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Evidence that platelets promote tube formation by endothelial cells on matrigel

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1 The involvement of platelets in neovascularization was investigated in the matrigel tube formation assay, an *in vitro* model of angiogenesis.

2 Platelets promoted the formation of capillary-like structures (expressed as relative tube area) number- and time-dependently. Relative tube area increased from 0.98 ± 0.02 ($n=8$) in the presence of 6.25×10^4, to 3.21 ± 0.12 ($n=8$) in the presence of 10^6 platelets/well compared to 0.54 ± 0.04 ($n=8$) in their absence. This increase was unaffected by acetyl salicylic acid (ASA), apyrase, and hirudin. Photographs from representative experiments, showed that platelets adhered along the differentiating endothelium.

3 Addition of α-thrombin (0.1–1 i.u. ml^{-1}), the nitric oxide (NO) donor sodium nitroprusside (SNP; 1–100 μM) or the NO synthase inhibitor, L-NG-arginine-methylester (L-NAME, 30–300 μM) to the assay, had no effect on tube formation compared to that seen with platelets alone.

4 Neuraminidase (0.01 i.u./10^7 platelets), which strips sialic acid residues from membrane glycoproteins, abolished the promoting effect of platelets on tube formation. The relative tube area in the presence of neuraminidase-treated platelets was 0.81 ± 0.03 ($n=8$), in the presence of untreated platelets 1.69 ± 0.09, $P<0.001$ ($n=8$) and in the absence of platelets, 0.80 ± 0.04 ($n=8$). The tetrapeptide Arg-Gly-Asp-Ser (RGDS; 20–200 μM) which inhibits von Willebrand factor, fibrinogen and fibronectin-mediated adhesion, had no effect on the promoting effect of platelets on tube formation.

5 These results indicate that platelets promote angiogenesis *in vitro*. This effect is largely independent from activation by α-thrombin, is not modified by manipulating NO and prostaglandin metabolism and proceeds possibly through adhesion of the platelets to the differentiating endothelium.

Keywords: Angiogenesis; platelets; *in vitro* matrigel; endothelial cells; α-thrombin; sodium nitroprusside; nitric oxide; cyclic guanylate monophosphate (cyclic GMP)

Introduction

Angiogenesis, is a complex and highly regulated process involving activation proliferation and migration of endothelial cells to form new capillaries from pre-existing vessels (Folkman & Singh, 1992; Maragoudakis, 1993). In the mature human, under physiological conditions, this process is associated with the female reproductive cycle and wound healing. Non physiological and unregulated angiogenesis accompanies disease states such as tumour growth and metastasis. It is now known that in angiogenesis dependent diseases (tumour growth, psoriasis) there exists increased thrombogenesis (Rickles & Edwards, 1983; McDonald, 1991). Central to thrombogenesis is the activation and adhesion of platelets. Platelets therefore may be involved in the mechanisms underlying angiogenesis. Angiogenesis, tumor growth and metastasis are indeed processes where platelets are believed to play an important role (Gasic *et al*., 1968, 1973; Pearlstein *et al*., 1984; Karpatkin *et al*., 1988; Nierodzik *et al*., 1991, 1992; Honn *et al*., 1992; Tsopanoglou *et al*, 1993). Platelet activation and adherence to the endothelium may lead to increased permeability and to initiation of proliferative phenomena through the release of growth factors (Page, 1988) many of which are angiogenic (Folkman & Singh, 1992).

