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Macrophage Inflammatory Protein-1alpha mediates Matrix Metalloproteinase-9 enhancement in human adherent monocytes fed with malarial pigment

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ABSTRACT

Objective: To investigate the role of macrophage inflammatory protein-1alpha (MIP-1alpha) in the detrimental enhancement of matrix metalloproteinase-9 (MMP-9) expression, release and activity induced by phagocytosis of malarial pigment (haemozoin, HZ) in human monocytes. **Methods:** Human adherent monocytes were unfed/fed with native HZ for 2 h. After 24 hours, MIP-1alpha production was evaluated by ELISA in cell supernatants. Alternatively, HZ-unfed/fed monocytes were treated in presence/absence of anti-human MIP-1alpha blocking antibodies or recombinant human MIP-1alpha for 15 h (RNA studies) or 24 h (protein studies); therefore, MMP-9 mRNA expression was evaluated in cell lysates by Real Time RT-PCR, whereas proMMP-9 and active MMP-9 protein release were measured in cell supernatants by Western blotting and gelatin zymography. **Results:** Phagocytosis of HZ by human monocytes increased production of MIP-1 alpha, mRNA expression of MMP-9 and protein release of proMMP-9 and active MMP-9. All the HZ-enhancing effects on MMP-9 were abrogated by anti-human MIP-1alpha blocking antibodies and mimicked by recombinant human MIP-1alpha. **Conclusions:** The present work suggests a role for MIP-1alpha in the HZ-dependent enhancement of MMP-9 expression, release and activity observed in human monocytes, highlighting new detrimental effects of HZ-triggered proinflammatory response by phagocytic cells in falciparum malaria.

1. Introduction

Malarial pigment haemozoin (HZ) is a ferriprotoporphyrin IX crystal produced by *Plasmodium* parasite after host haemoglobin catabolism, present in late stages of parasitized red blood cells (trophozoites) and in residual bodies shed after schizogony, which is avidly phagocytosed by human phagocytic cells[1]. In human monocytes it persists undigested for at least 72 hours in the intact lysosomes[2]: as a consequence, it impairs a large number of their functions, including repeated phagocytosis, antigen presentation, oxidative burst, bacterial killing, coordination of erythropoiesis[3,4], and stimulates overproduction of several cytokines, chemoattractant molecules and stress-dependent anti-apoptotic molecules[5]. Recently, HZ was also shown to enhance the production of two enzymes stored in so-called gelatinase granules: lysozyme and matrix metalloproteinase-9 (MMP-9)[3, 6-8], a proteolytic enzyme related to degradation of basement membranes, cleavage of inflammatory molecule proforms and disruption of inter-endothelial tight junctions[9,10]. Such an enhancement appeared to be directly connected to HZ-dependent production of tumor necrosis factor (TNF)alpha and interleukin (IL)-1beta[3, 6-8]. However, due to the large amount of often redundant soluble factors produced after phagocytosis[5], it is likely that MMP-9 enhancement may not be limited to the action of these two cytokines only. Since literature data indicated macrophage inflammatory protein (MIP)-1alpha as a positive regulator of MMP-9[11], in the present study MIP-1alpha protein levels after HZ phagocytosis were measured in human monocyte

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supernatants; therefore, a possible role for this molecule in the induction of MMP-9 expression, release and activity was investigated by using two combined approaches: a mimicking approach with recombinant human MIP-1alpha and a blocking approach with anti-human MIP-1alpha antibodies. As it will be described in the following sections, HZ promoted the production of MIP-1alpha and enhanced the expression and release of proMMP-9 protein and active enzyme, and MIP-1alpha appeared causally connected to MMP-9 enhancement at all levels investigated.

