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ENDOTHELIAL MICROPARTICLES (EMP) BIND AND ACTIVATE MONOCYTES: ELEVATED EMP-MONOCYTE CONJUGATES IN MULTIPLE SCLEROSIS

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1. ABSTRACT

Elevated plasma endothelial microparticles (EMP) have been documented in MS during exacerbation. However, the role of EMP in pathogenesis of MS remains unclear. We investigated the formation of EMP-monocyte conjugates (EMP-MoC) and their potential role in transendothelial migration of inflammatory cells in MS. EMP-MoC were assayed in 30 MS patients in exacerbation, 20 in remission and in 35 controls. EMP-leukocyte conjugation was investigated flowcytometrically by employing α-CD54 or α-CD62E for EMP, and α-CD45 for leukocytes. EMP-MoC were characterized by identifying adhesion molecules involved and their effect on monocyte function. *In vivo* (clinical): EMP-MoC were markedly elevated in exacerbation *vs*. remission and controls, correlating with presence of GD+ MRI lesions. Free CD54+ EMP were not elevated but free CD62E+ EMP were. *In vitro:* EMP bound preferentially to monocytes, less to neutrophils, but little to lymphocytes. Bound EMP activated monocytes: CD11b expression increased 50% and migration through cerebral endothelial cell layer increased 2.6-fold. Blockade of CD54 reduced binding by 80%. Most CD54+ EMP bound to monocytes, leaving little free EMP, while CD62+ EMP were found both free and bound. These results demonstrated that phenotypic subsets of EMP interacted differently with monocytes. Based on our observations, EMP may enhance inflammation and increase transendothelial migration of monocytes in MS by binding to and activating monocytes through CD54. EMP-MoC were markedly increased in MS patients in exacerbation compared to remission and may serve as a sensitive marker of MS disease activity.

2. INTRODUCTION

Multiple sclerosis (MS) is an immune mediated disease of the central nervous system (CNS), which is characterized clinically by a relapsing course and pathologically by multiple areas of inflammatory demyelination. Endothelial cells dysfunction and disruption of the blood brain barrier (BBB) followed by adhesion and transendothelial migration of activated monocytes and T cells are crucial steps in the pathogenesis of MS (1-3). Monocytes and T cells are the major cell types found in the perivenular infiltrates characteristic of MS (4, 5). In a recent clinical trial,

treating MS patients with natalizumab, a humanized monoclonal antibody to integrin $\alpha_4\beta_1$ (VLA-4), expressed on lymphocytes and monocytes and an important mediator of cell adhesion and transendothelial migration, markedly reduced the number of new lesions and relapses (6). These results further confirm the important role of adhesion and transendothelial migration of lymphocytes and monocytes in the pathogenesis of MS.

Under physiological conditions endothelial cells provide an antithrombotic and anticoagulant surface to the flowing blood. However, when the endothelial cells are perturbed by cytokines such as TNF-α (7), shear stress (8), or as a result of interaction with leukocytes and/or platelets (9), they express procoagulant phospholipids, tissue factor (TF) (10-12) and adhesion molecules including P-selectin (CD62P), E-selectin (CD62E), intercellular adhesion molecule-1 (ICAM-1/CD54), and vascular cell adhesion molecule-1 (VCAM-1/CD106) (13). Activated endothelial cells release small membrane vesicles, termed endothelial microparticles (EMP), bearing many adhesion molecules from the parent endothelial cell, notably platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), vitronectin receptor (integrin $\alpha_V\beta_3$ or CD51), CD54, and CD106 (14, 15). Elevated plasma levels of EMP have been documented in several diseases involving endothelial disturbance. These include multiple sclerosis (MS) (16), lupus anticoagulant (14), thrombotic thrombocytopenic purpura (TTP) (15), acute coronary syndromes (ACS) (17, 18), malignant hypertension (HTN) (19), pre-eclampsia (20) and diabetes mellitus (21). It has also been demonstrated that EMP interact with monocytic cells to induce expression of the monocyte β2 integrins Mac-1 (CD11b) and TF expression *in vitro* (22, 23). CD11b, a counter-receptor for ICAM-1, has been shown to play an important role in leukocyte rolling and adhesion to endothelium (24).

