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Role of expression site switching in the development of resistance to human Trypanosome Lytic Factor-1 in *Trypanosoma brucei brucei*

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ABSTRACT

Human high-density lipoproteins (HDLs) play an important role in human innate immunity to infection by African trypanosomes with a minor subclass, Trypanosome Lytic Factor-1 (TLF-1), displaying highly selective cytotoxicity to the veterinary pathogen *Trypanosoma brucei brucei* but not against the human sleeping sickness pathogens *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense*. *T. b. rhodesiense* has evolved the serum resistance associated protein (SRA) that binds and confers resistance to TLF-1 while *T. b. gambiense* lacks the gene for SRA indicating that these parasites have diverse mechanisms of resistance to TLF-1. Recently, we have shown that *T. b. gambiense* (group 1) resistance to TLF-1 correlated with the loss of the haptoglobin/hemoglobin receptor (HpHbR) expression, the protein responsible for high affinity binding and uptake of TLF-1. In the course of these studies we also examined TLF-1 resistant *T. b. brucei* cell lines, generated by long-term *in vitro* selection. We found that changes in TLF-1 susceptibility in *T. b. brucei* correlated with changes in variant surface glycoprotein (VSG) expression in addition to reduced TLF-1 binding and uptake. To determine whether the expressed VSG or expression site associated genes (ESAGs) contribute to TLF-1 resistance we prepared a TLF-1 resistant *T. b. brucei* with a selectable marker in a silent bloodstream expression site (BES). Drug treatment allowed rapid selection of trypanosomes that activated the tagged BES. These studies show that TLF-1 resistance in *T. b. brucei* is largely independent of the expressed VSG or ESAGs further supporting the central role of HpHbR expression in TLF-1 susceptibility in these cells.

1. Introduction

Infection and pathogenesis of mammals by African trypanosomes is influenced by innate immune molecules present in the blood of primates. Initially described as a minor subclass of human high density lipoprotein (HDL), Trypanosome Lytic Factor-1 (TLF-1) [1,2] contains apolipoprotein A-1 (apoA-1) and two primate specific proteins apolipoprotein L-1 (ApoL-1) and haptoglobin related protein (Hpr) [3–7]. In addition, high specific activity killing by TLF-1 also requires Hpr bound hemoglobin (Hb)[8]. This HDL subclass is highly toxic to the veterinary pathogen *Trypanosoma brucei brucei*. However, TLF-1 has no activity against the human sleeping sickness parasites *T. b. gambiense* or *T. b. rhodesiense*. The cellular pathway for TLF-1 killing of *T. b. brucei* is now well established. TLF-1 binds to a high affinity haptoglobin hemoglobin receptor (HpHbR) that recognizes Hpr/Hb within the TLF-1 particle and allows endocytosis and lysosomal trafficking [9–12]. *T. b. brucei* can be spared from TLF-1 killing by competition for receptor binding, inhibition of trafficking through the endocytic pathway or by treatment with compounds that elevate lysosomal pH [9,11].

A second trypanolytic serum complex has been identified, TLF-2, which contains ApoA-1 and Hpr as well as IgM [13]. ApoL-1 was not initially detected in TLF-2 by N-terminal sequencing but recent studies support the presence of this apolipoprotein in TLF-2 [14]. In addition to the presence of IgM in TLF-2, another distinguishing feature of the two serum complexes is that TLF-2 is largely devoid of lipid. Aside from the shared apolipoproteins the relationship of these two human defense complexes is largely unknown. However, it is likely that both TLF-1 and TLF-2 play significant roles in the innate immunity that humans have against *T. b. brucei* infection [13].

Within the circulation of primates, TLF-1 and TLF-2 have acted as selective agents leading to the emergence of resistant trypanosomes that cause human disease. This selection resulted in

Abbreviations: BES, bloodstream expression site; ESAG, expression site associated gene; VSG, variant surface glycoprotein; TLF, Trypanosome Lytic Factor; Hygro, hygromycin; MITat, Molteno institute trypanosome antigen type; HpHbR, haptoglobin hemoglobin receptor; ApoA-1, apolipoprotein A-1; ApoL-1, apolipoprotein L-1; Hpr, haptoglobin related protein; SRA, serum resistance associated.

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diverse mechanisms of resistance to TLF-1. Beginning with the work of DeGreef and Hamers, it was shown that human serum resistant *T. b. rhodesiense* expressed a novel protein called the serum resistance associated protein (SRA) [15]. The predicted structure of SRA showed that it is a member of the variant surface glycoprotein (VSG) family containing an internal deletion and a unique apoL-1 binding domain [4,16]. Expression and co-localization of SRA within endocytic compartments of *T. b. rhodesiense* have been proposed to allow formation of a TLF-1/SRA binary complex leading to inhibition of trypanosome lysis [4,10,12,17]. It is likely that a gain of function mutation, to an existing VSG gene, gave rise to SRA in an ancestral *T. b. brucei* and was sufficient to confer human infectivity.

Both group 1 and 2 *T. b. gambiense* lack the *SRA* gene and therefore evolved SRA independent mechanism(s) to avoid TLF-1 killing. The mechanism of TLF-1 resistance in *T. b. gambiense* (group 1) is the loss of TLF-1 binding and uptake [18]. We found low-level expression of the HpHbR in seven different field isolates of *T. b. gambiense* relative to the levels found in *T. b. brucei* and *T. b. rhodesiense*. Furthermore, expression of the *T. b. gambiense HpHbR* in receptor deficient *T. b. brucei*, failed to restore TLF-1 binding suggesting that changes to the coding sequence of the *T. b. gambiense* HpHbR may also contribute to reduced TLF-1 binding and killing of *T. b. gambiense* (group 1). The mechanism of TLF-1 resistance in *T. b. gambiense* (group 2) is unknown but does not correlate with loss of receptor-mediated uptake of TLF-1 suggesting a second, SRA-independent mechanism [19].