2. Materials and methods

2.1. Materials

Unless otherwise stated, reagents were obtained from Sigma-Aldrich, St. Louis, MO. Sterile plastics were from Costar, Cambridge, UK; Panserin 601 monocyte medium was from PAN Biotech, Aidenbach, Germany; Percoll was from Pharmacia, Uppsala, Sweden; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; enzyme-linked immunoadsorbent assay (ELISA) kit, anti-human MIP-1alpha blocking antibodies and recombinant human MIP-1alpha were from R&D Systems, Minneapolis, MN; TRIzol, M-MLV, oligo-dT, sense and anti-sense primers and Platinum Taq DNA Polymerase were from Invitrogen, Carlsbad, CA; DNA-free kit was from Ambion, Austin, TX; Beacon Designer 2.1 software was from Premier Biosoft International, Palo Alto, CA; dNTPs were from Applied Biosystem, Foster City, CA; anti-human MMP-9 monoclonal antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA; ECL Kit and HRP-conjugated anti-mouse secondary antibodies were from GE-Healthcare, Milan, Italy; Geldoc computerized densitometer and electrophoresis reagents were from Bio-Rad Laboratories, Hercules, CA.

2.2. Culturing of Plasmodium falciparum and isolation of HZ.

Plasmodium falciparum (*P. falciparum parasites*) (Palo Alto strain, mycoplasma-free) were kept in culture as described. After centrifugation at 5 000 × *g* on a discontinuous Percoll-mannitol density gradient, HZ was collected from the 0%-40% interphase, washed five times with 10 mM HEPES (pH 8.0) containing 10 mM mannitol at 4 ℃ and once with PBS, and stored at 20% (v/v) in PBS at −20 ℃ or immediately used for opsonization and phagocytosis.

2.3. Preparation and handling of monocytes

Human monocytes were separated by Ficoll centrifugation from freshly collected buffy coats discarded from blood donations by healthy adult donors of both sexes provided by the local blood bank (Associazione Volontari Italiani Sangue, Torino, Italy)[6]. Separated lympho-monocytes were resuspended in RPMI 1640 medium and plated on wells of 6-well plates. Each well received 2 mL of cell suspension containing 8×10^6 cells/mL in RPMI 1640. The plates were incubated in a humidified CO_2/air-incubator at 37 ℃ for 60 min. Thereafter non-adherent cells were removed by 3 washes with RPMI 1640 and adherent cells reincubated at 37 ℃ overnight in RPMI 1640. Shortly before starting phagocytosis, wells were washed with RPMI 1640 and Panserin medium added (2 mL/well).

2.4. Phagocytosis of opsonized latex particles or HZ and treatment with recombinant human MIP-1alpha or anti-human MIP-1alpha blocking antibodies

Latex particles and HZ washed once and finely dispersed at 30% (v/v) in PBS were added to the same volume of fresh human AB serum (AVIS blood bank) and incubated for 30 min at 37 ℃ to reach opsonization as described[6]. Phagocytosis was started by adding to adherent monocytes opsonized latex particles or HZ (50 RBC equivalents, in terms of heme content, per monocyte). The plates were then incubated in Panserin 601 medium in a humidified CO_2/air-incubator at 37 ℃ for 2 h. This time period maximized phagocytosis and was not sufficient to induce heme-oxygenase-mediated degradation of ingested heme[2]. As an average, each monocyte ingested HZ equivalent approx. to 8-10 trophozoites in terms of ingested heme, as shown previously. After phagocytosis, cells were washed and incubated in Panserin 601 medium in presence/absence of anti-human MIP-1alpha blocking antibodies (30 ng/mL) or recombinant human MIP-1alpha (20 ng/mL) in a humidified CO_2/air-incubator at 37 ℃ for the indicated times.

2.5. Measurement of MIP-1alpha production by ELISA

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37 ℃ for 24 h. The level of secreted MIP-1alpha was assayed in monocyte supernatants by specific ELISA, according to the manufacturer's instructions. A standard calibration curve was generated with recombinant human MIP-1alpha.

