Discussion
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Malaria is a parasitic disease caused by *Plasmodium*. Among four species that cause human malaria, *P. falciparum* is the most important in terms of number of clinical cases as well as mortality rate. Approximately 2 million people die every year due to malaria (WHO, 1997). Following the invasion of the malaria parasites into erythrocytes, it develops from ring to trophozoite to schizont stages. Infected erythrocytes at trophozoite and schizont stages are found to sequester in microvasculature of various organs. Sequestration in brain capillaries and placenta is associated with adverse clinical outcomes of cerebral malaria and complications in pregnancy suggesting that organ specific parasite sequestration is critical for the pathogenesis of malaria. The correlation of parasite sequestration with severe malaria was strengthened by postmortem examinations of patients who died of severe malaria, in which parasite accumulation was found in brain microvasculature. Parasite sequestration is mediated by the interaction of parasite derived family of proteins, PfEMP1, expressed on the surface of infected erythrocytes with receptors present on the surface of endothelial cells, a phenomenon known as cytoadherence. Numerous host endothelial receptors, such as TSP, CD36, ICAM-1, CSA, VCAM1, ELAM, CD31 and HA have been identified that can mediate cytoadherence, although other molecules present on host endothelial cells may potentially serve as receptors. The relative contribution of each of these receptors to malaria pathogenesis remains unclear. Several studies have established the importance of CSA in placental malaria and ICAM-1 in cerebral malaria. *In vitro* binding studies showed that most of the *P. falciparum* isolates can adhere to CD36 (Newbold et al., 1997 and Rogerson et al., 1999), which is widely expressed on various endothelial cells. Binding to ICAM1 by *P. falciparum* field isolates is also frequently observed. The expression of such endothelial receptors e.g. ICAM-1, CD36 and VCAM-1 are reported to be upregulated by inflammatory
cytokines, such as TNFα and IFNγ during malaria. Ligands that can bind to such endothelial receptors are expressed by the parasites on the infected erythrocyte surface and are referred to as Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1). PfEMP1 are high molecular weight clonally variant multidomain proteins that are encoded by var genes. The extracellular region of PfEMP1 contains multiple cysteine-rich domains that have been shown to mediate binding of infected erythrocyte to the various endothelial receptors. A single P. falciparum infected erythrocyte expresses a single var gene.

Screening of Indian field isolates of P. falciparum by in vitro binding to different known receptors revealed that most of the isolates either bound to CD36 or ICAM-1 or both and few of them showed binding to CSA. But, some of those isolates could not bind to any of the identified receptors, like CD36, ICAM-1 and CSA (unpublished observation, Chattopadhyay and Chitnis). This suggests that there might be some unidentified endothelial receptor(s), which could support adhesion of these apparently non-adherent isolates.

Our effort towards the identification of novel cytoadherence receptors was successful. In this study, we have not only characterized a novel endothelial cell surface protein, HABP1/p32/gC1qR that can serve as a potential receptor for cytoadherence by P. falciparum, but also identified the PfEMP1 gene that is predominantly expressed on infected erythrocyte, which may mediate cytoadherence of that strain to endothelial cells through HABP1.

HABP1 is a cell adhesive protein that is expressed in various cell types in different organs. It has been shown by different investigators that HABP1 is ubiquitously expressed on the surface of different endothelial cells, such as human umbilical vein endothelial cells (HUVEC) (Mahdi et al., 2002; Peerschke et al., 1996; Joseph et al., 1996) and bone marrow endothelial cells (BMEC) (Guo et al., 1999). The surface expression of
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HABP1 on endothelial cells has been shown to be upregulated by TNF-α and INF-γ (Guo et al., 1999). Both TNF-α and INF-γ are known to be elevated during severe malaria (MacPherson et al., 1985). Some pathogenic microorganisms are reported to use HABP1 as a cell surface receptor for their invasion into host cells and pathogenesis. For example, HABP1/gC1qR has been identified as a specific receptor for internalin B (InlB) mediated invasion of *Listeria monocytogenes* into various mammalian cells (Braun et al., 2000). All these observations allowed us to speculate that HABP1 may also be a receptor for cytoadherence of parasite to endothelial cells.

