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Differential apoptotic signaling in primary glial cells infected with herpes simplex virus 1

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Microglial cells and astrocytes are glial cell types that perform distinct functions and generate innate immune responses to counter invading pathogens. Herpes simplex virus 1 (HSV-1) is a neurotropic virus that is capable of causing severe, necrotizing encephalitis. HSV-1 infects both of these glial cell types. Microglial cells undergo an abortive infection, yet respond to viral infection by inducing a burst of proinflammatory cytokine and chemokine production. Following this cytokine burst, they rapidly succumb to cell death. In contrast, astrocytes do permit productive viral replication, but do not generate these same innate immune mediators. Although apoptosis has been implicated in a number of acute and chronic neurological disorders, little is known about apoptosis during viral encephalitis. In the present study, the authors investigated the effect of HSV-1 infection on cell survival and studied the mechanisms of cell-death in virus-infected, primary murine glial cells. The authors report that although apoptosis occurred rapidly in microglia, it was delayed during productive infection of astrocytes. Furthermore, microarray studies revealed significant variations in the expression of apoptotic genes between these two types of glial cells, indicating crucial differences in signaling pathways. Intrinsic as well as extrinsic pathways of apoptosis were found to be activated in both glial cell types. Specifically, genes involved in the tumor necrosis factor (TNF) signaling pathway were predominantly up-regulated in microglia, whereas genes of the Fas pathway were induced during HSV infection of astrocytes. *Journal of NeuroVirology* (2006) **12,** 501–510.

Keywords: apoptosis; astrocytes; herpes simplex virus; microglia

Introduction

Innate immune responses against viral infection are generated by a complex network of cells, including natural killer cells, macrophages, and dendritic cells. In the brain, microglia function as key immune macrophages leading the primary line of defense against a myriad of infectious agents (Rock *et al*, 2004). Together with astrocytes, the most abundant glial cell type, they maintain homeostasis. In the developing brain, activated microglia phagocytose surplus cells undergoing apoptosis and play crucial roles in determining the fate of developing neurons. Microglial cells are highly mobile and perform brain surveillance functions while being in direct contact with astrocytes, neurons, and blood vessels, implying that they communicate with cortical cells to coordinately monitor the integrity of the brain (Davalos *et al*, 2005; Nimmerjahn *et al*, 2005). Although the role of microglia in mounting a rapid immune response to viral infection is well established, accumulating evidence suggests that astrocytes may also contribute to both innate and adaptive immune responses through the production of proinflammatory cytokines and chemokines, which counter viral infection (Lieberman *et al*, 1989; Dong and Benveniste, 2001; Palma and Kim, 2004; Carpentier *et al*, 2005).

Herpes simplex virus (HSV)-1 is a neurotropic virus that causes a variety of infections in humans

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and is capable of infecting most mammalian cell types. Following primary infection in the mucosal epithelia, HSV migrates to the trigeminal ganglia, where it establishes a prolonged latency that can last the lifetime of the host. Reactivated HSV-1, in response to various stimuli such as stress, trauma, fever, and ultraviolet (UV) irradiation, may gain access to the central nervous system (CNS) and lead to severe, necrotizing encephalitis, which results in high morbidity and mortality (Rock *et al*, 2004).

One of the first hurdles that HSV-1 must overcome is evasion of the host's innate immune responses that are induced upon infection. Such responses include production of proinflammatory immune mediators, activation of acquired antigen-specific responses, and induction of apoptosis. Each of these responses is aimed at limiting the initial spreading of the virus and its subsequent propagation. Extensive studies on HSV biology have shown that the virus has evolved a number of mechanisms to circumvent the host's immune response by shutting down host cell RNA, DNA and protein synthesis. For example, HSV immediate-early (IE) protein ICP0 binds to cellular proteins involved in the ubiquitin pathway and causes protein degradation (Van Sant *et al*, 2001), ICP27 regulates splicing of cellular mRNAs by inhibiting RNA maturation (Hardy and Sandri-Goldin, 1994), ICP22 binds to and modifies the host RNA polymerase II (Long *et al*, 1999), and the virion host shut-off protein has been shown to selectively degrade cellular mRNA (Smiley, 2004).

In the present study, we investigated the mechanisms of HSV infection and cell death in primary glial cells. We report that HSV replicated productively in astrocytes, whereas viral infection was abortive in microglia. In their resting state, both microglia and astrocytes displayed identical expression profile of apoptotic genes, but when activated in response to HSV infection, markedly contrasting differences in gene expression were observed.

Results

Permissiveness of glial cells to HSV infection

We have previously reported that murine microglial cells are susceptible to HSV infection and that expression of the IE protein ICP4 can be detected within 4 h (Aravalli *et al*, 2005). To determine whether HSV infection of these glial cells is productive, we first examined viral replication and growth kinetics in cultures of primary murine microglia, as well as astrocytes, in a time course experiment. As seen in Figure 1, HSV replication in microglial cells was severely attenuated and the virus titer dropped below initial input levels within 96 h post infection (p.i.), demonstrating the occurrence of an abortive infection. In contrast, astrocytes permitted viral replication and high titers were detected, reaching a 3.5 log increase in viral titer within 24 h p.i. Thus, HSV efficiently entered both microglia and astrocytes, but failed to replicate productively in microglia as no rise in virus titer was observed in the freeze-thaw lysates (Aravalli *et al*, 2005). On the other hand, the virus was able to multiply productively in astrocytes.