2.6. Measurement of MMP-9 mRNA levels by real-time RT-PCR

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37 ℃ for 15 h. Total cellular RNA from 2×10^6 cells was isolated from monocytes by TRIzol, according to the manufacturer's instructions, and eluted in 20 μL diethyl pyrocarbonate water. To remove any contaminating DNA, RNA was treated with Ambion's DNA-free kit. Subsequently, 6 μg of RNA were reverse transcribed into single-stranded cDNA using M-MLV (200 U/μL final concentration) and oligo-dT (25 μg/μL final concentration). Real Time RT-PCR analysis was performed with the iCycler Instrument and the iCycler iQ Real Time Detection System Software version 3.0 (Biorad, Hercules, CA) as previously described[6]. MMP-9 (GenBank accession no. NM_004994) primers (forward: 5′-CCT GGA GAC CTG AGA ACC AAT C-3′, reverse: 5′-CTC TGC CAC CCG AGT GTA AC-3′) were obtained from Invitrogen. Oligonucleotide sequences were identified using Beacon Designer Software package (PREMIER Biosoft International, Palo Alto, CA) and designed to be intron-spanning allowing the differentiation between cDNA and DNA-derived PCR products. As housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

was used; primer sequences were from the Bio-Rad library: forward: 5′-GAA GGT GAA GGT CGG AGT-3′ and reverse: 5′-CAT GGG TGG AAT CAT ATT GGA A-3′. For each 25 μL PCR reaction mix: 1 μL cDNA (corresponding to 10^5 cells); 1.0 μL sense primer (10 μM); 1.0 μL anti-sense primer (10 μM); 0.5 μL dNTP (10 mM); 1.5 μL $MgCl_2$ (50 mM); 1.25 U Platinum Taq DNA Polymerase; 2.5 μL Buffer (10×); 1.7 μL SYBR Green (stock 1:10 000); and 14.55 μL PCR-grade water were mixed together. DNA polymerase was pre-activated for 2 min at 94 ℃ and the amplification was performed by 50 cycles (MMP-9) or 35 cycles (GAPDH) with denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 30 s. Relative quantification for MMP-9, expressed as -fold variation over untreated control cells, was calculated with the efficiency-corrected quantification model[12] after determination of the difference between C_T of the given gene A (MMP-9) and that of the calibrator gene B (GAPDH). C_T values are means of triplicate measurements. To validate the use of the method, serial dilutions of cDNA from monocytes, stimulated for 15 h by 20 ng/mL rhTNFalpha, were tested. Analyzed transcripts exhibited high linearity amplification plots (r>0.98) and similar PCR efficiency (99.7% for MMP-9 and 92.2% for GAPDH), confirming that the expression of each of these genes can be directly compared to one another. The specificity of PCRs was confirmed by melt curve analysis. The melting temperatures for each amplification product were 85.8 ℃ for MMP-9 and 86.5 ℃ for GAPDH.

2.7. Measurement of proMMP-9 protein release levels by Western blotting

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37 ℃ for 24 h. Thereafter cell supernatants were collected and 15 μL cell supernatants/lane were added to the loading buffer. The samples 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-9 monoclonal antibodies at 1/1 000 final dilution. Bands were visualized by enhanced chemiluminescence. Densitometric analysis of the bands was performed using a computerized densitometer.

2.8. Measurement of active MMP-9 protein release levels by gelatin zymography

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37 ℃ for 24 h. Thereafter the levels of active MMP-9 were evaluated by gelatin zymography in the cell supernatants as indicated[13]. Briefly, 15 μL cell supernatants/lane were loaded on 8% polyacrylamide gels containing 0.1% gelatin under non-denaturing and non-reducing conditions. Following electrophoresis, gels were washed at room temperature for 2 h in milliQ water containing 2.5% (v/v) Triton-X100 and incubated for 18 h at 37 ℃ in a collagenase buffer containing (mM): NaCl, 200; Tris, 50; $CaCl_2$, 10; and 0.018% (v/v) Brij 35, pH 7.5, with or without 5 mM EDTA to exclude aspecific bands. At the end of the incubation, the gels were stained for 15 min with Coomassie blue (0.5% Coomassie blue in methanol/acetic acid/water at a ratio of 30:10:60). The gels were destained in milliQ water. Densitometric analysis of the bands, reflecting total levels of active MMP-9, was performed using a computerized densitometer.

