Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular & Biochemical Parasitology 148 (2006) 117–124
MOLECULAR & BIOCHEMICAL PARASITOLOGY

VAR2CSA is the principal ligand for chondroitin sulfate A in two allogeneic isolates of *Plasmodium falciparum*

Michael F. Duffy [a,*], Alexander G. Maier [b], Timothy J. Byrne [a], Allison J. Marty [b,c], Salenna R. Elliott [a], Matthew T. O'Neill [b], Paul D. Payne [a], Stephen J. Rogerson [a], Alan F. Cowman [b], Brendan S. Crabb [b], Graham V. Brown [a]

[a] *The Department of Medicine, The University of Melbourne, The Royal Melbourne Hospital, Parkville, Vic. 3050, Australia*
[b] *The Walter and Eliza Hall Institute of Medical Research, 19 Royal Parade Parkville, Vic. 3050, Australia*
[c] *Microbiology Department, Monash University, Clayton, Vic. 3800, Australia*

Received 10 October 2005; received in revised form 6 March 2006; accepted 13 March 2006
Available online 7 April 2006

Abstract

Malaria during pregnancy causes serious disease that is associated with sequestration in the placenta of *Plasmodium falciparum* infected erythrocytes that adhere to several host receptors, including chondroitin sulfate A (CSA). The principal CSA binding ligand associated with placental sequestration is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the *var2csa* gene. We disrupted the *var2csa* gene in two allogeneic parasites and ablated CSA binding. However, in one parasite line we were able to re-select for adhesion to bovine trachea CSA associated with transcription of two *var* genes, *var-CS2* and *var*P. Parasites transcribing parts of *var-CS2* and *var*P were present in the placentae of some infected women but the mutant parasites that transcribed *var-CS2* and *var*P were recognized by sera from men and pregnant women independent of parity. This work raises the possibility that the PfEMP1 molecules encoded by *var-CS2* and *var*P may be minor contributors to placental malaria but also confirms the importance of the immunodominant, conserved *var2csa* PfEMP1s in pregnancy associated malaria and strengthens the case for *var2csa* as a pregnancy-specific malaria vaccine.

Keywords: Malaria; Pregnancy; CSA; *var2csa*; Adhesion; Transfection

1. Introduction

In areas of endemic transmission the burden of malarial disease falls primarily on children and pregnant women. Malaria in pregnancy causes mortality and morbidity principally through maternal anemia and low birth weight [1]. Susceptibility during pregnancy of previously immune women is associated with sequestration within the placenta of parasitised red blood cells (pRBCs) that express unique variant surface antigens (VSAs) [2]. The restriction of these VSAs to pregnant women correlates with the restricted adhesion phenotype observed in placental parasites. The host receptors implicated in adhesion of pRBCs to placenta are CSA, hyaluronic acid and non-immune globulins [3–5] whereas other host receptors, including CD36 and ICAM-1, mediate sequestration of pRBCs in non-pregnant individuals. Presumably the placenta selects for parasites that bind CSA by providing a novel host receptor in a form of CSA that is absent or not accessible to pRBCs in non-pregnant individuals. Because pRBCs expressing the CSA adhesion ligand are not selected for in non-pregnant individuals [6], malaria-exposed women do not possess immunity to pRBCs expressing the CSA adhesion ligand prior to pregnancy.

Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1) molecules are expressed on the surface of the pRBCs and mediate adhesion of the pRBCs to various host receptors. Each of the approximately 60 members of the *var* multigene family present within a single parasite encodes a different PfEMP1 [7]. *var* genes are subject to transcriptional control such that a single full-length *var* gene transcript is dominant at one time within a parasite [8,9]. Switching between the expressed *var*

Abbreviations: pRBCs, parasitised red blood cells; CSA, chondroitin sulfate A; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; VSA, variant surface antigen; Q-RT-PCR, quantitative RT-PCR; GalNAc, *N*-aetylgalactosamine

* Corresponding author. Tel.: +61 3 8344 3267; fax: +61 3 9347 1863.
E-mail address: mduffy@unimelb.edu.au (M.F. Duffy).

0166-6851/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2006.03.006

Table 1
Parasite adhesion to immobilized receptors expressed as pRBCs bound mm^{-2}

	CSA (mean ± S.E.M.)	Chondroitinase ABC treated CSA (mean ± S.E.M.)	CD36 (mean ± S.E.M.)
CS2	4094 ± 887	0 ± 0	2 ± 2
ItG CS3	2776 ± 253	n.a.	1 ± 1
CS2Δ*var2csa*	5 ± 3	n.a.	1443 ± 488
CS2Δ*var2csa*CS3	207 ± 64[a]	116 ± 28[b]	702 ± 133[c]
3C	2891 ± 396	n.a.	2 ± 2
3D7 CS3	2472 ± 303	n.a.	1 ± 2
3CΔ*var2csa*	3 ± 3	n.a.	657 ± 197
3CΔ*var2csa*CS3	1 ± 2[c]	n.a.	724 ± 149

Mean of three replicates, S.E.M. is the standard error of the mean. n.a., not available.
[a] Mean of six replicates from two experiments.
[b] Mean of 15 replicates from five experiments.
[c] Mean of nine replicates from three experiments.

genes allows parasites to evade the immune response through antigenic variation and also to change the adhesion phenotype of the pRBC.

