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Interaction between endoplasmic reticulum stress and caspase 8 activation in retrovirus MoMuLV-*ts*1-infected astrocytes

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Abstract

The murine retrovirus, MoMuLV-*ts*1, induces progressive paralysis and immune deficiency in FVB/N mice. We have reported previously that *ts*1 infection causes apoptosis in astrocytes via endoplasmic reticulum (ER) and mitochondrial stress (Liu, N., Kuang, X., Kim, H.T., Stoica, G., Qiang, W., Scofield, V.L., Wong, P.K.Y. Wong. 2004. Possible involvement of both endoplasmic reticulum- and mitochondria-dependent pathways in MoMuLV-*ts*1-induced apoptosis in astrocytes. J. NeuroVirol. 10, 189–198). In the present study, we show that caspase 8 activation in these cells is mediated through ER stress-associated elevation of death receptor DR5 and the C/EBP homologous protein (GADD153/CHOP), an ER stress-initiated transcription factor, rather than through TNFα and TNF-R1 interactions on the cell surface. Treatment with Z-IETD-FMK, a specific inhibitor of caspase 8 enzymatic activity, reduced ER stress by two mechanisms: by inhibiting caspase 8 activation, and by preventing cleavage of the ER-associated membrane protein BAP31 into BAP20, which exacerbates the ER stress response. These findings suggest that caspase 8- and ER stress-associated apoptotic pathways are linked in *ts*1-infected astrocytes.

Keywords: Endoplasmic reticulum stress; Caspase 8; Apoptotic pathways; Astrocytes; DR5; GADD153/CHOP; Retrovirus; BAP20

Introduction

The Moloney Murine Leukemia virus (MoMuLV)-*ts*1 retrovirus, a naturally occurring mutant of MoMuLV-TB, causes a neuroimmunodegenerative syndrome in FVB/N mice. Clinical manifestations of *ts*1 infection include hindlimb paralysis, wasting, and immunodeficiency (Wong et al., 1998). Pathological changes in the central nervous system (CNS) include spongiform degeneration, demyelination, gliosis, and neuronal loss (Stoica et al., 1993), all of which are also present in the brains of humans with a variety of neurodegenerative diseases, including HIV-associated dementia (Gonzales-Scarano et al., 1995), prion diseases (Diedrich et al., 1991), and amyotrophic lateral sclerosis (Mourelatos et al., 1994).

Amounts of tumor necrosis factor (TNFα) are markedly increased in astrocytes in spongiform lesions in the CNS in *ts*1-infected mice (Choe et al., 1998), and the TNFα pathway is implicated in the development of reactive astrocytes and astrocytic apoptosis in HIV-infected human brains (Elovaara et al., 1999). These observations suggested to us that TNFα may participate in retrovirus-induced neurodegeneration, but it was unclear how the TNFα pathway might contribute to these events in *ts*1 infection.

In CNS tissues of *ts*1-infected mice, astrocytes, microglia, oligodendrocytes, and endothelial cells are infected with *ts*1, but neurons are not (Stoica et al., 1993). As the most abundant cell type in the brain, and as the primary providers of neuronal support, astrocytes play a crucial role in the maintenance of normal neuronal function, especially by providing trophic and thiol redox compounds (Dringen and Hirrlinger, 2003; Furuya

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et al., 2000; Shih et al., 2003; Wang and Cynader, 2000; Wang and Cynader, 2001). Not surprisingly, astrocyte dysfunction contributes to neuronal death in many neurodegenerative diseases (Friedlander, 2003; Galey et al., 2003; Liu et al., 2004; Wong and Lynn, 1997).

In cultured murine astrocytes infected by *ts*1, the envelope precursor protein $gPr80^{env}$ accumulates in the ER (Shikova et al., 1993; Szurek et al., 1990). This causes an ER stress response and ER stress-associated apoptosis, as indicated by activation of the ER-resident transmembrane protein kinase PERK, hyperphosphorylation of eIF2α, upregulation of GRP78 and GADD153/CHOP, and cleavage activation of the procaspase 12 enzyme (Kim et al., 2005; Liu et al., 2004). In addition to ER stress-associated apoptosis, mitochondrial-driven apoptosis also contributes to astrocytic apoptosis caused by *ts*1 infection, both by mitochondrial transmembrane potential dissipation, and by caspase 9 activation (Liu et al., 2004). Prior to the present study, the mechanisms leading to mitochondrial stress had not been elucidated for *ts*1-infected astrocytes.

