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Short communication

Group B streptococcus induces trophoblast death☆

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ABSTRACT

Group B streptococcus (GBS) is one of the leading causes of neonatal infection; however the molecular mechanisms involved are not clearly known. Here we used high and low hemolytic GBS isolates and mutant GBS that lacks β-hemolysin expression and showed that GBS infection or exposure to GBS hemolysin extract induces primary human trophoblast, placental fibroblast and JEG3 trophoblast cell line death, and that GBS-induced trophoblast death was β-hemolysin dependent. The fibroblasts and trophoblasts provide an innate immune barrier between fetal and maternal circulation in the placenta. These data suggest that GBS may disrupt this barrier to invade fetal circulation.

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1. Introduction

GBS plays an important role in neonatal infection and preterm delivery [1]; however the microbial factors involved in the pathogenesis are not clearly known.

Trophoblasts are the outermost cell layer of the blastocyst. Once attached into the uterine wall, trophoblasts invade and proliferate into the decidua to develop the placenta and provide the barrier between the maternal and fetal circulation. Placental fibroblasts provide a supportive cellular network and play an important role for placental development. Currently there are no data on the effect of GBS infection on trophoblast or placental fibroblast survival.

GBS-induced host cell death was suggested to be important for the initiation of infection, bacterial survival, and suppression of the host immune responses [2–4]. Here we hypothesized that GBS infection may induce cell death in the placenta and this may play a role in microbial invasion and ultimately fetal and maternal infection and preterm delivery.

We exposed the JEG3 human trophoblast cell line and primary human placental trophoblasts and fibroblasts to GBS isolates with various hemolytic activity and showed that GBS infection and exposure to GBS extract are toxic to human trophoblasts and fibroblasts and that GBS β-hemolysin plays the key role in GBS toxicity.

2. Materials and methods

2.1. Cells lines and reagents

JEG3 human trophoblast cell line was obtained from American Type Tissue Culture Collection (Manaaas, VA) and cultured in MEM

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(Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate and 100 nM of penicillin/streptomycin (Invitrogen Life Technologies). Antibiotic free media was used during the attachment phase of the infection experiments.

2.2. Isolation and culture of primary trophoblasts

Trophoblasts were isolated from placentas obtained according to NIH, CSMC and Planned Parenthood IRB protocols. Briefly, tissue specimens were washed with cold HBSS (Invitrogen Life Technologies) to remove excess blood. Cells were scraped from the membranes, transferred to trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, CA) digestion buffer and incubated at 37 °C for 10 min with shaking. An equal volume of DMEM medium (Invitrogen Life Technologies) containing 10% FBS was added to inactivate the trypsin. This mixture was vortexed for 20 s, allowed to sediment, and the supernatant was collected. This was repeated twice and the collected supernatant was centrifuged at 1500 rpm for 10 min. Contaminating RBC were removed by resuspending the cellular pellet with HBSS, layering this over the same volume of Lymphocyte Separation Media (ICN Biomedicals, Aurora, OH), and centrifuging at 2000 rpm for 25 min. The cellular interface containing the trophoblast cells was collected and resuspended in DMEM supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA) and cultured at 37 °C/5% CO_2 for three passages. This technique provides >98% pure trophoblast cells.

2.3. Isolation and culture of human placenta fibroblast

Primary fibroblasts were isolated from human placentas obtained from consenting full-term-pregnant healthy women who underwent elective cesarean section according to the CSMC IRB protocol. Minced placenta was washed in sterile phosphate buffer saline (PBS), trypsinized in 0.25 Trypsin (Gibco) at 37 °C for 3 h and centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was removed and the cells were resuspended in Dulbecco Modified Essential Medium (DMEM) with 4 g/ml of glucose (Gibco), 20% fetal bovine serum and 1% antibiotics. The cells were plated in 100 mm petri dishes and cultured at 37 °C. The fibroblasts adhered at the bottom of the plate. The non-adherent non-fibroblast cells were washed and removed. Passage 10 cells were used for the studies.

