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Effect of hepatocyte growth factor signaling pathway activation on *Plasmodium berghei* infection

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ABSTRACT

Objective: To explore the effect of hepatocyte growth factor signaling pathway activation on *Plasmodium berghei* infection. **Methods:** In this study, hepatocyte growth factor was detected by ELISA and Western blotting assay. Hepatocyte injury was detected by FITC–dextran absorption assay, and hepatocyte growth factor expression was shown to be expressed in the same injury cells by immunofluorescence against hepatocyte growth factor. In addition, Activation of hepatocyte growth factor and its receptor signaling pathway was detected with immunoprecipitation and detection of phosphorylation status. **Results:** It was found that injury of hepatocytes by sporozoite migration induced the secretion of hepatocyte growth factor and it was hepatocyte growth factor that rendered hepatocytes susceptible to *Plasmodium* sporozoite infection. In addition, hepatocyte infections depended on activation of the hepatocyte growth factor and its receptor signaling pathway. **Conclusions:** Our results indicate that hepatocyte growth factor and its receptor may possibly be potential targets for new approaches to malaria treatment.

1. Introduction

The infection of hepatocytes by sporozoites within the mammalian host is an important step in the *Plasmodium* life cycle[1]. Several studies have reported that host cell infection is preceded by the traversal of several hepatocytes by the malarial parasites[2,3]. As a result, these hepatocytes are wounded due to membrane rupturing. After malaria sporozoites are injected into the mammalian host by infected mosquitoes, sporozoites must firstly infect hepatocytes to establish mammalian cycle of a malarial infection[1]. Sporozoites initially traverse the cytosol of some hepatocytes until finally invading one of them by forming a parasitophorous vacuole[2–4]. During the migrated process, some of the migrated hepatocytes die, but most of them survive. When the plasma membrane of a hepatocyte is wounded, some growth factors and other proteins stored in the cytosol are released to the extracellular environment[5–7]. In addition, hepatocyte injury may also accelerate the expression of certain growth factors, which can be secreted and further influence sporozoite development[6]. Therefore, hepatocyte wounding by sporozoite migration promotes

synthesize and release growth factors and other proteins that may have the capability of influencing the neighboring environment and helping sporozoite development in mammalian host. In previous studies, hepatocyte growth factor (HGF) has been shown to be one of the well-characterized growth factors that are released after injury[5–8]. Therefore, it is reasonable to think that HGF may render neighboring hepatocytes susceptible to infection. In the current study, we found that HGF and its receptor are involved into the sporozoite development in liver and that activation of HGF signaling pathway is necessary for malaria infection.

2. Materials and methods

2.1. Parasites and preparation of hepatocytes

Plasmodium berghei (*P. berghei*) (MR4, USA) sporozoites were obtained from dissection of *Anopheles stephensi* mosquito (breed in our laboratory) salivary glands infected with *P. berghei* as previous report[9]. Primary hepatocytes were prepared as in a previously described study[10]. Briefly, hepatocytes were isolated by collagenase H perfusion of mouse liver lobules and purified over a 60% Percoll gradient. Cells (in William's medium with 10% FCS) were seeded in chamber slides and incubated at 37 °C with 5% CO₂ for 24 h.

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2.2. Sporozoite infection

Sporozoites (5×10^4) were added to hepatocyte monolayers (2×10^5) for 24 h, before fixation and staining with monoclonal antibody (Santa Cruz biotechnology, USA) to detect the exoerythrocytic forms (EEFs) and infection was quantified by counting the number of EEFs as reported in previous study^[11]. All infections were done in triplicate and repeated three times. To detect the infection rate affected by HGF or antibodies against HGF, recombinant murine HGF (R&D Systems, USA) or monoclonal antibodies against HGF (Santa Cruz biotechnology, USA) were added and the EEFs were observed.

2.3. Determination of HGF levels

Western blot and ELISA were used to determine the levels of HGF in the media of infected hepatocyte monolayers and performed as we reported before^[12–14]. For ELISA, sporozoite-infected media were collected at different time points. Ninety-six-well plates were coated with monoclonal anti-HGF antibodies ($100 \mu\text{L}/\text{well}$, $1 \mu\text{g}/\text{mL}$) in coating buffer (carbonate-bicarbonate pH 9.6) overnight at 4°C . Plates were washed with PBST (0.05% Tween 20 in PBS) and blocked 1 hour at 37°C with $200 \mu\text{L}/\text{well}$ 1% bovine serum albumin (BSA) in PBST. After washing, sporozoite-infected media diluted serially in PBS were added and incubated for 2 hours at 37°C , followed by a dilution of anti-mouse IgG subclass antibodies (Santa Cruz biotechnology, USA) conjugated with alkaline phosphatase. Enzyme activity was measured with an ELISA reader (Bio-tek Laboratories Inc., USA). For Western blot analysis, sporozoite-infected media were separated by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE). The proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane blots were blocked at 4°C in 5% nonfat dry milk, washed, and probed with anti-HGF antibodies at 1 : 500. The blots were then washed and incubated with a biotinylated secondary antibody (biotinylated rat anti-mouse IgG), followed by transfer to Vectastain ABC (Vector Laboratories, USA).

