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ENDOTHELIAL MICROPARTICLES AS MARKERS OF ENDOTHELIAL DYSFUNCTION

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1. ABSTRACT

Endothelial microparticles (EMP) are small vesicles released from disturbed endothelial cells (EC). Owing to the central importance of EC injury in thrombotic and inflammatory conditions, assay of EMP as a marker of EC disturbance has come under intensive development by several laboratories. The review begins with established

markers of EC injury, commonly soluble markers such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, von Willebrand factor (vWF), etc., pointing out that many of these are in fact mixtures of true soluble molecules with membrane-bound forms, i.e., EMP. Assays of EMP from different labs are reviewed and standardization of assay is recommended. EMP are heterogeneous: those released in activation *vs*. apoptosis are distinctive in phenotypic markers and procoagulant properties. Application of EMP phenotype analysis can distinguish EC state of activation from apoptosis. Some EMP carry functional vWF with properties different from soluble vWF. Certain EMP bind to and activate monocytes; EMP-monocyte conjugates were found to be a marker of inflammatory disease such as multiple sclerosis (MS), and to enhance transendothelial migration of leukocytes *in vitro*. Clinical studies have revealed elevated plasma levels of EMP in lupus anticoagulant (LA), multiple sclerosis (MS), thrombotic thrombocytopenic purpura (TTP), coronary artery disease (CAD), hypertension, preeclampsia, and diabetes. Further refinement of EMP assay could open new windows for evaluating and monitoring endothelial injury in thrombotic and inflammatory disorders.

2. BACKGROUND: REVIEW OF PREVIOUS MARKERS OF ENDOTHELIAL INJURY

2.1. Foreword

It is now widely recognized that the vascular endothelium plays a pivotal role in the pathogenesis of numerous thrombotic and inflammatory disorders. Accordingly, much effort has been devoted to identifying plasma markers of endothelial disturbance.

The focus of this review is on recent advances in the analysis of EMP, a new approach to gaining insight on the status of endothelial cells (EC) in various pathologies. Applications to specific diseases are covered only briefly (§5). It is appropriate to begin with a short review of the more established markers of EC injury.

Unlike circulating blood cells, EC cannot be directly sampled for laboratory analysis. Therefore, various proxies believed to reflect EC disturbance have been employed. Prior to EMP, these have been chiefly of two kinds, (i) circulating endothelial cells (CEC) and (ii) soluble markers of EC disturbance.

2.2. Circulating endothelial cells (CEC)

The presence of intact CEC as a marker of EC injury was first reported in 1978 in the setting of angina pectoris and acute myocardial infarction (MI) (1), and a decade later, in sickle-cell crisis (2). After another decade, Dignat-George *et al* reported a method to capture and concentrate CEC using beads coupled with S-endo (CD146) (3), leading to their series of reports on elevated CEC in infectious disease (4), in TTP (5), angina and acute MI (6), and as reviewed by them (7). Related studies of CEC by other authors include CEC in sickle cell (SC) (8), a study of bone marrow-derived CEC (9), and a finding that elevated CEC in systemic lupus erythematosus correlated with plasma complement component C3a (10).

Despite many interesting findings, assay of CEC was not widely adopted, chiefly because of the large volume of blood required to achieve statistical significance: normal donors have only about 2 CEC per mL and in disease states, rarely more than 20 per mL (e.g. (10)) and standard deviations are often large. Also, the method for CEC isolation / concentration is tedious. Thus, although CEC assay has useful applications for certain research purposes, such as the interesting study of EC outgrowth in recipients of bone marrow transplant (11), it has not found general acceptance in the clinical laboratory.

One issue relevant to this review which arose in CEC studies is the question of whether or not the elevated CEC in disease states reflects apoptotic endothelium. Although EC apoptosis has been proposed to play a key role in prothrombotic conditions such as CAD or acute coronary syndrome (ACS) (12), CEC from CAD patients did not show apoptosis as judged by TUNEL assay (6), prompting a letter strongly critical of that finding (13), repeated in a review (14). The possible role of EC apoptosis in various disorders has remained controversial but EMP analysis may offer the means to resolve it, as discussed later (§4.2).

Also relevant to this review is that CEC from ACS patients were reportedly macrovascular and did not express activation markers (ICAM-1, VCAM-1, E-selectin) (6) whereas those from SC crises were said to be predominantly microvascular (CD36 positive) and did express the above three activation markers as well as P-selectin (8). (For studies of markers of EC activation elicited by cytokines, see (15); of immunologic phenotypes of activated *vs*. resting human umbilical vein EC (HUVEC), see (16).) These are further examples of unresolved issues which may be amenable to analysis by EMP.

2.2.1. Circulating endothelial progenitor cells (CEPC)

Most recently, CEPC have been identified as a unique population of cells, and their abundance in terms of colony forming units (CFU), determined by an assay in tissue culture of CEPC derived from peripheral blood mononuclear cells of 20 mL blood, was shown to correlate well with brachial reactivity, though poorly with Farmingham risk score, in a population of 45 males with and without risk factors (17). Although certainly an interesting and promising study, little is yet known of the significance of CEPC, and the difficulty of measuring them is a drawback for clinical use.

2.3. Soluble markers of endothelial injury

By far the most widely used method of assessing endothelial injury has been by assay of soluble markers known or believed to derive from or reflect endothelial disturbance. For review of some adhesins employed for this purpose, see (18, 19); for soluble cytokine receptors, see (20, 21). Table 1 shows at a glance representative

Table 1. Representative studies employing soluble markers of endothelial injury

Number	Disease or Model System	Markers Measured	Reference
1	ACS, Peripheral artery disease	sP-selectin	22
2	Acute MI	ICAM-1, VCAM-1, E-selectin	23
3	APLS	IL-6, vWF, endothelin-1, sVCAM, sICAM-1, P-selectin, TF, VEGF	24
4	APLS	TF	25
5	APLS	TM	26
6	Atherosclerosis	ICAM-1, VCAM-1, E-selectin	27
7	Atherosclerosis	vWF, vWF(Ag), sTM	28
8	Atherosclerosis (early)	sTM	29
9	Atherosclerosis to MI	sP-selectin, vWF	30
10	Atherosclerosis/CAD	ICAM-1, VCAM-1, E-selectin	31
11	Behcet's dis. (vasculitis)	TM	32
12	CAD, atherosclerosis	TM	33
13	CAD	ICAM-1, VCAM-1, E-selectin	34
14	CAD (stable)	ICAM-1, E-selectin	35
15	Liver injury	TM	36
16	MS	PECAM-1	37
17	MS	sVCAM-1, sICAM-1, sE-selectin	38
18	Peripheral vascular disease	vWF, P- & E-selectin, ICAM-1, VCAM-1, TM	39
19	Preeclampsia	vWF	40
20	Preeclampsia	ICAM-1, VCAM-1	41
21	Preeclampsia	TNF-α, IL-1ß	42
22	Rheumatoid arthritis	ICAM-1, ICAM-3, VCAM-1, E-, L- & P-selectins	43
23	Several (review vWF marker))	vWF	44
24	Stroke	t-PA, PAI-1	45
25	TTP	TM	46
26	TTP	t-PA, PAI-1, TAT, PIC, D-dimer	47
27	TTP, HIT, DIC*, HUS, ITP	P-selectin, beta-TG	48
28	TTP, HUS	P-selectin	49
29	TTP, Vasculitis, DIC, DM	TF	50
30	Unstable angina	sP-selectin	51
31	Vasculitis	TM, ICAM-1, VCAM-1	52
32	Vasculitis	TM	53

studies of this kind, the wide range of markers employed and the breadth of pathologies assessed.

