Short Communication

Pathogenic Role of P-Selectin in Experimental Cerebral Malaria

Importance of the Endothelial Compartment

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P-selectin is a leukocyte adhesion receptor expressed on the surface of activated platelets and endothelial cells. Its role in the pathogenesis of cerebral malaria was explored in a murine model of cerebral malaria. Infection of mice with *Plasmodium berghei* ANKA led to P-selectin up-regulation in brain vessels of cerebral malaria-susceptible mice but not of cerebral malaria-resistant mice. Treatment of susceptible mice with anti-mouse P-selectin mAb failed to prevent the development of the neurological syndrome. However, P-selectin-deficient mice infected with *Plasmodium berghei* ANKA had a cumulative incidence of cerebral malaria which was significantly reduced compared to wild-type animals (4.5% versus 80%, respectively), despite identical levels of parasitemia, platelet and leukocyte accumulation. To determine whether P-selectin on platelets and/or endothelium was responsible for the microvascular pathology, cerebral malaria was assessed in chimeric mice deficient in platelet or endothelial P-selectin, which were generated by bone marrow transplantation. Mice deficient only in endothelial P-selectin did not show any sign of cerebral malaria (vascular plugging, hemorrhages, or edema), while mice lacking only platelet P-selectin showed

signs of cerebral malaria similar to that seen in wild-type mice. These results indicate that endothelial P-selectin plays an important role in the pathogenesis of cerebral malaria. (Am J Pathol 2004, 164:781–786)

The prevalence of malaria is 300 to 500 million cases per year. One of the two major complications observed in *Plasmodium falciparum*-infected patients is cerebral malaria (CM). Epidemiological and public health aspects of this syndrome have been reviewed recently.¹ One of the experimental models of CM that reproduces several features of human CM is the infection of susceptible mouse strains with *Plasmodium berghei* ANKA (PbA).² Histopathological alterations of murine CM are vessel plugging by leukocytes, platelets and, to a lesser extent, parasitized red blood cells (pRBC),³ perivascular edema, and hemorrhage.^{4,5}

The pathogenesis of human and murine CM remains incompletely understood, although several factors and cell types appear to play a role. First, as demonstrated in the experimental model, the release of excessive amounts of tumor necrosis factor (TNF) induced by the parasite,⁶ and amplified by the CD4⁺ T-cell-dependent host response⁷ via cytokine interactions,^{8,9} is an important pathogenic step. In turn, TNF activation of endothelial cells (EC), results in ICAM-1 up-regulation^{10,11} and increased adhesiveness for leukocytes and platelets. Second, platelets appear to be effector cells of endothelial damage, via LFA-1/ICAM-1 interaction. This has been

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shown *in vivo* in murine CM⁵ as well as in other pathological conditions such as pulmonary fibrosis and irritant reaction in the skin.^{12–14} Furthermore, deleterious effects of platelets on TNF-stimulated EC have been demonstrated *in vitro*.¹⁵ Third, interactions of leukocytes with endothelium and the associated cytotoxicity play a role in the pathogenesis of CM, although diapedesis of leukocytes is not observed.^{6,11}

