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Differential apoptosis in BeWo cells after infection with highly (RH) or moderately (ME49) virulent strains of *Toxoplasma gondii* is related to the cytokine profile secreted, the death receptor Fas expression and phosphorylated ERK1/2 expression

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ABSTRACT

Introduction: Alterations of apoptosis are commonly associated with pregnancy complications and abortion. Modulation of apoptosis is a relevant feature of *Toxoplasma gondii* infection and it is related to parasite strain types. The aim of the present study was to evaluate the possible factors that are involved in the differential apoptosis of BeWo cells infected with distinct *T. gondii* strain types.
Methods: Human trophoblastic cells (BeWo cell line) were infected with RH or ME49 strains, the cytokine production was measured and the phosphorylation of anti-apoptotic ERK1/2 protein was analyzed. Also, cells were treated with different cytokines, infected with RH or ME49 strain, and analyzed for apoptosis index and Fas/CD95 death receptor expression.
Results: ME49-infected BeWo cells exhibited a predominantly pro-inflammatory cytokine profile, whereas cells infected with RH strain had a higher production of anti-inflammatory cytokines. Also, the incidence of apoptosis was higher in ME49-infected cells, which have been treated with pro-inflammatory cytokines compared to cells infected with RH and treated with anti-inflammatory cytokines. Moreover, Fas/CD95 expression was higher in cells infected with either ME49 or RH strain and treated with pro-inflammatory cytokines compared to anti-inflammatory cytokine treatment. The phosphorylation of ERK1/2 protein increased after 24 h of infection only with the RH strain.
Conclusion: These results suggest that opposing mechanisms of interference in apoptosis of BeWo cells after infection with RH or ME49 strains of *T. gondii* can be associated with the differential cytokine profile secreted, the Fas/CD95 expression and the phosphorylated ERK1/2 expression.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that can invade and replicate in almost all nucleated cells of warm-blooded animals [1]. Approximately one-third of the human population from all over the world is estimated to be chronically infected by *T. gondii* and, for that reason, it is considered as one of the most successful parasites that is able to infect humans [2,3]. The population structure of *Toxoplasma* is dominated by three clonal lineages, designated as strains types I, II and III, according to the virulence to mice and humans, which are mainly found in Europe and North America. While strains of the type I (RH) are virulent and

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uniformly lethal to mice, causing severe clinical manifestations of toxoplasmosis, strains of the types II (ME49) and III (VEG) are of moderate and low virulence to murine hosts, respectively, which are able to control acute phase of the disease and are able to establish chronic infections [4]. In South America, however, *T. gondii* strains show a high diversity of genetic structure and are designated as recombinant strains [5], with virulence patterns yet to be determined. Although the majority of infected healthy individuals have no symptoms, during pregnancy *T. gondii* can be transmitted to the fetus transplacentally, causing severe disease or even fetal death [5,6].

Apoptosis has been recognized as an important defense mechanism against viral, bacterial and parasitic infections [7,8]. Previous studies demonstrated that *T. gondii*-infected cells were resistant to apoptosis induced by various proapoptotic stimuli [9]. This phenomenon is considered as a crucial adaptation of the parasite that allows sustained intracellular survival and long-term persistence within the host cells [10]. Also, apoptosis modulation during *T. gondii* infection is associated with the virulence characteristics of the parasite [11]. Accordingly, in our previous study we demonstrated that the incidence of apoptosis in human trophoblastic cells (BeWo cell line) was differentially modulated by highly (RH) or moderately virulent (ME49) strains of *T. gondii* since RH-infected BeWo cells had lower incidence of apoptosis compared to ME49 strain or uninfected cells, and that type I strains can inhibit host cell apoptosis [12].

