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Expression of transferrin receptor 1, proliferating cell nuclear antigen, p27^{Kip1} and calbindin in the fetal and neonatal feline cerebellar cortex ☆

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ABSTRACT

Cerebellar cortices from feline fetuses with estimated gestational ages of 40–66 days and from kittens aged 2 days to 2 months, all negative for feline panleukopenia virus (FPV) infection, were analysed for expression of the transferrin receptor 1 (TrFR1), proliferating cell nuclear antigen (PCNA), p27^{Kip1} and calbindin. TrFR1, the receptor used by FPV to enter target cells, was expressed in capillary endothelial cells in the cerebellum at all fetal stages investigated and in Purkinje cells of a 3-week-old kitten, but not in the neuroblasts in the external granule layer (EGL). PCNA was expressed in cells of the superficial layer of the EGL. The cyclin-dependent kinase inhibitor p27^{Kip1} was expressed in cells of the deep layer of the EGL. Purkinje cells expressed calbindin from the earliest fetal stage investigated. Co-expression of PCNA and calbindin could not be demonstrated, indicating that feline Purkinje cells are post-mitotic from at least 40 days gestation.

Introduction

Feline panleukopenia virus (FPV) infection of feline fetuses during the last 3 weeks of gestation or FPV infection of kittens during the first 3 weeks of life can result in granuloprival cerebellar hypoplasia (Parrish, 1994). FPV is an autonomous parvovirus with a 5 kb negative sense single stranded DNA genome that invades cells using transferrin receptor 1 (TrFR1) and is internalised through a clathrin-dependent process (Parker et al., 2001). Canine parvovirus (CPV) is freed from endosomes by the phospholipase A2 activity of its capsid VP1 protein (Vihinen-Ranta et al., 2000, 2002; Suikkanen et al., 2003a; Farr et al., 2005) and reaches the cell nucleus probably along microtubules through a dynein-dependent process (Suikkanen et al., 2003b).

Parvoviruses do not possess a DNA polymerase and are unable to induce cell division (Tattersall, 1982; Cotmore and Tattersall, 1987; Berns, 1990). They rely on the host cell division machinery, beginning with host cell DNA polymerase δ, for successful completion of their lytic cycle (Deleu et al., 1999). Expression of parvoviral proteins is preceded by synthesis of a DNA double stranded replicative form, which is strictly dependent on host cell cycle initiation (Cotmore and Tattersall, 1987).

During perinatal infection, dividing external granule cell layer (EGL) neuroblasts are thought to be the primary target of FPV. However, a constant feature of cerebellar hypoplasia due to FPV is the loss of Purkinje cells (deLahunta, 1971). FPV proteins are expressed in Purkinje cell nuclei of kittens following experimental infection with FPV (Csiza et al., 1971) and in naturally occurring cases of cerebellar hyperplasia due to FPV (Aeffner et al., 2006; Poncelet et al., 2012).

Using immunohistochemistry (IHC) on normal feline cerebellar cortices harvested from fetuses and neonatal kittens, this study evaluated expression of (1) TrFR1, the receptor used by FPV to invade target cells; (2) proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase δ; (3) p27^{Kip1}, a cyclin-dependent kinase inhibitor expressed in cells exiting their cell cycles, as reported in the EGL layer of mice (Miyazawa et al., 2000); and (4) calbindin, which is expressed in mature feline Purkinje cells (Résibois et al., 2007).

Materials and methods

Sources of tissues from fetuses and kittens

Uteri were collected from 11 clinically normal pregnant queens by laparotomy under anaesthesia at the time of elective neutering; all queens recovered uneventfully. Fetuses for histology and IHC (n = 12) or Western blot (WB) analysis (n = 1) were removed and crown-rump lengths were measured. The fetuses were beheaded and cerebella were harvested. Twelve cerebella were cut sagittally and fixed in 10% neutral buffered formalin for 4 days before being processed for histology and IHC. The cerebellum from one kitten was deep frozen for WB analysis. In addition, cerebella were collected from three kittens aged 2 days (n = 1), 3 weeks (n = 1) and

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2 months ($n = 1$) that were euthanased for accidental separation from the queen, acute pneumonia and accidental hemisection of the cervical spinal cord, respectively. Samples of small intestine from cats that died from feline infectious enteritis/feline panleukopenia were used as positive controls and segments of small intestine from adult cats euthanased for reasons unrelated to FPV infection were used as negative controls.