Despite our knowledge of high plasma EMP levels in MS patients, the functional significance of EMP in the pathogenesis of MS remains unclear. In the present study, we investigated a new dimension of EC-leukocyte interaction in the context of MS. The first part of the report explores correlations between plasma levels of EMP, EMP-monocyte conjugates, and the neuroradiological features of MS patients. The second part explores the differential binding of EMP to monocytes, lymphocytes, and neutrophils and their effect on transendothelial migration of monocytes in an *in vitro* model. We present evidence identifying the major adhesion molecules involved in this interaction and migration, and examine the implications of the *in vitro* findings to pathogenesis of MS.

3. MATERIALS AND METHODS

3.1. Patient population

We studied 50 patients who met the Poser criteria (25) for MS. Their mean age was 31years, and 75% were women. The Institutional Review Board (IRB) approved the study and all patients provided informed consent. All patients had been underwent neurological examination and exacerbation was confirmed in 30 patients. None had received any immunosuppressive or immunomodulatory treatments for at least 6 months prior to entry in the study. Additionally, 35 normal volunteers were recruited as controls with a mean age of 34 years, and 70% were women.

3.2. Neuro-imaging

Brain and spinal cord MRI were performed in all patients on a 1.5 T machine with standard head coils. The imaging protocol included sagittal T1-, axial T1-, T2weighted, and FLAIR images. All MRI scans were performed after infusion of gadolinium diethylenetriamine pentaacetic acid (Gd). Axial T2- and post contrast T1-weighted images were used for assessment of MS plaques. The images were independently interpreted by neuroradiologists blinded to the study, using visual inspection.

3.3. Laboratory investigations

3.3.1. Antibodies and other reagents

The following fluorescent-tagged anti-human monoclonal antibodies (mAbs) were obtained from commercial suppliers: from Pharmingen, anti-CD31 (Cat. #555446, PE label), anti-CD51/61 (Cat. #555504, FITC label), anti-CD45 (Cat. #555483, PE label), anti-CD11b (Cat. #555389, Cychrome label); from Sigma, anti-CD54 (Cat. #F-0549, FITC label), anti-CD62E (Cat. #F-0674, FITC label); from Ancell, anti-CD105 (Cat. #326-040, FITC label). The lectin, *Ulex europaeus,* FITC-labeled, was obtained from Sigma (Cat. #L9006). The following non-fluorescent anti-human mAbs described by the suppliers as blockers of their respective binding sites were purchased from Chemicon: anti-ICAM-1 (Cat. #MAB2146), anti-VCAM (Cat. #MAB2144), and anti-E-selectin (Cat. #MAB2150). All mAbs are used without dilution.

3.3.2. Assay of whole blood EMP-lymphocyte, EMP-monocyte, or EMP-neutrophil conjugates in clinical studies

5 µL of citrated blood plus 20 L of PBS is incubated with 4 µL of anti-CD45-PE plus 4 µL of anti-CD54-FITC or anti-CD62E-FITC, for 30 min, then diluted with 1 mL of PBS for 2-color flow cytometry. By using fluorescent triggering on the red (PE) signal, all CD45+ cells (lymphocyte, monocytes and neutrophils) are displayed. Lymphocytes, monocytes, and neutrophils are identified by their distinctive CD45 fluorescence (lymphocyte > monocytes > neutrophils) and side scattering (neutrophils > monocytes > lymphocytes). EMP-leukocyte conjugates are assayed by measuring the co-expression of CD54 or CD62E antigens. The cut-off for defining leukocytes positive for EMP binding is based on a value of CD54 or CD62E fluorescence such that <5% of normal lymphocytes exceed this value. The same setting is used for monocytes and neutrophils, where typically 10-20% were positive in normal controls.

3.3.3. Monocyte and endothelial cell culture and treatments

All studies are conducted with human cerebral microvascular EC (MVEC) and maintained in culture as

previously described. (15) Activation of MVEC is induced by 24 hour incubation with TNF-α, or by apoptosis induced by serum and growth factors deprivation (Ser/GF Dep), for 24 hours, also as previously described (15). These treatments consistently induce endothelial activation (judged by the emergence of activation-dependent surface markers) or endothelial apoptosis, as judged by TUNEL assay and other methods (15). Monocytic cell line U937 was obtained from ATCC (Cat. #CRL-1593.2). These cells are cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS).

