

Induction of Chemokines in Human Astrocytes by Picornavirus Infection Requires Activation of Both AP-1 and NF- κ B

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KEY WORDS human astrocytes; picornavirus infection; chemokines; NF- κ B; AP-1

ABSTRACT Infection with different picornaviruses can cause meningitis/encephalitis in humans and experimental animals. To investigate the mechanisms of such inflammatory diseases, potential chemokine gene activation in human astrocytes was investigated following infection with Theiler's murine encephalomyelitis virus (TMEV), coxsackievirus B3 (CVB3), or coxsackievirus B4 (CVB4). We report that all these viruses are potent inducers for the expression of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) genes in primary human astrocytes, as well as in an established astrocyte cell line (U-373MG). Further studies indicated that both activator protein-1 (AP-1) and NF- κ B transcription factors are required in the activation of chemokine genes in human astrocytes infected with various picornaviruses. Interestingly, the pattern of activated chemokine genes in human astrocytes is quite restricted compared to that in mouse astrocytes infected with the same viruses, suggesting species differences in gene activation. This may result in potential differences in the pathogenic outcome in each species. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Many different enteric picornaviruses, including coxsackievirus B (CVB), cause acute or chronic aseptic meningitis and/or encephalitis in young children and immunocompromised individuals (Muir and van Loon, 1997; Liow et al., 1999; Rotbart, 2000; Hinson and Tyor, 2001). Coxsackievirus B3 (CVB3) and B4 (CVB4) infections in mice have also been used to study myocarditis and pancreatitis, as these viruses are known to cause these diseases in humans as well as in mice (Ramsingh et al., 1997; Modlin et al., 1999). In addition, Theiler's murine encephalomyelitis virus (TMEV) belonging to the cardiovirus genus of the picornavirus family causes acute encephalitis and/or chronic demyelinating disease in experimental mice; thus, this system has been used as a relevant model for virus-induced demyelinating disease (Kim et al., 2001). Many

of the previous studies have been focused on immune components involved in myocarditis and pancreatitis for CVB and demyelination for TMEV. However, numerous recent studies recognized the importance of innate immune responses to viral infections subsequently influencing the development of specific adaptive immune responses to the infectious agents (Liew and McInnes, 2002; Reiss et al., 2002). Despite the importance of innate immune responses, virtually nothing is known about the initial responses to CVB or other picornaviruses by human glial cells. Such studies

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Received 11 December 2002; Accepted 16 September 2003

DOI 10.1002/glia.10331

may be valuable in understanding the pathogenic mechanisms of encephalitis and demyelination induced by these viruses.

Chemokines are important mediators of the inflammatory response to many stimuli, including viral infections. Chemokines appear to have multiple biological functions in inflammatory responses, such as chemoattraction of a variety of cells, activation of certain cell populations, angiogenesis of endothelial cells and blood-brain barrier dysfunction (Kossmann et al., 1997; Kunkel and Butcher, 2002). In viral meningitis, interleukin-8 (IL-8), IFN γ -inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) appear to be responsible for the chemoattraction of neutrophils, peripheral blood mononucleated cells (PB-MCs), and activated T cells (Lahrtz et al., 1998). Similarly, significant elevation of MCP-1 and IL-8 is demonstrated in patients with demyelinating diseases (Van Der Voorn et al., 1999; Polyak et al., 2001). The production of cytokines and chemokines has also been shown in the CNS of mice following a variety of viral infections, including TMEV (Asensio and Campbell, 1997; Lane et al., 1998; Hoffman et al., 1999; Noe et al., 1999; Lane et al., 2000; Theil et al., 2000). Various chemokines were found in the CNS during the onset and late stages of TMEV-induced disease (Hoffman et al., 1999; Theil et al., 2000). More recently, we have shown that TMEV infection primarily upregulates regulated upon activation in normal T-cells, expressed, and secreted (RANTES) and IP-10 gene expression in primary murine astrocyte cultures and broader chemokine genes in oligodendrocyte and microglia cultures as early as 30 min after viral infection (Palma and Kim, 2001). These virus-induced chemokines overlap with those that are inducible by proinflammatory cytokines in human and mouse astrocytes (Wesselingh et al., 1990; Shrikant et al., 1995; Shrikant and Benveniste, 1996), suggesting that glial cells, astrocytes in particular, may be critical for early recruitment of inflammatory cells in the initiation of virus-induced, immune-mediated demyelination. However, very little is known about this chemokine gene expression in human astrocytes following infection with CVB, which is a potentially important mechanism involved in the initiation of inflammatory disease in target tissues, including the CNS.

In the present study, we have examined potential chemokine gene activation in human astrocytes following infection with TMEV, CVB3, or CVB4, which are known to cause acute or chronic disease in the CNS of humans and/or mice. We demonstrate that all these viruses induce IL-8 and MCP-1 selectively in primary human astrocyte cultures and a human astrocytoma line. Activation of both activator protein-1 (AP-1) and NF- κ B transcription factors appears to be necessary for the chemokine gene activation, suggesting that both factors are involved in the activation of chemokine genes in human astrocytes infected with various picornaviruses. This pattern of activated chemokine genes in human astrocytes is significantly different from that

in mouse astrocytes, and the differences between the species may differentially affect the pathogenic outcome by infection with the identical viruses.

