Enhanced platelet adhesion induces angiogenesis in intestinal inflammation and inflammatory bowel disease microvasculature

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Abstract

Although angiogenesis is viewed as a fundamental component of inflammatory bowel disease (IBD) pathogenesis, we presently lack a thorough knowledge of the cell type(s) involved in its induction and maintenance in the inflamed intestinal mucosa. This study aimed to determine whether platelet (PLT) adhesion to inflamed intestinal endothelial cells of human origin may favour angiogenesis. Unstimulated or thrombin-activated human PLT were overlaid on resting or tumour necrosis factor (TNF)-α-treated human intestinal microvascular endothelial cells (HIMEC), in the presence or absence of blocking antibodies to either vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, integrin αβ3, tissue factor (TF) or fractalkine (FKN). PLT adhesion to HIMEC was evaluated by fluorescence microscopy, and release of angiogenic factors (VEGF and soluble CD40L) was measured by ELISA. A matrigel tubule formation assay was used to estimate PLT capacity to induce angiogenesis after co-culturing with HIMEC. TNF-α up-regulated ICAM-1, αβ3 and FKN expression on HIMEC. When thrombin-activated PLT were co-cultured with unstimulated HIMEC, PLT adhesion increased significantly, and this response was further enhanced by HIMEC activation with TNF-α. PLT adhesion to HIMEC was VCAM-1 and TF independent but ICAM-1, FKN and integrin αβ3 dependent. VEGF and sCD40L were undetectable in HIMEC cultures either before or after TNF-α stimulation. By contrast, VEGF and sCD40L release significantly increased when resting or activated PLT were co-cultured with TNF-α-pre-treated HIMEC. These effects were much more pronounced when PLT were derived from IBD patients. Importantly, thrombin-activated PLT promoted tubule formation in HIMEC, a functional estimate of their angiogenic potential. In conclusion, PLT adhesion to TNF-α-pre-treated HIMEC is mediated by ICAM-1, FKN and αβ3, and is associated with VEGF and sCD40L release. These findings suggest that inflamed HIMEC may recruit PLT which, upon release of pro-angiogenic factors, actively contribute to inflammation-induced angiogenesis.

Keywords: angiogenesis • tumour necrosis factor • inflammation • inflammatory bowel disease

Introduction

Angiogenesis is an intricate process resulting in the generation of novel vessels from pre-existing ones, through the promotion of endothelial cell (EC) division, degradation of vascular base-...
inflammation [3]. In addition to pro-inflammatory actions, IL-8 has been implicated in tumour angiogenesis and may be secreted by colonic epithelial cells and microvessel EC [4]. Similarly, basic fibroblast growth factor (bFGF) may mediate tumour necrosis factor (TNF)-α-induced angiogenesis both in vitro and in vivo [5]. In this respect, we have recently shown that angiogenesis may be implicated in the pathogenesis of inflammatory bowel disease (IBD) [6]. In line with this assumption, microvessel density is increased in IBD mucosal tissues, a phenomenon that is mostly dependent upon heightened IL-8, bFGF and VEGF release [6]. In addition, angiogenesis blockade may represent a new and promising therapeutic target in experimental models of IBD [7, 8].

The CD40-CD40 ligand (CD40L) axis is a regulator and amplifier of immune reactivity and contributes to leucocyte and platelet (PLT) adhesion to inflamed intestinal EC [8–10]. CD40 expression is prominent in pathological conditions associated with angiogenesis and inflammation, and ligation of CD40 on EC and monocytes leads to production of bFGF and VEGF [11]. For instance, CD40 engagement on synovial fibroblasts by CD40L-expressing activated T cells up-regulates the production of VEGF, providing a potential explanation for the occurrence of neovascularization in rheumatoid arthritis [12]. CD40L is over-represented in patients with IBD, mainly reflecting enhanced expression on the PLT surface and spontaneous release into the circulation [10, 13]. Plasma sCD40L is particularly elevated in patients with active Crohn’s disease and ulcerative colitis compared with patients with inactive disease and with healthy controls [10, 13].

