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Cytoadherence of *Plasmodium falciparum*-Infected Erythrocytes to Human Melanoma Cell Lines Correlates with Surface OKM5 Antigen

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OKM5 antigen and thrombospondin are currently under investigation as potential receptors on the surface of human monocytes, endothelial cells, and melanomas responsible for the cytoadherence of *Plasmodium falciparum*-infected erythrocytes. We have studied the binding capacity of six human melanoma cell lines and related this property to the cytoplasmic and surface expression of the OKM5 antigen and thrombospondin by using indirect immunofluorescence assays on methanol-fixed and nonfixed melanomas. The presence of OKM5 antigen was detectable only in the melanoma lines which bound *P. falciparum*-infected erythrocytes. Thrombospondin was present in the cytoplasm of all the melanoma lines but was not detectable on the surface of any cells. Our work demonstrates a direct correlation between surface OKM5 antigen and cytoadherence in vitro. While our results do not exclude thrombospondin as a mediator of cytoadherence to endothelial cells in vivo, they showed no correlation between the presence of thrombospondin and the ability of melanoma cell lines to cytoadhere in vitro.

Two proteins of human endothelial cells, the OKM5 antigen and thrombospondin (TSP), are currently under investigation as potential receptors for the specific cytoadherence of *Plasmodium falciparum*-infected erythrocytes (IRBCs) (3, 13). The OKM5 antibody was raised against human monocytes and defines an 88-kilodalton glycoprotein antigen (17). OKM5 antibody also reacts with human platelets (17), endothelial cells (10), and C32 melanomas (3). TSP is a multifunctional 450-kilodalton glycoprotein found in many human cells including monocytes, platelets, and endothelial cells (reviewed in references 11 and 16). Cytoadherence to endothelial cells accounts for the sequestration of IRBCs containing mature asexual parasites from the peripheral circulation into the postcapillary venules. Occlusion of blood capillaries by sequestered IRBCs has important pathological consequences, including the symptoms of cerebral malaria. Sequestration is also an important factor in parasite evasion of host immune responses, since mature IRBCs avoid passage through the spleen, where, as grossly altered RBCs, they would be destroyed (1). An understanding of the relative importance of the OKM5 antigen and TSP as receptors for IRBCs could lead to methods for blockage or reversal of cytoadherence in vivo. One or both of these proteins may also prove useful for identification of the ligand on the surface of IRBCs.

A convenient laboratory model for the study of cytoadherence was developed by using monolayers of C32 amelanotic human melanoma cells (14), which exhibit the same specificity for cytoadherence as cultured human umbilical cord endothelial cells. Another human melanoma cell line, G361, lacks the receptors for *P. falciparum*-IRBCs (14) and may be used in comparative studies to identify the host molecules which mediate cytoadherence. OKM5 was associated with cytoadherence by several properties (3), including its presence on endothelial cells, monocytes (which also bind IRBCs [12]), and C32 melanoma cells, and by its absence from G361 cells. TSP has several properties consistent with a role in cytoadherence (13, 15). The most compelling of these is the ability of purified TSP immobilized on plastic to specifically bind IRBCs while other adhesive proteins such as laminin and fibronectin do not (12, 14). However, TSP alone may be insufficient for cytoadherence, since other cells such as fibroblasts which do not bind IRBCs (20) also secrete TSP (7).

To explore the contributions of these two proteins as potential receptors, we characterized the cytoadherence properties of six additional human melanoma lines and compared their reactivity with monoclonal antibody (MAb) OKM5 (17) and a specific anti-TSP MAb, A6.1 (5). Indirect immunofluorescence assays (IFAs) with these antibodies on both methanol-fixed and nonfixed melanoma cells demonstrate a clear correlation between the presence of cell surface OKM5 antigen and cytoadherence.

MATERIALS AND METHODS

Parasites. Knob-positive, binding-positive (K^+B^+) lines of Malayan Camp (MC) (4), St. Lucia (SL) (2) (from El Salvador), and FMG (FCR-3) (8) (from The Gambia) were maintained in intact Colombian or Bolivian night monkeys (*Aotus trivirgatus*) (6). Knob-negative, binding-negative (K^-B^-) MC and SL lines were derived from the K^+B^+ isolates by passage in culture and in splenectomized *Aotus* monkeys (2). Parasitized monkey blood was cryopreserved when the parasites were at ring stages (1), thawed and cultured by established methods (18), and used as a synchronous population of mid- to late-stage trophozoites.