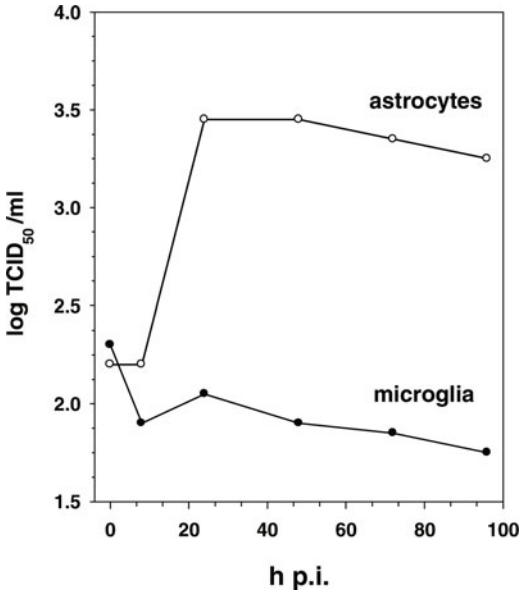

Figure 1 HSV replication in primary murine glial cells. Microglial (>99% MAC-1$^+$) and astrocyte (>99% GFAP$^+$) cells were infected with HSV for 0, 6, 24, 48, 72 and 96 h. Infected brain cells were harvested at the indicated time points and viral titers in the cryolysates were determined by 50% tissue culture infectious dose ($TCID_{50}$) assay on rabbit skin indicator cells. The viral titer at time 0 represents the amount of HSV detected following the 2 h absorption and subsequent washings. Data presented are representative of three independent experiments using cells isolated from different brain specimens.

Apoptosis in HSV-infected microglia

Although nonproductively infected, we observed that microglial cells did undergo cell death following HSV infection. To understand the reason for this death, we performed a time course experiment to determine the extent of oligonucleosomal DNA fragmentation of HSV-infected microglial cells and astrocytes, a hallmark of apoptosis, using an enzyme-linked immunosorbent assay (ELISA)-based approach. Following infection with HSV, glial cells were harvested at different time points and ELISA was performed. A relatively high multiplicity of infection (MOI) of 1 was used to maximize contact between the virus and the glial cells. Although an MOI of 1 means that every cell could encounter one plaque-forming unit (PFU), many cells may encounter two or more virus particles and some may remain uninfected. As shown in Figure 2A, DNA fragmentation was rapidly induced in microglial cells. Apoptotic DNA fragmentation could be detected as early as 8 h p.i. and peaked at 24 h p.i. Interestingly, DNA fragmentation did not occur in virus-infected astrocytes at these same time points. However,

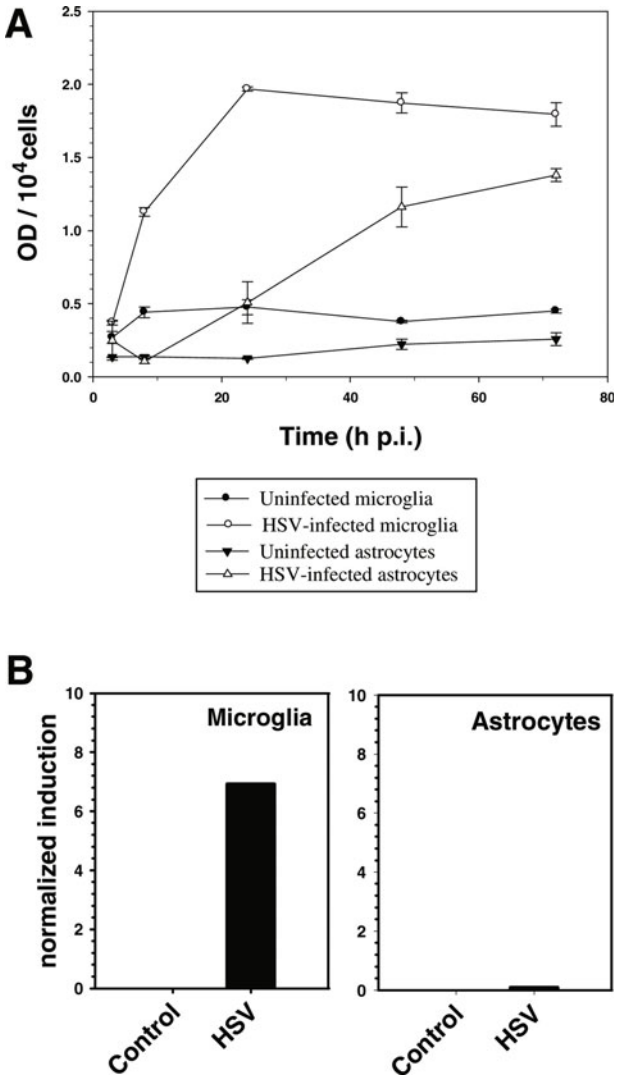

Figure 2 HSV infection induces rapid apoptosis in murine microglial cells, which is delayed in productively-infected astrocytes. (**A**) Primary glial cells were examined for apoptotic DNA fragmentation using a time course oligonucleosomal ELISA at 4, 8, 24, 48, and 72 h p.i. Data are presented as fold increase in DNA fragmentation in infected cells over uninfected control cells at the same time points. (**B**) Expression of proapoptotic caspase-3 was tested by real-time PCR using RNA isolated from uninfected and HSV-infected glial cells at 8 h p.i. Data are presented are representative of at least three independent experiments.

apoptosis was detected at later time points, between 24 and 48 h p.i., with a maximum occurring at the latter time point (Figure 2A). This result clearly demonstrates that apoptotic cell death did occur in astrocytes, but it was delayed relative to microglial cells, potentially by a virus-mediated mechanism operating during productive infection.