Although many of the papers cited in Table 1 indicate successful correlation between one or more such markers and clinical disease state, there is little agreement on the best marker and several objections to such studies have repeatedly been raised. For example, Mannucci, in his conclusion to a review of von Willebrand factor (vWF) as a marker of EC damage, notes that this marker suffers from poor specificity and that this is a problem shared by other soluble products of EC used as noninvasive aids to diagnosis (44). Another problem is that results by enzyme-linked immunoassay (ELISA) for soluble markers often vary from laboratory to laboratory, up to 50-fold (18). Furthermore, results by different markers are often or usually inconsistent; for example, in one study of antiphospholipid syndrome (APLS), only 2 of 8 different markers of EC damage were significantly elevated, vascular endothelial growth factor (VEGF) and soluble tissue factor (sTF) (24) (Table 1, entry 3).

Probably the most serious objection to the above methods is not yet appreciated since it has only come to light by study of EMP: many or most of the so-called soluble markers are in reality bound to EMP, at least in part. This is definitely true for at least some of the markers in Table 1, notably, PECAM-1 (CD31), ICAM-1 (CD105), E selectin (CD62E) and P selectin (CD62P) because, as indicated later, these are routinely used to identify EMP. That is, they are known to be carried on EMP, and can be largely removed by filtration through 0.1 μm, and so are not truly soluble. In further support of this, detection of any such marker by flow cytometry implies that the marker is microparticle-bound (MP-bound) since flow cytometry

cannot detect true soluble molecules of this size. (Most flow cytometers require a minimum of ~500 fluorescent molecules per particle for threshold detection.).

On the other hand, it is known that some fraction of many of these markers of EC perturbation is indeed truly soluble. Proteolytic mechanisms are known which cleave them from the extra-cellular matrix (ECM), or ectodomain, including but not limited to matrix metalloproteases (MMP) and related disintegrins and metalloproteases such as ADAM-10 (a disintegrin and metalloprotease-10), ADAM-17 (a disintegrin and metalloprotease-17) (54, 55), and as cited in (56), often collectively called "sheddases." Together with post-translational release of true soluble forms, and phospholipases specific for GPI-anchored proteins, these proteolytic mechanisms of release of soluble proteins are entirely different from those inducing EMP budding and vesiculation of the membrane.

In the majority of studies on Table 1, no special care was taken to distinguish between true soluble vs. MP-bound species. An exception is Katayama *et al* (49) who found significant P-selectin in plasma of TTP patients even after high-speed centrifugation (100,000g) and 0.22 μm filtration, consistent with Osmanovic *et al* (57). However, 0.22 μm filtration may not be sufficient to remove the majority of microparticles (MP). We have found that EMP bearing marker CD51 (integrin $\alpha_V\beta_{III}$, vitronectin receptor) is readily detected by flow cytometry, yet is only partly removed by filtration through 0.1 μm. In summary, it is evident that the majority of studies such as those on Table 1 have in reality measured markers bound to EMP or other MP, or mixtures of true soluble and MP-bound forms (as with selectins). This may explain some of the observed inconsistencies. For example, the disparate correlations of different "soluble markers" with clinical state may reflect EMP phenotypes, discussed later.

3. HISTORY AND METHODS OF ENDOTHELIAL MICROPARTICLE ANALYSIS

3.1. What are EMP?

As their name implies, EMP are sub-microscopic membranous particles (vesicles) of size range below about 1.5 μm, which are shed from the parent EC upon stimulation by activating agents such as TNF-α or during apoptosis. The process of MP release is termed vesiculation, and follows an earlier stage of membrane budding or blebbing before pinching off as free particles. They carry with them many of the membrane proteins and phospholipids of the parent cell, but as reviewed below, the set of proteins on a given EMP may not be representative of their relative abundances on the parent cell.

In many respects, EMP resemble the microparticles shed into plasma from the circulating blood cells, i.e. platelet microparticles (PMP), leukocyte microparticles (LMP), and erythrocyte microparticles (RBCMP). A 1999 review article on PMP covers many topics relevant also to EMP (58).

3.2. Historical perspective

To the best of our knowledge, EMP were first explicitly studied by Hamilton *et al* in 1990, who used flow cytometry to detect their release from HUVEC in tissue culture following stimulation by complement (C) (59). These methods were developed by members of the same group in earlier studies on platelets and PMP, e.g. (60, 61). EMP were detected by fluorescent antibody against the stimulus, C, leading to their finding that much or most of the added C was shed off from the HUVEC along with the EMP, depleting the parent cell of added C.

However, reliable measurement of EMP in patient blood is more challenging than studies in cell culture because blood contains many kinds of MP. Also, at least some types of EMP may adhere to other blood cells. Thus, clinical applications of EMP assay went unexploited for almost a decade.

Since 1990, our lab has been investigating PMP, particularly in certain subgroups of idiopathic thrombocytopenia purpura (ITP) patients (58). ITP is an autoimmune disorder in which autoantibodies bind to platelets, inducing their immune-mediated destruction (62). Patients with ITP are usually at risk of bleeding due to low platelets but we identified a subgroup who rarely bled despite severe thrombocytopenia, and showed that they were characterized by high levels of procoagulant PMP, which apparently functioned to protect them against bleeding. Follow-up of these patients revealed that in later years, those with high PMP tended to suffer ischemic small vessel disease of central nervous system (CNS), verified by magnetic resonance imaging (MRI), manifesting clinically in transient ischemic attack (TIA)-like syndrome, memory loss, leading to advanced dementia (63, 64). In related studies, we found that PMP can bind to restricted classes of leukocytes, activating them, suggesting a role of PMP in inflammation as well as hemostasis (65).

Around 1998, our interest shifted from PMP to EMP due to increasing awareness that assay of endothelial disturbance is essential to a fuller understanding of thrombosis and inflammation.

The first reported clinical application of EMP assay appeared in 1999 by Combes *et al* (66), as reviewed below. Our first studies of EMP appeared in early 2000 (67), followed later that year by findings *in vivo* (68, 69) and full publications soon after (70, 71). Since then, an exponentially growing number of publications on EMP have appeared, and steady progress has been made in measurement methodology and in understanding their clinical significance, to be reviewed.

3.3. Assay methods

3.3.1. General flow cytometry

The majority of EMP studies to date have employed flow cytometry as the primary method. For orientation, we have classified the methods of some representative clinical studies in Table 2. Additional important details are given later in the course of reviewing specific papers. (For example, Mallat *et al* measured EMP quantities by means of prothrombinase activity (72), making it difficult or impossible to compare with flow cytometric enumeration.)