It has been previously shown that α-thrombin, a potent activator of platelets (Fenton *et al*., 1991) stimulates angiogenesis in the chick chorioallantoic membrane (CAM) (Tsopanoglou *et al*., 1993). Moreover, nitric oxide (NO) which interferes with both platelet aggregation and adhesion (Radomski & Moncada, 1991), was found to be an inhibitor of angiogenesis in the CAM and of tumour growth and metastasis in mice implanted with Lewis Lung carcinoma (Pipili-Synetos, *et al*., 1994, 1995). However, both α-thrombin and NO exhibit a different profile when they are examined in the corneal assay of angiogenesis. In this normally avascular preparation, α-thrombin alone is ineffective (Knighton *et al*., 1982) whereas NO potentiates neovascularization (Ziche *et al*, 1994). In the cornea, α-thrombin can induce new vessel growth only when combined with platelets. The above observations indicate that platelets may indeed play a role in the angio- or anti-angiogenic effects of α-thrombin and NO respectively. To test this hypothesis the effect of platelets was examined on the matrigel tube formation assay (Kubota *et al*., 1988) and *in vitro* model of angiogenesis where endothelial cells are plated on laminin-rich basement membrane substratum and they form capillary-like structures.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously described (Jaffe *et al*., 1987). The cells were grown as monolayers in medium M199 supplemented with 20% fetal calf serum (FCS), 200 μg ml^{-1} endothelial cell growth supplement (ECGS), 4 u ml^{-1} heparin sodium, 100 u ml^{-1} penicillin-streptomycin and 50 μg ml^{-1} gentamicin. Cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

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Matrigel tube formation assay

The tube formation assay was performed as previously described (Kubota *et al*., 1988). Briefly, matrigel was used to coat the wells of 24-well plates (0.25 ml per well) and was left to polymerize at 37°C for 1 h. After polymerization, HUVECs (40,000 cells) suspended in 0.9 ml of M199 supplemented only with 2% FCS and antibiotics, were added to each well. The platelet suspension (10^7 platelets ml^{-1}) was then added in volumes ranging from 6.25–100 μl resulting in final concentrations of $6.25\times10^4-10^6$ platelets/well (or ml^{-1}). The final volume per well was adjusted, where necessary, to 1 ml with platelet suspension buffer. The test materials were either added to the wells just prior to addition of platelets or platelets were incubated with them prior to being added to the endothelial cells. In some experiments, platelets were removed by centrifugation after preincubation under different conditions and their supernatants were added to the HUVEC cultures. The effects of platelets on tube formation were evaluated under the following conditions: (a) platelets preincubated in resuspension buffer, (b) platelets preincubated in culture medium (c) platelets preincubated in culture medium on top of Matrigel, (d) platelets preincubated in medium from a HUVEC monolayer, (e) platelets preincubated in the presence of α-thrombin in resuspension buffer. The preincubations of platelets were performed for 15–30 min.

After 18 h of incubation with platelets or platelet supernatants with HUVECs on matrigel, the medium was removed and the cells were fixed and stained with Diff quick (Baxter Scientific). Photographs were then taken through a stereoscope (OLYMPUS SZ-PT) at a 1.5 magnification. These were then scanned and the resulting images were improved by enhancement, restoration and adjustment of the grey level. They were subsequently digitized to 650×450 pixels. The length of the tubes was measured after having adjusted all tubes to one pixel thickness. The tube area was then expressed as the percentage of pixels occupied by the 1-pixel-thick tubes in the 650×450 image and was quantitated using an image analysis software (ImagePC, Scion Corporation).

Platelet preparation

Suspensions of washed human platelets were prepared the day of the experiment as previously described (Pipili, 1985) and were used within 2 h after isolation. Briefly, whole blood was drawn from healthy drug-free donors in acid-citrate-dextrose anticoagulant (9 : 1 volume) and platelet-rich plasma (PRP) was obtained by centrifugation at $700\times g$ for 5 min. The PRP was removed and centrifuged at $350\times g$ for 20 min. The platelet pellet was gently resuspended in the suspension medium (mM) HEPES 10, pH 7.4, NaCl 145, KCl 5, $MgSO_4$ 1 and glucose 10, prewarmed at 37°C. 20 $\mu g\ ml^{-1}$ apyrase and 100 μM acetyl salicyclic acid (ASA) were always added at all steps, unless otherwise indicated. Where indicated, washed platelets were preincubated with neuraminidase (0.01 i.u./10^7 platelets) from *Vibrio cholera* and then washed again. Under these conditions, the effect of α-thrombin or collagen-induced aggregation has been shown to be reduced by 70% (van Ijzendoorn *et al*, 1996).

