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Survivin prevents apoptosis by binding to caspase-3 in astrocytes infected with the BeAn strain of Theiler's murine encephalomyelitis virus

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Abstract This paper reports the upregulation of the gene coding for the apoptosis regulator family member "surviving" in SJL/J mouse brain astrocyte cultures infected with the BeAn strain of TMEV. cRNA from mock- and TMEV-infected SJL/J astrocytes was hybridised to an Affymetrix whole murine genome DNA microarray. Analysis revealed the upregulation of two sequences coding for the survivin protein in infected cells; this was confirmed by RT-PCR and qPCR. Western blotting showed an increase in the synthesis of survivin and caspase-3 after infection. Unexpectedly, no enzymatic activity was detected in BeAn-infected cell lysates in caspase-3-specific colorimetric assays. Cross-linking experiments showed survivin and caspase-3 to exist as a complex containing one molecule of caspase-3 (17 kDa) and one of either 16 kDa or 14 kDa survivin. The neutralization of caspase-3 by survivin-containing lysates was demonstrated using recombinant caspase-3. Brains from TMEV-infected mice, but not from naïve mice, contained survivin mRNA during the acute phase of encephalitis. The present results suggest that astrocytes infected by the BeAn strain do not undergo apoptosis due to the production of survivin.

Keywords TMEV infection · Astrocytes · Apoptosis · Survivin · Caspase-3

Introduction

A number of DNA and RNA viruses trigger apoptosis in a range of cells, e.g., Herpex simplex virus (Puttur et al. 2010; Vanden Oever and Han 2010), Semliki forest virus (Barry et al. 2010), human cytomegalovirus (McCormick et al. 2010), avian influenza virus H5N1 (Ueda et al. 2010) and Ebola virus (Bradfute et al. 2010). Apoptosis in astrocytes can be caused by Moloney murine leukaemia virus (Liu et al. 2004), human acquired immunodeficiency virus (HIV) (Eugenin et al. 2007), the H1N1 and H5N1 influenza viruses (Wang et al. 2008) and Theiler's murine encephalomyelitis virus (TMEV). TMEV is a member of the family Picornaviridae that naturally infects the mouse central nervous system (CNS; Theiler 1937). When injected intracerebrally into genetically susceptible mouse strains, the low-neurovirulence BeAn strain of TMEV induces a chronic demyelinating disease reminiscent of human multiple sclerosis (MS), and in so doing, provides a classical animal experimental model for this disease (DalCanto and Lipton 1975). Mouse astrocytes possess some 2.5×10^3 receptors for TMEV on their surfaces (Sierra and Rubio 1993) and, along with and other glial cells and macrophages, provide a cell population in which these viruses can persist (Brahic et al. 1981; DalCanto and Lipton 1982; Zheng et al. 2001; Trottier et al. 2004). This persistence could be due to the induced production of survivin by the infected cells, preventing their apoptosis—a mechanism that might underlie MS in human patients. The present work examines whether survivin is upregulated in mouse astrocytes infected with the BeAn strains of TMEV.

Materials and methods

Viruses and infections

The virus strains used in the present work were the high-neurovirulence TMEV strain GDVII and the demyelinating BeAn 8386 strain. BHK-21 cells were grown at 37 °C in

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Dulbecco's modified Eagle medium (DMEM) containing 10 % FCS and gentamicin (Gibco BRL, Paisley, Scotland). Cell cultures were infected, incubated at 33 °C for 48 h and then subjected to sonication and centrifugation at 4 °C to remove cell debris. Supernatant stock titres (10^8 plaque-forming units [PFU]/mL) for both viruses were determined by standard plaque assay using 1 % Noble agar (Difco Laboratories, Detroit, MI) and staining with 0.2 % crystal violet in 20 % methanol.

Purified astrocytes were infected with one or the other TMEV strain in 75 cm^2 tissue culture flasks, using several multiplicities of infection (MOI). Infections were allowed to take place at room temperature over a period of 1 h. After infection, the cells were washed with 10 mL of DMEM plus 10 % FCS and the flasks incubated at 37 °C for different periods of time. Cells used for mock infections were incubated with a virus-free BHK-21 cell lysate.

Astrocyte cultures

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex of newborn SJL/J mice purchased from Harlan Laboratories (Indianapolis, IN) and maintained at the *Instituto Cajal* in specific pathogen-free conditions following USA National Institutes of Health guidelines. All experiments were approved by the Experimental Animal Use Committee of the *Instituto Cajal*. The cerebral cortex was isolated under a dissecting microscope and cleaned of the choroid plexus and meninges. Cell suspensions were filtered through a 135-μm pore size mesh into DMEM containing 10 % foetal calf serum (FCS) and gentamicin (Gibco BRL, Paisley, Scotland). After centrifugation, cells were filtered through a 40-μm nylon cell strainer (Falcon-Becton Dickinson, Le Pont De Claix, France) and cultured in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) at 37 °C. The medium was changed after 4 days of culture and twice per week thereafter. Cultures were enriched for astrocytes by the removal of the less adherent microglia and oligodendrocytes by shaking overnight at 37 °C at 250 rpm in a table top G 24 shaker (New Brunswick Scientific Co. Edison, N.J.). Cellular confluence was observed 10 days after plating, producing around 1×10^7 cells per flask; all showed a polygonal, flat morphology. A mean content of 98 % astrocytes was determined by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (Dakopatts, Glostrup, Denmark). The lack of noticeable mature oligodendrocytes or microglial/macrophage cells was confirmed using a guinea pig anti-myelin basic protein (MBP) antiserum and monoclonal anti-Mac-1 antibodies (Serotec, Oxford, UK; Rubio et al. 2003). Secondary fluorescein-labelled antibodies were purchased from Sigma Chemical Co (St. Louis, MO).