3.3.4. EMP pre-labeling and binding with monocytic cell line U937

EMP are generated by treating brain MVEC culture for 24 hr with TNF-α (10 ng/mL), then collecting the supernatants by centrifugation for 15 min at 42,000 x g. The resulting pellet is re-suspended in 1/10 the original volume of culture medium. Then to each 100 µL aliquot of EMP, 10 µL of each mAb of interest including CD31, CD54, CD62E, CD105, or CD106 are added. After 30 min incubation, the labeled EMP are washed twice in PBS (15 min, 42,000 x g), then re-suspended in 100 µL of PBS. Following this, 50 µL of labeled EMP are incubated with 20 µL of U937 cells ($1x10^6$ cells/mL) for 30 min. The EMP-monocyte conjugates are then assayed by flow cytometry and degree of binding expressed as the mean fluorescent intensity of the EMP label co-expressed with the cells.

For the blocking experiments, 100 µL of EMP produced as above are first fluorescently labeled by incubation with 10 µL of FITC-conjugated lectin, *Ulex europaeus* (100 µg/mL), for 30 min, washed as above and 50 µL of the labeled EMP are exposed to the above mentioned non-fluorescent blocking antibodies (10 µg/mL final conc.). The mixture is then incubated with U937 cells as above and binding of EMP determined by the fluorescent intensity of the FITC-*Ulex europaeus*.

3.3.5. *In vitro* assay of whole blood EMP-lymphocyte, EMP-monocyte, and EMP-neutrophil conjugates in experiments

5 µL of citrated blood plus 20 µL of PBS is incubated with 4 µL of anti-CD45-PE, as above, then 50 µL of EMP from activated brain MVEC pre-labeled with FITC-*Ulex europaeus* as explained above, see (3.3.4), was added. After 30 min, the mixtures were diluted with 1 mL of PBS for assaying EMP-leukocyte conjugates with 2-color flow cytometry. By using fluorescent triggering on the red (PE) signal, all CD45+ particles are displayed. Lymphocytes, monocytes, and neutrophils are identified by their distinctive CD45 fluorescence and side scattering. The conjugates are assayed by measuring the fluorescent intensity of CD54 or CD62E of EMP adhering to the leukocytes, and results expressed either in terms of mean fluorescent intensity (arbitrary units displayed on flow cytometer) or as percentage of leukocytes positive for an EC marker, as indicated.

3.3.6. Transendothelial migration (TEM) assay

These experiments are done using monocytic U937 cells, either control (untreated) or pretreated by exposure to EMP as described below. They were not otherwise deliberately activated. EMP prepared and preincubated with U937, and untreated U937 cells (controls), are added to a confluent monolayer of brain microvascular EC (MVEC) at $1x10^5$ U937 cells/mL. The EC were grown on a membrane filter (Corning Transwell) allowing recovery of transmigrated cells. After 60 min, the filtrate (lower chamber) and the supernatant (above the EC) are counted for monocytes and the percent of the total that migrated across the MVEC layer is determined.

Assay of endothelial microparticles (EMP) (15): 50 µL PPP are incubated with 4 µL of anti-CD31-PE plus 4 µL anti-CD42-FITC or 4 µL of anti-CD62E for 20 min with gentle (100 RPM) orbital shaking. 1 mL of PBS buffer is then added for flow cytometry on a Coulter EPICS XL flow cytometer. The rationale for the two-color method (CD31 and CD42) is that significant CD31 occurs on both EMP and PMP, whereas CD42 is restricted to platelets, allowing discrimination between them. On the other hand, since CD62E is a specific marker for endothelium, multiple labels are not needed. Detection of particles is set to trigger by CD31 or CD62E fluorescence signal greater than noise, and fluorescent particles are further separated on a histogram based on size. CD31+ EMP are defined as CD31+/CD42- particles with size < 1.5 µm. CD62E+ EMP are defined as CD62E+ particles with size < 1.5 µm.

3.4. Statistical analysis

One-way ANOVA (analysis of variance) was used to analyze the difference among three or more groups. If there were significant differences ($p < 0.05$) among these groups, then student's t-test was used to evaluate significance between pairs of groups. In cases where the data failed the normality test, then the Mann-Whitney rank sum test was used. The Chi-square test was used to compare frequency data between groups. All data analyses were performed using the Windows-based program, Statmost.

