

# Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage

Janka Kisucka\*<sup>†</sup>, Catherine E. Butterfield<sup>‡</sup>, Dan G. Duda<sup>§</sup>, Sarah C. Eichenberger\*, Simin Saffaripour\*, Jerry Ware<sup>¶</sup>, Zaverio M. Ruggeri<sup>||</sup>, Rakesh K. Jain<sup>§</sup>, Judah Folkman<sup>\*\*\*</sup>, and Denisa D. Wagner\*<sup>†,††</sup>

\*CBR Institute for Biomedical Research and <sup>†</sup>Department of Pathology, Harvard Medical School, Boston, MA 02115; <sup>‡</sup>Vascular Biology Program, Department of Surgery, Children's Hospital, Harvard Medical School, Boston, MA 02115; <sup>§</sup>Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02115; <sup>¶</sup>Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205; and <sup>||</sup>The Scripps Research Institute, La Jolla, CA 92037

Contributed by Judah Folkman, December 7, 2005

Platelets contain both pro- and antiangiogenic factors, but their regulatory role in angiogenesis is poorly understood. Although previous studies showed that platelets stimulate angiogenesis *in vitro*, the role of platelets in angiogenesis *in vivo* is largely uncharacterized. To address this topic, we used two *in vivo* approaches, the cornea micropocket assay and the Matrigel model, in four animal models: thrombocytopenic, *Lyst*<sup>bg</sup> (platelet storage pool deficiency), glycoprotein (GP) Ib $\alpha$ /IL4R transgenic (lacking extracellular GPIb $\alpha$ , the receptor for von Willebrand factor as well as other adhesive and procoagulant proteins), and *Fc $\gamma$ R*<sup>-/-</sup> (lacking functional GPVI, the collagen receptor) mice. Adult mice were rendered thrombocytopenic by i.p. administration of an antiplatelet antibody. The number of growing vessels in the thrombocytopenic mice was lower in the cornea assay, and they showed significantly increased appearance of hemorrhage compared with mice treated with control IgG. The thrombocytopenic mice also showed more protein leakage and developed hematomas in the Matrigel model. GPIb $\alpha$ /IL4R transgenic mice presented increased hemorrhage in both assays, but it was less severe than in the platelet-depleted mice. *Fc $\gamma$ R*<sup>-/-</sup> and *Lyst*<sup>bg</sup> mice showed no defect in experimental angiogenesis. Intravital microscopy revealed a >3-fold increase in platelet adhesion to angiogenic vessels of Matrigel compared with mature quiescent skin vessels. Our results suggest that the presence of platelets not only stimulates angiogenic vessel growth but also plays a critical role in preventing hemorrhage from the angiogenic vessels. The adhesion function of platelets, as mediated by GPIb $\alpha$ , significantly contributes to the process.

thrombocytopenic mice | blood vessel |  $\alpha$ -granule | cornea | collagen receptor

Although the best-defined function of platelets is in hemostasis and thrombosis, platelets also participate in other processes, such as inflammation and atherosclerosis (1). Platelets are anucleated cellular fragments rich in organelles including three types of secretory vesicles: dense granules,  $\alpha$ -granules, and lysosomes (2). Under physiological conditions, circulating platelets do not interact with the vessel walls. However, in response to endothelial activation or vascular injury when underlying extracellular matrix (ECM) is exposed, platelet adhesion and subsequent thrombus formation occur. Two major adhesion receptors, glycoprotein (GP) Ib-IX-V and GPVI, are primarily responsible for regulating this initial platelet adhesion (3–6). The binding of GPIb-IX-V to von Willebrand factor (VWF) establishes a transient bond, which reduces platelet velocity and thus facilitates their adhesion and activation (7, 8). A rapid conversion to stable platelet adhesion is required to promote thrombus formation. This process is primarily mediated by the interaction of platelet integrins and GPVI with collagen. Platelet activation also leads to secretion of platelet agonists, such as ADP and thromboxane A<sub>2</sub> (secreted from dense granules), to

reinforce the platelet aggregation, and of adhesion molecules, such as VWF, and growth factors from  $\alpha$ -granules.