cRNA target preparation, hybridisation and data analysis

Three independent culture replicates of sham-infected or BeAn-infected (MOI=1) SJL/J astrocytes were harvested 8 h post-infection, washed with phosphate-buffered saline (PBS) and total RNA isolated using TRIzol reagent (Gibco BRL). This was followed by a further purification round using the RNeasy Mini Kit (Qiagen, Valencia, CA.). Ten micrograms of RNA were converted to cDNA using the SuperScript Choice System kit (Gibco BRL). Second-strand synthesis was performed using T4 DNA polymerase and cDNA isolated by phenol-chloroform extraction. Isolated cDNA was transcribed using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY) with biotin-labelled UTP and CTP to produce biotin-labelled cRNA. Labelled cRNA was isolated using the RNeasy Mini Kit and fragmented in 100 mM potassium acetate-30 mM magnesium acetate-40 mM Tris–acetate (pH 8.1) for 30 min at 94 °C. Hybridisation performance was analysed using Test 2 arrays (Affymetrix, Santa Clara, CA) employing spike and housekeeping controls. Target cRNA was hybridised to the murine genome U74v2 microarray (Affymetrix) according to the manufacturer's recommendations. Briefly, 15 μg of fragmented cRNA was hybridised for 16 h at 45 °C with constant rotation (60 rpm). The microarrays were then washed and stained with streptavidin-conjugated phycoerythrin (SAPE) using an Affymetrix GeneChip Fluidic Station 400 apparatus. All hybridisation steps were performed by Progenika (Derio, Spain). Each gene on the U74v2 array was represented by 20 different 25-base cDNA oligonucleotides complementary to a cRNA target transcript (perfect match). As a hybridisation specificity control, an oligonucleotide containing a single base substitution corresponding to each perfect-match cDNA oligonucleotide (mismatch) was also represented on the array. Using Affymetrix-defined absolute mathematical algorithms describing perfect-match and mismatch intensities, each gene was defined as absent or present and assigned a binding intensity value. Virus-induced changes were quantified by the signal log ratio corresponding to $\log_2$ of the fold change for each gene. Standard errors for the mean transcriptional expression of genes were also calculated.

RT-PCR analysis of survivin and caspase-3 expression in infected cells

Total RNA from mock- or TMEV-infected astrocyte cultures was purified using the RNeasy Mini Purification Kit (Qiagen). Samples were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (RT; Promega,

Madison, WI). The primers used for mouse survivin and caspase-3 amplification were synthesized by Sigma-Aldrich (Tres Cantos, Spain).

Survivin:

Sense (5′-TCGCCACCTTCAAGAACTGGCCCTTCCTGGA-3′)
Antisense (5′-GTTTCAAGAATTCACTGACGGTTAGTTCTT-3′)

The sense primer corresponds to the srv86 primer and the antisense to the srvas311 primer described by Conway et al. (2000). The generation of a 225-bp amplicon was expected.

Caspase-3: (Gene Bank accession number NM_009810)

Sense (5′-TGGACGCAGCCAACCTCAGA-3′)
Antisense (5′-GCATACAGGAAGTCAGCCTC-3′)

It was expected that these primers would generate an amplicon of 382 bp (Lee et al. 2008).

A primer set for mouse β-actin, used as a house keeping positive control, was also supplied by Sigma-Aldrich:

Sense (5′-GTGGGCCGCCCTAGGCACCA-3′)
Antisense (5′-CTCTTTGATGTCACGCACGATTTC-3′)

The cycling conditions for the RT-PCR reactions were: 5 min at 94 °C followed by 30 cycles of 40 s at 55 °C plus 1 min at 72 °C, and a final 10 min extension period at 72 °C. All reactions were performed in a Perkin Elmer Cetus 480 DNA thermocycler. The resulting PCR products were purified using S-400 MicroSpin columns (Pharmacia Biotech, Uppsala, Sweden) and electrophoresed in 2 % NuSieve agarose gels (FMC Bio Products, Rockland, ME) in TAE buffer, stained with ethidium bromide and photographed in a Gelstation system (TDI, Barcelona, Spain). The DNA molecular weight markers used were produced by the digestion of DNA-MspI by pBR322 (New England Biolabs, Beverly, MA).

Quantitative real-time RT-PCR

Total RNA was extracted from mock- or TMEV-infected astrocyte cultures or from brains of uninfected or intracranially TMEV-infected mice, using the RNeasy Mini purification kit (Qiagen). cDNA was prepared from RNA using Moloney murine leukemia virus RT (Promega) and the above 3′ antisense primers. Quantitative RT-PCR (qPCR) was performed using an ABI PRISM 7000 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). The primers for survivin, caspase-3 and the control house keeping gene β-actin were the same as those used in RT-PCR. qPCR was performed in 20 μL reaction volumes using the Sybr® Green PCR Master Mix from Applied Biosystems, following the manufacturer's instructions. All reactions were performed in triplicate. The standard curves were generated using serial dilutions of cDNA. Survivin and caspase-3 expression were normalized to β-actin expression using the standard curve protocol provided by the manufacturer. Data were analysed using 7000 System SDS software (Applied Biosystems) and the results expressed as mean± SEM.

