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Effects of *Plasmodium falciparum*-infected erythrocytes on matrix metalloproteinase-9 regulation in human microvascular endothelial cells

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ABSTRACT

Objective: To investigate the regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human microvascular endothelium (HMEC-1) exposed to erythrocytes infected by different strains of *Plasmodium falciparum (P. falciparum)*. **Methods:** HMEC-1 cells were co-incubated for 72 h with erythrocytes infected by late stage trophozoite of D10 (chloroquine-sensitive) or W2 (chloroquine-resistant) *P. falciparum* strains. Cell supernatants were then collected and the levels of pro- or active gelatinases MMP-9 and MMP-2 were evaluated by gelatin zymography and densitometry. The release of pro-MMP-9, MMP-3, MMP-1 and TIMP-1 proteins was analyzed by western blotting and densitometry. **Results:** Infected erythrocytes induced *de novo* proMMP-9 and MMP-9 release. Neither basal levels of proMMP-2 were altered, nor active MMP-2 was found. MMP-3 and MMP-1 secretion was significantly enhanced, whereas basal TIMP-1 was unaffected. All effects were similar for both strains. **Conclusions:** *P. falciparum* parasites, either chloroquine-sensitive or -resistant, induce the release of active MMP-9 protein from human microvascular endothelium, by impairing balances between proMMP-9 and its inhibitor, and by enhancing the levels of its activators. This work provides new evidence on MMP involvement in malaria, pointing at MMP-9 as a possible target in adjuvant therapy.

1. Introduction

Despite valuable progress achieved by the recently established global eradication program, malaria still remains a major cause of morbidity and mortality, with 300-500 million clinical cases and almost one million deaths occurring each year[1]. In severe malaria, besides primary therapy including quinine and artesunate, which are effective against the parasite, in the recent years several new adjunctive therapies have undergone trials. The aim was to reduce malaria-induced pathophysiology, to reduce clinical symptoms and increase survival. However, results have been disappointing, with albumin being so far the only adjunctive therapy associated with reduced mortality in children with severe malaria[2]. Thus, a better understanding of mechanisms underlying the pathophysiology of complicated malaria in order to define new affordable treatment appears quite urgent.

In this context, convincing evidence on the involvement of human matrix metalloproteinases (MMPs) in malaria pathogenesis emerged very recently, thereby offering a new kaleidoscope of potential targets for adjunctive therapy[3-6]. MMPs are proteolytic enzymes able to disrupt subendothelial basement membrane and tight junctions and to modulate the activity of several pro-inflammatory mediators[7]. All these functions could be particularly crucial in facilitating CM development. *In vivo*, enhanced levels of MMP-1 in the brain[8], of MMP-9 in the whole blood[9], and of MMP-8 in the plasma[10] of human patients with severe malaria were described, whereas increased mRNA or protein levels of MMP-2, MMP-7 and MMP-9 were found in the brain of mice with CM[11,12]. *In vitro, P. falciparum*-infected red blood cells (RBCs) and malarial pigment haemozoin (HZ), a parasite waste product of haemoglobin digestion, have been shown to alter MMP production by phagocytic cells: in human monocytes, the phagocytosis of parasitized RBCs

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or of HZ has been reported to enhance the expression and activity of MMP-9[13-17]; in HZ-fed murine macrophages, altered balances between MMP-9 and its endogenous inhibitor [tissue inhibitor of metalloproteinase-1 (TIMP-1)] were shown[18].

In addition to phagocytes, another primary source of MMPs is represented by the endothelium[19,20]. To date, however, not many data on malaria-dependent regulation of endothelial MMPs are available. Two recent papers from our group demonstrated that HZ can upregulate MMP-9 production by human endothelial cells obtained from either small[21] or large[3] vessels. In the present study, we extended the previous observations by investigating the regulation of MMP-1, -2, -3, -9 and TIMP-1 in human microvascular endothelium after exposure to whole erythrocytes infected by two different strains of *P. falciparum*.

