SHORT ARTICLE

Reactivation of Nedd-2, a developmentally down-regulated apoptotic gene, in apoptosis induced by a street strain of rabies virus

SUKATHIDA UBOL and JITRA KASISITH

Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, Thailand

A laboratory strain of rabies virus has been reported to induce apoptosis in experimental animals. The present study demonstrated that a bat strain and a primary canine rabies virus isolate also induced apoptosis in vivo. This death process involved reactivation of the caspase gene, Nedd-2, a developmentally down-regulated apoptotic gene. Expression of Nedd-2 was significantly up-regulated in infected adult and suckling mice. Reactivation of Nedd-2 in infected adult mice started at around day 3 and was prominent on day 5. The level of expression was constantly high up to the time that mice showed signs of illness. Expression of Nedd-2 correlated with the appearance of apoptotic nuclei within the infected brain, suggesting that reactivation of a developmentally down-regulated gene, Nedd-2, may be required for apoptotic elimination of cells damaged by infection.

Introduction

Rabies virus, a member of the family Rhabdoviridae, is the causative agent of hydrophobia. Although this disease has been recognised for several centuries, its pathophysiology is only partially understood. Recently, neuronal apoptosis was reported in an infection by rabies virus [1]. Apoptotic death was demonstrated in both immunosuppressed and immunocompetent mice infected with a fixed strain of rabies virus, CVS-11 [2]. Furthermore, the degree of apoptotic death in suckling mice infected by CVS-11 correlated with severity of infection [2]. Whether this process of cell death is activated during natural rabies infections or during experimental infections with street strains of rabies virus is unknown.

Genes in the bcl-2 and caspase families such as Bax, ICE (a caspase I) and CPP-32 (a caspase III) have been demonstrated to play a direct role in rabies virus-induced death in vitro [3]. Nedd-2 (a caspase II), is of particular interest as this gene product has been shown to mediate apoptotic death of neurons during brain development [4]. Expression of this gene is significantly down-regulated after complete neural maturation. Whether this gene plays any role in neuronal apoptosis due to wild-type rabies virus infection is unknown.

The present work examined whether a street strain of rabies virus could induce apoptosis in vivo. The correlation between Nedd-2 expression and the appearance of apoptosis during infection with wild-type rabies virus was also investigated.

Materials and methods

Viruses

A street strain of rabies virus isolated from the brain of a rabid dog was obtained from the Queen Saovabha Memorial Institute. The brain was homogenised as a 10% suspension in phosphate-buffered saline and then clarified by centrifugation. The supernate was removed, divided into small volumes and kept at −70°C until use. The titre of primary isolated virus was determined by the mouse inoculation assay and found to be c. 10⁶ LD50/ml. A bat strain of rabies virus was obtained from Dr Charles Rupprecht, CDC, Atlanta, GA, USA.

Experimental mouse inoculation

Swiss albino mice, both adult and suckling, were inoculated with 10 µl of an 10⁶ LD50/ml suspension of either the primary isolate or the bat strain of rabies virus. Brains from these infected mice were used for
apoptosis detection and monitoring of Nedd-2 expression.

**In-situ apoptosis detection by TUNEL assay**

Adult mice were experimentally infected with either the bat strain or the street strain of rabies virus. On day 3, day 5 and when infected mice showed signs of illness, mice were killed and brains were fixed in formalin, embedded in paraffin and sectioned. The paraffin sections were deparaffinised and rehydrated in a series of down-grading alcohol solutions. Chromosomal DNA fragmentation of infected brain sections was detected by TUNEL assay (Boehringer Mannheim) as described previously [2]. Briefly, DNA strand breaks were detected in situ by the incorporation of fluoroscein-dUTP to the opened ends of DNA strands. The fluorescein signal was amplified by staining with anti-fluorescein-conjugated alkaline phosphatase and the colour was developed by specific substrate. Apoptotic nuclei were identified morphologically by the presence of condensed brown nuclei when examined by light microscopy.

**Detection of Nedd-2 gene up-regulation by RT-PCR**

Suckling and adult mice were infected by the street strain of rabies virus. Infected mice were killed and brains were taken for examination at various times. Brains were homogenised and total RNA was extracted as described previously [3]. The extracted RNA was then subjected to RT-PCR to monitor the expression of the Nedd-2 gene as described previously [3]. Briefly, 20 μl of RNA were reverse transcribed into cDNA with AMV reverse transcriptase. The cDNA product was used as a template for PCR to amplify the Nedd-2 gene. The Nedd-2 primers were: sense 5’ GTA TGA AAC ATA AGG ATG GCG 3’, anti-sense 5’ CTG AAC ACA AGG GAA CCA ATG 3’. The PCR products were confirmed by hybridisation with a 32p-labelled specific probe. The oligonucleotide probe specific to Nedd-2 was 5’ GTG AAC ACA GTA TTA TTG TGG GAA GAG GCC 3’.

