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In Vitro Model of Platelet-Endothelial Activation Due to Cigarette Smoke Under Cardiovascular Circulation Conditions

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Abstract—Cigarette smoke has been shown to increase platelet activation and endothelial cell (EC) adhesion molecule expression. In the present study, we utilized a hemodynamic shearing device (HSD) to investigate the above effects *in vitro* in a combined system of platelets and cultured HUVECs (Human Umblical Vein ECs) under physiological shear stress. We investigated the alteration of E-selectin expression on ECs upon exposure to: (1) platelets and nicotine-free smoke extract (NFE), (2) platelets alone, (3) NFE alone, under physiological shear stress. We additionally confirmed the protective effect of nicotine on platelet activation. We found that: (i) surface expression of E-selectin on ECs was significantly increased upon simultaneous exposure of ECs and platelets to NFE relative to exposure of ECs to either platelets or NFE alone ($p < 0.05$). (ii) Platelet activation was significantly increased in the presence of NFE ($p < 0.05$). (iii) Nicotine (200 nM) when added to NFE, significantly reduced platelet activation due to NFE ($p < 0.05$), an effect additionally confirmed by conventional cigarette extracts which contain nicotine ($p < 0.05$). We therefore conclude that: (a) NFE and platelets additively increase EC E-selectin surface expression, and (b) nicotine modulates platelet activation regardless of ECs.

Keywords—Cardiovascular diseases, Inflammation, Nicotine, Couette, Cone and plate, Viscometers.

INTRODUCTION

Cigarette smoking has been well established as a risk factor for development of cardiovascular disease.[1,9] A major part of this risk involves increased platelet activation due to cigarette smoke, as has been investigated both *in vivo*[18] and *in vitro*.[23] In two studies involving platelets alone, we have shown that this smoke-induced platelet activation is largely due to the non-nicotine smoke components and their effects can be modulated in the presence of nicotine.[22,23] While the direct desensitizing effect of nicotine on platelet activation is likely associated with reduced cardiovascular disease risk, such investigations need to be extended to a more physiologically relevant scenario to include the presence of the vasculature (endothelial cells (ECs), smooth muscle cells, and extracellular matrix). Additionally, this should be investigated under physiological shear stresses, as those play an important role in the effects of cigarette smoke and nicotine on platelets.

Prior *in vitro* studies have shown that high concentrations of cigarette smoke extract[6,28,29] and nicotine[32–34] increase adhesion molecule expression on ECs. These studies preclude the involvement of physiological shear stresses. Whether inflammatory effects due to smoke extract exposure may be attributed to its nicotine or nicotine-free components also remains unknown. Thrombin-activated platelets when introduced over cultured ECs bind to the cultured cells by several adhesion (receptor–ligand pairs) molecules[4,5,16,31] and may promote increase in EC adhesion molecule expression by downstream signaling. A link has been established between platelet factor-4 and endothelial E-selectin expression,[35] resulting from activation of nuclear factor-kappa B. The same mechanism has been reported for E-selectin expression on ECs due to cigarette smoke.[28] It is likely that if smoke extract and activated platelets both increase E-selectin expression on ECs by similar or distinct mechanisms, the levels of E-selectin surface expression on ECs may be significantly enhanced by their combined activity. Following such hypothesis, ECs simultaneously exposed to smoke extracts and smoke-activated platelets will be expected to increase EC adhesion molecule expression as a result of their combined activity.

The mechanical stress conditions (both fluid shear stress and cyclic strain) largely dictate the development of either pro- or anti-inflammatory phenotype of ECs.[7,12] Investigation of dynamic flow-dependent effects on cultured cells *in vitro* involves the use of devices that impart minimal inertial effects to the fluid

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and avoid re-circulation of fluid. The cone and plate viscometer and the annular Couette viscometer have been extensively used to study spatio-temporal effects of fluid shear stresses on cultured and circulating cells, respectively. Of note is the unique ability of the cone-plate viscometer to study both physiological and pathophysiological flow regimes within the same device,[8] while controlling any secondary flow effects.[27] If the two devices can be combined, both cultured cells and cell suspensions can be subjected to the same shear stress and the effects of interaction between them can be investigated by periodic sampling and subsequently analyzed.

In the present study, we utilize a hemodynamic shear device (HSD)[19] that combines features of the cone-plate with those of the annular Couette viscometer (cross-sectional view, Fig. 1a). The basic advantage of this device is that uniform and identical shear stress levels are maintained in both the cone-plate and Couette regions, and it facilitates convenient periodic cell sampling from the Couette region. We have recently used this device to study fluid shear-induced platelet activation under programmed shear stress.[19] In the present study, we test the hypothesis that platelets and the nicotine-free extract would confer an additive or enhanced inflammatory effect on ECs, by investigating the effects of cigarette smoke extract and platelets together on ECs under physiological shear stresses within the HSD. We additionally hypothesize that in contrast to conventional cigarette extracts, nicotine-free smoke extract would increase platelet activation more significantly, and that this effect may be independent of the presence of ECs.