4. RESULTS

4.1. EMP-Monocyte conjugate (EMP-MoC)

Using two endothelial markers, CD54 and CD62E, we assayed EMP conjugated with monocytes or lymphocytes as well as free EMP in MS patients during exacerbation and remission, and normal controls. The major findings are summarized in Figure 1. During MS exacerbations, 64 ±14% of monocytes are conjugated with CD54+ EMP, as compared to 30 ±13% in patients in remission ($p<0.001$) and 21 ±6% in normal subjects ($p<0.001$) (Figure 1A). In contrast, only 6 ±3% of lymphocytes from MS patients in exacerbation are conjugated with CD54+ EMP, and this value does not differ significantly from those in remission or controls (Figure 1A). These findings are consistent with the results of EMP binding to leukocytes *in vitro,* see section 4.4. (Figure 3).

Results for CD62E+ EMP-MoC were similar to those for CD54+ EMP-MoC except that the absolute values are lower (Figure 1B). CD62E+ EMP-MoC are significantly elevated during-MS exacerbations (47 ± 11%)

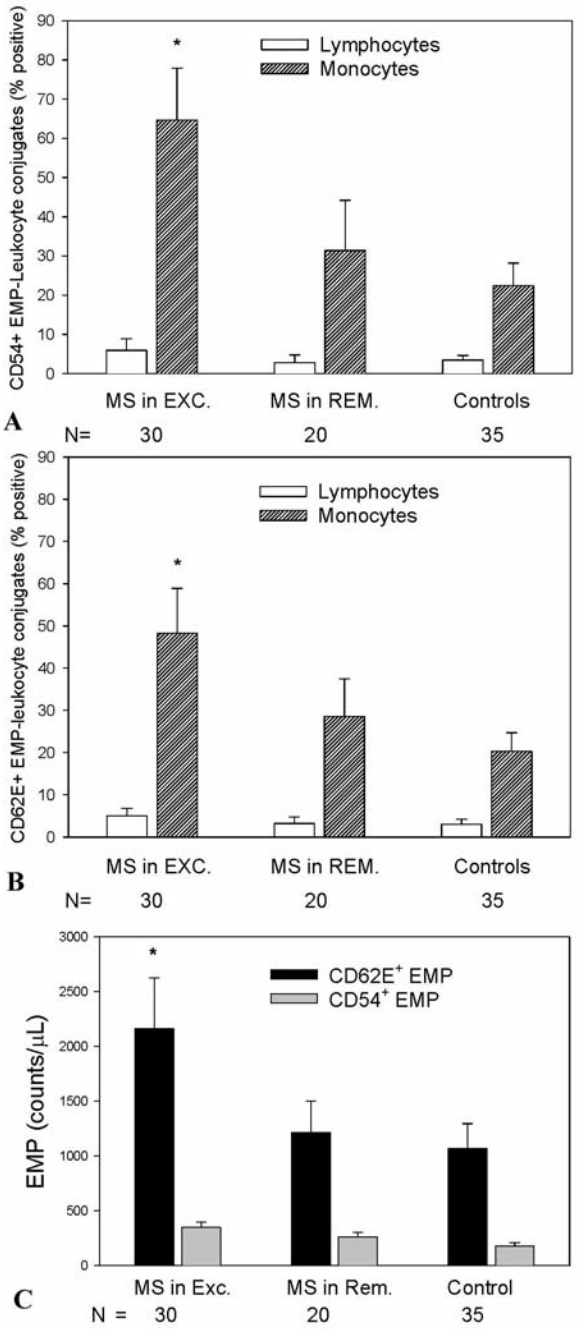

Figure 1. Comparison of EMP-leukocyte conjugates and EMP in MS patients in exacerbation *vs*. remission. (A) CD54+ EMP-leukocyte conjugates; (B) CD62E+ EMP-leukocyte conjugates; (C) CD54+ and CD62E+ free EMP. * indicates $p < 0.01$ as compared to controls or MS in remission. Error bars represent mean ± standard deviation.

compared to those in remission (27 ±9%) or to normal controls (19 ±4%). There is no significant difference in CD62E+ EMP-LymC between MS in exacerbation *vs*. remission or controls.