To further confirm this result, we performed quantitative real-time polymerase chain reaction (PCR) assays using total RNA isolated from uninfected as well as HSV-infected microglia and astrocytes (8 h p.i.) using primers specific for caspase-3, an effector protein activated during the execution stages of apoptosis. Interestingly, caspase-3 was up-regulated in virus infected microglial cells, but not in astrocytes (Figure 2B), indicating that activation of apoptotic pathways did occur in microglia but not in astrocytes at this early time point.

Apoptosis in HSV-infected astrocytes

To further validate the results obtained with astrocytes, a time course experiment was performed and changes in the cell membrane that occur during the initial stages of cell death were evaluated using flow cytometry. In normal cells, the cellular membrane component phosphatidylserine (PS) is localized mainly on the cytoplasmic surface of the cell membrane. However, during apoptosis, PS gets translocated to the outer leaflet of the membrane (Fadok *et al*, 1992). Treatment of dying cells with annexin V allows for detection of PS exposure as an early indicator of apoptosis. In these experiments, astrocytes were infected with HSV and membrane changes were measured using flow cytometry. As shown in Figure 3, 10% of astrocytes showed membrane changes at 5 h, 32% at 16 h, 44% at 24 h, and 58% at 48 h following viral infection. Moreover, cell death was complete in 29.8% of the cells at 48 h p.i. These findings demonstrate that membrane changes indicative of apoptosis were seen at early times when nuclear manifestations of apoptosis were not observed.

Differential expression of apoptotic genes in HSV-infected glial cells

In order to identify critical genes regulating apoptosis during HSV infection in glial cells, expression profiles of both pro- and antiapoptotic genes as well as other genes implicated in various known apoptotic pathways were examined using a murine-specific microarray. Identical sets of arrays were used for both glial cell types. Total RNA prepared from uninfected and infected glial cells was used to prepare cRNA for probing with arrays. This RNA was tested for its integrity by performing real-time PCR for caspase-3 expression prior to its use in microarrays (Figure 2B). As shown in Figure 2A, apoptosis occurred in microglia and astrocytes at different times post infection. Therefore, different time points were selected for microarray studies to reflect initial and peak apoptotic events. Because gene expression and protein production precede several hours ahead of DNA fragmentation, 8 and 16 h p.i. time points were chosen for microglial cells, and 8 and 32 h p.i. were selected as time points to examine pathway activation in astrocytes. Interestingly, in uninfected microglia and astrocytes, expression levels for most genes on the arrays were similar. However, when infected with HSV, significant variations in gene expression patterns occurred in these glial cells. Expression of proapoptotic genes was not induced in microglial cells at 8 h p.i. (Table 1). However, at 16 h p.i. a number of key

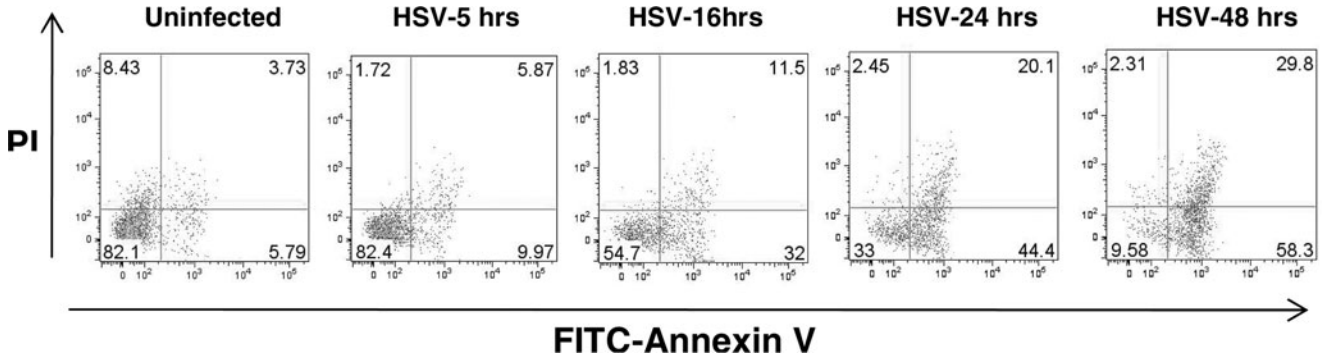

Figure 3 Early membrane changes associated with apoptosis in HSV-infected astrocytes. Exposure of phosphatidylserine (PS) was analyzed using FITC-labeled annexin V, which has strong affinity to PS, and cell death was assessed using propidium iodide (PI), in a time course experiment. HSV-infected astrocytes were harvested at different time points, together with the uninfected control at time 0, and the extent of cell death studied by flow cytometry. Data are representative of at least three independent experiments.

proapoptotic genes were significantly up-regulated in microglia, indicating that the cells were actively undergoing apoptosis. Similarly, astrocytes showed induction of a significant number of proapoptotic genes by 32 h p.i., once again demonstrating the delay in apoptosis in astrocytes (Table 1). Only the genes that were either up-regulated or down-regulated at least two-fold at any time point were listed in Table 1. Expression profiles of pro- and antiapoptotic genes differed significantly in both cell types, indicating that apoptosis was induced by a different mechanism in each glial cell type. HSV-infected microglia showed induced expression of caspase-1, -11, Bid, Bim, CD40, Ripk2, TRAIL, and Birc1e; whereas caspase-14, Sphk2, Birc6, Lhx4, p53, interleukin-10 (IL-10), nuclear factor (NF)-κB, Ltβr, Pycard, Pim2, Fas ligand and its receptor were highly expressed in virus-infected astrocytes (Table 1). In addition, both cell types showed elevated expression of caspase-2, -3, -8, -12, Mcl-1, Polb, Traf5, CIDE-B, and Cflar. Interestingly, expression of tumor necrosis factor (TNF)-α was down-regulated by 50% from 8 to 32 h p.i. in astrocytes, whereas it was elevated in microglial cells.