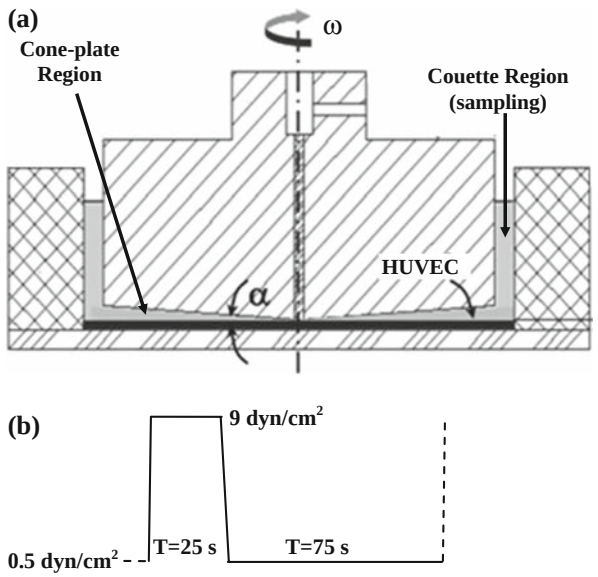

FIGURE 1. (a) (Adapted and modified from Nobili et al (2008) [19]) Cross-sectional view of the hemodynamic shear device (HSD). Platelets ± ECs are exposed to identical shear stress in the HSD. ECs are cultured on disks on top of the base-plate, platelets are sampled from the Couette region for PAS. The cone angle is represented by α, and rotational speed of the cone by ω. (b) Shear-stress waveform applied repeatedly for 33 cycles (100 s each) to the ECs and platelets in HSD.

METHODS

Reagents

Thrombin Receptor Activator Peptide-6 (TRAP-6), nicotine hydrogen tartrate salt, human fibronectin, EDTA, bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4), 10% paraformaldehyde (PFA, EM grade), and Trypsin-EDTA from Sigma (St. Louis, MO); FITC-conjugated mouse anti-human-E-selectin antibody from BD Biosciences (Franklin Lanes, NJ); Human Umbilical Vein Endothelial Cells (HUVECs) from Lonza (Walkersville, MD); Cigarettes—Quest 1 and 3 (Vector Tobacco, Mebane, NC) and Marlboro Red (Philip Morris, Richmond, VA) from a local pharmacy, glycol-modified polyethylene terepthalate (PETG) sheets from McMaster Carr (Robbinsville, NJ), Tumor Necrosis Factor-α (TNF-α; R&D Systems, Minneapolis, MN).

Smoke Extracts

Mainstream smoke extracts from two cigarettes of the same type were prepared in 100 mL of Hepes Buffered Saline (HBS, pH 7.4) as described previously,[23] with a small modification—the smoke was being bubbled through HBS in a vertical column (100 mL measuring cylinder) instead of a beaker used earlier, to facilitate a more efficient transport by increasing smoke-HBS contact time. The following three types of cigarettes corresponding to zero, medium, or high nicotine were used to prepare the three extract solutions: Quest-3 (nicotine-free extract or NFE, 10 mg tar, 0.05 mg nicotine), Quest-1 (medium-nicotine extract, 10 mg tar, 0.6 mg nicotine), Marlboro Red (high-nicotine extract, 16 mg tar, 1.2 mg nicotine). A final concentration of 10% extract was used in all studies.

Platelets

Thirty mL of citrated whole blood was obtained from adult human volunteers after informed consent. Platelet-rich plasma (PRP) was obtained after centrifugation at $300 \times g$ for 6 min, and loaded onto a Sepharose 2B-CL column to separate platelets from plasma proteins. Platelets at a concentration of 2×10^8/mL were eluted in citrated buffer as described previously[23] and used within 3 h of purification. The

order of experiments involving platelets from the same batch on a given day was randomly chosen.

Endothelial Cell Culture

HUVECs (passages 5–10) were cultured on human fibronectin (5 μg/mL)-coated PETG disks (diameter: 3 mm) until complete confluence was obtained as indicated by cobblestone morphology and absence of intracellular gaps. Functionality of HUVECs was confirmed by release of von-Willebrand factor (vWf) in culture and under shear stress. For this purpose, matched antibody-pair (primary and secondary, anti-human vWf) was purchased from Affinity Biologicals (Ontario, Canada) for analysis in an ELISA assay. HUVECs did not detach upon exposure to a constant shear stress of 9 dynes/cm^2 and remained confluent throughout the duration of experiments.

