REVIEW

New horizons in the analysis of circulating cell-derived microparticles

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Abstract. Analysis of circulating cell-derived microparticles (MP) is becoming more refined and clinically useful. This review, stemming from lectures given at Tokyo late 2003, does not repeat prior reviews but focusses on new horizons. A major theme is the rising recognition of platelets and their MP (PMP) as key mediators of inflammation/immunity. Among the major concepts developed are that (i) many so-called soluble markers of inflammation are in reality MP-bound; (ii) PMP and other MP appear to serve important signaling and immune functions including antigen presentation. In conclusion, MP analysis is poised to enter the mainstream of clinical testing, measuring specific antigens rather than gross levels. However, more research is needed to decisively establish their functions, and international standards are needed to allow comparing results from different laboratories. (Keio J Med 53 (4): 210–230, December 2004)

Key words: platelet microparticles, cell-derived microparticles, inflammation

Scope and Background

Scope

This review is based on Part I of lectures delivered in Tokyo, Japan, Nov. 29 (Part I) and Dec 1 (Part II) 2003, graciously sponsored by Otsuka Pharmaceuticals, Ltd. Part I centered on platelet microparticles (PMP), being an update with new perspectives since our 1999 review[1] while Part II focused on endothelial microparticles (EMP). Since our reivew of EMP is now published,[2] that topic is not emphasized here. Helpful reviews by others, each with distinctive perspective, include those by Nomura[3] and by Freyssinet.[4]

As the title indicates, this article features new horizons or challenges in research and applications of PMP rather than listing established findings covered in prior reviews. Other cell-derived microparticles (MP), notably leukocyte MP (LMP) and erythrocyte MP (RBCMP), are not a focus of this review but many of the same principles apply.

This is a critical review in the sense that it highlights pitfalls in certain methods and assumptions. Because of the rapidly expanding scope of relevant literature, no attempt has been made to exhaustively review all relevant work. The review begins with background to orient readers unfamiliar with MP studies.

What are cell-derived MP?

It is now established that all circulating blood cells, as well as endothelial cells (EC), are capable of releasing membranous fragments (vesicles), of size less than ~1 μm, bearing on their surfaces at least some of the antigenic markers distinctive of the parent cell. Several circumstances occurring in many disease states are known to stimulate their release such as (i) activation or apoptosis induced by numerous agents, (ii) partial or complete lysis such as by complement, (iii) oxidative injury, or (iv) other insults such as high shearing stress.[5] Fig. 1 shows schematically these *modes* of MP generation, as distinct from the detailed *mechanisms* by which they are released, discussed later and here labeled "*black box*" to signify that these mechanisms remain obscure. However, a rise in cytosolic calcium concentration, either from internal stores (dense tubules) or from the plasma, appears to be a necessary triggering event or common pathway for vesicle release.

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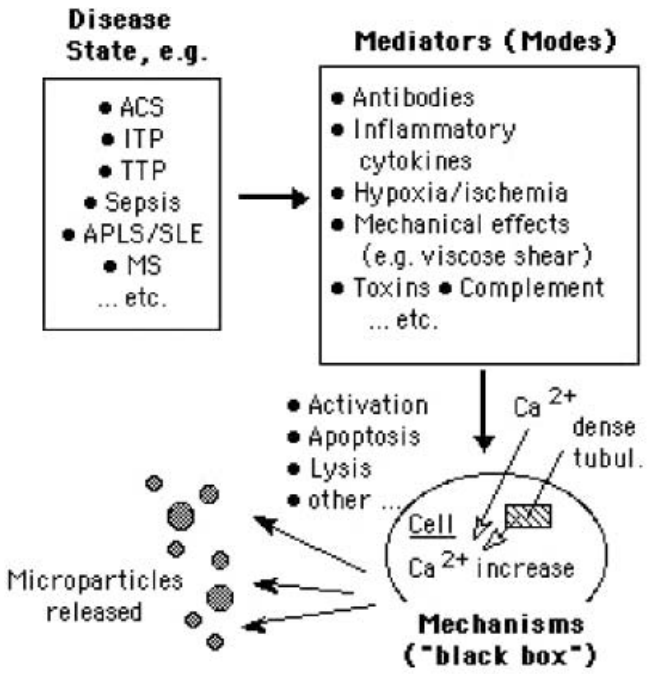

Fig. 1 Modes and mechanisms of cell-derived microparticle (MP) release. A wide variety of disease states can stimulate release of MP by mediators known to include antibodies, complement, cytokines, hypoxia, viscose shear and toxins. The MP phenotype may reflect the mode of insult, such as activation vs. apoptosis. Detailed mechanisms of vesiculation internal to the cell are incompletely understood. Abbreviations: ACS acute coronary syndrome; ITP, TTP immune and thrombotic thrombocytopenic purpura; APLS antiphospholipid syndrome; SLE systemic lupus erythematosus; MS multiple slerosis.

In contrast to other circulating lipoprotein particles (HDL/LDL, chylomicrons, various others), electron micrographic studies from many laboratories reveal that cell-derived MP are quite heterogeneous in density and size, most of the larger ones being true vesicles (hollow with lamellar membrane) as opposed to onion-like. Many of these shapes and dynamics can be replicated in synthetic liposomes.[6] We observed by differential centrifugation and electron microscopy that EMP are also heterogeneous in density, ranging from a protein-rich fraction (electron-dense in osmium staining) sedimenting at low-speed to a protein-poorer fraction sedimenting at high-speed, and all fractions contained heterogeneous sizes (unpublished). This heterogeneity is confirmed in many published electron micrographs of PMP.[7] As discussed later, there may be overlap between cell membrane-derived MP and other MP such as exosomes released by exocytosis from multivesicular bodies (MVB).

How are they measured?

Introduction: PMP and other MP are measured by a variety of methods or combinations of them. Flow cytometric and other methods were detailed in our previous review,[1] and more recently for the case of endothelial MP.[2] A variety of less common but ingenious methods have also been devised, *e.g.* immunoelectrophoresis.[7] Reliable ELISA methods have been developed, particularly by S. Normua *et al.*,[8,9] recently extended to LMP,[10] and by others.[11] Flow cytometry is inherantly quantitative but ELISA is not, therefore several authors quantitate by prothrombinase activity of the particles on the plate;[12] however, this method is dubious for reasons below and results cannot be directly compared to flow cytometry measurements. Measurement of total phosphate to establish phospholipid (PL) concentration has often been used,[1,13] being reliable but difficult to compare with flow cytometric counts. A forum on methods by six leading laboratories is currently in press in Journal of Thrombosis and Haemostasis (October issue).