Leukocyte adhesion receptors have been shown to be important in cell-cell interactions leading to leukocyte and platelet accumulation on the vessel wall. Members of the selectin family (P-, L-, and E-selectin) are important in the initial step of leukocyte rolling on the vessel wall and P-selectin is required for platelet rolling on the endothelium.¹⁶ The ligands for selectins are carbohydrate structures carrying a sialyl-Lewis^x epitope¹⁶ and the ligand for P-selectin has been identified as PSGL-1. The β 2 integrin-ICAM-1 pair, such as LFA-1/ICAM-1, which is crucial for CM pathogenesis, is involved in firm adhesion of leukocytes to the endothelium. LFA-1/ICAM-1 may also contribute to slow selectin-mediated rolling through inflamed vessels, a step that may favor firm adhesion.¹⁷ P-selectin is stored in the α granules of platelets and in the Weibel-Palade bodies of endothelial cells of some tissues such as lung but not in others, such as brain parenchyma¹⁸ and kidney.¹⁹ It is redistributed to the cell surface after activation with agonists such as thrombin, histamine, or calcium ionophore A23187²⁰ and can be up-regulated at the transcriptional level by cytokines.^{16,18} In particular IL-1 and TNF-stimulated brain microvascular endothelial cells (BMEC) demonstrate sustained P-selectin expression over 24 hours¹⁸ and *in vivo* administration of TNF induces P-selectin expression in the brain microvasculature.²¹ The expression of P-selectin has functional relevance: neutrophil adhesion to cytokine-stimulated BMEC deficient in P-selectin was 40% less than that to wild-type BMEC.¹⁸ P-selectin mediates leukocyte adhesion to activated endothelium and platelet and endothelial P-selectin is important in platelet rolling.²² Furthermore, P-selectin-dependent pRBC rolling may be required for the subsequent engagement of pRBC with endothelial CD36, an interaction previously observed *in vitro*²³ in human cells infected with *P. falciparum*. The murine CD36, which has been characterized only recently, may play a similar role in mice.²⁴ P-selectin knockout (KO) mice display a defect in leukocyte rolling and extravasation.¹⁶ Despite numerous data suggesting a role for P-selectin in pathogenesis of several vascular disorders, its role in CM has not been directly studied.

The aim of this study was to further investigate the mechanisms underlying the interactions between platelets, leukocytes, and endothelial cells in the pathogenesis of CM. We analyzed the expression of P-selectin in brain vessels during CM and the occurrence of CM in mice treated with functional blocking antibody to P-selectin and in P-selectin KO mice. Also, using mice chimeric for P-selectin expression generated by bone marrow transplantation between P-selectin KO and WT mice, we attempted to determine whether endothelial and/or the platelet P-selectin contribute to the CM-associated neurological syndrome.

Materials and Methods

Mice

Female CBA and BALB/c were purchased from IFFA-CREDO (les Oncins, France). P-selectin KO and WT counterparts generated on a mixed background (C57BL/6 and 129/SV)²⁰ were backcrossed eight times with C57BL/6 mice and housed in a virus/antibody-free facility at Longwood Medical Research Center (Brigham and Women's Hospital, Boston, MA). Control WT mice were also generated from mixed background C57BL/6 and 129/SV. Bone marrow (BM) chimeric mice were generated by bone marrow transplantation using P-selectin-deficient and WT mice as donors and/or recipients, as previously described.¹⁹ WT recipients grafted with KO bone marrow are referred to as PLT-/EC+. KO recipients grafted with WT bone marrow are referred to as PLT+/EC-, whereas KO recipients grafted with KO bone marrow are referred to as PLT-/EC-. Successful generation of appropriate animals was assessed by flow cytometric analysis of platelets for P-selectin expression as previously described.¹⁹ Mice were 8- to 12-weeks-old at the time of PbA infection.

Parasite

Infections were initiated by i.p. injection of 10⁶ *P. berghei* ANKA-pRBC per mouse, as described.⁷ Parasitemia and mortality were monitored daily and parasitemia was determined on blood smears after Giemsa staining.

Treatment with Antibodies

CBA/J mice were treated intravenously with 100 μ g of anti-mouse P-selectin monoclonal antibody (mAb)²⁵ once a day from day 4 to 7 of PbA infection. This time period was prior to the development of the neurological syndrome, ie, the second week of infection. Parasitemia and mortality were monitored daily.

Histopathology and Immunohistochemistry

For the study of histopathological signs of CM, brain and lungs from BALB/c and CBA-J mice were sampled in buffered formalin and stained with hematoxylin and eosin according to routine procedures. For immunohistochemical studies, organs were snap-frozen in liquid nitrogen; sections were acetone-fixed and stained with mAbs to mouse P-selectin (PharMingen, San Diego, CA and Ref. 25) or mouse GPIIb/IIIa (clone MWReg30)²⁶ using the ABC Elite peroxidase kit (Vector, Burlingame, CA). Secondary detection steps and washes were performed according to the manufacturer's instructions.