4.2. Levels of free EMP

We also measured and compared two species of free EMP (CD54+ and CD62E+) in plasma in the three groups. These markers are different from those used in the previous study (16) (CD51 and CD31). Free CD62E+ EMP are significantly elevated in exacerbation as compared to MS in remission or controls (Figure 1C). However, free CD54+ EMP are low and do not differ significantly between any pairs of the three groups, in contrast to the CD54+ EMP-MoC (Figure 1A). This suggests that most CD54+ EMP circulate bound as EMP-MoC.

4.3. Relation of EMP-MoC with MRI findings

A comparison of clinical, laboratory, and MRI data are presented in Table 1. Twenty-two of 30 MS patients (73%) in exacerbation had gadolinium enhancing lesions (Gd+). All 22 of these Gd+ patients (100%) also exhibited elevated CD54+ EMP-MoC. Indeed, assay of EMP-MoC appears to be somewhat more sensitive to exacerbation than Gd+ since only 3 of 8 MS patients who were in clinical exacerbation but did not have enhancing lesions on their MRI, did not have high levels of EMP-MoC. In all patients combined (Gd+ and Gd-), presence or absence of high levels of EMP-MoC is consistent with data of Gd± MRI in 41 of 50 cases (82%).

4.4. Binding of EMP from tissue culture to leukocytes in whole blood

EMP released by EC, treated for 24 hr with TNF-α to induce activation, or deprived of serum and growth factors (Ser/GF Dep) to induce apoptosis, displayed distinctive patterns of surface adhesion molecules (Figure 2). The levels of CD54+ EMP induced by TNF-α were 10- to 20-fold greater than those induced by Ser/GF Dep. Conversely, the levels of CD31+ EMP induced by TNF-α were significantly less than those induced by Ser/GF Dep (Figure 2). When EMP from these two type of experiments (TNF- or Ser/GF Dep) are pre-labeled with FITC-*Ulex europaeus*, washed, adjusted to equal concentrations, and then incubated with fresh whole blood (citrated, unstimulated, from normal controls), those from TNF-α treatment result in 2-fold greater binding to monocytes and neutrophils compared with EMP from Ser/GF Dep treatment, as defined by percent leukocytes positive for EMP (Figure 3). Clearly, EMP bind preferentially to monocytes as compared to neutrophils or lymphocytes.

4.5. Binding of EMP to monocytic U937 cells

To explore the nature of this interaction, we incubated monocytic U937 cells with aliquots of EMP pre-labeled with a panel of endothelial surface markers. Those EMP pre-labeled with ICAM-1 (CD54) exhibit the greatest apparent binding (Figure 4A). Because it might be argued that this results from the intensity of the ICAM-1-labeled signal or other artifact, we also measured the remaining free EMP in the cell-free supernatant: CD54-labeled EMP was most depleted, consistent with the majority having bound to the U937 cells (Figure 4B). Binding of EMP labeled at CD31 or at CD105 was approximately equal and both were significantly less than CD54-labeled EMP. In contrast, EMP labeled with CD51, CD62E or CD106 bound only weakly to U937. All of these results are consistent with the relative amounts of non-bound EMP found in the cell-free supernatants (Figure 4B).

Table 1. Correlation of CD54+ EMP-monocyte complex with clinical evaluation and MRI findings

Clinical evaluation	**MS in exacerbation**		**MS in remission**	
MRI findings	**MRI +**	**MRI -**	**MRI +**	**MRI -**
EMP-Monocyte Conjugates +	22	5	4	3
EMP-Monocyte Conjugates -	0	3	1	12

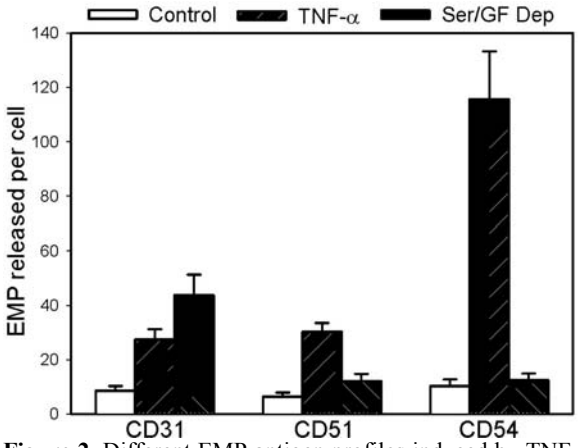

Figure 2. Different EMP antigen profiles induced by TNF-α or serum/growth factor depletion (Ser/GF Dep). EMP, derived from brain EC treated with TNF-α (10 ng/mL) or Ser/GF dep for 24 hours, were labeled with fluorescent markers of CD31, CD51, or CD54 for 20 min, then were assayed with flow cytometry. Error bars represent mean ± standard deviation; n = 6.