Pitfalls: (1) Annexin V/prothrombinase. In this increasingly popular method,[14–21] the primary means of detection or capture of MP depends on their binding annexin V (AnV) in the presence of calcium; secondary identification of MP types (PMP, EMP, LMP) is by ELISA of cell-specific antigens; and quantitation is often by prothrombinase activity (PF3 activity). The most serious objection to this method is that we have demonstrated, at least for EMP, that only a fraction of all MP are positive for AnV binding.[2] Specifically, EMP from activated endothelial cells (EC) were rarely positive by AnV binding, since 35-fold more were counted by anti-CD62E in the same samples; and even EMP from apoptotic cells, though much richer in AnV binding, gave only half as many positives by AnV as by CD31. Since AnV binding measures mainly the anionic PL, phosphatidyl serine (PS), which is largely responsible for prothrombinase activity, that method of quantitation will also give misleading results. In sum, therefore, the criterion of AnV binding and quantitation by prothrombinase selectively measures only a subset of MP, potentially giving grossly misleading results. Similar objections may apply to measurement of PMP by this method. Likewise, in quantitating RBC MP by acetyl cholinesterase activity,[22] there is no assurance that all RBC MP have this activity. (2) Centrifugation. Nearly all MP studies rely on preliminary centrifugations to remove red cells and whole platelets, giving platelet-poor plasma (PPP) in which the MP are measured. However, the centrifugation conditions are not standardized, potentially giving rise to grossly discordant results. For example, one study of MP in preeclampsia[21] reported results very different from findings from our lab,[23] probably because the former authors prepared PPP by centrifuging 2 minutes at 13,000× g, which we find sediments ~80% of all MP

detectable in flow cytometry. On the other hand, no amount of centrifugation is sufficient to sediment 100% of MP because, as noted above, some MP are no more dense than plasma.

The challenge of measuring total MP is therefore unmet. We have attempted lipophilic fluorescent dyes but found that they tend to form micelles, giving false counts even in the absence of MP. More promising has been the use of lectins such as FITC-labeled *Ulex europaeus* since this gives by far the higest counts with pure cell-derived EMP and PMP; however, it may bind to other plasma constituants making it unsuitable for *in vivo* applications. Other lectins such as *Bandeiraea simplicifolia* (a.k.a. isolectin B4, or *Griffonia simplicifolia*) may offer superior specificity for EMP but have not been tried.

A promising alternative would be density gradient centrifugation as used for separation of HDL/LDL fractions but that is suitable only for research purposes, being time- and labor-intensive and requiring expensive equipment. (3) Blood collection. Often under-appreciated is the importance of blood collection in MP studies. Platelet activation readily occurs *ex vivo* with release of artifactual MP, and some anticoagulants are known to affect antigens important for measurement, *e.g.* EDTA extracts calcium required for the structural integrity of GP IIb/IIIa,[24] making it invisible to fluorescent antibodies often used to identify PMP, and induces activation as defined by CD62P.[25] Even citrate, commonly preferred for platelet studies, differs significantly in effects on platelets between different manufacturers and tubes.[26] Ahnadi *et al.* recently compared effects of anticoagulants on platelet activation, finding significant differences.[27] Some workers place the blood on ice in the belief that this will be protective,[25] but in fact chilling activates platelets, causing MP release and loss of GP 1b (or glycocalicin).[1,28–30]

Furthermore, since many or most platelet membrane glyocoproteins contain disulfide links which are functionally important, lessons learned from preservation of homocysteine in sample handling[31,32] should apply to platelet/PMP studies. The -SH/-S-S- redox state is normally governed mainly by glutathione which, as might be expected, is associated with homocysteine[33] and presumably the thiol redox state of platelets as well. Redox state of platelet GPIIb/IIIa has been investigated.[34] The thiol protease calpain appears critical to a variety of platelet functions including PMP production,[35,36] more recently concerning conversion to inflammatory phenotype with production of PMP and PAF.[37] Anti-oxidants inhibit P-selectin expression[38,39] and platelets normally possess an array of anti-oxidants,[40] but platelet/PMP redox state in relation to various functions, such as tissue factor (TF) activity and MP shedding, has not been much investigated.

Need for standardization For reasons below, it is likely that MP analysis will soon enter the mainstream of clinical testing. Therefore, in view of the profusion of idiosyncratic methods now in use, few of which can be quantitatively compared to others, there is a pressing need to hammer out reference standard methods. Flow cytometry is probably the reference method of choice but agreement must be reached on instrument settings, sample handling, and standard markers for basic quantitation, *e.g.* CD144 for EMP, CD42b for PMP, glycophorin for RBC MP, CD45 for LMP, each of defined target epitope (clone) and fluoresence. There is, however, ample room for debate on preferred markers. For example, GPIbα (CD42b) is shed from platelets during vesiculation[41] but as a marker of PMP in ITP[42] gives counts different from CD41 (unpublished). CD61 (GPIIIb, integrin β3) has been suggested as a superior marker of platelets,[43] as might be GP VI[44] (CD49, collagen R), or certain lysosomal markers, *e.g.* CD68-positive MP were studied in ITP[45] or CD63. Since flow cytometry is unavailable in many laboratories, ELISA methods referenced above are often used but improved quantitation is needed, as on the model of quantitative titers now standard in measuring anticardiolipin antibodies.[46,47]

Why measure them? Clinical applications

At the present time, the most solidly established function of MP is their procoagulant activity (PCA) as reviewed[1] but even this is not yet proven to be a critical determinant of thrombosis, the evidence being largely circumstantial (associational), and as noted above, not all MP are procoagulant. A major purpose of this review is to indicate potentially vital functions of MP not yet widely appreciated.

To date, the main clinical application of MP analysis has been the simple correlation of their levels or quantities with various disease states, as amply referenced to 1999[1] and still continuing.[48] That is, they serve as general indicators of cell injury, stress, thrombosis, or inflammation. More specifically, dating from about 1990, PMP levels have been viewed as a marker of platelet activation.[49]

However, if this is their only significance then one may doubt the importance of measuring them since easier methods are available, such as CD62P expression on platelets,[50–52] detection of actived GPIIb/IIIa by the mAb PAC-1,[53] free thromboxane,[54–56] or circulating markers of inflammation.[57,58]

On the other hand, recent developments to be reviewed sugest that MP assay can supply crucial and specific information. In the first place, it now appears that many so-called soluble mediators/markers of inflammation are in reality MP-bound (next section). In

the second place, we have observed that PMP levels do not necessarily correlate with CD62P on platelets. There may exist distinct subtypes (phenotypes) of PMP, as has been shown for EMP.[59,60] For example, we observed clear correlation of PMP levels with absence of bleeding in ITP when PMP were measured by CD42b (GP Ib)[42] but not when measured by CD41 (GP IIb) (unpublished).

For these and other reasons below, it is our working hypothesis that PMP and other MP perform important signalling functions not yet appreciated, and that when such properties are better understood, assay of specific markers on PMP and other MP, rather than gross quantity alone, will enable improved diagnostics and insights to underlying pathophysiologies.

Microparticles vs. soluble markers

As a final but important introductory topic, it is now clear that many circulating markers of inflammation regarded as soluble are in reality bound to MP, at least in part. This was defended in some detail in our recent review.[2] For example, PECAM-1 (CD31) is widely accepted as a soluble marker[61] but we have routinely used it to assay PMP and EMP by flow cytometry.[23,62–64] Similarly, E-selectin is widely measured and regarded as a soluble marker of endothelial stress[65–68] but we routinely use it to identify endothelial MP (EMP),[59,60] demonstrating that it is actually MP-bound, at least in part, because flow cytometry cannot detect true soluble molecules. ELISA methods do not distinguish true soluble forms from MP-bound. Furthermore, filtration of plasma through 0.1 um often eliminates some or nearly all of such agents from flow cytometric detection.

