

HCoV-OC43-INDUCED APOPTOSIS OF MURINE NEURONAL CELLS

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1. INTRODUCTION

Apoptosis is a selective process for cell deletion and can be induced by a variety of physiological and non-physiological stimuli, including viral infections.¹ A growing number of viruses have been shown to actively promote apoptosis, representing a culmination of a lytic infection that serves to spread virus progeny to neighboring cells while evading host inflammatory responses.² Various groups of molecules are involved in the apoptotic pathway. One set of mediators implicated in apoptosis belongs to the cysteine dependent aspartate-specific family of proteases referred to as caspases. Caspase-3 has been identified as the key pro-apoptotic protease in neuronal apoptosis.³

HCoV-OC43 causes acute encephalitis in the central nervous system (CNS) of mice. Following intracerebral (IC) inoculation, mice developed disseminated infection and mortality seemed to be related to the amount of infectious virus in the CNS.⁴ Using a mouse primary cortical cell cultures, we showed that these cultures were productively infected by HCoV-OC43 and that neurons underwent nuclear fragmentation associated with activated caspase-3 positive staining, indicating that HCoV-OC43 infection induced neuronal apoptosis. We also demonstrated apoptosis of neurons takes place during encephalitis in the CNS of infected mice. These findings illustrate that HCoV-OC43 is responsible for neuronal cell death and suggest that apoptosis could play a role in the dissemination of HCoV-OC43.

2. MATERIALS AND METHODS

2.1. Virus, Mice, and Cortical Cell Cultures

The HCoV-OC43 strain was originally obtained from the American Type Culture Collection (ATCC), plaque-purified and grown on the human rectal carcinoma cell line

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HRT-18 as previously described.⁵ HCoV-OC43 virus stocks (10^6 TCID₅₀/mL) were kept at -80°C.

C57Bl/6 (MHV-seronegative female) mice (Jackson Laboratories), aged 21 days post-natal (DPN) were inoculated by the intracerebral (IC) route, using 10 µL containing 10 TCID₅₀ of HCoV-OC43.

Cortical cell cultures were obtained following the modified methods of Brewer and collaborators.⁶ Cells from mouse embryos at 16 to 18 days of gestation were dissected in HBSS without Ca²⁺ and Mg²⁺, supplemented with 1.0 mM sodium pyruvate and 10 mM HEPES, and dissociated by trituration. Supernatants were then transferred and centrifuged for 1 min at 1000 x g. The pellets were resuspended in 1 mL HBSS per brain. Cells were plated in neurobasal medium (Invitrogen) supplemented with 0.5 mM L-glutamine, 25 µM glutamate and B27 supplement (Invitrogen) and grown on glass coverslips, pretreated with poly-D-Lysine and plated at approximately 5×10^5 /cm². After 4 days, medium was replaced with Neurobasal/B27 without glutamate.

2.2. Infection of Cell Cultures and Infectious Virus Assays

Cortical cultures were infected with HCoV-OC43 at an MOI of 1, incubated at 37°C for 2 hr, then washed in warm PBS and incubated at 37°C. Supernatants were collected at 12, 24, 48, 72, and 96 hr postinfection (hpi). Collected supernatants were centrifuged for 5 min at 1000 x g and then immediately frozen at -80°C and stored until assayed. The extracts were processed for the presence and quantification of infectious virus by an indirect immunoperoxidase assay, as previously described.⁷ HCoV-OC43-susceptible HRT-18 cells were inoculated with serial logarithmic dilutions of each tissue sample in a 96-well Linbro plate. After 4 days of incubation at 33°C in 5% (v/v) CO₂, cells were washed in PBS and fixed with 0.3% (v/v) hydrogen peroxide (H₂O₂) in methanol for 30 min. After washing with PBS, they were incubated for 2 hr at 37°C in 1/1000 dilution of a mouse ascites fluid containing MAb 1-10C.3, directed against the spike protein of HCoV-OC43.⁸ Afterwards, cells were washed in PBS and HRP-conjugated goat anti mouse immunoglobulins were added and incubated for 2 hrs at 37°C. Antibody complexes were detected by incubation in DAB solution, with 0.01% (v/v) H₂O₂.

