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In vivo study on splenomegaly inhibition by genistein in *Plasmodium berghei*-infected mice

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ABSTRACT

Spleen plays an important role in removing old and damaged red blood cells and malaria-infected erythrocytes. When malaria parasites invade the spleen and induce splenomegaly, splenic function tends to be impaired. Thus, the inhibition of splenomegaly is strongly required to protect the spleen. In this study, malaria-induced splenomegaly is inhibited by injecting genistein into a *Plasmodium berghei*-infected ICR mouse. To explain this phenomenon, the effect of genistein in spleen and liver of malaria-infected mice was evaluated by histological examination. Malaria parasites disrupted splenic architecture. After treating genistein, the disrupted architecture in which red and white pulp regions were clearly separated in recovered to uninfected ones. Changes in biophysical properties of blood were studied by measuring the viscosity of blood collected from malaria-infected and uninfected mice using a microfluidic viscometer. Genistein also had a negligible influence on variation in blood viscosity. The enzymatic activity and expression pattern of proteins were then investigated to explain the genistein effect on malaria-induced splenomegaly. Genistein is a potential drug for splenomegaly in *P. berghei*-infected mouse.

1. Introduction

Malaria is one of the most severe infectious diseases in humans [1]. *Plasmodium berghei* is a *Plasmodium* species in African murine rodents [2]. The parasite *P. berghei* ANKA causes the development of neurovascular diseases and experimental cerebral malaria [3]. Malaria leads to respiratory distress, metabolic acidosis, hypoglycemia, renal failure, and multiorgan failure, such as liver, kidney, and spleen [1,4,5]. Among them, the spleen severely responds by becoming enlarged, i.e., a condition called splenomegaly [6]. Thus, to determine the amount of malaria transmitted in an endemic region, the size of the spleen has been considered as an index of its development [6].

Spleen is composed of red and white pulps separated by a marginal zone [7]. These complex structures confer the spleen with various functions, including immune response and phagocytosis [6]. Splenic red pulp region filtrates damage erythrocytes and recycle iron component from old erythrocytes. Meanwhile, the white pulp region has similar morphological structures with lymph nodes containing T and B cells, thereby giving rise to antigen-specific immune responses that protect the body from blood-borne bacterial, viral, and fungal infections [7]. The marginal zone is also a transit area through which circulating blood is efficiently surveilled [6]. When damaged and malaria-infected erythrocytes pass through the spleen, they are ruptured and removed [5]. However, malaria parasites could induce chronic infections through evasion and modulation of immune responses, thereby remodeling the spleen by which some of the syndromes of severe malaria might be caused [6]. Malaria infection has been reported to enhance macrophage spreading [8], activity of leukocytes [9], and reticular cells [10] in spleen. These cellular responses contribute to splenic-clearance capacities [11]. Phagocytes act as a protector by identifying pathogen, cellular stress, and dying cells. Lymphocytes scan the antigen-presenting cells [7]. In addition, reticular cells form the filtration beds to provide a modest level of basal clearance [11]. Thus, to protect such malaria-induced diseases, study on the role of spleen with respect to malaria parasites is very important [6].

Genistein is an isoflavone [12] that has several biochemical targets inside the cell [13]. It has various biological properties that can be used for treating diseases [13]. Genistein is a tyrosine kinase inhibitor which has been used to inhibit cancer by inducing G2/M arrest and apoptosis [14]. It also affects proliferation and angiogenesis of endothelial cells *in vitro* by down-regulating cell adhesion through regulation of VE-cadherin, connexin 43, and multimerin [15]. In addition, genistein was found to attenuate lipopolysaccharide-induced vascular permeability by regulating the toll-like receptor 4 pathway in chick embryos [12]. It also acts as phytoestrogen showing overlapping effects with 17β-estradiol and improves

Abbreviations: ECM, extracellular matrix; TEMED, tetramethylethylenediamine; PALS, periarteriolar lymphatic sheath; MMP, matrix metalloproteinases.

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endothelial dysfunction induced by ovariectomy [16]. In relation to malaria parasites, genistein inhibits the intraerythrocytic development of *P. falciparum* and *P. chabaudi*, especially under *in vitro* condition [17]. Genistein also inhibits the development of sporozoite in the liver under *in vitro* and *in vivo* conditions [17]. The reduction of malaria infection in the liver relieves the severity of diseases in subsequent blood stages [17]. Therefore, genistein plays an important role in reducing the development of malaria disease [17]. However, the relationship between genistein and splenomegaly has not yet been investigated under *in vivo* condition.

In this work, the effects of genistein on the reduction of splenomegaly caused by *P. berghei* were experimentally investigated, especially under *in vivo* condition. This study was the first to investigate the critical role of genistein as a feasible regulator of malaria-induced splenomegaly. The inhibition of malaria-induced splenomegaly by genistein was confirmed by examining the regulation of malaria-infected spleen using an *in vivo* mouse model.

2. Material and methods

2.1. Chemicals

Genistein was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals and parasites

Male ICR mice (Hsd:ICR[CD-1®]) aged 6–8 weeks (Koatech, Pyeongtaek, Gyeonggi-do, Korea) were acclimatized to laboratory conditions for a week. They were housed under standard conditions of light, temperature, and humidity. The rodent malaria parasite, *P. berghei* ANKA strain, was used to infect normal mice (five to six mice per group) and was provided by Kyungpook National University (Dr. Yeongchul Hong, Department of Parasitology, School of Medicine, Daegu, 700–422, South Korea). Mice were infected by intraperitoneal injection of parasitized RBCs. Blood was collected from tail vein for analysis. Drug was treated after 2 days of malaria infection. Drug was administered by intraperitoneal injection with a cycle consisting of 4 consecutive days of infections and 2 days of rest. Mice were sacrificed on day 7.

