Endothelial microparticles released in thrombotic thrombocytopenic purpura express von Willebrand factor and markers of endothelial activation

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Received 23 June 2003; accepted for publication 12 September 2003

Summary. It has been suggested that endothelial apoptosis is a primary lesion in the pathogenesis of thrombotic thrombocytopenic purpura (TTP). We tested this hypothesis by examining the phenotypic signatures of endothelial microparticles (EMP) in TTP patients. In addition, the effect of TTP plasma on microvascular endothelial cells (MVEC) in culture was further delineated. EMP released by endothelial cells (EC) express markers of the parent EC; EMP released in activation carry predominantly CD54 and CD62E, while those in apoptosis CD31 and CD105. We investigated EMP release in vitro and in TTP patients. Following incubation of MVEC with TTP plasma, EMP and EC were analysed by flow cytometry for the expression of CD31, CD51, CD54, CD62E, CD105, CD106 and von Willebrand factor (VWF) antigen. EMP were also analysed in 12 TTP patients. In both EC and EMP, CD62E and CD54 expression were increased 3- to 10-fold and 8- to 10-fold respectively. However, CD31 and CD105 were reduced 40–60% in EC but increased twofold in EMP. VWF expression was found in 55 ± 15% of CD62E+ EMP. Markers of apoptosis were negative. In TTP patients, CD62E+ and CD31+/CD42b− EMP were markedly elevated, and preceded and correlated well with a rise in platelet counts and a fall in lactate dehydrogenase. CD62E+ EMP (60 ± 20%) co-expressed VWF and CD62E. The ratio of CD31+/CD42b− to CD62E+ EMP exhibited a pattern consistent with activation. In conclusion, our studies indicate endothelial activation in TTP. EMP that co-express VWF and CD62E could play a role in the pathogenesis of TTP.

Keywords: von Willebrand factor, endothelial microparticles, thrombotic thrombocytopenic purpura, endothelium, endothelial cell activation.
used to measure EMP as follows: (a) to measure CD62E + of PPP (30 min and assayed for EMP within 4 h of venipuncture. Aliquots to obtain platelet-poor plasma (PPP). PPP was prepared by centrifuging 10 min at 1600 g to prepare platelet-poor plasma (PPP). PPP was prepared and assayed for EMP within 4 h of venepuncture. All aliquots of PPP (30 μl) in 12 × 75 mm polypropylene tubes were used to measure EMP as follows: (a) to measure CD62E+ EMP, PPP was incubated with 5 μl of PE-labelled anti-CD62E; (b) to measure CD31+/CD42b+ EMP, PPP was incubated with 5 μl each of PE-labelled anti-CD31 and FITC-labelled anti-CD42b. The purpose of the anti-CD42b was to exclude platelet microparticles (PMP), as explained previously (Jimenez et al., 2001).

Statistical analysis. Student’s t-test was used to evaluate significance between pairs of groups. In cases where the data failed the Kolmogorov–Smirnov normality test, the Mann–Whitney rank sum test was used. Statistical significance was defined as *P < 0.05*. All data analyses were performed using the software SigmaPlot 4.0 and Statmost.

RESULTS

Effect of TTP plasma on EMP and remnant whole EC in cultures. GFD-induced apoptosis in MVEC, as indicated by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) positivity, and viability by trypan blue was 72 ± 12% and 8 ± 3% for renal MVEC, and 53 ± 12% and 15 ± 4% for brain MVEC respectively. Exposure of MVEC to

TNF-α did not result in apoptosis as indicated by TUNEL positivity of <2% and viability of >93% for all cultures, confirming our previous reports (Jimenez et al., 2001, 2003; Jy et al., 2002a).

