Chondroitin Sulfate A Is a Cell Surface Receptor for *Plasmodium falciparum*-infected Erythrocytes

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Summary

Adherence of *Plasmodium falciparum*-infected erythrocytes to cerebral postcapillary venular endothelium is believed to be a critical step in the development of cerebral malaria. Some of the possible receptors mediating adherence have been identified, but the process of adherence in vivo is poorly understood. We investigated the role of carbohydrate ligands in adherence, and we identified chondroitin sulfate (CS) as a specific receptor for *P. falciparum*-infected erythrocytes. Parasitized cells bound to Chinese hamster ovary (CHO) cells and C32 melanoma cells in a chondroitin sulfate–dependent manner, whereas glycosylation mutants lacking chondroitin sulfate A (CSA) supported little or no binding. Chondroitinase treatment of wild-type CHO cells reduced binding by up to 90%. Soluble CSA inhibited binding to CHO cells by 99.2 ± 0.2% at 10 mg/ml and by 72.5 ± 3.8% at 1 mg/ml, whereas a range of other glycosaminoglycans such as heparan sulfate had no effect. Parasite lines selected for increased binding to CHO cells and most patient isolates bound specifically to immobilized CSA. We conclude that *P. falciparum* can express or expose proteins at the surface of the infected erythrocyte that mediate specific binding to CSA. This mechanism of adherence may contribute to the pathogenesis of *P. falciparum* malaria, but has wider implications as an example of an infectious agent with the capacity to bind specifically to cell-associated or immobilized CS.

Cytoadherence of *Plasmodium falciparum*-infected erythrocytes (IEs)[1] to cerebral postcapillary venular endothelium is a striking feature of the pathology of cerebral malaria (1, 2). The phenomenon has been studied in vitro using cell lines such as human endothelial cells (3, 4) and C32 melanoma cells (5), and a variety of purified glycoprotein receptors. Thrombospondin (6), CD36 (7), intercellular adhesion molecule 1 (ICAM-1) (8), vascular cell adhesion molecule 1, and E-selectin (9) have all been implicated as receptors on host cells, but the adherence patterns observed in some studies (4, 10, 11) suggest that additional, as yet unidentified, receptors exist. Sulfated glycoconjugates, including the glycosaminoglycan (GAG) heparin, interfere with attachment of *Plasmodium* sporozoites to the hepatocyte surface and may decrease sporozoite infectivity in mice (12, 13).

We tested the hypothesis that GAGs may be involved in cell–cell interactions in asexual stages of *P. falciparum* and found that IEs bound to cell-associated chondroitin sulfate (CS) and to immobilized chondroitin sulfate A (CSA) and that binding was inhibited by hydrolysis of cell surface CS, or by free CSA.

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CS, chondroitin sulfate; CSA, CSB, and CSC, chondroitin sulfates A, B, and C, respectively; GAG, glycosaminoglycan; HS, heparan sulfate; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IE, infected erythrocyte; PE, dipalmitoylphosphatidylethanolamine; TM, thrombomodulin.

Materials and Methods

Chemicals. Heparin (porcine intestinal mucosa), heparan sulfate (HS) (bovine kidney), dextran sulfate, fucoidan, CSA (porcine rib cartilage), chondroitin sulfate B (CSB) (bovine mucosa), chondroitin sulfate C (CSC) (shark cartilage), chondroitinase ABC (from *Proteus vulgaris*), heparitinase (from *Flavobacterium heparinum*), chymotrypsin, and pronase were from Sigma Chemical Co. (Sydney, Australia), and L-1-tosylamido-2-phenylchloromethyl ketone–treated trypsin (trypsin-TPCK) was from Worthington Biochemical Corp. (Freehold, NJ).

Parasitized Erythrocytes. Parasite strains FAF-EA8, D7, and E10 were derived from the Brazilian isolate ItG2F6. FAF-EA8 was cloned and selected on endothelial cells (10, 14), and D7 and E10 were cloned and selected for rosetting. FAF-EA8CHO5 was selected five times on Chinese hamster ovary (CHO) cells and D7CHO6 and E10CHO6 were selected six times, as will be described. Primary isolates A–F were from children living in Madang, Papua New Guinea (provided by Dr. F. Al-Yaman, Papua New Guinea Institute of Medical Research). Primary isolates G–I were from Australian travellers who had acquired malaria in various countries. Parasites were cultured as previously described (15), but using 5% human serum. Cultures were synchronized every 1–2 wk by sorbitol lysis (16).