Similar considerations apply to many other markers now regarded as soluble including ICAM-1, VCAM-1, P-selectin, tissue factor (TF), von Willebrand factor (vWF; partly bound to EMP[69] and PMP), thrombomodulin,[70] and CD40L.[71] It is well established that some of these do exist in true soluble form, usually due to enzymatic cleavage from the membrane or by post-translational editing,[72] but it is equally well established, by our lab and others, that a significant fraction, up to 80–90%, of some of them occur on cell-derived MP, presumably with their transmembrane domains intact and normally adjacent proteins present. (Fig. 2)

The practical importance of this lies in the fact that release of true soluble species occurs by mechanisms entirely different from membrane vesiculation, hence reflect different pathophysiologies. Secondly, true soluble species often have properties functionally different from their MP-bound forms (as we have shown for vWF[69]). In view of these considerations, it is expected that when the MP-bound markers are clearly distinguished by independent measurement from the true soluble species, much clearer relations will emerge between disease states and the marker in question. In sum, what are now reported as soluble species are in many cases actually reports of MP. Thus, to the extent that such so-called soluble markers are recognized as valuable clinical tools, MP analysis deserves at least equal recognition.

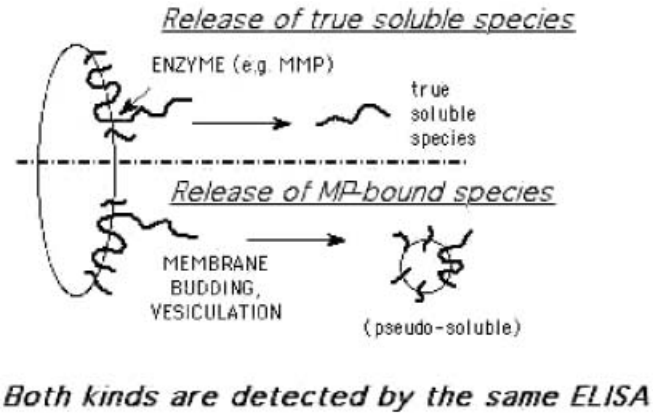

Fig. 2 True soluble proteins arise from mechanisms distinct from their MP-bound form. Immunologic methods such as ELISA will not distinguish true soluble proteins from their MP-bound forms (such as CD31 a.k.a. PECAM-1, or CD62E a.k.a. E-selectin), yet they reflect different pathophysiologies and may be functionally different. Soluble forms most often arise from proteolysis such as by matrix metalloproteases (MMP's), or by alternative splicing without transmembrane domains; whereas MP-bound forms arise by membrane vesiculation.

New Horizons for PMP

Introduction

Two main developments since our 1999 review are responsible for the new horizons in PMP analysis, (i) increasing recognition of platelets as mediators of inflammation and immunity, and (ii) discovery of platelet-associated tissue factor (TF). Table 1 roughly classifies some platelet-derived agents of interest, many of them recently discovered, others long-known but with newly recognized roles. This table is by no means comprehensive; others are listed in other sources[4,73] or are common knowledge; see any textbook.

Although many of the agents on Table 1 have already been identified on PMP, others have not. However, insofar as PMP are fragments of the cell membrane, we adopt the working hypothesis that many if not all platelet membrane-associated agents occur also on PMP, until shown otherwise. In some cases there is reason to believe that PMP are the sole or main functional vectors of those agents.

Table 1 The New Platelet: Some Agents of Recent Interest

I. Mediators of Inflammation/Immunity	
In general	73, 140, 141, 213, 214
RANTES, MIP-1, GRO-a, βTG, others	164–166, 215
IL-1	160, 161, 163; on LMP 162
CD40, CD40L	175, 176; on PMP 71
VEGF	216
BAFF	184
Fractalkine	178
Fas (on MP)	217
PAF (on PMP)	142
CCR1, 3, 4	179
CXCR4	166, 182; PMP 181
CX3CR1 (& pertussis tox.)	178, 218
Receptor heat-shock protein	208
Thrombospondin	129
Platelet factor 4 (PF4)	129
β-thromboglobulin	129
sPLA2 (PMP as subtrate)	13, 172
Nitric oxide synthase	219
MMP 1, 2, 9	referenced in 186
II. Coagulation/Clotting	
Tissue factor (TF)	87, 88; on PMP 89–91, 220, 221; generic or other MP 222, 223
TF pathway inhibitor (TFPI)	224
β2GP1 (on PMP)	94, 95, 98
Thrombomodulin (on LMP)	70
von Willebrand factor (on EMP)	60, 69
Factor V/Va	76
PAI-1	225, 226
III. Pro- and Anti-Angiogenic (see text for more):	
VEGF	216
Angiostatin	186
Endostatin	187
IV. Other Receptors	
For estrogen, androgen	227
For leptin?	228
For shiga toxin?	229
For erythropoietin (EPO)	82
For LDL?	230
For insulin?	219, 231
insulin R & NO synthase (NOS):	219, 231
For vasopressin (& DDAVP?)	169, 170, 232
β2 integrin (as in CD11a,b,c, CD18)	233
V. Miscellaneous	
Anti-oxidants	40
Amyloid precursor protein (APP)	234
Sialyl Lewis x (CD15s)	235
JAM proteins	236–238
CD59 (C inhibitor)	239, 240

Where do all these agents come from? On the one hand, platelets are notorious pack-rats, endocytosing numerous plasma proteins; for example, IgG/IgM, even virus. Much debate has been given to the source of platelet tissue factor (TF), possibly from leukocytes;[74] and on the source of platelet-derived coagulation factor V (originally called platelet factor 1),[75–78] which also occurs on PMP.[36,79,80]

On the other hand, an unexpectedly large variety of proteins are native to platelets and megakaryocytes,[81] as recently underscored by mRNA studies in which some 232 proteins additional to those known were identified[82] (TF not among them), unexpected because platelets are so tiny and lack nucleii. Similarly, proteomic approaches to platelet signalling proteins[83] and secreted soluble proteins[84] have yielded lengthy new lists. It would be most interesting to see the proteomic approach applied to PMP and other MP.

PMP in coagulation and thrombosis

As previously reviewed, the best studied role of PMP has been their procoagulant activity (PCA) by virtue of providing a suitably anionic phospholipid surface for assembly of the tenase and prothrombinase complexes, known as platelet factor 3 (PF3) activity.[1,85] PMP also carry FV/Va (cited above) from platelets.[86] However, these procoagulant properties are passive, meaning that PMP could support but not themselves initiate coagulation.