To study the evolution of TLF-1 resistance in African trypanosomes we reasoned that *in vitro* selection of *T. b. brucei*, by continuous cultivation in the presence of low concentrations of TLF-1, would lead to resistance and might provide insight into the events that led to TLF-1 resistance in the human sleeping sickness parasites. *T. b. brucei* 427-221 was treated with progressively higher concentrations of TLF-1 and over a nine month period parasites with differing levels of resistance to TLF-1 were identified, cloned and characterized [20]. Two striking traits were seen in the highly resistant *T. b. brucei* 427-800 cells. The first was a dramatic reduction in TLF-1 binding and uptake; the second was a periodic change in the expressed VSG in the increasing resistant cells [20]. Transcriptome analysis was consistent with activation of different BESs during the long-term selection. In subsequent studies, we found that HpHbR expression was reduced in TLF-1 resistant *T. b. brucei* 427-800^{R}, and other TLF-1 resistant lines, consistent with the loss of TLF-1 uptake [18]. However, the apparent correlation with VSG switching could not be excluded as contributing to the TLF-1 resistance phenotype of these cell lines. Here we present studies that address the role of the expressed VSG and ESAGs in TLF-1 resistance. In order to select for rapid BES switching we introduced the hygromycin resistance gene into an inactive expression site in TLF-1 resistant *T. b. brucei* 427-800^{R}. Treatment of *T. b. brucei* 427-800^{R} with hygromycin selected for cells that had switched to the newly activated BES. Using this tagged cell line we are able to show that susceptibility to TLF-1 is largely uninfluenced by the newly expressed VSG or ESAGs. These findings support our proposal that acquired resistance, in TLF-1 resistant *T. b. brucei*, was a consequence of the loss of the HpHbR expression.

2. Materials and methods

2.1. In vitro growth, generation and transfection of T. b. brucei cell lines

Bloodstream form *T. b. brucei* Lister 427 (MiTat 1.2) were used in these studies. TLF-1 resistant *T. b. brucei* 427-800^{R} cells were described previously [18]. Prolonged culturing in the absence of TLF-1 resulted in subpopulations of TLF-1 sensitive and resistant cells. Prior to subsequent experiments, cells were cloned by limiting dilution. Transfections were performed using the Amaxa electroporation system (Human T Cell Nucleofactor Kit, program X-001).

2.2. SDS-PAGE and northern blot analysis

Total cell protein from 2×10^6 trypanosomes was run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue. For northern blot analysis, radiolabeled probes containing entire open reading frames (ORF) were generated (Prime-It Random Primer Labeling Kit, Stratagene) and hybridized in a 40% (v/v) formamide hybridization mix with the addition of 10% (w/v) dextran sulfate. Final washes were performed with 0.1× SSC, 0.1% SDS at 65 °C for 20 min (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.4).

2.3. TLF-1 purification, labeling, lysis assays and flow cytometry

TLF-1 purification, labeling and lysis assays were performed as described previously [2,8]. Flow cytometry analysis was performed on samples with 3 μg/ml AlexaFluor-488 conjugated TLF-1. Cells were incubated for 1 h at 37 °C, washed 3 times with ice cold 1× PSG (1× PSG is 50 mM NaP_i, 45 mM NaCl, 55 mM glucose, pH 8.0) and analyzed with a Cyan Cytometer (DAKO).

2.4. TLF binding assays

Cells were harvested and resuspended at 3×10^7 cells/ml in pre-chilled HMI9 medium. Prior to the addition of Alexa488 conjugated TLF, cells were pre-incubated for 10 min at 3 °C. Cells were then incubated for 15 min at 3 °C in the presence of 3 μg/ml Alexa488 conjugated TLF and 10 μg/ml hemoglobin. After 3 washes with icecold PBS–glucose (1%), cells were fixed in 1% paraformaldehyde for 15 min on ice and analyzed by immunofluorescence microscopy. Image acquisition was carried out using a Zeiss Axio Observer microscope equipped with an AxioCam HSm Camera and analyzed with AxioVision v4.6 software (http://www.zeiss.com). Images were captured with the same exposure and were contrasted to the same extent.

2.5. RT-PCR of the expressed VSGs, ESAGs and HpHbR

Total RNA was isolated with Tripure Isolation Reagent (Roche). cDNA was generated in a Reverse Transcription (RT) reaction (Promega). Control reactions were performed with enolase, as well as reactions without added RT. For cloning and sequencing, PCR products were generated with Platinum High Fidelity Taq Polymerase (Invitrogen), gel purified and cloned into the PCR 2.1 vector (Invitrogen). Both strands were sequenced with M13 forward and reverse primers. VSG and ESAG sequences were compared to the *T. b. brucei* 427 data set (GeneDB).

3. Results

3.1. VSG switching is not required for gain or loss of TLF-1 susceptibility

The *T. b. brucei* Lister 427 (MITat1.2) cell line used in these studies is well characterized with respect to its susceptibility to TLF-1 and a complete description of its BESs, VSGs, and ESAGs is available [20,21]. Treatment of TLF-1 sensitive *T. b. brucei* 427-221, expressing the VSG221 (427-221^{S}), resulted in the gradual outgrowth of a population of highly resistant parasites that we called *T. b. brucei* 427-800^{R} [20] (Fig. 1A). A feature of *T. b. brucei* 427-800^{R} was the loss of TLF-1 binding and uptake. In addition, SDS-PAGE