Considering that the apoptosis process is critical for the development and homeostasis of placental tissues and the occurrence of apoptosis in trophoblastic cells is dependent on the *T. gondii* strain type, in the present study we investigated the profile of cytokines secreted by trophoblastic BeWo cells infected with RH or ME49 strains of *T. gondii*. Also, we analyzed the incidence of apoptosis in BeWo cells after infection with these *T. gondii* strains and treatment with different cytokines as well as the Fas death receptor expression and the ERK1/2 phosphorylated protein expression.

2. Materials and methods

2.1. Cell culture

BeWo cell line (human choriocarcinoma cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (GIBCO, Paisley, UK), supplemented with 25 mM HEPES, 2 mM L-glutamine, 10 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich, St Louis, MO, USA) and 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil) in a humidified incubator at 37 °C and 5% CO_2.

2.2. Parasites

Tachyzoites of the highly virulent RH strain of *T. gondii* were originally maintained in Swiss mice by intraperitoneal passages at 48 h intervals [13]. Parasites were obtained from mouse peritoneal exudates, washed twice (720× *g*, 10 min) in sterile phosphate-buffered saline (PBS) pH 7.2 and cultured in BeWo cells in order to obtain culture-derived parasites. Tachyzoites were harvested by scraping off the cell monolayer after 2–3 days of infection, passed through a 26-gauge needle to lyse any remaining intact host cells, washed (720× *g*, 5 min) in RPMI medium and the resulting pellet was resuspended in complete medium. Parasites were stained with 0.4% Trypan blue and counted in a hemocytometric chamber for further infection experiments.

Parasites of the moderately virulent ME49 strain of *T. gondii* were initially obtained by the pepsin digestion of brain cysts from chronically infected *Calomys callosus*, as previously described [14,12]. Parasites were purified by low centrifugation to remove host cell debris and the supernatant-contained tachyzoites were transferred and maintained in BeWo cell culture in order to obtain culture-derived parasites as described above for further infection experiments.

2.3. Cytokine treatment and T. gondii infection

BeWo cells were cultured in 96-well plates (1×10^5 cells/well/200 μl) for 24 h at 37 °C and 5% CO_2. Cells were then infected with *T. gondii*, RH or ME49 strain, at 5:1 (parasite:cell) ratio. After 2 h and 24 h of incubation, supernatants were collected and stored at −80 °C for cytokine assays. Cells incubated with medium alone served as controls.

In a second set of experiments, BeWo cells were cultured on 13-mm round glass coverslips into 24-well plates (1×10^5 cells/well/200 μl) for 24 h at 37 °C and 5% CO_2. Different concentrations of cytokines (15, 20, 25 and 30 ng/ml) were initially tested for cell viability and we selected for further experiments the concentration that showed no change of cell viability (15 ng/ml). Cells were washed with medium and treated with human recombinant cytokines: rMIF (BD Biosciences, San Diego, CA, USA) or rTNF-α (Millipore, São Paulo, SP, Brazil) or rIFN-γ (Invitrogen Life Technologies, Carlsbad, CA, USA) or rIL-6 (Peprotech, Rocky Hill, NJ, USA) or rTGF-β1 (R&D Systems, Minneapolis, MN, USA) or rIL-10 (R&D Systems), all of them at 15 ng/ml for 24 h at 37° and 5% CO_2. Cells were then infected with *T. gondii*, RH or ME49 strain, at 5:1 (parasite:cell). After 3 h of infection cells were again treated with cytokines as described above. As controls, cells were uninfected/untreated, treated/uninfected or infected/untreated. After 2 h and 24 h of incubation, medium was gently removed and cells were fixed in 10% buffered formalin for 24 h and were analyzed for apoptosis detection by immunohistochemistry.

In a third set of experiments, BeWo cells were cultured into 6-well plates (1×10^6 cells/well/ml) for 24 h at 37 °C and 5% CO_2. Cells were washed with medium and treated with human recombinant cytokines as described above. Cells were then infected with *T. gondii*, RH or ME49 strain, at 5:1 (parasite:cell). After 3 h of infection cells were treated again with cytokines as described above. After 2 h and 24 h of incubation, cells were analyzed for cell surface Fas/CD95 expression by flow cytometric analysis. As controls, cells were uninfected/untreated, treated/uninfected or infected/untreated.

