The expression of RANTES and chemokine receptors in the brains of scrapie-infected mice

Hyun-Pil Lee, Yong-Cheol Jun, Jin-Kyu Choi, Jae-Ill Kim, Richard I. Carp, Yong-Sun Kim

*Ilsong Institute of Life Science, Hallym University, 1605-4 Kwanyangdong, Dongangu, Anyang, Kyeonggi-Do 431-060, Republic of Korea

+Department of Microbiology, College of Medicine, Hallym University, Chuncheon, Kangwon-Do 200-702, Republic of Korea

+New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA

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Abstract

While chemokines play an important role in host defense, it has become abundantly clear that their expression is not solely restricted to immune cells. In this study, to investigate the role of chemokines in pathogenic mechanism of neurodegeneration in prion diseases, we determined the cerebral expression of RANTES, a major chemoattractant of monocytes and activated lymphocytes, and its receptors CCR1, CCR3 and CCR5 in ME7 scrapie-infected mice. The mRNA of RANTES gene was upregulated in the brains of scrapie-infected mice. Intense immunoreactivity of RANTES was observed only in glial fibrillary acidic protein (GFAP)-positive astrocytes of the hippocampus of the infected mice. In addition, the levels of mRNA expression of CCR1, CCR3, and CCR5 were increased in hippocampus of scrapie-infected brains compared to the values in controls. Immunostaining of CCR1, CCR3, and CCR5 was observed in reactive astrocytes of the hippocampal region of scrapie-infected brains. In addition, immunoreactivity of CCR5 was also observed in microglia of scrapie-infected brains. These results suggest that RANTES and its receptors may participate in amplifying proinflammatory responses and, thereby, exacerbate the neurodegeneration of prion diseases.

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1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders which are characterized by neuronal cell loss, vacuolation, astrocytosis and, in some cases, amyloid plaques (Carp et al., 1994). These diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker syndrome (GSS) in humans (Prusiner, 1998). The pathogenic agent of these diseases is thought to be composed predominantly of a post-translationally modified isoform (PrPSc) of the cellular prion protein (PrPC). Although it is unclear how PrPSc accumulation gives rise to the profound neurodegeneration characteristic of scrapie, it is believed that PrPSc is a major component of the infectious agent in prion diseases (Prusiner, 1998).

Although there is no detectable immune response accompanying scrapie infection (Aucouturier et al., 2000; Berg, 1994), the possible implication of the presence of inflammatory components in the brains of scrapie-infected animals is that there are highly localized cerebral host responses to the infection by the resident central nervous system (CNS) response cells, glial cells (Campbell et al., 1994; Duguid and
Chemoattractant protein (MCP)-1, and macrophage inflammatory protein 1α (MIP)-1α, interleukin-1 alpha (IL-1α), IL-1β, GFAP, and murine acute-phase response gene mRNA in the brain but not in peripheral tissue like spleen, kidneys, or liver (Campbell et al., 1994). IL-4, IL-5, gamma interferon (IFN-γ), IL-2, IL-6, and IL-3 mRNAs are absent or unaltered in prion diseases (Campbell et al., 1994). In addition to these cytokines, chemokines are present within and outside the CNS, where they function as soluble mediators possessing a spectrum of actions and chemotactic activities (Asensio and Campbell, 1999; Ransohoff et al., 1996; Wang et al., 2002). Localized production of chemokines is possible in astrocytes and neurons, two CNS cell types involved in conversion of PrPC to PrPSc.

Chemokine expression is invariably associated with inflammation at the onset of acute experimental allergic encephalomyelitis (EAE) and selectively elevated during relapses. The CC chemokine RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β are elevated following trauma (Grzybicki et al., 1998; Ghirnikar et al., 1996). Microglia produces MCP-1 in response to β-amyloid, suggesting a role in aging and Alzheimer’s disease (AD) (Meda et al., 1996). Baker et al. (2002) and Riemer et al. (2000) reported increased mRNA levels of CXC chemokine interferon-inducible protein 10 (IP-10) and B-lymphocyte chemoattractant (BLC) in CJD-infected microglia and in the brains of 263 K infected hamsters. The specific expression of CX3C chemokine fractalkine and its receptor (CX3CR1) have been investigated in a murine prion model (Hughes et al., 2002). However, the precise role of chemokines in prion diseases remains to be evaluated.