Hemodynamic Shear Device (HSD)

A combined annular Couette and cone-plate viscometer setup (Fig. 1a) was utilized to study the interaction between cultured HUVECs and circulating platelets under shear stress. HUVECs, cultured on PETG disks, occupy the base of the cone and the base of the annular Couette regions. The construction and characterization of this device has been described in our recent study.[19] The platelet suspension occupies the space (cone and annular Couette regions) above the HUVECs. The annular gap (740 μm) was determined to maintain identical shear stress levels under the cone and in the Couette regions. Sampling of platelets was performed from this annular Couette region. Capabilities of the cone-plate viscometer as a compact *in vitro* device to investigate a wide range of dynamic flow regimes experienced *in vivo* have been extensively discussed.[8,10] A repeated shear stress cycle of 100 s as indicated in Fig. 1b was used in the study, for a total of about 33 cycles or 54 min per experiment. All experiments were conducted within the HSD at the same shear stress. The objectives were to determine: (1) Expression of E-selectin on ECs upon exposure to platelets and nicotine-free smoke extract, and (2) Change in platelet activation upon addition of nicotine-free smoke extract and modulation of this effect by nicotine. TRAP-6 was used as a positive control in place of nicotine-free smoke extract, for objective 1. The parameters measured are further explained below, and summarized in Tables 1 and 2.

Platelet Activation State (PAS)

In experiments involving determination of platelet activation (Table 2), sampling was performed from the Couette region every 6 min and platelet activity (PAS or platelet activation state) was determined over the duration of the experiment (54 min), by a modified prothrombinase assay as described elsewhere.[13] This measure of platelet activation reflects primarily the release of factor Va from the α granules to the surface, plus the exposure of anionic phospholipid upon activation.[25] This method has been demonstrated to correlate well with platelet Annexin-5 expression,[14] as determined by flow cytometry. The results were normalized to maximum platelet activity (PAS determined after sonication of the platelet suspension for 10 W at 10 s) for the particular batch of platelets. Additionally, another parameter (Platelet Activation Rate or PAR) was determined to investigate whether PAS changes

TABLE 1. Summary of experiments for investigation of E-selectin expression on: (A) ECs with platelets and nicotine-free smoke extract and (B) ECs with platelets and TRAP-6.

Objective: Investigation of E-selectin expression on ECs by flow cytometry

Cells in HSD	Nicotine-free extract (10%)	Parameter
(A)		
EC	+[a]	E-selectin
EC	–[a]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin
Cells in HSD	**TRAP-6 (100 μM)**	**Parameter**
(B)		
EC	+[b]	E-selectin
EC	–[b]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin

[a]The signs (+/–) indicate the presence or absence of extract in the experiment.
[b]The signs (+/–) indicate the presence or absence of TRAP in the experiment.

TABLE 2. Determination of platelet activation state (PAS) for platelets exposed to the nicotine-free extract, two commercial cigarette extracts (Quest-1 and Marlboro), control and pure nicotine added to the nicotine-free extract.

Objective: Effect of nicotine on platelet activation state (PAS)

Cells in HSD	Type of smoke extract (10%)	Parameter
Platelets ± EC	NFE (Nicotine-free/Quest-3)	PAS, PAR
Platelets ± EC	NFE + 200 nM nicotine	PAS
Platelets ± EC	Medium-nicotine (Quest-1)	PAS
Platelets ± EC	High-nicotine (Marlboro)	PAS
Platelets ± EC	–	PAS, PAR

Platelet activity rate (PAR) was determined for selected conditions to exclusively determine the effect due to nicotine-free extract on platelets. Note that identical studies were performed for platelets alone and platelets + ECs, as indicated by ± sign.

more rapidly with time upon addition of nicotine-free extract.

E-Selectin Expression on ECs

At the end of studies outlined in Table 1, HUVECs were gently washed with pre-warmed (37 °C) PBS to remove any bound platelets, trypsinized, washed with FACS buffer (5 mM EDTA, 1% BSA, 4 °C, pH 7.4) and fixed with 4% PFA on ice. Prior to flow cytometry, the HUVEC samples were washed and resuspended (1×10^5/mL, FACS buffer) and incubated with saturating concentration (20 μg/mL) of mouse-anti-human-CD62E-FITC mAb for 45 min on ice. A total of 10,000 gated events were used for analysis on a BD FACScan flow cytometer. An IgG1-FITC isotype control was performed as a control to ensure binding specificity. Change in E-selectin expression for each of the four conditions in study (a) above was determined by fraction of total gated cells positive for E-selectin, relative to control. A total of eight experiments were conducted for each set of conditions (Table 1).