To further validate these microarray data, two genes that were up-regulated in microglia, two genes up-regulated in astrocytes, and two genes up-regulated in both microglia and astrocytes were selected for quantitative real-time PCR studies. PCR was performed for Cflar, caspase-3, TRAIL, Ripk2, Ltβr, and Sphk2. As shown in Figure 4, induced expression of TNF receptor-related apoptosis-inducing ligand (TRAIL) and Ripk2 occurred only in microglial cells confirming involvement of the TNF pathway, whereas Sphk2 and Ltβr were significantly upregulated in astrocytes. Although Sphk2 expression was not present at 8 h p.i. in microglia, it was induced at 16 h p.i. Both microglia and astrocytes showed increased expression of Cflar and caspase-3. Taken together, these findings clearly illustrate pivotal differences between microglial cells and astrocytes in generating apoptotic responses to HSV infection.

Putative apoptotic pathways operating in microglia and astrocytes during HSV infection

Having established the occurrence of apoptosis in HSV-infected primary glial cells, we then used the data obtained by microarray analysis to identify putative virus-induced pathways in both microglia and astrocytes (Figure 5). In general, two well-defined apoptotic pathways are triggered in cells infected with pathogens: one that emanates from death receptors present on host cell surface (extrinsic) and another that occurs from within the host cell via the mitochondrial pathway (intrinsic). In both cases, a death signal is transmitted, causing the activation of caspases and other cellular proteins in cascades and eventually culminating in the demise of the host cell. We found that HSV infection stimulated the up-regulation of genes involved in TNFR1 pathway in microglia (Table 1). TNFR1 ligands TNF-α and TRAIL were induced 60- and 137-fold, respectively, and TNFR1-associated proteins TRAF5, Tnfsf22, and Ripk2, which function proximal to the death receptor, were also significantly induced. Expression of other TNFR1 pathway proteins such as caspase-8 and -3, proapoptotic Bcl-2 proteins Bid and Bim, and Polb were also highly elevated. Interestingly, the expression of CD40 was induced 23-fold in these cells at 16 h p.i. In contrast, expression of proteins involved in the Fas and CD40 receptor-mediated pathways were activated in HSV-infected astrocytes. Expression of Fas receptor and its ligand were up-regulated 2.4- and 60.5-fold, respectively, and that of CD40 receptor was induced by 175-fold. Furthermore, downstream proteins that function in the Fas pathway such as caspase-3, -7, -8, -10, and NF-κB were also significantly up-regulated (Table 1). Based on these results, putative pathways of apoptosis were predicted for each of these glial cell types (Figure 5).

Discussion

A number of previous studies have described the mechanisms of immune response induction and

Table 1 Apoptotic genes expressed in HSV-infected glial cells actively undergoing apoptosis