The immediate appearance of platelets at the site of vascular injury and their importance in wound healing raised the hypothesis that platelets could also be important triggers of angiogenesis (9). Angiogenesis is a complex process involving proliferation and migration of endothelial cells to form new capillaries and blood vessels from preexisting vessels (10). It is essential in many biological processes such as development, reproduction, and wound repair. Angiogenesis is highly regulated, i.e., turned on for brief periods (days) and then completely inhibited in physiological conditions. However, many diseases, such as arthritis, diabetic retinopathy, and cutaneous and gastric ulcers, are driven by persistent, unregulated angiogenesis (11–14).

The potential role of platelets in angiogenesis was first suggested by the *in vitro* observations that platelets stimulated endothelial cell proliferation in culture and promoted formation of capillary-like structures in Matrigel assays (15). The early studies led to the identification of many pro- and antiangiogenic factors that are stored in platelets and released after platelet activation. Among the angiogenesis promoters found in platelets are VEGF (16, 17), platelet-derived growth factor (PDGF) (18), basic FGF (bFGF) (19), EGF (20), TGF (21), insulin-like growth factors (22), angiopoietin 1 (23), sphingosine-1-phosphate (24, 25), and matrix metalloproteinases (26–28). The angiogenesis inhibitors found in platelets include thrombospondin I (29), platelet factor 4 (30), plasminogen activator inhibitor I (31), and angiotensin (32).

It is now well established that tumor growth and metastasis require new blood vessel formation. Accumulating evidence indicates the contribution of platelets to the process of tumor angiogenesis as well. Increased numbers of activated platelets and increased expression of platelet adhesion receptors are common findings in cancer patients (33). Tumor cells can activate platelets (34), which release angiogenic factors, thereby directly affecting the tumor endothelium. Interestingly, an antiplatelet antibody was shown to suppress pulmonary metastasis (35).

Although the proangiogenic effects of platelets have been documented *in vitro* and in tumors (36, 37), little is known about platelets' precise role in regulating angiogenesis *in vivo*. To address this question, we examined the role of platelets in two different *in vivo* assays of angiogenesis in four animal models. The results suggest that platelets and their adhesive function

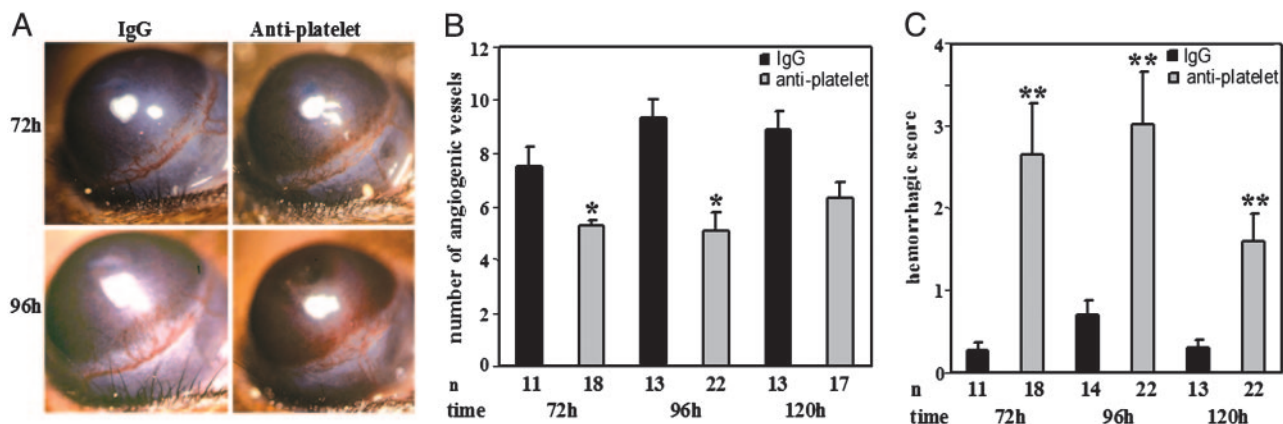
Conflict of interest statement: No conflicts declared.

Abbreviations: bFGF, basic FGF; ECM, extracellular matrix; GP, glycoprotein; VWF, von Willebrand factor; MP, microparticle.

\*\*To whom correspondence may be addressed. E-mail: judah.folkman@childrens.harvard.edu.

††To whom correspondence may be addressed at: CBR Institute for Biomedical Research, 800 Huntington Avenue, Boston, MA 02115. E-mail: wagner@cbr.med.harvard.edu.