Western blots

Mock- and TMEV-infected SJL/J mouse astrocyte cultures were collected with trypsin-EDTA (Gibco BRL) using a disposable cell scraper (Costar) and centrifuged in a conical tube for 10 min at 250 g. The supernatant was removed and the pellet solubilized with the lysis buffer provided in the caspase-3 Colorimetric Assay Kit from R&D Systems Inc. (Minneapolis, MN), at a ratio of 25 μL per 1×10^6 cells. After centrifugation and electrophoresis on 12 % polyacrylamide gels, separated proteins from cell lysates were transferred to nitrocellulose membranes and blocked with 10 % skimmed milk in Tris buffer. Membranes were incubated with either rabbit anti-survivin antibody (Catalogue # 2803) or rabbit anti-cleaved caspase-3 (Catalogue # 9661), both from Cell Signaling Technology Inc. (Danvers, MA). All primary antibodies were diluted 1:1,000. Stripped membranes were reprobed with a mouse anti-GAPDH monoclonal antibody (MAB374, Chemicon-Millipore, Billerica, MA) as a loading control. After washing in Tris–HCl, pH 7.4, containing 0.1 % Tween-20, the membranes were incubated for 2 h at room temperature with the secondary antibodies, i.e., rabbit anti-mouse IgG or goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA), diluted 1:10,000 and both labelled with horse radish peroxidase. After three more washes with Tris–HCl buffer containing 0.1 % Tween-20, immunodetectable bands were visualized by chemiluminiscence (ECL, Amersham Biosciences, UK). Bands were quantified by computing densitometry using a Molecular Dynamics 300A densitometer (Sevenoaks, Kent, UK). The molecular weights of the bands were determined using a Dual Color Precision Plus Protein Standard Ladder Kit from BioRad (Hercules, CA).

Chemical cross-linking and PAGE electrophoresis

The chemical cross-linking homobifunctional reagent disuccinimidyl suberate (DSS, Sigma, Catalogue number: S1885) was used to study the interaction of survivin and caspase-3 molecules in astrocyte cell homogenates. Twenty microlitres of homogenate from astrocytes infected at an MOI of 1 for 8 h were incubated for 2 h on ice with a freshly prepared solution of DSS in DMSO, to reach a final reagent concentration of 1 mM. The samples were then monitored by electrophoresis on SDS-12 % polyacrylamide

gels and Western blotted as above with anti-survivin and anti-cleaved caspase-3 antibodies.

Caspase-3 assay

Caspase-3 activity was determined in astrocyte cell lysates using the caspase-3 Colorimetric Assay Kit from R&D (Catalogue number: BF3100) according to the manufacturer's protocol. Briefly, infected cell cultures were washed with ice cold PBS, lysed with cell lysis buffer, centrifuged at 15,000×*g* for 20 min and assessed for caspase-3 activity in 96-well polystyrene plates (Nunc-Immno plates, Nunc, Roskilde, Denmark) based on absorbance at 405 nm. Apoptosis-positive controls were obtained by incubating astrocyte cultures with 200 nM staurosporine (Sigma, Catalogue number: S4400) for 6 h. Recombinant human caspase-3 (rcasp-3; R&D Systems, Catalogue number: 707-C3) was used as an additional positive control at a concentration of 50 ng per well. To demonstrate the specificity of the staurosporine treatment and the rcasp-3 controls, both were inhibited with 50 μM of the irreversible caspase inhibitor Z-VAD-FMK (R&D Systems, Catalogue number: FMK001).

In vitro inhibition of caspase-3 by cell extracts

Cell extract rcasp-3 concentrations were determined by titration using the caspase-3 Colorimetric Assay Kit. Recombinant molecule activity was inhibited by increasing amounts of mock-, BeAn- or GDVII-infected astrocyte lysates (2 mg of total protein/mL). The inhibitory activity of the cell lysates on rcasp-3-produced absorbance at 405 nm was neutralized by preincubation with a monoclonal anti-survivin antibody (Cell Signaling Technology Inc., Catalogue number: 71G4B7).

Immunocytochemistry and TUNEL assays

Ninety-eight percent pure astrocyte monolayers in culture chambers (Lab-Tek Chamber slide, Nunc, Neperville, IL) were incubated with rabbit anti-glial fibrillar acidic protein (GFAP) (Dakopatts, Golstrup, Denmark) diluted 1:1,000. After several rinses in PBS, goat anti-rabbit Cy-3-conjugated antibody (Amersham Biosciences) diluted 1:1,000 was added. Apoptotic cells were detected using the TUNEL method. Samples were processed for this using the Fluorescein *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany, Catalogue number: 1684817) following the manufacturer's instructions. All preparations were examined using a Leica TCS NT confocal laser scanning microscope equipped with an argon/krypton-mixed gas laser with an excitation peak of 647 nm for Cy-3 and 488 nm for TUNEL staining. Specificity was controlled by omission of the primary antibody or the terminal deoxynucleotidyl transferase (TdT) in the first step of TUNEL labelling. To determine the total number of cells, the nuclei were labelled with propidium iodide (1 mg/mL in PBS; Sigma).

In vivo expression of survivin mRNA

Six-week-old male SJL/J mice from Harlan Laboratories were infected by intracerebral injection and infection allowed to proceed for 12 days. They were anaesthetized with Fluothane® and their brains, testes and spleens removed and washed in PBS for total RNA purification (RNeasy Mini Kit; Qiagen). For the intracerebral inoculations, 20 μL of a suspension of the BeAn virus (2×10^6 PFU) was injected into the right cerebral hemisphere using a 25-μL Hamilton syringe. qPCR was performed with the above samples using five animals per time point. RT-PCR was performed as for astrocyte culture RNA.

Results

TMEV-induced alterations in gene expression

The purpose of the present DNA hybridisation assays was to study the transcriptional response of astrocytes to the perturbation of cell regulation induced by BeAn infection. The SE437 and SE438 Affymetrix GeneChip microarrays revealed two upregulated sequences: 98433at and 102734at (Table 1). These sequences had the Gene Ontology Database hit description of "Apoptosis regulator", the Reference Sequence description of "Death agonist" and the SCOP (Structural Classification of Proteins) description of "Anti-apoptotic protein survivin" (Table 1).

Sequence 102734at showed only marginal expression in sham-infected astrocytes. The latter sequence and 98433at showed a 3.0- and 5.6-fold upregulation in BeAn-infected cells respectively. These sequences were located in mouse chromosomes numbers 6 and 9 (Table 1). No evidence of any change in caspase-3 regulation was seen in any microarray analysis. The DNA array results therefore seem to indicate an overexpression of survivin-related proteins in BeAn-infected astrocytes.