Table 2. Representative methods of EMP analysis in clinical studies

Analysis	Application	Authors	Year	Reference
Flow Cytometric				
CD31+, CD51+	Lupus anticoagulant	Combes *et al*	1999	66
CD31+, CD42-	TTP	Jimenez *et al*	2001	70
CD31+, CD42-	Multiple sclerosis	Minagar *et al*	2001	71
CD31+, CD42-	Hypertension	Preston *et al*	2002	74
CD31+, CD42-	Acute coronary syndrome	Bernal-Mizrachi *et al*	2003	75
CD31+, CD42-	Preeclampsia	Gonzalez-Quintero *et al*	2003	76
CD51+	Diabetes	Sabatier *et al*	2002	77
CD62E+	TTP	Jimenez *et al*	2003	123
AnnexV+/CD146+	Sickle cell disease	Shet *et al*	2002, 03	78, 79
Immuno-capture/ELISA				
AnnexV+/CD146+	Acute coronary syndrome	Mallat *et al*	2000	72

The preferred method in our laboratory has become the 2-color combination of phycoerythrin (PE)-labeled anti-CD31 with fluorescein isothiocyanate (FITC)-labeled anti-CD42, see references on table. Because CD31 occurs also on platelets, platelet-specific CD42 allows counting the PMP population (CD31+, CD42+) distinct from EMP (CD31+, CD42-), giving both counts in a single run. This pair of markers has the advantage of being quite bright (abundant) and therefore sensitive. Not mentioned in the table are methods of assay of coagulant activities, which are also sometimes used to quantify EMP (as in (72)), or the possibility of using ELISA methods for primary detection of EMP; see next article. Certain markers used in other laboratories may have advantages in some applications, for example, VE-cadherin (CD144) used by Berckmans *et al* (73) is a highly specific single marker; we have not compared its brightness to the markers we regularly use.

There is yet no generally accepted single method. Our laboratory and others continue to explore other and possibly superior markers, including for purposes of enumerating subsets of EMP phenotypes which may be important in particular disease conditions, reviewed later. Of course, special applications such as EMP phenotype analysis or studies of a specific agent carried on EMP will require alternative markers or combinations.

3.3.2. ELISA methods for EMP

There are two reasons why ELISA methods may be a viable option for assay of EMP: (i) many laboratories do not have or cannot afford flow cytometry; (ii) ELISA is likely to be more sensitive for detecting very small particles and/or weakly expressed antigens. ELISA methods have been developed for measurement of PMP (80-82) and should be applicable to EMP. Indeed, some authors employ ELISA for the secondary identification of EMP and other MP subsets, e.g. (72). As noted earlier, ELISA methods which are now routinely used to detect "soluble markers" evidently also detect those markers when EMP-bound, since at least some of those markers are known to exist principally in MP-associated form (e.g. PECAM-1, tissue factor (TF), etc.). To quantitate both true soluble and MP-associated forms, the tests may be performed in duplicates with / without filtration of plasma through 0.1 um, as remarked above. We have not investigated quantities of EMP in serum as distinct from plasma, but it is known that PMP, at least, are abundant in serum (58).

3.3.3. Validation of detection specificity

A way of ascertaining if a given method gives a true count of EMP distinct from other MP is to mix known quantities of EMP with PMP or other MP, then determine if measuring the mixtures gives the same answer as measuring the pure components individually. Data of this kind were given by Bernal-Mizrachi *et al* in the Methods section (75). Briefly, known quantities of pure EMP from cell culture and PMP from activated isolated platelets were mixed; it was found that counts of EMP and PMP in the mixture were the same as when measured independently, within 5 - 8%.

Similarly, to test whether leukocyte microparticles might be confused with EMP, pan-leukocyte marker anti-CD45-FITC was substituted for the CD42 platelet marker (normally used with the CD31 marker as explained above) in several test series: negligible CD31+, CD45+ particles were detected. Additionally, use of non-immune mouse FITC-IgG gave negligible false EMP counts.

Some markers used for EMP detection may not be strictly specific for endothelium. If, however, it can be shown that their brightness on EMP is such that MP from other cells cannot be detected by that marker, then it may be acceptable. Tests of this kind are important to validate any method that purports to quantitate EMP distinct from other MP.

3.3.4. Total EMP

A difficult problem in EMP studies has been comparing results between laboratories using different methods and markers. Since EMP are now known to exist as heterogeneous species, in proportions varying with the nature of the EC injury (83), it is unlikely that any single marker will efficiently label total EMP. A number of workers have assumed, implicitly or explicitly, that annexin V (AnV) can capture or label essentially all MP (72, 84,

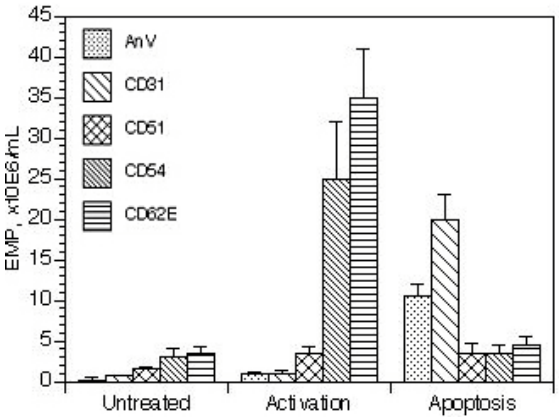

Figure 1. EMP numbers detected by five different markers. Results shown are from experiments conducted "*in vitro*" (tissue culture) using renal microvascular endothelial cells (RMiVEC). Values are expressed in terms of thousands of EMP per microliter of tissue culture supernatant following exposure to conditions causing either Activation (TNF-α) or Apoptosis (deprivation of growth factor). AnV = annexin V (PE labeled) as marker. Numerical values are from the same original table used to construct Table 3.

85). However, we have observed both *in vitro* and *in vivo* that only a fraction as many MP exhibit AnV binding as compared to other markers.

In tissue culture experiments, this fraction depended on the stimulating agonist or conditions such as apoptosis *vs*. activation (see §4.2). For example, as shown in Figure 1, EMP from TNF-α-stimulated EC cultures (activation) were 35-fold more numerous measured by anti-CD62E than by AnV. When EMP were generated by inducing EC apoptosis by deprivation of growth factors, known to favor particles which bind AnV, the number of EMP which bound AnV (10,500 ±1,500/μL), although ~10-fold higher than in activation, was still only about half the number detected by anti-CD31 (20,000 ±3,00/μL; Figure 1). Thus, the assumption that AnV positivity defines all EMP is unwarranted, especially in those cases where endothelial activation rather than apoptosis is the predominant pathology. In most conditions that we have explored, activation and not apoptosis was the predominant pathology; see §5.0 below.