In mammalian cells, apoptosis can be activated through two distinct sets of cascades, which are the intrinsic or the extrinsic pathways. Intrinsic pathways involve either the ER stress response or mithochondrial-driven apoptosis. The ER stress response activates the ER membrane-associated caspase 12 enzyme, which triggers events culminating in apoptosis. Mitochondrial apoptosis is governed by the BCL-2 family of proteins (the anti-apoptotic proteins BCL-2 and $BCL\text{-}_{XL}$, and the pro-apoptotic proteins BAX and BAK). The mitochondrial apoptotic cascade activates mitochondrial membrane-associated caspase 9, resulting in release of cytochrome *c* and other apoptogenic proteins from the intermembrane space of the mitochondria into the cytosol. Extrinsic pathways are initiated at the cell surface, and involve the cell membrane death receptors DR3, DR4, DR5, or the prototype cell surface membrane death receptors, TNF-R1 and Fas, which are activated by the cytokine tumor necrosis factor-alpha (TNFα) and the Fas ligand (FasL), respectively. Apoptotic signals originating from these receptors are transmitted by death domains (intracellular regions of death receptors), converging in the activation of caspase 8, activation of downstream caspases, such as caspase 3, 6, and 7, and cell death (Ashkenazi and Dixit, 1998). In addition, activated caspase 8 cleaves Bid, a BH3-only protein that modulates functions of proteins in the Bcl-2 family. Bid cleavage generates tBid, which then translocates from the cytosol to mitochondria, changing mitochondrial membrane permeability and causing cytochrome release (Breckenridge et al., 2003). By these mechanisms, extrinsic apoptosis pathways intersect with intrinsic pathways via caspase 8.

In human cells, which lack functional caspase 12 (Fischer et al., 2002), it has been reported that caspase 8 participates in ER stress-mediated apoptosis (He et al., 2002; Momoi, 2004; Sheikh et al., 1998). Recent studies have shown that the transcriptional factor CHOP, when activated by the ER stress inducer thapsigargin, can enhance expression of the cell membrane death receptor DR5 by binding to the CHOP-binding site in the 5′-flanking region of the DR5 gene, which couples ER stress signals to a DR5/caspase 8-related apoptotic cascade (Yamaguchi and Wang, 2004). We have shown previously that intrinsic pathways are involved in *ts*1-induced astrocytic apoptosis (ER stress and mitochondrial stress), but it was not clear prior to the present study whether or not extrinsic pathways, involving TNFα, were also involved. We show here that the extrinsic TNFα pathway is not required for caspase 8 activation in *ts*1-infected astrocytes. Our data instead identify interactions between ER stress and caspase 8 in infected cells, including (1) *ts*1-induced ER stress and CHOP activation, which initiates the DR5/caspase 8-related apoptotic cascade, and (2) activated caspase 8 signals to the ER, by cleavage of BAP31 into BAP20 (a molecule that exacerbates the ER stress response).

Results

Caspase 8 cleavage and activation in ts1-infected astrocytes

In primary cultured astrocytes, increased activated caspase 8 (the p10 subunit product of procaspase 8 cleavage) was detected at 3 and 6 days after *ts*1 infection (Fig. 1), although amounts of procaspase 8 (p50) did not differ between control and *ts*1-infected cells. The p10 subunit band was identified by two different antibodies: anti-procaspase 8 from Biovision (which detects both procaspase 8 and activated caspase 8) and anti-activated caspase 8 from Oncogene (which detects activated caspase 8 only). Amounts of activated caspase 8 were also increased after *ts*1 infection of cells of the immortalized astrocyte line C1 (data not shown). C1 cells have been used in our previous studies on *ts*1 infection (Liu et al., 2002, 2004; Qiang et al., 2004).

Activation of caspase 8 in astrocytes is independent of TNFα

We have reported that TNFα production is elevated in astrocytes of *ts*1-infected mice (Choe et al., 1998). In most mammalian cell types, TNFα induces apoptosis by extrinsic pathways involving TNFα binding to TNF-R1, resulting in activation of caspase 8, and downstream events initiated by caspase 8 activation. It seemed likely, therefore, that *ts*1-

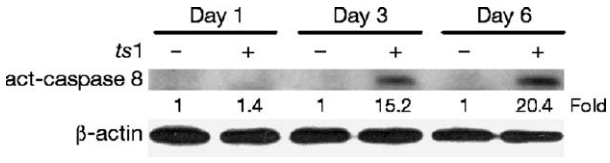

Fig. 1. Increased activated caspase 8 in primary cultured astrocytes induced by *ts*1 infection. Primary cultured astrocytes were infected by *ts*1 at MOI of 10 for 40 min in 100-mm dishes, and cells were harvested at various times after infection. Act-caspase 8 indicates activated caspase 8 (p10), and the times shown are days after infection. The same blots were stripped and immunoblotted with anti-β-actin antibody as a protein loading control. Blots shown are representative of four independent experiments. The bands in all the blots were scanned for densitometry analysis, normalized to β-actin, and the fold density shown under each band. Statistical analysis shows significant *ts*1-induced increases in activated caspase 8 levels at 3 and 6 dpi ($P < 0.005$).

