References

1. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-versus-host disease lethality. Blood. 2002;99:3493-3499.
2. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4+CD25+ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J Exp Med. 2002;196:389-399.
3. Stanzani M, Martins SL, Saliba RM, et al. CD25 expression on donor CD4+ or CD8+ T cells is associated with an increased risk for graft-versus-host disease after HLA-identical stem cell transplantation in humans. Blood. 2004;103:1140-1146.
4. Ng WF, Duggan PJ, Ponchel F, et al. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. Blood. 2001;98:2736-2744.
5. Komanduri KV, Viswanathan MN, Wieder ED, et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nat Med. 1998;4:953-956.
6. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25 high regulatory cells in human peripheral blood. J Immunol. 2001;167:1245-1253.
7. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057-1061.
8. Ziman JM. Information, communication, knowledge. Nature. 1969;224:318-324.

To the editor:

On the roles of cGMP and glycoprotein Ib in platelet activation

In a recent publication, Marshall et al[1] reported that they were unable to confirm our findings[2] that cyclic guanosine monophosphate (cGMP) and the cGMP-enhancing drug sidenafil promote platelet activation induced by von Willebrand factor (VWF) and low-dose thrombin. They also contradict our findings[3,4] on the role of extracellular signal-responsive kinase (ERK) in platelet activation. We would like to highlight some differences between their studies and ours that may explain this controversy.

(1) Marshall et al question the physiologic significance of the platelet VWF receptor, glycoprotein Ib-IX (GPIb-IX), in mediating signaling, mainly because VWF induces "weaker" signals compared with agonists such as collagen. However, whether a signal pathway is important cannot be determined by the strength of detectable "signals" but only by the outcome of the signals. In fact, it is well documented that GPIb-IX signaling is required for stable platelet adhesion and spreading on VWF under both flow and static conditions, and that it also induces integrin activation, platelet secretion, and aggregation.[5-11] Thus, GPIb-IX signaling is physiologically significant.

(2) Marshall et al fail to induce full-scale platelet aggregation with 0.1 U/mL thrombin, which was thus referred to as "subthreshold." However, in our studies, platelets fully aggregated in response to a much lower dose of thrombin (0.025 U/mL).[2] This difference suggests that the platelets used in their study were desensitized. One possible desensitization mechanism is activation of the platelets during their isolation (point no. 3). In our study,[2] protein kinase G (PKG) knock-out mouse platelets showed a reduced aggregation response to low-dose thrombin (0.025 U/mL) compared with full-scale aggregation of wild-type platelets. Platelet aggregation in response to high concentrations of thrombin was not significantly affected. Thus, the inability of wild-type platelets to respond to low-dose thrombin may represent a possible cause for the inability of Marshall et al to repeat our finding that PKG knock-out platelets had reduced platelet aggregation in response to low-dose thrombin.

(3) The inability of the platelets prepared by Marshall et al to respond normally to GPIb-IX–dependent agonists is also documented in another recent paper published by the same group,[12] in which VWF failed to induce functional responses in washed platelets, in contradiction to other investigators in the field.[5,7,11] The different responsiveness of platelets to VWF stimulation in their studies thus provides a possible explanation why Marshall et al failed to show cGMP elevation, ERK phosphorylation, and stimulatory effects of cGMP in VWF-stimulated platelets. It is not clear why platelets prepared by Marshall et al respond differently from platelets prepared by other investigators. However, we note that the basal platelet cGMP level determined by Marshall et al is dramatically higher than that reported by us using the same detection method and by other investigators (Table 1). It is therefore possible that the cGMP pathway was inadvertently activated during platelet preparation in their study, which may explain why no further elevation in cGMP levels was observable in response to VWF. We have observed that the effects of cGMP on platelet activation are biphasic. If cGMP is already elevated, exogenous cGMP or cGMP-enhancing drugs would not further increase platelet activation but would rather exert the secondary phase inhibitory effect.

(4) Marshall et al show that PKG inhibitors failed to inhibit thrombin-induced ERK phosphorylation. This result contradicts not only our data but also data from their companion paper[18] (by Gambaryan et al), in which PKG inhibitors attenuated thrombin-induced ERK phosphorylation and VWF caused a "variable" increase in platelet cGMP levels. Despite the differences in data interpretation and conclusions, Gambaryan et al[18] clearly show that PKG inhibitors attenuate platelet aggregation induced by ristocetin and thrombin, and inhibit thrombin-induced ERK phosphorylation, which is consistent with our data.