In a fourth set of experiments, BeWo cells were cultured into 6-well plates (1×10^6 cells/well/ml) for 24 h at 37 °C and 5% CO_2. Cells were washed with medium and infected with *T. gondii*, RH or ME49 strain, at 5:1 (parasite:cell). After 2 h and 24 h of incubation cells were lysed and subjected to Western blotting assays for phosphorylated ERK1/2 detection. As control, cells were incubated with medium alone. Six independent experiments were performed in triplicate for each condition.

2.4. Cytokine assays

2.4.1. Cytokine measurements by enzyme-linked immunosorbent assay (ELISA)

Human cytokines (MIF, TNF-α, IL-12, TGF-β1 and IL-10) were measured using sandwich ELISAs according to the manufacturer's instructions (R&D Systems for MIF, TGF-β1 and IL-10; BD Biosciences for TNF-α and IL-12). Briefly, 96-well plates were coated with capture monoclonal antibody to each cytokine, blocked and incubated with the samples and the respective standards for each cytokine. After washing, plates were incubated with biotinylated detection polyclonal antibody to each cytokine. The assay was developed using streptavidin–horseradish peroxidase and revealed with TMB substrate reagent set (BD Biosciences). Cytokine concentrations were determined via extrapolation from a standard curve obtained from known concentrations of each recombinant cytokine. The sensitivity limits of these assays were 31.3 pg/ml for MIF, IL-10 and TGF-β, and 7.8 pg/ml for TNF-α and IL-12.

2.4.2. Cytokine measurements by cytometric bead array (CBA)

Human IL-6 and IL-17A were measured using cytometric bead array (CBA; BD Biosciences) according to the manufacturer's instructions. Briefly, samples were mixed with cytokine capture beads and incubated with PE-conjugated detection antibody for 3 h at room temperature. After centrifugation, supernatants were carefully aspirated and discarded, and bead pellets were resuspended. Samples were examined under BD flow cytometry (FACSCanto II, BD Company, San Diego, CA, USA) and data were analyzed using BD Cell Quest and CBA software.

2.5. Apoptosis detection by immunohistochemistry

Fixed BeWo cells were incubated for 10 min at room temperature with 5% acetic acid and then with 2.5% normal goat serum diluted in 20 mM Tris–HCl buffered saline (TBS, pH 7.2) for 30 min at 37 °C to block non-specific binding sites. Cells were incubated with mouse monoclonal antibody to cytokeratin 18 neo-epitope (clone M30, CytoDEATH, Roche Diagnostics, Mannheim, Germany) diluted 1:250 in TBS for 12 h at 4 °C. Next, cells were incubated with biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Heidelberg, Germany) diluted 1:600 in TBS for 1 h at 37 °C. The reaction signal was amplified using avidin–biotinylated alkaline phosphatase complex at 1:100 (ABC system, Vectastain, Vector Labs Inc., Southfield, MI, USA), developed with fast red-naphthol (Sigma Aldrich) and counterstained with Mayer's hematoxylin. Coverslips were mounted on glass slides and cells were examined under a light microscope to assess immunostained apoptotic cells. Results of apoptosis index were expressed as the mean number of apoptotic cells in 100 counted cells. For the immunostained cells counting, round coverslips containing the adherent cells were divided to form four quadrants, in each quadrant 25 random cells were counted and it was determined how many cells were immunostaining positive for M30 using the 40× of magnification.

2.6. Fas (CD95) expression in BeWo cells by flow cytometric analysis

BeWo cells were stained with FITC-conjugated anti-Fas/CD95 antibody (Santa Cruz Biotechnology Inc.) or isotype-matched negative control (Millipore, São Paulo,