2.4. HGF staining in infected hepatocytes

For *in vitro* staining of HGF, sporozoites were added to monolayer of primary hepatocytes for 1 h in the presence of lysine-fixable dextran and $1 \text{ mg}/\text{mL}$ of FITC-conjugated. Primary hepatocytes were then washed, fixed with 4% paraformaldehyde and stained with monoclonal antibody to HGF (Sigma, USA) followed by rhodamine conjugated secondary antibody (Santa Cruz biotechnology, USA) as previous reported^[8].

2.5. Preparation of conditional media

Preparation of conditional media was done as previous reported^[8]. Conditional medium results from the incubation of *P. berghei* sporozoites (10^5) with hepatocyte monolayers (2×10^5) at 37°C for 1 h. The absence of infectious aporozoites was verified by adding conditional media to hepatocytes and determining infection.

2.6. HGF receptor (MET) immunoprecipitation and detection of phosphorylation status

Primary hepatocytes (8×10^5) were incubated or not with 4×10^5 *P. berghei* sporozoites and then lysed for 20 mins at 4°C in $200 \mu\text{L}$ of a buffer containing 5 mM EDTA, 50

mM HEPES (pH 7.4), 10% glycerol, 150 mM NaCl, 2 mM EGTA and 1% Triton $\times 100$ in the presence of phosphatase inhibitors and protease. Extracts were clarified at $15\,000 \text{ g}$ for 15 mins and incubated with protein antibody to mouse MET ($1 \mu\text{g}/\text{mL}$; Santa Cruz Biotechnology) and G-Sepharose for 4 h at 4°C with gentle rotation. Samples were then centrifuged, re-suspended in $50 \mu\text{L}$ of $2 \times$ Laemmli buffer and boiled for 5 mins. Extracts were electrophoresed on SDS-PAGE and transferred onto PVDF membranes. Blots were probed with antibodies to MET (Santa Cruz biotechnology, USA) and phosphotyrosine, and detected by the enhanced chemiluminescence system (Amersham, USA) as previous reported^[8]. Digital images were acquired and processed using Adobe Photoshop (Adobe, Mountain View, CA) and quantified as described previously^[15].

2.7. Statistical Analysis

Data was expressed as mean \pm standard deviation (SD) and analyzed by one-way ANOVA and *q* test using SPSS 12.0. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Release of HGF by sporozoite-infected hepatocytes

Primary hepatocytes were infected with sporozoites and the supernatants of sporozoite infected hepatocytes were collected after various incubation times. HGF levels, as determined by ELISA (Figure 1A) and Western blot analysis (Figure 1B), increased with time and reached its peak at about 3 h. The levels of HGF at time 0, 1, 1.5, 2, 2.5, 3 and 3.5 h were 79.1 ± 21.7 , 103.6 ± 32.7 , 207.4 ± 54.9 , 348.9 ± 82.5 , 515.3 ± 104.6 , 158.7 ± 38.3 , respectively. However, the control hepatocytes without infecting with sporozoites showed negative results in both ELISA and Western blot (data not shown). In order to directly demonstrate HGF production by sporozoite infection, we combined FITC-conjugated dextran assays for cell wounding with staining for HGF production in individual cells *in vitro*. FITC-conjugated dextran can only penetrate wounded cell membranes^[2]. Sporozoites were incubated with hepatocyte monolayer in the presence of FITC-dextran before staining for HGF. As shown in Figure 1C and D, dextran-positive cells were also HGF-positive, whereas dextran-negative cells did not express HGF (data not shown). These results suggested that hepatocytes infected by sporozoites were wounded and HGF was expressed and released during this procedure.