2. Materials and methods

2.1. Materials

All materials were from Sigma Italia, Milan, Italy, unless otherwise stated. MCDB 131 and RPMI 1640 medium were from GIBCO BRL, Paisley, Scotland; foetal calf serum was from HyClone, South Logan, UT; epidermal growth factor was from PeproTech EC, London, UK; Hepes buffer, Glutammine and Penicillin/Streptomycin were from EuroClone, Pero, Italy; monoclonal anti-human MMP-1, MMP-3, MMP-9, TIMP-1 Abs were from Santa Cruz Biotechnology, Heidelberg, Germany; computerized densitometer Chemidoc was from Biorad, Segrate, Italy.

2.2. Endothelial cell cultures

A long-term cell line of dermal microvascular endothelial cells (HMEC-1) immortalized by SV 40 large T antigen[22] was kindly provided by the Center for Disease Control, Atlanta, GA. Cells were maintained in MCDB 131 medium supplemented with 10% foetal calf serum, 10 ng/mL of epidermal growth factor, 1 g/mL of hydrocortisone, 2 mM glutamine, 100 units/mL of penicillin, 100 g/mL of streptomycin and 20 mM Hepes buffer, pH 7.4.

2.3. P. falciparum cultures

P. falciparum parasites (D10 and W2 strains; mycoplasma free) were kept in culture as described (21) at 5% hematocrit (human type A+ RBCs) at 37 ℃ in RPMI 1640 medium supplemented with 10% heat-inactivated A+ human plasma, 20 mM Hepes buffer, pH 7.4, in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2.

2.4. Isolation of trophozoite-stage parasitized RBCs

Parasite cultures (2%-4% parasitemia) were washed twice with serum-free culture medium, resuspended to 25% haematocrit and fractionated on a discontinuous Percoll/4% sorbitol (wt/vol) gradient (0%, 40%, 60%, 70%, 80%)[23]. After centrifugation at 1 075 *g*, trophozoite-stage parasitized RBCs were collected at 40%-60% gradient interphase, and washed three times with PBS.

2.5. HMEC-1 treatment

HMEC-1 were seeded at 10^5 cells/well in 24 well flat bottom tissue culture clusters. After overnight incubation to allow cell adhesion, monolayers were exposed to trophozoite-stage infected RBCs (2% final haematocrit, 5%-10% parasitemia) in a humidified CO_2/air-incubator at 37 ℃ for 72 h. In some experiments, cells were co-incubated with pRBC for 3 h, washed and replaced with medium for 72 h. All experiments were performed in serum-free medium. At the end of each treatment, supernatants were collected and used for MMPs assays.

2.6. Assay of proMMP-2/MMP-2 and proMMP-9/MMP-9 levels by SDS-PAGE gelatin zymography

The levels of MMP-2 and MMP-9 and their pro-forms were evaluated by SDS-PAGE gelatin zymography in the cell supernatants, as previously described[21]. Briefly, 15 μL of supernatants were loaded on 8% polyacrylamide gels containing 0.1% gelatin under non-denaturing and non-reducing conditions. Following electrophoresis, gels were washed with Triton TX-100 2,5% and incubated for 18 h at 37 ℃ in a collagenase buffer. After washings, bands were visualized by Comassie Brilliant Blue R-250 staining. Densitometric analysis of the bands was performed using a computerized densitometer.

2.7. Assay of MMP-1, MMP-3, proMMP-9, and TIMP-1 levels by Western blot analysis

Cell supernatants were loaded on 8% polyacrylamide gels under denaturing and reducing conditions, with addition of Laemmli buffer, blotted on a polyvinylidene difluoride membrane, and probed with anti-human MMP-1, MMP-3, MMP-9, and TIMP-1 monoclonal Abs at 1/500-1 000 final dilution. Bands were visualized by enhanced chemiluminescence. Densitometric analysis of the bands was performed using a computerized densitometer.

2.8. Statistical analysis

All data were obtained from three independent experiments with similar results. Results are shown as a representative image (gelatin zymography and Western blot analysis) or as mean values ± SD (densitometric analysis). All densitometric data were analyzed by Student's *t*-test.