In order to assess if HABP1 can serve as a cytoadherence receptor, it is necessary to have highly pure and functional active HABP1 for use in cytoadherence assays. The human gene encoding HABP1 has been cloned and sequenced in our laboratory (Deb and Datta, 1996). HABP1 was overexpressed in bacterial expression system and purified to homogeneity (Fig. 1B and 2A). Before using purified HABP1, its functionally active status was checked through its binding to HA under native conditions (Fig. 2B). In addition, gel filtration profile suggests that HABP1 predominantly exists as a homotrimer in solution, which is consistent with crystal structure data (Jiang et al., 1999). We have used this highly pure and functionally active recombinant HABP1 for *in vitro* binding studies. We have tested field isolates as well as laboratory isolates for binding to HABP1. These data reveal that most of the isolates tested showed specific binding to HABP1, although the extent of binding varies among isolates, suggesting that HABP1 may frequently act as a cytoadherence receptor. Binding of infected erythrocytes to HABP1 is increased linearly with the increase in the concentration of HABP1.

It is a common observation that parasite binding to different receptors is often linked. Parasites binding to ICAM1 can also bind to CD36. Thus, we tested the binding of HABP1 binding isolates to other cytoadherence receptors like CD36 and ICAM-1. It was observed that most of the HABP1
binding parasites bind strongly with CD36, whereas binding to ICAM-1 was low. However, to assess this pattern of binding conclusively, more isolates need to be tested.

In continuation, we attempted to investigate whether selection of parasite population for strong HABP1 binding has any effect on their binding to ICAM-1 and CD36. In other words, is binding to HABP1 and CD36 linked? We have used the laboratory strain 3D7, which also binds strongly with CD36 but comparatively weakly with HABP1, and very weakly to ICAM1 to study the linkage of cytoadherence phenotype. As a part of the programme, we have selected 3D7 on HABP1 coated plates and separated HABP1-binders from non binders and cultured them separately. This process of selection was repeated three times to separate two distinct populations, highly enriched HABP1-binders and HABP1-nonbinders. These HABP1-binder, nonbinder and unselected populations of 3D7 were allowed to bind on HABP1 coated plates; CHO-CD36 and CHO-ICAM-1. Interestingly, it was observed that HABP1-binder 3D7 line binds weekly to CD36, whereas CD36 binding of HABP1-nonbinder parasite remained unaltered as compared to unselected 3D7 parasite (Fig. 7A and B). HABP1 and CD36 binding are not linked and are likely to be mediated by distinct PfEMP1 molecules. The host cytoadherence receptors are divided into two groups, receptors with inducible expression, for example ICAM1, CD31 and VCAM1 and receptors that have constitutive expression like CD36, TSP and CSA. Guo et al. reported that TNF-α upregulates the surface expression of HABP1 on HBMC. We have used HUVEC for cytoadherence assays as HABP1 is reported to be expressed on the surface of HUVEC. We were also keen to find whether TNF-α treatment of HUVEC can upregulate the surface expression of HABP1 like HBMC and if so, whether upregulation of HABP1 expression can augment the binding of infected erythrocytes. HUVEC was stimulated with two different concentrations of TNF-α (15 ng/ml and 25 ng/ml), followed by flow cytometry using anti-HABP1 polyclonal antibodies.
antibodies. Data shows that TNF-α could upregulate the expression of HABP1 on HUVEC and the upregulation was more in case of stimulation with 15 ng/ml of TNF-α as compared to 25 ng/ml. Binding assays using *P. falciparum* IGH-CR-14 HABP1-binders were performed on HUVEC stimulated with 15 ng/ml of TNF-α and without TNF-α stimulation. Interestingly, there was a significant increase in the binding of infected erythrocytes with HUVEC, as compared to the binding with unstimulated HUVEC. Besides upregulating HABP1, TNF-α treatment of HUVEC also upregulates ICAM1. Soluble HABP1 blocks the binding of infected erythrocytes with HUVEC efficiently. Soluble ICAM1 also inhibits binding although to a lesser extent. The efficient inhibition of binding by HABP1 suggests that increase in the binding of infected erythrocytes to TNF-α stimulated HUVEC is mediated by enhanced level of HABP1.

