Induction of RANTES/CCL5 by herpes simplex virus is regulated by nuclear factor $\kappa$B and interferon regulatory factor 3

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Chemokines regulate migration of leukocytes to sites of infection. In this work, we have shown that the chemokine RANTES/CCL5 is produced after herpes simplex virus (HSV) infection of macrophages and fibroblasts and provide data on the underlying molecular mechanism. Reporter gene assays showed HSV-induced RANTES production to be regulated at the transcriptional level. Expression of RANTES was blocked by N-tosyl-L-phenylalanine, an inhibitor of the nuclear factor $\kappa$B (NF-$\kappa$B) pathway and also in cell lines stably expressing a dominant-negative mutant of I$\kappa$B kinase $\beta$. Cell lines stably expressing a dominant-negative mutant of interferon regulatory factor 3 (IRF-3) also produced dramatically decreased levels of RANTES after infection compared with the control cell line. In contrast, overexpression of dominant-negative p38 and also treatment with SB203580, an inhibitor of p38, did not significantly affect expression of RANTES. Taken together, these data suggest that HSV induces transcriptional activation of the RANTES gene through the transcription factors NF-$\kappa$B and IRF-3.

Herpes simplex virus (HSV) types 1 and 2 are ubiquitous human pathogens causing a number of diseases, including gingivostomatitis, cold sores, keratitis, genital herpes, meningitis and neonatal herpes (Whitley & Roizman, 2001). The pathology observed following HSV infection is due to the cytopathic effect of the infection as well as the host immune response (Skoldenberg, 1996). To eliminate the virus from infected areas, while at the same time avoiding immune-mediated tissue destruction, proper recruitment, activation and regulation of leukocytes is important. In this process, chemokines and their receptors play a central role through their activation and mobilization of leukocytes (Luster, 1998). The localized and controlled chemokine expression profile contributes to the shaping of the immune response. During HSV infections, chemokines play a part both in the immunopathology (Tumpney et al., 1998) and in elimination of the infection (Sin et al., 2000; Eo et al., 2001). Additionally, we have shown that RANTES is selectively induced after HSV infection of macrophages (Melchjorsen et al., 2002). Various sets of transcription factors are responsible for expression of RANTES depending on the cellular context and inducing agent. Several studies have shown that nuclear factor $\kappa$B (NF-$\kappa$B), the mitogen-activated protein (MAP) kinase system and the interferon regulatory factor (IRF) family are involved in virus-induced expression of RANTES (Lin et al., 1999; Genin et al., 2000; Kujime et al., 2000; Casola et al., 2001; Pazdruk et al., 2002).

In this study, we present studies on the molecular mechanism governing expression of RANTES during HSV infection. To investigate the signalling pathways, we used chemical inhibitors of specific signalling molecules and cell lines stably transfected with various dominant-negative mutants.

The murine macrophage cells J774A.1 and murine fibroblast cells NIH 3T3 were grown and maintained in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 5 and 10% foetal bovine serum, respectively. Fibroblasts were serum-starved for 2–4 h before further treatment. Cells were infected with $1 \times 10^6$ p.f.u. (m.o.i. of 2 or 4) or $3 \times 10^6$ p.f.u. (m.o.i. of 6) of either HSV-1 (KOS) or HSV-2 (MS) ml$^{-1}$. Where indicated, cells were treated with 3 µM N-tosyl-L-phenylalanine (TPCK; Sigma), or 10 µM SB203580 (Calbiochem) 20 min prior to infection. After the indicated periods of time, supernatants were harvested and RANTES levels were measured by ELISA (Peprotech). Alternatively, cells were lysed and luciferase activity was measured, or total RNA was harvested, reverse transcribed with oligo(dT) priming and analysed for the presence of RANTES, ICP27 and $\beta$-actin by PCR. For transfections, lipofectamin (Invitrogen) was used. To avoid single-clone abnormalities, the stably transfected clones were pooled. Stable transfections were carried out with dominant-negative (dn) I$\kappa$B kinase $\beta$ (IKK$\beta$), IRF-3, p38, or the empty vector pcDNA3. Selection was performed using G418 at a concentration of 300 µg ml$^{-1}$. Transient transfections were carried out with a luciferase-linked construct of the wild-type RANTES promoter (Casola et al., 2002).
Transfection efficiency and variation within plates was investigated using a GFP-vector (pEGFP-N1; Clontech), counting transfected cells by fluorescence microscopy. The variation of transfection efficiencies within plates was analysed by comparison of variation. Comparison of means was performed using the Student’s $t$-test. $P$ values $< 0.05$ were considered significant.

When looking at RANTES production after HSV infection, we found that both HSV-1 and -2 induced RANTES expression in macrophages and fibroblasts and that protein was detectable from 8 h post-infection (p.i.) (Fig. 1A–D). To examine whether the enhanced expression of RANTES was due to induced transcription, we performed a reporter gene assay using NIH 3T3 cells transfected with a luciferase-linked RANTES promoter construct. In parallel, the transfection efficiency and the possible variation within plates were investigated using a GFP vector. The variation of transfection efficiency between plates was shown not to be significant, with maximum efficiencies varying from 20 to 23% (data not shown), and therefore subsequent experiments were performed in triplicate without further normalization. As seen in Fig. 1(E), RANTES transcription was significantly induced when cells were infected with HSV. Thus, expression of RANTES after HSV infection is regulated at the transcriptional level.