3. Results

3.1. P. falciparum-infected RBCs induce release of active MMP-9 but not of active MMP-2 protein from HMEC-1

HMEC-1 were treated with RBCs infected by late stage trophozoites of two different strains of *P. falciparum* (D10, chloroquine-sensitive; and W2, chloroquine-resistant) for 72 h. Further investigation on cell supernatants was performed by SDS-PAGE gelatin zymography followed by

densitometric analysis to assess the levels of MMP-2/MMP-9 precursors and activated forms. Since the assay is equally sensitive for the pro-enzymes (inactive in solution) and the active forms, and because TIMPs and other endogenous inhibitors are dissociated from the enzyme during the SDS-electrophoresis, the intensity of the bands reflects the levels of the different forms of the gelatinases and not their net activities.

As shown in Figure 1, proMMP-9 and MMP-9 were absent in untreated HMEC-1 supernatants, whereas they were induced de novo by infected RBCs. Moreover, untreated control cells showed low levels of proMMP-2, which were not significantly altered by infected RBCs. The parasite effects on gelatinases production were not strain-specific, since no significant differences were found between the levels of gelatinases production after treatment with D10- or W2-infected erythrocytes. Moreover, when HMEC-1 were co-incubated with infected RBCs for 3 h, washed and kept in culture for other 72 h, results were similar to those obtained with 72 h of co-incubation (data not shown).

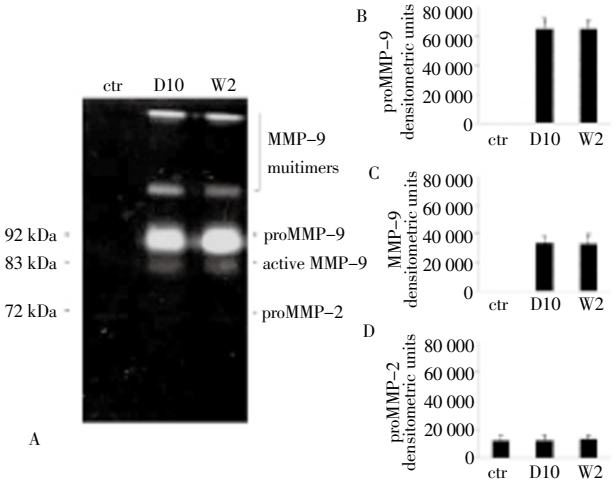

Figure 1. Effects of trophozoite-stage *P. falciparum*-infected RBCs on gelatinase levels in HMEC-1 supernatants.
A: representative gelatin zymogram from three different experiments, with 92 kDa, 83 kDa, and 72 kDa band representing proMMP-9, MMP-9, and proMMP-2 protein levels, respectively; B-D: densitometric analysis of gelatin zymography data, shown as means + SD. All densitometric data were analyzed for significance by Student′s *t*-test, with 3*vs*2: *P* not significant in all panels; 2/3*vs*1: $P<0.001$ in B-C and *P* not significant in D.

3.2. *P. falciparum*-infected RBCs impair the balance between pro-MMP-9 and TIMP-1 proteins released from HMEC-1

HMEC-1 was treated with RBCs infected by late stage trophozoites of D10 and W2 strains of *P. falciparum* for 72 h. Thereafter, cell supernatants were collected and protein levels of proMMP-9 and TIMP-1, the endogenous inhibitor of MMP-9, were analyzed by western blotting followed by densitometric quantification. As shown in Figure 2, proMMP-9 protein was absent in untreated HMEC-1 supernatants, whereas it was induced de novo by both parasite strains. On the contrary, the basal levels of TIMP-1 were not significantly altered by either parasite strain-infected RBCs. The effects of *P. falciparum*-infected erythrocytes were not strain-dependent, since no significant differences between either parasite strain were found.