Sialic acid analysis was performed by HPLC according to the method of Karamanos *et al*. (1990) using per-O-benzoylated derivatives and UV detection at 231 nm. Briefly, supernatants from platelets (10^7) which had been subjected to neuroaminidase treatment were freeze-dried to reduce the final volume and subjected to mild acid hydrolysis with 20 mM trifluoracetic acid at 80°C for 2 h. These conditions are appropriate for the complete liberation of sialic acids from glycoproteins, ensure acyl group release and prevent destruction of sialic acids. N-acetylneuraminic acid and N-glycolyneuraminic acid were used as standards.

Statistical analysis

Each experiment included at least duplicate wells for each condition tested. All results are expressed as mean$\pm$s.e.m. relative tube area, from at least three independent experiments. Results were compared by unpaired *t*-test and *n* signifies the number of experiments. Sigmoidal curve fitting was performed by the SLIDEWRITEPLUS (Advanced Graphics Software, Inc. CA, U.S.A.) graphics program according to the equation: $y=a_0+a_1(1+\exp(-(x-a_2)/a_3))$.

Materials

Matrigel was a generous gift from Dr Hynda Kleinman (N.I.H., Bethesda, U.S.A.) and Dr Theodore Fotsis (Ioannina, Greece). Alpha-Thrombin was a generous gift from Dr J. Fenton (Albany, N.Y., U.S.A.). Sodium nitroprusside (SNP), L-NG-arginine-methylester (L-NAME), apyrase, acetyl salicyclic acid (ASA), hirudin, RGDS, heparin sodium and ECGS were purchased from Sigma. Medium M199 and the rest of its supplements were purchased from Biochrom KG (Seromed). Neuraminidase from *Vibrio cholera* was a kind gift from Dr N. Karamanos.

Results

When increasing numbers of platelets were included in the tissue culture medium of the tube formation assay, there was an increase in the capillary-like structures formed (expressed as relative tube area), compared to controls without platelets (0.54 ± 0.04) (Figure 1). For platelet numbers ranging from 6.25×10^4 to 10^6 per well, this increase ranged between 0.98 ± 0.02, $P<0.001$, ($n=8$) and 3.21 ± 0.12, $P<0.001$, ($n=8$). In these experiments, platelets were isolated in the presence of apyrase and ASA. Similar results were obtained in the absence of ASA or apyrase or when the α-thrombin antagonists hirudin was incorporated in the procedure of platelet isolation (Figure 1). Figure 2a, b, c and d, are photographs from representative experiments showing capillary-like structures formed in the absence (a, c) and presence (b, d) of platelets (5.0×10^5 per well) after 5 h (a, b) and 18 h (c, d) of incubation. It can be seen that in the presence of platelets, the capillary-like network is almost complete (Figure 2b) after only 5 h of incubation compared to the time control (in the absence of platelets, Figure 2a). In this latter case, the endothelial cells have only just started to align. It therefore appears that platelets affect both the magnitude (Figure 2c and d) and the kinetics (Figure 2a and b) of tube formation.

The effect of platelets on tube formation was greatly reduced when platelets were centrifuged and increasing volumes of supernatant were added to the assay (Figure 3). 12.5, 25 and 100 μl of supernatant from a platelet suspension of 10^7 platelets ml^{-1} (corresponding to 1.25×10^5, 2.5×10^{-5} and 10^{-6} platelets in the uncentrifuged suspension) were added to the assay. It can be seen that these supernatants resulted in 0.60 ± 0.08 ($n=6$), 0.64 ± 0.08 ($n=8$) and 0.82 ± 0.09 ($n=6$) relative tube area. These values were significantly lower ($P<0.001$) compared to those seen in the presence of 1.25×10^5, 2.5×10^5 and 10^6 platelets per well (1.26 ± 0.08, $n=16$, 1.75 ± 0.10, $n=16$ and 2.95 ± 0.18, $n=9$ relative tube

area). Finally, when the same volumes of supernatant from a platelet suspension (10^7 platelets ml^{-1}) pretreated with 0.3 i.u. ml^{-1} of α-thrombin was added to the assay, the relative tube area was again significantly lower (0.56 ± 0.08, $P<0.001$, 0.71 ± 0.1, $P<0.001$ and 0.87 ± 0.13, $P<0.001$, $n=6$, relative tube area) compared to that seen in the presence of the same number of platelets as above but not different to that seen with platelet supernatants in the absence of α-thrombin. It therefore appeared that the physical presence of platelets themselves was necessary for the observed increases in tube formation to occur.

