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Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis

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Abstract

Background: Endothelial cells (EC) shed endothelial microparticles (EMP) in activation and apoptosis. *Objectives*: We compared the antigenic expression of EMP species released during activation as compared to apoptosis, in three cell lines. *Methods*: EC from renal and brain microvascular (MiVEC) and coronary macrovascular (MaVEC) origin were incubated with TNF-α to induce activation, or deprived of growth factors to induce apoptosis. Antigens expressed on EMP and EC were assayed flow cytometrically and included constitutive markers (CD31, CD51/61, CD105), inducible markers (CD54, CD62E and CD106), and annexin V binding. *Results*: It was found that in apoptosis, constitutive markers in EMP were markedly increased (CD31>CD105), with a concomitant decrease in expression in EC. Annexin V EC surface binding and annexin V+ EMP were more sharply increased in apoptosis than in activation. In contrast, in activation, inducible markers in EMP were markedly increased in both EMP and EC (CD62E>CD54>CD106). Coronary MaVEC released significantly less EMP than MiVEC. *Conclusion*: EC release qualitatively and quantitatively distinct EMP during activation compared to apoptosis. Analysis of EMP phenotypic signatures may provide clinically useful information on the status of the endothelium.

Keywords: Endothelial microparticles; Activation; Apoptosis; Endothelial cells; Phenotype; Adhesion molecules

1. Introduction

Endothelial cells (EC) provide a non-thrombogenic and non-adhesive surface but under pathologic conditions they become proadhesive and procoagulant [1,2]. The expression of surface antigens on resting and stimulated EC has been extensively studied [3]. Upon exposure to proinflammatory cytokines, such as TNF-α or IL-1β, EC synthesize and express on their surface numerous adhesins and other molecules which participate in leukocyte and platelet recruitment, coagulation and inflammation [3,4]. Apoptosis, on the other hand, elicits distinctive pathways, one prominent manifestation of which is the reversal of the EC membrane to expose anionic phospholipids, thereby providing a procoagulant surface [5,6]. In addition, apoptotic EC have been shown to shed membrane vesicles (EMP) with procoagulant activity [7].

More recently, it was shown that EC release membrane-derived microparticles (EMP) upon activation or apoptosis [8,9]. Hamilton et al. first reported the flow cytometric detection of microparticles released by umbilical vein EC (HUVEC) in response to complement C5b9 and calcium ionophore [10]. Subsequently, Combes et al. [8] partially characterized EMP released by HUVEC in response to TNF-α showing that they express membrane antigens PECAM-1 (CD31), vitronectin receptor (CD51), ICAM-1 (CD54) and E-selectin (CD62E).

Abbreviations: EMP, endothelial microparticles; EC, endothelial cells; TNF-α, tumor necrosis factor-α; IL-1β, Interleukin-1β; GFD, growth factor deprivation; TTP, thrombotic thrombocytopenic purpura; MS, multiple sclerosis; MiVEC, microvascular endothelial cells; MaVEC, macrovascular endothelial cells; CAMaVEC, coronary artery MaVEC; CD31, PECAM-1, platelet endothelial adhesion molecule-1; CD51/61, vitronectin receptor/$\alpha_V\beta_3$; CD54, ICAM-1, intercellular adhesion molecule-1; CD62E, E-selectin; CD105, endoglin; CD106, VCAM-1, vascular cellular adhesion molecule-1; AnnV, annexin V; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline.

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We previously reported that EC of two different cell lines (renal and brain microvascular EC) release EMP upon activation or apoptosis in vitro [9]. The EMP released expressed antigenic markers CD31 and CD51, and exhibited procoagulant activity as defined by platelet factor 3 activity and tissue factor [8,9]. In clinical studies, we reported elevated EMP in patients with thrombotic thrombocytopenic purpura (TTP), multiple sclerosis (MS), acute coronary syndromes, preeclampsia, and extreme hypertension [9,11–15]. In both TTP and MS, we found that EMP rose in relapses and normalized upon remission [9,11]. Others have reported elevation of EMP in patients with lupus anticoagulant [8] and coronary ischemia [15,16].

The present report concerns recent findings on the phenotypic characteristics of EMP released upon activation and apoptosis, defined in terms of antigenic expression. The aim of the study was to determine whether analysis of EMP can discriminate these types of endothelial injury, since such a method could offer new insights to the pathophysiology of thrombotic disorders, and possibly new avenues in their diagnosis and treatment.