RESULTS

IL-8 and MCP-1 Chemokine Genes Are Activated Selectively Upon Infection With Different Picornaviruses

To investigate the mechanisms involved in the initiation of inflammatory responses leading to meningitis/encephalitis following infection with CVB, the possibilities that these viruses are able to infect astrocytes and activate chemokine gene expression were examined using primary human astrocyte cultures and a human astrocyte cell line, U-373 after infection with CVB3 or CVB4 (Fig. 1). In addition, the chemokine gene activation patterns were compared to that induced by TMEV, which is known to induce various chemokine genes in primary murine astrocytes (Palma and Kim, 2001). The chemokine mRNAs expressed following infection with these viruses were identical with respect to the chemokines and kinetics. As early as 1–2 h after viral infection, the chemokine mRNAs were detectable and levels increased at 4–8 h. In addition, the chemokine genes activated by these viruses were very selective; only MCP-1 and IL-8 mRNAs of seven different chemokine mRNAs tested were significantly induced. Although the levels of chemokine gene activation were relatively low in the primary human astrocyte cultures, the pattern was virtually identical to that seen with the U-373 astrocyte cell line (Fig. 1B). The lower level of chemokine gene activation in the primary cultures may reflect lower infectivity and/or cell numbers used due to limited supply.

UV-Inactivated Virus Fails to Activate Chemokine Gene Activation

To verify that such chemokine mRNA expression leads to the production of chemokine proteins, accumulation of IL-8 and MCP-1 proteins in the culture supernatants of U-373 cells infected with virus for 6 h was assessed with enzyme-linked immunosorbent assay (ELISA) (Fig. 2). The results clearly indicate that the selective chemokine gene activation following infection with virus results in the production of the chemokine proteins. Thus, these chemokines induced upon viral infection are likely to affect the type and level of inflammatory responses by influencing the infiltration of select cell types. To determine further whether such chemokine gene activation requires viral replication, the chemokine levels in similar cultures exposed to ultraviolet (UV)-inactivated virus were compared to those of intact virus (Fig. 2). The results clearly indicate that UV inactivation abrogates the ability of virus to induce chemokine gene activation.

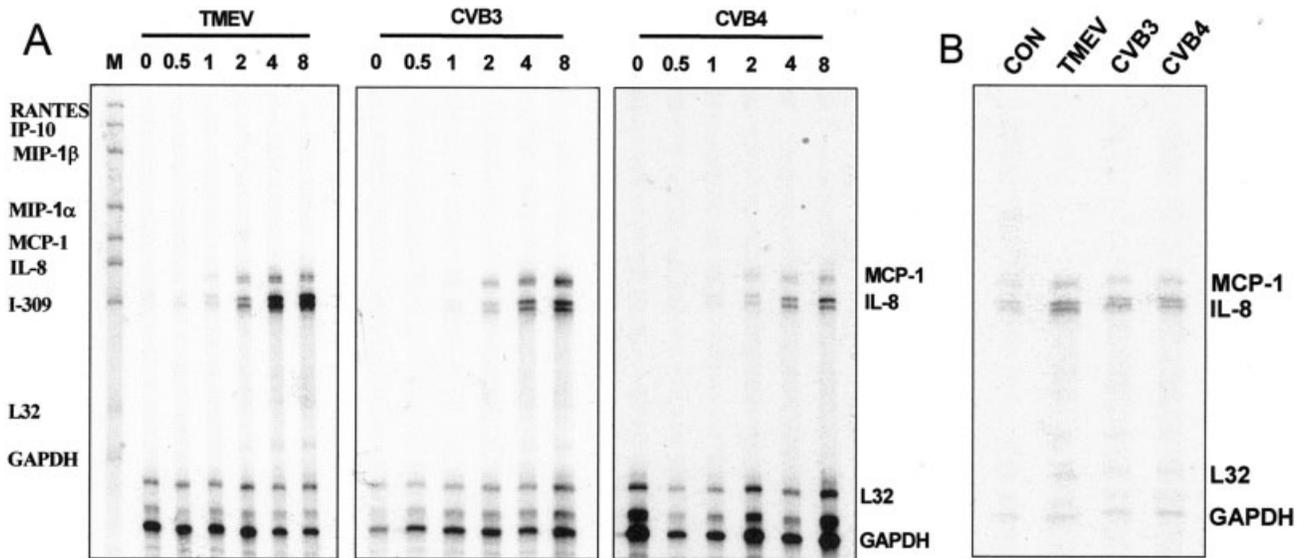


Fig. 1. Activation of select chemokine genes in human astrocytoma cells and primary astrocytes by Theiler's murine encephalomyelitis virus (TMEV), coxsackievirus B3 (CVB3), and coxsackievirus B4 (CVB4). Gene expression was assessed using RNase protection assay (RPA). A: Human astrocytoma cells were cultured with viruses for 0,

0.5, 1, 2, 4, and 8 h. B: Human primary astrocytes were incubated with viruses for 8 h. Cells not incubated with viruses were used as a negative control. L32 and GAPDH were used as an internal control for mRNA levels.

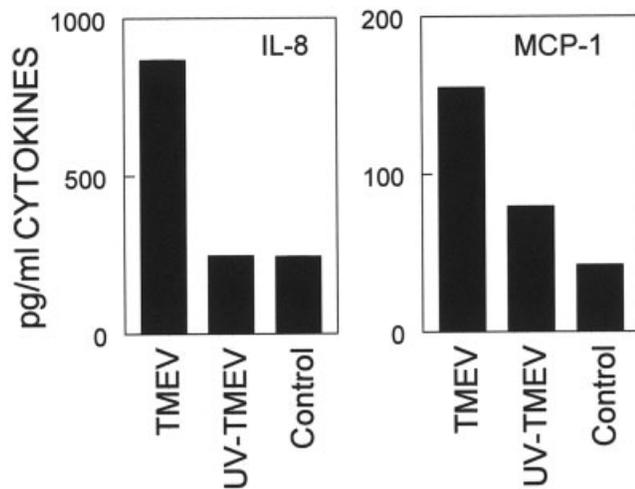


Fig. 2. Induction of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) proteins in human astrocytoma cells by Theiler's murine encephalomyelitis virus (TMEV) infection. Human astrocytoma cells were cultured with intact TMEV or UV-inactivated TMEV for 6 h. The presence of IL-8 and MCP-1 proteins in the culture supernatants was analyzed with ELISA. A representative of three separate experiments is shown. Similar results were obtained from U373 cultures after infection with coxsackievirus B (CVB) and UV-CVB (data not shown).

