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Modification of the Mechanical Properties of Red Blood Cell Membrane by Spent *Plasmodium falciparum* Culture Supernatant

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Abstract

The effect of spent culture supernatant on RBC membrane mechanical properties, RBC membrane deformability and stability, were assayed by ektacytometry after treating RBC ghost membranes with spent *Plasmodium falciparum* (*P. falciparum*) malaria culture supernatant fluid (Pf(+)), malaria culture medium which had not been exposed to *P. falciparum*-infected RBCs Pf(-), and malaria culture medium from an *in vitro* culture system containing uninfected RBCs (M). All treated RBC membranes were less deformable, and more stable than control, untreated RBC membranes. However, compared to controls, Pf(+)-treated RBC membranes were more deformable than both Pf(-)-treated and M-treated RBC membranes. Similarly, Pf(+)-treated RBC membranes were more stable than membranes treated with both Pf(-) and M. The deformability and stability of Pf(+)-treated RBC membranes were significantly different from membranes treated with both Pf(-) and M ($P \leq 0.05$). However, there was no significant difference between deformability and membrane stability for Pf(-)-treated and M-treated RBC membranes. Our results suggest that spent *P. falciparum* culture supernatant may significantly modify RBC membrane mechanical properties.

Keywords: RBC membrane, deformability, stability, *P. falciparum*, parasite culture supernatant.

1. Introduction

The red blood cell (RBC) membrane has been well described in terms of its structure and composition (Snyder and Sheafor, 1999). It is composed of a lipid bilayer, integral proteins, a sub-membranous skeletal protein network of spectrin, and peripheral proteins (Chasis and Mohandas, 1986). Deformability and stability are two essential qualities of the RBC membrane that play a crucial role in the maintenance of normal blood flow and supply to tissues (Arai *et al.*, 1990; Chien, 1987; Mohandas and Chasis, 1993). Consequently, any variation in RBC membrane deformability, as in the case of malaria (Miller, Baruch, Marsh & Doumbo, 2002) or sickle cell diseases (Platt, 1995) can potentially compromise the micro-circulatory function. Studies have demonstrated that the protein network underlying the RBC membrane, together with the membrane bilayer and the network of membrane-associated proteins, play a key role in regulating RBC membrane deformability and stability (Takakuwa, 2001). Considerable effort and time have been invested in the *in vitro* cultivation of the erythrocytic stages of *Plasmodium*, the stages most often associated with the pathogenesis of malaria.

A major accomplishment in this area was defining ideal *in vitro* conditions for continuous cultivation of *P. falciparum* strains from different geographical areas, using HEPES-buffered RPMI 1640 culture medium that is supplemented with human serum, RBCs, and sodium bicarbonate (Trager and Jensen, 1976; Trager and Jensen, 1977; Trager and Jensen, 1980). RBCs are an obligatory requirement in this *in vitro* medium. Indeed, fresh and stored erythrocytes appear to be equally suitable for continuous *in vitro* cultivation provided that adenosine triphosphate (ATP) levels of RBCs are within the normal range (Capps and Jensen, 1983, Schuster, 2002). While this unique medium has been shown to be suitable for *in vitro* cultivation of *P. falciparum* malaria stages, the effect of malaria culture medium which has not been exposed to *P. falciparum*-infected RBCs (iRBCs) and spent malaria parasite culture supernatant on RBC membrane mechanical properties has not been fully explored. *In vitro* cultivation of *P. falciparum* is usually carried out through

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the continuous preservation of iRBCs deposited in static thin layers of settled infected cells. However, not all aspects regarding how the culturing methodology affects the development and growth of the parasite are fully understood.

Although some agents have been shown to alter the RBC membrane mechanical properties (Beder *et al*., 2002), it is uncertain how *P. falciparum* spent parasite culture supernatant will affect both RBC membrane deformability and stability. The aim of this study was to assess the effect of spent parasite culture, and malaria culture medium which has not been exposed to iRBCs, on membrane deformability and stability of RBC.