That view changed dramatically with the discovery of platelet-associated tissue factor (TF).[87,88] TF antigen and activity also occur on PMP in significant amounts.[89–91] It was previously shown that PMP play a special role in engendering TF activity of monocytes[92,93] and other cells[71] but the recent finding that TF is intrinsic to platelets and PMP was a surprise in view of the long history of studies of platelets and TF. (We observed TF activity of PMP but dismissed it as an artifact, not suspecting that such a key property could have been overlooked for so long.) Thus, PMP are now known to be capable of initiating coagulation as well as passively supporting it. These two procoagulant activities of PMP, passive and active, are qualitatively distinct.

Other modulators of coagulation or clotting have also been identified on PMP, for example, β2GP-1,[94,95] closely associated with anti-phospholipid antibodies (APLA) in APS.[96,97] It exhibits calcium-dependent membrane binding despite absence of gla domain.[98] Theories purporting to explain the correlation between anti-β2GP-1 with APS have been proposed in terms of the protein C system;[99–101] see next section. We have shown that EMP carry significant vWF,[60] that PMP also carry it, and that EMP-vWF is functionally more active than free soluble (manuscript submitted).

PMP and the protein C anticoagulant system

As stated in our earlier review,[1] Tans *et al.* proposed that PMP could perform an anti-coagulant rather than

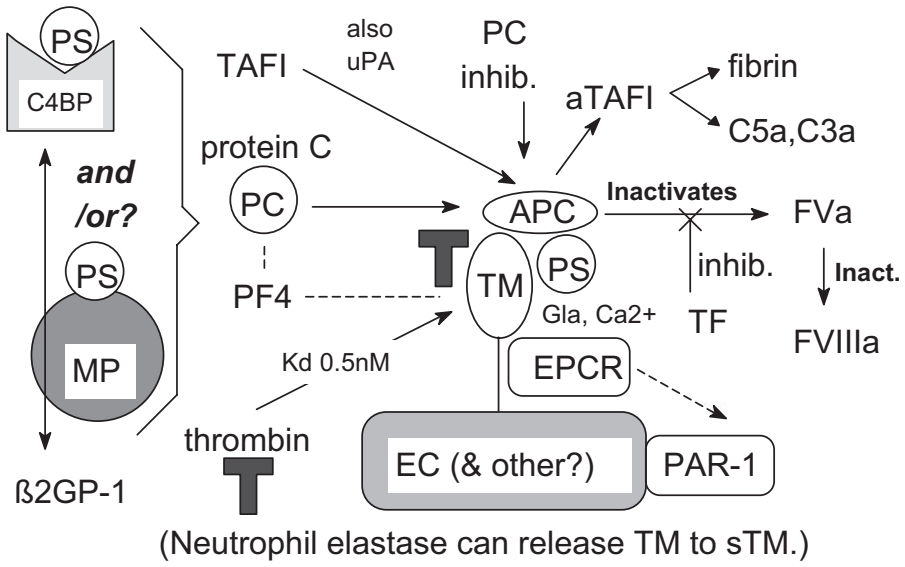

Fig. 3 Potential sites of interaction of cell-derived MP with the protein C anticoagulant system. This diagram, adapted from several sources, illustrates the still-growing complexity of the protein C system, critically involved in many thrombotic pathologies, probably including antiphospholipid syndrome as well as normal hemostasis. Although not yet proved, there is evidence that MP may significantly modulate several of the interactions shown, including EMP as site of assembly of the central complex, as discussed in text. Abbreviatons: PC protein C; APC activated protein C; PS protein S; MP cell-derived microparticle; TM thrombomodulin; C4BP complement C4 binding protein; EPCR endothelial protein C receptor.

procoagulant function in some circumstances by supporting the protein C system.[102] More recently, a similar argument has been advanced by Berckmans *et al.* in their discussion.[103] Confirming other reports, the latter find that circulating MP in normal controls support low-grade thrombin generation, but by the contact pathway, not TF (however, new insights on the contact pathway raise doubts about its supposed function[104]). They argue that low-levels of thrombin are almost entirely bound to thrombomodulin (TM), leading to activation of protein C and thus inactivation of FVa and FVIIIa; hence MP-dependent thrombin generation serves an anticoagulant function in normal hemostasis.[103]

A cartoon of the main players in the protein C/S system are shown in Fig. 3, based on several sources,[105] the intention being to indicate likely roles for MP in this system additional to that mentioned above, suspected because most of the key steps, like those of coagulation, occur at membrane surfaces. This system is obviously extremely complex,[106] well reviewed elsewhere[107–109] and no attempt is made to describe it here. (TAFI in the figure, thrombin activable fibrinolysis inhibitor, is now more properly called carboxypeptidase U.[110])

Gris *et al.* observed that a substantial but variable fraction of protein S is MP-associated and that clinical assays using PEG precipitation for free *vs.* C4bp-bound underestimate it because much of it is precipitated by PEG with MP.[111] Incidentally, this suggests that PEG might be exploited as a method of MP isolation. The MP carrying the protein S was not identified. For recent review of protein S.[112] Recently, much interest has focused on the endothelial protein C receptor (EPCR).[113] A true soluble form (sEPCR) can arise by MMP proteolysis induced by inflammation, as cited in;[114] but in a recent study,[115] a thrombophilic genotype (A3 haplotype) with elevated sEPCR associated with thrombosis showed no evidence of lacking the transmembrane domain, suggesting that the authors actually measured MP-bound EPCR, probably on EMP. Similarly, a true soluble form of thrombomodulin (TM) is released by neutrophil elastase,[105] yet TM has been detected on leukocyte MP;[70] since endothelium is the main site of TM, it may be expected also on EMP. (Neutrophil elastase also acts directly on FV/Va.[116]) Circulating TM was elevated in TTP in correlation with vWF[117] but we have recently found that vWF is in part associated with EMP[69] (manuscript submitted).

Another point of involvement of MP with the protein C system is *via* inhibition of activated protein C by MP-associated tissue factor (TF),[118] likely to also involve TF pathway inhibitor (TFPI). Platelet factor 4 (PF4) is another known participant involved in regulation of clotting through protein C[119] and occurs on PMP. As referenced above, β2GP-1 is rich on PMP and is known to affect the protein C system, predisposing to thrombosis and APS. It is difficult, of course, to sort out the many influences on this system *in vivo*, owing to its extreme complexity, only partly shown; for exam-