Cell Lines. Wild-type (K1) CHO cells and CHO cell glycosylation mutants (gifts of Prof. Jeffrey D. Esko, University of Alabama at Birmingham, Birmingham, AL) and C32 amelanotic melanoma cells (ATTC CRL 1585; American Type Culture Collection, Rockville, MD) were cultured and plated as described (3, 5, 17).

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All cells were used unfixed. CHO cell glycosylation mutant 677 expresses no HS, with a compensatory increase in CS, and mutant 745 expresses neither HS nor CS (18).

Purified Ligands. Platelet-derived CD36 (7) (Dr. M. Berndt, Baker Institute, Melbourne, Australia) was used at 1 μg/ml, and recombinant soluble ICAM-1 (Dr. A. Boyd, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) was used at 10 μg/ml. Duplicate spots of CD36 or ICAM-1 (40 μl for laboratory strains; 10 μl for wild-type isolates) were adsorbed overnight onto 36-mm petri dishes, blocked with 1% BSA, and washed with RPMI-Hepes (MultiCel; Cytosystems, Castle Hill, Australia) before assays.

Cytoadherence Assays, Inhibition of Binding by GAGs, and Erythrocyte Rosette Formation. Assays were performed at 3–7% parasitemia and 2% hematocrit in RPMI-Hepes supplemented with 10% serum. IEs were incubated with purified ligands or cells for 1 h at 37°C before nonadherent cells were washed off with RPMI-Hepes. To assess binding, dishes were fixed with 2% glutaraldehyde, stained with Giemsa, and examined microscopically. At least 300 CHO cells or 500 adherent IEs were counted. GAGs and other glycoconjugates were tested for inhibition of binding by adding them to the parasite culture (at a final concentration of 10 μg/ml) immediately before the assay. Rosetting assays were performed as previously described (19).

Selection for Increased CHO Cell Binding. To select for increased CHO cell binding, after removal of nonadherent cells in the assay described, the adherent cells were incubated with fresh culture medium and erythrocytes. The next day, after trophozoite maturation, division, and reinvasion, ring IEs were transferred to a new dish. To prevent the introduction of viable CHO cells into the ongoing culture, CHO cells were irradiated (3,000 rads) immediately before use.

Enzyme Treatment of CHO Cells and of IEs. K1 CHO cells were incubated with chondroitinase ABC (which cleaves linkages in CSA, CSB, and CSC) or heparitinase (which specifically cleaves HS chains) in RPMI-Hepes for 45 min at 37°C and then washed three times with RPMI-Hepes. For protease treatment of erythrocytes, the FAF-EA8CHO5 culture was washed once in RPMI-Hepes and suspended at 3% hematocrit in 100 μg/ml protease at 37°C for 30 min. The reaction was quenched with 10% human serum, and cells were washed three times with PBS before the binding assay.

GAG Conjugates. CSA and heparin were linked to dipalmitoylphosphatidylethanolamine (PE) through the reducing terminus, using the method of Sugiura et al. (20). Concentration of PE-linked material was determined by hexuronic acid assay. PE-linked CSA (CSA–PE) was used at a concentration of 50 μg/ml, and heparin-PE was used at 260 μg/ml. Duplicate 25-μl spots (10 μl for primary isolates) were adsorbed onto petri dishes overnight and blocked for 30 min with 1% BSA. Adherence assays were performed and counted as previously described. For prevention of adherence, CSA or HS was added to parasitized cells at 10 μg/ml immediately before the assay.

Results

Selection for Binding to CHO Cells Selects for Binding to CS and Alters Adherence to Other Ligands. The three parasite lines were selected for increased binding to CHO cells. CS normally forms ~30% of GAG on the cell surface. Binding of the selected line FAF-EA8CHO5 was completely dependent on the presence of cell surface CS. IEs bound well to CHO cell mutant 677, which expressed excess CS and no HS, but adhesion to 745 CHO cells (with neither HS nor CS) was absent (Table 1). The binding of the selected lines D7CHO6 and E10CHO6 was mainly, but not completely, attributable to CS (Table 1).

