Differential activation of the c-Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase signal transduction pathways in the mouse brain upon infection with neurovirulent influenza A virus

Isamu Mori,1–3 Fumi Goshima,2 Tetsuro Koshizuka,2 Naoki Koide,1 Tsuyoshi Sugiyama,1 Tomoaki Yoshida,1 Takashi Yokochi,1 Yukihiro Nishiyama2 and Yoshinobu Kimura3

Correspondence
Yoshinobu Kimura
ykimura@fmsrsa.fukui-med.ac.jp

1Department of Microbiology and Immunology, Research Center for Infectious Disease, Aichi Medical University School of Medicine, Aichi, Japan
2Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Aichi, Japan
3Department of Microbiology, Fukui Medical University School of Medicine, 23-3 Matsuoka-cho, Yoshida-gun, Fukui 910-1193, Japan

Received 26 February 2003
Accepted 27 May 2003

The temporal and spatial distribution of active c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) in the brain was investigated in an experimental virus–mouse system in which neurovirulent influenza A virus caused lethal acute encephalitis. Following stereotaxic microinjection into the olfactory bulb, virus-infected neurons appeared in several midbrain structures, including the ventral tegmental area, amygdala and the pyramidal layer of the hippocampus. Infected neurons exhibited apoptosis on day 5, as demonstrated by in situ detection of DNA fragmentation and active caspase-3. The stress-responsive JNK signal transduction pathway was activated in virus-infected neurons. Activation of p38 MAPK was widespread and occurred in astrocytes on day 7 after infection. Active p38 MAPK in astrocytes showed no association with apoptosis but appeared to be involved in regulation of TNF-α production. These results indicate that these two stress-activated protein kinases may play distinct roles during the course of lethal acute influenza virus encephalitis.

INTRODUCTION

Increasing evidence has suggested that neuronal apoptosis plays an important role in the pathogenesis of neurodegenerative, ischaemic and prion diseases as well as virus infections of the brain (Bredesen, 1995). However, knowledge is scarce on molecular mechanisms underlying the process of neuronal apoptosis induced by a wide range of insults given to the CNS. Elucidation of intracellular events during the process of neuronal apoptosis in the brain is essential for the establishment of an effective therapeutic strategy against these disorders.

A mitogen-activated protein kinase (MAPK) superfamily protein functions as a serine/threonine kinase that regulates intracellular signalling cascades and transmits extracellular stimuli from the plasma membrane into the nucleus. Extracellular signal-regulated kinases, the so-called classical MAPKs, are activated by mitogens and survival factors and transduce signals to promote cell proliferation and survival (Matsuzawa & Ichijo, 2001). In contrast, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK become activated by a variety of stress signals encompassing UV irradiation, inflammatory cytokines and oxidative stimuli and are implicated in the induction of apoptotic cell death (Mielke & Herdegen, 2000; Matsuzawa & Ichijo, 2001). Intriguingly, dsRNA has also been shown to activate these kinases (Iordanov et al., 2000). Active JNK phosphorylates c-Jun and activating transcription factor 2 (ATF-2). The JNK signalling pathway stabilizes and activates the pro-apoptotic effector p53 and antagonizes the function of anti-apoptotic mediator Bcl-2 by phosphorylation (Mielke & Herdegen, 2000). Phosphorylated c-Jun stimulates the transcription of several key target genes, including Fas, the death-inducer ligand (Morishima et al., 2001). Recent studies in various in vitro experimental systems have provided strong evidence that the JNK signal transduction cascade mediates neuronal apoptosis (Luo et al., 1998; Morishima et al., 2001; Putcha et al., 2001; Whitfield et al., 2001). Activation of this pathway in vivo, especially in the brain, upon exposure to stress stimuli has
not yet been addressed enough (Yang et al., 1997; Xia et al., 2001). On the other hand, p38 MAPK regulates gene expression by phosphorylating some transcription factors, including cyclic AMP-responsive element–binding protein, myocyte-enhancer factor 2C and ATF-2 (Mielke & Herdegen, 2000). Significant function of the p38 MAPK pathway has been reported in the apoptotic cascade in cultured neurons (Kummer et al., 1997; Horstmann et al., 1998; Ghatan et al., 2000; Zou et al., 2002). Activation of p38 MAPK has also been attributed to axotomy-induced apoptosis of retinal ganglion cells in rats, although the role played by p38 activation in apoptosis regulation differs in a cell-type–or stimulation-dependent manner (Kikuchi et al., 2000).