Statistical Analyses

All statistical analyses for multiple comparisons of PAS, PAR, and E-selectin expression on ECs, were performed with two-way or one-way ANOVA (SPSS Inc., Chicago, IL). Post-hoc tests to determine individual statistical differences within groups were conducted after Bonferroni's correction. A significance level of $p = 0.05$ was used for all analyses. Specifically, the following parameters across experimental groups were compared:

Platelet Activation State (PAS): The PAS values at times $t = 0$ min and $t = 54$ min obtained for all experimental conditions (Table 2) were averaged over total number of experiments ($n = 5$). Separate comparisons were made for PAS differences at the start ($t = 0$ min) and the end of the experiment ($t = 54$ min) for each set of conditions. We conducted a two-way ANOVA to investigate whether PAS was affected by (a) smoke extracts or nicotine, and (b) presence of ECs.

Platelet Activation Rate (PAR): To examine the effect of nicotine-free smoke extract on platelet activation with time, the PAS values obtained during the 54-min experiment for four conditions (Table 2) were fit to a two-degree polynomial curve. We additionally compared goodness of fit of this data between a 2-degree polynomial and a linear model.

E-Selectin Expression on ECs: E-selectin positive ECs were determined from cytometry for each condition in Table 1. The data obtained from cytometry corresponding to % positive gated cells for each condition fit better to a log-normal distribution (Shapiro-Wilk and Kolmogorov-Smirnov test for normality). The data was therefore log-transformed before multiple comparisons were made with one-way ANOVA separately for the two groups (Table 1A and 1B).

RESULTS

Platelet Activation State (PAS) Increases with Nicotine-Free Smoke Extract

We investigated platelet activation (PAS) in the presence of 10% nicotine-free smoke extract (NFE) and shear stress, both for platelets alone (Fig. 2a) and platelets with ECs (Fig. 2b). The data for each plot represents an average of five experiments. The corresponding results for the average PAS have been reported in Table 3. We found that platelet activation (PAS) was significantly increased upon addition of the nicotine-free extract ($p = 0.001$).

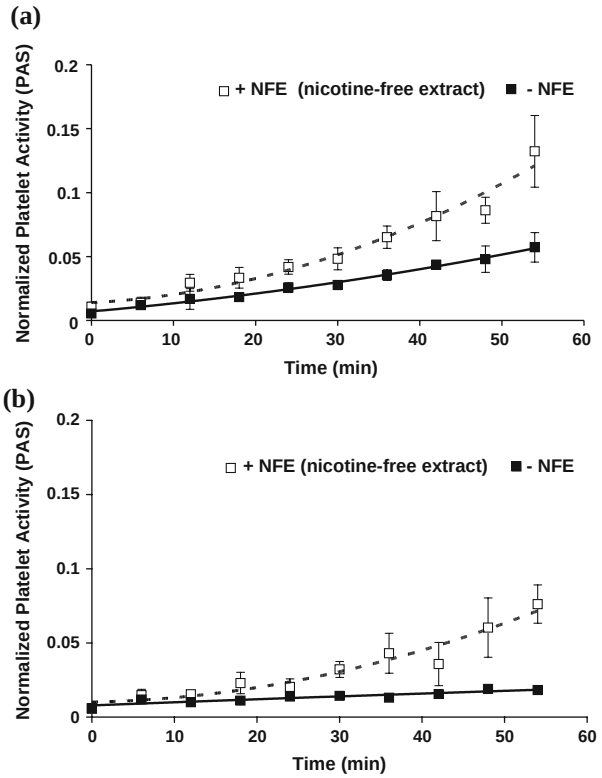

FIGURE 2. Platelet activation state (PAS) over 54 min in the HSD for (a) platelets (±smoke) and (b) platelets + ECs (± smoke). Note 10% nicotine-free smoke extract (NFE) was used. The data are fit to a 2-degree polynomial curve and the average platelet activation rates (PAR) are illustrated in Table 3.

TABLE 3. Summary of platelet activation (PAS and PAR, mean ± s.e.m.) upon exposure to nicotine-free smoke extract.

Condition	PAS at T = 54 min	PAR (1×10^{-5} min^{-1})
Platelets	0.06 ± 0.01	1.00 ± 0.60
Platelets + NFE	0.13 ± 0.03	3.02 ± 1.00
Platelets, ECs	0.02 ± 0.00	0.02 ± 0.15
Platelets, ECs + NFE	0.08 ± 0.01	3.00 ± 1.00

All experiments were done under the same shear stress in the HSD.

Platelet Activation Rate (PAR) Increases with Nicotine-Free Smoke Extract

We found that the 2-degree polynomial model was a better overall fit for our PAS data in 3 out of 4 cases shown in Fig. 2 and reported above: $p = 0.01$, 0.02 (platelets ± NFE) and $p = 0.87$, 0.04 (platelets + ECs ± NFE). To maintain consistency, we determined the PAR corresponding to the curvature or quadratic coefficient for the fit for each condition, prior to statistical test with two-way ANOVA. We found that PAR increased significantly due to the presence of nicotine-free smoke extract ($p = 0.006$). The average PAR values have been reported in Table 3.