		Fold change in microglia[§]		*Fold change in astrocytes*[§]	
Symbol	*Gene description* [¶]	8 h	16 h	8 h	32 h
Cflar	CASP8 and FADD-like apoptosis regulator (c-FLIP$_L$, MRIT, CLARP, CASPER, FLAME-1, CASH$_L$, USURPIN, I-FLICE)	n.i.	546.41	2.69	118.76
Tnfsf10	TNF superfamily receptor member 10 (TRAIL, APO-2L)	−1.75	137.16	n.i.	n.i.
Tnf	Tumor necrosis factor	0.30	61.49	22.28	10.00
Casp-11	Caspase 11	n.i.	46.00	n.i.	n.i.
Bircle[†]	Baculoviral IAP repeat-containing 1e	n.i.	30.57	n.i.	n.i.
Bid[*]	BH3 interacting domain death agonist	n.i.	30.95	n.i.	n.i.
Cd40	CD40	−3.06	23.82	3.29	175.99
Polb[†]	Polymerase (DNA directed), beta	n.i.	10.38	n.i.	16.26
Mcl-1[†]	Myeloid cell leukemia sequence 1	0.59	9.96	2.82	4.63
Tnfsf22	TNF superfamily receptor 22	−8.26	8.91	1.26	75.67
Casp-8[*]	Caspase 8 (FLICE, MACH, Mch5)	0.42	7.96	2.90	2.89
Traf5	Tnf receptor-associated factor 5	n.i.	6.80	1.79	95.08
Pak-7[†]	P21 (CDKN1A)-activated kinase 7 (PAK-5)	n.i.	6.48	2.74	130.48
Bcl2l11[*]	Bcl2-like 11 (apoptosis facilitator) (Bim, Bod)	0.53	5.65	n.i.	n.i.
Casp-12	Caspase 12	−5.00	5.32	n.i.	34.26
Bag-4	Bcl2-associated athanogene 4 (SODD)	−1.40	4.60	n.i.	n.i.
Casp-1	Caspase1 (ICE, IL1bc, p45)	0.45	2.94	n.i.	n.i.
Casp-3[*]	Caspase 3 (Apopain, Cpp32, LICE, YAMA, SCA-1, SREBP)	1.33	2.79	1.17	11.01
Casp-2	Caspase 2 (ICHII, NEDD-2)	0.80	2.59	2.06	9.77
Ripk2	Receptor (TNFRSF)-interacting kinase 2 (RICK, CARDIAK, CARD3, CLARP kinase)	1.83	2.23	n.i.	n.i.
Tnfsf11b	TNF receptor superfamily member 11b (TRANCE, Osteoprotegerin, Ly109l, ODF, OPG, Opgl, Rankl)	−0.09	12.08	n.i.	n.i.
Mapk8ip1[†]	MAP kinase 8 interacting protein (Ib-1, Jip-1, Skip. Mjip-2a, Prkm8ip)	−0.03	11.33	n.i.	n.i
Nme5	Non-metastatic cell protein 5 (NDK-H5n m23-M5 1700019D05Rik, NDP kinase homolog 5)	−0.07	11.12	n.i.	n.i.
Bok	Bcl2-related ovarian killer protein (Mtd, Matador)	−0.13	7.64	n.i.	n.i.
Bnip3	Bcl2/adenovirus E1B 19kD-interacting protein (Nip)	−0.18	4.05	n.i.	n.i.
Dad1[†]	Defender against cell death	−0.45	3.64	n.i.	n.i.
Tnfsf12a	TNF receptor superfamily member 12a (DR3L, (APO3L, Tweak, DR3LG)	−0.35	2.57	−0.52	−2.18
Casp-14	Caspase 14	n.i.	n.i.	n.i.	71.94
Fasl[*]	Fas ligand	n.i.	n.i.	n.i.	60.55
Ltβr[*]	Lymphotoxin B receptor	n.i.	n.i.	−1.60	44.47
Pim2[†]	Proviral integration site 2	n.i.	n.i.	n.i.	36.45
Casp-12	Caspase 12	n.i.	n.i.	n.i.	34.26
Sphk2[†]	Sphingosine kinase 2	n.i.	n.i.	n.i.	30.34
Lhx4[†]	LIM homeobox protein 4	n.i.	n.i.	n.i.	23.87
Birc6[†]	Baculoviral IAP repeat-containing 6	n.i.	n.i.	n.i.	19.01
Cide-b[*]	Cell death-inducing DNA fragmentation factor	n.i.	n.i.	0.93	16.67
Polb[†]	Polymerase (DNA directed), beta	n.i.	n.i.	n.i.	16.26
Il-10	Interleukin 10	n.i.	n.i.	1.29	13.51
Trp53[*]	P53	n.i.	n.i.	2.38	7.97
Tnfsf21	TNF receptor superfamily member 21	n.i.	n.i.	−2.04	4.10
Nfkb1	NF-kappa B1	n.i.	n.i.	2.69	4.01
Pycard	PYD and CARD domain containing protein	n.i.	n.i.	0.35	2.81
Fas[*]	Fas	n.i.	n.i.	2.14	2.40
Api5	Apoptosis inhibitor 5	n.i.	n.i.	−0.79	−22.88
Akt1	Thymoma viral proto-oncogene 1	n.i.	n.i.	−1.38	−9.62
Bad[*]	Bcl-associated death promoter (Bbc2, Bbc6)	n.i.	n.i.	−1.46	−5.14
Als2cr2	Amyotrophic lateral sclerosis 2 (juvenile) candidate 2 homolog (CALS-21, ILPIP, ILPIPA, MGC102916 PAPK, PRO1038)	n.i.	n.i.	−3.10	−5.00
Atf5	Activating transcription factor 5	n.i.	n.i.	−1.10	−3.01
Bag-3	Bcl2-associated athanogene 3 (Bis)	n.i.	n.i.	−0.66	−2.88
Tnfsf12a	TNF receptor superfamily member 12a	n.i.	n.i.	−0.52	−2.18

[*]Known proapoptotic genes; [†]known antiapoptotic genes; [¶]Synonyms are indicated in parentheses; [§]n.i. = gene expression was same as in uninfected controls; and negative values indicate down-regulation of gene expression.

apoptosis in HSV-infected cells, many using transformed cell lines, which may not accurately reflect primary cells. However, little is known about host cell responses or apoptotic induction during HSV infection in brain cells and much of the disease during herpes encephalitis may be due to the dysfunction of these innate defenses. We report here that microglial cells undergo an abortive HSV infection,