Computerized Image Analysis

Immunostained samples were analyzed on a Zeiss Axio-phot microscope coupled to a SAMBA quantitative image analysis software (Faculty of Medicine, Université de la

Figure 1. Expression of P-selectin in brain and lung on PbA infection. CM-resistant (BALB/c) and CM-susceptible (CBA/J) mice were sacrificed on day 8 post-infection (cerebral phase) for organ sampling. Right panels show P-selectin immunostaining on CM-susceptible mice and left panels show immunostaining on CM-resistant mice (**top** on brain and **bottom** on lung).

Méditerranée). Results were expressed as arbitrary units (a.u.) of stained surface per square micrometer of lumen surface, as determined by planimetry.

Statistical Analyses

Statistical analysis were performed using GraphPad Software. Kaplan Meier test was used to compare survival curves and Mann-Whitney tests were used to compare parasitemia, percentage of leukocyte sequestration, and GPIIb/IIIa immunostaining between KO and WT groups. For all statistical tests, $P < 0.05$ was considered significant.

Results

P-Selectin Expression During PbA Infection

P-selectin expression was analyzed in tissue of untreated and PbA-infected CBA/J and BALB/c mice. P-selectin

was not detected in brain vessels of uninfected mice or mice on day 4 of PbA infection (data not shown). However, CBA/J mice developing CM on day 8 of PbA infection exhibited significant staining for P-selectin in brain vessels of (Figure 1, top right). In contrast, at the same time of infection, P-selectin was undetectable in the brain vessels of BALB/c mice that are resistant to the neurological syndrome (Figure 1, top left). In the lung vessels, P-selectin was expressed in both strains of mice (Figure 1, bottom panels) in resting conditions²⁷ as well as on PbA infection.

Effect of in Vivo Blockade of P-Selectin in PbA-Infected Mice

To evaluate the role of P-selectin in CM pathogenesis, CM-susceptible CBA/J mice were treated with anti-P-selectin mAb (100 μ g per day on days 4, 5, 6, and 7 of PbA infection) before development of neurological signs, which typically begins at day 7. A functional blocking

Figure 2. Comparative features of P-selectin KO and WT C57BL/6 mice during PbA infection. **A:** Survival curves of WT (○) versus P-selectin KO mice (△) with area showing the length of the cerebral phase and arrowheads showing mice who died during cerebral phase without any neurological signs. On day 7 of the infection, in both WT and P-selectin KO mice, parasitemia (**B**), presence of platelets (**C**), and leukocyte sequestration (**D**) were determined. Platelets were detected after anti-GPIIb/IIIa labeling, quantitated by planimetry and expressed in square micrometers per 10 vessels, while the number of leukocytes were enumerated per 100 vessels.

mAb to selectin previously shown to be effective in blocking P-selectin-mediated leukocyte recruitment was used in this study.²⁵ Despite various treatment protocols, either on day 7 (the day before onset of CM), on day 6 and 7, or every day between day 4 and day 7, the incidence of CM was identical in treated and untreated mice (90%, data not shown).

P-Selectin KO Mice Are Protected Against CM

Parasitemia, occurrence of neurological manifestations, and mortality were assessed in a series of P-selectin KO mice on infection with PbA. Among control mice, 16 of 20 (80%) died of CM (Figure 2A). In contrast, among P-selectin KO mice, three died during the cerebral phase; although one of the three did develop clinical signs of CM, two (arrowheads) exhibited neither neurological signs nor histopathological lesions of CM, such as vascular occlusion, hemorrhage, and perilesional edema, as described below. Therefore, the incidence of CM-asso-

ciated mortality was 1 of 22 (4.5%) (Figure 2A). Deaths due to CM were confirmed by histopathological analysis of brain sections. In contrast to WT mice, which die mostly during the cerebral phase (days 6 to 12), the P-selectin KO mice (19 of 22) died from anemia and hyperparasitemia after 3 weeks of infection with PbA (Figure 2A). Parasitemia increased similarly in both WT and P-selectin KO mice and was identical in both sets of mice on day 7 (Figure 2B). In conclusion, P-selectin deficiency protected mice against CM despite the normal evolution of parasitemia in these mice at the time of CM (Figure 2B).

