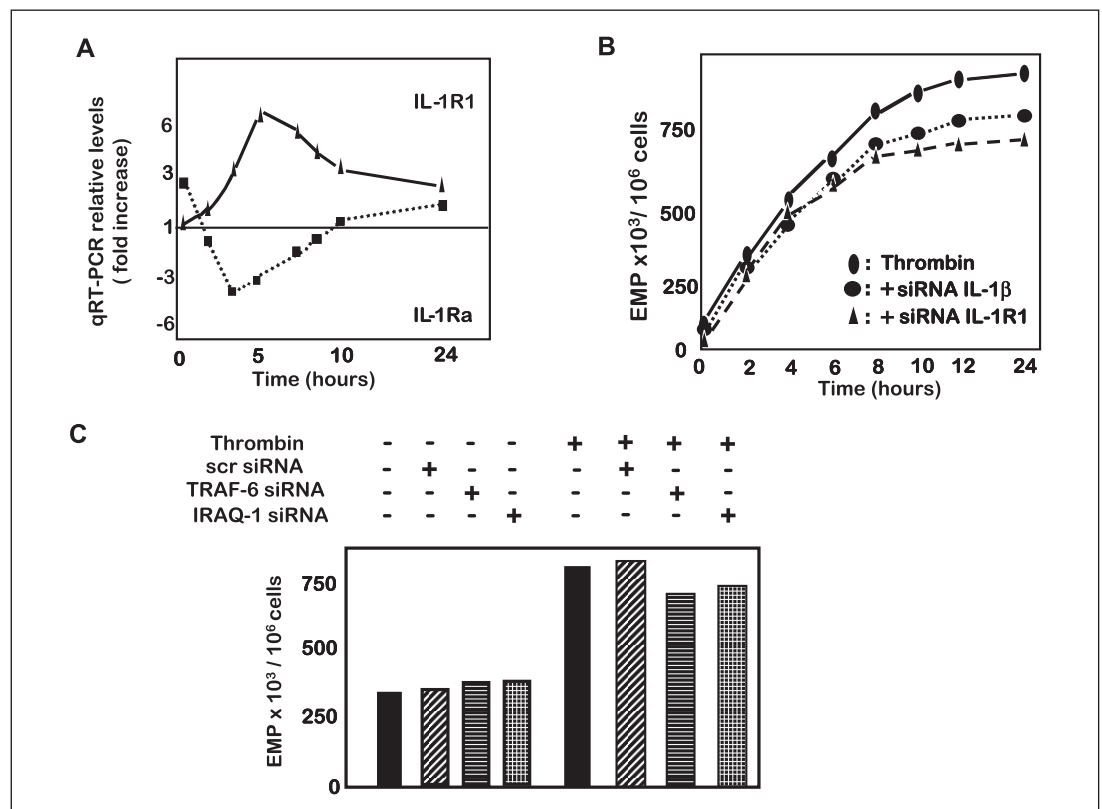
The *in vivo* mechanisms of EMP release were documented through a recent study showing that low shear stress is associated with *in vivo* EMP release in end-stage renal disease (12).

Molecular mechanisms involved in EMP release

Very few studies have analysed the intrinsic mechanisms leading to the formation and release of MP from endothelial cells. A study based on gene profiling analysis has identified an original pathway of endothelial vesiculation induced by thrombin, involving an activation of the Rho-kinase ROCK-II by caspase 2 in the absence of cell death (8). This mechanism of EMP generation depends on the nuclear factor (NF)- κ B activation and proceeds with several steps: a first phase that occurs early after thrombin binding to its receptor PAR-1, and a second phase that depends on transcriptional events mediated by thrombin and involving TRAIL/Apo2L, a cytokine that belongs to the TNF- α superfamily (13). This pathway implies the soluble form of TRAIL, secreted by endothelial cells under thrombin- or inflammatory- stimulation. The interaction between sTRAIL and its endothelial receptor TRAIL-R2 initiates the recruitment of death-domain-containing adaptor proteins TRADD and then TRAF2, RIP1 and NF- κ B, which participate to EMP release. Moreover, the engagement of this signalling pathway controls the thrombin-mediated up-regulation of the inflammatory mediators ICAM-1 and IL-8 (13). Moreover, gene profiling also identified IL-1 and IL-1Ra as additional players of thrombin-induced EMP. Indeed, thrombin stimulation of HMEC-1 resulted in an increase in IL-1R1 expression, a concomitant decrease in IL-1Ra and a low secretion of IL-1 (► Fig. 1A). Thrombin-induced EMP release was inhibited by specifically silencing of IL-1R1 or IL-1 (Fig. 1B). The engagement of IL-1R1 resulted in the recruitment of

Figure 1: Involvement of IL-1 and IL-1R1 in EMP generation mediated by thrombin.