2.3. Giemsa staining

To monitor the variation in infection rate (i.e., parasitemia), a drop of blood was collected from a tail vein, at predetermined times. Blood smears were performed 2 days after malaria parasite inoculation, once a day, up to day 8. After fixing the thin blood smear fixed with 99% methanol for 5 min on a slide, it was dried for 1–2 min. A diluted 4% Giemsa solution (Merck, Darmstadt, Germany) was prepared prior to staining. The slide was immersed in the prepared Giemsa stain solution for 25 min, rinsed with tap water, and then dried. The stained samples were examined using a microscope with a 100× oil-immersion objective lens.

2.4. Gelatin zymography

Gelatin zymography was used to detect matrix metalloproteinase (MMP)-2 and MMP-9 [18]. To investigate gelatinase activity, tissue samples were subjected to gelatin zymography. To filter cellular debris, samples were centrifuged at 16 000 × *g* for 20 min at 4 °C. The protein concentration of the collected supernatant solution was measured using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The samples were mixed with a 2× non-reducing sample buffer of the same volume. Then, 15–20 μl of mixed samples was loaded at each well. Polyacrylamide gels containing 10 ml of gelatin solution (2.65 mg/ml; Invitrogen, Carlsbad, CA, USA) was dispersed in a buffer solution (2.5 ml gel, 1.5 M Tris-HCl, pH 8.8; 165 μl of 10% SDS; 5.25 ml of 40% polyacrylamide; 165 μl of 50% glycerol; and 4 ml of distilled water). The staking gels contained 4% polyacrylamide in 1 M Tris-HCl (pH 6.8). They were polymerized by adding 100 μl of 10% ammonium persulfate and 10 μl of TEMED. The gels were then electrophoresized at 100 V. The electrophoresis gels were washed twice in 200 ml of 2.5% Triton X-100 (30 min each) by shaking and then incubated in 100 mM Tris-HCl, 5 mM $CaCl_2$, 0.005% Brij-35, and 0.001% NaN_3 (pH 8.0) for 6–48 h at 37 °C. The gels were stained with 0.25% Coomassie brilliant blue G-250 (50% methanol and 10% acetic acid) for 1 h at 25 °C and washed with destained solution (40% methanol and 10% acetic acid). The gels were then incubated for 1 h in a solution of 5% methanol and 7.5% acetic acid and kept under cellophane at 4 °C. The lower band was gelatinase-A (MMP-2), which corresponded with 66–72 kDa, whereas the upper band was gelatinase-B (MMP-9), which corresponded with 84–92 kDa.

2.5. Microfluidic chip for blood viscosity measurement

A microfluidic chip with a Wheatstone-bridge channel was used to measure the viscosity of blood samples with good accuracy [19]. As depicted in Fig. 2A, the microfluidic chip had two inlets and outlets, two identical parallel channels, and one bridge channel connecting the center of each parallel channel [20]. A silicon replica molder of 50 μm in depth initially was fabricated using conventional MEMS technologies, such as photolithography and deep reactive-ion etching. Using soft lithography, a polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, USA) block was then prepared after curing at 80 °C for 2 h. Finally, the microfluidic chip was fabricated by bonding the PDMS block onto a glass substrate using a oxygen plasma machine. To measure viscosity, a blood sample was supplied into the inlet (A) at a fixed flow rate of 1 mL/h (i.e., $Q_{Blood} = 1$ mL/h) using a syringe pump. Simultaneously, phosphate-buffered saline (PBS) solution was supplied into the inlet (B) with a syringe pump. Here, the viscosity of the PBS solution (1.00 ± 0.05 cP) was measured using a commercial viscometer (DV-II, Brookfield, USA). Blood viscosity was evaluated by monitoring a specific flow rate of the reference fluid (i.e., Q_{PBS}^{SW}) at which flow-switching phenomenon occurred in the bridge channel. Using a mathematical expression [19], blood viscosity (μ_{blood}) can be estimated with the following formula:

$$\mu_{\mathrm{Blood}} = \mu_{\mathrm{PBS}} \frac{{Q_{\mathrm{PBS}}}^{\mathrm{SW}}}{Q_{\mathrm{Blood}}} \quad (1)$$

where μ_{PBS} and Q_{Blood} denote the viscosity of the reference fluid and the flow rate of test blood, respectively. All experiments were conducted at 25 °C.

2.6. Western blot

A total of 40 μg protein was extracted from organs using tissue protein extraction reagent (Thermo Scientific, Wilmington, DE, USA). The proteins were loaded into a 10% acrylamide gel with sample buffer and then transferred to a polyvinylidene difluoride membrane, which was blocked with 5% milk TBS/T solution for 1 h at 25 °C. Anti-MMPs (Santi Cruz Technology, CA, USA), vascular endothelial growth factors (VEGFs; AbCam, Cambridge, UK), fibroblast growth factors (FGFs; AbCam, Cambridge, UK), and vascular endothelial (VE)-cadherins (R&D Systems, Minneapolis, USA) were used as primary antibodies. Monoclonal anti-β-actin (Santi Cruz Technology, Santa Cruz, CA, USA) was used for loading control. Bound antibody was detected with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Chemiluminescent signals were captured with the LAS 3000 system (Fuji, Tokyo, Japan).

2.7. Statistical analysis

All data were statistically analyzed by conducting SPSS *t*-test (IBM, Chicago, IL, USA) at 95% confidence level.