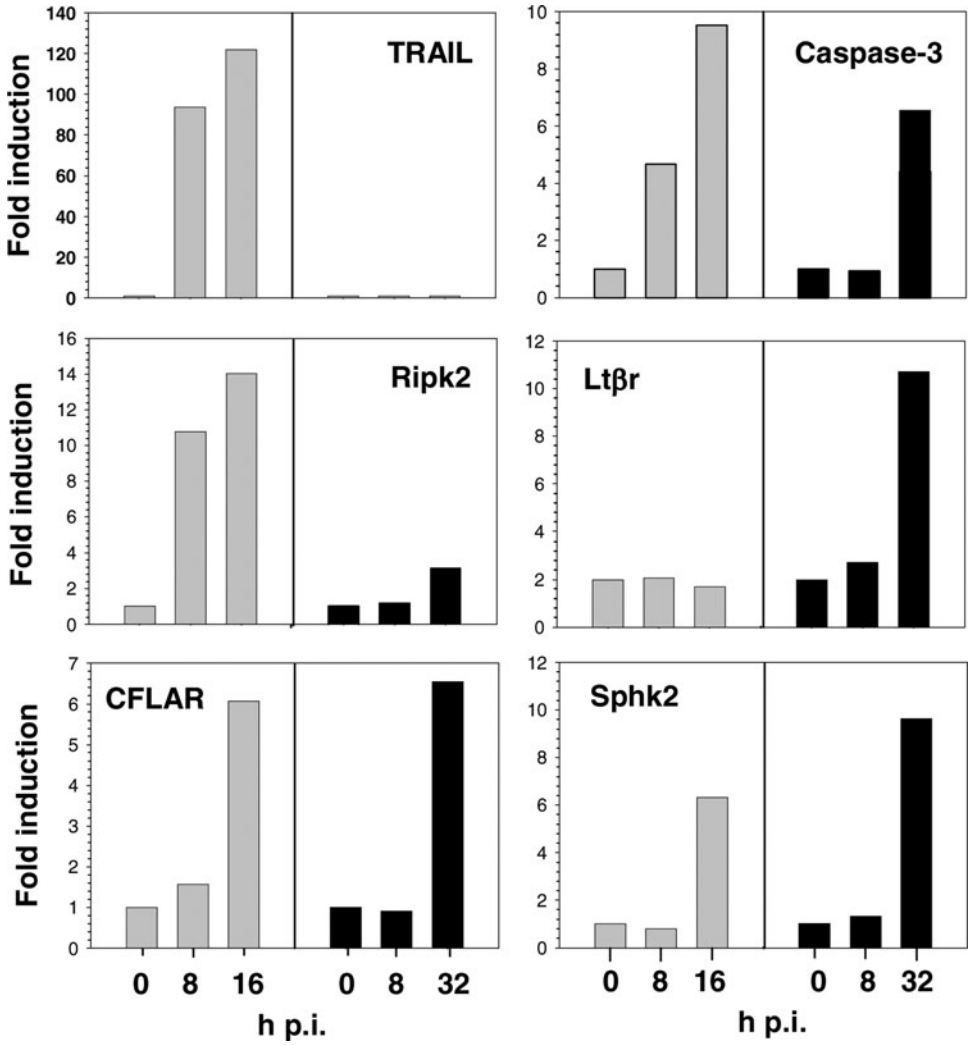

Figure 4 Differential expression of apoptotic genes in glial cells infected with HSV. Real-time PCR was performed with uninfected and HSV-infected microglial *(grey bars*) and astrocytes *(black bars)* using primers specific for apoptotic genes indicated. Data are representative of at least three independent experiments.

which rapidly results in cell death. Additional data obtained in this study demonstrate that HSV undergoes productive replication in astrocytes, but apoptotic progression in these cells was delayed relative to microglia. Membrane changes indicative of apoptosis do occur in astrocytes early during infection, at times when the nuclear manifestations of apoptosis are not apparent. The reason for this delay remains unknown, but it may be the result of direct viral-induced blockade, as HSV presumably needs to delay the nuclear manifestations of apoptosis until its replication cycle is complete (Smiley, 2004).

Recently, we have demonstrated that murine microglial cells respond to HSV by producing a large number of proinflammatory chemokines and cytokines through the toll-like receptor 2 (TLR2) signaling pathway (Aravalli *et al*, 2005). HSV-infected microglia were found to produce high amounts of TNF-α, IL-1β, IL-6, and CXCL2. In contrast, during the present study, none of these immune mediators were found to be expressed at high levels in HSV-infected astrocytes, except for TNF-α, indicating that productive viral infection may suppress their expression. Furthermore, TLR2 expression did not occur in virus-infected astrocytes and lack of TLR2 signaling might be a contributing factor for the lack of immune mediator production in astrocytes. Interestingly, TNF-α was down-regulated in astrocytes actively undergoing apoptosis at 32 h p.i., relative to its higher level expression at 16 h p.i. (Table 1). Furthermore, our array data show that resting microglial cells and astrocytes do express low levels TNF and Fas mRNA. Additionally, in preliminary studies, we found that signaling through TLR2 activated apoptosis in HSV-infected microglial cells, suggesting that apoptosis may be an innate immune defense mechanism to counter viral infection.

Apoptosis is a tightly regulated program of cell death carried out by a plethora of cellular proteins in response to various stimuli. Despite the notion