2.3. Immunofluorescence and TUNEL Assays Staining

To visualize viral antigens and cell markers, cultures were fixed with 4% (v/v) paraformaldehyde, for 30 min. Then, after washing, cells were permeabilized with 100% methanol for 5 min, incubated 2 hr with primary antibodies, as previously described.⁹ For viral antigens, we used 1/1000 dilutions of ascites fluid of the 4-E11.3 hybridoma that secretes monoclonal antibodies specific for the nucleocapsid protein of the serologically related hemagglutinating encephalomyelitis virus of pigs.¹⁰ Apoptotic cells were revealed by antibodies to active caspase-3 at 1/50 (Rabbit anti-human/mouse active caspase-3 antibodies; R&D Systems, Inc.). After several washes in PBS, cell coverslips were incubated for 1 hr in the dark with a combination of immunofluorescent secondary antibodies: Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes), both at a dilution of 1/1000. Then, after 3 washes in water, cells were incubated in 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Polysciences Inc.) at a 1/100 dilution for 5 min.

After final washes in water, coverslips from each well were removed and mounted on microscope slides in Immuno-mount and observed under a fluorescence microscope.

The In Situ Cell Death Detection FITC Kit (Roche Molecular Diagnostics) was used for TUNEL (transferase dUTP nick end labeling) assays. Fluorescent double labeling of brain slices with antibody to infected cells was performed in conjunction with the TUNEL assay to enable correlation of TUNEL-positive cells with the presence of viral infection. Briefly, mice were intracardially perfused with 4% (v/v) paraformaldehyde, as previously described.⁴ Brains were paraffin embedded and sectioned longitudinally. Sections were collected on slides, de-paraffined and incubated with primary antibodies for viral antigens and for TUNEL staining, as recommended by the manufacturer.

3. RESULTS

3.1. Apoptosis of Primary Neural Cell Cultures After Infection

Measurement of infectious viral titers in the supernatants revealed that mouse primary cortical cultures produced a significant viral titers (Fig. 1), with a peak at 48 hpi. With time, virus titers decreased in concomitance with disappearance of neurons in the infected culture. This illustrates that HCoV-OC43 induced neuronal cell death.

Immunofluorescent staining revealed that after infection of cortical cultures, neurons were positive for viral antigens (Fig. 2 A) and numerous infected cells were also positive for activated caspase-3 (Fig. 2 B). The nuclear fragmentation/condensation observed with DAPI staining colocalized with activated caspase-3 positive cells (Fig. 2 C and D), confirming that viral infection could directly trigger an apoptotic response.

3.2. Apoptosis of Neural Cells in Mouse Brains After Infection

As previously reported, intracerebral inoculation of HCoV-OC43 into mice led to a generalized infection of the CNS, which also affected the hippocampus.⁴ Double immu-

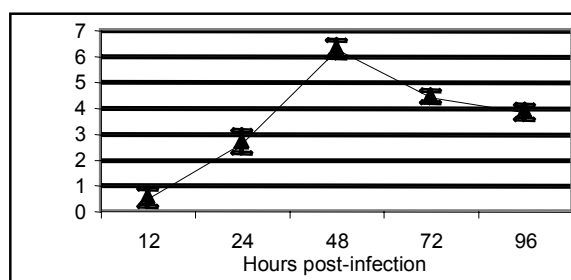


Figure 1. HCoV-OC43 replication in primary mouse cortical cultures. The results are expressed as the mean TCID₅₀ per milliliter for three independent experiments. Error bars indicate standard deviations of the means.

nostaining for viral antigens and TUNEL assays was performed on brain sections during the acute phase of the encephalitis, at 11 days post-infection (11 dpi). TUNEL positive cells could be seen in the hippocampus. Numerous neurons in the CA1 hippocampal layer were TUNEL-positive and numerous cells, in the same region, were also positive for viral antigens. Merged pictures illustrate that some of the infected neurons underwent apoptosis (Fig. 3 A) and that noninfected cells localized near the infected ones were also undergoing apoptosis (Fig. 3 B).

4. DISCUSSION

HCoV-OC43 was previously reported to induce apoptosis in MRC-5 cells, a lung cell line¹¹ but not in infected human monocytes/macrophages, unlike the infection observed with strain 229E.¹² The murine counterpart of HCoV-OC43, MHV, was reported to induce apoptosis in 17Cl-1 cells, a murine fibroblast cell line¹³ and in mouse brain neurons^{14,15} in addition to macrophage/microglial cells, astrocytes and oligodendrocytes.^{16,17} And recently, SARS-CoV was shown to induce apoptosis of Vero E6 cells.¹⁸