Survivin and caspase-3 RNAs in astrocytes

The results of the microarray analyses were compared with those provided by RT-PCR and qPCR. The specific mouse survivin and caspase-3 primer pairs were used to RT-PCR amplify two fragments in cultures infected with the BeAn strain at MOIs of 1 and 10 (Fig. 1). This was performed 8 h after infection.

In BeAn-infected cells, qPCR showed survivin mRNA synthesis at MOI of 1 and 10 to be 12-and 13-fold that seen

Table 1 Infection-upregulated sequences described as belonging to the Apoptosis inhibition or survivin family genes by the Gene Ontology database and others

Affymetrix sequence	Sham-infected astrocyte signal	TMEV-infected astrocyte signal	Signal log ratio	Fold change	Chromosome location	Gene Ontology	Reference sequence	Pathways	SCOP
98433 at	12.1 Absent	89.6 Present	2.8	5.6	6	Apoptosis regulator	Death agonist	Apoptosis	–
102734 at	60.0 Marginal	191.9 Present	1.5	3.0	9	Apoptosis inhibitor	Apoptosis inhibitor	–	Anti-apoptotic protein Survivin

All changes were significant based on triplicate analysis of signal log ratios. Standard errors in the mean expression (transcriptional) of each gene were calculated

SCOP "Structural Classification Of Proteins" database

in mock-infected cells respectively ($P<0.05$; Fig. 2, left panel; white bars). GDVII infection, however, induced no survivin mRNA synthesis (black bars). Caspase-3 production increased with MOI ($P<0.05$) in infections with both virus strains (Fig. 2, right panel). These qPCR expression increases were higher than those obtained by the microarray DNA hybridisation assay.

Western blot quantification of survivin and caspase-3

Figure 3 shows a representative Western blot of survivin from uninfected astrocytes (MOI=0) and from those infected at different MOI (0.1–10) with the BeAn virus. A Western blot of the housekeeping GAPDH gene product is also shown as a control for equal protein loading. The percentage optical density of the bands was quantified by densitometry and is shown as a bar chart at the bottom of the figure.

The 32 kDa precursor protein of caspase-3 undergoes proteolytic cleavage, mainly through the action of caspase-9, producing a final 17 kDa subunit that contains the enzyme active site (Li et al. 1997; Liu et al. 2004; Youle and Strasser 2008). The rabbit antibody used in these Western blots, directed to the 17 kDa caspase-3 cleaved product, does not recognize full length procaspase-3 or other precursor subunits. The presence of caspase-3 protein was significantly enhanced when the amount of virus used to infect astrocytes was increased, as shown in Fig. 4 (upper panel and bottom bar chart). These findings indicate that active caspase-3 is increasingly produced by astrocytes in response to TMEV infection.

Lack of caspase-3 activity

A colorimetric assay was used to measure the activity of rcasp-3, as well as caspase-3 activity induced in astrocyte cultures treated with staurosporine (STAU; both used as positive controls; Fig. 5). Activity was completely inhibited by the irreversible caspase inhibitor Z-VAD-FMK (Z-VAD). The absorbance obtained for the positive controls was roughly the same as that produced by cell lysate from astrocytes infected with the highly neurovirulent GDVII strain (Rubio et al. 2003).

Infection of astrocytes at different MOIs with the BeAn strain produced no detectable enzymatic activity in the corresponding colorimetric assay (Fig. 5). The samples showed the same absorbance at 405 nm as the uninfected control (0), despite the presence of caspase-3 proteins being previously demonstrated by Western blotting (Fig. 4). This suggests that caspase-3 molecules are neutralized by the survivin present in cell lysates (Fig. 3). This hypothesis was tested using cross-linking and in vitro inhibition experiments.

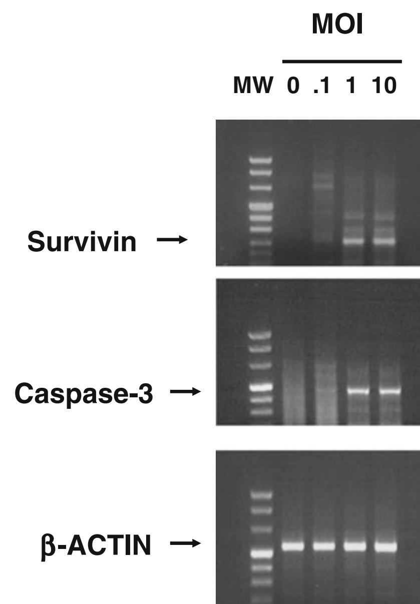

Fig. 1 Expression of survivin and caspase-3 mRNA in BeAn-infected astrocytes. Total RNA from astrocytes mock-infected (0) or infected at an MOI of 0.1–10 was reverse transcribed and PCR-amplified (at 8 h post infection [hpi]) using the primers for survivin, caspase-3 or β-actin (housekeeping gene control). *Lane MW* DNA molecular weight markers. Experiments were repeated three times and gave the same results