PCR for feline parvovirus

DNA was extracted from formalin-fixed, paraffin-embedded tissues from 10 fetal cerebella (one per queen), three postnatal cerebella (2 days, 3 weeks and 2 months of age), intestine from a FPV-infected kitten (positive control) and cerebellum and intestine from an uninfected adult cat (negative control) and tested for the presence of FPV using PCRs that amplified a 193 base pair (bp) product from the FPV VP1 gene and a 215 bp product from the FPV VP2 gene (Résibois et al., 2007). PCR for a 214 bp product from the histone 3.3 gene was used to assess DNA extraction and sample integrity (Résibois et al., 2007).

Histopathology

Fixed cerebella were dehydrated through a graded series of ethanol solutions and embedded in paraffin wax. Sections (5 μm thick for prenatal samples; 6 μm thick for postnatal samples) were stained with haematoxylin and eosin, or Luxol fast blue and Cresyl violet At least one section was used for each IHC examination, although two or three sections were used in most cases.

Immunohistochemistry

Mouse anti-human TrFR1 monoclonal antibody (clone H68.4, Zymed Laboratories) was used at a dilution of 1/500 for IHC and 1/1000 for WB analysis. This antibody targets amino acids 3–28 of human TrFR1. The corresponding sequence in cats (GenBank GI:8926605) differs at residue 11, in which asparagine is substituted for threonine. Antigen retrieval for TrFR1 IHC was performed by heating deparaffinised sections at 95 °C for 15 min in 1 mM ethylene diamine tetraacetic acid (EDTA).

Mouse anti-human PCNA monoclonal antibody (clone PC10, Abcam) was used at a dilution of 1/1000 for IHC and 1/5000 for WB analysis. This clone recognises PCNA in cats (Sarli et al., 1995). Antigen retrieval for PCNA IHC was performed by heating deparaffinised sections at 95 °C for 15 min in Tris–EDTA buffer (10 mM Tris base, 1 mM EDTA; pH 9.0).

Rabbit anti-human $p27^{Kip1}$ polyclonal antibody (catalogue number 7961, Abcam) was used at a dilution of 1/50 for IHC and 1/200 for WB analysis. The antibody is directed against amino acids 181–198 of human $p27^{Kip1}$; these amino acids are conserved in cats (GenBank GI:3913222). $p27^{Kip1}$ inhibits mitosis and has been used to demonstrate the exit of cerebellar EGL cells from the mitotic cycle in mice (Miyazawa et al., 2000).

Anti-rat calbindin D 28 kDa polyclonal antibody (catalogue number CB-38a, Swant) was used at a dilution of 1/5000 for IHC. This antibody stains Purkinje cell bodies and processes in adult feline cerebella (Résibois et al., 2007).

Details of the method for IHC are described in the accompanying paper (Poncelet et al., 2012). Non-specific binding sites were blocked with normal sheep serum at a dilution of 1/20 and the primary antibody was applied overnight. Goat anti-rabbit or goat anti-mouse serum was used as a secondary antibody. Binding was visualised using the peroxidase–antiperoxidase method, with diaminobenzidine as the chromogen. Phosphate buffered saline (0.01 M) was used for intermediary washing steps. A negative control (no primary antibody) was included in all runs.

Double immunolabelling for confocal microscopy

Antibodies against PCNA (1/500) and calbindin (1/50) were applied overnight at 4 °C, followed by Alexa 594 (red)-conjugated goat anti-rabbit secondary antibody (A-11012, Invitrogen, 1/100) and biotinylated horse anti-mouse antibody (BA-2001, Vector, 1/100), then Alexa 488 (green)-bound streptavidin (A-11227, Invitrogen, 1/100). Secondary steps were carried out for 30 min at room temperature. Sections were examined by confocal microscopy using an MRC 1024 (Bio-Rad) fitted on an Axiovert 100 (Zeiss) with an Argon–Krypton laser (emissions at 488 and 568 nm) and a 40× NA 1.2 water objective. Antibodies against PCNA (1/25) and anti-$p27^{Kip1}$ (1/20) were applied overnight, followed by FITC (green)-goat anti-mouse antibody (F-1010, Sigma, 1/12) and biotinylated goat anti-rabbit antibody (BA-1000, Vector 1/50), then Alexa 594 (red)-bound streptavidin (1/100). Secondary steps were carried out for 30 min at room temperature. Non-specific binding was blocked by incubation with normal sheep or horse serum (1/20 and 1/10, respectively; 15 min, room temperature). Sections were examined by confocal microscopy using an LSM 780 (Zeiss) fitted on an Axiovert 200 M (Zeiss) with an argon laser (emission at 488 nm), a helium neon laser (emission at 543 nm) and a 40× NA 1.2 water objective.