MAP Kinases and AP-1 Transcription Factor Are Activated After Viral Infection

To assess the mechanisms involved in the chemokine gene activation upon viral infection, the possibility of MAP kinase and consequent AP-1 activation was examined with U-373 cells infected with TMEV for varying (0–8-h) periods. Western blot analysis using antibodies specific for the phosphorylated form of the

molecules indicated that extracellular signal-regulated kinase (ERK-1/2) and c-Jun N-terminal kinase (JNK) were activated as early as 0.5–1 h and persisted for 4–8 h after viral infection (Fig. 3A). The levels of total ERK-1/2 were similar throughout the time periods indicating that the increases in the phosphoproteins were not due to the differences in the concentrations of proteins loaded to the gel. Similarly, activation of p38 mitogen-activated protein kinase (MAPK) was also detectable 1 h after viral infection (Fig. 3B). Therefore, infection of human astrocytes with picornavirus results in activation of the MAPK involved in inflammatory responses. To correlate the activation of MAPK with chemokine production, activation of a potential downstream transcription factor, AP-1 was assessed by electrophoretic mobility shift assay (EMSA), using the consensus AP-1-specific oligonucleotides (Fig. 3C). Our results clearly indicate that the AP-1 transcription factor is also activated soon after viral infection. AP-1 accumulation showed a peak at 0.5–1 h and was reduced significantly at 2 h and thereafter. The AP-1 binding assay was specific for the nucleotide sequence, as an excess amount of the unlabeled oligonucleotides successfully blocked the binding of nuclear extracts to the labeled nucleotide probe. These results strongly suggest that picornaviral infection leads to the activation of MAPK as well as AP-1 that is potentially associated with chemokine production.

IL-8 and MCP-1 Gene Expression Requires Activation of ERK-1/2 and p38

To examine whether MAPK is necessary for the activation of selected chemokine genes after viral infec-

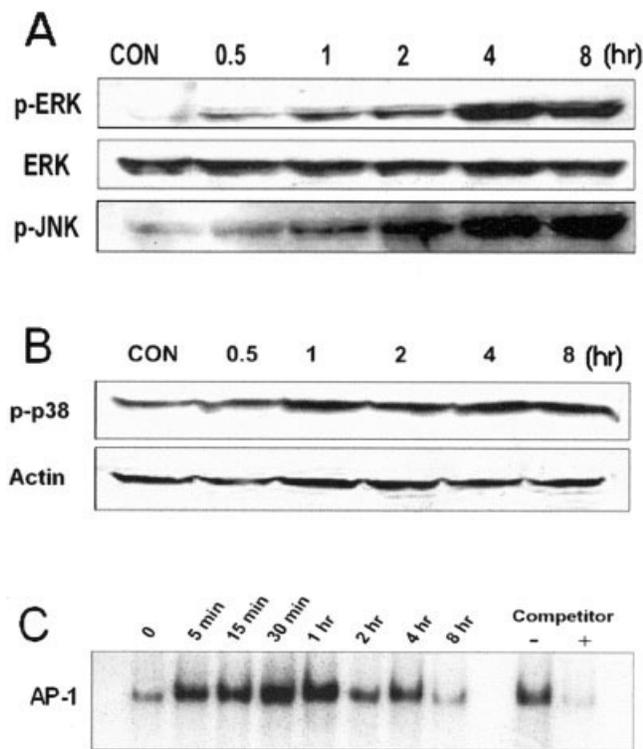


Fig. 3. Activation of MAP kinases and activator protein-1 (AP-1) in human astrocytoma cells following Theiler's murine encephalomyelitis virus (TMEV) infection. Human astrocytoma cells were cultured with TMEV for 0, 15, 30 min, 1, 2, 4, and 8 h. Activation of MAP kinases was assessed by determining the level of phosphorylation with Western blotting using antibodies specific for phosphorylated forms of kinases. Phospho-ERK and phospho JNK(A) and phospho-p38 (B) were determined. Either total ERK or actin was assessed using specific antibodies as internal controls for protein concentrations loaded. C: Human astrocytoma cells were cultured with TMEV for varying time periods before nuclear extraction to determine electrophoretic mobility shift assay (EMSA) for AP-1 binding. Unlabeled AP-1-specific oligonucleotide was used for competition analysis.

tion, cells pretreated for 1 h with specific inhibitors for ERK and p38 (U0126 and SB2024190, respectively) were subsequently infected with virus. Interestingly, these treatments inhibited MCP-1 and IL-8 mRNA production in a dose-dependent manner, while similar treatment with respective negative control chemicals, U0124 and SB202474, failed to inhibit the chemokine gene activation (Fig. 4A). The production of chemokine proteins paralleled the message levels; i.e., these inhibitors also suppressed the production of chemokines (Fig. 4B). These results suggest that activation of MAPK is essential for the selective chemokine production induced after infection with picornavirus in human astrocytes.

