Different apoptotic responses to Plasmodium chabaudi malaria in spleen and liver

Riyadh, Saudi Arabia.
Correspondence Author: Saad Alkahtani, Department of biology, Teachers college, King Saud University, P. O. Box 271942 Riyadh 11352
E-mail: salkahtani@ksu.edu.sa

Abstract—The purpose of this study was to determine whether the apoptotic responses to Plasmodium chabaudi malaria in spleen and liver via mRNA expression of three genes involved in apoptosis (Bax, Bcl-2 and Caspase-3) are similar or not and to detect if these genes could be a good marker for apoptosis due to infection with P. chabaudi of female C57BL/6 mice. Mice were injected intraperitoneally (ip) with 10⁶ P. chabaudi-infected erythrocytes and then sacrificed at days (0, 1, 4 and 8 respectively). Quantitative real-time-PCR was used to quantify apoptotic genes. The levels of Bax and Caspase-3 were significantly increased only at days 1 and 8 in the liver cells and at day 8 in the spleen. The level of Bcl-2 was significantly increased on all three days after infection of the liver cells whereas it was significantly decreased on all three days after infection of the spleen. In conclusion, the present data has shows that infection with P. chabaudi stimulated apoptotic genes in the liver and spleen cells in different ways. Based on the data obtained it can be hypothesized that the malaria outcome triggered different apoptotic pathways in the liver and spleen. Also, these apoptotic genes can be used to reliably detect apoptosis.

Keywords—Apoptosis, Malaria, Mice, Liver, Spleen.

I. INTRODUCTION

Malaria is one of the most devastating diseases in the world, particularly in tropical countries. Annually, about 400 million people worldwide are affected by malaria and around 2 million of them die (Guha et al., 2006). The few available anti-malaria drugs are becoming increasingly ineffective due to infection with Plasmodium parasites (Carlton et al., 2001). In particular, the Kupffer cells, which represent about 90% of all host macrophages, are able to eliminate parasite-derived hemoglobin and even Plasmodium-infected erythrocytes. During this process, Kupffer cells become increasingly activated, which manifests itself as a production of NO (nitrogen oxide) and diverse ROS (radical oxygen species). The NO and ROS in turn induce an oxidative stress which results in an increase in apoptosis of hepatocytes (Krücken et al., 2005a and Guha et al., 2006). On the other hand, the spleen is believed to participate in cleaning parasites from the circulation and to provide a strong hematopoietic response during acute infections (Dkhil, 2009).

Apoptosis is linked intimately with both physiology as well as pathology in a variety of cellular systems (Gourley et al., 2002; Alkahtani et al., 2009). The dysregulation of liver apoptosis during malaria is a critical event in liver pathology. Apoptosis does not only play an essential role in development and tissue homeostasis but is also involved in a wide range of pathological conditions (Oh et al., 2004). Reactive oxygen species (ROS) have been involved in the apoptosis induced by different stimuli as well as the pathologic cell death that occurs in many diseases (Oh et al., 2004).

Apoptosis may occur via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. The death receptor pathway comprises Fas (CD95/Apo-1) and TRAIL (Apo-2) and this pathway is activated when ligands specific for either Fas or TRAIL bind to their respective receptors, resulting in activation of Caspase-8, which finally activates Caspase-3 (Lyke et al., 2004). On the other hand mitochondrial pathway of apoptosis is initiated by the down-regulation of anti-apoptotic proteins (such as, Bcl-2,Bcl-xl) and/or up-regulation of pro-apoptotic proteins (such as, Bax, Bad, BID), resulting in the opening of mitochondrial permeability transition pores and the release of apoptosis inducing proteins (cytochrome c, apoptosis inducing factor, etc.) from mitochondria (Akannorti et al., 1996; Barton,1996; Gourley et al., 2002; Alkahtani et al., 2009).

The present study was undertaken to investigate whether the apoptotic responses to Plasmodium chabaudi malaria in spleen and liver via mRNA expression of three genes involved in apoptosis (Bax, Bcl-2 and caspase-3) are similar or not and, to detect if these genes could be a good marker for apoptosis during infection with Plasmodium chabaudi in the liver and spleen cells of female C57BL/6 mice.
II. MATERIAL AND METHODS

A. Animals and infection

Female C57BL/6 mice 10-14 weeks old were obtained from the central animal facilities of Heinrich Heine University, Düsseldorf, Germany, and housed in plastic cages. Mice were bred and maintained under specified pathogen free conditions. Mice were fed with a standard diet (Wohrlin, Bad Salzuflen, Germany) and water ad libitum. The experiments were approved by the State authorities and followed German law on animal protection.