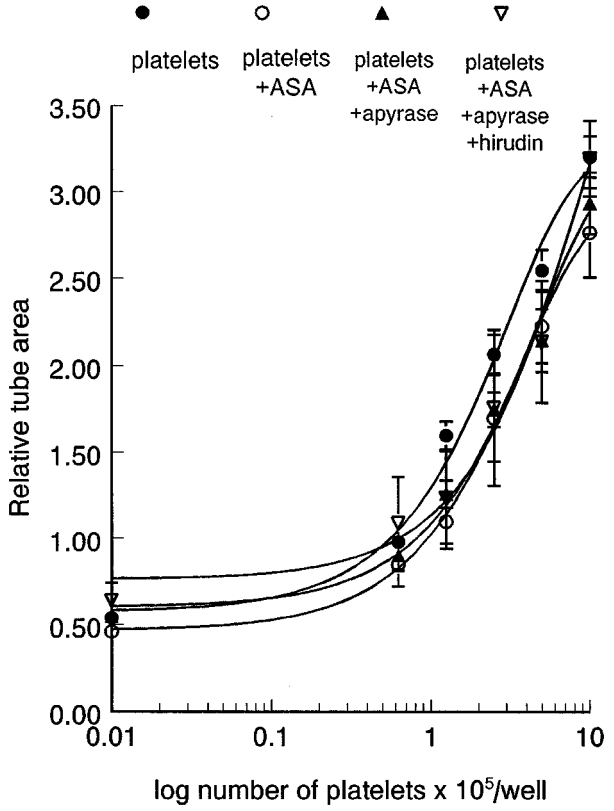

Figure 1 The effect of increasing number of platelets on tube formation by endothelial cells on matrigel in the absence and presence of ASA, ASA + apyrase and ASA + apyrase + hirudin. Results are the means ± s.e.mean of relative tube area.

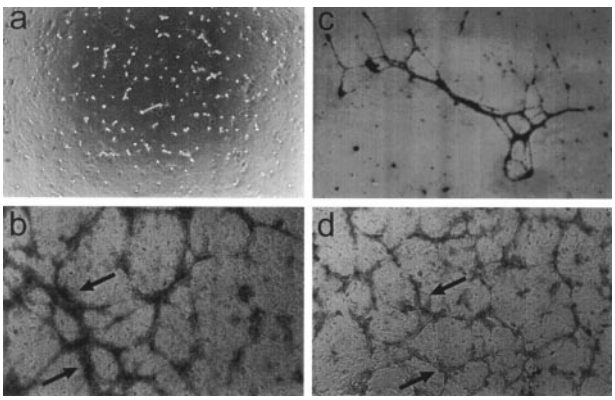

Figure 2 Representative experiment showing capillary-like structures formed at 5 h (a, b) and 18 h (c, d) in the absence (a, c) and presence (b, d) of 5×10^5 platelets. Arrows indicate the presence of platelets along the newly formed tubes.