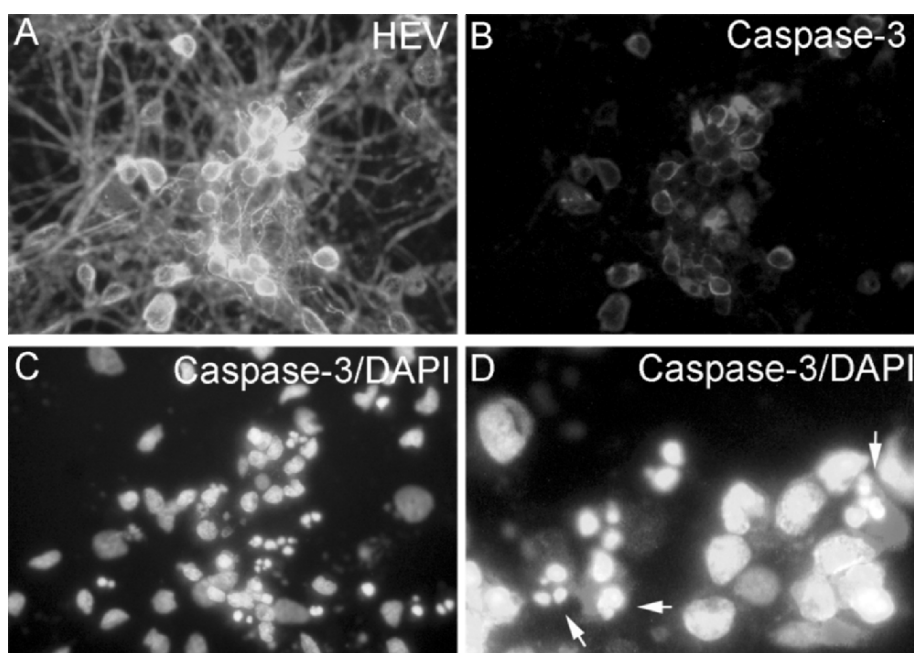


Figure 2. HCoV-OC43-induced apoptosis in primary mouse cell cultures. Cortical cells were infected by HCoV-OC43 at an MOI of 1. Twenty-four hpi, cells cultures were stained for viral antigens (A) and activated caspase-3 (B). In panel C and at higher magnification in panel D, activated caspase-3 positive cells staining were colocalized with the nuclear fragmentation observed with DAPI staining (arrows). All photographs were taken on the same cell culture field at a magnification of x200 for panels A, B, C and x400 for panel D.

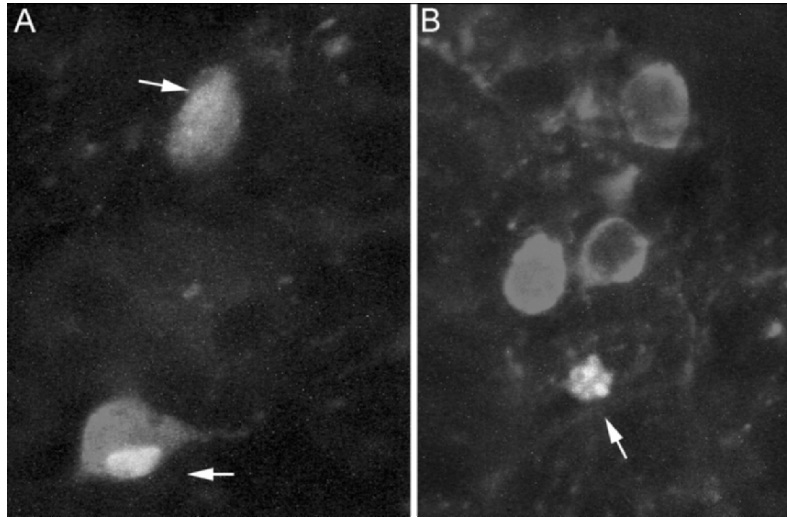


Figure 3. Caspase-3 is activated in hippocampal neurons from mice infected by HCoV-OC43. During the acute phase of the disease (11 dpi), CA1 hippocampal layer exhibited caspase-3 positive staining (arrows) colocalized with viral antigens staining (A) or in proximity of infected neurons (B). Magnification x200.

We have previously demonstrated the neuroinvasive properties of HCoV-OC43 in mice and that the severity of disease seemed to be linked to the amount of infectious virus in the CNS,⁴ illustrating that virus replication played a major role in the development of the pathology. Some viruses appear to use apoptosis as a mechanism for killing cells and spreading. This represents an important mechanism for efficient dissemination of progeny virions, as well as a means by which viruses can induce host cell death, while limiting inflammatory and other immune responses.² Here we report that HCoV-OC43 induced neuronal apoptosis *in vitro*, as well as *in vivo*, which could account for part of the neuronal cell death observed after infection. These results illustrate one of the possible mechanisms used by HCoV-OC43 to spread to the whole CNS, which was responsible for the development of an acute encephalitis in infected mice.

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