2.9. Statistical analysis

For each set of experiments, data are showed as Means ±SEM or one representative imagine of three independent experiments. All data were analyzed by Student's *t*-test (equal variances).

3. Results

3.1. HZ promotes production of MIP-1alpha by human adherent monocytes

Human adherent monocytes were unfed (control cells), fed with latex particles (control meals), or fed with HZ for 2 h. After termination of phagocytosis, cells were incubated for 24 h; therefore, supernatants were collected and MIP-1alpha protein levels were measured by specific ELISA. Figure 1 shows mean values ± SEM of MIP-1alpha ng/mL produced by human monocytes in three independent experiments. HZ (column 3) induced a ~20-fold enhancement of basal levels (column 1) of MIP-1alpha. Such an increase was not dependent on phagocytosis *per se*, since inert latex particles (column 2) did not promote any significant alteration. Data were analyzed by Student's *t*-test, and following differences were obtained. Latex-fed cells versus control cells: no significant differences; HZ-fed cells versus control/latex-fed cells: $P<0.05$.

3.2. HZ enhances MMP-9 mRNA expression in human adherent monocytes: role of MIP-1alpha

Human adherent monocytes were unfed (control cells), fed with latex particles (control meals), or fed with HZ for 2 h. After termination of phagocytosis, cells were incubated in presence/absence of recombinant human MIP-1alpha or anti-human MIP-1alpha blocking antibodies for 15 h. Therefore, MMP-9 mRNA expression was analyzed by Real-time RT-PCR in monocyte lysates. As shown in Figure 2, MMP-9 mRNA levels in HZ-treated cells (column 2) were ~60-fold higher than in control cells (column 1). Addition of recombinant human MIP-1alpha to unfed cells (column 3) induced a ~15-fold increase of MMP-9 mRNA levels compared to controls, following the HZ trend; the effect of HZ was abrogated when anti-human MIP-1alpha blocking antibodies were added (column 4), since cells showed MMP-9 mRNA levels similar to controls. As expected, latex particles did not alter basal levels of MMP-9 mRNA (not shown). Data were analyzed by Student's *t*-test, and following differences were obtained. HZ-fed cells versus control cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus control cells: $P<0.05$; HZ-fed cells treated with anti-human MIP-1alpha antibodies versus HZ-fed cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus HZ-fed cells: $P<0.05$; HZ-fed

cells treated with anti-human MIP-1alpha antibodies versus control cells: no significant differences.

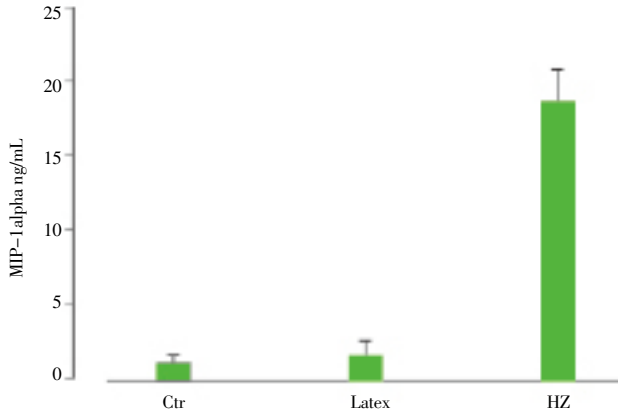

Figure 1. HZ-dependent enhancement of MIP-1alpha production by human adherent monocytes.