Control Experiments for Initial Platelet Activation State (PAS at t = 0 h)

To ensure that purified platelets did not activate significantly over the 3 h time course of use each day, we performed control experiments and measured the platelet activity overtime with the modified prothrombinase assay. We found that during this time course platelets activated less than 1% when left on the shaker. The mean (±s.e.m.) PAS for this control experiment (repeated 3 times with different platelet batches) at times $T = 0$, 1, 2, 3 h was found to be 0.0051 ± 0.0016, 0.0051 ± 0.0007, 0.0099 ± 0.0021, and 0.0071 ± 0.0009, respectively. Additionally, we compared the PAS data at the beginning of all our experiments as outlined in Table 2 (i.e., PAS at $t = 0$ min) and found that the initial level of platelet activation did not differ between different experimental groups and conditions ($p > 0.05$) and was less than 1% as found in control experiments above. The mean (±s.e.m.) starting PAS ($t = 0$ min) was calculated as 0.0082 ± 0.0008.

E-Selectin Expression on ECs

Three representative cytometry histograms for expression of E-selectin on ECs, when exposed to (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line), are shown in Fig 3a. In all cases, the shift in fluorescence intensity histogram is distinct upon simultaneous exposure of ECs to platelets and smoke (condition iv, thick solid line). The average increase in E-selectin expression for each of these conditions is illustrated in Fig. 3c. The data is an average of eight individual experiments, and mean ± s.e.m values for % positive gated cells are shown. This was statistically confirmed by ANOVA over the log-normalized data for the four conditions mentioned above. The pair-wise differences were then determined after post-hoc Bonferroni's correction after significance was established with ANOVA ($p = 0.001$), and the results are illustrated in Table 4. E-selectin expression obtained upon concurrent exposure of ECs to the nicotine-free extract and platelets, was significantly different from all other conditions and proves the hypothesis of current study.

To confirm this additive effect, another agonist (TRAP-6) which is known to maximally activate P-selectin expression on platelets,[11] was used in place of NFE and the above study was repeated. In contrast with smoke-activated platelets that had a final PAS of 6%, TRAP-6 activated platelets had a PAS of over 90% for all experiments. It should be noted that despite the high PAS, in the absence of fibrinogen there was no platelet aggregation in these studies (confirmed by flow cytometry to P-selectin). An identically statistically significant effect was observed with TRAP-6 ($p < 0.05$, statistical data not shown) as reported above for NFE. As above, three representative cytometry histograms for the expression of E-selectin on ECs when NFE is replaced with TRAP-6 are shown in Fig. 3b. The shift in fluorescence intensity when ECs are concurrently exposed to platelets and TRAP-6 is distinctly identified from individual exposure conditions (Fig. 3b). The average results for % E-selectin positive gated ECs (mean ± s.e.m.) from eight experiments are illustrated in Fig. 3c. Similar statistical results confirming the additive effect were found as in the case of nicotine-free extracts. This further supports the above conclusion that expression of E-selectin on ECs upon concurrent exposure of platelets in combination with the agonist that activates them was significantly increased relative to E-selectin expression obtained upon individual exposure of ECs to either platelets or agonists.

Conventional Cigarette Extracts and PAS

Extracts were prepared from two commercially available cigarettes (that contain nicotine): (1) Marlboro Red (1.2 mg nicotine); and (2) Quest-1 (0.6 mg nicotine) as outlined in the methods. Addition of these