To obtain a better measure of true total EMP *in vitro*, we have employed the FITC-labeled lectin *Ulex europaeus*, which yields EMP counts that are from 2-fold to 5-fold higher than by the next brightest marker. Unfortunately, this marker is unsuitable for *in vivo* assays owing to lack of specificity (unpublished). It has been reported that isolectin B4 from *Bandeiraea simplicifolia* (a.k.a. *Griffonia simplicifolia*) is specific for brain endothelia in homogenates (88) and may be worthwhile testing as an EMP marker. We have explored the use of lipophilic dyes for labeling total MP but were frustrated by finding that these dyes form micelles which are detected by flow cytometry as false MP even in the absence of MP.

3.3.5. EMP and other MP in normal controls

Although all studies of EMP in diseases have compared results to normal controls, Berckmans *et al* specifically addressed levels of various MP in normal individuals (73). Pursuant to their prior studies (89-91), they centrifuged total MP from normal plasma and reported that the number fraction of MP from platelets, red blood cells, granulocytes and EC were respectively 63.2%, 7.5%, 12.3% and 17.1% (converted to percentages by this reviewer), as determined by flow cytometry: positivity for AnV was the primary criterion, which in combination with various other antibodies established cell origin. We remarked above on the pitfalls of AnV. It may also be mentioned that the number of EMP counted depends on the marker used. They found that about 12.5% of all MP were positive for tissue factor (TF) antigen. They assayed TF activity by addition of MP to defribrinated plasma (Reptilase) and used a chromogenic thrombin substrate to show that a low level of thrombin generation occurs constantly in normal healthy persons, confirming other studies, some of which they cite. All TF activity was MP-associated. Interestingly, however, in contrast to patients, the procoagulant activity (PCA) in normal controls was by the contact pathway, not the TF pathway.

4. EMP STUDIES *IN VITRO*

4.1. Heterogeneity of EMP

The first clue that not all EMP are alike emerged from work by Jimenez *et al* (70, 93) in the course of studying EMP labeled with a panel of antibodies from cells treated either with TNF-α or deprived of growth factors (and later, mitocycin C). The numbers and proportions appeared to vary between these two treatments, depending on fluorescent markers used. This led to an extensive survey of immunofluorescent markers for the flow cytometric enumeration of EMP elicited by these two treatments, summarized in Table 3, (92, 93). It was subsequently realized that one of these treatments resulted in apoptosis, the other in activation (see §4.2 below). The table shows responses of both whole EC and their EMP to two classes of stimulation - activation (induced by TNF-α) and apoptosis (induced by deprivation of growth factors) - as measured by the seven markers indicated, in three EC lines: renal and brain microvascular (RMiVEC, BMiVEC) and coronary artery macrovascular (CMaVEC). The arrows indicate increase or decrease relative to untreated (baseline) values in the two right-most columns, as detailed in the Legend to this table. It should be noted that the coronary artery EC line gave only a weak apoptotic response compared to the microvascular lines, i.e. a smaller percentage of cells became apoptotic.

Further evidence of EMP heterogeneity emerged from clinical studies in MS (71) and CAD (75), where it was found that the diagnostic significance of EMP levels depended on which marker was used to identify and count them. For example, when CD51 positivity was the criterion, patients with MS showed chronic elevation irrespective of exacerbation or remission, but when CD31

Table 3. Responses of EMP release as judged by various markers

		Activation (TNF-α)		Apoptosis (GFD)		Untreated (control)	
Marker	Cell line	Whole EC (FL* units)	EMP (counts)	Whole EC (FL units)	EMP (counts)	Whole EC (FL units)	EMP (counts)
CD31	RmVEC	↓	↑↑	↓↓↓	↑↑↑	230 ±25	0.80 ±0.04
	BmVEC	↓↓	↑↑	↓↓	↑↑↑	225 ±30	0.7 ±0.1
	CMVEC	↓	↑	↓↓↓	↑↑	10 ±2	0.1 ±0.01
CD105	RmVEC	↓	↑	↓↓↓	↑↑	330 ±25	4.0 ±0.6
	BmVEC	↓↓	↑↑	↓↓↓	↑↑↑	255 ±25	4.0 ±1.0
	CMVEC	↓↓	↑↑	↓↓↓	↑↑	3.0 ±0.5	1.5 ±0.2
CD51	RmVEC	↑	↑↑	no change	↑↑	17 ±3	1.5 ±0.3
	BmVEC	↑↑	↑↑	no change	↑↑	15.5 ±4.0	2.0 ±0.5
	CMVEC	↑	↑↑	↓	no change	20.0 ±4.5	2.0 ±0.5
CD62E	RmVEC	↑↑↑	↑↑↑	no change	no change	3.0 ±0.6	3.5 ±0.7
	BmVEC	↑↑↑	↑↑	no change	no change	2.5 ±1.0	4.0 ±1.5
	CMVEC	↑	↑	↓	↓↓	2.0 ±0.5	4.0 ±0.5
CD54	RmVEC	↑↑↑	↑↑↑	no change	no change	6.0 ±1.5	3.0 ±1.0
	BmVEC	↑↑↑	↑↑	no change	no change	7.5 ±2.5	3.5 ±1.0
	CMVEC	↑↑	↑	no change	↓	3.0 ±1.0	3.5 ±0.5
CD106	RmVEC	↑↑↑	↑	no change	no change	1.5 ±0.2	1.0 ±0.5
	BmVEC	↑↑↑	↑↑	no change	no change	1.5 ±0.1	1.0 ±0.5
	CMVEC	↑↑↑	↑↑	no change	no change	2.0 ±0.5	0.5 ±0.1
Annexin V	RmVEC	↑↑	↑↑	↑↑	↑↑↑	3.0 ±0.7	0.20 ±0.04
	BmVEC	↑	↑↑	↑↑	↑↑↑	4.0 ±1.0	0.5 ±0.1
	CMVEC	↑	↑	↑↑	↑↑	1.5 ±0.2	0.5 ±0.1

The table shows responses as judged by fluorescent-labeled antibodies to markers indicated at left, on whole EC and EMP after 24 hr of treatment to induce either activation (TNF-α) or apoptosis (growth factor deprivation, GFD). Whole EC were measured by flow cytometry after detachment by trypsin in terms of mean fluorescent intensity; EMP are relative numbers detected per mL of media supernatant. Results are shown for three cell lines: renal microvascular (RmVEC), brain microvascular (BmVEC), and coronary artery macrovascular (CMVEC). The arrows indicate increase (up) or decrease (down) relative to untreated control values at right: ↑ = 15% to 2-fold; ↑↑ = 2 to 10-fold; ↑↑↑ = >10-fold; ↓= 15% to 25% reduction; ↓↓ = 25%-50%; ↓↓↓= >50% reduction. EMP results at right are in terms of numbers detected x10^6/mL. All experiments were repeated at least three times and reproducibility was good as shown by ±SD. The upper three markers are constitutive; the lower four (beneath heavy line) are inducible, i.e. respond to stimulation. Apoptosis was evaluated by TUNEL assay and checked for viability by trypan blue dye exclusion: only 0.5% to 2% of TNF-α-treated cells were positive for apoptosis; whereas 50% ±10% and 65% ±10% of RmVEC and BmVEC, respectively, were positive following deprivation of growth factor. However, CMVEC resisted apoptosis, only 5% ±3% being positive after this treatment. * Fluorescent

positivity was the criterion, significant elevation during exacerbations compared to remission was observed (71).