© 2006 by The National Academy of Sciences of the USA



**Fig. 1.** Effect of platelet depletion on angiogenesis in the cornea micropocket assay. (A) Photographs of corneas of mice injected with IgG (Left) or antiplatelet antibody (Right) 72 and 96 h after implanting hydron pellets containing 80 ng of bFGF. Note undefined borders of the vessels in the platelet-depleted eyes due to hemorrhage (red). (B) Number of angiogenic vessels counted 72, 96, and 120 h after bFGF pellet implantation. (C) Hemorrhage scores 72, 96, and 120 h after bFGF pellet implantation. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  compared with IgG controls.

support angiogenesis and prevent excessive leakage and hemorrhage from newly formed vessels.

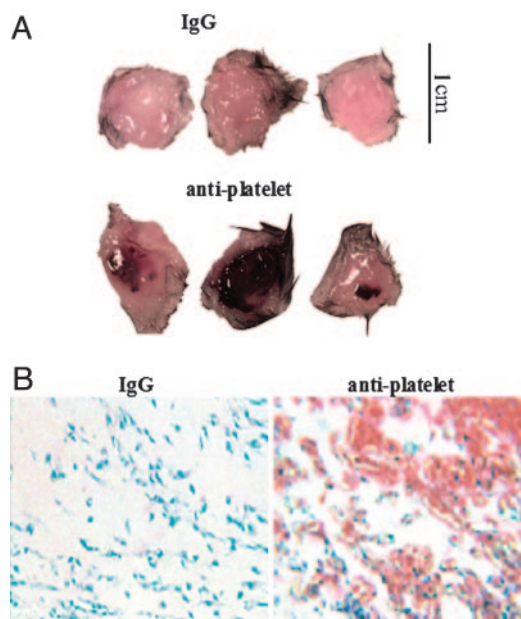
## Results

### Platelet Depletion Led to the Formation of Fewer Vessels and, Most Notably, Highly Hemorrhagic Vessels in a Cornea Angiogenesis Model.

To determine whether platelet depletion affects experimental angiogenesis, we used the cornea micropocket assay. Pellets containing the slow-release polymer hydron and bFGF were surgically implanted into the micropockets of mouse corneas, which are avascular. Thrombocytopenia was induced 1 h after implantation of the pellets. A single i.p. injection of antiplatelet antibody in the mice resulted in a profound thrombocytopenia within 1 h, with a >95% reduction in the number of circulating platelets. The thrombocytopenia induced by this single injection was transient, and platelet levels started to return to normal by day 3. Sustained thrombocytopenia was induced by a repeated injection of the antibody on the third day. Sprouting of the limbal vessel into the cornea was observed. Newly formed vessels in the corneas of the control mice were clearly visible and nonhemorrhagic at 72 and 96 h (Fig. 1A Left) and also at 5 days (data not shown). The angiogenic vessels in the corneas of the platelet-depleted animals were less well defined and were surrounded by extravascular RBCs (Fig. 1A Right). The length of new vessels was therefore difficult to define in corneas of the platelet-depleted animals. However, there was a significant decrease in clock hours (length of the limbal vessel showing sprouts) 5 days after pellet implantation in the platelet-depleted group when compared with the control group ( $2.21 \pm 0.16$  for IgG and  $1.80 \pm 0.11$  for antiplatelet group,  $P < 0.05$ ). Animals treated with the antiplatelet antibody also had lower numbers of corneal vessels when compared with the control animals (Fig. 1B). The number of vessels was significantly decreased in the antiplatelet antibody group at 72 and 96 h after pellet implantation ( $P < 0.05$ ), and the difference was close to significant at 120 h after pellet implantation ( $P = 0.057$ ). To compare the RBC leakiness of the newly formed vessels, a blinded observer assigned a hemorrhagic score to the eyes. The score was significantly different in the two sets of animals at all time points ( $P < 0.001$ ) (Fig. 1C).