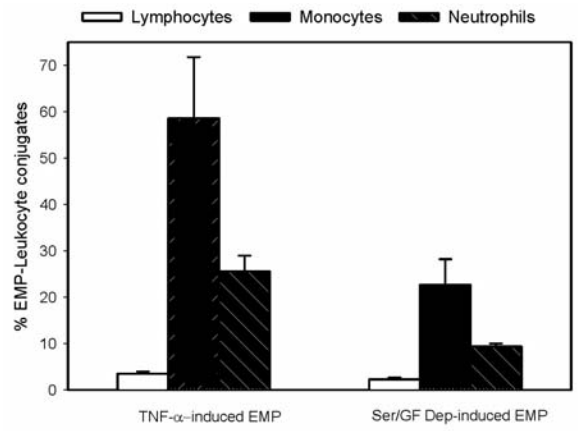

Figure 3. Binding of EMP to blood leukocytes. EMP, pre-labeled with FITC-*Ulex europaeus,* were incubated with normal blood for 30 min as described in "Methods" section 3.3.4**,** then the mixtures were assayed for EMP-lymphocyte, EMP-monocyte, or EMP–neutrophil conjugates by flow cytometry. Error bars represent mean ± standard deviation; n = 6.

4.6. Effect of blocking adhesive molecules on EMP binding

Addition of blocking mAbs against CD54 (ICAM-1), CD62E (E-selectin), and CD106 (VCAM-1) resulted in inhibition of EMP binding to U937 cells of 80%, 30%, and 22%, respectively (Figure 5) The combination of all three of these mAbs resulted in 90% inhibition of EMP binding. These results suggest that CD54 (ICAM-1) is the major adhesion molecule on EMP responsible for EMP-MoC formation.

4.7. Effect of EMP on transendothelial migration of U937 cells

To further explore possible effects of EMP binding on monocyte functional activity, we compared transendothelial migration of U937 cells through EC monolayer alone, *vs*. conjugated with EMP. As shown in Figure 6A, monocytes conjugated with EMP resulted in 2.6-fold greater transendothelial migration rate than U937 alone (sham-treated). See legend to the figure for details. We also assayed for CD11b expression on the monocytes in the presence and absence of EMP binding, with results summarized in Figure 6B. It was observed that CD11b expression is significantly elevated (by about 60%) after EMP binding, indicating monocyte activation by EMP (Figure 6B).

5. DISCUSSION AND SUMMARY

We previously reported that assay of plasma CD31+ EMP serves as a sensitive indicator of disease activity since MS plasma levels were elevated 2.85-fold in exacerbation compared to remission or controls, and were associated with presence of gadolinium-enhancing (Gd+) lesions on brain MRI (16); CD51+ EMP were slightly elevated in both remission and exacerbation. In the present study, free EMP bearing markers of CD54 and CD62E (Figure 1C) did not show superior discrimination of disease activity than CD31 in the previous report. In the present study MS patients showed marked increases in circulating EMP-MoC conjugates during exacerbations. The increases correlated with Gd+ MRI, whereas values were near normal in remission. Assay of EMP-monocyte conjugates (EMP-MoC) gave superior results when analyzed on an individual patient basis (Table 1) and their correlation with Gd+ MRI appeared to be superior to CD31+ EMP: with the majority of Gd+ patients exhibited elevated blood levels of EMP-MoC. Therefore this method, if suitably refined and standardized, may have valuable clinical application in monitoring disease activity of MS.