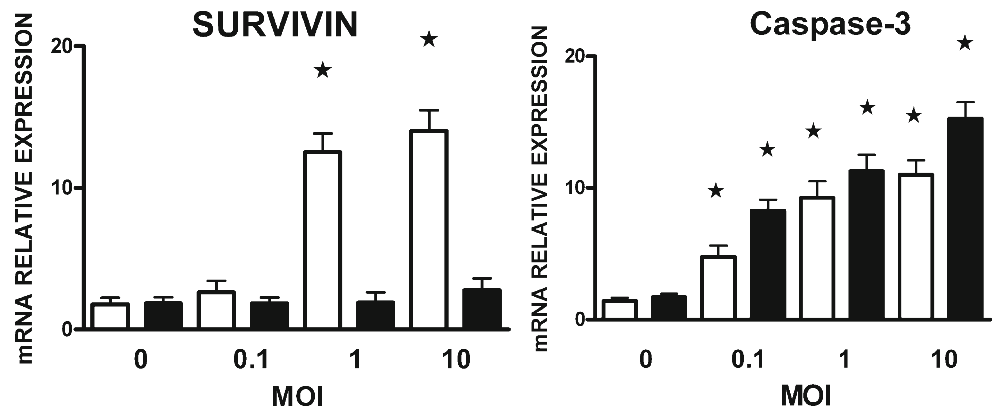

Fig. 2 Survivin and caspase-3 mRNA levels in astrocytes infected at different MOIs, as determined by qPCR. Total RNA was purified at 8 hpi. Data represent the mean±SD of experiments performed in triplicate. *Significantly different ($P<0.05$) to the mock infected control group (0), as determined by the Student *t* test. *White bars* BeAn-infected astrocytes RNA, *black bars* GDVII-infected astrocytes RNA

Cross-linking experiments

Cell extracts from astrocytes infected with the BeAn strain (MOI of 1), and containing both caspase-3 and survivin (Figs. 3 and 4), were subjected to cross linking experiments. The homobifunctional reagent disuccinimidyl suberate (DSS), which has amine reactivity, was used for cross linking. Western blotting of the linked samples was undertaken using specific anti-survivin (Fig. 6, left panel) or anti-caspase-3 antibodies (right panel). Dramatic auto cross-linking of two or more survivin molecules (due to the DSS cross-linker effect) was detected (the large dot in the upper part of left panel). This was probably due to the high percentage (14 %) of amine-rich lysine and arginine residues in the survivin molecule (Muchmore et al. 2000).

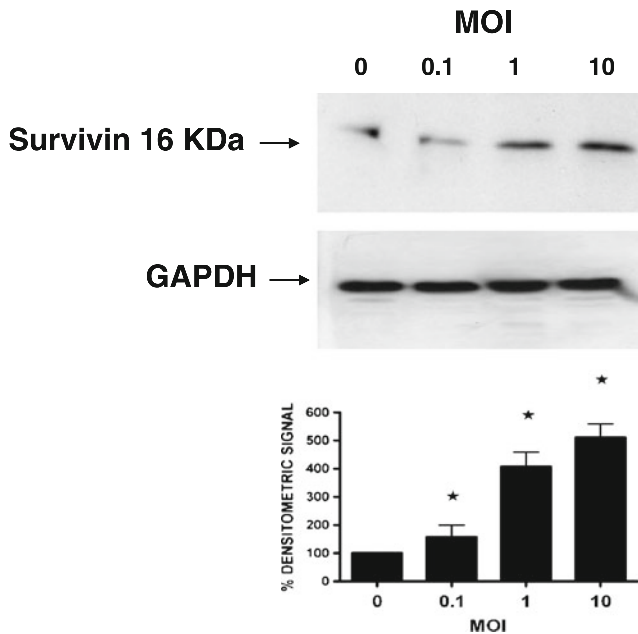

Fig. 3 SDS-PAGE and Western immunoblot analysis (with anti-survivin antibodies) of cell lysates from uninfected astrocytes (0) or cells infected at increasing MOIs (0.1–10; 8 hpi). Cell lysates from such astrocytes were boiled with SDS and examined by Western blotting as described in the "Materials and methods" section. A positive control with GAPDH was included to check the equal loading of the samples. The percentage density of the bands is shown in the bar chart at the *bottom* of the figure. Data represent the mean±SD of samples from blots repeated three times. *Significantly different ($P<0.05$) to the mock infected control group (0), as determined by the Student *t* test

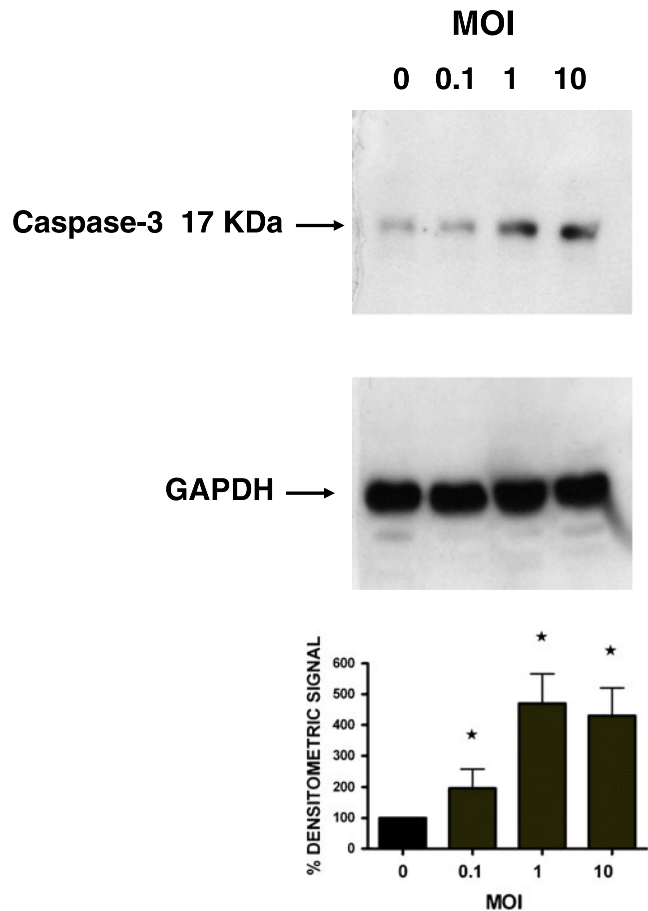

Fig. 4 SDS-PAGE and Western blot analysis of the cleaved, activated caspase-3 fragment (17 kDa) in cell lysates from uninfected astrocytes (0) or cells infected at different MOIs (8 hpi). A positive control with GAPDH was included to check for equal loading. The percentage density of the bands is shown in the bar chart at the *bottom* of the figure. *Significantly different ($P<0.05$) to the mock infected control group (0) as determined by the Student *t* test. The data represent the mean±SD of three samples