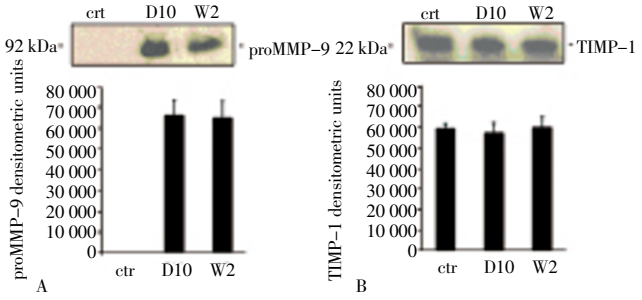

Figure 2. Effects of trophozoite-stage *P. falciparum*-infected RBCs on proMMP-9 (A) and TIMP-1 (B) protein release from HMEC-1. Upper panels: representative western blots, with 92 kDa and 22 kDa bands representing proMMP-9 and TIMP-1 protein levels, respectively; lower panels: mean densitometric values ± SD. All densitometric data were analyzed for significance by Student′s *t*-test, with 3*vs*2: *P* not significant in all panels; 2/3*vs*1: $P<0.001$ in A and *P* not significant in B.

3.3. *P. falciparum*-infected RBCs enhance protein release of MMP-3 and MMP-1 from HMEC-1

HMEC-1 was treated with RBCs infected by late stage trophozoites of D10 and W2 strains of *P. falciparum* for 72 h. Thereafter, cell supernatants were collected and protein levels of MMP-3 and MMP-1, two enzymes sequentially involved in proMMP-9 proteolytic activation, were analyzed by western blotting followed by densitometric quantification. As shown in Figure 3, both protein levels of MMP-3 and MMP-1 released from HMEC-1 were significantly enhanced after stimulation with *P. falciparum*-infected erythrocytes. Parasite-dependent upregulation of MMP-3/MMP-1 protein release was not strain-specific, and no significant differences were found whether D10- or W2-infected erythrocytes were used.

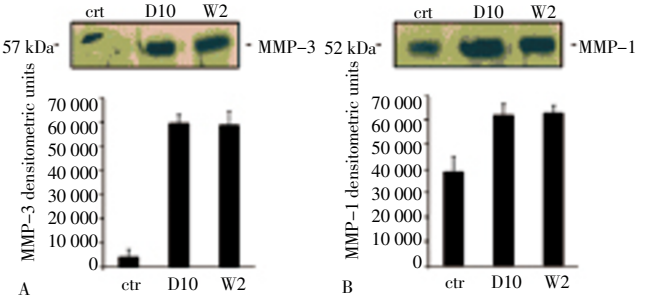

Figure 3. Effects of trophozoite-stage *P. falciparum*-infected RBCs on MMP-3 (A) and MMP-1 (B) protein release from HMEC-1.
Upper panels: representative western blots, with 57 kDa and 52 kDa bands representing MMP-3 and MMP-1 protein levels, respectively; lower panels: mean densitometric values ± SD. All densitometric data were analyzed for significance by Student′s *t*-test, with 3*vs*2: *P* not significant in both panels; 2/3*vs*1: $P<0.001$ in A and $P<0.01$ in B.

4. Discussion

The complications of *P. falciparum* malaria, including

CM, are associated to cytoadherence of RBCs to activated endothelial cells overexpressing several adhesion molecules, such as intracellular adhesion molecule-1 and vascular cell adhesion molecule-1[24]. Therefore, microcirculatory obstruction, tissue hypoxia and metabolic disturbances occur as a result of infected RBCs sequestration in human microvessels[25]. Parasite-dependent endothelial activation is also associated with modulation of the production of proinflammatory cytokines, including TNFalpha, IL-6 and IL-8[26], and vasoactive mediators, like constitutive and inducible endothelin-1[27,28]. Moreover, parasitized RBCs were shown *in vitro* to decrease trans-endothelial electrical resistance and permeability of the blood-brain barrier (BBB) [29], two features of CM which in vivo lead to brain oedema and ring haemorrhages[25]. Here we show that the treatment of a human microvascular cell line (HMEC-1) with RBCs infected by two different strains of *P. falciparum* (D10 and W2) modified the pattern and the levels of released MMPs and TIMPs. The percentages of parasitemia (5%-10%) used in these experiments are relevant to biological conditions associated with severe malaria. Hyperparasitaemia in falciparum malaria is defined as >4% parasitized RBCs in the peripheral blood, although locally in the small vessels of the brain it may be much higher due to sequestration[30].