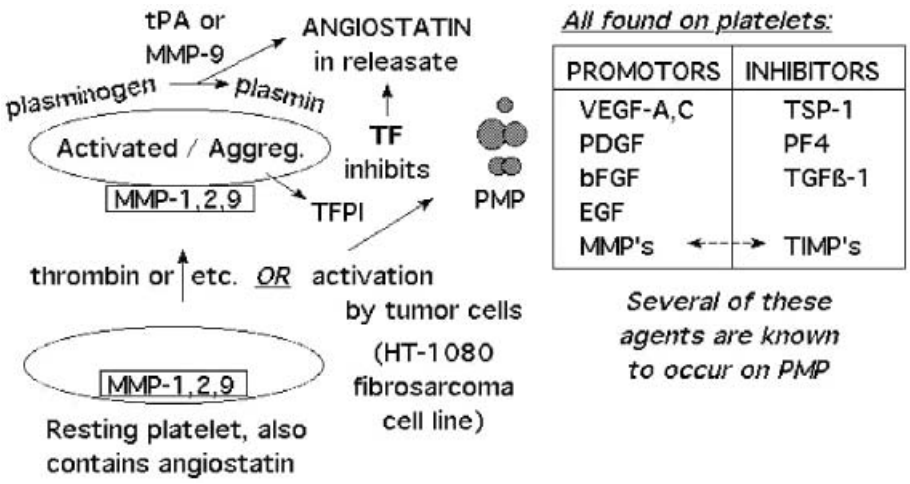

Fig. 4 Potential roles of platelet microparticles (PMP) in angiogenesis. This figure, adapted from several sources (especially ref's 186–188), illustrates another of the ''new horizons'' of MP studies, since all the promotors and inhibitors of angiogensis shown on the inset table are found on platelets, and several have been identified on PMP. It is likely that all membrane-asociated species on platelets also occur also on PMP (working hypothesis). See 3.3 of text. Abbreviations: MMP matrix metalloprotease (numbers 1, 2, 9 identified on platelets); TIMP tissue inhibitors of MMP; TF tissue factor (disseminated on microparticles); TFPI is TF pathway inhibitor; tPA tissue-type plasminogen activator; TSP thrombospondin; PF4 platelet factor 4; VEGF vascular endothelial growth factor; PDGF platelet derived growth factor; bFGF basic fibroblast growth factor; EGF endothelial growth factor; TGF transforming growth factor.

ple, not shown in Fig. 4 are the actions of PAI-1 and vitronectin.[120]

Nonetheless, in summary, there are numerous points at which PMP or other cell-derived MP are known or plausibly suspected to affect the protein C system, either positively or negatively. Further study is clearly needed and would certainly be warranted in view of the pivotal importance of this system to hemostasis, thrombosis, and inflammation. For example, activated protein C is the only therapy thus far shown to be effective in sepsis;[121–124] PMP in sepsis has been studied[16,125–127] including by our lab[128] (full manuscript submitted).

The New Platelet: Beyond Thrombosis

Introduction

The recent discovery of many additional bioactive agents on/in platelets and PMP such as those listed in Table 1 extend the likely role of PMP beyond thrombosis.

It should be emphasized that not all agents known or suspected to occur on PMP (or other MP) necessarily occur on all PMP, as it is now emerging that cell-derived MP can occur in multiple phenotypic species, that is, bearing certain restricted sets of proteins; and that the distribution of phenotypes can vary with the stimulus eliciting their release. For example, Fox noted different protein compositions on PMP elicted by dibucain *vs.* calcium ionophore A23187[129] and we have shown different phenotypic sets of EMP depending on whether the endothelial cells (EC) were activated or apoptotic.[59] Likewise, it was remarked above that PMP measured by two different markers (CD41, CD42) did not both correlate with disease activity, implying distinctive subsets.

It is relevant to add that platelets themselves appear to comprise multiple species. Examples other than those discussed in our prior PMP review[1,130–133] include differencces in electrophoretic mobility;[134] platelet age subpopulations by lectin affinity[135] (others have used bouyancy); uptake of thiazole orange;[136] and distinct subpopulations in chronic myeloid leuekemia (CML)[137] and other cancer hemostatic abnormalities.[138] We have shown distinctive electrophoretic mobility of platelets from patients with recurring TIA.[139] It may be expected that PMP would reflect such subpopulations in one or more differentiating markers or functional properties.

PMP in inflammation

Several roles of platelets in inflammation were well reviewed as of 1994 under editorship of M. Joseph, much of it summarized in Ch. 2 of that book,[140] but were not widely appreciated until recently.[73,141] We suggest that many of those roles may actually be served by PMP, to greater or lesser extent. Thumbnail sketches of some of the mediating agents follows.

Platelet activating factor (PAF) is carried mainly on

PMP[142] and is known to modulate leukocyte adhesion to endothelium,[142] to contribute to endotoxin-induced injury,[143,144] organ transplant injury,[145] and may be involved in some thrombocytopenias.[146] PAF can be significant in picomolar amounts[147] and may have special importance in stroke and brain injury.[148] It is responsible for the release of vWF in response to DDAVP therapy; see below on vasopressin.

Platelet factor 4 (PF4) has been known since the early days and has been shown to occur on PMP[129] but has received new attention lately because of a suspected role in atherosclerosis,[149] perhaps through its contribution to priming neutrophils *via* GM-CSF;[150,151] and because it has been identified as a key antigen in heparin-induced thrombocytopenia (HIT) and HIT with thrombosis (HITT).[52,152] For some updated references on PF4 and other inflammatory mediators from platelets, see Anitua.[73] When secreted upon activation of platelets, it localizes to the plasma membrane, at least in part,[153] as do other granule proteins identified on PMP (P selectin, thrombospondin,[154,155] CD63,[156] *etc.*), supporting our working assumption that many if not all platelet membrane-associated molecules occur also on PMP, until proven otherwise. Among many putative roles, PF4 is involved in regulation of clotting through protein C[119] (Fig. 3).

Thrombospondin (TSP) also has long been known on PMP[129,157] and has diverse activities including modulating vWF activity,[158] complement activity (it copurifies with factor H),[159] and angiogenesis. Interleukin 1 (IL-1) has been known for some 15 years to be present in platelets[160,161] and has been identified on monocyte MP,[162] hence likely occurs also on PMP. mRNA shows it is constitituve, not acquired from plasma.[163]

RANTES, a potent chemotactic factor for eosinophils from platelets,[164] has been immuno-histochemically localized to the α-granules, to the open canalicular system (OCS), and to the plasma membrane, as was MIP-1,[165] leading to the expectation that they are also present on PMP. Aside from their chemotaxis for eosinophils and basophils, both mediate release of histamine and thus are true inflammatory mediators. Three such mediators (PF4, RANTES, β-thromboglobulin) were shown to be slowly but significantly released from stored platelet concentrates and may contribute to nonhemolytic transfusion reactions;[166] these are probably on PMP, at least partly.

The platelet receptor(s) for vasopressin is of special interest to us because we showed that DDAVP (desmopressin, a vasopressin analog), now widely used as an anti-hemorrhagic drug, can act directly on platelet to induce a rise in cytosolic calcium and release of PMP,[167] contraray to two prior reports.[168,169] This work was overlooked by two subsequent reviews of DDAVP and by a recent paper on DDAVP action on platelets,[170] possibly because our results were not believed in view of the prior negative reports using supposedly more sensitive calcium reporters. In fact, the dye we used, CTC, is more sensitive, not less, in the vicinity of membranes such as dense tubules because it is lipophilic.[171] Although the effects shown *in vitro* were modest, *in vivo* DDAVP could act synergistically with PAF released from leukocytes in response to DDAVP, since this is believed to be the mechanism by which DDAVP elicits release of vWF from the vascular endothelium.[167]

Secretory phospholipase A2 (sPLA2), like C-reactive protein (CRP), is an acute phase reactant whose plasma levels increase up to 1000-fold in infections (inflammation). It is constitutively present in platelets and probably on PMP since it has been shown to act on PMP (but not on intact platelets) to produce lysophosphatidic acid (LPA), of diverse activities including platelet aggregating, which may be promoted by other products of sPLA2 activity such as arachadonic acid.[13] sPLA2 became tightly bound to added albumin, degraded phosphatidyl serine (PS), and was suggested to participate in ceramide production in response to other inflammatory mediators. The observed effects were augmented by sphingomyelinase, the beta toxin of *S. aureus*.[13] More recently, Billy *et al.*, adopting the view that sPLA2 is mainly anticoagulant, emphasized the role of sPLA2 in degrading phosphatidyl serine (PS) to abolish the prothrombinase activity of MP; this happened unexpectedly rapidly, *i.e.* only a small degree of PL membrane hydrolysis abolished support of coagulation.[172] In summary, the activities of human sPLA2 (as distinct from that from bee or cobra venoms), whether anti- or pro-thrombotic, are not yet entirely clear but it does seem clear that PMP are likely to be key participants in its actions.