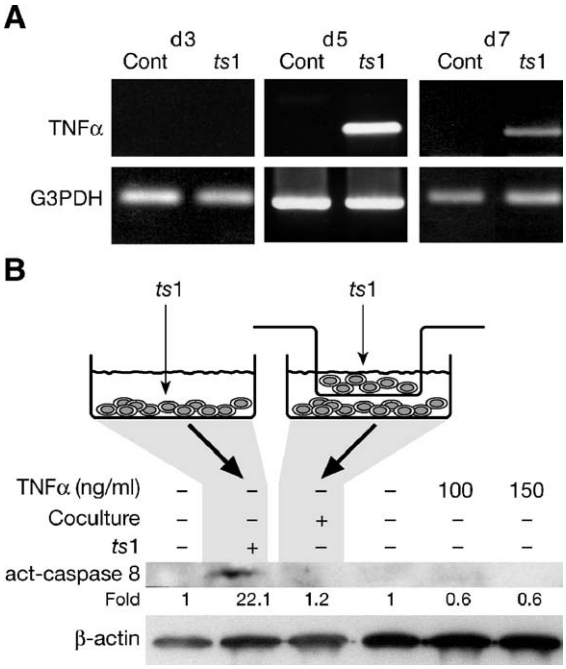

Fig. 2. Activated caspase 8 in uninfected primary cultured astrocytes vs. *ts*1-infected cells (as in Fig. 1), cocultured and after TNFα treatment. (A) Expression of TNFα was measured by RT-PCR of mRNA from *ts*1-infected astrocytes (*ts*1) and uninfected astrocytes (Cont) at 3, 5, and 7 dpi. RT-PCR of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to control for total mRNA loading. (B) The levels of activated caspase 8 in astrocytes with different treatments were measured by Western blotting. For *ts*1 infection, astrocytes were infected at an MOI of 10 for 40 min in 100-mm dishes, and cells were harvested at different time points after infection. For coculture, *ts*1-infected and uninfected control astrocytes were grown on Anopore culture inserts, and the inserts were placed on top of 100-mm dishes plated with a layer of uninfected astrocytes. After 7 days of coculture, the uninfected astrocytes in the bottom dishes were harvested for Western blotting. For TNFα treatment, astrocytes were plated in 100-mm culture dishes, and either left untreated or treated with 100 or 150 ng/ml TNFα. Cells were harvested after 7 days of treatment. Blots shown are representative of three independent experiments.

induced cleavage and activation of caspase 8 in cultured astrocytes might likewise be mediated by TNFα, which is produced by infected cells.

To test this possibility, we seeded primary cultured astrocytes into Anopore culture inserts, whose membranes are permeable to low-molecular-weight compounds, such as TNFα, but not to *ts*1 virus. After seeding, the cells were infected with *ts*1, and the inserts containing infected cells placed into culture dishes whose wells contained uninfected astrocytes. Fig. 2A shows that the peak time of TNFα mRNA production in infected astrocytes was 5 days after *ts*1 infection. In Fig. 2B, however, which shows data after 7 days of coculturing, the uninfected astrocytes in the bottom wells showed neither signs of apoptosis nor evidence of caspase 8 activation. Even when TNFα was added directly to the culture medium in contact with the uninfected astrocytes, at concentrations up to 150 ng/ml, neither cell death nor caspase 8 activation occurred (Fig. 2B). We conclude that activation of caspase 8 in *ts*1-infected astrocytes is due to intracellular responses to *ts*1 infection, rather than to extrinsic pathways requiring extracellular TNFα.