IBD has been associated with PLT dysfunction, and increased PLT aggregation to epinephrine, collagen and/or ADP [14]. Increased PLT expression of P-selectin [15] and elevation of PLT-derived microparticles have been previously reported in patients with IBD. In addition to qualitative abnormalities, PLT are numerically elevated in patients with IBD in relation with increased thrombopoietin and IL-6 serum levels [16, 17]. Interestingly, an increased tendency to form PLT-leucocyte aggregates has been described both in the affected colonic mucosa and in the peripheral blood of patients with active IBD, but not in other chronic inflammatory disorders [18, 19]. PLT-derived inflammatory and pro-angiogenic mediators such as sCD40L and VEGF may be instrumental in the in vitro migration and vessel-like organization of EC, pointing to a role for PLT in inflammatory neovascularization [20].

The present study was designed and conducted to determine whether activated PLT may contribute to angiogenesis through an enhanced adhesiveness to inflamed EC with subsequent release of pro-angiogenic growth factors. We also addressed the potential molecular determinants of PLT–EC interactions that may contribute to angiogenesis and inflammation in the IBD microvasculature [21]. We show herein that PLT adhesion to inflamed microvessel EC translates into an enhanced release of pro-angiogenic mediators, providing clues on the potential role of activated PLT in the promotion of inflammation-driven angiogenesis in the gut.

**Materials and methods**

**Patient population**

Patients with active IBD were studied after their informed consent. The investigations were reviewed and approved by the local Ethical Committee. All diagnoses were confirmed by clinical, radiological, endoscopic and histological criteria, as previously detailed [10, 13]. Anatomical disease extent was assessed by radiological and endoscopic examination. Peripheral blood samples were also obtained from consented healthy blood donors and were used to isolate PLT for control experiments, as reported [13, 22]. Patients’ characteristics were summarized in Table 1.

**Procurement and culture of HIMEC**

Surgical specimens of colonic origin were used to isolate human intestinal microvascular endothelial cells (HIMEC), as reported elsewhere [23, 24]. Briefly, after enzymatic digestion of intestinal mucosal strips, samples were gently compressed to extrude EC clumps, which adhered to fibronectin-coated plates, and were subsequently cultured in MCDB131 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% FBS, antibiotics, heparin, and EC growth factor. HIMEC were routinely plated on fibronectin-coated wells of a 24-well cluster plate at a density of $5 \times 10^5$/well. For HIMEC activation, cells were supplemented with 100 IU/ml TNF-α (R&D Systems, Oxon, UK). Cultures of HIMEC were maintained at 37°C in 5% CO₂ and cells were used between passages 3 and 10 [23].

**Table 1 Patients’ characteristics**

| Number of patients | 17 |
|--------------------|----|
| UC                 | 8  |
| CD                 | 9  |
| Sex                |    |
| Male               | 9  |
| Female             | 8  |
| Disease duration (years) | 8 (2–18) |
| Location           |    |
| Ileal              | 6  |
| Colonic            | 9  |
| Ileo-colonic       | 2  |
| Proximal           | 0  |
| Concomitant medications |    |
| Steroids           | 4  |
| Azathioprine       | 7  |
| Mesalamine         | 12 |
| Antibiotics        | 2  |
Isolation of PLT and PLT-HIMEC co-culture

PLT from normal donors were obtained by gel filtration of PLT-rich plasma (PRP) onto Sepharose 2B columns (25 × 1 cm) equilibrated with a buffer containing 20 mM Hepes, 135 mM NaCl, 5 mM KCl, 5 mM glucose, 0.2% bovine serum albumin (pH 7.4). Ethylenediaminetetraacetic acid (EDTA) disodium salt (1 mM final concentration) was added to the PRP prior to gel filtration to minimize PLT activation during washing procedures. The resulting PLT population was essentially free of contaminating erythrocytes (<0.1%) and peripheral blood mononuclear cells. In order to rule out PLT activation due to the isolation procedure, PLT activation state was assessed before and after isolation by measuring P-selectin expression levels, as previously detailed [10]. The PLT count was adjusted to 1 × 10^5/µl with washing buffer in all the experiments [13, 25].