A) Time-dependent mRNA expression of IL-1R1 and IL-1Ra in HMEC-1 in response to thrombin stimulation. B) EMP generation by thrombin in HMEC-1 transiently transfected with IL-1 or IL-1R1 siRNA. C) EMP generation by thrombin after engagement of IL-1R1 in HMEC-1 specifically knocked-down for TRAF6 and IRAQ-1 (n=3).



adaptors proteins TRAF6 and IRAQ1 that transduced a signalling pathway leading to amplification of EMP release by thrombin (Fig. 1C). Consequently, the inflammatory mediators regulated by thrombin are key partners linking coagulation and inflammation, and represent an autocrine pathway that amplifies endothelial vesiculation (► Fig. 2).

Whether intracellular pathways regulating EMP release and the general inflammatory response can be related remains an open question. A recent study identified p38 mitogen-activated protein kinase (MAPK) as a critical molecule in the production of pro-inflammatory EMP (14). Indeed, the authors showed that EMP triggered by TNF- α activation induced an increase of soluble ICAM-1 from endothelial cells, thus providing a paracrine loop enhancing the endothelial response to inflammation.

Another open question is whether EMP released from apoptotic cells differ in lipid and protein composition from those shed following cell activation, and could possibly have different physiological effects. Blebbing of the cellular membrane occurs rapidly after cells enter the apoptotic process. Bleb formation depends upon actin cytoskeleton and actin-myosin contraction, which is regulated by caspase3-induced Rho Kinase I and II activation (8, 15, 16). Rho kinase activation is required for re-localisation of DNA fragments from the nuclear region to membrane blebs, suggesting that MP from apoptotic cells may contain nuclear material (15, 17, 18). Among the classical apoptosis mechanisms, the signalling pathways controlling EMP formation still remain mostly unknown. It has been suggested that EMP generated in response to apoptosis are more likely to express the constitutive endothelial cell marker PECAM-1, whereas EMP released by endothelial cell activation are characterised by increased expression of inducible endothelial markers such as CD62E (19, 20). Due to this difference, the analysis of EMP phenotypic signatures could provide clinically useful information on the status of the endothelium.

EMP structure and functions

EMP composition

Endothelial-derived microparticles are complex vesicular structures that express a large repertoire of molecules representative of their parent cells. Their composition may vary depending upon the cell they originate from and the type of stimulus that caused their formation (21, 22). EMP harbour at their surface a panel of phospholipids and proteins including oxidised bioactive lipids, membrane receptors controlling the coagulation equilibrium (23–27) (endothelial protein C receptor [EPCR], thrombomodulin [TM] and tissue factor [TF]), adhesion molecules (5) (intercellular cell adhesion molecule-1 [ICAM-1], platelet endothelial cell adhesion molecule-1 [PECAM-1], vascular cell adhesion molecule [VCAM-1], endothelial-selectin [E-selectin]). In addition, enzymes such as matrix metalloproteases (MMP) (28), nicotinamide adenine dinucleotide phosphate (NADPH) oxydase (29) or proteolytic systems such as urokinase plasminogen activator/uPA receptor (uPA/uPAR) plasminogenic activity (30) and also growth factor receptors (19, 31) (endoglin, Hedgehog morphogen [32]) are also expressed on EMP surface. Presence of immunoglobulins, complement molecules or major histocompatibility complex (MHC) molecules were also reported at the MP surface (33–35). Moreover, recent studies have demonstrated that MP can enclose transcription factors, mRNA and microRNA (36–41), suggesting a possible regulatory role for MP. All together, the phospholipids, proteins, receptors and genetic information are selectively sorted during the vesiculation process. This selective enrichment of surface molecules may be due to the accumulation of membrane lipids and proteins in cholesterol-rich raft domains prone to blebbing during MP formation. Thus, EMP released after endothelial cell activation or apoptosis could not only be considered as "minia-