Western blot analysis

WB analysis was used to confirm the specificity of the antibodies used in this study. Feline fetal cerebellum and pelleted Crandell feline kidney (CrFK) cells were thawed on ice and covered with an equal volume of modified radioimmunoprecipitation (RIPA) buffer (Ando et al., 2011). Feline fetal cerebellum was dissociated with a tissue grinder, while CrFK cells were pipetted vigorously and the mixtures were rotated for 1 h at 4 °C. The suspensions were centrifuged at 4380 *g* for 20 min at 4 °C. Protein concentrations in the supernatants were determined colorimetrically against standard dilutions of bovine serum albumin (Bio-Rad Protein Assay). Loading buffer was added to adjust the protein concentration to 10 μg/μL and the preparations were boiled for 5 min.

Details of the method for WB analysis are described in the accompanying paper (Poncelet et al., 2012). Samples (50–100 μg protein/lane) were separated by 10% or 12% W/V glycine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), depending on the molecular weight (MW) of the analysed proteins, and transferred to nitrocellulose or polyvinylidene fluoride membrane using a liquid transfer system (Bio-Rad). Membranes were blocked in semi-skimmed powdered milk (10% W/V in Tris-buffered saline) for 1 h at room temperature, then incubated with primary antibodies overnight, followed by incubation with anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Sigma). Peroxidase activity was detected using the electrochemiluminescence (ECL) detection kit (Millipore) and visualised with CL-XPosure Film (Thermo Scientific).

Results

Fetuses and kittens

One or two fetuses were collected from each of 11 pregnant feline uteri (total $n = 12$ fetuses). The crown-rump lengths of 12 fetuses used for histology were 75–135 mm; from a regression line of gestational age as a function of crown-rump length (Johnson et al., 2004), fetal ages ranged from 40 to 66 days, a period that includes the last 3 weeks of pregnancy in cats; estimates of embryonic ages (E) were 40 ($n = 2$), 43 ($n = 1$), 44 ($n = 2$), 48 ($n = 1$), 52 ($n = 2$, same uterus), 54 ($n = 2$, same uterus), 60 ($n = 1$) and 66 ($n = 1$) d for the harvested fetuses. All fetal cerebella tested (one cerebellum from each of E40, E43, E44, E48, E60 and E66; two cerebella for each of E52 and E54), as well as the three postnatal cerebella (2 days, 3 weeks and 2 months of age) and the cerebellum and intestine from an uninfected adult cat (negative control), were negative for FPV VP1 and VP2 gene products by PCR. A 214 bp histone 3.3 gene product was amplified from all samples, indicating successful extraction of DNA. The positive control intestinal sample was positive by PCR for VP1 (193 bp) and VP2 (215 bp) (see Appendix A: Supplementary Fig. 1).

Western blot analysis

WB analysis of proteins extracted from feline fetal cerebellum and CrFK cells demonstrated a single band at 99 kDa with the anti-TrFR1 antibody (Fig. 1d), 27 kDa with the anti-$p27^{Kip1}$ antibody (Fig. 2b) and 36 kDa with the anti-PCNA antibody (Fig. 3b).

Immunohistochemistry for transferrin receptor 1

Expression of TrFR1 was evident in cerebellar capillary endothelial cells at all stages of gestation and postnatal ages investigated (Fig. 1a). Immunolabelling of TrFR1 in Purkinje cells was evident only in the 3-week-old kitten, in which speckled staining of the cytoplasm of most Purkinje cells was observed (Fig. 1b); this was similar to the labelling of epithelial cells of the intestinal crypts of Lieberkühn from a normal adult cat (Fig. 1c). Positive immunostaining for TrFR1 was not observed in Purkinje cells of fetuses, the 2-day-old neonatal kitten or the 2-month-old kitten. TrFR1 expression was not observed in EGL cells at any stage of gestation or postnatal age investigated.