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Apoptosis induced by Oropouche virus infection in HeLa cells is dependent on virus protein expression

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ABSTRACT

Oropouche (OROV) is a single-stranded RNA arbovirus of the family *Bunyaviridae*, genus *Orthobunyavirus*, which has caused over half a million cases of febrile illness in Brazil in the past 30 years. OROV fever has been registered almost exclusively in the Amazon region, but global warming, deforestation and redistribution of vectors and animal reservoirs increases the risk of Oropouche virus emergence in other areas. OROV causes a cytolytical infection in cultured cells with characteristic cytopathic effect 48 h post-infection. We have studied the mechanisms of apoptosis induced by OROV in HeLa cells and found that OROV causes DNA fragmentation detectable by gel electrophoresis and by flow cytometric analysis of the Sub-G1 population at 36 h post-infection. Mitochondrial release of cytochrome *C* and activation of caspases 9 and 3 were also detected by western blot analysis. Lack of apoptosis induced by UV-inactivated OROV reveals that virus-receptor binding is not sufficient to induce cell death. Results obtained in cells treated with chloroquine and cycloheximide indicated that viral uncoating and replication are required for apoptosis induction by OROV. Furthermore, treatment of the cells with pan-caspase inhibitor prevented OROV-induced apoptosis without affecting virus progeny production. The results show that OROV infection *in vitro* causes apoptosis by an intracellular pathway involving mitochondria, and activated by a mechanism dependent on viral replication and protein synthesis.

1. Introduction

Oropouche virus (OROV) is an emerging zoonotic arbovirus of the family *Bunyaviridae*, genus *Orthobunyavirus*, serogroup *Simbu* (Schmaljohn and Hooper, 2006), which causes outbreaks of human febrile illness in urban areas of tropical South America. The infection is clinically characterized by fever, myalgia, headache, arthralgia, skin rash and malaise, and can last several days (Pinheiro et al., 1981). While OROV fever has been registered almost exclusively in the Amazon region, global warming, deforestation and redistribution of vectors and reservoir animals increase the risk of OROV spreading to other areas of the Americas.

Apoptosis is an evolutionarily conserved mechanism of programmed cell death, which is critical for the embryo development and tissue homeostasis of multicellular organisms. Apoptosis is activated by a variety of extracellular and intracellular stimuli and involves different signals (Hengartner, 2000). Apoptotic cells display specific morphological and biochemical features that distinguish them from living cells and from necrotic cells. Apoptotic cells are characterized by detachment, shrinkage and rounding, plasma membrane blebbing, nuclear collapse and chromatin condensation. Other apoptosis hallmarks are a loss of the asymmetry in the distribution of phospholipids between cell membrane leaflets, nuclear fragmentation and caspase activation (Kroemer et al., 2009). Ultimately, cells are fragmented into apoptotic bodies that are rapidly phagocytosed by neighboring cells or phagocytes, without associated inflammation or tissue damage.

Apoptosis plays important roles in host protection by eliminating cells damaged by irradiation, chemical injuries or virus infections (O'Brien, 1998). Virus-infected cells can be eliminated by apoptosis, necrosis, or by cell lysis mediated by components of the innate and adaptive immune systems (Everett and McFadden, 2001; Roulston et al., 1999). On the other hand, viruses have evolved strategies to regulate apoptosis, either by blocking a specific step of the cascade, preventing premature death of the host cell and thus maximizing virus production, or by actively inducing apoptosis, which consequently facilitates the spreading of virus progeny to neighboring cells while limiting host immune-inflammatory responses (O'Brien, 1998).

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Virus replication in the host cell is required for the induction of apoptosis by several viruses (Roulston et al., 1999). Conversely, apoptosis induction in the absence of viral replication occurs much less frequently, and has been reported for type 3 reovirus (Tyler et al., 1995), avian leukosis virus (Brojatsch et al., 1996), human herpes simplex 1 (Aubert et al., 1999), bovine herpesvirus (Hanon et al., 1997), vaccinia virus (Ramsey-Ewing and Moss, 1998) and Sindbis virus (Jan and Griffin, 1999).

OROV causes cytolytical infection in cultured cells of several lineages, with cytopathic effect (CPE) appearing around 48 h post-infection. We investigated the role that apoptosis plays in the OROV-induced CPE in HeLa cells. The presently reported results indicate that OROV induces apoptosis *in vitro*, triggered by a mechanism that requires viral gene expression and this process of programmed cell death is not required for virus replication.

2. Materials and methods

2.1. Cells and virus

HeLa cells were cultured in minimum essential medium (MEM, Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco/Invitrogen), 1× Antibiotic–Antimycotic solution (Gibco/Invitrogen), 100 mM L-glutamine (Gibco/Invitrogen), and incubated at 37 °C in 5% CO_2. The OROV strain BeAn19991 kindly donated by Dr. Luiz Tadeu Moraes Figueiredo, from FMRP-USP, was propagated in Vero cells and titrated in Vero cells by the $TCID_{50}$ method (Reed and Muench, 1938). Cell cultures were infected (multiplicity of infection, MOI equal to 10) with OROV in a small volume of MEM supplemented with 2% bovine serum for 1 h at 4 °C. Then the inoculum was removed, cells were washed with PBS (Gibco/Invitrogen), replenished with fresh medium and incubated until the time for harvesting. Inactivated OROV (volume of 500 μL) was produced by exposure to 254 nm ultraviolet light at a distance of 5 cm for 5 h on ice. Inactivation was confirmed by titration of virus samples in HeLa cells before and after UV exposure. The control for this experiment (OROV C-) consisted of native OROV incubated on ice for 5 h without UV light.

