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Research article

Cytotoxic T Lymphocyte Granzyme-b mediates neuronal cell death during *Plasmodium berghei ANKA* induced experimental cerebral malaria

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ABSTRACT

Cerebral malaria is a complex, acute, neurological disease characterised by a sudden onset of cerebral symptoms. This disease is manifested as initial arousable stage that is followed by an unarousable coma and eventually death. Parasite burden and CD8+ T cell count in the brain determines the disease outcome. Cytotoxic CD8+ T cell-derived Granzyme-b is required for the development of experimental cerebral malaria (ECM), but the mechanism of pathogenesis is not known. Here, we show that CD8+ T cells infiltrate in to the brain during ECM releasing Granzyme-b that is cytotoxic to neuronal cells. Granzyme-b kills neuronal cells through direct cytotoxicity and also by activating neuronal caspase-3 and calpain1 via cytoskeletal breakdown. Our results showed the increased expression of cell adhesion molecules and chemokine receptors in the brain and their associated infiltration of T cells during ECM.

1. Introduction

Infection of *Plasmodium falciparum* and *Plasmodium ovale* results in severe pathological syndrome, cerebral malaria, in humans. Though the disease pathogenesis is not clearly understood, research data indicates the critical role of hypoxic conditions, immune system, infiltration of effector T cells [8,34] and toxins that are released by the parasites [4,25,37]. The role of activated T cells entering CNS and causing damage has drawn much attention [3]. Several studies report the role of T cells in *Plasmodium berghei* ANKA(*PbA*), parasite specific to C57BL/6 mice, induced pathology in the blood [32,46] and liver stages [11] of malaria. CD8+ T cells are protective during liver stages but are pathogenic in the brain during the blood stage [36,43], whereas CD4+ T cells are protective both during the liver and blood stages [20,54],as well as in the brain [21]. The function of CD8+ T cells depends on their location or the tissue in which they are present. Evidence is being accumulated on the infiltration of peripheral immune cells into the brain [12,34]. During experimental cerebral malaria (ECM) pathology, a series of events occur leading to unarousable coma and death. Initiation of these events occur due to the expression of increased chemokine receptors and adhesion molecules in the brain facilitating T cell infiltration [49] and occlusion of parasitized red blood cells (pRBCs) in blood vessels. These events can lead to insufficient supply of blood, oxidative stress, oedema [38,45,51] vascular permeability [3,24] and blood brain disruption [33,39]. The disruption of BBB causes infiltration of activated immune cells into the brain inducing neuronal cell death directly or indirectly through the activation of resident immune cells [40,41].

Cell death during ECM is associated with elevated levels of necrotic (cathepsin b and calpain1) and apoptotic (active caspase-3) cell death proteases, indicating apo-necrotic mode of death. We have earlier shown that these proteases induce neuronal cell death through cytoskeletal breakdown [14]. In addition, inhibition of caspase-3 and over expression of Bcl-2, an anti-apoptotic protein, prevented ECM induced cell death [22]. This might be stress induced rather than pathogen-induced signalling cascade as some reports indicate no role for TLRs in the pathology [52]. Moreover CD8+ T cells in the brain of human post-mortem cerebral malaria samples as reported in immunohistological studies of CD8+ T cells in the tissue from Malawian children who died of CM, Mackenzie et al., 1999 and intravascular leukocytes in the brain of Malawian children with fatal malaria, Proc. 48th meeting of the Am. Soc. Trop. Med. Hyg. Abstract No. 781 [16,42,44,53] as well as in mouse models [6,7], and the pathogenicity of cytotoxic T lymphocytes (CTLs) in other neurodegenerative diseases like cerebral ischemia [10] signifying the connecting link between T cells and activation of cell death proteases. CTLs kill the resident cells by direct cytotoxicity through Granzyme-b/perforin. Owing to the presence of T cells in the post mortem samples and pathogenic T cells in ECM, we aimed to study the role of T cells in neuronal cell death and their relation to caspase-3 and calpain1 mediated neuronal cell death. Exploring the role of each component of complex pathology helps to understand the mechanism of cell death that may help in pathological intervention.

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2. Materials and methods

2.1. Antibodies

For western blotting and co-immunoprecipitation, anti-Neurofilament light (NF (L), Sigma, N5139), anti-Caspase-3 (Cell Signalling and Technology, 9662), anti-calpain1 (Chemicon International, Termicula, USA, MAB3164), anti-Granzyme-b (Calbiochem, AM52) and anti-B-actin (Epitomics, 1879-1) were used. Immunohistochemistry was performed by biotinylated anti-mouse (Cat# BA 9200) and anti-rabbit (Cat# BA 1000) secondary antibodies (Vector laboratories), FACS and immunofluorescence using FITC conjugated anti-CD4 and PE conjugated anti-CD8 antibodies (eBiosciences, 11-003-81 and 12-0083-81, respectively), anti-NSE (Chemicon International, Termicula, USA, AB951), and Cy3 conjugated anti-mouse secondary antibody (kind gift from Dr. Shiva Kumar, UoH, India).

2.2. Ethics statement

C57BL/6J mice were purchased from NIN Hyderabad and were reared under strict, hygienic conditions. Experiments were conducted as per the guidelines of the institutional animal ethical committee (IAEC, University of Hyderabad), and surgical procedures were approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) registration no. 151/1999/CPCSEA dated July 1999, guidelines framed by the Indian Council of Medical Research, India. Our animal protocol approval nos. UH/IAEC/PPB-2-E1/2009 from 27/05/2009-10 and LS/IAEC/PPB/2010/12 from 18/03/10-17/03/11 is approved by CPCSEA. *Plasmodium berghei* ANKA (*PbA*) parasites were purchased from the parasite bank, National Institute of Malaria Research (NIMR), New Delhi.