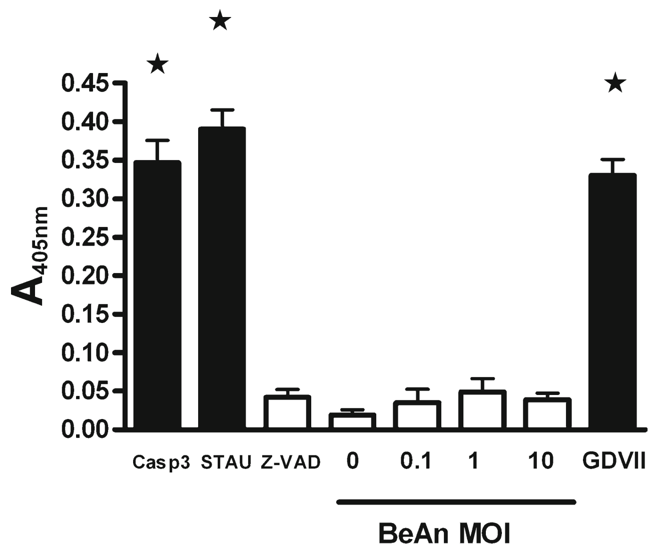

Fig. 5 Caspase-3 enzymatic activity of infected astrocyte lysates in colorimetric assays. Cells were mock-infected (0) or infected at different MOIs with the BeAn virus for 8 h. A positive activity control was provided by 50 ng of recombinant caspase-3 (Casp3). As further controls, cell cultures were treated with 200 nM staurosporine alone (STAU) or staurosporine plus 50 μM of the irreversible pan-caspase inhibitor Z-VAD-FMK (Z-VAD). A virus-specific control was also obtained by infecting cells with the GDVII strain (GDVII) at a MOI of 10. *Error bars* indicate the SD for three samples from three independent experiments. *Significantly different ($P<0.05$) to the mock infected control group (0), as determined by the Student *t* test

Neither survivin nor caspase-3 were detected in the 16–17 kDa range—where they would be expected to migrate if unlinked in the original cell extracts (Fig. 6, right). Rather, bands of 31–33 kDa were detected by both antibodies, corresponding to the cross-linking of caspase-3 (17 kDa) with the two described survivin forms (16 and 14 kDa; Conway et al. 2000; arrows). These results indicate that caspase-3 and survivin formed dimers with no enzymatic activity.

In vitro inhibition of rcasp-3 activity by survivin-containing extracts

Figure 7a shows the titration analysis of rcasp-3 activity. The limiting amount of 20 ng of recombinant enzyme detected was then used in inhibition experiments. Several dilutions of mock- or TMEV-infected astrocyte extracts were assessed for their effect on the enzymatic action of rcasp-3, detected as a reduction in absorption at 405 nm (Fig. 7b). The survivin-containing BeAn-infected extracts suppressed rcasp-3 activity in a dose-dependent manner. The absorbance plateau between the 100 and 50 μg BeAn-infected cell lysates is probably due to the high protein concentrations of the samples. Mock- and GDVII-infected astrocyte cell extracts contained no neutralizing survivin, as shown by the complete lack of inhibition of rcasp-3 (Fig. 7b).

Finally, anti-survivin monoclonal antibodies completely inhibited the rcasp-3 neutralizing capacity of the BeAn-infected cell extracts (Fig. 7c). This clearly shows that the above suppressive effect was mediated by survivin.

Identification of apoptotic astrocytes

Immunostaining of the astrocytes with antibodies to the GFAP astrocyte marker in the chamber slides revealed extensive staining of the cytoplasm (Fig. 8, right panels). TUNEL staining of cultures infected with the GDVII strain showed several green nuclei corresponding to cells undergoing apoptosis (Fig. 8, upper left panel). No staining was seen in cells infected with the BeAn virus in chamber cultures (Fig. 8, bottom left panel). Some cells showed plasma membrane blebbing and morphological features characteristic of apoptosis (Fig. 8, upper left panel). When treated by quantitative analytical imaging, mean percentages±S.E.M of TUNEL staining were 21.2 %±3.8 for GDVII- and 2.1 %±0.9 for BeAn-infected astrocytes, $p<0.01$ determined by the Student's *t* test.

TMEV induced survivin mRNA in infected SJL/J mouse brains

When total RNA from young, uninfected SJL/J mouse organs was extracted and RT-PCR amplified, only the spleens and testes—the organs with the most proliferative tissues—showed the spontaneous presence of survivin mRNA (Fig. 9, T and S). Brains were consistently negative (Fig. 9b). To investigate the possible pathological significance of these findings for the experimental model of MS, the survivin mRNA levels in the brains of infected animals were measured by qPCR at different times after intracerebral

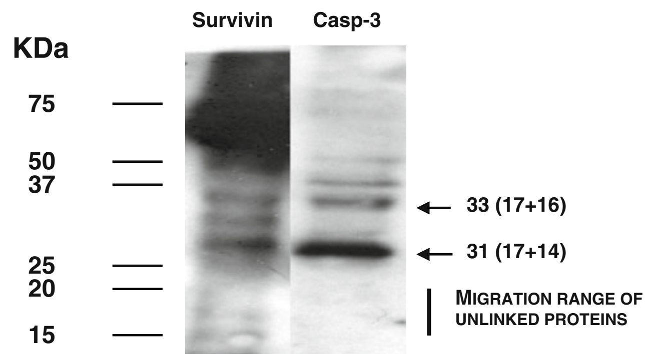

Fig. 6 Cross-linking of TMEV-infected astrocyte lysates (MOI=1; 8 hpi) using disuccinimidyl suberate (DSS). The left blot was developed with anti-survivin antibody, the right with anti-caspase-3 antibody. The molecular weights of protein standards ladder are shown on the *left*. The 33 and 31 kDa positions (survivin-caspase-3 dimers) are shown by the *arrows at the right*. The expected approximate migration of the 16 kDa survivin and of the 17 kDa caspase-3 unlinked proteins is also shown on the *right*