Confluent monolayers of HIMEC were incubated with either 2% bovine serum albumin alone or 0.5 U/ml thrombin alone, or they were overlaid with 100 × 10^5 resting or thrombin-activated PLT. Plates were immediately centrifuged at 700× g for 2 min. to bring PLT and HIMEC in close apposition. Each experiment was performed in duplicate. After 4 hrs at 37°C, supernatants were harvested, transferred to polypropylene tubes, centrifuged at 1300× g at 4°C for 10 min. to remove cell debris, and stored at −70°C until analysis. In preliminary experiments, we ensured, by visual microscopy and flow cytometry with PLT-specific CD42b antibodies, that washings had removed virtually all PLT from the HIMEC monolayers. At the end of the co-culture, HIMEC were washed five times in cold phosphate buffer saline (PBS) and a single cell suspension obtained using a detaching buffer (PBS, 20 mMol/l HEPES, pH 7.4, 10 mMol/EDTA and 0.5% bovine serum albumin) for 10 min. each on ice and at 37°C, followed by vigorous pipetting.

Release of pro-angiogenic factors

VEGF-A and sCD40L levels in culture supernatants were measured in triplicate with commercially available ELISA, following the manufacturer’s instructions. The limits of detection were as follows: <9 pg/ml VEGF-A and <10.1 pg/ml sCD40L.

In vitro tube formation assay

EC tube formation was assessed using Matrigel™, a solubilized extracellular basement membrane matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, as detailed elsewhere [26]. Briefly, multiwell dishes were coated with 250 µl of complete medium containing 5 mg/ml Matrigel™ and HIMEC re-suspended in complete growth medium were seeded at a density of 5 × 10^5. Cells were cultured on Matrigel™ for 16 hrs and inverted phase-contrast microscopy was used to assess formation of endothelial tube-like structures. Five high-power fields per condition were examined and experiments were performed in duplicate.

Statistical analysis

The approximation of data distribution to normality was preliminarily tested with statistics for kurtosis and symmetry. Results were presented as mean and S.D. All comparisons were performed with the Student's t-test for paired or unpaired determinations or with the ANOVA, as appropriate. The criterion for statistical significance was defined as P ≤ 0.05.

Results

The pro-inflammatory cytokine TNF-α enhances the expression of cell adhesion molecules (CAM) on HIMEC

In a first set of experiments, we determined how the exposure of HIMEC to TNF-α would affect the expression pattern of a spectrum of adhesion molecules deemed relevant for HIMEC interaction with PLT. To this end, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, αvβ3 integrin, tissue factor (TF) and fractalkine (FKN) expression was investigated by flow cytometry on HIMEC that were exposed to TNF-α in vitro for 16 hrs. As shown in Fig. 1A, TNF-α-treated HIMEC up-regulated ICAM-1, αvβ3 integrin and FKN, and expressed de novo VCAM-1 and TF when compared to CAM levels on untreated HIMEC. The analysis of the mean fluorescence intensity ratios indicated that the magnitude of adhesion molecule induction by TNF-α differed significantly, with αvβ3 integrin and FKN being particularly responsive to the pro-inflammatory stimulus applied to HIMEC (Fig. 1B).

Inflammation promotes PLT adhesion to HIMEC

To ascertain whether a prototypical inflammatory cytokine such as TNF-α was endowed with the ability to promote PLT adhesion to HIMEC, 10 × 10^5 PLT from healthy control patients were co-cultured with 50 × 10^5 HIMEC that were activated with TNF-α, as above detailed. To dissect the role of PLT activation status, if any, in the interaction with HIMEC, PLT were treated with thrombin prior to HIMEC co-culture.