Selection for binding to CHO cells altered the adherence profile to other ligands. Binding to ICAM-1 and CD36 decreased markedly, by >95% for parasite line FAF-EA8 (Table 1). Adherence to C32 melanoma cells increased and became more readily inhibited by CSA (Table 1), in keeping with the observation that between 2 and 11 CS chains are attached to a 250-kD surface proteoglycan of melanoma cells (21).

Free CSA Inhibits and Reverses Binding to CHO Cells. The CSA used (from porcine rib cartilage) is composed of predominantly 4-sulfated *N*-acetylgalactosamine and approximately equal amounts of glucuronic acid and iduronic acid (22). CSA significantly impaired binding of FAF-EA8CHO5

Table 1. *Binding of Infected Erythrocytes to Cells and Purified Ligands*

	Cells and purified ligands tested							
Line	K1 CHO	677 CHO	745 CHO	HUVEC	C32	C32 + CSA binding (inhibition %)	CD36	ICAM-1
FAF-EA8	82 ± 12	ND	ND	692 ± 60	1,133 ± 37	968 ± 45 (14.6%)	1,253 ± 262	642 ± 106
FAF-EA8 CHO5	585 ± 29	2,215 ± 491	2 ± 1	17 ± 9	2,149 ± 362	80 ± 25 (96.3%)	59 ± 9	6 ± 2
E10	13 ± 2	ND	ND	221 ± 63	227 ± 15	176 ± 48 (22.5%)	ND	184 ± 41
E10CHO6	430 ± 36	1,344 ± 193	149 ± 9	53 ± 6	1,285 ± 33	370 ± 77 (71.2%)	324 ± 80	100 ± 11
D7	40 ± 8	ND	ND	665 ± 112	559 ± 88	466 ± 96 (16.6%)	501 ± 95	76 ± 6
D7CHO6	635 ± 91	1,740 ± 344	84 ± 8	220 ± 39	1,605 ± 159	671 ± 147 (58.2%)	142 ± 16	73 ± 18

Comparison of selected lines and parent lines for adherence to wild-type CHO cells, CHO glycosylation mutants 677 and 745, CD36, ICAM-1, human umbilical vein epithelial cells (HUVECs), and C32. Values are mean ± SE binding for triplicate experiments. Binding to cells is per 100 cells; binding to purified ligands is per square millimeter.

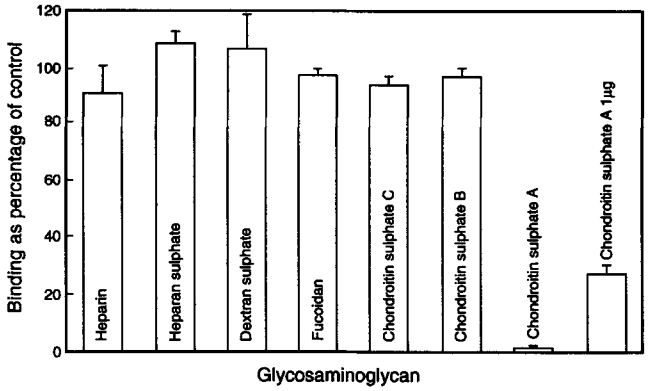

Figure 1. Inhibition of binding of FAF-EA8CHO5 to K1 CHO cells by GAGs and glycoconjugates. All chemicals were added to binding assays at 10 µg/ml; CSA was additionally used at 1 µg/ml. Results show binding as a percentage of control, with mean ± SE for triplicate experiments.

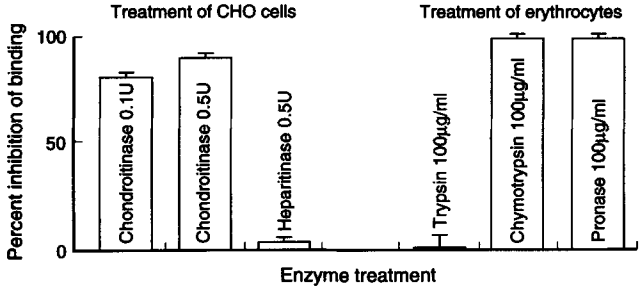

Figure 2. Effect of glycosidase treatment of CHO cells and protease treatment of IEs on cytoadherence. Results show the percent decrease in binding (±SE) compared with control for triplicate experiments.

to K1 CHO cells at 10 µg/ml (99.2 ± 0.2%) and 1 µg/ml (72.5 ± 3.8%). CSB contains ~85% iduronic acid, and CSC consists of 6-sulfated *N*-acetylgalactosamine and glucuronic acid. Neither inhibited binding at 10 µg/ml (Fig. 1). HS, the predominant CHO cell surface GAG, did not inhibit binding, even at 100 µg/ml. Free CSA had no effect on binding to ICAM-1 or CD36 (data not shown). Addition of free CSA at time points during an adherence assay substantially reversed cytoadherence. Free CSA (10 µg/ml) added 15 min before completion of a binding assay released >80% of bound parasites (data not shown).