2. Materials and methods

2.1. EC culture

Renal and brain microvascular EC (MiVEC) and coronary artery (macrovascular) EC were obtained from Cell Systems (Kirkland, WA, USA; Cat. Nos. ACBRI 376, ACBRI 128 and ACBRI 377, respectively) and were cultured as previously described [9]. Upon confluency, cells were detached with a passage reagent group (Cell Systems) following manufacturer's protocol, resuspended in CS-C medium and replated in 12-well tissue culture multi-well clusters (Corning, NY, USA), precoated with attachment factor (Cell Systems) at a density of 1×105 per well. Subsequently, they were maintained for 48 h in CS-C medium prior to the assays.

2.2. Cell treatments and assay of apoptosis

Cells were exposed for 24 h to either 10 ng/ml TNF-α (Sigma) or deprivation of serum and growth factors (GFD) using CS-1 medium (Cell Systems). Controls were untreated. Preliminary time-dependent studies were performed to estimate the optimal time for EMP and EC assay and collection, where it was determined that in 24 h EC released maximal EMP without evidence of degradation.

DNA fragmentation characteristic of apoptosis was evaluated with the TUNEL assay and Trypan blue dye exclusion as previously described [9]. In earlier studies, it was shown that the dose of TNF-α employed here does not induce early or late-stage apoptosis in primary EC cultures [8,9]. These results were confirmed in another of our studies, since incubation with 10 ng/ml of TNF-α did not induce caspase 3 upregulation [17].

2.3. Preparation of EMP samples

The EC culture wells contained 1.0 ml of supernatant and 30 μl was used per test. To the 30-μl samples were added, in 12×75 mm polypropylene tubes, 3 μl of each of the following fluorescent antibodies: anti-human CD31 (Pharmingen, 555446, PE), anti-human CD51/61 (Pharmingen, 555504, FITC), anti-human CD54 (Sigma, F-0549, FITC), anti-human CD62E (Sigma, F-0674, FITC), anti-human CD105 (Ancell, 326-040, FITC)or anti-human CD106 (Pharmingen, 555647, PE). To assess annexin V binding, samples were incubated with human FITC-labeled annexin V (Sigma). The samples were then incubated 20 min at room temperature with gentle shaking (orbital shaker, 120 rpm), then 0.5 ml of phosphate-buffered saline (PBS) was added, and the samples were ready for flow cytometry.

2.4. Detection of surface markers on endothelial cells (EC)

The above antigenic markers were also applied to the remnant EC. After 24 h treatment, supernatants were removed and assayed for EMP. Each plate was incubated with 10 μL of the above monoclonal antibodies for 30 min, then excess antibody was removed and the EC were washed thrice with 1 ml each of complete basal medium. The cells were detached and the resulting suspension of EC was examined by flow cytometry. For annexin V binding studies, EC were detached using 0.05% trypsin without EDTA.

2.5. Flow cytometry

EMP were analyzed on a Coulter EPICS XL (Beckman Coulter, Miami, FL, USA) at medium flow rate setting and 30-s stop time. Particle detection was set to trigger by a fluorescent signal greater than background threshold. Light-scatter and fluorescence channels were set at log gain. Fluorescent events were further separated on another histogram based on size (forward light scatter). EMP were defined as particles ≤ 1.5 μm size and bearing EC antigens. To convert flow cytometer counts under these conditions to an estimate of EMP/ml of original supernatant, it was determined using standard beads that 18 μl of sample was actually aspirated and counted for every 30 s, hence the conversion factor F

$$F = (1.04 \text{ ml}/0.018 \text{ ml})(1.0 \text{ ml}/0.03 \text{ ml}) = 1926,$$

was applied for 30 μl samples. Results for surface expression of antigens on whole EC is given in terms of mean fluorescent intensity units. Daily calibration using fluores-

cent beads (Beckman Coulter) ensured that fluctuations were less than 2%. Isotype-matched control values for each fluorophore were subtracted from EMP counts and from EC mean fluorescence. Non-specific binding accounted for less than 10% of total EMP counts.

2.6. 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. All data analyses were performed using the Windows-based program, Sigmaplot 2001.