Assessment of Platelet and Leukocyte Sequestration in WT and P-Selectin KO Mice

Brain samples were obtained from three P-selectin KO mice on day 11 post-infection, ie, at the end of the cerebral phase, and compared to that of six WT mice, which died of CM between day 8 and 11. Brain sections were immunostained for the GPIIb-IIIa platelet integrin and leukocyte nuclei were enumerated in at least 10 vessels per section. While GPIIb-IIIa was not found in brain vessels of non-infected mice, significant staining was seen in PbA-infected mice. Computerized image analysis revealed that the area of GPIIb-IIIa staining was similar in the two groups of mice (Figure 2C), indicating that P-selectin deficiency did not abrogate the sequestration of platelets in brain vessels. Furthermore, there was no reduction in the number of leukocytes per brain vessel in P-selectin KO compared to WT mice (Figure 2D).

Assessment of the Pathogenic Role of Platelet versus Endothelial P-Selectin in CM

Since P-selectin is expressed both on the platelet and the endothelial cell surface, we examined whether P-selectin on one or the other cell type played a prominent role in the pathogenesis of CM. WT mice were irradiated and transplanted with bone marrow cells from P-selectin KO mice and P-selectin KO mice were treated identically with BM cells from WT mice, to generate chimeric mice expressing P-selectin on platelets (PLT+/EC-) or on endothelial cells (PLT-/EC+). BM chimeric mice were then analyzed after infection with PbA. PLT+/EC- mice were protected against CM (Figure 3) to the same extent as P-selectin KO mice. In contrast, PLT-/EC+ presented a 57.1% incidence of CM (Figure 3). Among chimeras, histopathological evidence of CM was found only in infected PLT-/EC+ mice despite similar levels of parasitemia in both sets of chimeric mice (data not shown).

Discussion

In this study, it is shown that P-selectin plays a major role in the pathogenesis of CM, since P-selectin KO mice are protected against mortality and the neurological complications of malaria. Indeed, a recent study also reported a role for P-selectin in PbA infection-induced mortality.²⁸

Figure 3. Survival curves of BM chimeric mice. Open circles (○) represent chimeric WT mice grafted with bone marrow of P-selectin KO mice (PLT-/EC+), and open triangles (Δ) represent chimeric P-selectin KO mice grafted with P-selectin WT mice (PLT+/EC-).

The protection conferred by P-selectin gene deletion occurred despite unaffected levels of parasitemia and unaltered accumulation of leukocytes and platelets in the cerebral microvasculature. Finally, an important role for endothelial *versus* platelet P-selectin in CM pathogenesis is demonstrated.

Despite the dramatic protection from PbA-associated mortality in P-selectin-deficient mice compared to WT counterparts, treatment of WT mice with a well-characterized functional antibody to P-selectin did not prevent CM. The inability of anti-mouse P-selectin mAb treatment to prevent CM might be explained by the mode of expression of P-selectin. P-selectin is not expressed constitutively on cell surfaces. Due to the likely narrow time window during which P-selectin becomes expressed, it might be difficult to efficiently block its activity. It could be suggested also that the neutralizing activity of the anti-P-selectin mAb was insufficient, even at the doses used here.