A first screening by gelatin zymography measuring the levels of precursors and active forms of gelatinases revealed de novo induction by parasitized RBCs of either pro- or active MMP-9 protein release, without altering basal levels of proMMP-2 and without promoting any proMMP-2 activation. This evidence is consistent with previous data showing MMP-9 induction in human endothelial cells isolated from large calibre vessels after treatment with RBCs infected by *P. falciparum* trophozoites[3]. Moreover it was previously shown that in human monocytes, phagocytosis of trophozoite-stage parasitized RBCs, enhances the basal levels of constitutively secreted MMP-9[13,16]. Interestingly, trophozoites contain high amounts of HZ, a ferriprotoporphyrin Ⅸ crystal bound to a large spectrum of proteins and lipids of host or parasite origin, which has been directly related to MMP-9 overexpression[3,13-17,21] and activation[31].

The endogenous inhibitor of MMP-9 is TIMP-1, which acts by chelating the zinc of the metalloproteinase active site, thereby contrasting any effects of the MMP pro-peptide removal and blocking the enzymatic activity[32]. In previous studies, parasitized RBCs did not alter TIMP-1 mRNA in human brain microvascular endothelial cells[26], whereas HZ did not modify TIMP-1 protein levels secreted by human dermal microvascular endothelium[21]. This evidence is consistent with results from the present study, which did not highlight any modification of basal TIMP-1 protein levels released from HMEC-1 after treatment with parasitized RBCs. Thus, the parasite-mediated induction of MMP-9 might be the net effect of increased proMMP-9 and unaltered TIMP-1 release.

Furthermore, mechanisms underlying proMMP-9 activation require a proteolytic cascade sequentially involving plasminogen, MMP-3 and MMP-1[33]. In the present work, both basal MMP-3 and MMP-1 proteins were enhanced by parasitized RBCs; these increases might explain the parasite-induced levels of active MMP-9 previously described. A similar modulation of MMPs and their inhibitors has been demonstrated in the same cell line treated with HZ[21]; additionally, accumulation of MMP-1 and urokinase-type plasminogen activator receptor has been described in the brains of patients with fatal CM[8,34], suggesting that the proMMP-9 proteolytic machinery is activated in areas of intense parasite sequestration and vascular damage.

Nevertheless, a crucial point highlighted by this work is that the observed effects on endothelial MMP/TIMP regulation was promoted by both D10 and W2 parasite strains in a similar way, suggesting that modulation of MMP/TIMP production by endothelial cells is independent on parasite phenotype. This evidence makes MMPs potential targets for an adjunctive therapy since MMPs, as a result of their proteolytic activity, play a broad spectrum of effects directly connected to CM features. Indeed, it has been shown that several MMPs, including MMP-9, are able to process inter-endothelial tight junctions proteins, such as cerebral occludin and claudin-5[35] or the BBB protein beta-dystroglycan[36], and cleavage of these proteins could cause increased BBB permeability. Moreover, MMPs also modulate the activity of several pro-inflammatory molecules, such as TNFalpha, IL-1beta, IL-8 and ENA-78[7], thus amplifying the local inflammatory response.

In conclusion, the present study shows that *P. falciparum* parasites, either chloroquine-sensitive or -resistant, promote the release of active MMP-9 protein from human microvascular endothelial cells, as a result of impaired balance between proMMP-9 and its inhibitor TIMP-1, and of enhanced levels of its activators MMP-3 and MMP-1. This work provides new evidence on MMP involvement in malaria, and might help to better understand mechanisms of complicated malaria. Moreover, it strengthens previous evidence pointing at MMPs - MMP-9 in particular - as possible targets for new adjunctive therapies against falciparum malaria, aimed to prevent lethality associated with the severe form of the disease.

Conflict of interest statement

The authors declare no conflict of interest.

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