3. Results

3.1. Analysis of EMP and whole EC phenotypes

GFD induced apoptosis in MiVEC as evinced by TUNEL positive results and viability by Trypan blue (65 ± 10% and 10 ± 2% viable EC for RMiVEC, 50 ± 10% and 12 ± 3% viable EC for BMiVEC). Coronary artery (CA) MaVEC were comparatively resistant to GFD at 24 h, showing only modest increase in TUNEL positivity, confirmed by Trypan blue dye uptake (5 ± 3% and 95 ± 8%, respectively). Exposure of MiVEC or MaVEC to TNF-α did not result in apoptosis under our conditions, judged by TUNEL assay

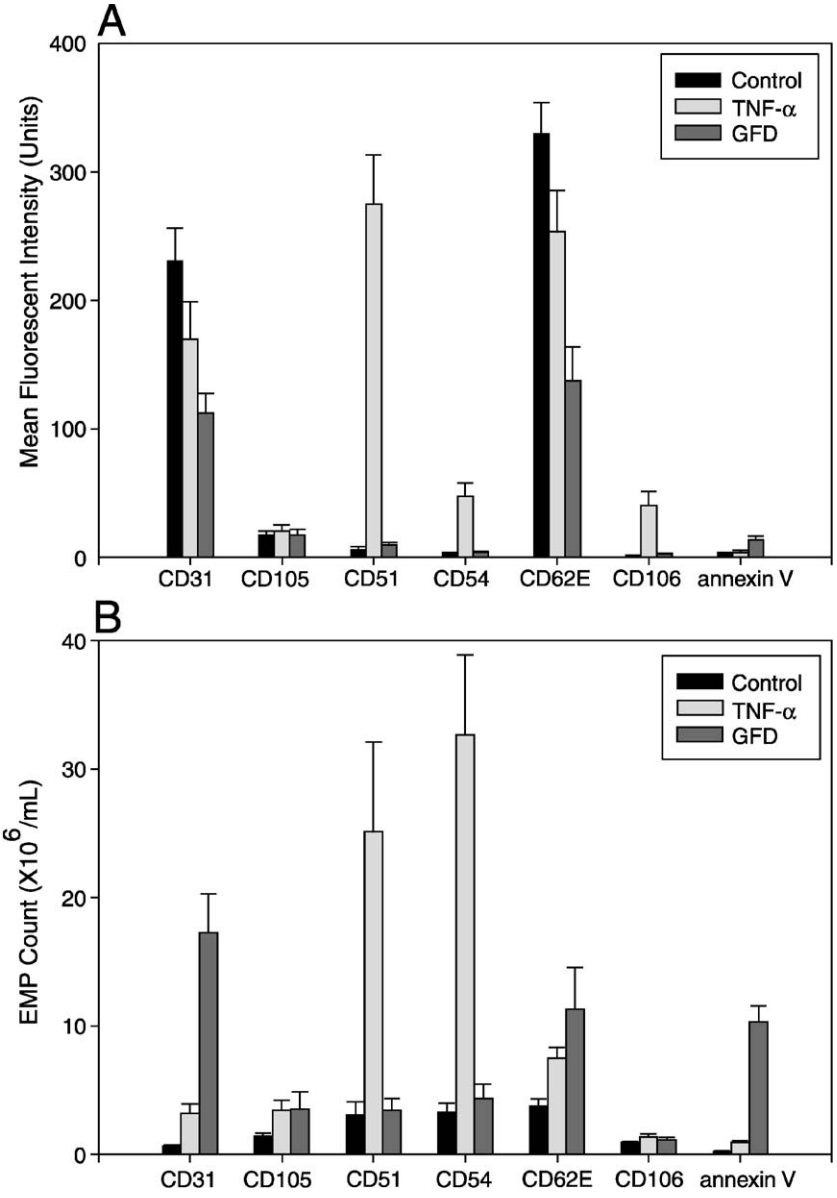

Fig. 1. Expression of antigens on renal MiVEC (A) and EMP (B). Results are shown after 24 h of no treatment (open bars, controls), or treatment with TNF-α to induce activation (lightly shaded middle bars) or growth factor deprivation (GFD) to induce apoptosis (dark bars). Units for whole cells (A) are expressed in arbitrary units of mean fluorescent intensity (FLIU), while results for EMP (B) are in counts × 10^6/ml. Similar values were obtained with brain MiVEC (not shown). Error bars are standard error of the mean. Values shown are the means of at least three experiments. Significance: *, ≤ 0.05–0.01; **, ≤ 0.01.

and Trypan blue dye exclusion (TUNEL positivity < 2% and viability >93% for all cultures).

Data presented in Fig. 1 show that EC release species of EMP exhibiting qualitative and quantitative differences in antigenic phenotypes in response to activation vs. apoptosis. Fig. 1A shows the results (fluorescent intensities) for whole renal MiVEC, and Fig. 1B shows the EMP counts. As shown in Fig. 1A, the mean FL intensity of constitutive markers (CD31, CD105) on renal EC are markedly reduced in apoptosis and less so in activation by TNF-α. These changes are in contrast to those for EMP, shown in Fig. 1B, where a sharp rise in EMP positive for these markers can be seen in apoptosis but a weaker rise in activation.