Figure 1 summarizes the data on immunophenotypes of renal EC (Fig 1A,B) and the EMP counts (Fig 1C,D). Figure 1A shows that constitutive markers CD31 and CD105 on whole EC were substantially reduced in GFD when compared with untreated EC (Un), while the EMP bearing these markers were greatly increased by GFD treatment (Fig 1C). However, as shown in Fig 1B, inducible markers CD54 and CD62E on the parent EC were increased by activation (TNF-α) but not by apoptosis (GFD), and likewise, the EMP released (Fig 1D). When these results were compared with those from plasma treatments, it was seen that TTP plasma from the acute stage (TTP-A) induced a phenotypic signature consistent with activation, not apoptosis, because of the expression of inducible markers on the EC surface and EMP (Fig 1B,D). Note that plasma from TTP patients in remission (TTP-R) had no such effect; nor did exposure to plasma from ITP patients or normal controls. Although CD31+ and CD105+ EMP were increased moderately by TTP plasma (Fig 1C), this effect was only marginally greater than that of TNF-α and the TTP plasma caused no decrease in CD105 surface expression (Fig 1A). We previously found a ratio of CD62E+ to CD31+ EMP of >4.0 in activation and <0.4 in apoptosis (Jimenez et al., 2003). In the present study, this ratio was 5.6 for MVEC stimulated with TTP plasma.

The above studies were conducted with renal MVEC. We also investigated brain MVEC by the same experiments and obtained data comparable with that given above (not shown).

EMP in TTP patients

Data on a representative patient are plotted in Fig 2A, together with the platelet counts (Fig 2B) and LDH (Fig 2C). All patients tested presented similar EMP patterns. It should be noted that both measures of EMP returned to normal after remission was achieved on day 18 (Fig 2).

As shown in Fig 3, both CD31+/CD42b and CD62E+ EMP counts were significantly elevated in TTP patients during the acute phase of TTP when compared with counts obtained in normal controls or in patients in remission. In three patients, EMP measurements were performed pre- and post-plasmapheresis. CD62E+ and CD31+ EMP counts fell...
sharply after the first treatment and thereafter declined steadily (Fig 4).

Association of VWF with EMP
As shown in Fig 5A, supernatants from cultured renal MVEC stimulated with plasma from five TTP patients yielded EMP that co-expressed VWF with CD62E at a level about 15-fold that of five normal controls. Figure 5B shows corresponding EMP plasma results in five TTP patients and five controls: VWF co-expressed with CD62E on EMP in the plasma of TTP patients was about fivefold of that found in normal control plasma.

DISCUSSION
Endothelial dysfunction plays a central role in the pathogenesis of many thrombotic and inflammatory disorders (Wu & Thiagarajan, 1996). However, existing laboratory tests to define or monitor endothelial disturbances in the clinical setting are limited. We have reported elevated CD31+/CD42b EMP in patients in active phases of TTP and multiple sclerosis (MS), and observed that the levels decreased to normal in remission (Jimenez et al., 2001; Minagar et al., 2001). We have also reported elevated EMP in pre-eclampsia, acute coronary syndromes and malignant hypertension (Bernal-Mizrachi et al., 2003; Gonzalez-Quintero et al., 2003; Preston et al., 2003). Others have reported elevated EMP in patients with lupus anti-coagulant, coronary artery disease and diabetes mellitus (Combes et al., 1999; Mallat et al., 2000; Boulanger et al., 2001; Sabatier et al., 2002a). Clinical applications of an EMP assay show promise for monitoring endothelial injury in thrombotic and inflammatory disorders.

We and others have shown that EMP are heterogeneous, as defined by antigenic phenotypes, and that their relative abundance and phenotype reflects the nature of the EC injury (Combes et al., 1999; Jimenez et al., 2001, 2003). In addition, EMP express tissue factor (TF) and provide a phospholipid surface, indicating procoagulant potential (Combes et al., 1999; Jimenez et al., 2001). Further investigation of
the mechanisms of generation and functional characteristics of EMP in different disorders involving endothelial perturbation could lead to the improved understanding of the endothelial lesion in specific vascular, thrombotic and inflammatory conditions.

Endothelial injury is believed to be the primary underlying lesion in TTP, initiating intravascular platelet adhesion, aggregation and formation of platelet rich thrombi in microvasculature (Ridolfi & Bell, 1981). However, the precise nature of the endothelial lesion in TTP is poorly understood. It has been reported that TTP plasma induced apoptosis of EC derived from brain, renal, or dermal microvascular origin, but not EC derived from larger vascular beds (Laurence et al., 1996; Mitra et al., 1997).

Thus, it has been argued that apoptosis may be the principal endotheliopathy in TTP (Laurence et al., 1996; Laurence & Mitra, 1997; Mitra et al., 1997, 1998).