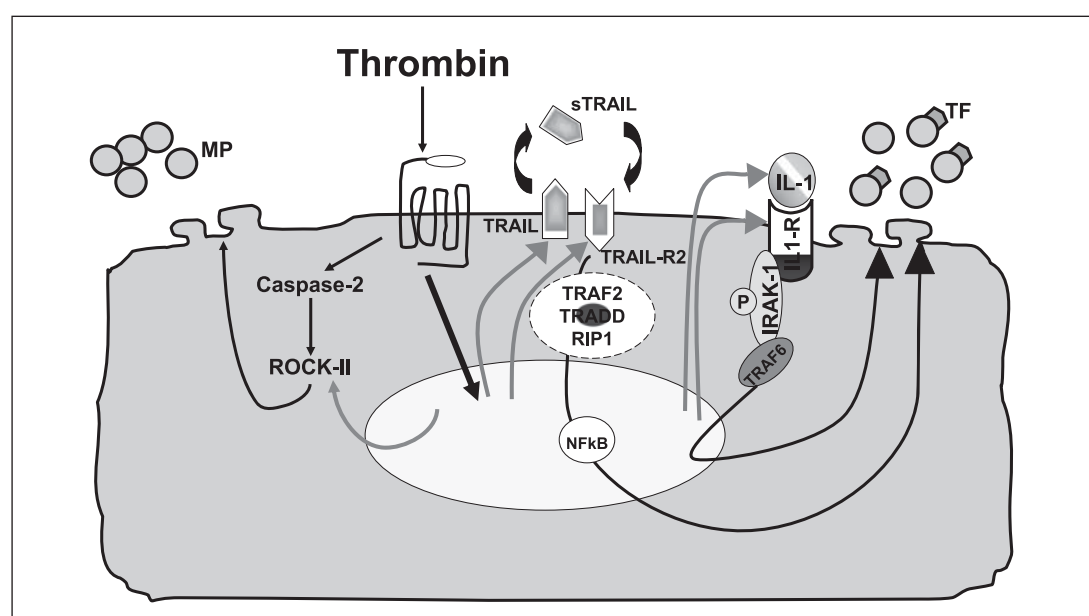


Figure 2: Mechanisms of EMP formation.

Thrombin stimulation of endothelial cells induces a complex release of EMP that requires kinase and NF- κ B activation, and the expression of pro-inflammatory cytokines, which amplifies the endothelial vesiculation.

ture versions of endothelial cells" but also as specific biological entities by their capacity to enrich specific molecules from parent cells and then to display different properties.

Expression of this large repertoire of molecules supports the multifaceted functions of EMP. In this section, we will summarise the proposed functions of EMP, in vascular homeostasis, that derived from *in vitro* and *in vivo* observations (► Fig. 3).

Role of EMP in thrombosis

A direct consequence of PS expression on EMP is that PS can bind to coagulation factors and promote their activation, consistent with a pro-coagulant potential for EMP. In addition to PS exposure, EMP harbour tissue factor (TF), the initiator of the extrinsic coagulation pathway, suggesting that EMP could promote the assembly of clotting enzymes leading to thrombin generation. This capacity of EMP to mediate thrombin generation was first demonstrated by the reduction of the clotting time of normal plasma incubated with increasing amounts of EMP released *in vitro* (5). The pro-coagulant activity of EMP was then confirmed by the demonstration that EMP from activated cells triggered TF-dependent thrombin formation *in vitro* and thrombus formation *in vivo* (42). Moreover, the contribution of endothelial cells to the circulating pool of TF-bearing MP was demonstrated in patients with endotoxaemia (43) and intravascular coagulopathies (27). Pro-coagulant EMP have also been found in atherosclerotic plaques (44, 45) and in patients with acute coronary syndrome (46). Thus, by exposing PS and TF, EMP behave as biological vectors in the dissemination of pro-coagulant potential. However, recent studies demonstrated that EMP can also expose endothelial protein C receptor and exhibit anticoagulant properties (25, 26), suggesting that EMP participate in the tuning of the pro-coagulant / anti-coagulant equilibrium.