2.4. Bacterial strains and culture conditions

We used wild type clinical GBS isolates: highly encapsulated type III strain COH1; which has low hemolysin activity [5,6] and highly hemolytic WT strain NCTC10/84 (1169-NT1), a serotype V isolated from the blood of a septic neonate [7]. For preparing the extract containing β-h/c (hemolysin/cytolysin) activity, the low hemolysin COH1, the highly hemolytic WT strain NCTC10/84 and the corresponding non-hemolytic, non-pigmented isogenic allelic exchange mutant NCTC:cylEΔ*cat* (NCTC10/84M)were used [8].

2.5. Preparation of GBS hemolysin extracts

NCTC10/84 WT, COH1 and NCTC10/84M GBS strains were grown in Todd–Hewitt broth (THB, Difco) at 37 °C overnight, diluted to 1/20 in 200 mL of THB, and grown to mid-log phase ($OD600 = 0.4 = 10^8$ cfu/mL). All strains grew equally well under the conditions used in these experiments. The bacteria (2×10^{10} cfu) were harvested and incubated for 2 h at 37 °C in PBS containing 2% starch and 1% glucose. The supernatant was collected and filter sterilized, and cold 100% methanol was added (1:1) to precipitate the hemolysin. Extracts were incubated at −20 °C overnight or at −80 °C for 1 h, and resuspended in 2 mL of PBS. The amounts of hemolysin extracts used in the exposure assays, 2, 1.5 and 0.5 μL corresponded to hemolysin extract from 2×10^7, 1.3×10^7 and 5×10^6 cfu respectively [2].

2.6. Hemolysin activity assay

Serial two-fold dilutions of 5 μL of β-hemolysin extract with PBS were prepared, starting with a 1:40 dilution. An equal volume of 1% sheep erythrocytes in PBS was added. Solutions were incubated 1 h at 37 °C/5% CO_2, then 30 min at 4 °C. PBS glucose alone and lysed RBC in 1% SDS were used for negative and positive control respectively. Hemolytic titer of a given strain was determined by the reciprocal of the greatest dilution producing 50% of the hemoglobin release compared with the SDS positive control. The hemolytic titer of NCTC10/84 WT was 1280.

2.7. GBS infection

Assays for GBS infection was performed essentially as described before [2]. Briefly, GBS strains, COH1 and NCTC10/84 were grown in THB to mid-log phase ($\sim 10^8$ cfu/ml; $OD_{600} = 0.4$), washed in PBS, resuspended in RPMI with 10% FBS, and used to infect trophoblast cell line and primary fibroblasts. Plates were centrifuged at 700 × g for 5 min to settle the bacteria on the monolayer surface then incubated at 37 °C in 5% CO_2 for 1 h. The concentration and purity of each GBS inoculum was confirmed by quantitative culture on THB agar plates.

2.8. Lactate dehydrogenase (LDH) assay

Cell death was assessed by measuring the supernatant lactate dehydrogenase levels by performing colorimetric lactate dehydrogenase (LDH) assay (Roche Diagnostics) according to the manufacturer's instructions. The data is reported as the percent of maximum LDH released upon Triton X-100 treatment of cells.

2.9. FACS analysis of apoptosis

Annexin-V is a Ca^{2+}-dependent protein with a strong affinity for phosphatidylserine (PS), which is externalized in the early stages of apoptosis. In non-apoptotic cells, PS is present only on the inner surface of the membrane and false positive results may occur if the membrane is damaged, as occurs in necrotic cells. Cell damage and false positive PS labeling can be assessed by staining with propidium iodide, which does not enter an intact cell. We assessed apoptosis by incubating cells with FITC-conjugated Annexin-V (Roche Molecular Biochemicals, Indianapolis, IN). Labeling procedures followed those suggested by the manufacturer's manual. Briefly, cells were resuspended in Annexin labeling solution containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$, and fluorescein-conjugated Annexin-V for 15 min. After being washed twice with PBS, cell pellets were resuspended in PI (propidium iodine) (2 μg/ml) containing PBS and analyzed by flow cytometry. Apoptosis was presented as percent positive cells stained with Annexin-V. Necrotic cells were presented as the percent positive cells stained with PI.

2.10. Statistical analysis

The experiments were set up in triplicate and were repeated on at least three separate occasions. For LDH assays, the average LDH measurement of wells from each treatment was divided by the LDH value of the lysis control to calculate a percentage of total cell lysis LDH secreted, as recommended by the manufacturer. Error bars represent the 95% confidence intervals calculated using the standard deviation. Student's *t*-test was used to compare the means between

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