At the time of CM, immunohistochemical analysis of the brain indicated that P-selectin is detectable in the vessels of susceptible CBA/J mice and absent in the brain of resistant BALB/c mice. It can be suggested that in the brain vessels of resistant BALB/c mice, endothelial cells are not activated and therefore do not induce the accumulation of platelets, confirming previous studies of radiolabeled platelet distribution in PbA-infected mice⁵ and of immunostaining with anti-GPIIb/IIIa.^{29,30} Indeed, ICAM-1 is absent on brain microvessels of BALB/c mice on day 8 of infection, ie, at the time of CM in susceptible animals,¹¹ and the absence of ICAM-1 up-regulation may be the mechanism of resistance to CM in mice specifically lacking the type 2 TNF receptor.³¹ In contrast, in lung vessels, a significant staining for P-selectin was observed in both strains on PbA infection. This confirmed the fact that although injection of anti-LFA-1 monoclonal antibody suppresses the development of the cerebral syndrome, the level of platelet sequestration seen in the lung is not modified.⁵ This implies that the lung pathology does exist in PbA infection but is not crucial for the development of a fatal outcome. The staining pattern of P-selectin in brain vessels, in-between sequestered leukocytes, suggests a predominant presence on platelets,

rather than on endothelial cells. It is conceivable that endothelial cells have been activated but that, at the time of brain sampling, they have down-regulated their expression of this molecule or have shed P-selectin. A pathogenic role of platelets can be envisaged at several levels, as previously suggested by the protective effect of platelet depletion and by the reduction of platelet sequestration on treatment with anti-LFA-1 mAb. Platelets can alter endothelial functions in numerous ways, directly and/or indirectly via a modulation of leukocyte functions and even effects on normal and parasitized red blood cells (reviewed in Ref. 32). We addressed the hypothesis that P-selectin deficiency prevents platelet accumulation in brain vessels on PbA infection. However, immunohistochemical analyses of GPIIb-IIIa in brain sections from P-selectin KO mice at the time of cerebral phase showed that platelets are accumulated to the same extent as in infected WT mice.

The role of platelet P-selectin *versus* endothelial P-selectin in the pathogenesis of CM was evaluated using chimeric mice. Mice with a deficiency solely in platelet P-selectin (PLT-/EC+ mice) still develop CM at 57%, while those lacking the molecule solely on the endothelial side (PLT+/EC- mice) are fully protected. Thus, the P-selectin on the endothelium plays a primary role in CM pathogenesis. Endothelial P-selectin could be involved in various ways in cerebral malaria. First it could participate in platelet accumulation and activation. The present study indicates that platelet sequestration alone is not sufficient for CM to occur nor is it dependent on P-selectin, because platelet accumulation was grossly normal in P-selectin null mice that were protected from CM. Thus, it is possible that endothelial activation leading to P-selectin expression is required for platelet activation and subsequent sequelae. This would be consistent with previous studies indicating that platelets can enhance the killing of activated but not of resting endothelial cells.¹⁵

Second, P-selectin on the endothelium may support PbA-infected erythrocyte rolling/adhesion. However, this has only been seen in the human system; this interaction is mediated by PfEMP-1, which does not have an ortholog in *P. berghei* and there is no evidence to suggest that *P. berghei*-infected erythrocytes adhere to this receptor. In murine CM, indeed, infected erythrocyte accumulation in brain vessels is not a major feature although it has been described.^{3,33} Third, soluble P-selectin, shown to be of endothelial origin in a model of kidney inflammation,¹⁹ is pro-coagulant³⁴ and has been shown to promote fibrin deposition in the lumen of injured vessels.³⁴ Thus, in P-selectin-deficient mice that lack soluble P-selectin, there may be less coagulation and subsequently less tissue damage. In falciparum malaria patients, elevated plasma levels of soluble P-selectin have been reported,³⁵ disseminated intravascular coagulation has been documented in severe cases of human malaria, and fibrin deposition has been reported in tissues from fatal malaria cases.³⁶

Taken together, our data show a novel implication of an endothelial surface molecule, P-selectin, in brain endothelial injury and vascular occlusion observed in CM. Our

studies showing an important role for endothelial P-selectin in CM pathogenesis suggests the possibility that endothelial P-selectin may trigger platelet sequestration and activation which, in turn, potentiate adhesiveness and endothelial killing by TNF, two sets of events that participate in the development of brain hemorrhages in CM. Another possibility is that P-selectin expressed on cytokine-activated endothelial cells may be shed and that the resulting soluble P-selectin may provide pro-coagulant activity that contributes to CM pathogenesis.

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