The baseline adhesion of resting PLT to untreated HIMEC is shown in Fig. 2, left part. The stimulation of HIMEC with TNF-α enhanced PLT adhesion (Fig. 2). Interestingly, activation with thrombin further increased the number of adhering PLT to resting HIMEC and, to an even greater extent, the number of PLT that adhered to TNF-α-activated HIMEC (Fig. 2). It should be pointed out that provision of TNF-α to HIMEC per se was capable of enhancing the adhesion of resting PLT compared to that recorded with unstimulated HIMEC, as shown in Fig. 2. Collectively, these studies suggest that a pro-inflammatory cytokine stimulus promotes the adhesion of resting PLT to HIMEC and that this effect is significantly enhanced by PLT activation prior to the co-culture.

PLT adhesion to inflamed HIMEC is mediated through ICAM-1, αvβ3 and FKN

We next attempted to dissect the mechanism(s) that govern PLT adhesion to HIMEC under the inflammatory conditions that we established herein. Because provision of TNF-α to HIMEC translates into the up-regulation of selected adhesion molecules, we maintained PLT-HIMEC co-cultures either in the presence or
absence of blocking antibodies directed against VCAM-1, ICAM-1, \( \alpha_v\beta_3 \) integrin, TF and FKN. Co-cultures were also performed with resting HIMEC and resting PLT (negative control for PLT adhesion) and with TNF-\( \alpha \)-stimulated HIMEC and thrombin-activated PLT (positive control for PLT adhesion). As shown in Fig. 3, neutralization of either surface ICAM-1, \( \alpha_v\beta_3 \) integrin, or FKN significantly, albeit not completely, inhibited PLT adhesion to HIMEC. Conversely, no changes in the number of adhering PLT were recorded in co-cultures established in the presence of blocking antibodies to either VCAM-1 or TF compared with the control co-cultures containing TNF-\( \alpha \)-activated HIMEC and thrombin-activated PLT. Collectively, neutralization studies suggest that a restricted panel of adhesion molecules is involved in the enhanced PLT adhesion to inflamed endothelial surfaces in our system model.

**PLT from patients with active IBD are particularly effective at releasing pro-angiogenic growth factors upon co-culture with HIMEC**

We also aimed to ascertain whether PLT interaction with inflamed HIMEC may promote the release of pro-angiogenic growth factors. To accomplish this goal, we initially measured the release of VEGF-A and sCD40L in the supernatant of co-cultures established with HIMEC and PLT from healthy donors. VEGF-A was undetectable in the supernatant of unstimulated HIMEC, but was readily measured in supernatants of resting PLT (Fig. 4). PLT activation with thrombin translated into a significant up-regulation of VEGF-A levels compared with those detected in the supernatant of resting PLT. Of interest, pre-treatment of HIMEC with the pro-inflammatory cytokine TNF-\( \alpha \) enhanced the release of VEGF-A in the co-culture with both resting and thrombin-activated PLT (Fig. 4).

We subsequently reproduced the above co-culture experiments using PLT from patients with active IBD, either Crohn’s disease or ulcerative colitis. PLT from patients with IBD released greater amounts of VEGF-A both spontaneously and following activation with thrombin compared with equal numbers of PLT from healthy controls (Fig. 4). Notably, VEGF-A production by resting PLT was further enhanced in the co-cultures containing TNF-\( \alpha \)-activated HIMEC. Not unexpectedly, the highest release of VEGF-A was detected in the co-cultures established with thrombin-activated PLT and TNF-\( \alpha \)-activated HIMEC.

The same culture conditions were applied in further experiments aimed at quantifying sCD40L production. As depicted in...
Fig. 5, PLT from healthy controls released sCD40L and this phenomenon was significantly enhanced by PLT activation with thrombin. Whereas TNF-α-activated HIMEC promoted sCD40L release from unstimulated PLT, no such increase in sCD40L production occurred in the co-cultures established with activated PLT and TNF-α-stimulated HIMEC compared with those containing activated PLT alone (Fig. 5). Interestingly, PLT from patients with active IBD released significantly greater quantities of sCD40L compared with PLT from healthy controls, both spontaneously and following thrombin activation. Finally, the co-culture of PLT from IBD patients with TNF-α-activated HIMEC further promoted sCD40L secretion, as depicted in Fig. 5. Collectively, PLT isolated from patients with active IBD were endowed with a greater capacity to secrete both VEGF-A and sCD40L either untouched or following their in vitro activation with thrombin. No differences were found between PLTs derived from Crohn’s disease or ulcerative colitis patients (data not shown).