NF- κ B Is Also Activated in Human Astrocytes Following Viral Infection

Inflammatory responses following infection with various infectious agents are highly dependent on activa-

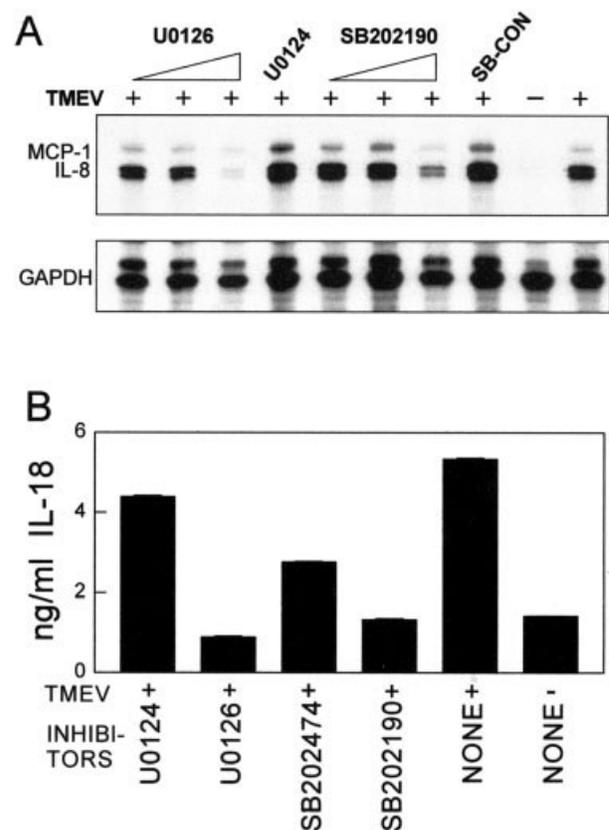


Fig. 4. Inhibition of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) production induced following viral infection by U0126 and SB202190. A: mRNA levels were determined by RPA. U373 cells pretreated with three different concentrations (0.1, 1, and 10 μ M) of MEK1/2 inhibitor U0126 or the p38 inhibitor SB202190 for 1 h prior to Theiler's murine encephalomyelitis virus (TMEV) infection for 8 h. As negative controls for U0126 and SB202190, U0124 (10 μ M) and SB202474 (10 μ M) were used, respectively. B: IL-8 levels were determined with ELISA. U373 cells were pretreated with 10 μ M of the inhibitors or controls for 1 h prior to TMEV infection. Culture supernatants were collected after 24 h and then assessed the levels of IL-8 protein.

tion of the NF- κ B transcription factor. Therefore, the possibility of NF- κ B activation in the astrocytoma cells following viral infection was next examined (Fig. 5). First, the levels of total I κ B- α and phosphorylated form of I κ B- α were assessed during viral infection in astrocytes by Western blot analysis (Fig. 5A). The results indicated that I κ B- α is rapidly phosphorylated (>2-fold) as early as 15 min upon viral infection, as compared to uninfected control cultures, and sustained for at least 8 h. In addition, the overall I κ B level was increased significantly 15 min after viral infection, declined rapidly at 30 min, and remained low thereafter. These results strongly suggest that I κ B is rapidly phosphorylated and subsequently degraded upon viral infection, leading to NF- κ B activation. To assess directly the activation of NF- κ B, accumulation of NF- κ B molecules in the nuclei of virus-infected astrocytoma cells was assessed by EMSA, using NF- κ B-specific oligonucleotides (Fig. 5B). The results indicate that nuclear translocation of NF- κ B starts at 15 min, peaks at 30

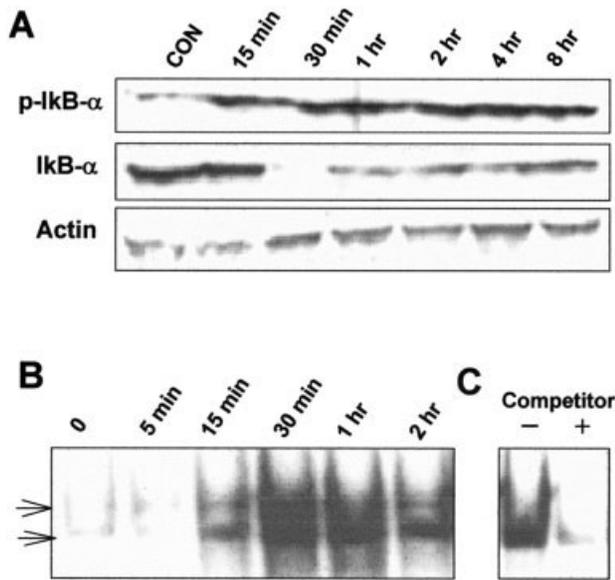


Fig. 5. Activation of NF- κ B in human astrocytoma cells following viral infection. **A:** Phosphorylation of I κ B- α in astrocytoma cells following Theiler's murine encephalomyelitis virus (TMEV) infection was assessed by western blot analysis using antibodies specific for I κ B- α and phospho-I κ B- α . **B:** Activation of NF- κ B was determined with electrophoretic mobility shift assay (EMSA) following NF- κ B binding to nuclear extracts from virus-infected cells. **C:** Specificity of the binding was further examined by a competition test in the presence of cold NF- κ B probe.

min, and then becomes reduced yet remains elevated at 2 h following viral infection. The binding was completely abrogated to background levels in the presence of excess amount of unlabeled NF- κ B-specific oligonucleotides (Fig. 5C). Altogether, these results clearly demonstrate that the NF- κ B pathway is rapidly activated in human astrocytes similar to the AP-1 transcription factor upon picornavirus infection (Fig. 3).