Our findings on leukocyte-EMP interaction indicate that EMP bind strongly to monocytes and minimally to lymphocytes. The EMP-binding process activates monocytes, as evidenced by upregulation of the adhesion molecule CD11b (Mac-1). Furthermore, EMP binding facilitates transendothelial migration, as illustrated by our model showing monocytes migration through human cerebral endothelial monolayer *in vitro*. In our previous study of MS patients, we found that measuring EMP using two different antigenic markers (CD31, CD51) gave different results: Elevated values of CD31+ EMP correlated well with exacerbation but CD51+ EMP did not. This led to *in vitro* studies where we found that different

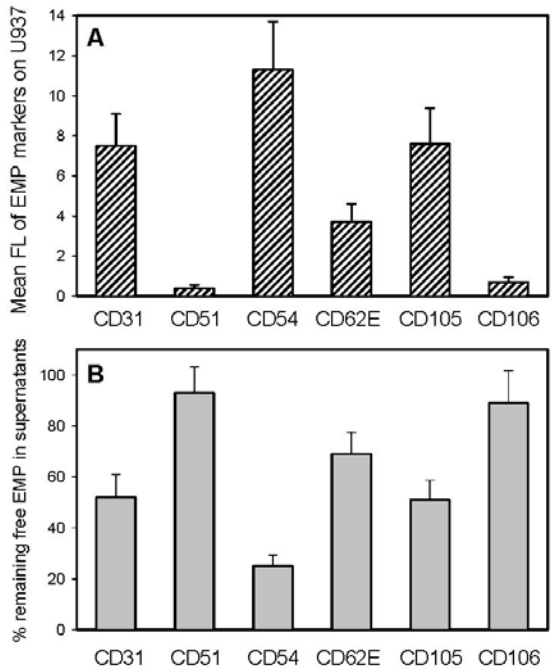

Figure 4. Binding of EMP with the indicated labels to U937 cells. EMP, pre-labeled with different endothelial markers including CD31, CD51, CD54, CD62E, CD105, or CD106, were incubated with U937 cells for 30 min as described "Methods" section, then the mixtures were centrifuged at 160 x g for 10 min. The supernatants were collected and used for assaying the remaining free EMP as shown in the bottom panel (B). The pellets were re-suspended in PBS and were assayed for EMP-monocyte conjugate formation as shown in the top panel (A). Error bars represent mean ± standard deviation; n = 4.

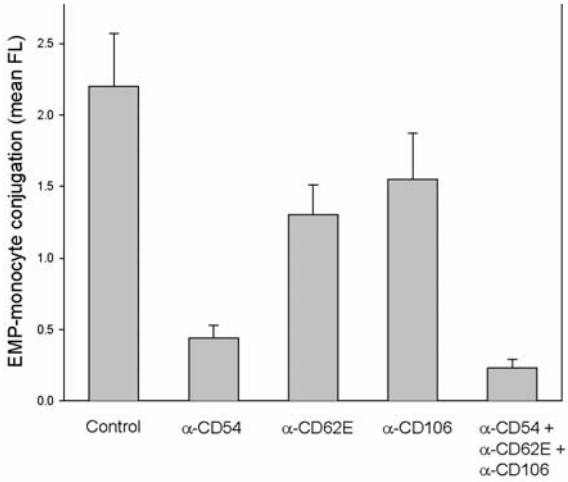

Figure 5. Effects of blocking antibodies against CD54, CD62E, or CD106 on EMP-monocyte interaction. EMP, pre-labeled with FITC-*Ulex europaeus,* were incubated with U937 cells in the presence or absence of blocking antibodies against CD54, CD62E, or CD106 (10 μg/mL final concentration). The degree of EMP-monocyte conjugation was expressed by the fluorescent intensity of labeled EMP on monocytes. Error bars represent mean ± standard deviation; n = 4.

kinds of endothelial injury (activation *vs.* apoptosis) gave rise to distinctive patterns of EMP phenotypes as defined by a panel of antigenic markers (Figure 2).

Endothelial microparticles (EMP) identified by different markers exhibit markedly different binding affinities for monocytes. Those identified by CD54 (ICAM-1) expression bind most avidly to monocytes, followed by EMP identified by CD31 (PECAM-1) or CD105 (endoglin), whereas those identified by CD51, CD62E (E-selectin) or CD106 (VCAM-1) bound only weakly to monocytes (Figure 4).

We hypothesize that these species arise from lipid rafts carrying distinctive assemblies of receptors and other markers (for review, see (26)) budding off as EMP species. Recently, we reported that membrane clustering (capping) of CD31 (PECAM-1) on EC exhibited morphology distinct from that of CD54 (ICAM-1) (27) and proposed that such membrane clustering results in release of EMP enriched in the respective species (CD31, CD54). However, it is likely that these EMP species overlap in some antigens, as may be implied by the similar behavior of CD31+ and CD105+ EMP (Figure 4).