In a subsequent series of experiments, platelets (10^7 platelets ml^{-1}) were resuspended in culture medium at the end of the isolation process, incubated at 37°C for 15 min and then centrifuged. 25 μl of the resultant supernatant (corresponding to 2.5×10^5 platelets in the uncentrifuged suspension) was again ineffective in increasing tube formation (0.86 ± 0.3, n.s., $n=8$ relative tube area) compared to that seen in the absence of platelets (1.17 ± 0.3, $n=9$ relative tube area). To check whether the release product of the interaction between platelets and matrigel might contribute to the stimulation of tube formation, platelets (10^7 platelets ml^{-1}) were preincubated with matrigel and culture medium for 15 min. They were then centrifuged and 25 μl of supernatant added to the tube formation assay. Under these conditions, the supernatant had no effect on the number of tubes formed (0.81 ± 0.27, n.s., $n=7$) compared to controls as above. To examine whether interaction between platelets and endothelial cell products might cause secretion of substances promoting tube formation, platelets in the same concentration as above, were added to conditioned medium from endothelial cell monolayers for 15 min at 37°C. The platelets were then centrifuged and 25 μl of supernatant added to the assay. Again the supernatant resulting from this procedure had no effect on the ability of endothelial cells to form tubes (1.60 ± 0.22, n.s., $n=13$ compared to 1.17 ± 0.3, $n=9$ control relative tube area). As stated above all these experiments, point to the necessity of the physical presence of platelets for the observed increases to occur.

When 0.1 – 1 i.u. ml^{-1} of α-thrombin were added to the assay together with the platelets (2×10^5 per well), only the 0.3 i.u. ml^{-1} concentration caused a small increase in the

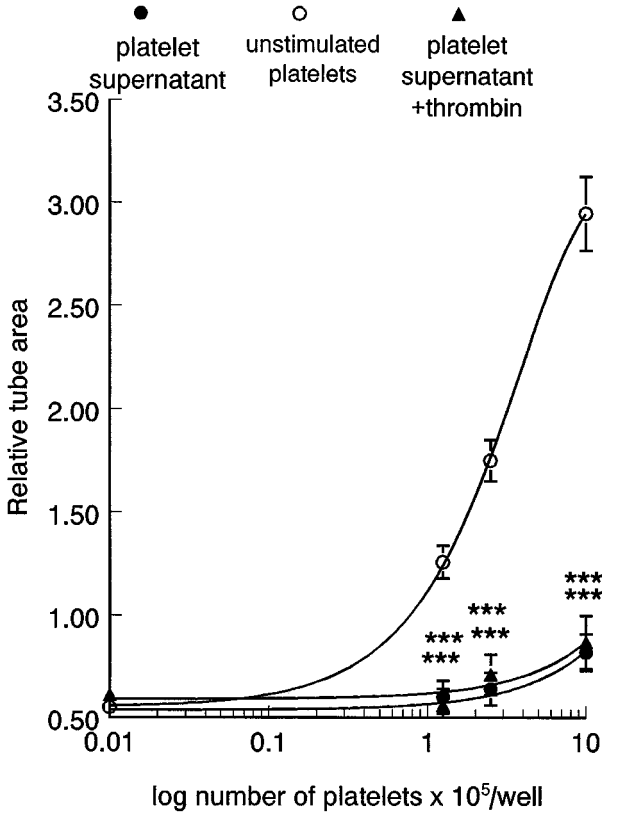

Figure 3 The effect of supernatants from preincubated (in the absence, and presence, of 0.3 i.u. ml^{-1} of α-thrombin) and centrifuged platelets on tube formation by endothelial cells on matrigel compared to that of unstimulated platelets. Results are the means ± s.e.mean of relative tube area and are compared by unpaired *t*-test. ***Denote statistical significance of $P<0.001$.