3.2. Correlation of HGF with malaria infection

To examine whether the malaria infection is mediated by HGF, primary hepatocytes were pre-incubated with recombinant murine HGF (rHGF) before addition of *P. berghei* sporozoites. As positive and negative controls, we pre-incubated primary hepatocytes with conditioned medium or fresh medium, respectively. rHGF increased infection rate to a greater extent than fresh medium (fMed), but similar to that found in the positive conditional medium (cMed, Figure 2A), 972.7 ± 203.5 in rHGF group versus 902.9 ± 179.2 in cMed group and 413.8 ± 84.9 in the fMed group. There was significant difference between rHGF group and fMed group ($P < 0.01$). In addition, we still observed that rHGF reached its peak activity in 3 h, consistent with the results found in the above Western blot results (Figure 2B). These results showed that HGF was correlated with malaria

infection, and suggested that HGF release was a prerequisite for sporozoite infection of hepatocytes.

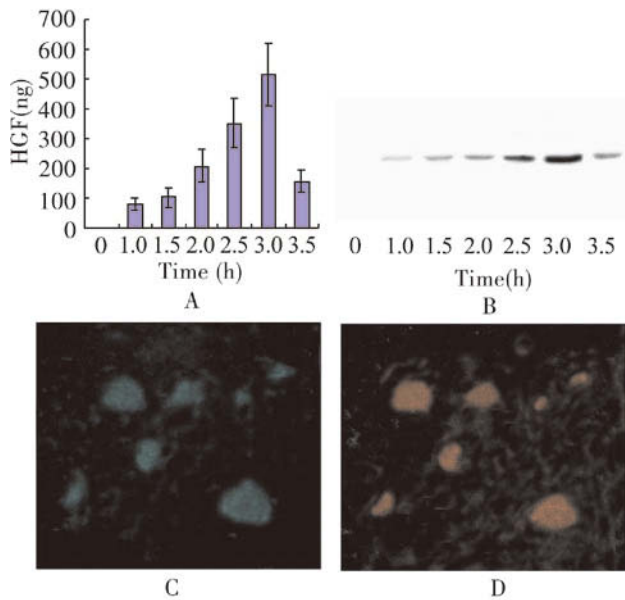


Figure 1. HGF expression detected by ELISA (A) and Western blotting (B) after hepatocyte injury by sporozoites. Injury cell was performed by FITC-dextran absorption (C) and HGF expression was shown to be expressed in the same injury cells by immunofluorescence against HGF (D).

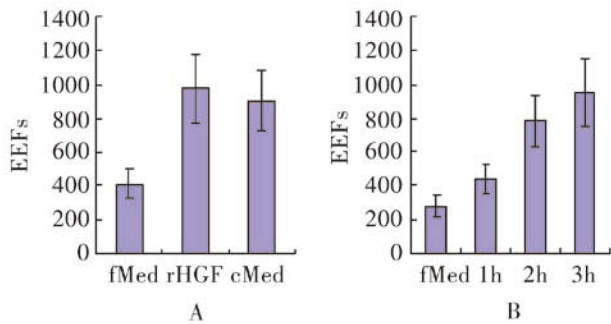


Figure 2. Recombinant HGF increased malarial infection (A), and the same concentration of recombinant HGF reached the best effects at about 3 h (B).

3.3. Neutralization of both HGF and MET inhibited malaria infection

To further confirm that HGF and its receptor MET were correlated with malaria infection, we added neutralizing monoclonal antibodies against HGF or MET to the culture medium before incubation with primary hepatocytes and sporozoites. In addition, we applied antibody against fibroblast growth factor (FGF) as a negative control. We found that the both antibodies against HGF or MET not only abolished the infectious effects of conditioned medium (cMed), but also decreased infection even below the basic level observed in fresh-medium cultures (fMed, Figure 3A and B). However, antibody against FGF showed no effects (Figure 3A). These results indicated that both HGF and MET involved the sporozoites infection and liver stage development.

3.4. Activation of HGF/MET signaling pathway was require for hepatocyte infection

MET is the tyrosine kinase receptor for HGF[16], we thus considered that HGF/MET signaling activation would be required for the malaria infection. In this study, we used MET immunoprecipitation and detection of phosphorylation. Primary hepatocytes were incubated with *P. berghei* sporozoites for 1 h as testing group (Figure 4B, t group) and the control group (Figure 4B, c group) were not incubated with *P. berghei* sporozoites, activation of MET kinase was observed which was indicated by tyrosine phosphorylation of the receptor (Figure 4A), the quantitated units in t group (Figure 4A, lane 2) and c group (Figure 4A, lane 1) were 21.8 ± 4.3 and 7.3 ± 1.6 , respectively, suggesting that activation of HGF/MET signaling pathway was require for hepatocyte infection.