2. Materials and Methods

2.1. In vitro cultivation of malaria parasites

P. falciparum (F32 strain) used for this study was a gift from Prof. Akira Kaneko of Tokyo Women's Medical University. *P. falciparum* F32 was maintained in a continuous culture as previously described by Trager & Jensen (1976). The culture contains type O^+ RBCs at 2% hematocrit in malaria culture medium, Pf(-) which consists of RPMI 1640 medium buffered with 25mM HEPES and 24 mM $NaHCO_3$ and supplemented with 10 % heat-inactivated human O^+ serum. Culture media were kept under a standard gas mixture of 5% O_2, 5%CO_2 and 90% N_2 at 37^{o}C. From a culture system containing mixed stage *P. falciparum*-infected RBCs at 10 % parasitemia, the supernatant was harvested and centrifuged for 15 minutes at ×500 g (GS-6KR: Beckman, Fullerton, California). This supernatant (Pf(+)), typically described as "spent", was used for subsequent experiments. Similarly, supernatant from malaria culture medium incubated with uninfected RBCs (Pf(-)), under conditions similar to that of spent culture system, was harvested and treated in the same manner described for spent *P. falciparum* culture supernatant. All solvents and chemicals were of analytical grade and were purchased from Wako Chemicals (Osaka, Japan). Water was purified using the milliQ pore system (Millipore, Bedford, MA, USA).

2.2. RBC lysis and 5T5K ghost preparation

Fresh type O^+ venous blood was drawn from healthy volunteers, collected in heparin-coated tubes and processed within minutes after collection. RBCs were separated from leukocytes by filtration through polyurethane filter kit (Terumo), and washed three times with 10 mM Tris-HCl buffer (pH 7.4) that contains 120 mM KCl to remove cytosolic components. RBCs were lysed and washed once in ice-cold 1:35 diluted 5T5K buffer (5T5K buffer: 5mM Tris-HCl, pH 7.4, containing 5mM KCl) by centrifugation at ×15,000 g to obtain 5T5K RBC ghost cells. Ghost-cell suspensions were incubated at 37°C for 40 min for membrane resealing.

2.3. RBC lysis and MgATP ghost preparation

MgATP ghost-cells were prepared in the same manner described for 5T5K ghost-cells with slight modifications, as described previously by Mohandas & Chasis (1993). The 5T5K buffer (lysing buffer) was supplemented with 10 mM ATP and 1 mM $MgCl_2$. To restore isotonicity, 100 µl of a mixture of 150 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol was added to RBC ghost suspensions and incubated at 37°C for 40 min for membrane resealing. MgATP-ghosts were prepared in the presence of MgATP throughout the process, whereas 5T5K ghosts were prepared in the absence of MgATP. The morphology of unfixed resealed ghosts was examined by dark-field light microscopy using a Nikon microscope.

2.4. Pre-treatment of ghost membranes

MgATP ghost suspensions were re-suspended in nine volumes of either spent culture supernatant (Pf(+)), malaria culture medium (Pf(-)), or a medium from *in vitro* culture containing uninfected static RBCs (M). MgATP ghost re-suspensions were incubated for 30 minutes at 37^{o}C. Untreated MgATP ghosts served as control. After incubation, all RBC ghosts were centrifuged once at ×500 g for 10 minutes and assayed by ektacytometry for changes in membrane deformability and stability.

2.5. RBC membrane stability assay

RBC membrane resistance to shear-induced fragmentation was measured by subjecting MgATP ghosts to a constant high shear stress of 750 dynes cm^{-2} in the ektacytometer, as described by Hardeman *et al*. (1994). For the present study, 500 µl of each test sample (treated and untreated ghosts) was thoroughly mixed with 3 ml of 50 % dextran (wt./vol.) in 10 Mm TBS buffer (pH 7.4), resuspended in the ektacytometer chamber, and rotated at 100 rpm to generate the desired shear stress. When the shear stress is first applied, RBC ghosts are maximally deformed into ellipsoids and produce a narrow elliptical pattern that generates a maximal value of the deformability index (DI). RBC ghosts are unable to withstand the large value of applied shear stress, and subsequently begin to fragment, with DI gradually decaying under the constant high shear stress. A decrease in the DI value reflects fragmentation of the intact membranes into small, undeformable spherical particles. T_{50} is the time required for DI to decay to half its maximum value. A decrease in the T_{50} value reflects a decrease in RBC membrane stability, and vice versa. Suspending RBC ghosts in a viscous medium is necessary for the ektacytometric method as cells suspended in a low viscosity fluid (e.g. Tris-buffered saline, TBS) tumble, and do not deform in response to the applied shear stress (Johnson, 1989). It has been reported that the use of dextran, a neutral polysaccharide, to increase the viscosity of the suspending medium, does not affect the integrity of RBC membrane or alter its mechanical behavior (Nash & Meiselman, 1984).