These studies with HUVEC along with direct binding of *P. falciparum* infected erythrocyte with soluble HABP1 shown earlier identifies HABP1 as a novel cytoadherence receptor. The major parasite protein that mediates cytoadherence is PfEMP1 encoded by *var* genes. We expect PfEMP1, that is expressed on HABP1 binder population, would be responsible for binding to HABP1. An attempt was made to clone the gene that encodes a PfEMP1, which mediates binding of infected erythrocytes to HABP1. *P. falciparum* 3D7 was used for this study because 3D7 binds to HABP1 and the whole genome of 3D7 has been sequenced. This will give us an advantage to find out the full length gene with sequence analysis. To achieve this, 3D7 was selected on HABP1 coated plates to separate HABP1-binding from the nonbonding population and this panning was repeated three times. Total RNA was isolated both from HABP1 binding and nonbinding 3D7 infected erythrocytes at trophozoite stage. The RNA was reverse transcribed into cDNA using random hexamers. The cDNA obtained from HABP1 binding and nonbinding parasite were subjected to PCR amplification using degenerate primers based on the conserved *var* gene sequence within
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DBL-1α domains, followed by separation of PCR products by PAGE. Two distinct bands were found in the HABP1 binding 3D7 parasite, whereas three bands were found in nonbinding population. All these five bands were purified from PAGE, cloned in pGEM-T easy vector and three colonies from each were sequenced. Sequences of the DNAs isolated from three different colonies, representing each of the two bands from binder population are identical. In nonbinder population, sequences obtained from the different colonies from each bands are heterogeneous. But, the two sequences present in the binder population are also detected in the nonbinder parasites. Blast search analysis of *P. falciparum* genome database with the sequences obtained results in the identification of specific genes, as represented in Fig. 4.2.

Analysis of the sequences, available from HABP1 non binding 3D7 population revealed identification of four distinct *var* genes denoted in PlasmoDB database as Chr7.glm_337, Chr10.glm_15, Chr5.glm_352 and Chr7.glm_6. As compared to that, two distinct *var* genes, Chr7 glm_337 and Chr5.glm_352 are expressed in HABP1 binding 3D7 population. One of these, Chr5.glm_352 is annotated as a truncated pseudogene lacking the predicted transmembrane domain. The other one isolated from binder is a complete *var* gene denoted as Chr7.glm_337, which could also be detected in the non-binder population. It is not surprising that the *var* gene identified in binders is also expressed in nonbinding population. Since the complete separation of binder from non-binder is not possible and a small fraction of binder may always be present in the non-binder population. Thus, we conclude that the gene, Chr7.glm_337, identified from the HABP1 binder population can be a candidate *var* gene for cytoadherence through HABP1.

In summary, the present study identifies a novel receptor, hyaluronan binding protein 1 (HABP1) for cytoadherence of different field and laboratory isolates of *P. falciparum*. Its specificity and linkage with other known receptors have been studied. Interestingly, upregulation of HABP1
expression on cell surface by inflammatory cytokines e.g. TNF-α on HUVEC allows more number of parasites to bind, indicating a direct correlation of parasite cytoadherence with the availability of HABP1 as a receptor on HUVEC. In severe malaria, the increased level of TNF-α has also been reported. Thus, the regulation of parasite sequestration by the expression of HABP1 on endothelial cell surface may have importance in severe malaria. Finally, we have identified a candidate *var* gene Chr7.glm_337 of 3D7 that is expressed in the HABP1 binder 3D7 parasite, which may mediate cytoadherence through this novel receptor HABP1 on endothelial cell surface. Our present observations provide valuable informations regarding cytoadherence in *P. falciparum* by a novel endothelial receptor and a *var* gene, unidentified yet. Therefore, further studies will be carried out toward the development of novel intervention strategies to prevent the severity of malaria.