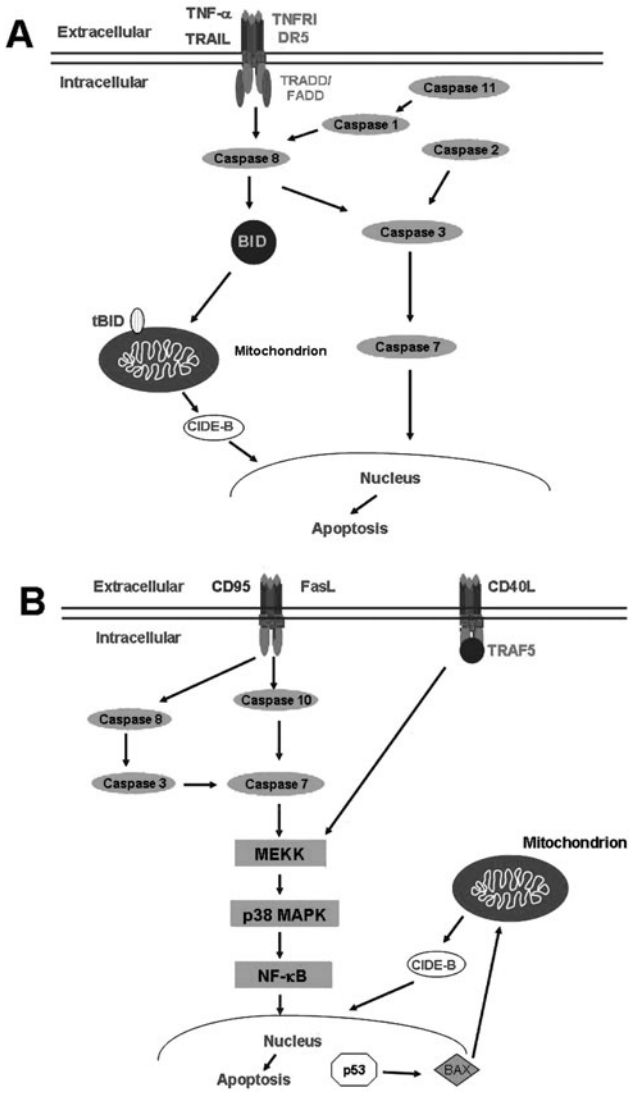

Figure 5 Putative apoptotic pathways operating in murine glial cells. Pathways were deduced from microarray analysis of gene expression in HSV-infected microglial cells (**A**) and astrocytes (**B**).

of an apoptotic component in herpes encephalitis, the molecular mechanisms and signaling pathways that lead to apoptosis during HSV infection in primary glial cells have not been identified. In our microarray studies, we found that resting microglia and astrocytes express a similar set of apoptotic genes. However, when activated by viral infection, these different glial cell types produce distinct apoptotic proteins at both early and late time points. It should be kept in mind that identification of pathway activation occurring in glial cells in response to HSV infection is an association and does not show direct cause-and-effect.

Apoptotic genes induced in HSV-infected microglial cells

Proapoptotic genes such as TRAIL, caspase-1, -11, Birc1e, Bid, Bim, and Bag-4 were induced in HSV-infected microglia, but not in astrocytes. Caspase-1 was originally identified as a protein that causes maturation of pro-IL-1β to active proinflammatory IL-1β protein. Although caspase-11 does not process pro-IL-1β directly, IL-1β was not made in mice lacking caspase-11, indicating that caspase-11 has an indirect effect on IL-1β synthesis. Thus, caspase-11 functions as an upstream regulator of caspase-1 and indirectly affects IL-1β maturation (Wang *et al*, 1998; Kang *et al*, 2000). We found recently that HSV-infected microglia produce IL-1β (Aravalli *et al*, 2005). From the microarray data presented here, it is plausible that activation of caspase-1 by caspase-11 and subsequent pro-IL-1β processing by caspase-1 takes place in virus-infected microglial cells.

TNF-α and members of the TNF superfamily, such as Fas and TRAIL, were shown to induce apoptosis in brain cells (Martin-Villalba *et al*, 1999; Nitsch *et al*, 2000). Activation of TNFR1, CD95, and DR4/DR5 by TNF-α, Fas, and TRAIL, respectively, trigger the activation of cell death involving caspase-8 and caspase-3 (Ashkenazi, 2002). Activated caspase-8 cleaves the Bcl2 inhibitory BH3-domain protein (Bid) to produce truncated Bid (tBid), which in turn induces the mitochondrial apoptosis where cytochrome *c* is released into the cytoplasm. Caspase-3 then cleaves its substrates such as CIDE-B to trigger chromosomal DNA fragmentation. Our results demonstrate that activation of TNF pathway was occurring in microglial cells as all these proteins were upregulated in response to HSV infection (Table 1).

Apoptotic genes induced in HSV-infected astrocytes

Unlike microglia, astrocytes induced the expression of Fas ligand and its receptor, Ltβr, caspase-14, Pim2, Sphk2, Lhx4, Birc6, Trp53, and Pycard genes. Fas ligand, its receptor, as well as downstream effector molecules of the Fas pathway such as caspase-8 and -3 were activated in HSV-infected cells (Table 1). The expression of TNF-α in virus-infected murine astrocytes could be due to Fas signaling, as was documented for human astrocytes. Trp53 was demonstrated to influence the sensitivity of transformed germ cell line cells to undergo Fas-mediated apoptosis by modulating the expression of Fas on their cell membranes and subsequently influencing the degradation of antiapoptotic protein Cflar (Chandrasekaran *et al*, 2006). It is therefore likely that p53 expressed in astrocytes might mediate the degradation of antiapoptotic Cflar protein.

Literature reports of caspase-8 activation in astrocytes are controversial. Although the lack of caspase-8 expression was reported as a possible reason for astrocyte survival (Wosik *et al*, 2001), caspase-8 was shown to be up-regulated during Fas- and TRAIL-induced apoptosis in human astrocytes (Song *et al*, 2006). In this study we found that caspase-8 expression was induced in HSV-infected murine microglia and astrocytes (Table 1), suggesting a role for caspase-8 in glial cell apoptosis.

Apoptotic genes expressed in both microglia and astrocytes

A significant number of genes were highly upregulated in virus-infected microglial cells as well as in astrocytes, indicating common mechanisms existing in these cell types. For example, RipK2, a serine/threonine kinase expressed at significant levels in the brain, promotes activation of caspase-8 and regulates Fas-mediated apoptosis (Inohara *et al*, 1998). Ripk2 expression was found to be elevated in HSV-infected glial cells (Figure 4), indicating its role in caspase-8 activation and interaction with Cflar to promote death receptor–induced apoptotic signaling. CD40 ligand, its receptor, as well as its downstream effector protein TNF receptor–associated factor 5 (Traf5) were expressed in both microglia and astrocytes, indicating a CD40-induced pathway involving c-Jun NH2-terminal kinase (JNK) and NF-κB activation is functional in both these cell types. Induced expression of CD40 in microglia as well as astrocytes suggests that CD40 may play a role in attracting $CD4^+$ and $CD8^+$ cells to counter HSV in the context of brain infection.