Simak *et al* has independently reported EMP heterogeneity in a study of paroxysmal nocturnal hemoglobinuria (PNH), finding that CD105/CD54 EMP were markers of chronic activation (failed to correlate with hemolysis) while CD105/CD144 detected acute states (94). This is consistent with findings by Jimenez *et al* cited above.

4.2. EMP analysis distinguishes apoptosis from activation

In the course of the work summarized in Table 3, it was noticed that the pattern of EMP phenotypes (defined by the markers used to identify them) was distinctly different in apoptosis compared to activation. (See also Figure 1.) Specifically, it was determined that the ratio of EMP counted by two markers, CD62E+ EMP / CD31+ EMP, is always >4.0 in activation, but <0.4 in apoptosis. Other pairs of markers give similar discrimination. The significance of this finding lies in potentially resolving the controversy concerning whether or not EC apoptosis is an important component of a given disease state (such as CAD, TTP, etc.), as the answer to this could influence treatment modalities or preventive strategies.

Accordingly, this criterion was applied to EMP in plasma of patients with TTP, leading to the clear conclusion that EC in TTP are activated, not apoptotic (83). Conversely, the same authors have more recently shown by the same method that the EC injury due to oxidized low density lipoprotein (LDL) and/or elevated cholesterol is indeed apoptotic (95).

A major reason for the interest of Jimenez *et al* in the above studies was their finding that neither TNF-α nor plasma from TTP patients induced apoptosis of EC cultures, in conflict with some but not other reports. The likely explanation for this inconsistency is given in the discussion of Jimenez *et al* (95), to which we may add a reference to a more recent clarification of the complex pathways initiated by TNF-α, leading via NF-κB either to activation or apoptosis depending on subtle conditions (96).

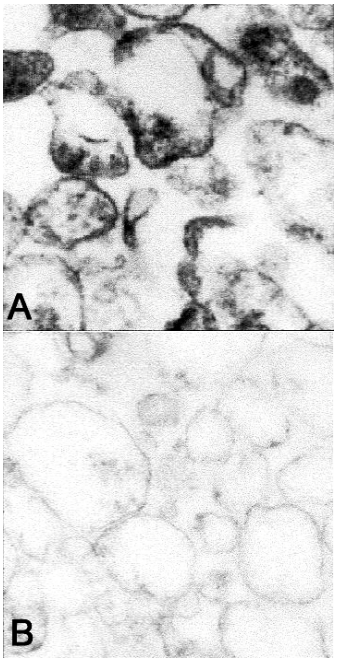

Figure 2. Electron micrographs of EMP. Cultured brain MVEC were deprived of growth factors or exposed to TNF-α for 18 hrs, conditions known to induce apoptosis (A) or activation (B), respectively, in our conditions (70, 83). Supernatants were centrifuged at 18,000 rpm for 30 min and pellets were fixed in glutaldehyde until processed for EM staining and viewing. Magnifications, 28,750X.

This method may be applicable to other diseases to resolve the same question, such as whether apoptotic EC are prominent in specific other coronary conditions, a continuing controversy. Further potential applications of EMP phenotype analysis are under investigation.

4.3. Electron micrographs of EMP

Representative electron micrographs of EMP from RmVEC are shown in Figure 2 (A, B), comparing EMP from apoptotic cells (A) to those from activated cells (B). Apoptosis was induced by growth factor deprivation (GFD) and activation was induced by TNF-α as described (70, 83). The EMP from apoptotic EC were more electron dense and contained granular and nuclear materials. In contrast, the EMP induced by TNF-α were membrane vesicles containing little electron dense materials. For other electron micrographs by other authors, see for examples (66, 97).

4.4. Plasma from patients induces EMP release

It was shown by Jimenez *et al* that plasma from TTP patients, pre-filtered through 0.l μm filter to remove pre-existing EMP, induced substantial release of EMP from cultured microvascular renal or brain EC, as defined by positivity for CD31 or CD51 (70) (Figure 3 in Ref 70). The agent in the plasma responsible for the observed activation of EC has not been identified but possibilities include cytokines or antibodies (anti-EC or anti-phospholipid), with or without mediation of complement (C). If C is involved, it might be detectable on EMP by the methods of Hamilton *et al* (59). The same group observed a similar effect ($p<0.001$), though of lesser magnitude, by plasma from MS patients: EMP positive for CD31 or CD51 were elicited (71) (Figure 5 in Ref 70). Since those experiments were conducted with microvascular EC (MiVEC), and since they observed that macrovascular EC (MaVEC) were less sensitive to several agonists in releasing EMP than MiVEC, it would be interesting to see if these effects are specific to micro- vs. macro-vascular EC, in so far as TTP is a disease largely confined to MiVEC, and likewise, MS is believed to affect mainly the MiVEC of the blood-brain barrier (BBB).

4.5. Plasminogen activator inhibitor-1 (PAI-1) induces EMP bearing uPAR

On the assumption that elevated PAI-1 is a hallmark of EC dysfunction and associated thrombotic disposition (e.g., (45)), Brodsky *et al* investigated the effect of PAI-1 on HUVEC cell culture (98). Using flow cytometry, they found that incubation of HUVEC with plasminogen activator inhibitor-1 (PAI-1) (1-10 ng/mL) for 1-3 hr induced significant release of EMP positive for urokinase-type plasminogen activator receptor (uPAR), and concomitant decrease of uPAR on the cell surface, as determined by laser confocal fluorescence microscopy. Results are calculated in terms of EMP population / mL of supernatant, and per parent cell.

4.6. Mechanism of EMP generation

It is likely that the mechanism of EMP release is similar to that of MP release from other cells such as platelets but at the time of our review on PMP (58), theories were rather vague. Jy *et al*, using fluorescent microscopy, has recently shown that release of CD31+ EMP is preceded by localized accretions of CD31 on the cell surface, a phenomenon also known as membrane capping (99). Specifically, they showed that EMP released correlated with percentage of fully capped cells, measured at intervals (0, 2hr, 4hr, 8hr, 24hr). Fluorescent images (1000x) of caps resolved numerous microparticles apparently being shed directly from microvilli (or pseudodia) protruding from the caps.

These observations suggest that capping of membrane proteins (or other focal adhesions) is a necessary prelude to the shedding of at least some species of EMP. It is now well known that membrane surface proteins are quite mobile and can agglomerate (or dimerize, etc.) by virtue of lipid rafts (100-102), areas of distinctive phospholipid (PL) composition with distinct embedded proteins, a topic overlapping with phenomena such as patching and capping, focal adhesions, and receptor clustering. A variety of enzymes, known or hypothetical, have been implicated in membrane blebbing and vesiculation, including scramblase, floppase, calpain, and PL translocase, briefly considered in our review (58). For updates on these topics, see (103-105).