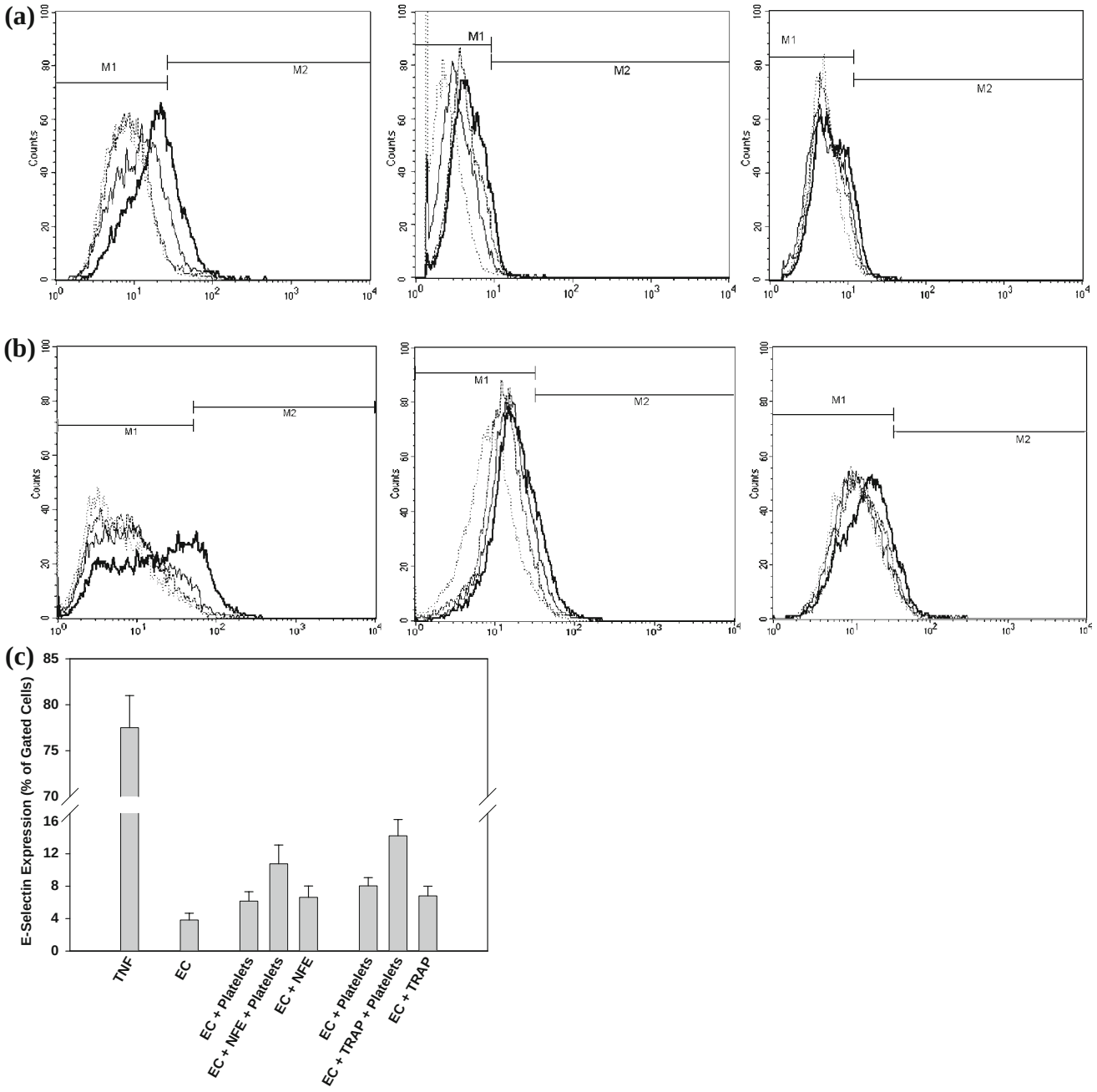

FIGURE 3. (a) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1A: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line). (b) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1B: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and TRAP-6 (dashed-dotted line), and (iv) shear stress and TRAP-6 and platelets (thick solid line). (c) Summary of % E-selectin positive gated ECs (mean ± s.e.m.) determined by flow cytometry. All experiments (n = 8) were done under shear stress in HSD as per conditions on horizontal axis, except TNF-α control. Smoke (10% NFE) and TRAP-6 (100 μM final) were used where stated.

extracts (10%) in place of the NFE, reduced platelet activation (PAS) relative to the latter ($p = 0.02$, Fig. 4). PAS was different in the presence of ECs (Fig. 4), but this difference was not influenced by the smoke extracts or nicotine ($p = 0.8$). The pair-wise differences between PAS for each condition (Table 2) were ascertained by post-hoc tests and the results are illustrated in Table 5. Despite the higher tar content of Marlboro cigarettes (16 mg) relative to the Quest cigarettes (10 mg), platelet activation was significantly

TABLE 4. Statistical summary for the expression of E-selectin on ECs.

Conditions	EC	EC + NFE	EC + platelets	EC + platelets + NFE
EC	–	1.00	1.00	0.01*
EC + NFE	1.00	–	1.00	0.03*
EC + platelets	1.00	1.00	–	0.01*
EC + platelets + NFE	0.01*	0.03*	0.01*	–

p-values from post-hoc pair-wise statistical tests for E-selectin expression in the presence of nicotine-free smoke extract, corresponding to conditions in Table 1A. Note that (*) indicates statistical difference.

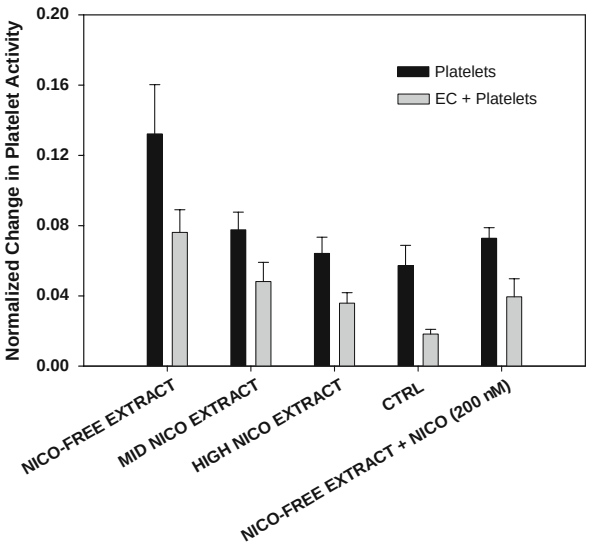

FIGURE 4. PAS for platelets ± ECs, in the HSD for (*left to right*): (1) nicotine-free (10% NFE), (2) medium-nicotine (10%), (3) high-nicotine (10%) extracts, (4) control (no smoke, no nicotine), and (5) condition (1) with pure nicotine (200 nM) added. Data (mean ± s.e.m.) in each bar is representative of five experiments.