Further stimulating interest in platelets/PMP as inflammatory/immune mediators are recent reports on CD40/CD40L[173–177] making it a hot topic; it has been identified on PMP *in vitro*[71] and *in vivo*, being another example of a supposedly soluble molecule which is in fact MP-bound, at least in part.

Chemokine receptor CX_3CR1 for fractalkine (and for pertussis toxin) has been identified on platelets,[178] as have other chemokine receptors.[179,180] The most abundant of these, CXCR4, was readily identified in PMP by its bright signal and may play a role in cancer metastasis[181] and juvenile ITP.[182] CXCR4 (and CD4) can be transferred by PMP to other cells, rendering them susceptible to HIV infection;[181] related observations were earlier made for MP-mediated transfer of CCR5.[183]

The recent identification of the above, and BAFF,[184] fractalkine[178] and other such mediators (Table 1) on platelets, underscores the role of platelets as

major inflammatory mediators. PMP are already known to carry several of these and may be their major vectors, particularly in the microenvironment of the site of inflammation where platelet activation occurs, with concommitant release of PMP at locally high concentrations.

PMP in cancer: *mediators of angiogenesis & metastasis*

Multiple lines of evidence suggest that PMP play a unique and critical role in cancers. A recent ASH abstract reported that PMP (including platelet exosomes) markedly stimulated metastatic potential of 5 cancer cell lines, as judged by at least four measures, and related effects were confirmed *in vivo*.[185] Anitua *et al.* classified many platelet-derived substances as pro- and anti-angiogenic.[73] Our Fig. 4 summarizes these and related properties, based mainly on Jurasz *et al.*[186] Of notable interest is the presence of matrix metalloproteases (MMP) 1, 2 and 9 in platelets, which become surface-expressed in response to activation, along with their TIMP inhibitors, and angiostatin, released in tandem with plasmin by the action of tissue plasminogen activator (tPA) or MMP-9. Platelets also express and release endostatin,[187] said to be a fragment of plasminogen.[188] Platelet activation can be caused by thrombin (which itself possesses angiogenic potential[189]) or by tumor cells and results in PMP known to carry at least some of the angiogenic mediators listed, *e.g.* VEGF and TF;[71] TF is also an angiogenic mediator.[190] Not shown in the figure is thrombospondin, another of the many platelet-derived regulators of angiogenesis[191] known to be vectored on PMP.

Consequently, if as proposed by Folkman and Kalluri[192] and similarly by Jurasz,[186] the difference between common and harmless *in situ* tumors and their progression to lethality hinges on the balance between pro- and anti-angiogenic factors in circulation, then MP may well be pivotal in this balance insofar as they are known vectors of several of these factors. MP from tumor cells have been shown to promote angiogenesis,[193] presumably via VEGF and other MP-associated angiogenic factors.[194] Oncogenes can be transferred *via* that species of MP known as apoptotic bodies.[195]

Exosomes: *platelets as antigen-presenting cells* (*APC*)

An exciting recent development in immunology has been the recognition that certain classes of microparticles, usually collectively called *exosomes*, perform crucial antigen-presenting and other cross-talk functions. A cartoon conveying some concepts and key experiments is shown in Fig. 5, based mainly on Wolfers *et al.*[196] and sources cited there,[197,198] with helpful commentary on their intracellular sources, known as multivesicular bodies (MVB)[199] (pg 625). They are

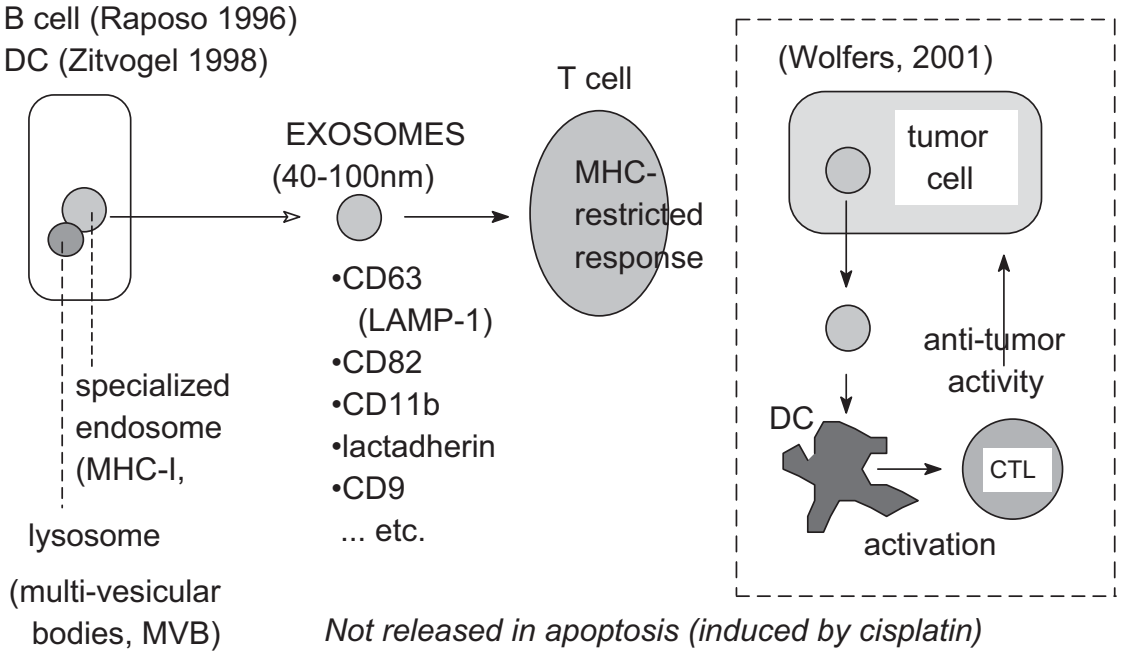

Fig. 5 Exosomes as a variety of cell-derived MP. This figure sketches some functional activities of exosomes, which may overlap in both detection and function with plasma membrane-derived MP such as PMP. It is adapted from text of ref's 196–198. Exosomes are expelled from multi-vesicular bodies associated with a specialized endosome and function to present antigens. They are best characterized from B cells and dendritic cells but are now recognized also in platelets. See 3.4 of text. Abbreviations: DC dendritic cell; MHC major histocompatability complex; CTL cytotoxic lymphocyte; MVB multivesicular bodies.

relevant here because it is now known that exosomes are released from platelets as well as leukocytes,[200,201] and therefore constitute a species of PMP.