4.7. Potential functional roles of EMP

4.7.1. Modulation of leukocyte function

Sabattier *et al* demonstrated that EMP can bind to and activate cultured monocytes, as judged by induced

expression of TF antigen, and that this effect could be largely inhibited by anti-CD54 (106).

Jy *et al*, building on previous work on PMP-leukocyte interactions (65), had independently found similar results (including selective inhibition of the interaction by anti-CD54) but by a different approach, studying leukocytes in whole blood and measuring leukocyte activation in response to added EMP by expression of CD11b (107), as first presented at a meeting (108). That report also showed that EMP interacted only weakly with neutrophils relative to monocytes, and hardly at all with lymphocytes.

Of particular interest was the finding that EMP generated from EC undergoing apoptosis were weak in this effect compared to equal concentration of EMP from EC activated by TNF-α (108). This may be due to higher levels of CD54+ EMP induced by TNF-α compared to apoptotic EC. In a binding study using U937 cells exposed to EMP labeled with various markers, the EMP pre-labeled with CD54 exhibited the greatest apparent binding, followed by EMP pre-labeled with CD31, CD62E, CD51. Accordingly, the CD54 labeled EMP was largely depleted in the cell-free supernatant, consistent with the majority having bound to the U937 cells (108). Those authors also proposed that the relatively low concentration of CD54+ EMP found in blood in various disease states is explained by the finding that these EMP preferentially and strongly bind to leukocytes, reducing their free concentration. Finally, they demonstrated that monocytes with adhering EMP were facilitated in their passage through an endothelial monolayer (108). Taken together, these findings suggest that at least one function of EMP is to modulate inflammation *via* leukocyte activation and transendothelial migration.

4.7.2. Procoagulant activity (PCA)

This term has been applied to two completely different activities: (i) anionic phospholipids (PL_{anion}), especially phosphatidyl serine (PS), are essential for catalyzing coagulation, an activity known as platelet factor 3 (PF3), and therefore qualifies as PCA even though PL_{anion} cannot by itself initiate coagulation; (ii) the term PCA is also applied to tissue factor (TF) activity known to occur on EMP (70, 72, 97, 109). However, it is not yet possible to ascertain the relative magnitudes (importance) of these activities on EMP vs. PMP, LMP, or whole cell surfaces (such as PF3 activity of activated platelets or TF expression on leukocytes or activated EC).

TF activity in particular has been difficult to quantitate, with conflicting reports, apparently because of variable expression of "cryptic" TF and masking by tissue factor pathway inhibitor (TFPI) (110, 111); we have attributed variable results partly to sensitivity to sulfhydryl redox state (112). Although more work is needed, much evidence suggests that EMP likely play important roles in coagulation, especially at local sites of injury, not only by virtue of PF3 and TF activities but possibly also by modulating the protein C/S-thrombomodulin (TM) anticoagulant system.

4.7.3. Anti-coagulant function

In our review of PMP (58) it was mentioned that Tans *et al* suggested that PMP could serve an anti-coagulant function by supporting the protein C/S/TM pathway (113). Gris *et al* demonstrated that a large part of protein S is MP-associated, and that clinical assays using polyethylene glycol PEG precipitation cause underestimation of protein S because much of it is precipitated with MP (114). More recently but in a similar spirit, Berckmans *et al* have shown that low levels of thrombin generation by MP in normal controls is via the contact pathway (independent of TF) and may serve an anti-coagulant function, on the grounds that protein C could thereby be activated by the trace of thrombin (73). They note, however, in their interesting discussion that their findings and others "challenge our understanding of the clotting mechanism" and that "data regarding the presence of tissue factor exposing microparticles ... require further experimental support" (73).

4.7.4. Decoy theory

The decoy principle holds that receptors released from the parent endothelial cell will act to tie up ligands destined for it, effectively neutralizing them and blunting the response. This principle is well accepted for soluble cytokine receptors (18, 20, 21) and has been exploited therapeutically by administering recombinant TNF-α receptor (115), complement receptor (116), and might apply also to Fc receptor (117). Likewise, soluble CD62P has been shown to inhibit CD18-dependent adhesion of activated neutrophils to EC (49). The same principle should hold for EMP insofar as they are known to bear several receptors from the parent EC, e.g. uPAR, see §4.5 above.

4.7.5. Expulsion of noxious agents

As earlier noted, Hamilton *et al*, observing that complement (C) added to induce platelet activation, was promptly shed off bound to PMP, proposed that one functional role of MP shedding is to rid the cell of noxious agents (59). That observation was extended to other cell types (118) and might apply, for example, to MS since EMP are known to be elevated in MS (71) and C-mediated injury to the myelin sheath has been reported (119). If expulsion of noxious agents by shedding is a general principle, then it may be possible to detect other agents on EMP that might have provoked their release from the parent cell, such as viruses, amyloid species, parasites, prion protein, or antibodies. In this regard, Simak *et al* have demonstrated that EMP express prion proteins (138). Plasma MP have been shown to correlate with anti-cardiolipin antibody titers in HIV (114).

4.7.6. Protective function

Circulating mRNA has been of great interest in cancer studies (120) (and see references there) but this presented a puzzle because mRNA is normally very labile in plasma due to enzymatic attack. This mystery may have been solved by the finding that most circulating mRNA is in fact microparticle-bound, leading to the proposal that this protects it from enzymatic degradation (121); the authors cite other work in support (122). This is relevant to the present review because of the recent finding that vWF is

associated with EMP, at least in part, and in this form exhibits properties different from free soluble vWF with respect to ristocetin-induced platelet aggregation (123, 124), as further commented in §5.3 below. Although this work is still in progress, the authors hypothesize that EMP-bound vWF is protected against proteolysis and therefore remains in the ultra-large (polymer) form, which would account for the greater stability of platelet aggregates induced by EMP-bound vWF relative to free vWF. It is not yet known if MP-bound vWF is associated also with factor VIII, as it otherwise normally is.

5. EMP IN CLINICAL STUDIES

5.1. Introduction

The following is a brief synopsis of the key papers known to us dealing with applications of EMP analysis to clinical conditions. Since each laboratory uses somewhat different methods, it is difficult to compare results among them. Measured levels of EMP depend significantly on the marker or markers used to identify them. Ideally, at least the basic EMP assay should be standardized, with availability of reference standard sets of fluorescent microparticles. In general, the most recent papers on a given topic may be expected to utilize the latest improvements in methodologies from a given laboratory.

5.2. Patients with lupus anticoagulant

The first report of clinical application of EMP assay was by Combes *et al* (66). They investigated EMP in 30 healthy controls and 30 patients with lupus anticoagulant (LA). EMP in patients with LA were significantly higher, by about 2-fold, than healthy controls. Patients with LA are at increased risk of thrombotic complication, thus LA may be regarded as a manifestation of a hypercoagulable state. They studied EMP in patients with and without thrombosis, observing significantly more EMP in those with thrombosis. Interestingly, the level of EMP was not reduced in those treated with anticoagulation for thrombosis.