2.2. DNA fragmentation assay by gel electrophoresis

At the indicated times, OROV- and mock-infected HeLa cells were harvested and centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatants were discarded, cell pellets were resuspended in 500 μL of lysis solution containing 10 mM Tris pH 7.4, 1 mM EDTA, and 0.2% Triton X-100 (Sigma–Aldrich), followed by incubation at 4 °C for 20 min and centrifugation at 12,000 × *g* for 20 min. The supernatants were treated with 100 μg/mL RNAse A (Invitrogen) for 1 h at 37 °C followed by 0.1 mg/mL proteinase K (Invitrogen) for 1 h at 57 °C and then nucleic acids were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma–Aldrich) and precipitated with ethanol following published procedures (Ausubel, 2003). After ethanol precipitation, nucleic acid was resuspended in TE (10 mM Tris–HCl pH 7.4, 10 mM EDTA), analyzed by electrophoresis on 1.5% agarose, stained with 0.5 μg/mL ethidium bromide, and examined under UV light.

2.3. DNA fragmentation assay by flow cytometric analysis

At indicated times and conditions, 10^6 cells were washed with PBS, fixed in 70% ethanol at 4 °C for 20 min, washed in PBS with 0.05% BSA (Sigma–Aldrich) and resuspended in 50 μg/mL propidium iodide (Sigma–Aldrich) containing 40 μg/mL RNase A. Cells were analyzed using a Becton–Dickinson FACScan Advantage to determine the percentage of apoptotic cells, gauged at the sub-G1 cell subpopulation (Nicoletti et al., 1991).

2.4. Western blot

Cells were harvested using a cell sacraper, washed in PBS, resuspended in lysis buffer containing 25 mM Tris–HCl pH 7.5, 0.1 mM PMSF (Sigma–Aldrich), 0.5 mM EDTA, 10 mM KCl (Synth), 5 mM $MgCl_2$ (Synth), 1 mM DTT (Sigma–Aldrich), 5% glycerol (Merck), 1 mM aprotinin (Sigma–Aldrich), and 5 mM leupeptin (Sigma–Aldrich). The suspension was lysed by 5 ultrasonic pulses in a cell disruptor (Fisher Scientific) on ice, and then centrifuged at 15,000 × *g* for 30 min. Protein concentration was determined by the bicinchoninic acid method with BCA Protein Assay Reagent (Pierce). Protein extracts (30 μg) were subjected to sodium dodecyl sulfate (SDS) 12.5% polyacrylamide gel electrophoresis, and then electroblotted onto nitrocellulose membranes (Protran, Schleicher & Schuell). The membranes were incubated with 1:200 mouse anti-caspase 9 antibody (BD Pharmingen) in PBS (Gibco/Invitrogen) for 12 h, followed by 1:1000 horseradish peroxidase (HRP)-conjugated secondary rabbit anti-mouse antibody (Molecular Probes) in PBS for 1 h. The membranes were then revealed by incubation with 0.03% DAB (3,3′-diaminobenzidine, Sigma–Aldrich). The membrane was then stripped using a stripping buffer (62,5 mM Tris–Cl pH 6.7, 2% SDS, 100 mM 2-mercaptoetanol) and incubated at 50 °C for 30 min, followed by the incubation with a 1:500 goat anti-actin antibody (BD Pharmingen) for 12 h, followed by 1:1000 horseradish peroxidase (HRP)-conjugated secondary rabbit anti-goat antibody (Molecular Probes) for 1 h. The membrane was then revealed using ECL Plus Western Blotting Detection Reagent (GE Healthcare) on Hyperfilm (GE Healthcare).

2.5. Cytochrome C detection

Cells were harvested using a cell sacraper, washed with PBS and resuspended on extraction buffer MB: 210 mM mannitol (Sigma–Aldrich); 70 mM sucrose (Sigma–Aldrich); 1 mM EDTA (Sigma–Aldrich); 10 mM HEPES pH 7.5 (Sigma–Aldrich), 0.1 mM PMSF (Sigma–Aldrich); 1 mM aprotinin (Sigma–Aldrich), 5 mM leupeptin (Sigma–Aldrich). Cell suspensions were passed through a 25G1 needle attached to a 5 mL syringe (BD-Becton, Dickinson Company) and then centrifuged at 500 × *g* for 5 min. The supernatants were separated and centrifuged one more time at 10,000 × *g* for 30 min in order to obtain a pellet of heavy membrane organelles, including mitochondria. This fraction of the cell extract was named "high membrane portion" (HM) and it was resuspended with the MB buffer with 1% Triton X-100 (Invitrogen). The supernatant obtained from the previous centrifugation was then centrifuged at 100,000 × *g* for 1 h at 4 °C and the supernatants were kept as "cytoplasmatic portion" (C). Protein extracts from the HM portion were then tested for cytochrome *C* by Western Blot with a 1:500 dilution of mouse anti-cytochrome *C* antibody (BD Pharmingen) for 12 h revealed with an 1:1000 horseradish peroxidase (HRP)-conjugated secondary rabbit anti-mouse antibody (Molecular Probes) for 1 h as described above.

2.6. Caspase 3 activation detection

Cells were harvested using a cell sacraper, washed once and resuspended in PBS at a concentration adjusted to 1 × 10^6 cells/mL. The caspase 3 fluorogenic substrate (Z-Asp-Glu-Val-Asp)2-rhodamine 110-bisamide (Calbiochem) was added to this suspension at 50 μM final concentration, followed by incubation at 37 °C for 10 min protected from light. Cells were then washed with PBS to remove excess of substrate and fluorescence was analyzed by flow cytometry in a Becton–Dickinson FACScan Advantage.