To clarify the underlying mechanisms whereby virus infection triggers neuronal apoptosis in the brain, we have investigated the activation kinetics of two apoptosis mediators, JNK/SAPK and p38 MAPK, during the course of lethal acute encephalitis caused by neurovirulent influenza A virus infection (Mori et al., 2002). Here we show that the JNK/SAPK signal transduction cascade becomes activated in virus-infected neurons, while delayed and widespread activation of p38 MAPK occurs in astrocytes and recruits inflammatory responses in the mouse brain.

**METHODS**

**Experimental infection of animals.** The R404BP strain of influenza A virus (a kind gift from S. Nakajima, The Institute of Public Health, Japan) was propagated as described elsewhere (Mori et al., 1999). R404BP virus ($10^7$ p.f.u.) in 1 mL sterile PBS was injected stereotaxically into the right olfactory bulb of specific-pathogen-free, 4-week-old, female C57BL/6 mice (Clea), as reported previously (Mori et al., 1999).

**UV inactivation of virus.** Stock virus suspension was exposed to a 15 W UV lamp at a distance of 30 cm for 30 min at 4°C with continuous and gentle stirring. After irradiation, infectivity was assayed by UV inactivation of virus.

**Tissue processing.** Under deep anaesthesia by intraperitoneal administration of 7·2 % chloral hydrate in PBS (0·05 ml g⁻¹ body weight), mice were perfused transcardially with 3 % formaldehyde in PBS. Brains were soaked in 20 % sucrose in PBS at 4°C for 24 h and frozen at −80°C. Coronal sections of 14 μm thickness of brain tissue were cut at the midbrain level on a cryostat.

**Dual immunofluorescent labelling.** Tissue slices were incubated in 5 % donkey serum (Chemicon International) containing 0·3 % Triton-X in PBS for 20 min and reacted in a mixture of two primary antibodies diluted in 2 % donkey serum containing 0·3 % Triton-X in PBS at 4°C overnight. The primary antibodies adopted in this study included goat polyclonal anti-influenza A virus antibody (working dilution of 1:500; Chemicon International), rabbit polyclonal anti-neuronal nitric oxide synthase (nNOS) antibody (working dilution of 1:100; Chemicon International), rabbit polyclonal anti-cleaved caspase-3 (Asp¹⁷⁵) antibody (working dilution of 1:50; Cell Signalling Technology), rabbit polyclonal anti-JNK antibody (working dilution of 1:200; Cell Signalling Technology), rabbit polyclonal anti-p38 MAPK antibody (working dilution of 1:400; Santa Cruz), rabbit polyclonal anti-phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) antibody (working dilution of 1:200; Cell Signalling Technology), anti-phospho-c-Jun (Ser⁶³) antibody (working dilution of 1:200; Cell Signalling Technology), rabbit polyclonal anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) antibody (working dilution of 1:200; Cell Signalling Technology), mouse monoclonal anti-glia fibrillary acidic protein (GFAP) antibody cocktail (working concentration of 10 μg ml⁻¹; Pharmingen), goat polyclonal anti-TNF-α antibody (working dilution of 1:200; Santa Cruz) and rabbit polyclonal anti-Fas ligand antibody (working dilution of 1:200; Wako Pure Chemical Industries). Then, tissue sections were incubated in secondary antibodies that had been affinity-purified and absorbed for dual immunolabelling (all diluted 1:100 in PBS supplemented with 0·3 % Triton-X; Chemicon International), including rodamine-labelled donkey anti-goat immunoglobulin, fluorescein-labelled donkey anti-rabbit immunoglobulin and rodamine-labelled donkey anti-mouse immunoglobulins. Binding was visualized under a confocal laser scanning microscope.