5.3. Thrombotic thrombocytopenic purpura (TTP)

The classical triad of TTP symptoms are consumptive thrombocytopenia, microangiopathic hemolytic anemia, and fluctuating neurological disturbances. These arise from systemic intravascular platelet aggregation. Large-multimer vWF is now considered pivotal in initiating platelet adhesion and aggregation in the intravascular space, a key step in the pathogenesis of TTP.

Continuing long-standing interest of our laboratory in TTP (125, 126), Jimenez *et al* reported elevated EMP in patients with TTP by measuring CD31+/CD42b- EMP species, observing that EMP levels rose in acute stages, returned to normal in remission, and correlated well with disease activity (70). EMP elevation appeared to precede other markers of disease activity such as platelet counts and lactate dehydrogenase (LDH) (70). In more recent studies, CD62E rather than CD31 was exploited as the main marker, yielding higher EMP counts and better correlation with other markers of TTP activity such as platelet counts, LDH, and hemolysis (123).

5.3.1. EMP and vWF

Most recently, the remarkable discovery was made that many of the EMP in TTP are positive for vWF (123). The significance of this lies with growing evidence that the underlying pathology in TTP stems from aberrant processing of vWF by the enzyme which normally cleaves it, ADAMTS-13 (127). It has now been shown that platelet aggregation induced by vWF-positive EMP are markedly more stable (resistant to dissociation) then aggregates formed by free soluble vWF, in the presence of ristocetin (124). This effect would be expected to enhance formation of platelet rich thrombi *in vivo*. Although that work is still in progress, preliminary indications suggest that EMP may play a vital role in the interaction of vWF with platelets at sites of injury.

5.4. Multiple Sclerosis

Minagar *et al* reported a sharp elevation of CD31+ EMP during exacerbations of MS in contrast to remissions; but CD51+ EMP, although always lower, were significantly above normal in both states, suggesting that the latter is a marker of chronic EC stimulation, the former a marker of acute stimulation (71). EMP levels defined by CD31 were significantly elevated in active MS, and returned to normal in remission. Elevated levels of CD31+ EMP were associated with the presence of gadolinium enhancing lesions on MRI of neuro-axis.

5.4.1. EMP-leukocyte conjugates in MS

In related studies, introduced above in §4.7.1, Jy *et al* undertook to assess a possible functional interaction between EMP and leukocytes (108, 128). They observed that EMP preferentially bind to monocytes, activating them. When monocyte-EMP conjugates were assayed in patients, they found significant elevations in patients with active MS compared to remission; and levels of these conjugates correlated well with Gadolinium enhancement in MRI (108, 128).

5.4.2. Transendothelial migration of leukocytes through BBB

Since MS is an immune-mediated disorder in which transendothelial migration (TEM) of activated leukocytes through the BBB into brain is believed to be central to initiation of CNS inflammation, Jy *et al* (108, 128) and Jimenez *et al* (129) utilized a simple *in vitro* model of TEM to investigate a possible role of EMP in this process. In this model, TEM of monocytic U937 cells through a monolayer of brain microvascular EC (BBB model) was the probe for investigating the effects of plasma from MS patients or normal controls, with and without EMP. The most recent work, as yet reported only in the above abstract (129), demonstrated that (i) plasma from MS patients sharply increases TEM, (ii) pre-treatment of the leukocytes with EMP further facilitated TEM, (iii) leukocytes after passing through the monolayer exhibited bound EMP (surface markers acquired from EC) and (iv) drugs such as interferon-beta-1b and danazol inhibited TEM.

5.5. Acute coronary syndromes

Mallat *et al* measured MP in ACS by first capturing MP with immobilized AnV, quantitating them by prothrombinase activity, then identifying cell origins by an ELISA method, i.e. by capture on microtiter wells coated with anti -CD3, -CD11a, -CD31, -CD146, or -GPIb (72). They refer to their earlier studies for details of methods (84,

109). They studied 39 patients, of whom 12 had stable angina (SA) and 27 had documented ACS (angiograms), compared to 12 patient controls (72). Their main finding was that EMP (particles captured by anti-CD146 or anti-CD31), not other MP, were elevated in acute MI, by 2.5-fold ($p<0.01$). Two patients suffering acute episodes exhibited the highest levels of all. In this study, neither EMP or other MP levels differentiated between SA and normal controls, between unstable angina (UA) and MI, or between UA and normal controls. We commented above on the method of AnV capture: it biases the measurement toward apoptotic bodies, tending to miss EMP arising from activation (see §3.3.4 and §4.2), as might predominate in SA. Their prothrombinase method of quantitation, standardized in terms of known amounts (nmol/L) of defined PL vesicles as in a previous study by that group (109), cannot be compared to results from other laboratories which directly count the MP by flow cytometry. Despite these cavils, theirs was the first study to demonstrate elevated EMP in ACS (72).

Bernal *et al*, working in a similar direction at the same time on a larger number of patients (n=84), confirmed elevated EMP in ACS, using flow cytometry of two measures of EMP, CD31+/CD42- and CD51+ (75). A number of original findings from that work are of interest. First, they found that EMP levels were much higher in those suffering a first MI compared to those with prior or recurring MI, $p=0.01$ (75). In new onset patients, CD31+ EMP was higher in MI than UA ($p<0.05$) but this effect was lost when patients with prior history were included. Second, they found that EMP results by the two markers, CD31 and CD51, were distinctly different. For example, CD51+ EMP, although clearly elevated in ACS relative to controls, did not distinguish new ACS from recurring ACS as CD31+ EMP did. The authors conclude that CD31+ EMP is mainly a marker of acute events while CD51+ EMP reflects chronic endothelial stress, as also observed in MS (71). Third, their method was able to discriminate between SA and normal controls ($p<0.001$) (75).

The same authors, speculating that the new-onset effect might be due to endothelial exhaustion in patients with recurring or chronic CAD, studied the effect of using a blood-pressure cuff to cause repeated temporary ischemia of the forearm in normal volunteers, and confirmed a fatigue phenomenon: EMP release was reduced following repeated applications of the cuff (130). More recently, a further advance was made in demonstrating a clear correlation between EMP levels and risk score of lesions determined by coronary angiography (131).

Morel *et al* have recently reported that vitamin C appears to protect MI patients against further endothelial injury as measured by reduced EMP in vitamin C patients compared to placebo (87). Their method depended on capture with AnV. They found that PMP were higher than EMP in MI, by more than 6-fold.

5.6. Hypertension

Preston *et al* investigated a possible relation between hypertension (HTN) and endothelial injury as measured primarily by EMP. They studied patients with untreated severe (diastolic blood pressure (BP) $\geq$120) or mild (BP >95, <100) HTN compared to normal controls, and observed that EMP was highest in severe HTN ($p=0.002$) and showed a significant positive correlation with both systolic and diastolic BP (74), first reported at meetings (132, 133). Interestingly, they found no correlation between BP and soluble markers of endothelial activation (sVCAM-1, sICAM-1, vWF), and so concluded that EMP assay appears to be the most sensitive method for assessing BP-induced effects on the endothelium, and consequent risk of impending hypertensive vascular and organ damage (74).