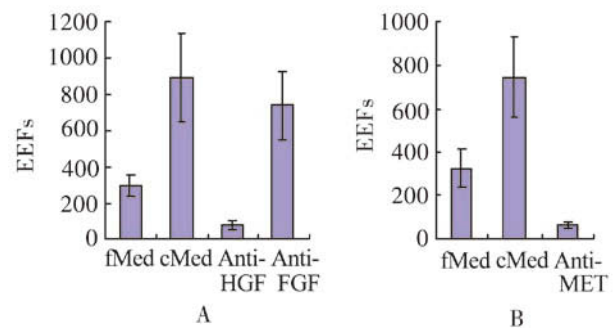


Figure 3. Both monoclonal antibody against HGF (A) and antibody against HGF receptor, MET (B), inhibited sporozoite infection of hepatocytes. However, monoclonal antibody against FGF did not effect on the sporozoite infection (A).

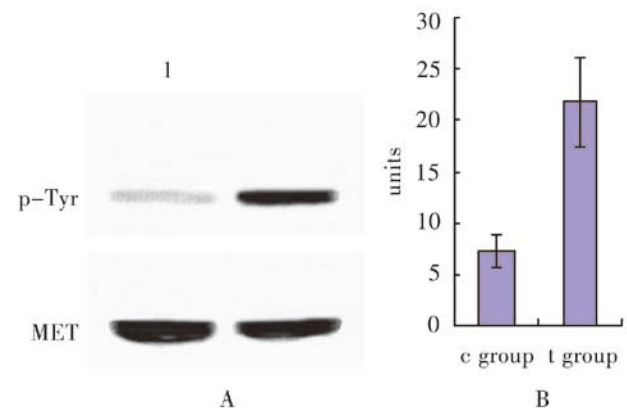


Figure 4. Activation of HGF/MET signaling pathway was require for liver infection by malaria. Sporozoites infected hepatocytes (B: t group) were phosphorylated (A: lane 2), non-infected hepatocytes (B: c group) show negative (A: lane 1), indicating HGF/MET signaling activation during the period of sporozoite infecting of host hepatocyt.

4. Discussion

Malaria infection begins with injection of *Plasmodium* sporozoites from a mosquito into human hosts. Thereafter, the sporozoites migrate to the host liver, wound and invade hepatocytes, and transform into EEFs that further develop

to produce schizonts containing thousands of merozoites that are released into the host blood stream and invade erythrocytes[1,16]. It is the blood stages of malaria that are responsible for developing the clinical symptoms of the malaria disease. It is thus obvious that infection of liver by sporozoites is an obligate stage for malaria invasion of human hosts. The liver stage of *Plasmodium* is an asymptomatic stage[16]. The liver stage of malaria parasites produces various red cell-infectious merozoites within its host hepatocytes. It is thought that the vacuole-enclosed parasite completely depends on the host cell for successful development but the molecular parasite - host cell interactions underlying this remarkable growth have remained unknown[17,18]. We hypothesize that it may be the liver specific microenvironment that provides sporozoites various unknown growth factors that further promote the sporozoites to transform and develop. In our current study, we demonstrated that HGF was released after sporozoites invade the hepatocytes. Thus, HGF is necessary for malaria sporozoite transforming and further developing in liver.

Recently, Mueller and his colleagues reported two putative small transmembrane proteins, UIS3 and UIS4 (up-regulated in infective sporozoites, UIS), in the liver stages of the rodent malaria parasite *P. berghei*[19,20]. Targeted gene deletion showed that UIS3 and UIS4 are only essential for liver stage growth and development.[19,20]. UIS4 was localized to the liver stage parasitophorous vacuole membrane[19]. However, UIS3 interacts directly with liver-fatty acid binding protein (L-FABP) and down-regulation of L-FABP expression in hepatocytes and over-expression of L-FABP promotes malaria sporozoites growth[21]. These study results indicate that malaria parasites do express certain proteins to interact with host molecules and it is such interactions that promote the malaria parasites further developments in the hepatocytes.

Malaria infection needs the entrance of sporozoites into hepatocytes through the formation of a parasitophorous vacuole[17, 18]. This process remains poorly understood. Its initial steps involve CD81 and heparan sulfate proteoglycans[12,23]. In our current study, we test a well-known growth factor(HGF) that is reported to be released after hepatocyte injury. We found that injury of hepatocytes by sporozoite migration induces secretion of HGF and it was HGF that rendered hepatocytes susceptible to *Plasmodium*-sporozoite infection. In addition, hepatocyte infections depend on activation of the HGF and its receptor signaling pathway. Our results indicate that HGF and its receptor may possibly be potential targets for new approaches to malaria treatment.

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Conflict of interest statement

We declare that we have no conflict of interest.

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