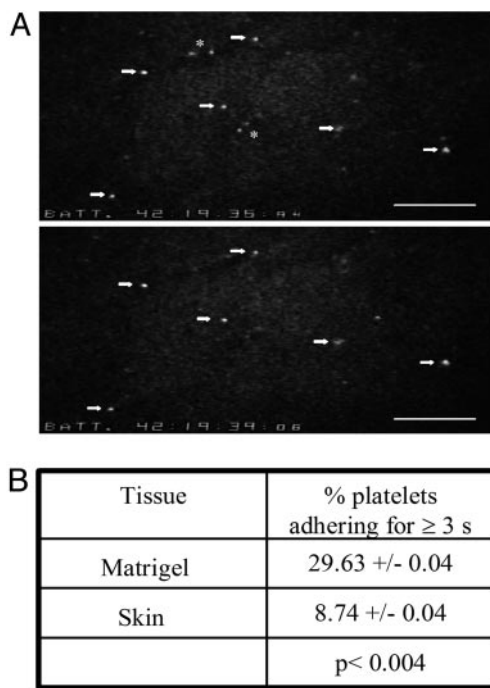
**Platelet Depletion Caused Hemorrhage and Fragility of the Implanted Matrigel.** To test the role of platelets in angiogenesis further, we used the Matrigel assay (38). One group of mice was injected with the antiplatelet antibody 1 h after Matrigel implantation and reinjected on the third day, and the control group was injected with IgG. The Matrigels were dissected 7 days later. We

found readily discernable differences between the two groups in the gross morphology of the Matrigel (Fig. 2A). Matrigel plugs of platelet-depleted mice were very fragile, and various degrees of hemorrhage were found in every plug in the antiplatelet group, but no obvious hemorrhage was detected in the plugs from the control group (Fig. 2A). Infusion of 2% Evans blue dye (binds to albumin) intravenously 3 h before Matrigel isolation showed greater protein leakage in platelet-depleted angiogenic vessels (OD per g was  $13.5 \pm 0.7$  for the antiplatelet group and  $10.2 \pm 0.6$  for the IgG group;  $n = 7-8$ ,  $P < 0.02$ ). Hematoxylin and eosin staining of Matrigel sections showed a marked presence of extravascular RBCs and many hematomas in the antiplatelet antibody-treated gels as compared with those from IgG-treated mice (Fig. 2B). To evaluate the RBC content in the



**Fig. 2.** Effect of platelet depletion on angiogenesis in Matrigel assay. Matrigels containing 80 ng of bFGF were implanted s.c. and recovered on day 7 after implantation. (A) Representative photographs of gross morphology of Matrigel implants from mice injected with IgG (Upper) and mice injected with antiplatelet antibody (Lower). (B) Hematoxylin and eosin staining of Matrigel sections from the mice injected with IgG (Left) and from the platelet-depleted mice (Right). Note the numerous extravascular RBCs in Right.





**Fig. 5.** Visualization of platelets *in vivo*. Platelet–endothelium interactions were investigated in angiogenic and mature quiescent vessels in a dorsal skin fold chamber by *in vivo* fluorescence microscopy. (A) Two representative images taken 3 s apart show the same field within the Matrigel. Arrows indicate platelets that remained adherent during this period. Asterisks in *Upper* indicate examples of nonadherent platelets (moved away in *Lower*). It is important to note that only  $\approx 5\%$  of the circulating platelets were fluorescent. Thus, the actual number of adherent platelets is possibly much higher than shown. (Scale bars, 50  $\mu\text{m}$ .) (B) Quantitative analysis of platelet adherence. Percentage of adherent platelets from total number of platelets observed in the field was determined as described in *Materials and Methods*.  $n = 4$  animals in each group.

lation, the angiogenic vessels in the Matrigel showed significantly enhanced platelet–vessel wall interactions. The number of platelets firmly attached to the vascular wall (Fig. 5A) was increased  $>3$ -fold in angiogenic vessels when compared with mature skin vessels (Fig. 5B,  $P < 0.004$ ).

## Discussion

Based on clinical and preclinical findings, Folkman and colleagues (9) proposed that tumor angiogenesis depends not only on endothelial cells and tumor cells but also on platelet–endothelium interaction. The basis of this hypothesis was that platelets are a rich source of stimulators and inhibitors of angiogenesis and that they interact with the endothelium to change its properties. This hypothesis appears to apply to other angiogenic processes given that our current study indicates that the platelets and their adhesive function are critical because they stimulate the growth of new blood vessels while preventing excessive hemorrhage.