Melanoma cell lines. C32r human amelanotic melanoma cells (ATCC CRL 1582) were obtained from the American Type Culture Collection, Rockville, Md. Six other melanoma lines were obtained from patients at Memorial Sloan-Kettering Cancer Center, Rye, N.Y. The following cell lines were studied: SK-MEL-23, SK-MEL-28, SK-MEL-93II,

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SK-MEL-93III, SK-MEL-147, and MeWo. All of the lines can be distinguished morphologically. SK-MEL-23 is heavily pigmented. The melanoma lines were cultured in RPMI 1640 medium with L-glutamine and without $NaHCO_3$ (GIBCO Laboratories, Grand Island, N.Y.) and supplemented with 5.95 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), 50 mg of hypoxanthine, 2 g of glucose, 32 ml of 7.5% $NaHCO_3$, and 5 mg of gentamicin (GIBCO) per liter and 10% fetal calf serum (FCS) (GIBCO).

Cytoadherence assays. Cytoadherence assays were performed in Lux 8-well tissue culture plates (Flow Laboratories, Dublin, Va.) by the method of Schmidt et al. (14) with parasitemias of 7 to 20%. In all cytoadherence experiments, the melanoma cells were fixed with 1% Formalin (37% [wt/vol] formaldehyde; Fischer Scientific) in phosphate-buffered saline, pH 7.2 (PBS), which does not alter their cytoadherence (19). Bound IRBCs were fixed in 0.08% glutaraldehyde (diluted from 8% glutaraldehyde; Polysciences, Inc., Warrington, Pa.) and stained in 2% Giemsa in PBS for 20 min. Assays were quantitated by counting the number of melanomas binding ≥1 IRBC in a field of 100 melanomas. The total number of IRBCs bound per 100 melanomas was also enumerated. These assays were performed for duplicate wells on at least two occasions for each parasite-melanoma cell combination.

IFA. MAb OKM5 (1 mg/ml) was a generous gift of G. Goldstein, Ortho Diagnostics, Raritan, N.J. Anti-TSP MAb A6.1 (1 mg/ml) was kindly provided by W. Frazier, Washington University School of Medicine, St. Louis, Mo. Indirect IFAs were performed on melanoma cells cultured on 4-well glass-bottomed tissue culture chambers (American Scientific Products, Columbia, Md.) and fixed in absolute methanol for 20 minutes at −20°C. After rehydration in PBS, the fixed cells were incubated with a 1/1,000 dilution of OKM5 (1 μl/ml) or a 1/100 dilution of A6.1 (10 μl/ml) in PBS with 5% FCS and 0.2% sodium azide (PBS-FCS-azide) or with the diluent alone for 30 min at room temperature, washed three times in PBS-FCS as above, and incubated with a 1/30 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (kindly provided by R. Asofsky, Laboratory of Microbial Immunity, National Institutes of Health) as above. The slides were washed three times in PBS and mounted in 70% glycerol–PBS, pH 8.0, containing 1 mg of the antiquenching agent *p*-phenylenediamine (9) per ml and viewed under UV illumination on a Leitz Ortholux microscope.

IFAs of nonfixed melanomas were performed with cells released from culture flasks with a sterile scraper and suspended in ice-cold PBS-FCS-azide. All procedures, including incubations and centrifugations, were carried out at 4°C with chilled buffers. The cells were washed in PBS-FCS-azide and pelleted in Eppendorf tubes (500 × *g* for 1 min). A 50-μl amount of 1/100 OKM5 MAb or 1/50 A6.1 MAb or diluent alone was added to each tube, and the tubes were incubated for 30 min. The cells were pelleted, the MAb was removed, and the cells were washed three times in PBS-FCS-azide. A 50-μl amount of 1/5 goat anti-mouse immunoglobulin was added per tube, and the tubes were incubated and washed as above. The cells were suspended in 0.05% paraformaldehyde in PBS, pH 7.2.

RESULTS

Cytoadherence of K^+B^+ and K^-B^- strains to six melanoma cell lines. We characterized the cytoadherence ability of six human melanoma cell lines by comparing their binding of K^+B^+ MC and SL parasites with that of K^-B^- parasites derived from the knobby lines of each strain. All of the parasite lines were simultaneously tested for binding to C32 melanomas, which (along with in vivo sequestration) defines their B^+ or B^- character. MC K^+B^+ parasites bound to the majority of C32 and SK-MEL-23 cells and to a small number of SK-MEL-93II cells compared with the MC K^-B^- parasites, which bound to very few cells in any line. Similarly, the SL K^+B^+ parasites bound to the C32 and SK-MEL-23 lines and to a small number of SK-MEL-93III cells, while SL K^-B^- IRBC binding was negligible (Fig. 1).