In the present study, we investigated the phenotypic profiles of EMP that were released upon incubation of TTP plasma with EC in vitro as well as EMP in blood samples from patients with acute TTP. Our previous studies showed that CD62E and CD31 are the most useful markers of EMP found to date. In activation, CD62E+ EMP are the most abundant, while in apoptosis, CD31+ EMP predominate. Accordingly, the ratio of CD62+ EMP to CD31+ EMP provides a useful measure for discriminating a state of endothelial activation from apoptosis: this ratio is high in activation, low in apoptosis (Jimenez et al., 2003). This conclusion was based on findings obtained with TNF-α, a known activator, and deprivation of growth factor, conditions known to induce apoptosis (Pober et al., 1986; Hogg et al., 1999).

When plasma from TTP patients was incubated with MVEC, TTP plasma induced release of EMP expressing predominantly CD62E, hence the CD62E/CD31 ratio was consistent with endothelial activation, not apoptosis. Parallel clinical studies (in vivo) on EMP in TTP patients revealed that CD62+ EMP were more abundant than CD31+ EMP, indicating that EMP in TTP are released from
activated, not apoptotic. EC. Praprotnik et al (2001) recently reported that stimulation of EC with TTP plasma resulted in a state of endothelial activation.

The present report also includes data on EMP pre- and post-plasmapheresis. Interestingly, all four patients for whom this data were available showed a dramatic drop in EMP after the first treatment; thereafter, a more gradual reduction after each plasma exchange was observed. It is possible that the first plasmapheresis may remove large quantities of thrombogenic EMP accumulated in the circulation while subsequent procedures may clear only the newly released EMP. Alternatively, plasmapheresis may eliminate a factor present in TTP plasma that perturbs the endothelium, possibly an antibody or cytokine such as TNF-α. In this regard, Wada et al (1993) reported that TNF-α was elevated in the circulation of TTP patients during the onset of TTP. This would be consistent with our finding that TNF-α acts selectively on the microvasculature, eliciting a potent response in MVEC but having little effect on coronary artery EC at the same concentration (Jimenez et al, 2003).

Our study raises the question of the potential role of EMP in TTP. EMP are procoagulant, by virtue of both TF and procoagulant phospholipids (platelet factor three activity) (Combes et al, 1999; Jimenez et al, 2001). Our finding in vitro that TTP plasma induces release of EMP rich in VWF, and in vivo that EMP in TTP patients also express VWF, suggests a role of EMP in the pathogenesis of TTP. Impaired proteolysis of ULVWF multimers in TTP patients due to the decreased activity of the metalloprotease ADAMTS-13 (a disintegrin and metalloproteinase with thrombospondin motif-1) is currently a favoured hypothesis for the pathogenesis of TTP (Moake, 2002). Our finding that EMP are carriers of VWF suggest their possible involvement in aberrant VWF processing in TTP.

Studies are underway to clarify the issue of VWF abnormalities as related to EMP in TTP. It has been observed that microparticles, in general, act as activators of leucocytes. We, and others have documented that EMP bind to and activate monocytes, eliciting expression of TF (Jy et al, 2002b; Sabatier et al, 2002b). Thus, EMP may modulate the inflammatory response of monocytes by regulating the release of cytokines such as TNF-α or IL-1β, which could in turn result in a state of endothelial activation, as seen in TTP.

To summarize: (i) TTP plasma incubated with MVEC in vitro induced the release of EMP with phenotypes indicative of activation, and approximately 60% of them carried VWF; (ii) in parallel clinical studies, EMP levels in patients with TTP correlated well with the severity of TTP. EMP measured in TTP patients carried VWF and exhibited the phenotype of EC activation. The factor in TTP plasma responsible for release of EMP remains to be elucidated. The expression of VWF on EMP further implicates EMP in the pathophysiology of TTP.

ACKNOWLEDGMENTS

We would like to thank R.N. Micheline Aristide and M.T. Ariel Rodriguez from Transfusion Services at Jackson Memorial Hospital for their support. This work was supported by the Wallace H Coulter Foundation. We are also grateful for support from the Roz & Cal Kovens Research Fund, the Charles & Jane Bosco Research Fund and the Mary Beth Weiss Research Fund.

REFERENCES