reduced by the 2-fold higher nicotine content in the former ($p = 0.001$). Specifically, the PAS measured with Marlboro extract was less than that measured with the NFE by 48% (platelets only) and 47% (platelets + ECs). The PAS measured with the Quest-1 extract was also significantly lower compared to the NFE extract ($p = 0.03$). These findings clearly support the role of nicotine as a key modulator of platelet activation in smokers, at least within the short experimental durations as in the current study.

Further, when 200 nM nicotine was added in addition to 10% NFE, the resulting PAS decreased by 45% (platelets only) and 49% (platelets + ECs) relative to activation in the presence of NFE as reported above ($p = 0.004$). This confirms our earlier findings that nicotine is a key modulator of smoke-induced platelet activation.

DISCUSSION

Hemodynamic Shear Device

Platelet activation and aggregation at elevated shear stresses has been successfully investigated previously in the cone-plate viscometer.[15,30] The interaction between platelets and ECs has also been investigated in parallel-plate flow chambers[17] and also the cone-plate viscometer.[20] Equally well described is the annular Couette viscometer with a narrow annular gap and inner rotating cylinder, which has been used to investigate shear-induced blood damage and hemolysis of red blood cells.[21] The HSD design incorporates features of these two viscometers to maintain the same shear stress and facilitate sampling, as discussed under methods and in our recently published study.[19] In the present study, we used an intermittent shear stress of 9 dynes/cm^2 which is representative of flow in a normal coronary artery. Under these conditions, the flow is laminar ($\tilde{Re} < 0.5$) and secondary flow effects in the cone region therefore may not impose significant

TABLE 5. Statistical summary for the effect of nicotine on platelet activation (PAS at *T* = 54 min).

Conditions	Nicotine-free	Medium-nicotine	High-nicotine	Control	NFE + 200 nM nicotine
Nicotine-free	–	0.03*	0.00*	0.00*	0.00*
Medium-nicotine	0.03*	–	1.00	0.36	1.00
High-nicotine	0.00*	1.00	–	1.00	1.00
Control	0.00*	0.36	1.00	–	1.00
NFE + 200 nM nicotine	0.00*	1.00	1.00	1.00	–

The experimental conditions are described in Table 2. The table shows *p* values, determined from post-hoc pair-wise tests after significant difference for effect of experimental condition on PAS was established with two-way ANOVA ($p = 0.02$). Note that (*) indicates statistical difference.

localized forces and edge effects in the present system.[27] The present system can therefore be utilized to investigate EC-platelet or EC-leukocyte interactions under physiological or pathophysiological shear stresses.

E-Selectin Expression on ECs

We show that nicotine-free smoke extract or TRAP-6, when introduced together with platelets over ECs, result in a relatively greater increase in E-selectin expression on ECs. Although the mechanisms are unknown, it is likely that both smoke extract (or TRAP-6) and platelets contribute to the enhanced expression: (1) Downstream signaling resulting from engagement of receptor–ligand pairs on apposite cells may increase E-selectin expression on ECs. For instance, the presence of endothelial vWf in the cell suspension may act as bridging molecule between platelets and ECs, and this could be supplemented with integrin-like interactions between adhesion receptors on both cell types.[4] (2) ECs have been previously shown to increase E-selectin expression in response to smoke extracts[6] and also by TRAP-6 binding receptors on the surface.[26] We did not get significantly different E-selectin expression with platelets or nicotine-free extract introduced alone over ECs relative to ECs exposed to shear stress (control). However upon simultaneous introduction of platelets and nicotine-free extract on ECs, the E-selectin expression obtained is significantly different from the other three conditions (Table 4). We obtained similar results with TRAP-6 (in place of NFE), thus confirming the above mechanism of additive E-selectin expression.

Peak E-selectin expression on ECs in the presence of pro-inflammatory cytokines such as TNF-α and IL-1-β typically occurs at 4–6 h. The duration of our experiments was 54 min, after which ECs were immediately fixed. This may explain the lower E-selectin expression rates after 54 min in our experiments, but is comparable to levels found after TNF-α treatment beyond 2 h.[3] Such levels may be sufficient to induce leukocyte adhesion and progression of inflammation. The time limitation in our study arises from maintaining identical experimental conditions with the same passage of ECs and batch of platelets for one set of results (Table 1, four conditions) involving four experiments of 54 min each, done on the same day. Nevertheless, a relatively smaller but significant increase in E-selectin is observed and confirms our hypothesis. We may therefore conclude that such expression of E-selectin observed when activated platelets and agonists are introduced together over ECs, may represent the initial inflammatory response similar to the one induced by cytokines.