Recently, it was shown that platelet releasate promotes endothelial cell migration and that the addition of platelets into the Matrigel solution before injection induces angiogenesis in a dose-dependent manner (41). Our result showed that, when the platelets were depleted *in vivo*, there was a significant reduction of neovascularization as determined by the cornea micropocket assay (Fig. 1). This finding is in agreement with the result reported by Rhee *et al.* (42) showing a reduction of retinal neovascularization by thrombocytopenia, although excessive hemorrhage was not noted in that study. Rhee *et al.* (42)

employed the hypoxia-induced retinal angiogenesis model in newborn mice, whereas in the current study we used two different models of angiogenesis in adult mice. These results also corroborate the study by Ma *et al.* (13), demonstrating that thrombocytopenia in rats caused a significant inhibition of gastric ulcer healing, a process known to depend on angiogenesis similar to wound healing. Recently Brill *et al.* (43) showed that infusion of platelet-derived microparticles (MPs) generated from thrombin-activated platelets induced angiogenesis and improved revascularization after chronic ischemia *in vivo*. This work extends the *in vitro* study by Kim *et al.* (44) in which platelet-derived MPs promoted proliferation and survival of endothelial cells, as well as tube formation. Although GPIb, which we show to be important, is present and ready for adhesion on resting platelets, the results suggest that, in addition, platelet activation generating MPs is involved in the process of angiogenesis. GPIb $\alpha$  could be involved also in platelet activation and generation of MPs. There is evidence that GPIb $\alpha$  binds  $\alpha$ -thrombin and modulates its function (45). Interestingly, the binding site for thrombin on GPIb was shown to play a role in the exposure of negatively charged phospholipids on the platelet surface (46), a step in MP generation.

The above results indicate that platelets' overall effect on angiogenesis is stimulatory. In our experiments, the occurrence of hemorrhage and excessive protein leakage in platelet-depleted animals also shows platelets' role in the stabilization of newly formed vessels. It is possible that the platelet–endothelial interaction or platelet adhesion to exposed ECM during the endothelial cell sprouting is essential to prevent leakage and hemorrhage from the angiogenic vessels. Our intravital microscopy results indicate that platelets preferentially adhere to the newly formed vessels in Matrigel in the skin chamber angiogenesis assay (Fig. 5). It will be critical to establish whether platelets adhere to endothelial cells activated by the angiogenic process (47) or to ECM components exposed during angiogenesis. Platelets adhere to collagen mainly through GPVI. However, our results indicate that, in contrast to GPIb, GPVI-mediated adhesion does not affect angiogenesis (Fig. 3). Rhee *et al.* (42) found platelet remnants and microvesicles at the sites of angiogenic sprouts, and platelet microthrombi were also seen accumulating in the retinal neovasculature of the diabetic rats (11). These microthrombi were necessary to suppress the breakdown of the blood–retinal barrier. Taken together, these results suggest the importance of platelets (or their MPs) in the stabilization of angiogenic vessels.

The effects of platelets in angiogenesis might be mediated by platelet granular growth factors and cytokines released from platelets in addition to platelet–vessel wall interactions. Using a model for platelet-dense granule and lysosomal-secretion deficiency, we investigated their role in angiogenesis. We did not find any defects in angiogenesis in *Lyst*<sup>tg</sup> mice, suggesting that the dense granule contents and lysosomal secretion may not be critical for the formation of new blood vessels. Although our study demonstrates that the dense granules and secretory lysosomes of platelets are not essential in angiogenesis, it is likely that  $\alpha$ -granules play an important role in angiogenesis. Because thrombocytopenia produced a stronger phenotype than lack of platelet adhesion, activation of platelets after adhesion may trigger secretion of  $\alpha$ -granule contents such as growth factors (e.g., VEGF, TGF $\beta$ , and platelet-derived growth factor), which stimulate endothelial sprouting and formation of new vessels. At present, however, no model for  $\alpha$ -granule deficiency is available to test this hypothesis directly.