Table 1. Basal cGMP concentrations in human platelets (pmol/10^8 platelets)

Basal cGMP	Method	Reference no.
Approximately 180	EIA	Marshall et al[1]
0.63	EIA	Li et al[2]
0.2 ± 0.06	RIA	Eigenthaler et al[13]
0.9 ± 0.2	RIA	Radomski et al[14]
0.14 ± 0.06	EIA	Moro et al[15]
0.09 ± 0.01	Prelabeling technique	Jang et al[16]
1 ± 0.3	RIA	Mullershausen et al[17]

All samples used were washed platelets. EIA indicates enzyme immunoassay; and RIA, radioimmunoassay.

Xiaoping Du, Jasna A. Marjanovic, and Zhenyu Li

Correspondence: Xiaoping Du, Department of Pharmacology, University of Illinois College of Medicine, 835 S Wolcott Ave, Chicago, IL 60612; e-mail: xdu@uic.edu.

References

1. Marshall SJ, Senis YA, Auger JM, et al. GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase. Blood. Prepublished on December 18, 2003, as DOI 10.1182/blood-2003-09-3319. (Now available as Blood. 2004;103:2601-2609).
2. Li Z, Xi X, Gu M, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cell. 2003;112:77-86.

3. Li Z, Xi X, Du X. A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin αIIbβ3. J Biol Chem. 2001;276:42226-42232.
4. Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. Blood. 2003;101:4423-4429.
5. De Marco L, Girolami A, Zimmerman TS, Ruggeri ZM. Interaction of purified type IIB von Willebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and initiates aggregation. Proc Natl Acad Sci U S A. 1985;82:7424-7428.
6. Gralnick HR, Williams SB, Coller BS. Asialo von Willebrand factor interactions with platelets: interdependence of glycoproteins Ib and IIb/IIIa for binding and aggregation. J Clin Invest. 1985;75:19-25.
7. Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. J Clin Invest. 1991;88:1568-1573.
8. Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors: a dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. J Biol Chem. 1992;267:11300-11306.
9. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell. 1996;84:289-297.
10. Gu M, Xi X, Englund GD, Berndt MC, Du X. Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin αIIbβ3 using a reconstituted mammalian cell expression model. J Cell Biol. 1999;147:1085-1096.
11. Jackson SP, Schoenwaelder SM, Yuan Y, Rabinowitz I, Salem HH, Mitchell CA. Adhesion receptor activation of phosphatidylinositol 3-kinase: von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. J Biol Chem. 1994;269:27093-27099.
12. Marshall SJ, Asazuma N, Best D, et al. Glycoprotein IIb-IIIa-dependent aggregation by glycoprotein Ibalpha is reinforced by a Src family kinase inhibitor (PP1)-sensitive signalling pathway. Biochem J. 2002;361:297-305.
13. Eigenthaler M, Nolte C, Halbrugge M, Walter U. Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets: estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. Eur J Biochem. 1992;205:471-481.
14. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci U S A. 1990;87:5193-5197.
15. Moro MA, Russel RJ, Cellek S, et al. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. Proc Natl Acad Sci U S A. 1996;93:1480-1485.
16. Jang EK, Azzam JE, Dickinson NT, Davidson MM, Haslam RJ. Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. Br J Haematol. 2002;117:664-675.
17. Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, Koesling D. Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. J Cell Biol. 2003;160:719-727.
18. Gambaryan S, Geiger J, Schwarz UR, et al. Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase. Blood. Prepublished on November 26, 2003, as DOI 10.1182/blood-2003-09-3349. (Now available as Blood. 2004;103:2593-2600).

Response:

On the roles of cGMP and glycoprotein Ib in platelet activation: a response

We are grateful for the opportunity to reply to the letter from Du et al in regard to our recent publication in *Blood*.[1]

Our study was designed to compare the role of Src family kinases, protein kinase G (PKG), and p42/44 mitogen-activated protein (MAP) kinases in platelet aggregation by glycoprotein Ib-IX-V (GPIb-IX-V). The study involved experiments in washed platelets, platelet-rich plasma, and whole blood (both in vitro and in vivo). Our observations support a role for Src kinases in mediating platelet aggregate formation in whole blood on collagen at an intermediate rate of shear. In contrast, all of the experimental data indicate that the role of cyclic guanosine monophosphate (cGMP) is inhibitory, whereas we were unable to find a major role for p42/44 MAP kinases in modulating platelet activation.

In their letter, Du et al have questioned whether the methods used to prepare platelets in these studies have influenced the findings. In this context, we wish to emphasize the internal consistency of the observations in washed platelets, platelet-rich plasma, and whole blood. Furthermore, our study and the accompanying paper by Gambaryan et al[2] have shown that the 2 PKG inhibitors, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, are able to inhibit platelet function through a mechanism other than that of PKG blockade.[1,2] This was illustrated in our study by the observation that the 2 PKG inhibitors inhibit aggregation to thrombin in PKG-deficient murine platelets.[1] This challenges one of the major lines of evidence in support of a role for PKG in mediating platelet activation reported by Li et al.[3]

Our comments on the individual points raised by Du et al are as follows:

(1) We are in agreement with Du et al in regard to the ability of von Willebrand factor (VWF) to promote platelet activation but believe that this alone is not proof of its physiologic relevance.