B. Blood stage malaria

We used a non-clonal line of *P. chabaudi* (Wunderlich et al., 1988) exhibiting a very similar, but not identical restriction length polymorphism pattern to *P. chabaudi chabaudi* (Kruecken et al., 2005b). Erythrocytic stages of *P. chabaudi* were passaged weekly in NMRI mice. From these mice, blood was taken and 10⁶ *P. chabaudi*-infected erythrocytes were injected i.p. in the mice. Parasitemia was evaluated in Giemsa-stained blood smears. The total number of erythrocytes was determined in a Neubauer chamber. A total of five mice were sacrificed each day (0, 1, 4 and 8, respectively) by cervical dislocation. Livers were removed and cut into smaller pieces and kept at -80°C.

C. RNA-Isolation

Approximately 250 mg frozen liver and spleen were homogenized with an ultraturrax in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany) for one minute. After mixing with 1 ml chloroform for 15 s, the suspension was incubated for 15 min at room temperature and centrifuged at 3,000 g for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol and air-dried and dissolved in 200 µl RNase-free water. RNA concentrations were determined at 260 nm, and the purity of RNA was checked in 1% agarose gel.

D. Quantitative real-time PCR

All RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 h and then converted into cDNA following the manufacturer’s protocol using the Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Mastermix from Qiagen (Hilden, Germany). We investigated the genes encoding the mRNA for the following proteins: 18S, Bax, Bcl-2, Caspase-3. All primers used for qRT-PCR were commercially obtained from Qiagen. PCR reactions were conducted as follows: 2 min at 50°C to activate uracil-N-glycosylase, 95°C for 10 min to deactivate UNG, 40 cycles at 94°C for 15 s, at 60°C for 35 s and at 72°C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA was normalized to 18S rRNA. The threshold Ct value is the cycle number selected from the logarithmic phase of the PCR curve in which an increase in fluorescence can be detected above background. The ΔCt is determined by subtracting the Ct of 18S rRNA from the Ct of the target (ΔCt = Ct-target – Ct-18S rRNA). The relative mRNA levels of non-infected mice are described as a ratio of target mRNA copy to 18S rRNA copy = 2^-ΔCt. The fold induction of mRNA expression on days (0, 1, 4 and 8 p.i.) was determined using the 2^-ΔCt-method (ΔΔCt = ΔCt day 0 p.i. - ΔCt day 8 p.i.).

E. Statistical analysis

The two-tailed Student’s t-test and Fisher’s exact test were used for statistical analysis.

III. RESULTS

A. Characteristics of *P. chabaudi* infection

The parasitemia of mice with 10⁶ *P. chabaudi*-infected erythrocytes became evident on day 4 and rocketed to reach its peak (48%) on day 8 and then reduced declined to about 0.4% on day 12 (Fig. 1).

B. Apoptotic genes expression

Quantitative real-time PCR was used to detect changes in mRNA levels of different apoptotic genes. The level of mRNA expression for Bax as a pro-apoptotic gene was significantly (P<0.05) increased on days (1 and 8) after infection in the liver and on day 8 in the spleen compared with day 0 (Fig. 2). The level of Bcl-2 as an anti-apoptotic gene was significantly (P<0.05) increased at all three days after infection in the liver cells and in contrast, significantly (P<0.05) decreased at all three days after infection in the spleen cells compared with day 0 (Fig. 3). The level of mRNA expression for caspase-3 as executioner gene was significantly (P<0.05) increased at days (1 and 8) after infection in the liver and at day 8 in the spleen compared with day 0 (Fig. 4).

IV. DISCUSSION

The present data shows that infection with *P. chabaudi* induced different apoptotic responses at different time points during malaria infection. These data connect with previous investigations in our laboratory focused on the apoptotic effect (Alkahtani, 2010). Apoptosis is a complex cellular process triggered as a response to a wide variety of stimuli (Bergmann-Leitner et al., 2009). Apoptotic processes are regulated by many of the same biochemical intermediates, including death factors and reactive oxygen species (Sanchez-Alonso et al., 2004). Presented data agreed with previous results that malaria infection significantly induces liver apoptosis mediated by oxidative stress mechanisms (Guha et al., 2007). The pathogen of malaria, Plasmodium, enters erythrocytes and thus escapes recognition by the immune system and then induces oxidative stress to the host erythrocytes and triggers erythropoiesis (Alkahtani, 2009; Föller et al., 2009).