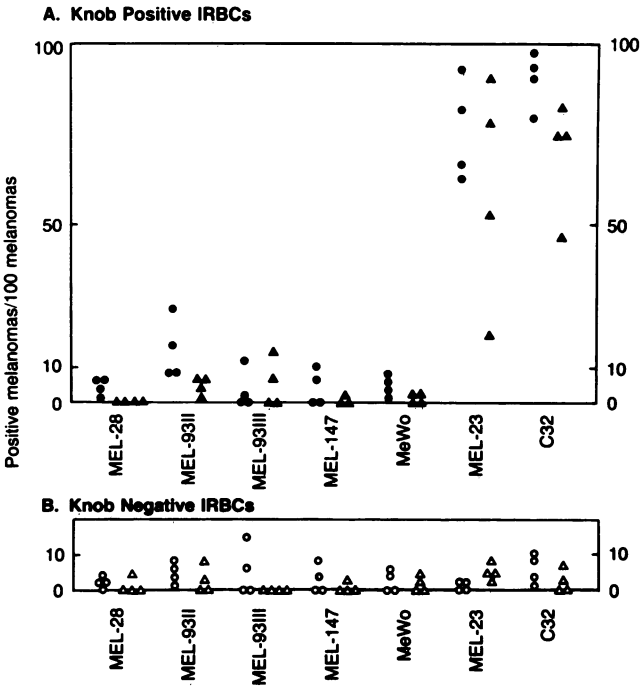

FIG. 1. Binding of *P. falciparum*-IRBCs to melanoma cells. (A) Knob-positive parasites MC K^+ (●) and SL K^+ (▲); (B) knob-negative parasites MC K^- (○) and SL K^- (△). Results are shown as positive melanoma cells (binding one or more IRBC) per 100 melanoma cells. Each point is the result of an individual assay.

K^-B^- *P. falciparum* parasites do not bind to melanoma cells (19). In individual experiments, depending on the precise washing conditions, the number of K^-B^- parasites in association with any melanoma cell is generally zero but may vary to a maximum of three. Uninfected RBCs did not bind to any cell line.

Figure 2 shows an estimate of the number of IRBCs bound per melanoma (IRBCs bound divided by binding melanoma cells). SK-MEL-23 cells bound both MC and SL K^+B^+ IRBCs in numbers comparable to those found for the C32 line. Binding of IRBCs to the SK-MEL-93II line was variable, especially with SL K^+B^+ parasites—one SK-MEL-93II cell bound 100 IRBCs. The small proportion of binding cells in the other four melanoma lines bound uniformly low numbers of IRBCs, comparable with the number bound by their K^-B^- controls, and these lines were regarded as cytoadherence negative.

A further cytoadherence assay was carried out on all seven melanoma lines concurrently with the IFAs described below (Table 1). FMG K^+B^+ parasites reacted in a similar fashion to the MC and SL K^+B^+ IRBCs, binding to the

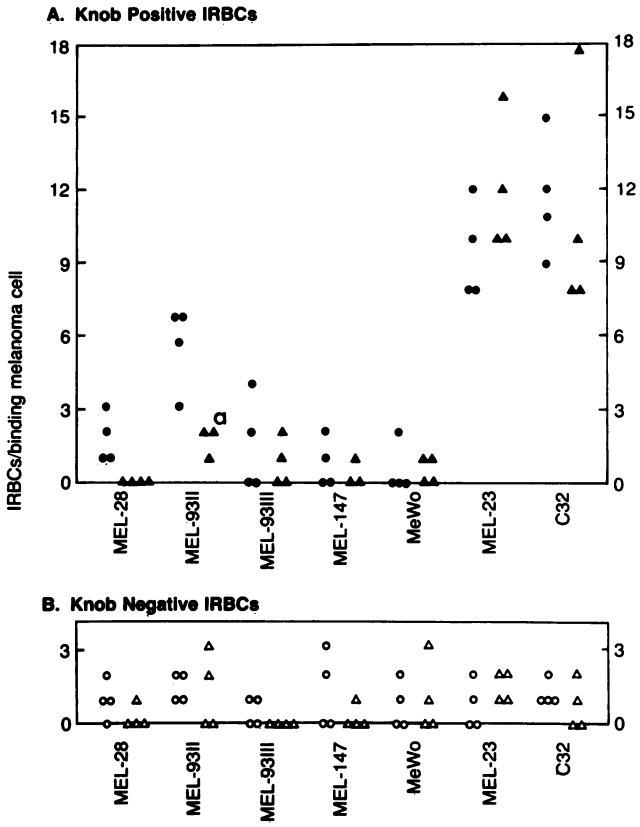

FIG. 2. IRBCs bound per melanoma cell. (A) Knob-positive parasites MC K^+ (●) and SL K^+ (▲). (B) Knob-negative parasites MC K^- (○) and SL K^- (△). Zero represents a lack of binding melanoma cells. Each point is the result of an individual assay. (a) One melanoma cell in this preparation bound 100 IRBCs.

majority of SK-MEL-23 and C32 cells but not to the other lines (with the exception of 1/100 SK-MEL-93II cells).