Modulation of PAS by Nicotine

The addition of pure nicotine to nicotine-free smoke extract has been previously shown to reduce platelet activation due to smoke extracts and shear stress.[22,23] We confirmed this protective effect of nicotine in our present study, albeit in the presence of ECs (Fig. 4). ECs have been known to express a subset (alpha-7) of cholinergic receptors on the surface, which can bind nicotine.[24] A recent *in vivo* study has demonstrated that nicotine may confer immunosuppressive effects on ECs by this mechanism.[24] Possible modulatory effects of nicotine on ECs (as mentioned above) may influence the differences observed with respect to reduction in PAS relative to nicotine-free extract (Fig. 4), however longer experiments are needed to investigate such effects. Platelet activation (PAS) was not statistically significant in the presence of medium- and high-nicotine extract relative to control ($p = 0.36$ and $p = 1.00$, respectively, Table 5). This shows that the presence of nicotine in the cigarette at concentrations above 200 nM may compensate for the harmful effects of non-nicotine cigarette components on platelet activation. Additionally, our conclusions regarding the protective effects of nicotine on platelet activation are well supported by our recent study with adult smokers whereby smoking zero-nicotine cigarettes caused a significantly higher (~3-fold) increase in platelet activation, than smoking medium-nicotine cigarettes.[11]

It should, however, be noted that the conclusion holds only for the short experimental duration of the study. Longer experiments with purified platelets are not feasible and the effects of nicotine and cigarette smoke extract over extended periods can only be investigated in animal models (*in vivo*). An alternative approach that involves pre-treatment of ECs with nicotine and cigarette smoke extract prior to exposure with platelets, under physiological conditions, will be investigated in the future.

PAS in Presence of ECs

Endothelial cells protect platelets from shear-induced activation by a variety of mechanisms.[2] Such mechanisms may be initiated by mechanotransduction under shear stresses, and may be influenced by the topography and the net-forward magnitude of the stresses.[7] Our shear stress condition is laminar and atheroprone effects associated with disturbed flow conferring an inflammatory phenotype to ECs may therefore not be expected.[7,12] Given the short duration and lower shear stress conditions in our study, antithrombotic effects due to release of NO and prostacyclins may be significant only during the acceleration and peak shear stress phase (~25% experimental time or 14 min). We believe that the difference in PAS arise

due to a combination of the above factor and the fact that ECs may form a passivating layer for the platelets, which may be less thrombogenic than the material surfaces of our device. As observed in Fig. 4, PAS was different in the presence of ECs, however this difference was not influenced by nicotine or smoke extracts. This is indicated by the lack of significance of the effect of interaction between group (platelets, ECs and platelets) and experimental conditions (i.e., nicotine and smoke) in two-way ANOVA ($p > 0.05$).

Our primary focus here is to demonstrate the pro-inflammatory effects of nicotine-free smoke on platelets in the presence of ECs, thereby bringing the system close to physiology. We clearly show that PAS increases significantly in the presence of the nicotine-free extract, relative to control (Fig. 2). Additionally, we observe that the rate of increase of PAS in the presence of such extracts (i.e., PAR) is non-linear. This fact corroborates our earlier findings of non-linear increases in PAS due to platelet-platelet co-operativity at physiological (2×10^8/mL) concentrations. We attributed such increase to the enhanced Factor Va release by activated platelets. In the present study, we observed that the PAR was statistically more non-linear (Figs. 2a and 2b) when smoke extract was added to the platelets (irrespective of the presence of ECs). Such non-linearity may be enhanced by external agonists such as cigarette smoke which may induce much higher Factor Va release by platelets.

In summary, we show that the HSD is a compact *in vitro* device to study the interaction between circulating and cultured cells under physiological shear stresses. More importantly, we also show that E-selectin expression on ECs is enhanced significantly only upon simultaneous exposure to platelets and nicotine-free cigarette smoke extract. We additionally confirm that nicotine decreases platelet activation due to cigarette smoke, in the presence of ECs. In this *in vitro* study this effect of nicotine was studied within a combined system of ECs and platelets, which brings this study closer to a physiologically relevant scenario. Such a system may therefore be used to develop precursor models to study inflammation *in vitro*. More studies that predispose the ECs to an inflammatory phenotype, before platelets or agonists are added are clearly needed.

In conclusion, our study supports the hypothesis that cigarette smoke is likely associated with increased cardiovascular disease risk, and this may be effectively modulated by nicotine.

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