**In situ detection of DNA fragmentation.** DNA fragmentation was detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labelling (TUNEL) method using the ApoTag Direct In Situ Apoptosis Detection kit (Intergen). Dual imaging for virus antigens and TUNEL reactions was carried out as described in a previous report (Mori & Kimura, 2000).

**Detection of mRNA for influenza A virus membrane protein 1 (M1) and TNF-α.** Total mRNA was extracted from brain tissue at the midbrain level using a mRNA Isolation kit (Roche). cDNA synthesis and successive PCR were carried out with the One-Step RNA PCR kit (AMV) (TaKaRa), according to the manufacturer’s instructions. Sequences of primer pairs were as follows: influenza A virus M1, 5’-GAGATGCCACAGAGA-3’ and 5’-TGGTGTGATCTG-CAC-3’ (Urabe et al., 1993); mouse TNF-α, 5’-CCTGTAGCCCAATGTGCTAGC-3’ and 5’-TTGACCTAGCGCTAGTGGT-3’ (Tucker & Sack, 2001); and mouse β-actin, 5’-ATCTGGACACCACACCT-CTACA-3’ and 5’-GTTTACAGGTGACAGAGATT-3’ (Meyding-Lamade et al., 1998). PCR conditions were as follows: 50°C for 30 min and 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. The final products were separated on 1·2 % agarose gel, stained with ethidium bromide and visualized under a UV lamp. Amplified DNA fragments from influenza A virus M1, TNF-α and β-actin transcripts were expected to be 684, 374 and 567 bp, respectively.

**RESULTS**

**Clinical observation.** C57BL/6 mice, after stereotaxic microinjection with the R404BP strain of influenza A virus into the right olfactory bulb, displayed decreased motor activity on day 5, manifested hunching and emaciation on day 7 and died within 10 days. This demonstrated the lethal effect of virus infection of the CNS. The mean survival day was 7·9 ± 0·6 (averages ± SD, n = 10).

**Infection of mouse brain with the R404BP strain of influenza A virus.** R404BP virus introduced into the right olfactory bulb spread to the midbrain level of the amygdala, ventral tectal area, pyramidal layer of the hippocampus, substantia nigra zona compacta and mammillary nucleus on day 5 after infection, as reported in a previous report (Mori et al., 2002). Double immunolabelling of virus antigens and nNOS, which is a marker for neurons, demonstrated that the virus infected neurons selectively in...
these brain structures (Fig. 1a–c). Neuronal morphology was retained relatively well on day 5. In situ dual labelling showed that 90% of virus-infected neurons displayed TUNEL-specific signals ($n = 50$) (Fig. 1d–f). Furthermore, 90% of infected neurons exhibited immunoreactivity for cleaved caspase-3 ($n = 50$) (Fig. 1g–i). Collectively, these findings demonstrated the occurrence of caspase-dependent neuronal apoptosis upon virus infection. On day 7, infected neurons increased in number (Fig. 2), began to shrink and lost neurite structures, which showed a process of apoptotic neurodegeneration. In the normal brain of age-matched C57BL/6 mice, the levels of both TUNEL-specific and active caspase-3-specific signals were not detectable.

**Activation of the JNK/SAPK signal transduction cascade**

In the normal brain, JNK molecules were detectable occasionally in a cytoplasmic staining pattern (Fig. 3a). On day 5 after infection, signals for JNK increased in intensity and showed nuclear accumulation in some infected neurons (Fig. 4a–c). Most neurons infected with the virus displayed phospho-JNK- and phospho-c-Jun-specific signals in a nuclear staining pattern (Fig. 4d–i). The brains of mice inoculated with UV-inactivated virus showed no activation of the pathway. These results proved unequivocally the activation of the JNK/c-Jun cascade in infected neurons. Upregulated expression of Fas ligand, a transcriptional target of c-Jun, was undetectable.