ER stress causes activation of caspase 8 via DR5 upregulation in infected astrocytes

From our earlier studies, we know that *ts*1 infection causes ER stress both in primary astrocytes and C1 cells (Kim et al., 2005; Liu et al., 2004; Qiang et al., 2004). We also know that *ts*1 infection induces subsequent mitochondria-driven apoptosis, as indicated by transmembrane potential dissipation and caspase 9 activation (Liu et al., 2004). However, the mechanism by which *ts*1-induced ER stress is linked to *ts*1-induced mitochondrial stress remained unclear. We therefore hypothesized that if caspase 8 activation is indeed initiated intracellularly, rather than by an extrinsic pathway involving TNFα (as suggested in Fig. 2), then ER stress might initiate this by upregulating CHOP and DR5 expression. Fig. 3A shows that primary astrocyte cultures did exhibit increasing levels of the ER stress markers GRP78 and CHOP, DR5, and increased activated caspase 8 level, by 3 days

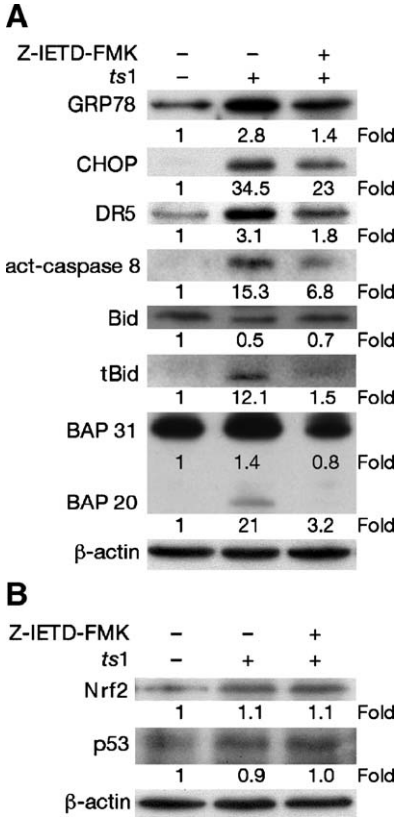

Fig. 3. ER stress is coupled to caspase 8 activation, and activated caspase 8 promotes the ER stress response, in *ts*1-infected astrocytes. (A) Levels of GRP78, CHOP, DR5, activated caspase 8, Bid, tBid, and BAP31 cleavage in astrocytes infected by *ts*1, with and without treatment with Z-IETD-FMK. (B) Levels of Nrf2 and p53 in astrocytes infected by *ts*1, with and without treatment with Z-IETD-FMK. Primary cultured astrocytes were infected by *ts*1 at MOI of 10 for 40 min in 100-mm dishes, and then treated with 2.5 μmol/L Z-IETD-FMK. Controls and treated cells were harvested 3 days after infection for Western blotting. The same blots were stripped repeatedly and immunoblotted with the series of antibodies. Blots shown are representative of three independent experiments. GRP78, CHOP, DR5, activated caspase 8, tBid, and BAP20 levels were significantly increased in infected vs. control cultures, and significantly decreased in infected cultures treated with Z-IETD-FMK ($P < 0.05$). There was no difference in Nrf2 and p53 levels between in infected untreated vs. infected+ Z-IETD-FMK-treated cultures.

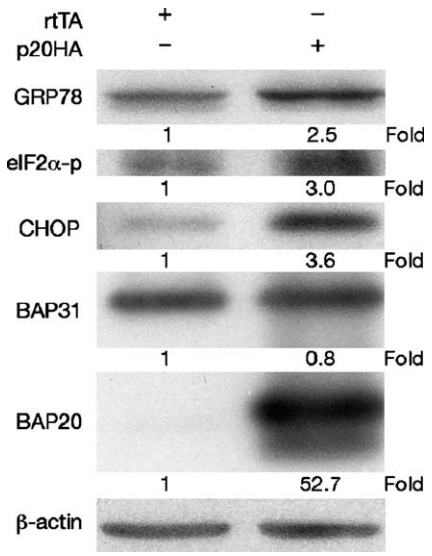

Fig. 4. Levels of GRP78, eIF2α phosphorylation, CHOP, BAP31, and BAP20 in C1 cells transfected with adenovirus p20HA (encoding BAP20) and rtTA (encoding control vector). C1 cells were plated into 6-well-plates, transfected with adenovirus at 100 pfu/ml, and harvested for Western blotting 48 h after transfection. The same blots were stripped repeatedly and immunoblotted with the series of antibodies. Blots shown are representative of three independent experiments. Amounts of GRP78, CHOP, phosphorylated eIF2α, and BAP20 were significantly increased in p20HA vs. rtTA (empty vector)-infected cells ($P < 0.05$). BAP31 levels did not differ between cultures receiving the empty vector or cultures overexpressing BAP20.

after infection with *ts*1. Notably, activated caspase 8 also caused Bid cleavage into tBid (Fig. 3A), an event known to induce cytochrome *c* release and caspase 9 activation, leading in turn to mitochondrial apoptosis (He et al., 2002; Sheikh et al., 1998). The data suggest that activated caspase 8 connects ER stress to mitochondrial stress, both of which are intracellular apoptotic pathways, following *ts*1 infection.