**Results**

**Rabies virus-induced apoptosis in vivo**

To find out whether infection by a naturally isolated strain of rabies virus could induce apoptosis, paraffin sections of brain derived from symptomatic mice on day 12 after infection were examined for DNA fragmentation. TUNEL assay of mouse brain tissue revealed massive apoptotic death in both the cerebellum and cerebrum after infection with a primary isolate of a street strain of canine rabies virus (Fig. 1a). Similar results were obtained with a bat variant of rabies virus (Fig. 1c).

**Reactivation of Nedd-2 during infection with a street strain of rabies virus**

To determine whether Nedd-2 is involved in apoptosis induced by a street strain of rabies virus, expression of this gene was monitored in both adult and suckling mice by RT-PCR (Fig. 2). Expression of Nedd-2 was clearly shown in uninfected suckling mouse brains. However, it was suppressed significantly in uninfected adult mouse brains when compared with the level of β-actin gene expression (Fig. 2a and b). Reactivation and up-regulation of Nedd-2 were clearly evident after

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*Fig. 1. Detection of apoptosis in brains of symptomatic mice on day 12 after infection. (a) mouse brain infected with a primary isolate from a rabid dog; (c) mouse brain infected with a bat strain rabies virus; (b, d) uninfected brains.*
infection of both the suckling and adult mice (Fig. 2a and b, respectively). Reactivation of this gene in adult mice was detected weakly by day 3 and up-regulation was significant by day 5. Expression of Nedd-2 was sustained at high levels until the end of the study (the day that mice developed symptoms), 11–12 days after infection (Fig. 2b). The appearance of apoptotic death was not detected on day 3, but was clearly shown by day 5 (data not shown), at which time high levels of Nedd-2 expression were found (data not shown).

Discussion

The occurrence of apoptotic cell death in brain tissue after laboratory infection with adapted strains of rabies virus has been reported previously. The present study demonstrated that infection with a primary isolate of a street strain of rabies virus also induced apoptotic death. These data strengthen a previous report in which apoptotic neurons were found in the hippocampus and brain stem of a patient with AIDS who developed rabies, and suggest that in that case report, apoptosis was at least partly due to rabies virus induction rather than being caused by HIV alone [5]. This evidence demonstrates that apoptotic death of neurons occurs during natural infection by rabies virus. Infection by a bat strain of rabies virus in experimental animals caused destruction of neurons by the same mechanism. Whether bats can survive rabies infections is unclear. If they do, it would be very interesting to discover the molecular mechanism involved.

Apoptosis constitutes a mechanism to control development and homeostasis. It occurs through the activation of a cascade of genes and the caspase family is a group of genes that orchestrates this process. Activation and overexpression of this gene family can drive cells into the apoptotic process. Neuronal death triggered by different initial causes is mediated by distinct caspase family members. For example, death of sympathetic neurons due to trophic factor deprivation is independently mediated by caspses II and III, whereas death of the same cells due to SOD-1 down-regulation proceeds through caspase 1 (ICE) activation [6–8]. Although Nedd-2, a caspase II, and CPP-32, a caspase III are both up-regulated during the death of neurons caused by trophic factor withdrawal, the role of CPP-32 does not seem to be significant for death under such conditions [9].

Death of neuroblastoma cells during infection with a laboratory strain of rabies virus involved the action of ICE and CPP-32 [3]. Whether these two caspases are activated independently is unknown. In the present study, death of neurons induced by the street strain of rabies virus involved reactivation of Nedd-2. This reactivation was found to correlate with the appearance of apoptotic death, as at high levels of Nedd-2 expression significant levels of apoptotic nuclei were detected. The fact that apoptosis was not detectable when Nedd-2 was weakly detected at day 3 after infection may be due to the very low number of apoptotic nuclei and the rapid clearance of those dead cells. In addition to Nedd-2 reactivation, ICE was also up-regulated during wild-type rabies virus infection (data not shown). Whether ICE and Nedd-2 are activated independently is not clear. If so, it is possible that activation of only one pathway may not be sufficient for death and that reactivation of the developmentally down-regulated apoptotic gene may be required to ensure complete elimination of rabies virus-infected neurons.

The outcome of apoptotic death of rabies virus-infected neurons is controversial. Some evidence suggests a protective role, but other evidence supports a destructive role [1,2,10]. It is generally accepted that apoptosis of virally infected cells serves as a defence mechanism, as it can reduce the number of infected cells. However, neurons are special cells in a non-renewable population. Therefore, massive apoptotic death of such a population may result in malfunction of the central nervous system (CNS), as well as disease development. For example, fatal encephalitis in suckling mice due to Sindbis virus infection is correlated with the degree of apoptotic death of neurons and mortality can be prevented by inhibition of neuronal apoptosis by bcl-2 overexpression [11–13]. In the case of rabies virus infection, it remains to be determined whether the defence process becomes destructive.

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References