Activated PLT favour tubule formation in HIMEC cultures

In a further set of functional studies, we wanted to determine whether thrombin-activated PLT may induce tubule formation in HIMEC. To answer this, HIMEC were cultured on a solubilized extracellular basement membrane extract and used to assess the formation of endothelial-like structures by inverted phase-contrast microscopy [26]. As clearly shown in Fig. 6A, tube-like structures could be seen after co-culturing fluorescently labelled resting HIMEC with thrombin-activated PLT derived from healthy controls but not in cultures established with HIMEC alone. Thrombin alone failed to induce tubule formation by HIMEC (data not shown). Fluorescent microscopy experiments with PLT-HIMEC co-cultures stained with different fluorochromes indicated that activated PLT preferentially localized at endothelial luminal surfaces, as shown in a 3-dimensional adhesion assay on matrigel (Fig. 6B).
Discussion

It is presently believed that IBD results from the interaction of genetic, environmental, microbial and immune factors. Accumulating evidence suggests that non immune cells such as mucosal EC, fibroblasts, neurons and PLT actively contribute to IBD pathogenesis [27]. In particular, we and others have previously provided novel and substantial evidence that PLT are dynamic participants in the multi-component system responsible for mucosal inflammation and injury [28]. Since the number and...
activation state of PLT are markedly increased in IBD patients. PLT contained within the systemic circulation represent a potential risk factor for triggering an inflammatory response at the intestinal level. For these considerations, PLT are currently viewed as an attractive target for therapeutic intervention [13, 22, 29]. In a different disease context such as multiple sclerosis, PLT-derived VEGF reportedly sustains angiogenesis, a process that may be exacerbated as a result of PLT interaction with the injured ECs [30].

CD40L, also referred to as gp39, is a cell surface molecule largely restricted to activated CD4+ T cells, and is also expressed by human vascular EC, smooth muscle cells and macrophages. The CD40-CD40L axis has been implicated in the pathogenesis of several human diseases, including atherosclerosis [31], rheumatoid arthritis [12] and IBD [8]. PLT have been reported to trigger CD40-dependent inflammatory responses in the IBD microvasculature and to induce endothelial CAM up-regulation, chemokine secretion and leucocyte recruitment [10]. However, the role of activated PLT in inducing mucosal angiogenesis has not been explored yet, although extensive data on PLT-microvasculature interactions have been reported in mice [32]. Since PLT aggregation and microthrombi are frequent findings in the IBD microvasculature [28, 33, 34], it would be particularly important to gain insights into the molecular determinants of such interaction in human beings.

Our data show that human PLT adhesion to HIMEC mainly occurs when PLT, HIMEC or both are activated, and that PLT-HIMEC interactions are mediated by ICAM-1, avβ3 integrin, and FKN as suggested by antibody blockade experiments. Not unexpectedly, TNF-α, a potent pro-inflammatory stimulus, induced adhesion molecule expression on HIMEC, leading to the promotion of PLT adhesion. FKN and avβ3 integrin manifested the highest degree of induction in response to TNF-α stimulation, suggesting that these CAM may be exquisitely sensitive to a pro-inflammatory milieu. Remarkably, FKN has been shown to mediate the adhesion of FKN receptor-expressing T cells to HIMEC, pointing to this molecule as a determinant of HIMEC interaction with both immune and non-immune cells [35, 36].

In addition to adhering to the activated endothelium, PLT are known to secrete a wide array of pro-inflammatory mediators [28]. In the present study, we focused mainly on angiogenic molecules such as VEGF-A and sCD40L. As shown by the co-culture experiments, PLT were the main source of both VEGF-A and sCD40L, and PLT secretion was higher when PLT were activated with thrombin, or maintained in co-culture with TNF-α-treated HIMEC, a finding compatible with higher PLT adhesion and with enhanced PLT activation. Notably, PLT from patients with active IBD were particularly prone to release pro-angiogenic VEGF-A and sCD40L, either spontaneously or in response to thrombin, when compared with PLT.