In contrast, inducible markers (CD54, CD62E) are sharply increased on whole renal MiVEC only in activation, not in apoptosis (Fig. 1A) and this is accompanied by impressive increases in the EMP carrying these markers (Fig. 1B). CD54 and CD62E are abundant on both the whole EC and on the

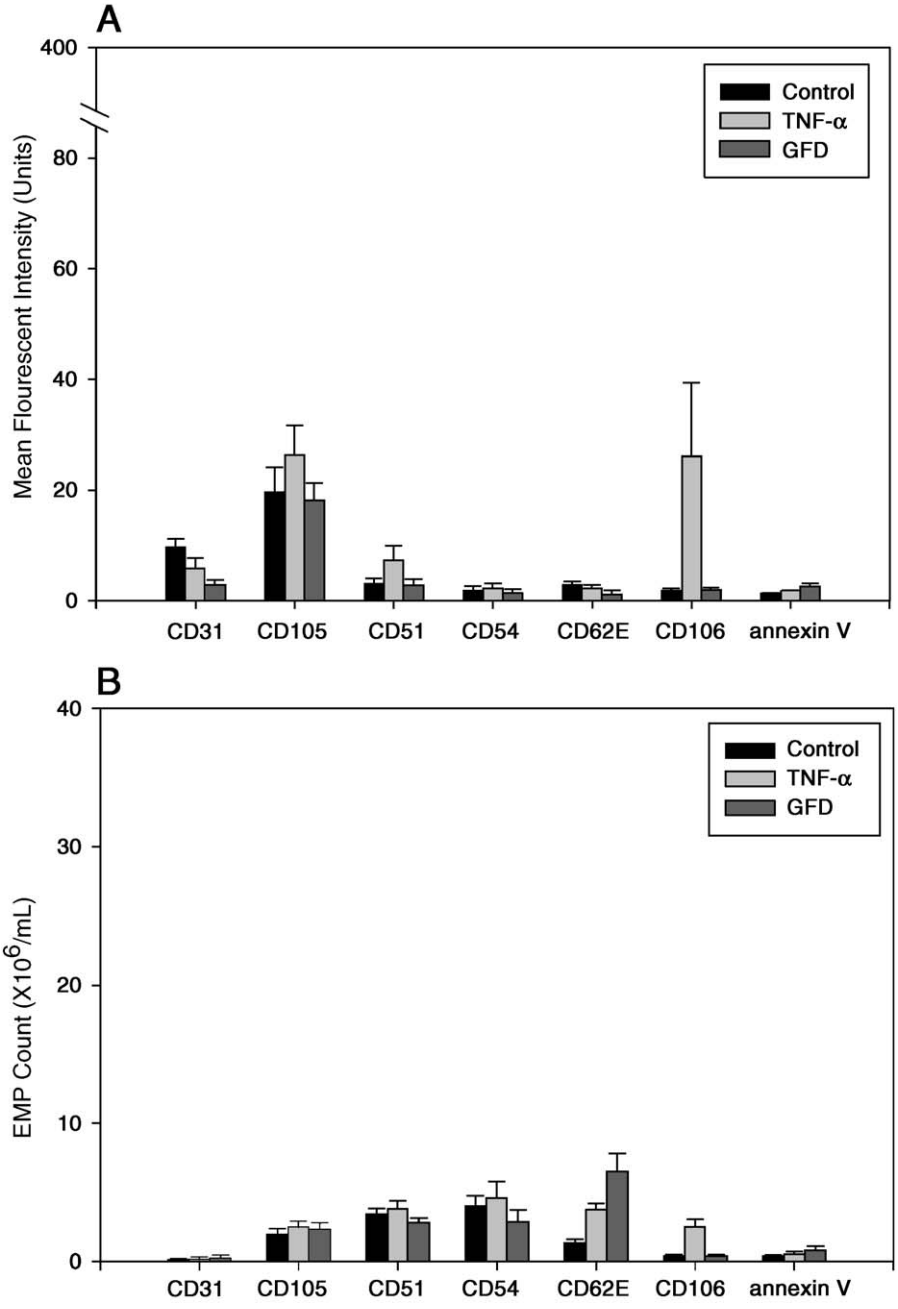

Fig. 2. Expression of antigens on coronary artery (macrovascular) MaVEC (A) and EMP (B). Results are shown after 24 h of no treatment (open bars, controls), or treatment with TNF-α to induce activation (lightly shaded middle bars) or growth factor deprivation (GFD) to induce apoptosis (dark bars). Units for whole cells (A) are expressed in arbitrary units of mean fluorescent intensity (FLIU), while results for EMP (B) are in counts × 10^6/ml. Error bars are standard error of the mean. Values shown are the means of at least three experiments. Significance: *, ≤ 0.05 – 0.01; **, ≤ 0.01.