NF- κ B Inhibitors Suppress Virus-Induced Transcription of IL-8 and MCP-1 Genes

To correlate NF- κ B activation and its role in chemokine gene expression induced by viral infection, the effect of varying concentrations of an I κ B kinase inhibitor, PDTC, on chemokine production was examined (Fig. 6A,B). The experimental results showed that blocking I κ B phosphorylation inhibits transcription of both MCP-1 and IL-8 messages induced after viral infection, leading to complete inhibition at 50 and 100 μ g/ml of PDTC. The levels of chemokines secreted by the virus-infected cells in the presence of the inhibitor corresponded with transcription levels. Because these chemical inhibitors may also inhibit the activation of NF- κ B without involving I κ B, the effect of a dominant negative form of I κ B, a replication-deficient adenovirus expressing I κ B substituted at two phosphorylation sites required for NF- κ B activation, was also examined (Fig. 6C). Chemokine gene expression in astrocytes

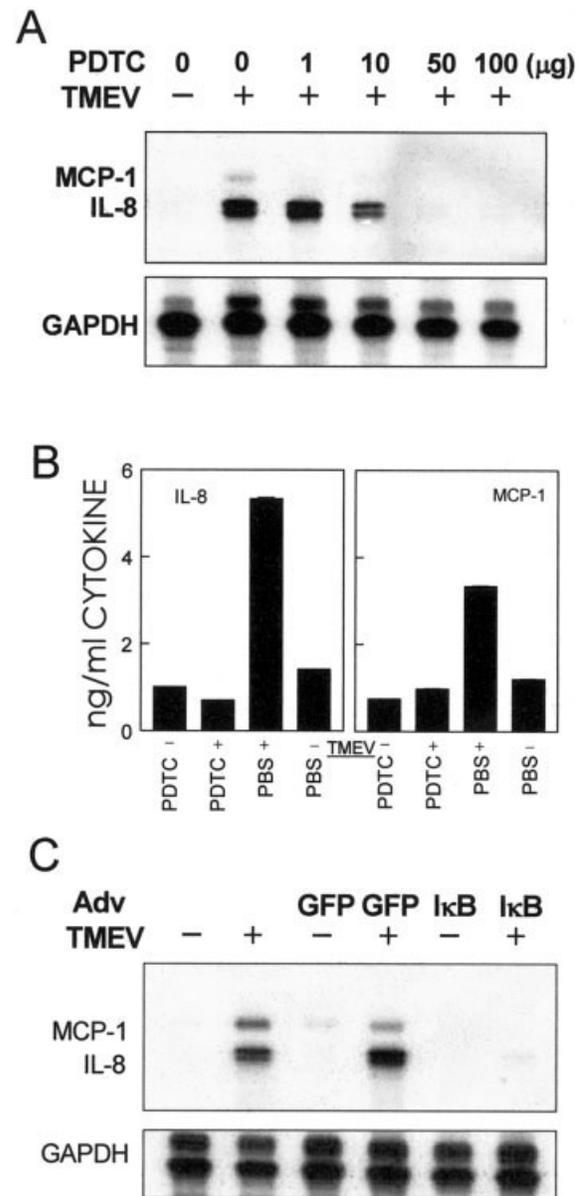


Fig. 6. Inhibition of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) mRNA induction by blocking the NF- κ B pathway. **A:** U373 cells were pretreated with PDTC for 1 h prior to Theiler's murine encephalomyelitis virus (TMEV) infection for 8 h. **B:** Supernatants from PDTC-treated and control cultures were assessed for the presence of IL-8 and MCP-1 using ELISA. **C:** Cells were infected with adenovirus expressing dominant negative mutant form of I κ B before viral infection. Adenovirus-GFP was used as a negative control.

infected with the adenovirus-I κ B super-suppressor prior to picornavirus infection was compared to that infected with control GFP-adenovirus. Astrocytes preinfected with the I κ B super repressor, but not control GFP-adenovirus, completely inhibited IL-8 and MCP-1 gene expression inducible by TMEV. These results clearly demonstrate that the activation of NF- κ B pathway is also necessary for IL-8 and MCP-1 gene expression induced by the picornavirus infection.

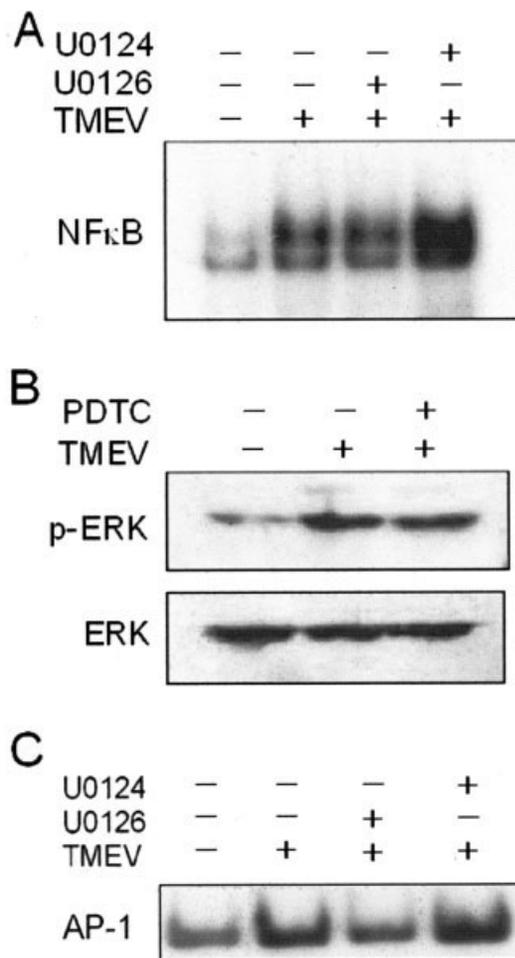


Fig. 7. Independence between activation of ERK and NF- κ B for induction of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) gene expression. **A:** Human astrocytoma cells were pretreated with a MEK1/2 inhibitor U0126 or a control U0124 (10 μ M) for 1 h prior to Theiler's murine encephalomyelitis virus (TMEV) infection for 30 min and subjected to EMSA for NF- κ B binding. **B:** Cells were pretreated with PDTC (100 μ M) for 1 h before TMEV infection and then analyzed by western blot assay using antibodies specific for ERK and phospho-ERK. **C:** Cells were pretreated for 1 h with either U0126 or U0124 prior to infection with TMEV and then nuclear extracts of control and infected cells were assessed for binding to the activator protein-1 (AP-1) nucleotide sequence.