Greater EMP binding affinity for monocytes, compared to lymphocytes, may be due to specific ligands expressed on monocytes while are absent from lymphocytes. Because integrin CD11b (Mac-1) is expressed on monocytes and neutrophils but minimally on lymphocytes, and is a *counter*-receptor for ICAM-1 (24), it is consistent with our observation of EMP binding to monocytes but not lymphocytes, and confirmed by inhibition of binding by CD54 blockade. A functional role of EMP-monocyte may be implicit. A recent study demonstrated that binding of EMP to monocytes induced monocyte activation, including enhanced tissue factor activity (23). Supporting this argument, we found that EMP binding induced significantly increased expression of monocyte CD11b, which in turn may lead to increased binding to activated endothelium.

Transendothelial migration of monocytes and CD4+ T lymphocytes across the BBB is believed to be a major step in formation of demyelinating lesions in MS. Mechanisms of leukocyte transendothelial migration have been studied extensively but details remain elusive. Multiple adhesion molecules have been proposed to play specific roles in these events. Adhesion molecules, such as endothelial leukocyte adhesion molecule-1 (ELAM-1) and VCAM-1, have been implicated in leukocyte adhesion to endothelium. (28-30) Also, PECAM-1, has been shown to be an important player in monocytes transendothelial migration (30-32), particularly in MS (33).

Migration of activated leukocytes endothelial cell monolayer involves a sequence of steps; first, rolling then adhesion, followed by passage through the endothelial junction. However, the present study suggests additional complexities, insofar as we have shown that EMP binding significantly increases the expression of adhesion molecules CD11b on monocytes and enhances the rate of

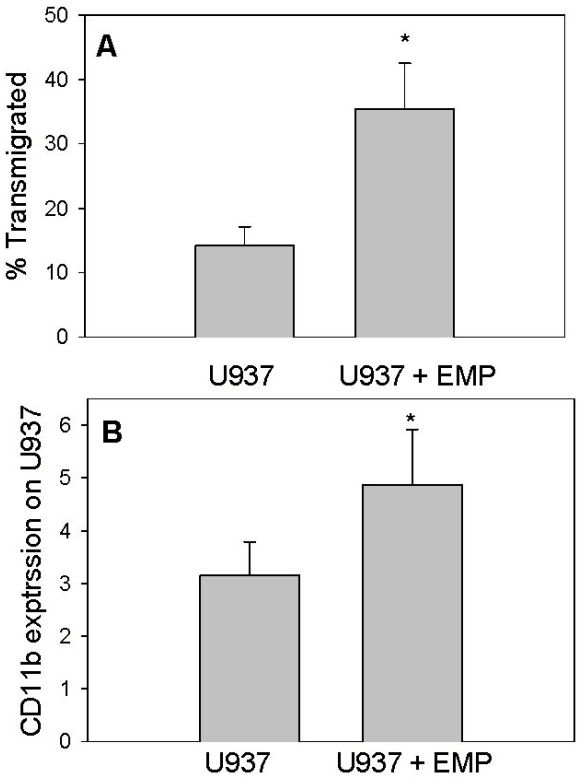

Figure 6. Effect of EMP binding on monocyte transmigration and CD11b expression. U937 cells were incubated with or without EMP for 30 min, then were assayed for monocyte transmigration (A) or CD11b expression (B). * indicates $p < 0.05$ as compared to controls. Error bars represent mean ± standard deviation; $n = 4$.

monocyte transendothelial migration through brain microvascular endothelial cell monolayer. Specifically, our data suggests that monocytes conjugated with EMP have enhanced adhesion to endothelial cells. These events indicate the "positive feedback" or amplification of adhesion events, once adhesion has been initiated. However, further studies are needed to gain clearer insights on the role of EMP in transendothelial migration of leukocytes in pathogenesis of MS.

In summary, EMP are heterogeneous species of membrane vesicles released from injured endothelium. They preferentially bind to and activate monocytes, and when so bound, enhance monocyte migration through the endothelial monolayer *in vitro*. In clinical studies, levels of EMP-monocyte conjugates were significantly elevated during exacerbations of MS relative to remission or controls (Figure 1), and this correlated with Gd+ MRI lesions (Table 1) Measurement of endothelial micro particle conjugates (EMP-MoC) has promise as a sensitive new marker of disease activity in MS.

6. ACKNOWLDEGMENTS

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