![Graph showing release of sCD40L upon co-culture of HIMEC with PLT from healthy controls and patients with active IBD.](image)

**Fig. 5** Release of sCD40L upon co-culture of HIMEC with PLT from healthy controls and patients with active IBD. PLT from either healthy controls (n = 8, empty columns) or patients with active IBD (8 UC and 9 CD; shaded columns) were co-cultured with HIMEC that were either left untouched or activated with TNF-α. The release of sCD40L was measured with conventional ELISA in culture supernatants. The bar graph (mean ± S.D.) summarizes the results obtained in six independent experiments.
from healthy volunteers. In addition, VEGF-A and sCD40L production by PLT from patients with IBD were maximal upon co-culture with TNF-α-activated HIMEC. Indeed, these in vitro experiments might recapitulate an in vivo scenario, where PLT circulate in the inflamed IBD microvasculature, encounter an activated endothelium, adhere and get activated themselves, contributing to foster intestinal inflammation [28]. When translating our findings to an in vivo context, it is conceivable that neither PLT adhesion to EC nor PLT release of pro-angiogenic mediators perturb intestinal homeostasis under physiological conditions. This is backed by our observation that both the number of resting PLT adhering to HIMEC and the release of VEGF and sCD40L by resting PLT are significantly lower compared with the data recorded with thrombin-activated PLT, suggesting that in vivo PLT activation occurring in patients with IBD is required to trigger PLT recruitment and adhesion [9], and to foster the release of pro-angiogenic cytokines upon interaction with the inflamed EC. Unequivocal evidence is now available in favour of PLT activation in patients with IBD, including an increased tendency to form PLT-leucocyte aggregates [37], an increased PLT aggregation response to epinephrine, collagen and ADP [14], and the ability of activated PLT to induce the formation of reactive oxygen species by polymorphonuclear leucocytes [38]. It is thus tempting to speculate that PLT activation status will dictate the outcome of PLT–EC interaction in vivo.

In addition, we explored the role of PLT as a novel cell type involved in mediating angiogenesis. EC tube formation on basement membranes replicates many of the steps in angiogenesis, encompassing adhesion, migration, protease activity, alignment and tube formation [39]. In our tube formation assays with a solubilized extracellular membrane matrix, the presence of thrombin-activated PLT prompted robust angiogenesis, suggesting a potential active role of PLT in mediating inflammation-induced angiogenesis. We have recently shown that CD40L and VEGF-A are crucial molecules that act at the cross-road between angiogenesis and inflammation [7]. In support of this, preclinical models of experimental IBD have shown that blockade of either CD40L or VEGF-A may induce significant amelioration of experimental colitis, associated with dramatic inhibition of inflammation-induced angiogenesis. Therefore, it is tempting to speculate that such therapeutic effect could also result from the inhibition of PLT-derived pro-angiogenic and pro-inflammatory sCD40L and VEGF-A.

Collectively, our study indicates that PLT may serve as partners for the promotion of angiogenesis and sheds some light into the molecular determinants that drive inflammation-induced angiogenesis. Whether PLT may be successfully targeted to limit pro-angiogenic factor release and dampen inflammation in clinical IBD will have to be tested by future clinical trials.

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**Fig. 6** Tubule formation assay and visualization of PLT-HIMEC interaction. (A) PLT from healthy controls were either left untouched or activated with thrombin. Tubule formation was evaluated as detailed in 'Materials and methods'. One representative experiment out of six with similar results is shown. (B) PLT from healthy controls were either left untouched or activated with thrombin. The interaction of HIMEC (green) and PLT (red) was visualized by confocal microscopy. One representative experiment out of four with similar results is shown.
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Conflict of interest

The authors declare no competing financial interests.

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