2.5.1. RBC membrane stability assay

The Maximum Deformability Index (DI_{max}) and initial rate of change of the deformability index ($DI_{initial}$) which is a direct measure of membrane deformability, were determined at various shear stress values by laser diffraction analysis using an ektacytometer (LORCA: RR Mechantronics; Hoorn, The Netherlands). Details of ektacytometry have been described elsewhere (Johnson, 1989; Hardeman *et al*., 1994). Briefly, 100 µl of each test sample was re-suspended in 3.0 ml of 50% dextran in 10 mM TBS (wt./vol.), pH 7.4, and subjected to increasing shear stress up to 200 dynes cm^{-2}. Changes in laser

diffraction patterns were analyzed to derive DI_{max}. A decrease in $DI_{initial}$ indicates a reduction in RBC membrane deformability. Suspending RBC ghosts in a viscous medium is necessary for the ektacytometric method as cells suspended in a low viscosity medium (e.g. TBS) tumble and do not deform in response to the applied shear stress (Johnson, 1989).

3. Results

3.1. Morphology of resealed ghosts

Resealed ghosts prepared without MgATP were predominantly echinocytic in shape (Fig1.A). However a few discoid-shaped ghosts were observed. Ghosts prepared in the presence of MgATP were all discoid in shape (Fig. 1B), indicating that a minimum concentration of ATP is essential for maintenance of normal discoid shape. Pre-treatment with the various media did not alter the discoid shape of MgATP ghosts (Fig.1C, D and E).

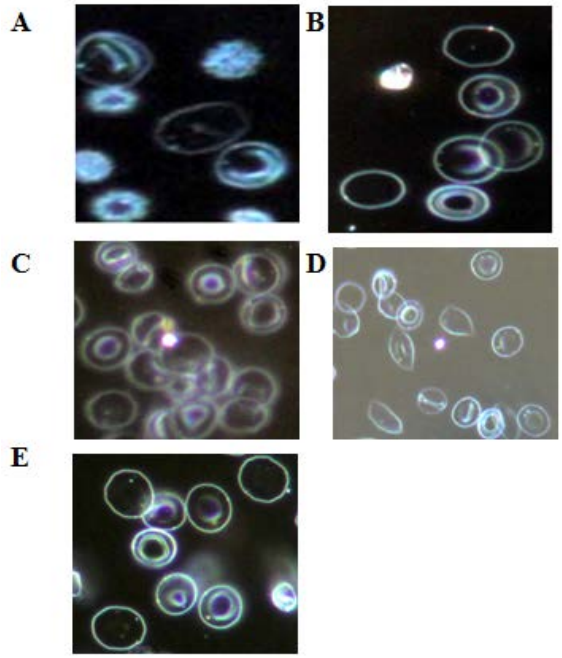

Figure 1. Dark-field light microscopy of ghosts. Panel A: 5T5K ghosts, Panel B: MgATP ghosts, Panel C: Pf(+)-treated MgATP ghosts, Panel D: Pf(-)-treated MgATP ghosts, Panel E: M- treated MgATP ghosts.

3.2. Membrane stability profile of MgATP and 5T5K RBC ghosts

Membrane stability profile of 5T5K ghosts and MgATP ghosts is shown in Fig. 2. When ghosts were constantly subjected to a high shear stress of 750 dynes/cm^2, ghost membranes were maximally deformed and fragmented soon after attaining the maximum deformability index (DI_{max}), resulting in decreased DI value. The rate of decrease of DI is a direct measure of membrane mechanical stability. 5T5K ghosts underwent fragmentation faster than MgATP ghosts which resulted in the reduction of DI value.

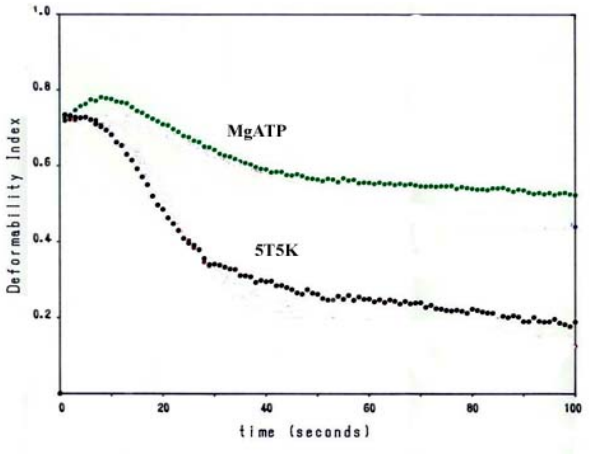

Figure 2. Deformability profile of 5T5K and MgATP ghosts

3.3. Duration for DI to reach half its maximum value (T_{50})

The Mean T_{50} values for the various treatments were 96, 40, 31 and 28 seconds for control, Pf(+), Pf(-) and M-treated MgATP ghosts, respectively (Fig. 5). Control MgATP had the longest fragmentation time, whereas M-treated MgATP ghosts had the shortest. Treated RBC ghosts exhibited varying decline in T_{50} values to about 58%, 68% and 71% of that of the control ghosts. These reductions correspond to Pf(+), Pf(-) and M-treated MgATP ghosts.