Moreover, because of the expression of endothelial adhesive molecules, EMP can bind to other cell types and transfer bioactive molecules such as TF (31), so participating to the dissemination

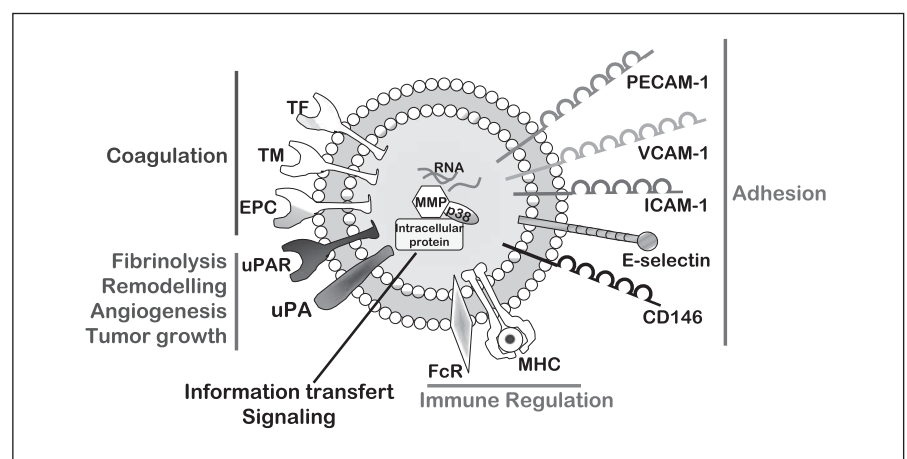
and amplification of pro-inflammatory and pro-coagulant responses. Indeed, it was reported that TF-positive EMP bind to the monocytic cell line THP-1 and then induce monocyte TF expression and pro-coagulant activity (31). A possible TF transfer from TF-bearing EMP to activated platelets could be also involved in this pro-coagulant response, since such a mechanism was reported for TF-exposing leukocyte MP (47). Consequently, EMP can induce pro-coagulant pathways leading to thrombin generation, directly due to their intrinsic TF and PS-dependent pro-coagulant activity, but also indirectly, through dynamic interactions with monocytes able to trigger TF-dependent pathways. As a whole, EMP can amplify the bi-directional relationship between inflammation and thrombosis by conveying cell information.

Involvement of EMP in the tuning of angiogenesis

Besides their pro-coagulant activity, EMP also behave as a surface supporting plasmin generation by expressing the urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (30), which could counteract the thrombin generated by EMP. In this context, EMP support a link between haemostasis and angiogenesis. Indeed, the plasminogen activation system plays a pivotal role in maintaining vascular patency and facilitating cell migration and angiogenesis. This proteolytic potential affects the angiogenic potential of endothelial progenitor cells (30). By conveying plasmin, EMP activate matrix metalloproteases (MMP) which are involved in the extracellular matrix degradation and the release of growth factors that play a crucial role in tissue remodelling, angiogenesis and cancer spreading. Other *in vitro* studies provided evidence that EMP convey angiogenic messages supported by proteases belonging to the MMP family (28) but also by a horizontal transfer of mRNA, able to activate a pro-angiogenic program in endothelial cells (37). These findings were confirmed *in vivo* by a recent study demonstrating that EMP derived from ischaemic muscle promote *in vitro* endothelial proliferation and *in vivo* post natal vasculogenesis (29). However, the involvement of EMP in angiogenic re-

Figure 3: EMP harbour a panel of receptors, enzymes and molecules which confer them a role in coagulation, inflammation, angiogenesis and immune regulation .

TF, tissue factor; TM, thrombomodulin; EPCR, endothelial protein C receptor; uPAR, urokinase plasminogen activator receptor; uPA, urokinase plasminogen activator; FcR, Fc receptor; MHC, major histocompatibility complex; E-selectin, endothelial selectin; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1; MMP, matrix metalloproteases.



ate and perpetuate endothelial dysfunction as well as coagulation dissemination. Consequently, pathophysiological functions attributed to EMP may contribute to the tuning of vascular homeostasis (► Fig. 4).