relative tube area (2.28 ± 0.31, n.s., $n=13$) which although consistent, it was not statistically significant compared to that seen with platelets alone (1.78 ± 0.20, $n=6$). When platelets were preincubated with α-thrombin there was no further increase in tube formation (1.34 ± 0.07, n.s., 1.73 ± 0.12, n.s. and 2.91 ± 0.20, n.s., $n=8$, relative tube area) compared to that seen with the same number of platelets alone (1.26 ± 0.08, 1.75 ± 0.10 and 2.93 ± 0.18, $n=9-16$). In this experiment, a platelet suspension (10^7 platelets ml^{-1}) was incubated with 0.3 i.u. ml^{-1} of α-thrombin. Subsequently, a volume between $25-100$ μl of platelet suspension (containing 1.25×10^5, 2.5×10^5 and 10^6 platelets) was added to the assay in a final volume of 1 ml. It follows that under these conditions, α-thrombin was virtually absent from the assay. When the NO donor SNP ($1-100$ μM) was added to the assay together with platelets (2.5×10^5 per well), there was no effect on tube formation compared to that seen with platelets alone. Under these conditions, the relative tube area ranged from 1.5 ± 0.17, n.s., $n=6$ to 1.36 ± 0.28, n.s., $n=6$ compared to controls with platelets alone (1.27 ± 0.15, $n=22$). Moreover stimulation of tube formation by platelets (2.5×10^5 per well) was not affected by the NOS inhibitor L-NAME in concentrations ranging from $30-300$ μM. Under these conditions, the relative tube area in the presence of L-NAME ranged between 1.4 ± 0.05, n.s., $n=6$ and 1.3 ± 0.07, n.s., $n=6$, relative tube area, compared to controls (platelets alone, 1.2 ± 0.13, $n=12$).

In order to examine the possibility that the platelet-promoting effect on tube formation involved adhesive events with endothelial cells *via* their surface glycoproteins, the platelets were preincubated with neuraminidase (0.01 i.u. 10^7 platelets), which strips sialic acid residues from these glycoproteins, as described by Crook & Crawford (1988). Under these conditions the platelets (2.5×10^5 per well) failed to promote tube formation compared to controls in the absence of platelets (relative tube area in the presence of neuraminidase treated platelets was 0.81 ± 0.03, n.s., $n=8$, in the presence of untreated platelets 1.69 ± 0.09, $P<0.001$, $n=8$ and in the absence of platelets, 0.80 ± 0.04, $n=8$, Figure 4). To verify that neuroaminidase had indeed removed sialic acid residues, sialic acid determination was performed in the supernatant of platelets preincubated with the enzyme. It was found that these supernatants contained 3 nmoles/10^7 platelets of N-acetylneuroaminic acid indicating that neuroaminidase had removed sialic acid residues from platelets.

The tripetide sequence RGD (Arg-Gly-Asp) is recognised by several integrins (Hynes, 1992). When the platelets were preincubated for 5 min with $20-200$ μM of the RGDS tetrapeptide (Arg-Gly-Asp-Ser, which suppresses RGD-dependent ligand binding to integrins) and then added to the assay (the medium also contained the same amount of RGDS), they promoted tube formation as in the absence of neuraminidase treatment (data not shown).

Throughout the description of the Results section it becomes evident that the control relative tube area in the absence of platelets, as well as that in their presence, varies between series of experiments. This is due to the fact that different batches of matrigel were used. These may vary considerably in their ability to initiate tube formation by endothelial cells (Dr Hynda Kleinman, personal communication). The observed differences however between control and test are comparable, irrespective of the particular batch used.