Although said to be somewhat smaller at 40–80 nm[201] or 60–90 nm[196] than PMP normally detected in flow cytometry (~100 μm), there is some overlap. For example, in one platelet study, the isolated size fraction > 100 nm of putative exosomes exhibited the same key marker CD81 (a.k.a. TAPA-1) as the smaller high-speed fraction[202] but that large size fraction was not further investigated. Both fractions exhibited lysosomal marker CD63 (LIMP or LAMP-1), one of the markers often used to identify endosomes but also a well-known surface marker of platelet activation found on PMP.[49,156,203–205] Perhaps relatedly, if lysosomal markers are signatures of exosomes, Nomura *et al.* found MP positive for CD68, a lysosomal marker, in ITP at levels higher than controls, $p < 0.01$.[95] In view of the size overlap, and since they derive from platelet MVB or alpha-granules, the latter known to mirror the platelet surface,[155] it appears that at least some platelet exosomes are detected along with PMP in normal flow cytometry; and likewise for exosomes from other cells and their respective surface membrane MP since their granule proteins, too, often mirror the identifying proteins of the plasma membrane.[206] Indeed, direct evidence that PMP may function in this way has been reported.[207] If a research-grade flow cytometer is available with high-power laser, it should be possible to specifically detect and measure exosomes more easily than by conventional tedious ultracentrifuge methods of isolation.

The immunologic role of exosomes is probably related to other agents recently identified on platelets such as the receptors for heat shock proteins (HSP), now known to exert pleiotropic effects including activation of dendritic cells. Thus in the report by Hilf *et al.*,[208] the HSP called Gp96, which activates dendritic cells (DC) to induce release of pro-inflammatory cytokines, is shown to bind to thrombin-activated platelets 10-fold increased relative to resting platelets, attenuating its effects, as in wounds or trauma, and reducing potential autoimmune responses.

Such work has been widely extended, as in work on the EAE mouse model of MS employing MBP as antigen,[209] of special interest to our lab because of our long interest in microparticles in MS, initially PMP[210] but more recently EMP.[63,11,212]

Possibly Related or Unidentified MP

One often encounters mention of microparticles (MP) in the literature without clear attribution of their kind or source. For example, we earlier mentioned the finding by Gris *et al.* of MP associated with protein S but the MP remain to be identified. In this article we cite other such cases warranting further investigation, particularly as they imply new functions of MP.

MP bearing mRNA

Circulating mRNA has been of interest in cancer research, but is highly unstable when free in plasma, thereby raising the question of its persistence, answered by finding it is protected against degradation by association with MP.[241,242] It had earlier been shown that mRNA on so-called apoptotic bodies is protected.[243] The source and type of MP in question was not fully addressed. This observation raises the possibility that a general function of MP may be to protect agents caried on them against degradation. We have postulated that vWF associated with EMP is thereby protected against proteolytic degradation, maintaining it in active high-multimer form[69] (full manuscript submitted).

Filtration affects von Willebrand factor (vWF) assay

vWF was shown to be depleted in some patients as a result of filtration of the plasma, causing errors in clinical hematology tests.[244] This suggests that vWF (and possibly other factors affected in that reference) are MP-associated, to a degree varying with individual patients. We have directly demonstrated that vWF can occur bound to EMP and in that form is partly filterable;[69] and that significant vWF associates also with PMP (by electrophoresis and Western blot; unpublished). Of related interest, the same authors (Favaloro *et al.*) have shown that storage of blood on ice can also lead to errors in tests for vWD;[245] insofar as cold is known to induce platelet activation and PMP formation, one may conjecture that cold-generated PMP are binding platelet vWF in some patients, explaining the errors through loss of vWF with PMP in centrifugation.

Nucleosomes, etc

Jiang *et al.* noted an abundance of so-called nucleosomes in patients with SLE, and in that paper[246] reports measuring them after administering necrotic or apoptotic Jurkat cells to mice. This term may be equivalent to the poorly defined MP called apoptotic bodies released from apoptotic cells, but may be equivalent to what we term EMP from apoptotic EC, which are distinct from EMP released from activated EC.[59] They have been shown to be active in the horizontal transfer of oncogenes.[195] Incidentally, it has recently been demonstrated that exposure of phosphatidyl serine (PS), which is particularly rich on MP from apoptotic cells, is a true receptor for signalling phagocytosis,[247] hence is involved with the clearance of MP exposing

this phospholipid. However, as indicated in that survey, the phagocytosis signal is complex, also involving such as C1q of complement[247] as well as LOX-1, β2GP1-R, vitronectin R, and Mer receptor tyrosine kinase.[247]

Other examples abound, such as the transport of LPS on an MP-like lipid binding protein to its receptor CD14 and its regulation by TFPI.[248] It may be noted, by the way, that a cytosolic receptor for LPS is now recognized, through work by G. Nonez and colleagues, involving NOD1 and NOD2 (now called CARD-4 and CARD-15), independent of the Toll-like receptors recognizing extra-cellular ligands, all acting on NF-κB genes; see perspective with references.[249]

High- and low-density lipoproteins (*HDL, LDL*)

Best known for their cardiovascular risk (LDL) or benefit (HDL), these particles occur in a narrow range of small sizes (LDL 20–23 nm, HDL 7–13 nm[250]), also defined by characteristic apolipoproteins, hence are unlikely to be confused with cell membrane-derived MP. Nevertheless, they may be relevant in this context, because of evidence that they can fuse with or otherwise directly act on platelets.[230,251] LDL and HDL are known to exhibit many activities, some of which are also found on cell-derived MP, a few examples being thrombin/AT III complexes,[252] procoagulant activity,[253] C reactive protein (CRP),[254] PAF degrading enzyme,[255] TFPI (formerly called LACI, lipoprotein-associated coagulation inhibitor),[256,257] and β2GP1.[258] Some of these agents are evidently promiscuous (non-selective) with regard to the phospholipids to which they bind, hence could associate with more than one kind of circulating MP. For example, TFPI is heterogeneously distributed in lipoprotein fractions, not necessarily associated with apo-B or apo-A1[257] and is found also in other plasma fractions and in platelets[224] (By analogy, the coagulation proteins assemble to active form with liposomes of many kinds and sources, not just platelets or PMP or cephalin; *e.g.* we find vegetable lecithin to be as potent as brain cephalin in coagulation). In sum, HDL/LDL may be relevant to cell-derived MP studies *via* interactions such as fusion and influence on cell-derived MP sheddding by lipid exchanges altering the PL composition of lipid rafts involved in MP shedding.[259]

Mechanisms of Microparticle Release

Background

Despite many efforts, detailed biophysical knowledge of the mechanisms of MP release remains elusive. This article surveys some major paradigms now current and other suggestive clues. Our previous review[1] briefly surveyed this topic as of then, in particular, work by Fox *et al.*, White *et al.*, and especially by Sims, Shattil, Wiedmer and colleagues in connection with their series of studies of PMP release by complement,[260] where they achieved only partial success on the mechanism question, *e.g.* use of protein kinase inhibitors.[261] However, a few principals have been repeatedly confirmed, notably the requirement for elevation of cytosolic calcium,[36] either from internal stores or through the plasma membrane. Metabolic energy was not required. Haynes *et al.* of this institution has well reviewed and diagrammed calcium handling in platelets,[262] although knowledge of the relevant calcium channels has since improved.