Z-IETD-FMK reduces the ER stress response

Activated caspase 8 is known to cleave the ER membrane-associated protein BAP31, and the cleavage product BAP20 increases mitochondria-driven apoptosis by elevating the influx of Ca^{2+} into mitochondria after its release from the ER (Chandra et al., 2004; Ng et al., 1997). However, no relationship between caspase 8 and ER stress has been reported previously. Fig. 3A shows that treatment of *ts*1-infected astrocytes with Z-IETD-FMK, a specific inhibitor of activated caspase 8, reduced both activated caspase 8 and BAP20 levels, in association with downregulation of the ER stress markers GRP78 and CHOP. This observation suggests that intact activated caspase 8 (in the absence of inhibitor) may promote and amplify the ER stress response. To exclude the possibility that Z-IETD-FMK globally inhibits protein levels, we also measured levels of the transcription factor NF-E2-related factor 2 (Nrf2) and p53, neither of which is associated with caspase 8 activation. Fig. 3B shows that there was no change in these proteins after Z-IETD-FMK treatment.

Z-IETD-FMK inhibits not only the catalytic activity of activated caspase 8, but also reduces the amounts of many cleaved procaspases in treated cells, thereby reducing the availability of the corresponding activated enzymes (Scoltock and Cidlowski, 2004). In addition to its indirect reduction of caspase 8 levels via ER stress, therefore (Fig. 3A), this direct effect of Z-IEDT-FMK may affect levels of caspase 8, as well as caspase 8 activity, in *ts*1-infected cells.

BAP20 overexpression induces the ER stress response

To determine whether activated caspase 8 promotes the ER stress response by increasing BAP20, we overexpressed BAP20 in C1 cells using adenovirus vectors either containing or lacking BAP20-encoding sequences. In these experiments, BAP20-transfected C1 cells (transfected with the p20HA vector) showed significantly increased death compared to control C1 cells transfected with the empty vector rtTA (data not shown). This is consistent with work by others showing that BAP20 overexpression promotes cell death (Breckenridge et al., 2003). Fig. 4 shows that the BAP-20-overexpressing cells also had elevated levels of the ER stress markers GRP78, CHOP, and phosphorylated eIF2α, compared to C1 cells infected with the empty vector, at 48 h after transfection, and that C1 cells overexpressing BAP20 exhibited elevation of BAP20 only, but not of BAP31 (confirming the specificity of transfection). Correlation of BAP20 overexpression with ER stress markers in these transfected cells implies that activated caspase 8 promotes ER stress in *ts*1-infected astrocytes by cleaving BAP31 to yield BAP20.

The absence of TNF-R1 does not change disease development in ts1 infection

In the CNS of *ts*1-infected mice, regions containing spongiform lesions show increased expression of TNFα (Choe et al., 1998). For this reason, and in light of the data presented above,

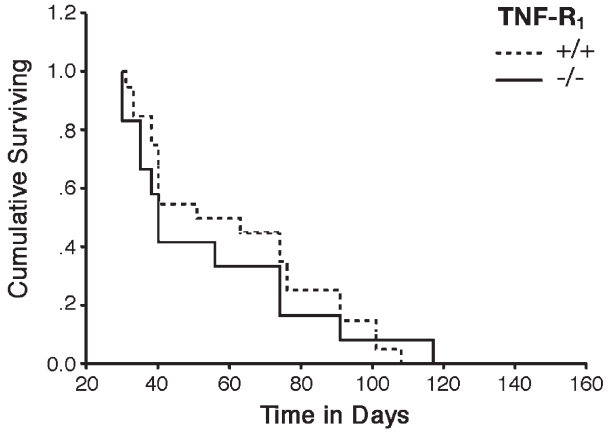

Fig. 5. Survival vs. time curves for homozygous TNF-R1 knockout vs. wild-type littermate mice infected at birth with *ts*1. 20 wild-type mice and 12 TNF-R1 littermate knockout mice were infected with *ts*1 and followed for construction of survival curves. The Kruskal–Wallis test identified no statistical difference between the knockout vs. wild-type mice.

we used *ts*1-susceptible TNF-R1 knockout mice (see Methods) to ask whether TNFα contributes to *ts*1-induced neurodegeneration in intact CNS tissues, even though it apparently does not do so in *ts*1-infected astrocyte cultures in vitro. As shown in Fig. 5, TNF-R1 knockout mice did not show an altered timecourse for disease development, compared to wild-type mice, following *ts*1 infection.