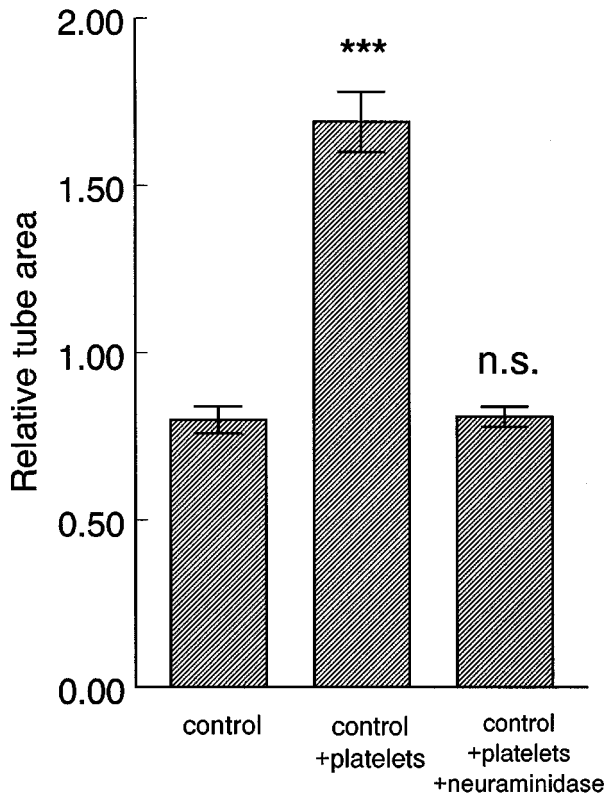

Figure 4 The effect of neuraminidase-treated platelets on tube formation. Controls contained only HUVECs, controls + platelets contained HUVECs + 2.5×10^5 platelets per well, controls + neuraminidase-treated platelets contained HUVECs + 2.5×10^5 platelets/well which had been pretreated with neuraminidase (0.01 i.u./10^7 platelets). Results are the means ± s.e.mean of relative tube area and are compared by unpaired *t*-test. ***Denote statistical significance of $P<0.001$ compared to tube formation by HUVECs alone.

Discussion

The correlation between increased thrombogenesis and angiogenesis-dependent diseases is an extensively studied phenomenon (Rickles & Edwards, 1983; McDonald, 1991). Furthermore, α-thrombin and NO (which have opposing actions on platelet activation) were shown to have angio- and antiangiogenic properties respectively (Tsopanoglou *et al*., 1993; Pipili-Synetos *et al*., 1994). Since thrombogenesis involves platelet activation and adhesion, the question was raised as to whether platelets may directly affect the angiogenic process.

In the present study it was shown that, in an *in vitro* model of angiogenesis, unstimulated human platelets were potent promoters of tube formation by endothelial cells on matrigel. Tube formation was accelerated in the presence of platelets, suggesting that platelets affect both the magnitude and the kinetics of this process. The promoting effect required the physical presence of platelets since their release products alone had no measurable effect on tube formation. These observations suggest that the phenomenon involves events requiring direct cell to cell interaction between platelets and endothelial cells. These could result in cytoskeletal changes which are involved in tube formation (Grant *et al*., 1991).

The quiescent healthy endothelium produces autocoids such as prostacyclin and NO, which prevent the adhesion and aggregation of platelets by raising intracellular levels of cyclic GMP and cyclic AMP (Moncada & Vane, 1978; Azuma *et al*., 1986; Radomski *et al*., 1987; Furlong *et al*., 1987; Sneddon & Vane, 1988; Venturini *et al*., 1992). This has been shown in experiments in the present study and in that by van Ijzedoorn *et al*. (1996) where platelets did not adhere to HUVEC monolayers but remained in suspension in the culture medium.

In the matrigel tube formation model of angiogenesis however, the endothelium is no longer quiescent but is actively differentiating to form capillary-like structures. Differentiating endothelial cells release substances such as plasminogen activator (Schnaper *et al*., 1995) which is known to increase adhesion of platelets on endothelial cells by compromising their anti-adhesive properties (Chen *et al*., 1997). It is therefore likely that under differentiating conditions, the endothelial cells acquire a different set of properties which facilitates their interaction with platelets. This is supported by the fact that neither ASA (which inhibits prostacyclin formation) nor L-NAME (a NO synthase inhibitor) modified the effect of unstimulated platelets on tube formation. Moreover, as evidenced by the photographic records (area around the tubes indicated by arrows), platelets appeared to adhere to the differentiating endothelium. Platelet aggregation or release had no effect on tube formation. This was evidenced by the fact that α-thrombin-preactivated platelets did not alter the platelet potentiating effect. Moreover the platelet release products had no effect on tube formation in the absence of platelets.