**Activation of p38 MAPK**

It should be noted that p38 MAPK was undetectable in the normal brain parenchyma (Fig. 3b) (Maruyama et al., 2000) and that both p38 MAPK and phospho-p38 MAPK did not become visible anywhere in the brain parenchyma, even on day 5 after virus infection (Fig. 4j–l). However, on day 7 after infection, weak p38 MAPK-specific signals appeared in...
brain cells (Fig. 5a–c). Cells exhibiting phospho-p38 MAPK immunoreactivity appeared widespread in the brain parenchyma, notably in the cerebral cortex, the dentate gyrus and the CA1, CA2 and CA3 regions of the hippocampal formation (Fig. 5d–f). Of interest, these cells did not overlap with virus-infected neurons at all but did with GFAP-positive cells. This indicated that activation of p38 MAPK occurred in GFAP-positive astrocytes in the brain upon virus infection (Fig. 5g–i). Phospho-p38 MAPK-specific signals appeared occasionally in a nuclear staining fashion. Although p38 MAPK has been implicated in the induction of apoptosis, GFAP-immunopositive cells that emerged in virus-infected regions did not show any TUNEL-specific signals (Fig. 5j–l). Since p38 MAPK is involved in post-transcriptional regulation of IL-1β-induced TNF-α production in cultured human astrocytes (Lee et al., 2000), we examined the in vivo production of this cytokine in phospho-p38-immunopositive astrocytes. TNF-α immunoreactivity overlapped with phospho-p38 MAPK (Fig. 6). RT-PCR detected TNF-α transcripts on days 5 and 7 after infection (Fig. 7). These results raise the possibility that p38 MAPK may regulate synthesis of TNF-α molecules in astrocytes in response to neuronal infection with the neurovirulent influenza A virus in vivo.

**DISCUSSION**

In this study, we have investigated activation patterns of JNK/SAPK and p38 MAPK in the mouse brain following infection with the neurovirulent influenza A virus. Our results provide the first evidence that the JNK cascade can be activated in CNS neurons upon virus infection and the p38 counterpart in astrocytes with a significant time-lag. Recently, we reported apoptosis of murine olfactory receptor neurons with activation of the JNK/c-Jun/Fas ligand pathway following intranasal infection with the R404BP strain of influenza A virus (Mori et al., 2002a). In the present study, however, we could not detect upregulated expression of Fas ligand molecules in the mouse brain following infection with the same virus. Alternatively, JNK activation in neurons may lead to induction of BIM, a member of the BH3-only pro-apoptotic subfamily of the Bcl-2 protein family, as well as BAX-dependent cytochrome-c release from mitochondria, which induces caspase activation (Putcha et al., 2001; Whitfield et al., 2001). These findings underscore the importance of the JNK cascade in induction of neuronal apoptosis in vivo.

Neuronal apoptosis induced by influenza A virus infection in the brain is multifactorial (Mori & Kimura, 2001). Cytotoxic lymphocytes, especially through the perforin/granzyme system-mediated mechanism, play a pivotal role in killing CNS neurons that are infected with the WSN strain of influenza A virus, as demonstrated in mice with targeted disruption of transporter associated with antigen presentation 1 and perforin genes (Mori et al., 1999; Mori & Kimura, 2000). Granzyme B released from effector lymphocytes enters the target cells and activates caspase cascades directly (Trapani et al., 2000). On the other hand, subacute induction of apoptotic neurodegeneration in the brain of influenza virus-infected perforin−/− mice could be attributable to sustained activation of the JNK pathway (Mori et al., 1999; Mori & Kimura, 2000). Prolonged JNK activation has been demonstrated in various apoptotic paradigms (Tobiume et al., 2001). Thus, influenza virus-induced neuronal apoptosis in the brain involves activation of the intrinsic (i.e. apoptosome) and extrinsic (i.e. death receptors and perforin/granzyme) pathways (Putcha et al., 2001).

Astrocytes with active p38 MAPK produced TNF-α molecules (Fig. 6), synchronous with the development of clinical symptoms in virus-infected mice (hunching and emaciation). Activation of p38 MAPK in astrocytes mediates...
IL-1β-signalling and results in post-transcriptional production of TNF-α (Lee et al., 2000). Interestingly, IL-1β-stimulated activation of the p38 MAPK cascade in cultured astrocytes has been reported in connection with prostaglandin E₂ production, which gives another example of the relationship between p38 MAPK and neuroinflammation (Molina-Holgado et al., 2000). Global forebrain ischaemia leads to neuronal death in the hippocampus, activation of p38 taking place in microglia in the vicinity of the affected area (Walton et al., 1998). Similar to our findings, delayed induction of p38 MAPK has been noted in reactive astrocytes in the regions of the brain undergoing selective neuronal death induced by kainic acid (Che et al., 2001). In addition, phosphorylation of p38 MAPK has been detected in glial cells within senile plaques in the brain of patients with Alzheimer’s disease (Hensley et al., 1999).