Discussion

We report here that intrinsic ER stress response pathways, not the extrinsic TNFα pathway, cause caspase 8 activation in *ts*1-infected cultured astrocytes. When added to cultures of *ts*1-infected astrocytes, the inhibitor Z-IETD-FMK, which specifically inhibits the activity of the caspase 8 enzyme, reduces the ER stress response that normally follows infection, as shown by reduced GRP78 and CHOP expression. We suggest that this result is a consequence of caspase 8 downstream cleavage of BAP31 into BAP20. The data presented here strongly suggest that caspase 8 activation modulates ER stress by cleaving BAP31 to yield BAP20. This novel pathway for caspase 8 activation has not been described previously, and these data are the first to show that BAP20 overexpression amplifies the ER stress response.

Our previous work has shown that *ts*1 infection causes ER stress and apoptosis via activation of ER stress-associated caspase 12 in astrocytes (Liu et al., 2004). In addition to *ts*1, other viral infectious agents also cause retention of viral proteins in the ER, leading to ER stress-associated cell death in infected cells. These include $FrCas^E$ (Dimcheff et al., 2004), the Tula hantavirus (Li et al., 2005), Japanese encephalitis virus (Su et al., 2002), bovine diarrhea virus (Jordan et al., 2002), and hepatitis C virus (Waris et al., 2002; Chan and Egan, 2005).

In human cells, which lack functional caspase 12, ER stress induces CHOP expression, which then induces DR5 expression and caspase 8 activation, leading to mitochondrial stress and cell death. In mammalian species having functional caspase 12, including mice, ER stress may activate apoptosis by two routes: (1) by ER stress leading to caspase 12 activation, followed by apoptosis (Liu et al., 2004), and (2) by ER stress leading alternatively to caspase 8 activation, which activates mitochondria-driven apoptosis and amplifies the ER stress response (Fig. 6). In *ts*1-infected murine astrocytes, it appears that both apoptotic pathways are activated after *ts*1 infection. These results confirm and extend our previous results from *ts*1-infected astrocytes (Liu et al., 2004) by showing that activated caspase 8, like activated caspase 12, is connected to ER stress, and that both enzymes initiate intracellular signaling events linking ER stress and subsequent mitochondrial stress.

BAP31 is part of a complex in the ER that connects procaspase 8 with the anti-apoptotic regulators Bcl-2 or Bcl-X_L. In the absence of Bcl-2, or in the presence of activated caspase 8, BAP31 is cleaved into BAP20. This triggers ER Ca^{2+} release and subsequent increased mitochondrial uptake of Ca^{2+}, leading to mitochondrial-associated apoptosis (Chandra et al., 2004; Ng et al., 1997). Disruption of ER homeostasis, leading to release of Ca^{2+} from ER stores, appears to be the primary initiator of the