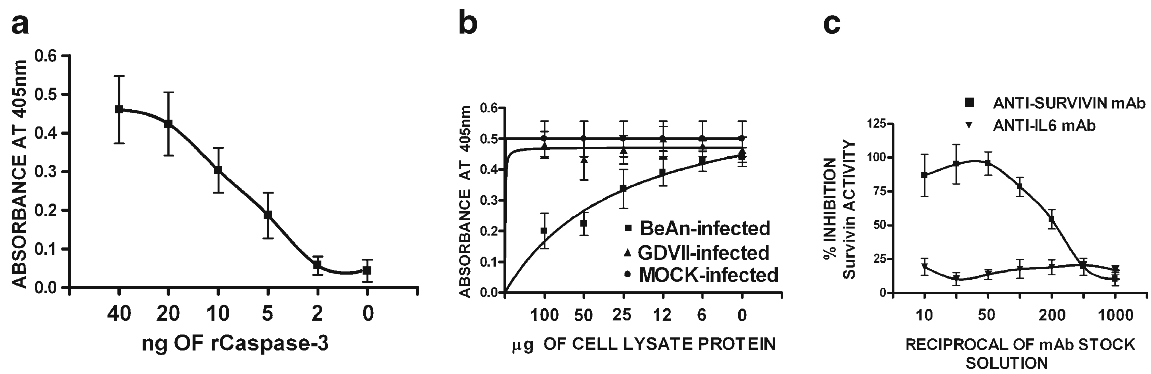

Fig. 7 Inhibition of recombinant caspase-3 activity by survivin-containing lysates. In **a**, rcaspase-3 enzymatic activity was determined by titration, measuring the absorbance of the colour produced at 405 nm. In **b**, 20 ng of rcaspase-3 per well were preincubated for 30 min at 37 °C with different amounts of BeAn-, GDVII- (MOI=1) or mock-infected astrocyte lysates. In **c**, the inhibition demonstrated by the BeAn-infected lysates in **b** was neutralized by increasing amounts of specific anti-survivin mAb antibody. A negative control isotype mAb was also included (anti-interleukin 6 mAb antibody)

injection of BeAn. A time course increase in survivin mRNA was detected, peaking on day 5 post injection and then decreasing to non-significant levels (see box plot at the bottom of Fig. 9). This agrees with the viral replication reported by other authors (Trottier et al. 2004) (peaking on day 5 post infection, declining by 1,000-fold on days 10–11, as determined by qPCR).

Discussion

Apoptosis is a highly controlled phenomenon that eliminates unwanted or damaged cells in multicellular organisms (Vaux and Korsmeyer 1999). The low-neurovirulence BeAn strain of TMEV has been shown to induce apoptosis in restrictive cell lines but not in permissive cell lines (Jelachich and Lipton 1996) nor in SJL/J mice astrocytes (Zheng et al. 2001; Rubio et al. 2003). The survival of these cells may be due to the induced production of survivin. In contrast, the high-neurovirulence strain GDVII causes devastating encephalitis *in vivo* (DalCanto and Lipton 1975), possibly because no survivin production is induced. The present results provide evidence that supports this hypothesis.

Mammalian survivins are members of the “inhibitors of apoptosis” (IAP) protein family, and *in vitro* bind to and

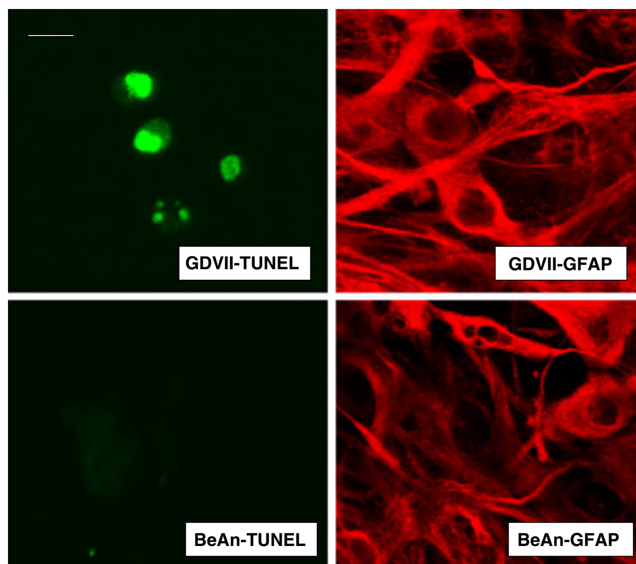

Fig. 8 Confocal images from three independent astrocyte cultures infected for 8 h at an MOI of 10 with GDVII or BeAn virus. Staining for TUNEL (*left panels*) or GFAP (*right panels*) were shown. *Scale bar*, 10 µm