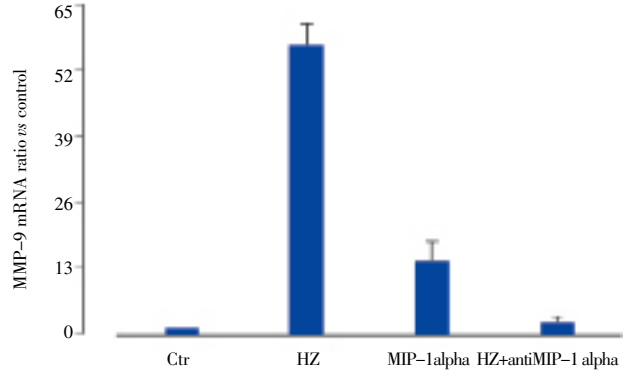

Figure 2. Role of MIP-1alpha in the HZ-dependent enhancement of MMP-9 mRNA expression in human adherent monocytes.

3.3. HZ enhances proMMP-9 protein release by human adherent monocytes: role of MIP-1alpha

Human adherent monocytes were unfed (control cells), fed with latex particles (control meals), or fed with HZ for 2 h. After termination of phagocytosis, cells were incubated in presence/absence of anti-human MIP-1alpha blocking antibodies or recombinant human MIP-1alpha for 24 h. Therefore, proMMP-9 protein release was analyzed by Western blotting and optical densitometry in monocyte supernatants. Figure 3 shows a representative blot (upper panel, 92-kDa positive bands corresponding to proMMP-9) and mean values ± SEM of arbitrary densitometric units from three independent experiments (lower panel). ProMMP-9 protein levels in HZ-treated cells (lane/column 2) were ~7-fold higher than in control cells (lane/column 1). Addition of recombinant human MIP-1alpha to unfed cells (lane/column 3) mimicked the HZ trend, showing a ~6-fold increase of proMMP-9 protein levels compared to controls. The effect of HZ was abrogated when anti-human MIP-1alpha blocking antibodies were added (lane/column 4), cells showing proMMP-9 protein levels similar to controls. As expected, latex particles did not alter basal levels of proMMP-9 protein (not shown). Data were analyzed by Student's *t*-test, and following differences were obtained. HZ-fed cells versus control cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus control cells: $P<0.05$; HZ-fed cells treated with anti-human MIP-1alpha antibodies versus HZ-fed cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus HZ-fed cells: $P<0.05$; HZ-fed cells treated with anti-human MIP-1alpha antibodies versus control cells: no significant differences.

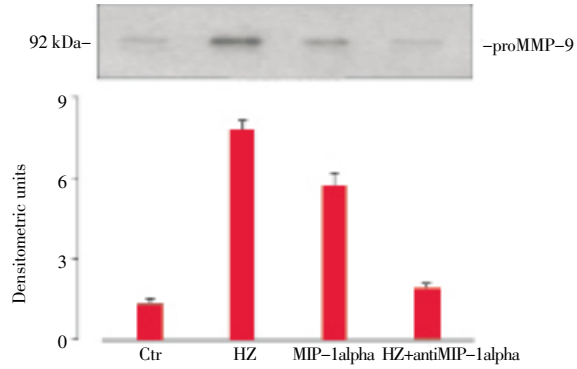

Figure 3. Role of MIP-1alpha in the HZ-dependent enhancement of proMMP-9 protein release from human adherent monocytes.