The T_{50} for all treatments were significantly different from each other, except for Pf(-) and M-treated MgATP ghosts ($P \leq 0.05$).

3.4. Maximum deformability index (Di_{max})

MgATP ghosts attained higher DI_{max} values than 5T5K ghosts (Fig. 3), and because of that they were used for subsequent experiments. When MgATP ghosts were subjected to increasing values of shear stress, the deformability index value increased, reaching a maximum of 0.79, 0.73, 0.77 and 0.74 for control, Pf(+), Pf(-) and M-treated ghosts, respectively (Fig.3). Although Pf(+)-treated ghosts recorded the lowest DI_{max} value, the Newman-Keuls Multiple Comparison Test showed that there was no significant difference between the mean DI_{max} values of Pf(+)- and M-treated ghosts ($P \leq 0.05$). Similarly, a comparison between untreated and Pf(-)-treated ghosts showed no significant difference between their mean DI_{max} values ($P \leq 0.05$).

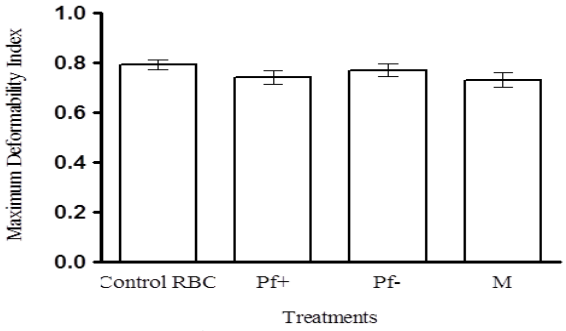

Figure 3. DI_{max} values for the various treatments. All values are reported as Mean ± S.D.

3.5. Initial rate of change of the deformability index ($Di_{initial}$)

$DI_{initial}$ values for the various treatments are shown in Fig. 4. Mean $DI_{initial}$ values obtained were 2.9, 2.5, 1.5 and 1.4 for control, Pf(+), Pf(-) and M-treated 5T5K ghosts, respectively. The mean $DI_{initial}$ value was highest for control RBCs, and lowest for M-treated RBCs ($P \leq 0.05$). Compared to control ghosts, treated RBCs exhibited reductions in $DI_{initial}$ values to about 14%, 48% and 52% of that of the control ghosts, corresponding to Pf(+), Pf(-) and M-treated MgATP ghosts, respectively. Stastical analysis indicates that the $DI_{initial}$ for Pf(-) and M-treated ghosts were not significantly different from each other. The mean $DI_{initial}$ values of all other treatments were significantly different from each other ($P \leq 0.05$).

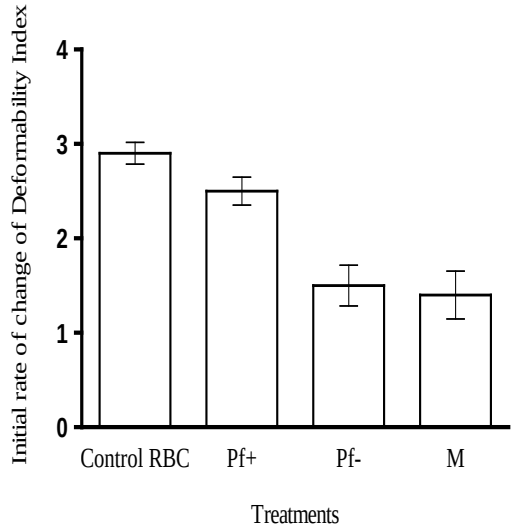

Figure 4. DIinitial values for the various treatments. All values are reported as Mean ± S.D

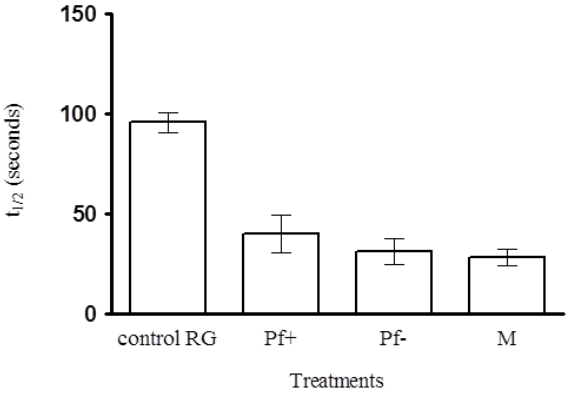

Figure 5. T_{50} values for the various treatments. All values are reported as Mean with Range