Treatment of CHO Cells with Chondroitinase or IEs with Protease but Not Trypsin Reduces Cytoadherence. Treatment of CHO cells with chondroitinase ABC markedly reduced binding, but heparitinase had no effect (Fig. 2). Treatment of IEs with chondroitinase or heparitinase did not alter binding (data not shown). Trypsin treatment of the IEs had no effect on binding to CHO cells, whereas both chymotrypsin and pronase reduced adherence (Fig. 2), suggesting that a trypsin-resistant protein mediates adherence to CSA on CHO cells.

Binding to Immobilized CSA. CSA and heparin were conjugated to PE to allow immobilization of GAG on polystyrene plates. Parental strains bound to CSA–PE, and binding was increased in selected lines (Table 2), but there was no significant binding to heparin–PE. Binding was almost totally inhibited by free CSA, but HS had no effect (Table 2).

Patient Isolates May Bind to Immobilized CSA. Five of nine primary isolates tested bound to CSA–PE (Table 3), suggesting that this phenomenon is likely to be of biological relevance in natural infections. The isolates were also examined for rosette formation and for binding to CD36, ICAM-1, and CSA. All formed rosettes and adhered to CD36, and six adhered to ICAM-1 at >5/mm² (Table 3). In this small study, there was no correlation between CSA binding and other phenotypic characteristics. Although levels of CSA binding were modest, isolate F was successfully adapted to in vitro culture and then underwent three rounds of selection on CSA–PE. This selected line bound very well to CSA–PE (>2,000 IEs per mm²).

Discussion

Several infectious agents are known to adhere to HS (23–25), and proteoglycans containing HS are widely distributed on vascular endothelium (26). The intravascular distribution of CS proteoglycans has not been well studied, and to our knowledge, binding of infectious agents to CS has not been previously described.

Variation in expression of CS on endothelia in sites of parasite sequestration could represent a host factor in malaria patho-

Table 2. *Binding of Infected Erythrocytes to Immobilized Glycosaminoglycans*

Line	CSA–PE	Heparin–PE	CSA–PE + CSA	CSA–PE + HS
FAF-EA8	90 ± 9	0.5 ± 0.2	0.8 ± 0.3	105 ± 9
FAF-EA8CHO5	2,350 ± 269	7 ± 4	0.25 ± 0.2	2,488 ± 143
E10	32 ± 6	0.5 ± 0.2	0.7 ± 0.4	39 ± 7
E10CHO6	987 ± 108	0.7 ± 0.5	0.7 ± 0.5	1,033 ± 83
D7	36 ± 8	0.5 ± 0.2	0.1 ± 0.1	ND
D7CHO6	3,400 ± 187	0 ± 0	0.9 ± 0.5	3,091 ± 199

Adherence of IEs to CSA–PE or heparin–PE and to CSA–PE in the presence of free CSA or HS (10 µg/ml). Results are expressed as parasites bound per mm² and show mean ± SE for triplicate experiments.

Table 3. *Binding of Patient Isolates to Purified Ligands and to Uninfected Erythrocytes (Rosetting)*

Isolate	Parasitemia	Rosetting	CD36	ICAM-1	CSA–PE
A	1.0%	1%	69, 93	54, 36	0, 0, 0, 1
B	0.9%	0.7%	334, 337	4, 4	12, 10, 12, 12
C	1.3%	1.7%	335, 347	3, 5	0, 1, 4, 1
D	0.7%	4.5%	288, 309	2, 2	2, 1, 0, 0
E	1.6%	1.7%	541, 192	37, 16	8, 5, 6, 13
F	13.0%	7.5%	3780, ND	83, 122	55, 50, 32, 82
G	11.2%	5.9%	3540, 2995	21, 44	8, 8, 23, 6
H	3.8%	15%	2385, 2267	327, 255	8, 24, 27
I	2.2%	1.7%	469, 524	16, 14	0, 1, 0, 0