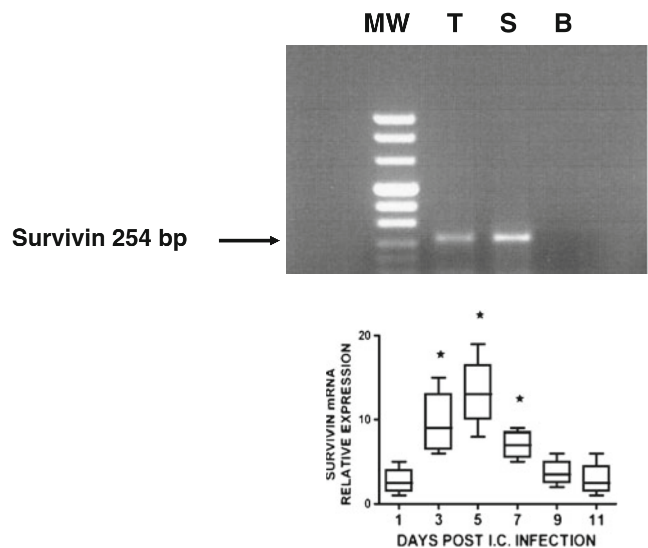

Fig. 9 Tissue distribution of survivin mRNA detected by RT-PCR and qPCR. Upper panel: total RNA from testicles (T), spleens (S) and brains (B) was amplified by RT-PCR using survivin primers. The lower box plot shows the presence of significant amounts of virally-induced survivin mRNA in the brain, as demonstrated by qPCR. Five individual animal samples per time point were used in two different experiments. *Significantly different ($P<0.05$) to uninfected brains (sample B in the *upper panel*), as determined by the Student *t* test

inhibit caspase-3 when in molar excess (Uren et al. 1998; Conway et al. 2000). Human X chromosome-linked IAP protein inhibits 70 % of caspase-3 activity when in 2-fold molar excess (Deveraux et al. 1997). Neural progenitors are well protected from apoptosis by survivin expression (Gualco et al. 2010). Some viruses induce apoptosis while others attenuate or inhibit it—perhaps by inducing cellular survivin—to circumvent the host response of initiating apoptosis in infected cells (Brune 2011). Indeed, Cowpox virus, Baculovirus, Rotavirus and Marek's disease virus appear to go one step further and code for their own apoptosis-preventing IAPs which strongly resemble those of the mammalian cells they infect (Thornberry and Lazebnik 1998; Cerio et al. 2010; Bagchi et al. 2010; Xu et al. 2011).

The infection of astrocytes from mouse strains susceptible to TMEV-induced demyelination causes the upregulation of a number of genes coding for cytokines, (Sierra and Rubio 1993; Palma et al. 2003), chemokines (Palma and Kim 2004; Rubio and Sanz-Rodriguez 2007), GTPases (Rubio et al. 2008), adhesion molecules such as VCAM-1 (Rubio et al. 2010a) and interferon α/β (Rubio et al. 2010b). The aim of the present work was to demonstrate that the BeAn strain, but not the GDVII strain, upregulates survivin in infected astrocytes, preventing their apoptosis. Since BeAn-infected astrocytes do not undergo apoptosis, the BeAn virus is not cleared from the spinal cord in infected SJL/J mice (Trottier et al. 2004). This may contribute to long-term, immune-mediated demyelination, thus providing the above-mentioned experimental analogue to MS. The present findings may therefore be important for the understanding of human neuroinflammatory diseases such as viral encephalitis or MS.

The present results, plus the fact that the activation of caspase-3 is required for the induction of encephalitis by Reovirus (Berkham et al. 2010) suggest that BeAn-infected astrocytes produce survivin that neutralize caspase-3. mRNA specific for survivin and the effector caspase-3 were produced in BeAn-infected cells (Figs. 1 and 2), and both proteins were present in cell lysates, as demonstrated by Western blotting (Figs. 3 and 4). In addition to the absence of any cytopathic criteria of apoptosis (e.g., increased nucleus size, DNA condensation, or the loss of the normal polygonal, flat cellular morphology etc.), Fig. 5 shows that that caspase-3 enzymatic activity was blocked in BeAn-infected astrocytes. Further, TUNEL and GFAP staining showed that while GDVII-infected astrocytes underwent apoptosis, those infected with the BeAn strain did not (Fig. 8).

The results of the cross linking experiments suggest that each caspase-3 molecule becomes bound to survivin in a 1:1 ratio in the cytoplasm of BeAn-infected astrocytes (Fig. 6). Figure 7 shows that rcasp-3-specific enzyme-colorimetric activity was progressively neutralised by increasing amounts of survivin-containing cytoplasmic BeAn homogenates; such neutralization was inhibited by anti-survivin antibodies.

mRNA for the apoptosis-inhibitor survivin was detected in testicles and spleen but not in uninfected brain tissue (Fig. 9). Interestingly, immunohistochemical studies performed on 20 brain samples from humans with progressive multifocal leukoencephalopathy (PML) have also shown the upregulation of survivin in astrocyte cultures infected with the neurotropic JC polyomavirus (Piña-Oviedo et al. 2007). Nonetheless, astrocytes cannot be declared the only brain cell type infected between 1 day and 12 days post infection, despite the fact that immunochemical analyses have shown most of the astrocytes in the brains of mice intracerebrally infected with the BeAn strain to carry heavy viral loads (Zheng et al. 2001).

Together, the present results suggest that the high-neurovirulence GDVII strain has no ability to prevent apoptosis; *in vivo* this strain causes severe, acute encephalitis. In contrast, in BeAn-infected cells, the induction of survivin neutralizes caspase-3 to prevent their undergoing apoptosis. Certainly, both TMEV strains induced casp-3, but GDVII did not induce the expression of survivin, as demonstrated by the absence of mRNA (Fig. 2). This explains how the BeAn strain can persist in infected cells; *in vivo*, the low neurovirulence and scant encephalitis associated with the BeAn strain is likely linked to this. Indeed, the percentage of TUNEL-positive nuclei in the astrocytes of BeAn-infected SJL/J mouse spinal cords is only 0.56 %±0.55 % (Schlitt et al. 2003). This persistence of the BeAn strain in the CNS of SJL/J mice, however, appear to induce slow, immune-mediated demyelination, providing us with an experimental animal model of human MS.

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