(2) The experiments on the PKG-deficient platelets were designed to investigate whether the PKG inhibitors have effects that are unrelated to PKG blockade. This was found to be the case. We have also questioned the argument that thrombin mediates platelet activation through the same pathway as GPIb-IX-V (Marshall et al[1]). In this context, the studies by Li et al[3] in PKG-deficient platelets would be of increased relevance if undertaken with VWF.

(3) It is misleading to imply that we are the only group to report the absence of platelet activation by GPIb-IX-V in washed platelets, as illustrated by a recent publication in *Blood*.[4] It is widely recognized that the preparation of VWF can have an important influence in such studies. Further, Du et al have failed to emphasize that our study also reported GPIb-IX-V–mediated platelet activation in platelet-rich plasma.[5] This is a more relevant assay than washed platelets in that there is no need to add VWF.

Du et al have understandably questioned the high level of cGMP that was reported in the online prepublished version of our paper.[1] This has been corrected in the final published version. Thus, the level of cGMP is similar to that reported by others, including the group of Du et al. Further, we measured a similar level of cGMP in plasma and in washed platelets and observed cGMP elevation in response to a number of agents but not to VWF.

(4) The papers by Marshall et al[1] and by Gambaryan et al[2] also describe inhibition of platelet activation by the PKG inhibitors, 8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, in agreement with the observations of Li et al.[3] However, these 2 studies attribute the inhibitory action to a non–PKG-dependent action. The third PKG inhibitor used by Li et al,[3] KT5823, is unable to inhibit purified PKG.[6] Additionally, neither we nor Gambaryan et al observed activation of p42/44 MAP kinases by VWF.[1,2] Further, we reported in 1996 that the p42/44 MAP kinase pathway is not required for platelet activation by thrombin,[7] in contrast to the later study by Li et al.[8]

While we recognize that experimental conditions can have an important bearing on results, we do not believe that this is the explanation for the opposing observations/conclusions made by us[1] and by Gambaryan et al,[2] to those made by Du et al. We emphasize that our conclusions are based on the use of several distinct

experimental approaches and include studies performed in whole blood, platelet-rich plasma, and washed conditions.

Yotis Senis, Stuart Marshall, and Steve Watson

Correspondence: Yotis Senis, Centre for Cardiovascular Sciences, Division of Medical Sciences, Institute of Biomedical Research, Wolfson Drive, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; e-mail: y.senis@bham.ac.uk.

References

1. Marshall SJ, Senis YA, Auger JM, et al. GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase. Blood. 2004;103:2601-2609. (Prepublished on December 18, 2003, as DOI 10.1182/blood-2003-09-3319.)
2. Gambaryan S, Geiger J, Schwarz UR, et al. Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase Blood. 2004;103:2593-2600. (Prepublished on November 26, 2003, as DOI 10.1182/blood-2003-09-3349.)
3. Li Z, Xi X, Gu J, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cell. 2004;112:77- 86.
4. Wu Y, Suzuki-Inoue K, Satoh K, et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. Blood. 2001;97:3836-3845.
5. Marshall SJ, Asazuma N, Best D, et al. Glycoprotein IIb-IIIa-dependent aggregation by glycoprotein Ibalpha is reinforced by a Src family kinase inhibitor (PP1)-sensitive signalling pathway. Biochem J. 2002;361:297-305.
6. Bain J, McLauchlan H, Elliott M, et al. The specificities of protein kinase inhibitor: an update. Biochem J. 2003;371:199-204.
7. Börsch-Haubold AG, Kramer RM, and Watson SP. Inhibition of mitogen-activated protein kinase kinase does not impair primary activation of human platelets Biochem J. 1996;318:207-212.
8. Li Z, Xi X, Du X. A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin αIIbβ3. J Biol Chem. 2001;276:42226-42232.

To the editor:

Irreversible myelosuppression after fludarabine-melphalan conditioning: observations in patients with graft rejection

"Nonmyeloablative" conditioning regimens, including the fludarabine-melphalan regimen, are thought to induce engraftment by virtue of host immunosuppression and to spare myeloid precursors.[1-4] At our institution, there were 4 graft rejections among more than 100 patients conditioned with fludarabine and melphalan. Three of these cases occurred in patients with relatively normal hematopoiesis prior to transplantation, providing an opportunity to assess the myelosuppressive properties of fludarabine-melphalan in vivo.