Platelets are surrounded by a membrane that consists of phospholipids. Three angiogenic phospholipids (lysophosphatidate, phosphatidic acid, and sphingosine-1-phosphate) have mitogenic activities and stimulate migration, proliferation, adherence, junction assembly, liberation of endothelial cells from



8. Mazzucato, M., Pradella, P., Cozzi, M. R., De Marco, L. & Ruggeri, Z. M. (2002) *Blood* **100**, 2793–2800.
9. Pinedo, H. M., Verheul, H. M., D'Amato, R. J. & Folkman, J. (1998) *Lancet* **352**, 1775–1777.
10. Folkman, J. & Shing, Y. (1992) *J. Biol. Chem.* **267**, 10931–10934.
11. Yamashiro, K., Tsujikawa, A., Ishida, S., Usui, T., Kaji, Y., Honda, Y., Ogura, Y. & Adamis, A. P. (2003) *Am. J. Pathol.* **163**, 253–259.
12. Knighton, D. R., Ciresi, K. F., Fiegel, V. D., Austin, L. L. & Butler, E. L. (1986) *Ann. Surg.* **204**, 322–330.
13. Ma, L., Elliott, S. N., Cirino, G., Buret, A., Ignarro, L. J. & Wallace, J. L. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 6470–6475.
14. D'Amore, P. & Shepro, D. (1977) *J. Cell. Physiol.* **92**, 177–183.
15. Pipili-Synetos, E., Papadimitriou, E. & Maragoudakis, M. E. (1998) *Br. J. Pharmacol.* **125**, 1252–1257.
16. Mohle, R., Green, D., Moore, M. A., Nachman, R. L. & Rafii, S. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 663–668.
17. Wartiovaara, U., Salven, P., Mikkola, H., Lassila, R., Kaukonen, J., Joukov, V., Orpana, A., Ristimaki, A., Heikinheimo, M., Joensuu, H., *et al.* (1998) *Thromb. Haemost.* **80**, 171–175.
18. Heldin, C. H., Westermark, B. & Wasteson, A. (1981) *Biochem. J.* **193**, 907–913.
19. Kaplan, D. R., Chao, F. C., Stiles, C. D., Antoniadis, H. N. & Scher, C. D. (1979) *Blood* **53**, 1043–1052.
20. Ben-Ezra, J., Sheibani, K., Hwang, D. L. & Lev-Ran, A. (1990) *Am. J. Pathol.* **137**, 755–759.
21. Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K. & Ichihara, A. (1985) *Biochem. Biophys. Res. Commun.* **133**, 1042–1050.
22. Karey, K. P. & Sirbasku, D. A. (1989) *Blood* **74**, 1093–1100.
23. White, R. R., Shan, S., Rusconi, C. P., Shetty, G., Dewhirst, M. W., Kontos, C. D. & Sullenger, B. A. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 5028–5033.
24. English, D., Welch, Z., Kovala, A. T., Harvey, K., Volpert, O. V., Brindley, D. N. & Garcia, J. G. (2000) *FASEB J.* **14**, 2255–2265.
25. Hla, T. (2004) *Semin. Cell Dev. Biol.* **15**, 513–520.
26. Galt, S. W., Lindemann, S., Allen, L., Medd, D. J., Falk, J. M., McIntyre, T. M., Prescott, S. M., Kraiss, L. W., Zimmerman, G. A. & Weyrich, A. S. (2002) *Circ. Res.* **90**, 1093–1099.
27. Fernandez-Patron, C., Martinez-Cuesta, M. A., Salas, E., Sawicki, G., Wozniak, M., Radomski, M. W. & Davidge, S. T. (1999) *Thromb. Haemost.* **82**, 1730–1735.
28. Jurasz, P., Chung, A. W., Radomski, A. & Radomski, M. W. (2002) *Circ. Res.* **90**, 1041–1043.
29. Iruela-Arispe, M. L., Bornstein, P. & Sage, H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5026–5030.
30. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F. & Sharpe, R. J. (1990) *Science* **247**, 77–79.
31. Booth, N. A., Simpson, A. J., Croll, A., Bennett, B. & MacGregor, I. R. (1988) *Br. J. Haematol.* **70**, 327–333.
32. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994) *Cell* **79**, 315–328.
33. Sierko, E. & Wojtukiewicz, M. Z. (2004) *Semin. Thromb. Hemost.* **30**, 95–108.