5.7. Preeclampsia

Preeclampsia (PE) is a pregnancy-associated disorder in which endothelial damage is believed to play a role. Elevations of soluble markers of EC injury such as VCAM-1, ICAM-1, E-selectin, fibronectin and vWF lend support to this theory. Gonzalez-Quintero *et al* applied EMP analysis to a prospective, case-controlled study of twenty preeclamptic and twenty healthy pregnant women as controls, and demonstrated a significant elevation of CD31+CD42- EMP in PE (76). In more recent follow-up work employing also marker CD62E, results were clearer and more dramatic. Additionally, it was found that EMP correlated closely with proteinuria in that condition (unpublished data). They also observed a correlation between EMP and mean arterial BP, as in the work by Preston *et al* cited above (74).

In a series of related reports, Van Wijk *et al* (134-136) also investigated microparticles (MP) in preeclamptic women. One deals with the coagulant properties of generic MP, not EMP per se, and will not be considered here (134). Another deals with the effect of patient MP on endothelium-dependent artery relaxation (136); though it does not deal specifically with EMP, it is cited because it provides evidence supporting the hypothesis that endothelial dysfunction is involved with PE, and that MP can modulate this activity (overnight incubation with patient MP but not normal MP abolished bradykinin-mediated relaxation). Their other paper investigated MP in PE of various cellular origins, including EMP (135), but found no difference in EMP (or PMP) between PE and normal pregnancies, in conflict with the above cited work of Gonzalez-Quintero *et al* (76). We suggest that the explanation for this discrepancy may lie with the method: Van Wijk *et al* used positivity for AnV as the primary criterion in flow cytometry, which as previously explained (§3.3.4) misses the majority of activation-derived EMP; and even if the EC are apoptotic, detection efficiency by annexin V is limited.

Bretelle *et al* also recently studied MP in PE, normal pregnancy (NP), and intrauterine growth restriction (IUGR) (137). The relevant results were that no significant difference in EMP was found, or in "total MP" (AnV-positive), but a significant decrease, not increase, in PMP was observed in PE compared to NP. These findings, too, conflict with those of Gonzalez-Quintero *et al* (76). Possible explanations for the discrepancy include loss of

the majority of MP by excessively high-speed centrifugation (2 min at 13,000xg) and/or choice of fluorescent markers (137).

5.8. Diabetes

Sabatier *et al* compared MP numbers and PCA in controls vs. diabetes types 1 vs. 2, finding that type 1 had elevated PMP, EMP and total MP ("TMP", defined as annexin V-positive MP) as well as elevated PCA associated with the TMP (77). Type 2 showed elevation only in TMP numbers, not PMP, EMP or PCA. In the type 1 patients, the PCA activity level correlated with glycosylated hemoglobin, HbA(1c). The authors suggest that these findings may be related to vascular complications in diabetes mellitus.

5.9. Paroxysmal nocturnal hemoglobinuria and sickle cell crisis

Simak *et al* (94, 138) found marked elevations of MP of endothelial origin in paroxysmal nocturnal hemoglobinuria and SC disease, not in aplastic anemia. Interestingly, as observed by our laboratory (see §4.1), they observed different diagnostic significances depending on the marker used to identify EMP: they interpreted CD105+/CD54+ EMP to reflect chronic activation, since it failed to correlate with hemolysis indicated by LDH.

5.10. Work in progress

Results which follow are recent and thus far reported only in abstract.

5.10.1. Metabolic syndrome

Metabolic syndrome, a concurrent disturbance of insulin or glucose with obesity, dyslipidemia and hypertension, now affects approximately 10% of middle aged men in the United States and presents a serious challenge in public health since it predisposes to atherosclerosis and CAD. Present guidelines call for no intervention in early stages of this syndrome but we found clear-cut evidence that even in early stages, there is significant EC activation reflected in elevated EMP (CD31 or CD54 positive) and platelet activation reflected in increased PMP and activation marker CD62P (139). Increased platelet-leukocyte conjugates were also observed, suggesting inflammation in this syndrome. These results were taken to suggest that early prophylactic intervention may be warranted, especially because EMP assay may afford an easy and sensitive method of tracking patient progress. However, prospective study will be required to evaluate the cost/benefits of such a proposal aimed at minimizing development of CAD in this syndrome.

5.10.2. Hyperlipidemia

Recent work has demonstrated good correlation ($p=0.003$) between cholesterol levels and EMP measured by CD31+/CD42- EMP (95). However, EMP measured by CD62E showed no such relation, in further evidence of distinct EMP species. In the same study, it was shown that oxidized low-density lipoprotein (oxLDL) induced slow but continued release of CD31+ EMP from coronary artery EC; few CD62E+ EMP were detected (95). Plasma from patients with high cholesterol had an effect similar to oxLDL, including inducing apoptosis of the coronary artery EC.

5.10.3. Sepsis

High mortality in sepsis has been attributed to uncontrolled hyper-inflammation but recent findings challenge this, indicating that immunosuppression and impaired inflammatory reaction is to blame. Soriano *et al* studied EMP profiles in sepsis, along with intracellular nitric oxide (NO) and other markers, in 36 patients with sepsis (140). The 28 day mortality was 51%. Those who died had had much lower levels of EMP (and platelet-leukocyte conjugates), but increased NO in leukocytes, compared to survivors. When fitted to a model, the levels of these markers were highly predictive of mortality in sepsis. These data support the recent opinion that reduced endothelial cell activation and impaired inflammatory response is related to mortality in sepsis.

6. PERSPECTIVE

In summary, it appears that EMP analysis is emerging as the method of choice for assessing endothelial involvement in disease states. Although few studies have yet undertaken direct comparison of results by EMP *vs*. soluble markers, our EMP results for TTP, CAD, and MS appear to offer superior discrimination of clinical states as compared to other published studies employing soluble markers of endothelial disturbance.

On the other hand, there is ample room for improvement of EMP assay methodology. There is a need for inter-laboratory standardization of EMP assay. ELISA techniques may also be useful, especially in affording greater sensitivity than flow cytometry for weakly expressed markers, but must be adapted to distinguish between soluble and particle-bound antigens (such as by 0.1 um filtration). Alternative markers or combinations of them may improve flow cytometric analysis, and certain markers may be specific for particular disease states, kinds of endothelial injury, or regions or types of vasculature (e.g. micro- *vs*. maco-). That is, EMP phenotype analysis holds promise beyond discriminating EC apoptosis from activation.

The functional role(s) of EMP (discussed above §4.7) remain to be firmly established. Interaction of EMP with leukocytes, and their vWF-dependent interaction with platelets, suggests important functions in inflammation and thrombosis. Elucidation of mechanism of EMP generation could lead to pharmacological manipulation of EMP levels for therapeutic purposes.

We are confident that when such refinements are ushered in over the next few years, EMP analysis will find acceptance as a useful clinical method for assessing endothelial status.

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