The membrane flip/flop paradigm

This concept, due mainly to Zwaal *et al.* beginning in the mid-1980s, holds that the process of MP shedding relies on specific enzymes (floppase, scramblase[263]) which induce the normally in-facing and more anionic phospholipids of the inner side of the membrane bilayer to switch, flip, or scramble to the outer (plasma) side; this causes the platelet (or other cell) to become procoagulant, since anionic phospholipids such as PS are essential to supporting coagulation, and is accompanied by the sheddding of procoagulant MP.[264,265] (It had long been known that inside-out erythrocytes could be prepared,[266] and for that matter, mitochondria,[267] the chief novel insight of Zwaal *et al.* being to draw a connection between procoagulant activity and membrane sidedness; and the hypothesis that this was controlled by one or more enzymes.)

This was surveyed in our prior PMP review but is reiterated here because of what we believe to be unwarranted generalizations now commonly drawn from that concept, specifically, that a defining feature of MP is exposure of PS. For example, as remarked earlier, many laboratories now define, measure, capture, or otherwise isolate MP on the basis of annexin V binding, which is quite specific for PS; but we find that only a fraction of MP are PS-positive, depending on their mode of production, *e.g.* apoptosis *vs.* activation.[2] The review by J.M. Freyssinet is properly skeptical of claims about certain enzymes (scramblase, floppase) inducing the flip-flop and associated MP release,[4] yet makes that hypothesis a central organizing principle, as we have criticized in a letter-to-the-editor.[268] Other evidence that platelet activation is not necessarily coupled to membrane flip-flop is seen, for example, in work by Leo *et al.*, who found that knock-out mice lacking SLP-76 responded to agonists with 10-fold increased CD62P, yet showed no increase in surface PS expression;[44] unfortunately, they did not measure MP release.

The lipid raft concept

It had been known for a long time that many if not all proteins of the plasma membrane exhibit a high degree of lateral mobility,[269] manifested in such phenomena as receptor clustering, dimerization, and capping of leukocytes.[270–272] Recently, the concept of lipid rafts has emerged, in part to account for aspects of this mobility, being floating islands of distinctive lipid composition bearing restricted sets of proteins (such as GPI-anchored), and are studied by their differential solubility in detergents.[273–280] Erythrocytes (RBC) have been good models for study[281] and their rafts help us to understand earlier findings such as the selective shedding of RBC MP enriched in acetyl cholinesterase.[282] The latest ASH meeting included new findings on rafts related to TF activity[283] and platelet cytoskeleton.[284] Some authors speaks of just one kind of raft but others imply several. Sun *et al.* has sought to relate scramblase activity to lipid rafts.[285]

We have observed by fluorescence microscopy the clustering (capping) of PECAM-1 as a prelude to the shedding of MP enriched in it,[286] suggesting that specific kinds of rafts are preferentially shed as MP. This would explain our finding of multiple phenotypes of EMP,[59] *i.e.* on the assumption that they reflect distinctive raft species. Millan *et al.* has studied rafts in the uropods of leukocytes,[287] further hinting at a link between rafts and blebbing, vesiculation. Relatedly, we have observed that the shedding of endothelial MP (EMP) *in vivo* is highly sensitive to the lipid profile of the volunteer before and after a high-fat meal[259] (in press), consistent with the fact that the composition and stability of rafts are very sensitive to levels and compositions of particular plasma lipid particles and transfer proteins. For example, Frenkel *et al.*, following earlier studies investigating release of erythrocyte microparticles (RBC MP) in response to incubation with liposomes of various compositions, which induce various shape changes (such as to echinocytic), demonstrated that concentration of cholesterol in the membrane is key to vesicle shedding, and does not depend on ATP.[22] Thus, the now emerging elucidation of lipid rafts promises better understanding of the shedding of MP with distinctive antigen composition.

Liposome models and complement (*C*)

Using artifical liposomes as models of MP, it has been shown that those bearing negative or positive charge bind and activate complement (C) leading to lysis by the classical and alternative pathways, respectively, while neutral liposomes have no effect.[288] They readily identified activated C on the liposomes. Such studies are important for application of liposomes as drug delivery systems but may have limited relevance to natural MP since physiologic MP often or usually bear agents which modulate the action of C, such as sialyl groups known to bind the C inhibitor, factor H,[288] and probably other more specific membrane-associated inhibitors of C-mediated autologous lysis such as DAF and CD59, which we briefly studied;[240] for authoritative review.[289] On the other hand, C-mediated lysis of opsonized platelets, whose study was pioneered by Sims *et al.*[1] and which we have also investigated,[290] results in the membrane attack complex (MAC) being largely carried away with the resulting PMP so that the cell recovers. C was readily detected on the resulting PMP (also EMP[291]), suggesting, by the way, that C-mediated platelet or endothelial injury, as may occur in some patients with ITP, TTP, APS, *etc.*, could be identified by measuing C-associated MP. Perhaps relatedly, Taylor *et al.*, using a baboon model of thrombosis induced by TNF and anti-protein C in the setting of partial stasis of the superficial femoral vein, showed that infusion of phospholipid vesicles greatly amplified the thrombotic response,[292] although for reasons above, the physiologic relevance of artificial liposome infusion is doubtful. We trust, however, that current liposome-based drug delivery systems are at last aware of these pitfalls.[293]

Parting remarks

Although a detailed understanding of MP shedding is not yet at hand, it is likely to be furthered by recent advances in understanding cytoplasmic vesicle traffic, uropod formation,[294] inter-cellular membrane fusions, granule secretions and related events, including oft-drawn parallels between neurons and platelets; several of these topics are reviewed by Reed *et al.*[295] As long appreciated,[129] all these events seem to involve the cytoskeleton (although the striking models of Baumgart *et al.* show such properties without any protein[6]).

Summary & Conclusions

The central message of this review has been to indicate new horizons for research and clinical applications of PMP and other MP. Specifically, we envision a second generation of MP assays in which specific functional agents on MP rather than gross quantities of MP constitute the analyte. This expected new generation of assays will most likely focus on the putative immune and inflammatory roles of MP, and will benefit from the realization that many so-called soluble markers are in reality MP-bound, at least in part.

However, the word putative should be emphasized because it remains to be proved that MP are indeed crucial carriers of such agents, whose quantitation could

be of real clinical value. In other words, realization of this vision entails the considerable preliminary challenge of demonstrating exactly which of the many likely functions outlined above are in fact important and are directly reflected by quantitation of the MP carrying the agents in question.

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