MAPK Activation by Picornaviral Infection Is Not Associated With NF- κ B

Since activation of both MAPK and NF- κ B pathways is necessary for the chemokine gene expression in human astrocytes induced by picornaviral infection (Figs. 4 and 6), it would be important to delineate whether MAPK activation is somehow associated with NF- κ B activation. To examine this possibility, activation of NF- κ B was assessed following TMEV infection in the presence of inhibitors for ERK and NF- κ B, respectively (Fig. 7). NF- κ B activation was apparently independent of ERK-1/2 activation (Fig. 7A), since the presence of inhibitor of upstream MEK, U0126, did not affect the activation of NF- κ B induced by viral infection. Simi-

larly, the presence of NF- κ B inhibitor, PDTC, did not affect the activation of ERK-1/2 (Fig. 7B), which appears to be necessary for induction of chemokine gene expression by viral infection (Fig. 4). Inhibition of ERK-1/2 activation with pretreatment with U0126, but not control U0124, significantly reduced the nuclear translocation of AP-1, suggesting that an ERK-1/2 pathway is required for AP-1 activation (Fig. 7C). Together, these results indicate that activation of both NF- κ B as well as AP-1 transcription factors are necessary for the human chemokine gene expression, although the activation pathways for these transcription factors are independent of each other.

Different Chemokine Genes Are Activated in Human and Mouse Astrocytes

We have previously observed that TMEV infection in primary mouse astrocytes induces the expression of several chemokine genes, including RANTES, MIP-1 α/β , MIP-2, MCP-1, and IP-10 (Palma and Kim, 2001). Therefore, the chemokine gene induction limited to MCP-1 and IL-8 in human astrocytes by the same viruses appears to be significantly different from that of mouse astrocytes. To verify the possible differences in the chemokine gene expression between human and mouse astrocytes, chemokine gene expression in mouse primary astrocytes was also assessed after infection with TMEV, CVB3, or CVB4 (Fig. 8). As previously shown with TMEV infection (Palma and Kim, 2001), primary mouse astrocytes expressed a wide range of chemokines following infection with these viruses. Thus, the pattern of chemokine genes activated in mouse astrocytes by these viruses is significantly different from that in human astrocytes, where no significant levels of RANTES and IP-10 genes are activated. These results clearly indicate that the pattern of chemokine gene activation by picornaviruses in human astrocytes is much more restricted as compared to that in mouse astrocytes.

DISCUSSION

Various picornaviruses, including CVB and poliovirus in the enterovirus genus, as well as TMEV in the cardiovirus genus, are known to cause acute or chronic meningitis/encephalitis in humans and/or mice (Muir and van Loon, 1997; Ramsingh et al., 1997; Liow et al., 1999; Modlin et al., 1999; Rotbart, 2000; Hinson and Tyor, 2001; Kim et al., 2001). To understand the pathogenic mechanisms involved in the initiation of inflammatory responses in the CNS following infection with these picornaviruses, chemokine production in human primary astrocyte cultures and an astrocytoma line were analyzed upon infection with CVB or TMEV. Our results demonstrated that only select chemokines, IL-8 and MCP-1 are activated in human astrocytes by viral infection (Figs. 1 and 7). Recent studies reported that

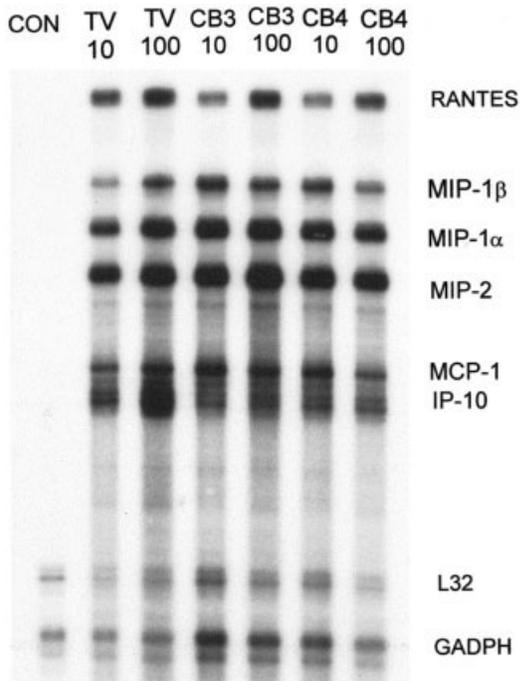


Fig. 8. Differences in chemokine gene induction in the primary mouse astrocytes by the picornaviruses. Mouse primary astrocyte cultures derived from neonatal brains were infected with Theiler's murine encephalomyelitis virus (TMEV), coxsackievirus B3 (CVB3), or coxsackievirus B4 (CVB4) and the chemokines genes activated by these viruses for 8 h were compared by RPA.

either human primary astrocytes or astrocytoma lines produce IL-8 or MCP-1 following infection with HIV-1 or coronavirus, respectively (Cota et al., 2000; Edwards et al., 2000). In addition, infection with certain picornaviruses, such as rhinovirus and CVB3, can induce IL-8 in various human fibroblast cells (Johnston et al., 1998; Heim et al., 2000). These results strongly suggest that induction of highly restricted chemokine gene activation is common to many different viral infections in various human cell types.