Understanding how cells respond to chemokines is complex because most chemokines bind to more than one receptor and most receptors bind to several chemokines (Rossi and Zlotnik, 2000). Thus, the ability of cells to respond to chemokines depends on the set of chemokine receptors that they express which may vary depending on the state of activation of the cell. In addition, the type of response elicited by a chemokine is dependent on the level of the mediator and the responding cell type. In this work, to investigate the role of CC chemokines in prion diseases, we have analyzed scrapie-infected mice for the expression of CC chemokine RANTES and its receptors CCR1, CCR3, and CCR5 in the brain (Pakianathan et al., 1997).

2. Materials and methods

2.1. Mouse strain and scrapie strain

Mice were bred and maintained in our Experimental Animal Center of Hallym University, and male C57BL mice 4–6 weeks of age were used. The ME7 scrapie strain was kindly provided by Dr. Alan Dickinson of AFRC and MRC Institute (Edinburgh, Scotland, UK). This scrapie strain was maintained by serial intracerebral passage of brain homogenate from terminally affected mice. Inocula were prepared from brain tissue homogenized in sterile phosphate-buffered saline (PBS) solution at 1% (w/v). The animals were inoculated intracerebrally with either 30 μl of 1% (w/v) ME7 brain homogenate or normal brain homogenate (control animals). In the ME7-infected mice, the onset of clinical symptoms occurred reproducibly between 150 and 160 days post-inoculation, and mice were sacrificed at 150 days. Mice inoculated with normal brain homogenate remained healthy over the same period.

2.2. Tissue preparation

The animals were sacrificed under 16.5% urethane at 150 days after inoculation with ME7 scrapie strain, a time when clinical manifestations of disease were evident. For immunohistochemistry and immunofluorescent studies, mice were then perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. The brains were immediately removed, cut into blocks, post-fixed in the same period.

2.3. RT-PCR analysis

Total RNA from whole brains of control (n=3) and infected groups (n=3) was isolated using TRI Reagent (GIBCO BRL, USA) according to the manufacturer’s protocol. Purified RNA (2 μg) was used for the amplification of mRNA using the Access RT-PCR system (Promega, USA) with the primers described in Table 1. Reverse transcription was done for 60 min at 42 °C followed by 25 cycles of amplification (each consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 57 °C, extension for 1 min at 72 °C) in a thermal cycler (Perkin Elmer Cetus, USA). To normalize the amount of each PCR product, RT-PCR of β-actin was carried out in a separate reaction with mouse specific primers. The products were separated on 1.8% agarose gels and visualized with ethidium bromide. The RT-PCR product was gel-purified, TA-cloned in pGEM-T vector (Promega). DNA sequencing of the amplicon of each gene demonstrated that it was the expected gene fragment (data not shown) for the particular chemokine and its receptors. The specificity of RT-PCR products of RANTES gene was...
verified by Southern hybridization with nested oligonucleotides (Table 1).

### 2.4. Immunoprecipitation and Western blot analysis

Samples (1.5–2 mg total protein) were incubated with goat anti-RANTES antibody (15 μl) overnight at 4 °C. Protein A sepharose was then added for 1 h at 4 °C. Samples were centrifuged for 5 min at 12,000 x g and the supernatant saved for measurement of total RANTES levels. Protein A immunocomplexes were washed three times with buffer containing 50 mM Tris–HCl (pH 8.0) and 150 mM NaCl. Samples were separated by 15% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Immunostaining of RANTES was performed using a chemiluminescent-based detection scheme involving an polyclonal goat anti-RANTES antibody (1: 300, Santa Cruz Biotechnology, USA), horseradish peroxidase (HRP)-conjugated anti-goat IgG (Zymed, USA) for signal amplification and detection.

### 2.5. RNase protection assay (RPA)

A pGEM-T clone containing the coding region of mouse RANTES (301 bases) was treated with SalI and used as a DNA template to generate a $^{32}$P-labeled antisense riboprobe. For β-actin, a linearized pTRIPLEX-script containing β-actin gene was used to produce an antisense riboprobe. $^{32}$P-labeled antisense cRNA probes for the RANTES and β-actin were synthesized according to the manufacturer’s instructions (Ambion, USA). After purification and precipitation the probes were added to tubes containing 20 μg of the total RNA in hybridization buffer. After hybridization for 16 h at 56 °C, the unhybridized RNA was digested with RNase A and T1 using the kit, following the manufacturer’s instructions. The RNA duplexes were extracted, precipitated and dissolved in gel loading buffer, and resolved on a 5% polyacrylamide gel containing 8 M urea. Autoradiography was performed at −70 °C (Konica, USA).