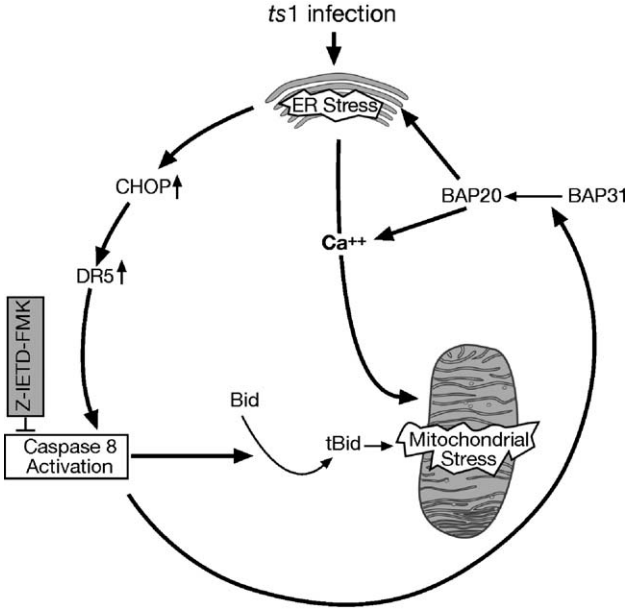

Fig. 6. Schematic diagram showing two pathways by which TNFα-independent, intrinsic caspase 8 activation might contribute to apoptosis in *ts*1-infected astrocytes. (1) ER stress may cause caspase 8 activation by upregulation of upregulation CHOP, which activates DR5 transcription, after which DR5 transcription leads to caspase 8 activation. In turn, activated caspase 8 cleaves Bid into tBid, initiating mitochondria-driven apoptosis via tBid translocation. (2) Alternatively, intrinsic caspase 8 activation may lead to, or amplify, ER stress, cleaving BAP31 into BAP20, thereby triggering Ca^{2+} release into the ER and mitochondrial uptake of Ca^{2+}, and resulting in ER stress and mitochondrial stress, with both leading to apoptosis. The caspase 8 inhibitor Z-IETD-FMK inhibits caspase 8 enzymatic activity, preventing BAP20 production and ER stress.

ER stress response (Cardozo et al., 2005; Rutkowski and Kaufman, 2004), but BAP31 cleavage into BAP20 may participate in this disruption, since BAP20 is an important regulator of the egress of certain newly synthesized integral membrane proteins from the ER (Stojanovic et al., 2005). Overexpression of BAP20 thus not only may amplify pre-existing ER stress caused by Ca^{2+} release, but also could modulate ER protein processing or intracellular "detection" of protein misfolding. This interesting question warrants further investigation.

In this paper, we show that the caspase 8 enzymatic activity inhibitor Z-IETD-FMK reduces the ER stress response (GRP78 and CHOP expression) in *ts*1-infected cultured astrocytes, possibly by inhibiting the activity of the caspase 8 enzyme and the downstream cleavage of BAP31 into BAP20 by caspase 8. Our earlier work has documented that *ts*1-induced spongiform lesions contain elevated levels of cytokines, including TNFα and FasL, with increased TNFα being most evident in astrocytes and microglia. In some studies, TNFα has been shown to be cytotoxic for neurons (McGuire et al., 2001; Venters et al., 2000), but other evidence suggests that TNFα is neuroprotective in some contexts (Barger et al., 1995; Cheng et al., 1994; Noorbakhsh et al., 2005). Because TNFα is also implicated in astrocyte activation and apoptosis in HIV-infected human brains (Elovaara et al., 1999), the extrinsic TNFa-TNF-R apoptotic pathway is thought generally to be the only event

leading to caspase 8 activation inside cells. Work described here and recent studies by others now indicate that this is not universally the case.

A recent report by Jolicoeur et al. (2003) compared the course of *ts*1 disease development in TNF-R1 knockout mice to that in wild-type and TNF-R1 heterozygotic mice. The data demonstrated clearly that paralysis (indicative of neurodegeneration) occurs precisely on schedule in knockout mice. Another recent study has shown that the course of neurodegeneration caused by the FrCasE murine retrovirus is not affected by the absence of the gene encoding TNFα (Peterson et al., 2004). Our data from TNF-R1 knockout mice are consistent with these two sets of results, in showing that neither TNFα nor its receptor TNF-R1 is required for the normal timing and development of *ts*1-induced spongiform neurodegeneration. It is interesting that both FrCasE- and *ts*1-infected mice develop spongiform neurodegeneration after induction of ER and mitochondrial stress, apparently without apoptotic pathways involving TNFα (Liu et al., 2004; Kim et al., 2001; Jolicoeur et al., 2003; Peterson et al., 2004), and that both models involve abnormal accumulations of viral proteins in infected cells of the CNS. We suggest that similar mechanisms cause the death of infected glial cells, and of uninfected neurons, in both systems. The results reported here, which link caspase 8 to ER stress, provide new information about intracellular events that accompany ER stress in mammalian cells, since ER stress does not involve activated caspase 12 in humans, as it does in mice. The data here indicate that caspase 8 could be a new target for treatment for ER stress-related diseases in humans.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), penicillin, streptomycin, Fungizone, trypsin, and TNFα were purchased from Invitrogen Life Technologies (Carlsbad, CA). All other reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Primary murine astrocyte culture, TNFα treatment, and ts1 infection

Primary cortical astrocytes were isolated from 1- to 2-day-old FVB/N mice. Pups were sacrificed using carbon dioxide, and the cerebellum of each mouse removed and minced separately in ice-cold DMEM/F12 medium by a modification of methods described previously (Shikova et al., 1993). The cells were plated onto poly-L-lysine-coated flasks and grown in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone, at 37 °C in a humidified atmosphere of 5% CO_2 in air until reaching confluence (typically 7 to 9 days). The cells were then passed four or five times before use for *ts*1 infection or TNFα treatment. At this time, the cultures contained more than 99% glial fibrillary acidic protein (GFAP)-positive cells, as detected by rabbit anti-mouse GFAP antibody (DAKO, Carpinteria, CA).