3.4. HZ enhances active MMP-9 protein release by human adherent monocytes: role of MIP-1alpha

Human adherent monocytes were unfed (control cells), fed with latex particles (control meals), or fed with HZ for 2 h. After termination of phagocytosis, cells were incubated in presence/absence of anti-human MIP-1alpha blocking antibodies or recombinant human MIP-1alpha for 24 h. Therefore, active MMP-9 protein release was analyzed by gelatin zymography and optical densitometry in monocyte supernatants. Figure 4 shows a representative gel (upper panel, 83-kDa negative bands corresponding to active MMP-9) and mean values ± SEM of arbitrary densitometric units from three independent experiments (lower panel). Active MMP-9 levels in HZ-treated cells (lane/column 2) were ~7-fold higher than in control cells (lane/column 1). Addition of recombinant human MIP-1alpha to unfed cells (lane/column 3) mimicked the HZ trend, showing a ~6-fold increase of active MMP-9 levels compared to controls. The effect of HZ was abrogated when anti-human MIP-1alpha blocking antibodies were added (lane/column 4), since active MMP-9 levels were similar to controls. As expected, latex particles did not alter basal levels of active MMP-9 protein (not shown). Data were analyzed by Student's *t*-test, and following differences were obtained. HZ-fed cells versus control cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus control cells: $P<0.05$; HZ-fed cells treated with anti-human MIP-1alpha antibodies versus HZ-fed cells: $P<0.001$; unfed cells treated with recombinant human MIP-1alpha versus HZ-fed cells: $P<0.05$; HZ-fed cells treated with anti-human MIP-1alpha antibodies versus

control cells: no significant differences.

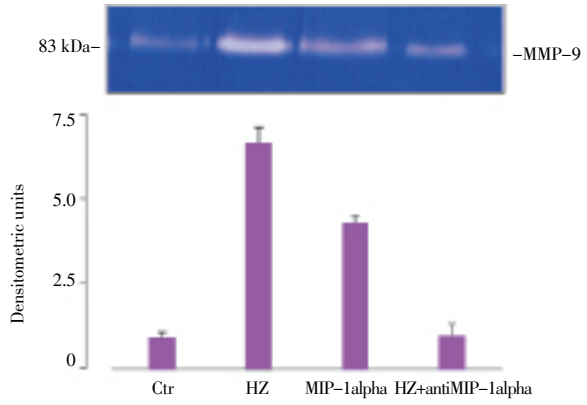

Figure 4. Role of MIP-1alpha in the HZ-dependent enhancement of active MMP-9 protein release from human adherent monocytes.

4. Discussion

During the recent years, growing evidence on the involvement of MMPs in malaria pathogenesis has been shown[3, 14, 15]. These proteolytical enzymes appear causally connected to clinical progress towards severe malaria complications such as cerebral malaria (CM), an encephalopathy associated with cytoadherence of parasitized red blood cells (pRBCs) to the endothelium, recruitment of immune cells, release of inflammatory and vasoactive mediators, blood brain barrier damage, brain oedema, ring-haemorrhages, and occasionally presence of pRBCs and leukocytes in the extravascular space[16, 17]. Enhanced levels of MMPs have been described in either human or murine malaria models: MMP-9 mRNA was higher in full blood of human patients with severe malaria[18], whereas serum MMP-8[19] and brain MMP-1[20] were enhanced in human patients with CM; additionally, mRNA or protein levels of several MMPs were found elevated in brains from murine models of CM[14, 21].

Interestingly, HZ was shown in vitro to promote MMP-9 production by several human or murine cell types, including adherent or immunopurified monocytes isolated from human peripheral blood[6], human THP-1 monocytic cell line[22], human microvascular endothelial cells[23], human umbilical cord vein endothelial cells[3] and murine RAW264.7 macrophage cell line[24]. The ferric moiety of HZ primes the activation of proMMP-9 by other MMPs through directly binding of its haemopexin domain[25], while the lipid moiety of HZ seems to be related to the inflammation-mediated enhancement of MMP-9 gene induction and protein release[5, 7,8,23,24].

The pattern of genes of proinflammatory molecules induced after phagocytosis of HZ by human monocytes is quite extended, and includes TNFalpha and IL-1beta (whose production has been directly related to HZ-dependent MMP-9 enhancement)[6-8], as well as several chemokines[5]. Interestingly, to this list belongs also MIP-1alpha, which is able to induce MMP-9 gene expression[11].