### 2.6. Immunohistochemistry

Immunohistochemical procedures were performed on 6 μm sections of brain. Endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide in methyl alcohol for 30 min. The sections were incubated overnight at 4 °C in PBS containing 10% normal horse serum (Vector Laboratories, UK) with primary antibodies added at the following dilutions: goat anti-RANTES, 1:100; rabbit anti-CCR1, 1: 300; rabbit anti-CCR3, 1:300; goat anti-CCR5, 1:100. All of the antibodies used in this study were purchased from Santa Cruz Biotechnology. Subsequently, sections were washed in PBS and incubated for 1 h at room temperature with appropriate secondary biotinylated anti-goat IgG or anti-rabbit IgG (1:1000 in PBS; Vector Laboratories, USA). After washes in PBS, antibody binding was detected using the avidin–biotin peroxidase (HRP)-conjugated anti-goat IgG (Zymed, USA) for signal amplification and detection.

### 2.7. Immunofluorescent staining of brains for RANTES and GFAP

To double-immunostain for RANTES and glial fibrillary acidic protein (GFAP), sections were incubated with primary antibodies, goat anti-RANTES (1:100, Santa Cruz Biotechnology) and rabbit anti-GFAP (1:800, DAKO, Denmark) overnight at 4 °C. Sections were washed with PBS, pH 7.2, three times for 20 min each wash. Secondary antibodies, TRITC-labeled donkey anti-goat IgG (1:100, Jackson Laboratories, USA) and FITC-labeled donkey anti-rabbit IgG (1:100, Jackson Laboratories), were added to the slides for another 2 h incubation at room temperature in the dark. Slides were washed with PBS, pH 7.2, three times for 20 min each and were mounted using Vectashield mounting medium.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Product size (bp)</th>
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<td>ACTIN</td>
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<td>ACCTTCACACCCAGCCATG</td>
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<tr>
<td></td>
<td>Antisense</td>
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<td></td>
</tr>
<tr>
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<td>301</td>
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<tr>
<td></td>
<td>Antisense</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>GTCACCTGAGAAGGCAACGAGTCAGTGCTG</td>
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<td></td>
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<td>CGTGACGACGAAATCGATCAAAC</td>
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<tr>
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<tr>
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<tr>
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<td>Antisense</td>
<td>GCGTTGACCATGTGTCTGGAAGAACACT</td>
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medium (Vector Laboratories) to help protect the fluorescence. Slides were stored at 4 °C and were later observed using a laser confocal microscope (Zeiss, Germany).

3. Results

3.1. Expression of RANTES mRNA and protein

We analyzed expression levels of the RANTES mRNA in the brains of scrapie-infected and control mice by RT-PCR and RPA. Amplification using specific primers yielded bands of expected size (RANTES, 301 bp; β-actin, 309 bp). The level of RANTES gene expression in the infected mice was upregulated by scrapie infection, whereas β-actin mRNA levels, as constitutively expressed controls, were virtually identical in the two groups of mice (Fig. 1A). To further explore this issue, we analyzed the level of RANTES gene expression by RPA. Using RPA, RANTES expression was also detected only in the brains infected with scrapie (Fig. 1C). The expression levels of RANTES protein were investigated by immunoprecipitation analysis (Fig. 1B). Immunostaining of RANTES in scrapie-infected mice was much higher than that of control mice, which is in accordance with RT-PCR and RPA results as shown in Fig. 1A and C. But Fig. 1B seems to show some RANTES protein in control brain. In addition, Bakhiet et al. (2001) have reported the ability of resident brain cells to constitutively express chemokine genes. Therefore, we performed Southern blot analysis of PCR products in order to verify RT-PCR products of RANTES gene in the control mice. There was marked induction of RANTES mRNA in the brains of scrapie-infected group (n=3), as compared to control group (n=3) (Fig. 2).