To characterize further the induced transcription and the signalling pathways responsible for HSV-induced RANTES production, macrophage J774A.1 cells or fibroblast NIH 3T3 cells were seeded, left overnight to settle and treated with inhibitors 20 min prior to infection to block two cellular signal transduction pathways. HSV-2-induced RANTES mRNA accumulation was measured at 5 h p.i. and RANTES secretion at 7 h p.i. To assess whether expression of viral immediate-early genes was affected by the drug treatment, we included immediate-early ICP27 and saw that the inhibitors did not disrupt viral gene expression (Fig. 2B, D). Therefore, the effects on RANTES expression were assumed to be on the RANTES promoter activity, rather than indirectly via disruption of viral gene expression.

To evaluate the role of NF-κB in virus-induced RANTES expression, cells were treated with TPCK, a serine protease inhibitor, which blocks degradation of the NF-κB inhibitor IκB and hence prevents NF-κB activation. This inhibitor strongly impaired the ability of HSV-infected cells to produce RANTES mRNA and protein (Fig. 2). NF-κB-dependent HSV-induced production of antiviral substances such as IL-6, IL-12, TNF-α and NO has been established (Paludan et al., 1998; Malmgaard et al., 2000; Paludan, 2001; Paludan et al., 2001) and the present finding suggests that expression of RANTES in HSV-infected cells, also relies on NF-κB.

The MAP kinase system and p38 are activated after infection with many viruses and have been connected to regulation of RANTES production (Kujime et al., 2000; Pazdrak et al., 2002). Previous studies from this laboratory have shown that production of IL-6 and TNF-α in HSV-infected macrophages is dependent on p38 (Paludan, 2001; Paludan et al., 2001). To investigate the role of p38 in the expression of RANTES we used SB203580, a highly specific inhibitor of p38 MAP kinase. Treatment with SB203580 did not affect RANTES mRNA accumulation in HSV-infected macrophages and only slightly reduced mRNA levels in fibroblasts (Fig. 2). A modest reduction in protein levels was observed just above 0-05 in some experiments and just below in

![Fig. 1. Production of RANTES by macrophages and fibroblasts after HSV infection. J774A.1 and NIH 3T3 cells were infected with $3 \times 10^6$ p.f.u. HSV-1 (KOS) or HSV-2 (MS) m$^{-1}$ (m.o.i. of 6). (A–D) After incubation for the indicated period of time, supernatants were harvested and analysed by ELISA. Results are shown as means from triplicate cultures ± SEM. Similar results were seen in at least three independent experiments. (E) HSV-induced RANTES promoter activity. NIH 3T3 cells were transiently transfected with a construct of the wild-type RANTES promoter. After 16 h the cells were infected with $1 \times 10^6$ p.f.u. HSV-1 m$^{-1}$ (m.o.i. of 4) for 24 h and luciferase activity was measured. Uninfected cells (UT/mock) served as control. Data are expressed as mean of three cultures ± SEM of measured luciferase activity.](image-url)
others. These results suggest that p38 is not involved in HSV-induced RANTES transcription, but may play a minor role at the post-transcriptional level.

Given the above-described dependency on NF-κB and the unsolved role of p38 in HSV-induced RANTES expression, we further investigated these factors using J774A.1 macrophages stably transfected with dominant-negative mutants of IKKβ and p38. We also included dominant-negative IRF-3, known to be involved in expression of RANTES in response to some viruses (Genin et al., 2000; tenOever et al., 2002). The results showed that cells stably transfected with the empty vector (pcDNA3) retained the ability to produce RANTES mRNA and protein after HSV infection (Fig. 3A, E). The p38 mutant cell line displayed only slightly reduced RANTES mRNA accumulation and protein secretion (Fig. 3D, F). In contrast, the IRF-3 and IKKβ mutant cell lines were strongly disabled in their capacity to produce RANTES following HSV infection (Fig. 3B, C, E).

In this study, we have found that HSV induces expression of RANTES through up-regulation of gene transcription and that this is dependent on NF-κB and IRF-3. We and others have previously found that these two transcription factors become activated during HSV infection (Preston et al., 2001; Paludan, 2001). NF-κB and IRF-3 have also been shown to be essential for RANTES expression in response to some virus infections (Lin et al., 1999; Genin et al., 2000; Casola et al., 2001), whereas alternative mechanisms, notably MAP kinase-dependent pathways, play major roles in RANTES expression during other virus infections (Kujime et al., 2000). Our results did not support the idea that p38 is involved in RANTES transcription, but the data do not exclude a minor role for p38 at the post-transcriptional level. Work by others has demonstrated that p38, through action at the post-transcriptional level, regulates RANTES expression in respiratory syncytial virus-infected cells (Pazdrak et al., 2002).

Taken together, the results presented here show that cells respond to HSV infection by producing RANTES and that this proceeds through a mechanism dependent on NF-κB and IRF-3. Production of RANTES and other chemokines by virus-infected cells may allow the organism to recruit leukocytes rapidly to the site of infection, thereby initiating an efficient antiviral response.
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REFERENCES