In terms of useful melanoma lines for in vitro cytoadherence assays, SK-MEL-23 may be compared with the C32 line, containing a majority of binding cells and large numbers of IRBCs per melanoma cell. It should be noted that SK-MEL-23 cells grew more slowly than C32 cells under the conditions described. The SK-MEL-93II line contains a small subpopulation of binding cells which is not sufficiently stable to be useful.

TABLE 1. Reactivity of melanoma cells with MAbs OKM5 and A6.1

Melanoma cell line	Binding of *P. falciparum* FMG (%)	Reactivity			
		OKM5		A6.1	
		Fixed cells	Live cells	Fixed cells	Live cells
C32	+ (82)	+	+	+	−
SK-MEL-23	+ (93)	+	+	+	−
SK-MEL-28	−	−	−	+	−
SK-MEL-93II	−[a]	−	−	+	−
SK-MEL-93III		−	−	+	−
SK-MEL-147	−	−	−	+	−
MeWo	−	−	−	+	−

[a] One melanoma in 1,000 scanned bound 8 IRBCs.

Comparison of binding and nonbinding melanoma cell lines for intracellular TSP and OKM5 antigen. Indirect IFA of methanol-fixed melanoma cells with an anti-TSP MAb (A6.1) and the OKM5 MAb identified TSP as an intracellular component of all seven melanoma cell lines, whereas the OKM5 antigen was only detected in the C32 and SK-MEL-23 melanoma lines (Table 1). There were no obvious differences in fluorescence intensity between the lines with the anti-TSP MAb. C32 and SK-MEL-23 cells exhibited some cell-to-cell variation in intensity with OKM5 MAb but were all positive compared with their respective negative controls.

Comparison of binding and nonbinding melanoma cell lines for surface TSP and OKM5 antigen. Nonfixed melanomas in suspension were compared in an indirect IFA with MAbs A6.1 and OKM5 for the presence of the appropriate antigen on the cell surface. Intact cells from all melanoma cell lines failed to fluoresce with MAb A6.1, while broken cells in the same preparations exhibited strong cytoplasmic reactivity. Figures 3c and d show this result for C32 cells under bright-field and UV illumination, respectively.

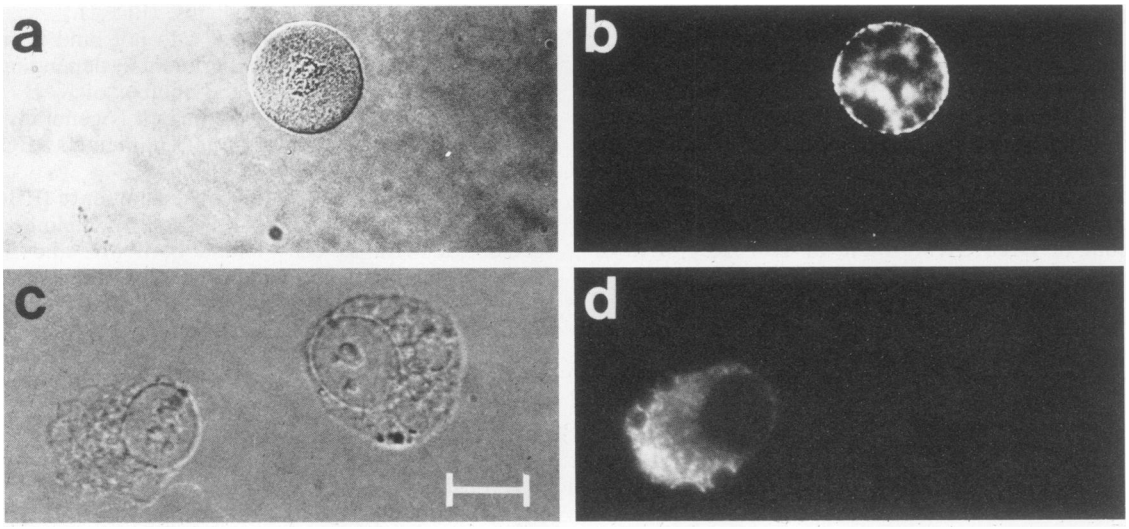

FIG. 3. IFA of unfixed C32 cells reacted with the OKM5 (a and b) or A6.1 (c and d) MAb under bright-field illumination (a and c) and UV illumination (b and d). Bar, 10 μm.