Cell apoptosis is regulated via two major pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Both pathways converge at the level of active effector caspases. Caspase-independent mechanisms
may also contribute to cell death. Effector caspases cleave various cellular target proteins, finally leading to apoptosis (Alkahtani et al., 2009). Apoptosis could result from a combination of both pathways: the intrinsic apoptosis pathway by generation of oxidative stress and the extrinsic apoptosis pathway by activation of Kupffer cells which can secrete TNF (Alkahtani, 2009). In the spleen, splenomegaly and changes in splenic cell numbers were the most common changes linked to the changes in parasitemia of *P. chabaudi* and this may reflect lymphocyte migration to the peripheral blood. On the other hand, rapid change in cellular necrosis proving the role of the spleen in protective immunity against blood-borne infection due to *P. chabaudi* malaria (Dkhil, 2009).

The intrinsic apoptotic pathway is initiated when Bcl-2 leads to the release of cytochrome c and other pro-apoptotic cofactors from the inter-membrane space of mitochondria which is considered an essential cofactor required for the activation of caspases and is regulated by the Bcl-2 family of proteins upstream of caspase activation (Baliga and Kumar, 2002). Once in the cytosol, cytochrome c interacts with the apoptotic protease activating factor-1 (Apaf-1) and the procaspase-9 forming the apoptosome complex (Yuan and Yankner, 2000). The result is the cleavage and activation of procaspase-9 and other procaspases that are responsible for the executive stages of apoptotic cell death. The Bcl-2 family of proteins localize at membrane compartments during apoptosis and can either promote or inhibit apoptosis. Substantial evidence suggests that the primary role of Bcl-2 family proteins is to regulate the release of cytochrome c from mitochondria. Briefly, Bcl-2 is an apoptosis suppressing factor that heterodimerizes with Bax and neutralizes the effects of the latter. When Bcl-2 is present in excess, cells are protected against apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed death. Therefore, it is the ratio of Bax to Bcl-2 which determines the fate of a cell (Sanchez-Alonso et al., 2004). Infections with intracellular pathogens may provide an appropriate stress-related signal which would normally trigger the intrinsic pathway of apoptosis and, thereby, disturb or even prevent the further development of the affected host cells as a response to intracellular pathogens (Graumann et al., 2009). Recently, it has been reported that the p18/Bax fragment cleaved from full-length Bax (21-kDa) is as efficient as full-length Bax in promoting Cyt c release (Oh et al., 2004).

Gene expression analysis using RT-PCR indicates the significant up-regulation of Bax expression in liver of malaria infected mice suggesting the involvement of mitochondrial pathway of apoptosis (Guha et al., 2006b). However, besides the fact that different apoptotic markers cannot always be detected at the same time, very little is known of the exact timing of the successive steps in the apoptotic pathways and for this reason it is suitable to confirm this at the protein level. This is in agreement with the opinion of more and more researchers arguing that the detection of mRNA differences can only indicate a biological significance if protein expression and activity can confirm the results and this is certainly true for caspases because they are secreted as inactive procaspases, which are only active after further modification (Huerta et al., 2007; Vandeaele et al., 2008).

As observed in this study overexpression of apoptotic genes leads to apoptosis. Up-regulation of Bax and/or down-regulation of Bcl-2 mRNA have been observed in several experimental models indicating that the expression of Bax and Bcl-2 genes may be regulated by p53 (Mishra et al., 2006). Under our experimental conditions, malaria infection with *P. chabaudi* induces apoptosis in the liver and spleen cells via Bax, Bcl-2 and Caspase-3 tests in different ways. So this study suggests more investigations are needed to confirm apoptosis by using other tests such as, studying morphological changes, apoptosis mechanism in both liver and spleen cells, ultrastructure of cells that may undergo apoptosis and measure the proteins level as well.

REFERENCES


Figure 1. Parasitemia (48%) of female C57BL/6 mice (n= 20) infected with 106 P. chabaudi-infected erythrocytes.

Figure 2. Quantitative real time-PCR of Bax gene expression in the liver and spleen of Plasmodium chabaudi infected mice. The expression of Bax was measured at different times. The data present are the mean ±SE (n=5). *: significant value at (P<0.05).
Figure 3. Quantitative real-time PCR of Bcl-2 gene expression in the liver and spleen of *Plasmodium chabaudi* infected mice. The expression of Bcl-2 was measured at different times. The data present are the mean ±SE (n=5). *: significant value at (P<0.05).

Figure 4. Quantitative real-time PCR of Caspase-3 gene expression in the liver and spleen of *Plasmodium chabaudi* infected mice. The expression of caspase-3 was measured at different times. The data present are the mean ±SE (n=5). *: significant value at (P<0.05).