It is interesting to note that IL-8, a member of the CXC family of chemokines, is a potent chemoattractant and activator of neutrophils, basophils, monocytes, and endothelial cells. Furthermore, IL-8 is known to enhance the cytopathic effect of encephalomyocarditis virus (EMCV), a member of picornavirus on a human cell line (Khabar et al., 1997b). This chemokine appears to play a major negative role in the early defense against viruses by inhibiting interferon- α (IFN- α) activity resulting in increases in viral RNA synthesis and persistence (Khabar et al., 1997a). In contrast, MCP-1 is a member of the CC family of chemokine, directing neutrophil and monocyte/macrophage infiltration to the site of infection. These chemokines have also been found in various inflammatory responses and at the sites of inflammatory diseases, including demyelinating lesions of multiple sclerosis (Van Der Voorn et al., 1999; Polyak et al., 2001). Therefore, these chemokines, in particular, are most likely to play profound

roles in the initiation and expansion of inflammatory responses to various picornaviruses in the target tissues of viral infection, including the CNS.

The signal pathways responsible for the highly selective chemokine gene activation in human astrocytes infected with these picornaviruses have also been assessed in the present study. We primarily used TMEV as a representative picornavirus for these studies since identical patterns of chemokine gene activation are observed upon infection with TMEV, CVB3, or CVB4 in both human (Fig. 1) and murine astrocytes (Fig. 8), suggesting that common mechanisms are involved in chemokine gene activation by these viruses, and perhaps by picornavirus in general. Interestingly, activation of both MAPK and NF- κ B appears to be necessary for the chemokine gene activation after viral infection (Figs. 4 and 6). In particular, U0126, a specific inhibitor for ERK activation, exerted a powerful suppression of both IL-8 and MCP-1 gene expression. Recently, it has been shown that IL-8 production is linked to activation of ERK-1/2 by other viruses (Chen et al., 2000; Alcorn et al., 2001). In addition, HIV-1 Tat protein is capable of inducing primarily IP-10, MCP-1, and IL-8 chemokine gene expression in a human astrocyte line (CRT-MG), and the expression of both MCP-1 and IL-8, but not IP-10, is inhibited by U0126 (Kutsch et al., 2000). However, it remains unclear how the ERK-1/2 activation pathway is associated with chemokine gene expression. Previously, it has been established that MEKK1 (ERK kinase kinase-1) induces activation of both IKK- α and IKK- β leading to NF- κ B activation. (Lee et al., 1997, 1998). Thus, ERK activation may be necessary for NF- κ B activation following infection with picornavirus. However, our results show that activation of ERK induced after viral infection is independent from activation of NF- κ B (Fig. 7). In addition, it appears that the downstream target of ERK is AP-1 for chemokine gene expression in human astrocytes (Fig. 7C), similar to human microglia following stimulation with IFN- β (Kim et al., 2002). Recently, activation of p38 and JNK was shown in fibroblast cells following infection with EMCV (Iordanov et al., 2000). Since p38 MAP kinase is involved for NF- κ B-dependent gene expression by regulating the DNA binding of TATA-binding protein to the TATA box (Carter et al., 1999), this MAPK may also play a role in this pathway.

Viral replication appears to be required for activation of chemokine gene expression, as UV-inactivated virus failed to activate chemokine production in human astrocytes (Fig. 2). This is consistent with our earlier reports that chemokine gene activation is abrogated by UV-inactivation of virus (Palma and Kim, 2001) and that only mouse astrocytes producing viral RNA produce cytokines after TMEV infection (Palma et al., 2003). These results strongly suggest that viral infection and replication are required for cellular gene activation by picornavirus. Interestingly, our preliminary study suggests that overlapping but different signaling pathways are required for viral replication and chemokine gene activation in human astrocytes. For example,

NF κ B and p38 inhibitors, but not ERK inhibitor, suppressed viral message production, which is different from chemokine gene activation. We do not know why MAPK remain activated up to 8 h in contrast to AP-1, which decreases after 2 h. Similarly, we do not know why phospho-I κ B remains high, and yet overall I κ B levels recover. Obviously, additional studies are required to unravel the complex signaling relationship between viral replication and activation of chemokine genes, as well as other cellular survival genes.

The promoter regions of the both MCP-1 and IL-8 chemokines activated by CVB or TMEV infection contain both AP-1 and NF- κ B binding sites (Mukaida et al., 1990; Martin et al., 1997). In addition, IL-8 gene expression induced by cytomegalovirus (CMV) involves concurrent binding of AP-1 and NF- κ B to the promoter sites of the IL-8 gene (Murayama et al., 1997), suggesting that the combination of these transcription factors is a powerful inducer for selective chemokine gene expression (Ondrey et al., 1999). Similarly, induction of MCP-1 by IL-1 β in human endothelial cells depends on the cooperation between AP-1 and NF- κ B (Martin et al., 1997). However, it is also conceivable that undefined additional regulatory elements in the IL-8 and MCP-1 genes may be involved in the expression of these chemokine genes, as described for the respiratory syncytial virus (RSV) response element in the IL-8 promoter region (Casola et al., 2000). Nevertheless, it is very likely that chemokine genes responsive to a combination of AP-1 and NF- κ B transcription factors are preferentially activated in human astrocytes by certain viral infections, including picornaviruses. This preferential activation of select chemokine gene expression may function as a mechanism favoring viral host evasion by lowering antiviral IFN function and promoting cellular infiltration, ultimately supporting viral expansion and inflammation.