4. Discussion

The effects of a variety of agents on RBC membrane deformability and stability have been studied previously (Hardeman & Ince, 1999; Beder *et al*., 2002; Tadesse *et al.*, 2004). However, the effect of *P. falciparum* culture supernatant on RBC membrane mechanical properties of deformability and stability, has not been established. The present study shows that RBC ghosts prepared in the presence of MgATP attained higher DI_{max} values than ghosts prepared without MgATP, as indicated by the membrane stability profile (Fig. 2). This may possibly have to do with the ATP-dependent discocyte configuration, as ATP depletion of RBCs has been associated with discocyte-echinocyte transformation (Palek, Stewart, & Lionetti, 1974). The mechanism by which RBCs maintain their biconcave shape has been the subject of numerous studies. One of the critical factors for the maintenance of biconcave shape is the level of RBCs ATP (Weed *et al.* 1969; Szasz, 1970). We have used a laser diffraction method (ektacytometry) to directly measure changes in RBC membrane indices of deformability and stability. This technique was validated by subjecting resealed RBC ghosts to manipulations known to modify the membrane shear modulus (Heath *et al.*, 1982). Our results show that, compared to control untreated RBC membranes, ~~a~~ there was a reduction in RBC membrane deformability upon pretreatment with spent parasite culture supernatant (Pf(+)), malaria culture medium (Pf(-)), and malaria culture medium containing uninfected RBCs (M). The reduction in RBC membrane deformability by spent supernatant from *P. falciparum* cultures is consistent with the observation that in *falciparum* malaria, the deformability of the entire RBC population is reduced in proportion to disease severity (Nuchsongsin *et al.*, 2007). It has been found that in patients with severe *falciparum* malaria, the entire RBC mass, containing both uninfected and infected RBCs, becomes rigid (Cooke, Mohandas, & Coppel, 2004; Parker *et al.*, 2008; Nuchsongsin *et al.*, 2007). Several mechanisms such as hemin-induced oxidative damage of the RBC membrane, alterations in the phospholipid bilayer, and attached spectrin network have been proposed as being responsible for the increased rigidity and reduced deformability of the RBCs infected with *falciparum* malaria (Parker *et al.*, 2008; Nuchsongsin *et al.*, 2007). The cause of rigidity of RBCs under *in vitro* culture conditions, however, is not well-defined, but has been attributed, according to one study, to the discharge of a multitude of proteins from infected RBCs into the culture media (Naumann *et al.*, 1991), some of which could be involved in modulating the deformability of uninfected RBCs *in vitro*. Our results indicate that both Pf(-) and M-treated RBCs caused a greater reduction in RBC membrane deformability than Pf(+)-treated RBCs. This difference in membrane deformability is most likely due to the depletion of one or more of these components during parasite cultivation. As expected, our results reveal that the control RBC ghosts had the highest membrane stability. This is due to the intact membrane skeleton that is devoid of any modulation. However, the RBC membrane stability was reduced significantly after exposure to malaria culture medium, but was partially restored as time progressed. Data from both pathologic membranes and biochemically perturbed membranes have elucidated that RBC deformability and stability are not related to one another, as decreased deformability can be associated with either increased or decreased membrane stability. (Chasis and Mohandas, 1986). These findings imply that different skeletal protein interactions may regulate RBC membrane deformability and stability.

Although multiple parasite ligand-erythrocyte receptor interactions must occur for successful *Plasmodium* invasion of the human red cell (Montero, Rodriguez, Oksov & Lobo, 2009), it is probable that a transient change in RBC membrane stability is essential for *P. falciparum* invasion of the RBC or its survival within the micro-environment of the *in vitro* culture. This change may occur independently of changes in the RBC membrane deformability.

5. Conclusion

In this study, we have shown that malaria culture media and spent parasite culture supernatant can modify the stability and deformability of the RBC membrane. However, these two parameters do not seem to be related. Although medium change is essential for the sustenance of *P. falciparum in vitro*, the premise that modulation of RBC membrane mechanical properties may be indispensable for *P. falciparum* invasion, requires further elucidation.

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Authors' Contributions

NAA and YT designed the study. NAA was responsible for the study implementation and data collection. NAA and YT analyzed the data. NAA wrote the paper and all authors reviewed the manuscript.

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