For TNFα treatment, astrocytes were seeded in 100-mm culture dishes. The next day, TNFα was added into the culture media at concentrations of 100 and 150 ng/ml. After 7 days, the cells were harvested for Western blotting.

For *ts*1 infection, astrocytes were seeded in 100-mm plastic tissue culture dishes. The next day, the cells were incubated 2 h in DMEM/F12 medium containing 1% FBS and 10 μg/ml polybrene (to enhance viral absorption), and then infected with *ts*1 for 40 min at a multiplicity of infection (MOI) of 10 (in DMEM/F12 medium containing polybrene, as before) at 34 °C. The infected cells were then washed and fed again with fresh DMEM/F12 medium containing 10% FBS, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Z-IETD-FMK (to a final concentration of 2.5 μmol) was added to the culture medium immediately after *ts*1 infection.

Coculture of astrocytes

Astrocytes were seeded into 0.02 μm Anopore culture inserts, whose cell-attachment surfaces are permeable only to low-molecular-weight compounds (including TNFα), but not to *ts*1 virus. After mock infection or infection with *ts*1, the inserts were placed into 100-mm culture dishes whose wells contained uninfected astrocytes, to allow restricted exchange of molecules between the astrocytes in the two compartments. At 7 days of coculture, the uninfected cells in the bottom wells of the cocultures were harvested for Western blotting.

Transfection and BAP20 overexpression

Adenovirus shuttle vectors encoding either P20HA (for BAP20 expression) or rtTA (the empty vector control) were kindly provided by Drs. Gordon C. Shore and Mary Sutherland of McGill University. The C1 astrocyte cell line, established in our laboratory, has most of the characteristics of primary astrocytes (Lin et al., 1997; Liu et al., 2002, 2004; Qiang et al., 2004), and was used in transfections. For this study, C1 cells were grown in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2 in air until reaching confluence. The cells were then seeded into 6-well-plates at a density of 1×10^5 cells/well overnight. The next morning, the cells were infected either with the empty vector or with P20HA, according to instructions from the providers (Breckenridge et al., 2003). The cells were harvested for Western blotting at 48 h after transfection.

Western blotting analysis

Total proteins were isolated using RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.25 mM phenylmethylsulfonyl fluoride, and 1 mM sodium fluoride in phosphate-buffered saline) supplemented with protease inhibitor cocktail (Sigma), according to a modified

protocol as previously described (Liu et al., 2004). Protein concentrations were measured using the Bio-Rad *DC* Protein Assay Reagent (BioRad lab, Hercules, CA) following the manufacturer's suggestions. Cell lysates containing 50 μg of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% gel for Bid detection). After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). The membranes were sequentially probed, stripped, and then re-probed with different antibodies against activated caspase 8 (Oncogene), procaspase 8 (BioVision), CHOP, GRP78, DR5 (Santa Cruz, CA), tBid (kindly provided by Dr. Xiaodong Wang (from University of Texas Southwestern Medical Center), BAP31 (kindly provided by Dr. Gordon C. Shore), and β-actin. After reaction with appropriate horseradish peroxidase-conjugated secondary antibodies, immune complexes were detected by chemiluminescence (NEN Life Science Products, Inc., Boston, MA).

Establishment and infection of ts1 susceptible TNF-R1 knockout mouse strains

TNF-R1 knockout mice on the C57BL/6 background (a strain resistant to *ts*1) were purchased from Immunex (Seattle, WA). These mice were backcrossed with FVB/N mice (the most susceptible strain to *ts*1) over five generations. Homozygous TNF-R1 knockout and wild-type progeny were identified by PCR analysis of mouse-tail DNA, and were tested and confirmed to be *ts*1 susceptible at this point. Heterozygous mice were then mated and their pups were used for *ts*1 infection experiments (100 μl containing 10^7 infectious units of virus/ml). After infection at birth, the animals were followed, and survival curves constructed for TNF-R1 homozygous knockout vs. wild-type sibling mice. Four litters of pups, including total 20 wild-type and 12 homozygous TNF-R1 knockout mice, were used in this study.

Statistical analysis

Data are presented as mean ± SD. Statistical significance of the results was determined by Student's *t* test. A probability of less than 5% was considered significant. The survival curves of TNF-R1 mice were analyzed by Dr. David Johnson, using the Log Rank Statistical test (Kruskal–Wallis test).

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