A proportion of intact cells from the C32 and SK-MEL-23 cell lines were strongly fluorescent with the OKM5 MAb. Of 100 intact C32 cells counted by eye, 83 were strongly positive, as were 53 of 100 SK-MEL-23 cells. The other cell lines were completely negative. Cytospin preparations of these negative cell lines did reveal a single strongly surface-positive SK-MEL-93II cell in approximately 2×10^4 cells and several weakly positive cells in a similar number of cells of the SK-MEL-28 line. Figures 3a and b show these results for the C32 line in suspension.

DISCUSSION

We have identified the heavily pigmented SK-MEL-23 melanoma line as a second cell line which, like the amelanotic C32 line (14), bears a specific surface receptor(s) for *P. falciparum*-IRBCs. The majority of SK-MEL-23 and C32 cells bound at least eight IRBCs per melanoma. Nonspecific binding of K^-B^- parasites was similar in all melanoma lines. We defined SK-MEL-28, SK-MEL-93III, SK-MEL-147, and MeWo as nonbinding lines because they rarely contained any cells with greater numbers of adherent K^+B^+ IRBCs than the K^-B^- controls (Fig. 1 and 2). They may be compared with the G361 melanoma line, previously shown to lack the cytoadherence property (14). SK-MEL-93II is essentially a nonbinding line containing a small subpopulation of binding-positive cells.

There was a positive correlation between the ability of melanomas to bind IRBCs and detection of OKM5 antigen. Internal OKM5 antigen was detected by indirect IFA of methanol-fixed cells only in the two cytoadherence-positive lines, SK-MEL-23 and C32. Indirect IFA of live cells detected OKM5 on the surface of the two binding lines, and weak reactivity was seen in a very small subpopulation of SK-MEL-28 cells.

TSP was detected by MAb A6.1 in methanol-fixed cells in every melanoma cell line tested, irrespective of its cytoadherence capacity. TSP was not detectable by IFA on the surface of C32 cells or on the surface of cells of any of the other melanoma lines. This may be a function of weak surface binding and removal during the numerous washing steps or may reflect the sensitivity of the IFA system. MAb A6.1 recognizes only some conformations of TSP, and binding of the antibody has been shown to be very low in the presence of millimolar concentrations of Ca^{2+} (5). Thus, since extracellular Ca^{2+} is higher than intracellular levels, it is likely that cell surface TSP would bind A6.1 with much lower affinity than intracellular TSP.

This limited analysis has shown no simple explanation for the role of TSP in cytoadherence. However, pure TSP undoubtedly binds *P. falciparum*-IRBCs in a specific manner (13, 15), and soluble TSP (200 μg/ml) blocks binding of IRBCs to both immobilized TSP and melanomas by more than 80% (13). Anti-TSP MAb 2.5 (50 μg/ml) reduces binding to TSP by 85% and to melanomas by 49%. MAb OKM5 (2 μg/ml) inhibits binding of IRBCs to melanomas, monocytes, and endothelial cells (3). It is possible that two mechanisms of binding exist, but a tempting hypothesis is that TSP acts as a bridging molecule between the parasite ligand and OKM5 antigen on the melanoma-endothelial cell surface in a similar way to its proposed stabilizing function, between fibrinogen and glycoproteins IIb and IIIa, in platelet aggregation (recently reviewed by Lawler [11] and Silverstein et al. [16]). In support of this hypothesis is the recent demonstration of binding of TSP to the OKM5 antigen (A. S. Asch, J. Barnwell, R. L. Silverstein, and R. L. Nachman, Clin. Res. 34:450A, 1986). It remains to be seen whether cells in the process of binding IRBCs in vivo or in vitro express detectable surface TSP.

In conclusion, our work with seven human melanoma cell lines demonstrates a direct correlation between surface expression of OKM5 antigen and cytoadherence of *P. falciparum*-IRBCs in vitro. While our results do not exclude TSP as a mediator of cytoadherence to endothelial cells in vivo, our work showed no correlation between the presence of TSP and the ability of melanomas to specifically bind *P. falciparum*-IRBCs in vitro.

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