DNA fragmentation into oligonucleosomal units is a hallmark of apoptosis and is triggered by caspase-3 activation of one or more of several DNA fragmentation factors. Four such proteins, cell death–inducing DFF45-like effectors CIDE-A and CIDE-B, and DNA fragmentation factors DFF-A and DFF-B, were present in microarrays used in this study. Among these, only CIDE-B was expressed by glial cells. This protein, localized in the mitochondria, induces apoptosis in a caspase-dependent manner that causes cytochrome *c* release from the mitochondria (Chen *et al*, 2000; Erdtmann *et al*, 2003). Upon infection with HSV, CIDE-B expression was highly elevated in astrocytes (Table 1) and marginally increased in microglia, demonstrating a key role for CIDE-B in generating DNA fragmentation of glial cells.

In this study we show for the first time that a large number of apoptotic genes are differentially activated in two distinct glial cell types upon infection with HSV. Most of these genes have never been reported to be expressed in primary brain cells. Evidently, presence or lack of gene expression is a cell type–specific feature, as we identified a number of key pro- as well as antiapoptotic genes for each glial cell type in this study. Furthermore, the analysis of their expression revealed putative pathways operating in glial cells during the course of HSV-induced apoptosis. Further characterization of the apoptotic pathways described here may be valuable in developing new strategies for modulating the mechanisms of apoptosis to counter herpes encephalitis.

Materials and methods

Preparation of glial cell cultures

Wild-type Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA). Purified microglial cell and astrocyte cultures were prepared from these mice as described previously (Chao *et al*, 1993). Growth medium for microglial and astrocyte cultures was Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Logan, UT) and antibiotics. Murine microglial cell cultures used in these experiments were >99% pure, as determined by MAC-1 antibody staining (Roche Applied Science, Indianapolis, IN), and the astrocyte cultures were >99% pure, as determined by glial fibrillary acidic protein (GFAP) staining (Aravalli *et al*, 2005).

Virus

HSV-1 17 syn^+ was propagated in rabbit skin fibroblasts and used for infection studies at the indicated multiplicities of infection (MOI). For microarrays, an MOI of 1 was used and for PCR as well as fluorescence-activated cell sorting (FACS) studies an MOI of 2.5 was used. Time points for astrocytes were 8 and 32 h p.i. for microarray and PCR, and 8, 16, 24 and 48 h p.i. for FACS analysis.

Oligonucleosomal ELISA assays

A sandwich ELISA-based system (Roche Applied Science) was used to detect nucleosomes generated due to DNA fragmentation during apoptosis. The assay was performed at the indicated time points as per the manufacturer's instructions.

Microarray analysis

The OligoGEArray mouse apoptosis microarrays (OMM-012) (SuperArray, Frederick, MD) were used for our studies. Total RNA was isolated from uninfected and infected microglial cells at 8 and 16 h p.i., as well as uninfected and infected astrocytes at 8 and 32 h p.i., using the RNeasy Mini Kit (Qiagen, Valencia, CA). Hybridization steps and detections were performed according to manufacturer's instructions. Chemiluminescent detection was carried out and positive spots on the arrays were scanned using a Kodak Image Station 2000R (Molecular Imaging Systems, Rochester, NY) and were quantified using GEArray Analysis Suite software (SuperArray). Data are presented as relative induction of each gene, normalized to the housekeeping gene GAPDH.

Real-time PCR

The cDNA from uninfected and infected glial cells at each time point was synthesized with 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo dT_{6-12} primers (Sigma-Genosys, The Woodlands, TX). Quantitative real-time PCR was performed using the FullVelocity SYBR Green QPCR master mix (Stratagene, La Jolla, CA) following the manufacturer's specifications. The 25-μl final reaction volume consisted of pre-made reaction mix (SYBR Green I dye, reaction buffer, Taq DNA polymerase, and dNTPs), 0.3 mM of each primer, and 0.5 ng cDNA in

water. Reaction conditions for PCR for the Mx3000P QPCR System (Stratagene) were as follows: polymerase activation at 95°C for 5 min, 40 denaturation cycles of 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 10 s. A melting curve was performed to assess primer specificity and product quality using the same conditions. The relative levels of products were quantified using the 2(-Delta Delta C(T)) method (Litvak and Schmittgen, 2001) β-Actin was used as positive control and for normalizing values of cytokine and apoptotic genes tested. Primer sequences will be provided upon request.

Evaluation of apoptosis in astrocytes by flow cytometry

Flow cytometry was performed with Vybrant apoptosis assay kit (Molecular Probes, Eugene, OR) following the manufacturer's instructions. 2×10^4 astrocytes were infected with HSV and were harvested at 5, 16, 24, and 48 h p.i. Following washes in cold phosphate-buffered saline (PBS) and resuspension in 100 μl 1× annexin-binding buffer, 0.75 μl fluorescein isothiocyanate (FITC)–annexin V and 0.1 μg propidium iodide (PI) were added to the cells and incubated at room temperature for 15 min. Stained cells were then analyzed by flow cytometry using FACS Canto System (BD Biosciences, San Jose, CA). Cells stained with annexin V but not with PI were defined as apoptotic and those stained with both annexin V and PI as necrotic. Quantitative data were obtained by determining the percentage of stained cells in total cell population. Astrocytes treated with 10 μM camptothecin (Sigma) and cells killed by heating for 5 min at 56°C were used as positive controls for apoptosis and necrosis, respectively.

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