Patient isolates were tested for rosette formation and for binding to CD36, ICAM-1, and CSA–PE. Binding is expressed as parasites bound per square millimeter for duplicate assays (each of two spots for CSA–PE), and rosetting is given as the percentage of trophozoite-IEs in rosettes. Parasitemias represent the percentage of all erythrocytes infected with trophozoites at the time of assay.

genesis. One or more CS chains can be linked to thrombomodulin (TM) (27), an integral membrane protein of vascular endothelium. These chains, composed predominantly of CSA, have functional activity in thrombin–TM interactions. CS may also be attached to the endothelial proteoglycan ryudocan in the rat (28). We speculate that CSA attached to TM, or potentially to human ryudocan, may be involved in adhesion in vivo. Our preliminary data (Rogerson, S. J., unpublished observations) show that HUVECs from some but not all individual donors have detectable surface CSA. This agrees with the observations of Lin et al. (29) that HUVECs from pooled donors contain less TM, and a lower proportion of TM that has CS attached, than arterial endothelial cells from a single donor. The expression of other receptors that mediate adherence of IEs to endothelium has recently been shown to correlate with patterns of sequestration (30), but the distribution of CSA on microvascular endothelium representing preferred sites of sequestration was not examined by Lin et al. (29).

We have recently described trypsin-resistant adherence to C32 melanoma cells of a parasite line, T4, that has a trypsin-resistant, chyomotrypsin-sensitive, high molecular weight protein detectable by surface radioiodination (10). Binding of T4 to C32 melanoma cells was reduced by free CSA (Chaiyaroj, S. C., unpublished observations). As with the CHO-selected lines, T4 binds less effectively than the parent FAF-EA8 to CD36 and ICAM-1. We postulated that this trypsin-resistant ligand on IEs mediates adherence (10), and we now believe that it may be interacting with CSA as receptor on the melanoma cell surface. It is unclear whether this represents a variant form of the putative cytoadherence molecule *P. falciparum* erythrocyte membrane protein 1 (31), but multiple ligands on the IEs are thought to mediate endothelial cell adherence in vitro (32), and more than one may be expressed simultaneously (10).

Our finding that primary isolates and laboratory strains of *P. falciparum* adhere to CSA may explain the previously observed adherence of parasite line FC27 to an unidentified ligand on microvascular endothelial cells (4), and it is consistent with the observations of Hasler et al. (11) that one of five Malawian isolates bound at significant levels to wild-type CHO cells. These data suggest that ICAM-1 and CD36 transfected CHO cells (11) may not be appropriate substitutes for the immobilized ligands in assessing adherence, as the contribution of CSA binding cannot readily be determined. Although binding of primary isolates was modest, in vitro selection increased this binding, and similar selection for adherence to CSA may operate in vivo.

The marked difference in inhibition of binding by CSA compared with CSB or CSC was surprising. The sulfation pattern and the proportions of glucuronic acid and its epimer iduronic acid in CS chains may be very variable (22). The porcine rib cartilage CSA used contains predominantly 4-sulfated *N*-acetylgalactosamine and approximately equal amounts of glucuronic acid and iduronic acid (22), whereas CSB contains a low proportion of glucuronic acid, and *N*-acetylgalactosamine residues in CSC are predominantly 6-sulfated. Although the current study did not elucidate the specific oligosaccharide motif that supports binding to the ligand on IEs, it appears to involve structures present in CSA that occur infrequently in the CSB or CSC preparations used. The relative involvement of glucuronic acid and iduronic acid in such a motif can be clarified only by studies using well-defined oligosaccharides.

The novel finding that CSA may act as a specific receptor for an infectious agent is of general interest, expanding the established roles of GAGs in cell–cell interactions. The discovery that CSA can be an adherence ligand for asexual stages of *P. falciparum* adds an extra level of complexity to attempts to unravel the interplay of IEs and host adhesion molecules and their relevance to the pathogenesis of severe malaria. These data suggest that malarial cytoadherence, like leukocyte adhesion, may involve both glycoprotein and carbohydrate receptors and offer a new approach to investigate the relationship between adherence and disease severity that has proved so elusive.

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