This pattern of restricted chemokine gene activation induced in human astrocyte cultures after viral infection is quite distinct from that induced in mouse primary astrocyte cultures (Figs. 1 and 8). Species differences in the chemokine gene activation may represent differences in the requirements for chemokine gene activation. For example, the RANTES gene does not contain an AP-1 binding site and yet is activated predominantly in mouse astrocytes infected with these viruses. Thus, NF- κ B transcription factor alone appears to be sufficient for mouse chemokine/cytokine gene activation, perhaps leading to broader gene expression (Palma et al., 2003). The significance of this difference in chemokine gene activation between human and mouse astrocytes is unclear. However, the difference in the chemokine gene activation pattern is species specific and not dependent on the viruses in the picornavirus family, as demonstrated using different picornaviruses in the present study (Figs. 1 and 8). The differences in chemokine gene expression are not likely to reflect a difference in the level of infection between human and mouse astrocytes, since a similar infectivity (20–30%), as well as unaltered patterns by varying

viral dose, are observed in these cell types. Thus, an identical virus may be able to manifest a different disease in different species. The type of initial chemokines produced alone may not be sufficient to direct the outcome of inflammatory diseases since these viruses induce different diseases in the same species. Perhaps, combinations of the level and site of viral persistence and the type of infiltrating cells may shape the adaptive immune response leading to either the protection or further development of specific inflammatory diseases.

MATERIALS AND METHODS

Cell Culture

BHK and MEK cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human U-373MG cells, a human astrocytoma cell line, were cultured in modified Eagle's medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 2 mM L-glutamine, and penicillin/streptomycin. Human fetal primary astrocytes were prepared by Dr. In-Hong Choi (Seoul, Korea) and were cultured in the identical medium as U-373MG cells.

Reagents

The MEK-1/2 inhibitor U0126, the p38 MAPK inhibitor SB202190, and the respective controls U0124 and SB202474 were purchased from Calbiochem (San Diego, CA). The NF- κ B inhibitor (PDTC) was obtained from Sigma (St. Louis, MO).

Viral Infection

TMEV was expanded *in vitro* using permissive BHK cells, and CVB3 or CVB4 was expanded using MEK cells. Viral titer was determined by plaque assay on BHK or MEK cells and subsequently used for *in vitro* infection. Astrocytes were infected with TMEV or CVB at 10 multiplicity of infection (MOI) for varying time periods. Either infection medium or BHK lysates was used as control for viral infection in the mock-infected cultures. To inhibit specifically the activation of NF- κ B, U-373MG cells were infected with the adenovirus super-repressor for I κ B 24 h before TMEV infection. Greater than 90% of the astrocytes were infected by adenovirus based on assessment with a control GFP-adenovirus.

Total RNA Isolation and RNase Protection Assay

Total cellular RNA was extracted using guanidinium isothiocyanate and phenol followed by ethanol precip-

itation (Chomczynski and Sacchi, 1987). RNase protection assay was used to determine the expression levels of various chemokines, using the RiboQuant multiprobe (hCK-5) system (Pharmingen, San Diego, CA). The probe set was transcribed with T7 RNA polymerase, hybridized overnight, and then analyzed according to the manufacturer's instructions.

ELISA

The levels of IL-8 and MCP-1 secreted by U-373 cells after viral infection were assessed using ELISA kits (BD-Pharmingen, San Diego, CA). Supernatants from U-373MG cultures infected with TMEV were measured for the levels of IL-8 and/or MCP-1 production.

Western Blot Analysis

Uninfected control and virus-infected U-373MG cells were sonicated, centrifuged, and then protein concentration in the supernatant was measured by Bradford method (Pierce, Rockford, IL). The proteins (100 μ g per lane) were electrophoresed on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ), and then probed with horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibodies specific for ERK-1/2, phospho-ERK-1/2, phospho-JNK, or phospho-p38 (New England Biolabs, Beverly, MA). The levels of specific proteins were visualized with the ECL technique (Amersham Biosciences, Piscataway, NJ).

Electrophoretic Mobility Shift Assay

Nuclear extracts from U-373 cells infected with virus were prepared as previously described (Nguyen et al., 1998). The nuclei were pelleted at 3,000g for 10 min and resuspended in 200 μ l of high salt buffer (10 mM HEPES, pH 7.9, 1 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 400 mM NaCl, 15% glycerol, 1 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM sodium orthovanadate). The suspension was rocked gently for 30 min at 4°C followed by microcentrifugation at 12,000g for 10 min at 4°C. The protein concentration in the supernatant was determined with the Bradford method. Double-stranded oligonucleotides containing the consensus NF- κ B or AP-1 sequences (Santa Cruz, Santa Cruz, CA) were used in the EMSA. Oligonucleotides were end-labeled with [γ -³²P] ATP (Dupont-NEN, Boston, MA) using T₄ polynucleotide kinase. Typically, 10 μ g of nuclear extracts were equilibrated for 15 min in binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT) and 1 μ g of poly dI/dC (Amersham Pharmacia

Biotech). ³²P-labeled oligonucleotide probe (~20,000 cpm) was added to the extracts and incubated for an additional 20 min at 4°C. Bound and unbound probes were then separated by electrophoresis on a 5% native polyacrylamide gel at 10 V/cm and subsequently analyzed using a phosphor imager (Bio-Rad, Richmond, CA). For competition analysis, a 50-fold molar excess of unlabeled oligonucleotides was added to the nuclear extracts 30 min prior to the addition of the corresponding labeled probe.

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