Various studies have proposed different PfEMP1 molecules as the CSA adhesion ligands important in pathogenesis of malaria in pregnancy, as reviewed elsewhere [10]. However, the most consistent data implicates the PfEMP1 molecule VAR2CSA [11]. Transcription of *var2csa* is associated with adhesion to CSA and hyaluronic acid [11,12] and also with reactivity to serum raised against CSA binding pRBCs in allogeneic parasite lines [13]. Recombinant domains of *var2csa* bind to CSA [14] and react with sera from residents of endemic areas in a gender-specific, parity-dependent fashion [15]. Recent evidence that VAR2CSA was exclusively responsible for CSA adhesion in a single parasite line [16] corroborated these data but it is possible that the parasite line studied lacked CSA binding PfEMP1(s) present in other parasites because of the tremendous diversity in the genomic *var* repertoire between allogeneic parasites. We disrupted *var2csa* in two allogeneic CSA binding parasite lines to determine whether other PfEMP1 in either isolate could bind CSA. Adhesion to bovine trachea CSA was ablated by *var2csa* deletion but was recovered in one mutant parasite that transcribed at least two other *var* genes. However, the mutant parasites did not react with sera in a gender-specific, parity-dependent fashion, suggesting that most malaria in pregnancy arises from parasite adhesion to CSA in association with expression of the unique epitopes of VAR2CSA.

2. Results and discussion

To determine the importance of *var2csa* during malaria in pregnancy we disrupted *var2csa* in both *P. falciparum* 3D7 derived 3C parasites, that are isogenic with NF54 parasites, and ItG derived CS2 parasites. Both the 3C and CS2 parasites were repeatedly selected for a high level of adhesion to CSA (Table 1) and transcribed predominantly *var2csa* [12]. Both CS2 parasites and *var2csa* transcribing NF54 parasites are recognized by sera from residents of malaria endemic areas in a gender-specific and parity-dependent fashion [11,17] consistent with a critical role for expression of *var2csa* in malaria in pregnancy. The *var2csa* gene was disrupted by double cross-over homologous recombination [18] in CS2 parasites and by single cross-over recombination in 3C parasites (Fig. 1). Integration of the human *dhfr* gene into *var2csa* was shown by Southern blotting hybridization to derive the parasite lines CS2Δ*var2csa* and 3CΔ*var2csa* (Fig. 1).

Disruption of *var2csa* in the CS2Δ*var2csa* and 3CΔ*var2csa* parasite lines ablated binding to CSA and these parasites bound

Fig. 1. Disruption of the *var2csa* gene. (a) Strategy for disruption of the *var2csa* gene. The pHHT-TKΔ*var2csa* (ItG) and pHHT-TKΔ*var2csa* (3D7) plasmids contain the human *dhfr* gene cassette flanked by two *var2csa* sequences that targeted homologous recombination within the *var2csa* gene of both isolates to a region spanning the DBL2x domain, inter-domain and DBL3x domain. The plasmids also contained the *Herpes simplex thymidine kinase* (TK) gene cassette for negative selection with ganciclovir. The transfected cell lines were derived by homologous recombination of the plasmids into the *var2csa* wildtype locus leading to a disruption of the gene. CS2Δ*var2csa* was derived by double cross-over recombination and 3CΔ*var2csa* was derived by single cross-over recombination from the 5′ flank. The positions of the 5′ flank and 3′ flank probes used for the Southern blot, the *Hpa*I (H) sites within the CS2 and 3C *var2csa* sequences and the *Bam*HI (B) and *Eco* RV (E) sites within the 3C *var2csa* sequence are shown as are the positions of the c10 and *id2&3* Q-RT-PCRs. (b) Southern blot analysis of genomic DNA from: CS2 and 3C wildtype parasites; CS2Δ*var2csa* and 3CΔ*var2csa* mutant parasites; CS2Δ*var2csa*CS3 and 3CΔ*var2csa*CS3 parasites, that had been sequentially selected three times on CSA, showing integration of pHHT-TKΔ*var2csa* (ItG) and pHHT-TKΔ*var2csa* (3D7) plasmid sequences into the cognate *var2csa* gene locus. The 5′ flank probe hybridized to a 5484 bp fragment in *Hpa*I digested genomic DNA from wildtype CS2 parasites and a 1147 bp fragment in *Bam*HI digested genomic DNA from wildtype 3C parasites, these fragments were the sizes predicted for an intact *var2csa* gene. The same probe hybridized to a 1471 bp fragment in *Hpa*I digested genomic DNA from CS2Δ*var2csa* and CS2Δ*var2csa*CS3 parasites and a 1976 bp fragment in *Bam*HI digested genomic DNA from 3CΔ*var2csa* and 3CΔ*var2csa*CS3 parasites. These were the sizes predicted for the fragments following successful disruption of the *var2csa* locus. The 5′ flank probe also hybridized to 9211 and 8386 bp *Bam*HI fragments in 3CΔ*var2csa* and 3CΔ*var2csa*CS3 parasites that were the predicted sizes for linear, intact plasmid and integrated plasmid backbone. The 3′ flank probe hybridized to the predicted wildtype *var2csa* 6469 bp *Eco* RV and 5484 bp *Hpa*I/*Sac*I fragments in 3C gDNA. The 3′ flank probe hybridized to 6469 and 5666 bp *Eco* RV fragments from 3CΔ*var2csa*CS3 gDNA which were the predicted sizes for wildtype *var2csa* and plasmid backbone, respectively. It also hybridized to 5737 and 6231 bp *Hpa*I/*Sac*I fragments in 3CΔ*var2csa*CS3 gDNA, these were the predicted sizes for a single cross-over recombination from the 5′ flank.