34. Folkman, J. & Klagsbrun, M. (1987) *Science* **235**, 442–447.
35. Gasic, G. J., Gasic, T. B. & Steward, C. C. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 46–52.
36. Banks, R. E., Forbes, M. A., Kinsey, S. E., Stanley, A., Ingham, E., Walters, C. & Selby, P. J. (1998) *Br. J. Cancer* **77**, 956–964.
37. Olas, B., Mielicki, W. P., Wachowicz, B. & Krajewski, T. (1999) *Thromb. Res.* **94**, 199–203.
38. Passaniti, A., Taylor, R. M., Pili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S. & Martin, G. R. (1992) *Lab. Invest.* **67**, 519–528.
39. Kanaji, T., Russell, S. & Ware, J. (2002) *Blood* **100**, 2102–2107.
40. Jain, R. K., Munn, L. L. & Fukumura, D. (2002) *Nat. Rev. Cancer* **2**, 266–276.
41. Brill, A., Elinav, H. & Varon, D. (2004) *Cardiovasc. Res.* **63**, 226–235.
42. Rhee, J. S., Black, M., Schubert, U., Fischer, S., Morgenstern, E., Hammes, H. P. & Preissner, K. T. (2004) *Thromb. Haemost.* **92**, 394–402.
43. Brill, A., Dashevsky, O., Rivo, J., Gozal, Y. & Varon, D. (2005) *Cardiovasc. Res.* **67**, 39–38.
44. Kim, H. K., Song, K. S., Chung, J. H., Lee, K. R. & Lee, S. N. (2004) *Br. J. Haematol.* **124**, 376–374.
45. Celikel, R., McClintock, R. A., Roberts, J. R., Mendolicchio, G. L., Ware, J., Varughese, K. I. & Ruggeri, Z. M. (2003) *Science* **301**, 218–221.
46. Dormann, D., Clemetson, K. J. & Kehrel, B. E. (2000) *Blood* **96**, 2469–2478.
47. Melder, R. J., Koenig, G. C., Witwer, B. P., Safabakhsh, N., Munn, L. L. & Jain, R. K. (1996) *Nat. Med.* **2**, 992–997.
48. English, D., Garcia, J. G. & Brindley, D. N. (2001) *Cardiovasc. Res.* **49**, 588–599.
49. Hirschi, K. K. & D'Amore, P. A. (1997) *EXS* **79**, 419–428.
50. Frenette, P. S., Johnson, R. C., Hynes, R. O. & Wagner, D. D. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7450–7454.
51. Andre, P., Denis, C. V., Ware, J., Saffaripour, S., Hynes, R. O., Ruggeri, Z. M. & Wagner, D. D. (2000) *Blood* **96**, 3322–3328.
52. Chen, J. & Lopez, J. A. (2005) *Microcirculation* **12**, 235–246.
53. Hartwell, D. W., Butterfield, C. E., Frenette, P. S., Kenyon, B. M., Hynes, R. O., Folkman, J. & Wagner, D. D. (1998) *Microcirculation* **5**, 173–178.
54. Simon, D. I., Chen, Z., Xu, H., Li, C. Q., Dong, J., McIntire, L. V., Ballantyne, C. M., Zhang, L., Furman, M. I., Berndt, M. C., *et al.* (2000) *J. Exp. Med.* **192**, 193–204.
55. Jurk, K., Clemetson, K. J., de Groot, P. G., Brodde, M. F., Steiner, M., Savion, N., Varon, D., Sixma, J. J., Van Aken, H. & Kehrel, B. E. (2003) *FASEB J.* **17**, 1490–1492.
56. Andrews, R. K., Gardiner, E. E., Shen, Y., Whisstock, J. C. & Berndt, M. C. (2003) *Int. J. Biochem. Cell Biol.* **35**, 1170–1174.
57. Ni, H., Denis, C. V., Subbarao, S., Degen, J. L., Sato, T. N., Hynes, R. O. & Wagner, D. D. (2000) *J. Clin. Invest.* **106**, 385–389.
58. Nieswandt, B., Bergmeier, W., Rackebrandt, K., Gessner, J. E. & Zirngibl, H. (2000) *Blood* **96**, 2520–2527.
59. Kenyon, B. M., Voest, E. E., Chen, C. C., Flynn, E., Folkman, J. & D'Amato, R. J. (1996) *Invest. Ophthalmol. Vis. Sci.* **37**, 1625–1632.
60. Bergmeier, W., Burger, P. C., Piffath, C. L., Hoffmeister, K. M., Hartwig, J. H., Nieswandt, B. & Wagner, D. D. (2003) *Blood* **102**, 4229–4235.
61. Leunig, M., Yuan, F., Menger, M. D., Boucher, Y., Goetz, A. E., Messmer, K. & Jain, R. K. (1992) *Cancer Res.* **52**, 6553–6560.