EMP of activated but not apoptotic MiVEC. Annexin V binding sites are modestly but significantly increased on the EC only in apoptosis, and are preferentially enriched on the EMP.

Similar trends in constitutive and inducible antigens in EMP and EC surface are observed with brain MiVEC (not shown).

3.2. Coronary artery (macrovascular) EC

As seen in Fig. 2 results with macrovascular coronary artery (CA MaVEC) differed in many respects from both of the MiVEC lines. In the resting state, they exhibited $\leq 10\%$ as much CD31 and CD105 as MiVEC. Under GFD conditions, which induced apoptosis in $\geq 50\%$ of MiVEC, only $\sim 5\%$ of CA MaVEC became apoptotic (accounting for their relatively low EMP shedding). Under conditions of activation (TNF-α), their EMP release was significantly reduced compared to MiVEC, especially CD54 and CD62E. However, it is clear that they responded to TNF-α since this is evidenced by a sharp upregulation of CD106 on the cell surface.

4. Discussion

In the present study, we investigated a wider spectrum of EMP markers than in previous studies, and report antigenic profiles of the parent whole EC as well as EMP in resting, activated, and apoptotic states in three EC lines. We have demonstrated that EMP are phenotypically distinct in apoptosis vs. activation. This paves the way for further studies of possibly distinctive EMP phenotypes in various kinds of endothelial injury.

In general, EMP expressing constitutive markers, such as CD31 and CD105, were markedly increased in apoptosis, while those expressing inducible markers such as CD54 and CD62E were increased only in activation. Accordingly, analysis of phenotypes of EMP may provide insight into the nature of endothelial injury.

TNF-α was selected for this study because of its ability to induce endothelial activation. TNF-α induces transcription factor NF-κB resulting in morphological rearrangement and expression or release of an array of adhesins and cytokines [4,18–20]. Transcription factor NF-κB has been shown to affect pro-survival genes of the iap and xiap family that may render human EC less sensitive to TNF-α-mediated apoptosis [20,21]. Resistance to TNF-α-induced apoptosis in EC is abrogated by the synergistic action of TNF-α with protein synthesis inhibitors [22]. On the other hand, EC have been shown to undergo apoptosis due to hypoxia, hyperoxia or oxidation [23]. We found that EC expression of inducible adhesins CD54, CD62E and CD106 in apoptosis remained similar to controls, as did EMP bearing these antigens; however, annexin V binding was increased in both apoptosis and activation, though more dramatically in apoptosis.

It is well established that endothelial cells are heteregenous according to their organ of origin. EC exhibit differences not only in growth and morphology, but also in responsiveness to growth factor, cytokines or other activators such as PMA, thrombin or cytokines [24–26]. In the present study, we found that coronary artery MaVEC when compared to MiVEC, were less responsive to GFD after 24 h, which probably accounted for their comparatively weak release of EMP. This is consistent with previous reports of differences in susceptibility of MiVEC vs. MaVEC to damage in response to different stimuli [27,28].

Our data show that in apoptosis, EMP positive for CD31 and CD105 are greatly increased relative to EMP positive for CD62E, CD54, or CD106, while in activation, this trend was reversed. Accordingly, we adopted the ratio of CD62E/CD31 populations, rather than their absolute numbers, as a criterion for distinguishing activation vs. apoptosis: a ratio ≥ 10 identifies activation while ratio ≤ 1.0 identifies apoptosis. We also observed that the ratio of annexin V binding EMP to CD54+ EMP is another candidate signature in vitro since in activation this ratio is always >2.0, whereas in apoptosis it is <0.1.

Our clinical studies showed that EMP levels can provide useful information on the status of endothelial injury (cited in Introduction). For example, during the acute phase of TTP, circulating CD62E+ EMP were significantly higher than CD31+ EMP, indicating a ratio suggestive of EC activation, not apoptosis, in TTP [29]. On the other hand, EMP profiles in acute coronary syndrome (ACS) suggested the signature of apoptosis.

In conclusion, EC releases phenotypically and quantitatively distinct EMP in activation and apoptosis. The antigenic phenotypes of EMP tend to reflect those of the endothelial cells. Therefore, phenotypic analysis and quantitation of EMP can provide useful information reflecting the nature of endothelial injury. EMP can be a useful marker of EC injury. Further characterization and a better understanding of the biology of EMP could open new avenues for monitoring endothelial disturbances non-invasively in various disorders.

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