Patient 1 had sickle cell disease. She received 20 mg/m^2 fludarabine daily for 5 days and 140 mg/m^2 melphalan for 1 day. Stem cells from her HLA-identical brother were incubated with alemtuzumab. She received no posttransplantation graft-versus-host disease (GVHD) prophylaxis. Neutrophil and platelet recovery of donor origin occurred by day 16. On day 18, a febrile syndrome was followed by rapid decline in blood counts. On day 30, back-up autologous stem cells were reinfused. Autologous recovery occurred by day 40 after the initial donor transplantation.

Patient 2 had refractory anemia with ringed sideroblasts (RARS). White blood cell and platelet counts were normal and marrow was normocellular (Figure 1A-B). Conditioning consisted of 25 mg/m^2 fludarabine daily for 5 days, 20 mg alemtuzumab daily for 5 days, and 140 mg/m^2 melphalan for 1 day. Stem cells from her one antigen-mismatched brother were infused on day 0. Tacrolimus was given after transplantation. By day 13 the absolute neutrophil count (ANC) recovered to 0.8×10^9/L, without platelet recovery. On day 16 a febrile syndrome occurred, followed by decline in blood counts. A bone marrow aspirate on biopsy on day 21 was hypocellular without hematopoiesis. On day 28 after conditioning with antithymocyte globulin, she received additional donor cells. She never recovered her counts. A bone marrow aspirate on day 46 was hypocellular without hematopoiesis (Figure 1C-D). She died on day 53 after the transplantation.

Patient 3 had mantle cell lymphoma. Blood counts were normal and bone marrow was normocellular (Figure 1E-F). Small lymphoid aggregates occupied 5% of the marrow space. Conditioning and GVHD prophylaxis for his unrelated donor transplantation were identical to patient 2. The ANC recovered to 6.0×10^9/L by day 13 and the platelet count, to 116×10^9/L by day 20. The counts declined thereafter and he developed profound pancytopenia. A bone marrow biopsy on day 26

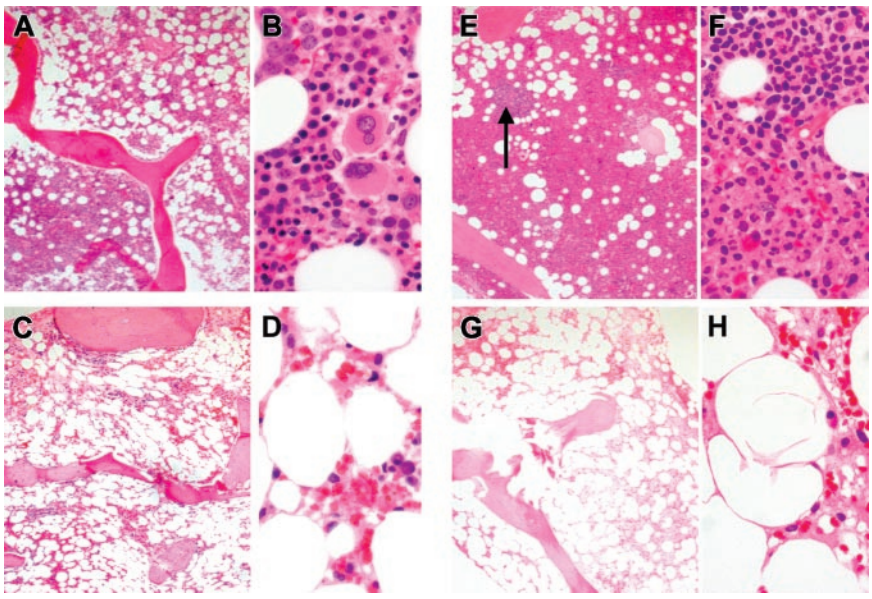

Figure 1. Bone marrow morphology before and after transplantation in patients 2 and 3. (A-B) Bone marrow of patient 2 prior to conditioning (original magnification, × 20 [A]; × 100 [B]). The marrow is normocellular with an abundance of hematopoietic precursors. (C-D) Bone marrow at day 46 after transplantation (original magnification, × 20 [C]; × 100 [D]). The marrow is profoundly hypocellular. The only nucleated elements are residual plasma cells. (E-F) Bone marrow of patient 3 prior to conditioning (original magnification, × 20 [E]; × 100 [F]). The marrow is normocellular with an abundance of hematopoietic precursors. Notice also the presence of nodular lymphoid infiltrates (arrow). (G-H) Bone marrow of patient 3 at day 26 after transplantation (original magnification, × 2 [G]; × 100 [H]). The marrow is profoundly hypocellular. Hematoxylin and eosin stain was used for each panel.