The present study investigated the effects in vitro of HZ on MIP-1alpha protein release from human adherent monocytes and the potential role of this chemokine in the HZ-dependent upregulation of MMP-9 gene expression, protein release and activity.

Results showed that basal levels of MIP-1alpha released by monocytes were enhanced by HZ. Such an enhancement was not a consequence of phagocytosis per se, since inert latex particles did not reproduce HZ effects. This evidence is consistent with previous data obtained from in vitro[26, 27] and in vivo[28,29] models showing increased production of MIP-1alpha by mononuclear cells. Moreover, the increased production of MIP-1alpha appeared to be causally connected to the HZ-dependent enhancement of MMP-9 mRNA expression and of proMMP-9 and active MMP-9 protein release. Indeed, anti-human MIP-1alpha blocking antibodies abrogated the effects of HZ on MMP-9 at all levels investigated, whereas unfed cells treated with recombinant human MIP-1alpha showed higher levels of MMP-9 mRNA expression, protein release and enzyme activation, mimicking the trend of HZ phagocytosis effects. These results are analogous to those obtained from blocking/mimicking experiments performed for TNFalpha and IL-1beta in previous studies[6,7]: thus, they underline the redundant role of proinflammatory molecules produced by monocytes as a response to HZ stimulus and justify future research on the possible use of broad-spectrum instead of specific anti-inflammatory drugs as adjuvant therapy for complicated malaria.

Unfortunately, the mechanisms through which HZ exerts its stimulatory action on MMP-9 and related inflammatory molecules MIP-1alpha, TNFalpha and IL-1beta are not yet fully understood. Indeed, the HZ-dependent enhancement of MIP-1alpha has been related to the ferric moiety of malarial pigment either in human[5] or murine[28] models, whereas the increased production of TNFalpha, IL-1beta and MMP-9 is triggered by the lipid moiety of HZ, and particularly by 15- hydroxyeicosatetraenoic acid (15-HETE), a potent lipoperoxidation derivative generated by HZ through haem autocatalysis[7,8]. Additionally, either human[30] or murine[29] models suggest that HZ enhances MIP-1alpha expression through MAP kinases pathways (specifically ERK1/2 phosphorylation), while the HZ-dependent enhancement of the triade IL-1beta/TNFalpha/MMP-9 requires the activation of NF- κ B pathway[8,22]. However, these data might be not conflicting, since the mechanisms of HETE-dependent NF- κ B activation have been suggested to involve also MAP kinases (ERK1/2, JNK and p38 cascades), which might be required for stabilized messenger transcripts[31]. Meanwhile, in the murine system, HZ induced MIP-1alpha production through ERK1/2- either dependent or independent mechanisms[29], suggesting that concomitant activation of some parallel pathways which might synergize rather than compete may occur. Nevertheless, future studies are certainly required in order to clarify the mechanisms underlying cell responses to HZ.

In conclusion, the present work supplies new evidence on the inflammation-dependent enhancement of MMP-9 by HZ. As a future perspective, the use of anti-inflammatory drugs, as well as the use of synthetic MMP inhibitors, might be useful to prevent or cure complicated malaria. Interestingly, recent studies using anti-inflammatory drugs in rodent models of complicated malaria led to promising results: high doses of dexamethasone blocked malaria-associated acute respiratory distress syndrome[32] and beta-interferon suppressed the development of experimental CM[33]. Similarly, the use of BB-94, a broad-spectrum MMP inhibitor, significantly increased the survival rate of mice with CM[21]. Therefore, a better understanding of dynamics of pro-inflammatory response and MMP regulation during malaria should be strongly encouraged in a reasonable perspective to look for new adjuvant therapies for complicated malaria.

Conflict of interest statement

We declare that we have no conflict of interest.

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