**Fig. 4.** Activation of the JNK signal transduction pathway. Amygdala on day 5 after infection. (a) Infected neurons. (b) JNK immunoreactivity. (c) Overlapped confocal image. Note the occasional upregulation of JNK and its nuclear translocation upon infection (arrows). (d) Infected neurons. (e) Phospho-JNK immunoreactivity. (f) Overlapped confocal image. Infected neurons display phospho-JNK immunoreactivity in a nuclear staining pattern (arrows). (g) Infected neurons. (h) Phospho-c-Jun. (i) Overlapped confocal image. Infected neurons display phospho-c-Jun immunoreactivity in a nuclear staining pattern (arrows). (j) Infected neurons. (k) Phospho-p38 MAPK immunoreactivity. (l) Overlapped confocal image. Note the undetectable level of phospho-p38 immunoreactivity at this time-point. Bar, 20 μm.
These descriptions are consistent with the present observation that activation of p38 MAPK parallels the neuro-inflammatory process during virus infection of the brain. It is expected that in combination with antiviral treatment (Mori et al., 2002b), a therapeutic intervention against the MAPK pathways, using a JNK inhibitor and a p38 inhibitor (Kikuchi et al., 2000; Lee et al., 2000; Mielke & Herdegen, 2000), may suppress apoptotic neuronal death and neuro-inflammation during acute virus encephalitis and minimize neurological sequelae.

Fig. 5. Activation of p38 MAPK on day 7 after infection. (a) Infected neurons in the ventral tegmental area (VTA). (b) p38 MAPK immunoreactivity. (c) Overlapped confocal image. Note the upregulated expression of p38 MAPK in brain cells, including virus-infected and degenerating neurons. (d) Infected neurons in the hippocampal formation where infection predominates in the pyramidal layer (Py). (e) Phospho-p38 MAPK immunoreactivity. (f) Overlapped confocal image. Note the specific signals for phosho-p38 MAPK appear conspicuous in the CA1 region, which is not a major target of the virus. (g) GFAP-specific signals in the VTA, which is a major target of the virus. (h) Phospho-p38 MAPK immunoreactivity. (i) Overlapped confocal image. Note that phospho-p38 MAPK immunoreactivity emerges inside of some GFAP-positive cells and occasionally appears in a nuclear staining manner (arrowheads). (j) GFAP-specific signals in the VTA. (k) TUNEL-specific signals. (l) Overlapped confocal image. Note the two signals do not co-localize with each other. Bar, 20 μm.
Fig. 6. Evidence of TNF-α production in phospho-p38 MAPK-immunopositive cells. Cerebral cortex. (a) Phospho-p38 MAPK immunoreactivity. (b) TNF-α immunoreactivity. (c) Overlapped confocal image. Phospho-p38 MAPK-immunopositive cells frequently manifest TNF-α-specific signal (arrows). The arrowhead points to a TNF-α-producing cell without phospho-p38 MAPK expression. Bar, 20 μm.

Fig. 7. Detection of TNF-α-specific mRNA by RT-PCR at the midbrain level. M1, Influenza A virus membrane protein 1.

ACKNOWLEDGEMENTS

We would like to thank Eiko Iwata and Tama Tsuruguchi for technical assistance. This work was supported in part by a grant from the Waksman Foundation of Japan. Isamu Mori was the recipient of an Encouragement of Young Scientist Award from the Nakanihon Infectious Diseases Research Foundation, Japan.

REFERENCES


http://vir.sgmjournals.org


Tobiume, K., Matsuzawa, A., Takahashi, T. & 7 other authors (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep 2, 222–228.