EMP pathological implications

Increased EMP levels in diseases

Because EMP can compromise vascular homeostasis, they may represent new players in the initiation / amplification of several inflammatory and thrombotic diseases. Circulating EMP are detectable in the plasma of healthy subjects and their amount increase under pathological conditions associated with increased thrombotic risk and endothelial dysfunction. Thus, elevated levels of EMP were reported in acute coronary syndromes (46, 63, 64), acute ischaemic stroke (65, 66), diabetes (67–70), metabolic syndrome (71), severe hypertension with endorgan damage (72–74), thrombotic thrombocytopenic purpura (75), antiphospholipid syndrome (76, 77), lupus anticoagulant (5), vasculitis (78), chronic venous insufficiency (79), venous thromboembolism (80), paroxysmal nocturnal haemoglobinuria (81), all conditions defined by deregulated inflammatory and homeostatic responses.

Relationship between EMP levels and endothelial dysfunction

The release of EMP has also been associated with endothelial dysfunction assessed by abnormal flow mediated dilatation in pathological settings such as end stage renal failure or preeclampsia (51, 82) but also in obese women (83) and multiple sclerosis (84). Accumulating evidence showing pro-coagulant, pro-inflammatory and pro-angiogenic role for EMP indicate that EMP not only constitute an emerging marker of endothelial injury useful to identify patients with vascular risk, but can also be considered as pathogenic vectors. Accordingly, sustained elevated amount of EMP after myocardial infarction or diabetes are indicative of a poor clinical outcome (85). In a recent study, EMP levels were reported to be an independent predictor of the presence of coronary artery disease (69).

EMP as biomarkers of vascular risk

Accumulating data showed that elevated levels of EMP have been detected in several diseases, particularly cardiovascular diseases including acute coronary syndrome (46, 63, 64), stroke (65, 66) and hypertension (73), but also in other vascular settings associated with a thrombotic propensity (86). Elevated EMP are associated with most of the cardiovascular risk factors such as obesity, hyper-

tension, diabetes (87), and appear indicative of a poor clinical outcome. In the future, provided that standardisation of available methodologies could be achieved, EMP measurement will offer new perspectives to assess vascular risk. Indeed, EMP measurement remains a technical challenge due to the lack of standardisation. First, several pre-analytical variables such as blood collection, sample processing, transportation and centrifugation may have a major impact on MP measurement. Second, flow cytometry measurement presents some limitations such as threshold for particle size detection or standardised instrument settings. However, this limit will be overcome with the new generation of flow cytometers. Moreover, the choice of specific antibodies against endothelial antigens is critical for EMP quantification in pathological settings (88, 89). Therefore, developing standardised protocols for EMP measurement is a prerequisite for the full definition of their clinical interest as prognosis markers.

Moreover, another critical point, still debated, is whether EMP can be considered as a cause or a consequence in these diseases. In patients with end-stage renal disease, it was recently suggested (90) that EMP could both reflect and induce endothelial dysfunction. Indeed, EMP could promote vascular dysfunction by decreasing NO production, and thus trigger the disease. On the other hand, patients with end-stage renal failure present low shear stress and increased concentrations of uremic toxins, both participating in endothelial vesiculation.

EMP as new therapeutic targets?

The recognition of EMP as vectors in the transcellular exchange of pathogenic information offers new pharmacological perspectives. Indeed, several therapies beneficial in cardiovascular disorders were reported to reduce circulating MP levels. For example, type 2 diabetic patients treated with the calcium antagonist nifedipine showed a reduced level of platelet-, monocyte- and endothelial-cell derived MP (91). Administration of benidipine, another channel calcium blocker, decreased concentrations of EMP in hypertensive patients with type 2 diabetes (92). Moreover, diabetic patients treated with eicosapentaenoic acid showed a significant decrease of their EMP plasma concentrations (93). In patients with metabolic syndrome, pioglitazone administration diminished EMP levels (94). Intra-vitreal anti-VEGF injection decreased vitreous EMP shed following proliferative diabetic retinopathy (49). Thus, these studies bring new insights into the understanding of the mechanisms of EMP generation and the development of associated diseases.

In conclusion, EMP can be considered as complex structures expressing a large repertoire of endothelial molecules and biological functions that are related to their potential involvement in the tuning of vascular homeostasis. The notion that EMP are conveyors of biological activities with major role in inflammation, thrombosis and angiogenesis is an exciting prospect. However, proof of this concept remains to be fully established *in vivo* using animal models, a critical step to better understand the pathophysi-

ological implications of EMP. Moreover, extending our knowledge on the mechanisms controlling endothelial vesiculation will certainly open novel therapeutic perspectives based on the inhibition of EMP release.

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