2.3. Induction of cerebral malaria

C57BL/6J mice received an intraperitoneal injection of 10^6 *PbA* parasites. Control mice were injected with the same amount of PBS. Infection was monitored by staining caudal blood smears with Giemsa and Jaswant Singh and Bhattacharyya (JSB)-I and JSB-II, and the cerebral symptoms were monitored through behavioural changes. Percentage of cerebral symptoms was approximately 85–90% with parasitemia < 20%, and the infected mice died between 7 and 15 days post-infection. Mice that showed convulsions and coma between 6 and 9 days post-infection were considered as cerebral malaria mice. Mice that died at later stages with a parasitemia > 20% are due to severe anaemia. Depending on the behavioural symptoms, mice were sacrificed 6–9 days post-infection and the samples collected were used for further studies.

Mice were sacrificed 6–9 days post-infection by transcardial perfusion with 4% paraformaldehyde in PBS and brain were dissected out. Brains obtained from the sacrificed mice were divided into two groups. One group consisted of samples that were stored at −80 °C and were later grinded in liquid nitrogen to isolate RNA and DNA using TRIZOL method. The second group of samples were used for histochemical studies. They were fixed in perfusion solution for 12 h and then processed in a graded alcohol series, followed by chloroform. The processed samples were embedded in wax, and 3-μm sections were cut using a microtome. The tissue sections were mounted on poly-L-Lysine coated slides and were used for immunohistochemical and immunofluorescence studies.

2.4. Western blots

Control and infected ECM mice were sacrificed with an overdose of pentobarbital. Brains were dissected (n = 5) and stored at −80 °C. These samples were homogenised in modified radio immunoprecipitation assay buffer containing 1 mM PMSF, a pan protease inhibitor, and the phosphatase inhibitors β-glycerophosphate (10 mM), NaF (10 mM) and Na_3VO_4 (0.3 mM). Lysates were sonicated, centrifuged and 50 μg of protein estimated by Lowry was then separated by using 10%, and 12% for caspase-3 SDS-PAGE gel, transferred onto a nitro-cellulose membrane and immunoblotted with 1:1000 dilutions of respective anti-caspase-3, anti-calpain1, anti-NF (L) and anti-Gra-b primary antibodies. Anti-mouse and anti-rabbit secondary antibodies either with ALP or HRP tag were used at a dilution of 1:1000 and developed using ECIL method or BCIP-NBT substrate.

2.5. H&E histological staining

Tissue sections were stained with haematoxylin and eosin following a standard protocol, briefly dewaxed in graded alcohol series followed by xylene clearing, stained with Eosin and Haematoxylin, air dried, mounted in DPX and observed under light microscope (Nikon-ALPHAPHOT-2).

2.6. Real-time PCR and PCR

Total RNA and DNA was isolated from the immediately liquid nitrogen snap frozen samples after brains were taken out from −80 °C. Total RNA was obtained by the TRIZOL reagent method (Invitrogen), and the cDNA was synthesised using a first-strand cDNA synthesis kit (Blueprint-TAKARA). Specific forward and reverse primers for the cell adhesion molecules ICAM-1 and VCAM-1 and for the chemokine receptor CXCR4 were selected by obtaining their sequences from the NCBI nucleotide database using online OligoCalC software. Forward and reverse primers for CXCR4 (5′GGAACCGATCAGTGTGAGTAT3′ and 5′CACCAATCCATTGCCGACTAT3′) and for ICAM-1 and VCAM-1 (5′GAGATCACATTCACGGTGCTG3′ and 5′AGCTGGAAGATCGAAAGT CCG3′, and 5′GACATCTACTCTTTCCCCAAGG3′, 5′TGTTCATGAGCTGG TCACCCT3′, respectively) were produced. The presence of T cells in the brains of control and infected mice were detected by normal PCR using the specific forward 5′GGGGGTTTGTTCTCTATCTCTTCC3′ and reverse 5′TATCCACAGGAGATGATGGTGCAC3′ primers for TCR-α segment of the TCR gene. Forward 5′ATCTTCTTGTGCAGTGCCAGC3′ and reverse 5′TTGAGGTCAATGAAGGGGTCG3′ GAPDH primers were used as a control gene for real-time and normal PCR.

2.7. Immunohistochemistry

Immunohistochemical and immunofluorescence analysis was done by processing 3-μm-thick paraffin sections of ECM and control mice in a series of graded alcohol, dehydrated in 100% alcohol, cleared in xylene, antigen retrieved in citrate buffer (pH 6.0) by heating in micro oven for 11 min and permeabilised in 0.1% Triton-X 100 for 15 min. Endogenous peroxidase was inhibited by incubating sections in 3% H_2O_2. Sections were washed and blocked using 10% normal goat serum for 60 min. The sections were incubated with anti-CD4 and anti-CD8 (1:1000) antibodies overnight at 4 °C, followed by washes in PBS and incubation with goat anti-mouse and rabbit biotinylated secondary antibodies (1:200) for 90 min. Sections were developed using a Vectastain Elite ABC kit (Vector Laboratories).

2.8. Triple immunofluorescence

Triple immunofluorescence was carried out on sections that were processed in a series of alcohols and permeabilised with 0.1% Triton-X 100. Sections were incubated with mouse anti-Granzyme-b primary antibody overnight at 4 °C. Sections were washed in PBS, blocked with 10% goat serum and then incubated with anti-CD4 and anti-CD8 (1:200) primary antibody cocktail conjugated with FITC and PE fluorochromes respectively for 90 min. Following primary incubation, sections were washed in PBS, stained and mounted in 90% glycerol. Analysis was performed using a Leica laser scanning confocal microscope (Leica TCS SP2)