The fact that neuroaminidase abolished the promoting effect of platelets suggests that this effect involves interactions between platelet surface glycoproteins and endothelial cells *via* integrins mediating cell-cell adhesion. Alpha$_v\beta_3$ on endothelial cells and $\alpha_{11b}\beta_3$ on platelets share common ligands such as the von Willebrand factor, fibrinogen and fibronectin (Hynes, 1992). These could mediate adhesion between the two cell types as it has been shown in HUVECs in culture and platelets (Bombeli *et al*., 1998). The interaction between the two integrins in the above study however proceeded through RGD recognition. In the present study RGDS was ineffective, suggesting that a different set of interactions took place. Alpha$_{11b}\beta_3$ is thought to be the principal integrin on platelets involved in platelet-HUVECs interaction (Bombeli *et al*., 1998). This integrin also recognizes RGD sequences on its ligands (Hynes, 1992). However in addition to this sequence it recognizes alternative sequences on both fibrinogen (Hynes, 1992) and von Willebrand factor (William & Gralnick, 1987). It is possible therefore that $\alpha_{11b}\beta_3$ may be involved in the interaction between platelets and the differentiating endothelium. Alpha$_{11b}\beta_3$ in unstimulated platelets is normally inactive requiring signalling which will evoke the necessary conformational changes to the alpha subunit and convert it to the activated form (Kieffer & Phillips, 1990, Phillips *et al*., 1991). In the matrigel tube formation system platelets are in contact with extracellular matrix proteins (collagen, laminin) as these are not only components of the matrigel but they are also secreted by the endothelial cells during tube formation. Collagen and laminin may therefore interact with platelets and activate $\alpha_{11b}\beta_3$ *via* outside in signalling independent of aggregation and secretion.

Thrombin can also activate attachment of platelets to endothelial cells (Czervionke *et al*., 1978; Fry *et al*., 1980; Hoak *et al*., 1980). Kaplan *et al*. (1989) have shown that pretreatment of endothelial cells with α-thrombin, in the absence of platelets, results in the attachment of platelets to endothelial cells after the removal of α-thrombin from the fluid phase. The data of that report support the contention that endothelial cells bind and retain α-thrombin in a manner in which it remains active and available for platelet activation. In the present study, the presence of α-thrombin along with platelets and HUVECs, caused a small increase in tube formation only at the concentration of 0.3 i.u. ml^{-1}. This small increase, although consistent, was not statistically significant. These data suggest that platelet activation by α-thrombin, is not required in the potentiation of tube formation by platelets. Neovascularization occurring in pathological conditions such as tumour growth and metastasis or physiologically in embryogenesis (as in the case of the CAM) however, is initiated in intact vessels where a thrombogenic stimulus may provide a powerful initiator of the angiogenic process (Tsopanoglou *et al*., 1993). Under these conditions extracellular matrix may not be available to platelets and interaction with α-thrombin may be important and lead, among other things, to adhesive events supporting neovascularization.

Nitric oxide is known to inhibit adhesive cell to cell interactions involving endothelial cells and leucocytes or platelets (Schmidt *et al*., 1994; Horstrup *et al*., 1994). This mechanism may account for the inhibition of angiogenesis in the CAM and tube formation in the matrigel assay system (in the absence of platelets) (Pipili-Synetos *et al*., 1994) and for the maintenance of platelet antiadhesive properties under normal conditions. The presence of extracellular matrix however in this study, imitates the situation where the vascular endothelial integrity is compromised and subendothelial basement membrane components are exposed to interact with platelets. Under these circumstances, NO released by SNP (in the concentrations used in the present study) was probably unable to counteract the collagen/laminin-stimulated adhesion of platelets to the differentiating endothelium.

In conclusion, unstimulated platelets strongly promote tube formation in the *in vitro* matrigel assay of angiogenesis possibly through adhering to the differentiating endothelium *via* their surface glycoproteins. This effect is not affected substantially by α-thrombin or NO. Platelet adhesion is important in tumour growth and metastasis (Gasic *et al*., 1968; Pearlstein *et al*., 1984; Karpatkin *et al*., 1988; Nierodzik *et al*., 1991, 1992; Honn *et al*., 1992). The results of the present study indicate an additional mechanism by which platelets may interact with the vascular wall and affect tumour progression, *via* promotion of a key step in the angiogenic cascade. Further studies are warranted in order to determine the precise mechanism(s) involved.

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