3.2. Cellular localization of RANTES

To determine the distribution of RANTES in scrapie-infected brain, we immunostained the brain sections using the avidin–biotin peroxidase method. Intense immunoreactivity of RANTES was observed in reactive astrocytes of the hippocampus of scrapie-infected mice (Fig. 3B), but not in control brains (Fig. 3A). Immunohistochemical staining of both control and scrapie-infected mice in the absence of a primary antibody displayed minimal background staining, indicating the specificity of the positive staining seen with the RANTES antibody (data not shown). In double-immunofluorescent staining of RANTES (Fig. 3C) and GFAP (Fig. 3D) in scrapie-infected mice, intensive immunoreactivity of RANTES was colocalized within GFAP-positive astrocytes of the hippocampus of scrapie-infected mice (Fig. 3E).

To date, the majority of research investigating the role of chemokines in CNS disease has addressed either their secretion by brain parenchymal cells, including microglia and astrocytes, or their leukocyte attractant properties (Glabinski and Ransohoff, 1999). However, the effects that these mediators have on the responses of brain parenchymal cells are less clear. In order to understand how brain parenchymal cells respond to RANTES, we examined the expression and localization of their receptor genes.

3.3. Chemokine receptor expression and cellular localization

The ME7 murine scrapie model produces severe, pathological changes (Jeffrey et al., 2000), notably accumulation of abnormal PrP throughout the CNS, widespread vacuolation, which is particularly intense in thalamus and hippocampus, reactive gliosis especially in areas of vacuolation,
and neuronal loss, which can be most clearly identified in CA1 pyramidal neurons of the hippocampus. Therefore, we examined the expression of CCR1, CCR3, and CCR5 chemokine receptors in whole brain and in hippocampus of scrapie-infected mice by RT-PCR analysis. In whole brain samples, RT-PCR analyses showed no significant alteration in expression levels of each receptor mRNAs (Fig. 4A). In contrast, there was significant upregulation of each receptor gene in hippocampus of the scrapie-infected group compared to the control group (Fig. 4B).

![Image](image1)

Fig. 3. Immunostaining for RANTES in the hippocampus of control and scrapie-infected mice. Control (A), scrapie-infected (B) brain sections: the astrocytes of the hippocampus of infected mice show intense RANTES immunoreactivity. Intense RANTES immunoreactivity (C: TRITC) colocalizes (E: merge) with GFAP-positive astrocytes (D: FITC) in the hippocampus of scrapie-infected mice. C–E is confocal microscopy image. ×400.

![Image](image2)

Fig. 4. RT-PCR analysis of RANTES receptors (CCR1, CCR3, and CCR5) in total RNA from whole brains (A) and hippocampus (B) of control and scrapie-infected mice. PCR products of RANTES receptors (CCR1, 456 bp; CCR3, 357 bp; CCR5, 459 bp) are detected in control (n=3) and scrapie-infected mice (n=3). As a control, RT-PCR products were standardized by analysis of constitutive expression of β-actin gene (309 bp). M, molecular weight marker; C, control; I, infected. In the lower portion of A and B, data are expressed as mean±S.D. of values obtained for three control vs. three scrapie samples. There is significant elevation of the levels of RANTES receptors in hippocampus of scrapie-infected mice (Student’s t-test).
To examine which cells respond to RANTES, we conducted an immunohistochemical study by using antibodies to its receptors. Intense immunoreactivities of CCR1, CCR3, and CCR5 were observed in reactive astrocytes of the hippocampus of scrapie-infected mice (Fig. 5D, E, and F) but not in control mice (Fig. 5A, B, and C). In serial section staining with anti-GFAP antibody (Fig. 5G and H), GFAP-positive astrocytes also contain CCR1 (Fig. 5D) and CCR3 (Fig. 5E). In double-immunohistochemical staining with anti-GFAP and anti-CCR5 antibodies, strong immunoreactivities of GFAP and receptors were co-localized within astrocytes in the brains of scrapie-infected mice (G, H; filled arrows). In double-immunohistochemical staining for CCR5 and for astrocytes-specific GFAP, strong immunoreactivity of GFAP and CCR5 were colocalized within astrocytes in the brains of scrapie-infected mice (I, filled arrow). In addition, immunoreactivity of CCR5 was also shown in microglia-like cells which do not contain GFAP (I, blank arrow). * Indicates landmark blood vessel in adjacent sections. ×400.

4. Discussion

The pathogenesis of scrapie is poorly understood. However, elevated levels of chemokines have been observed in several brain diseases, suggesting that these molecules function as regulators of brain inflammation (Karpus and Ransohoff, 1998; Glabinski and Ransohoff, 1999). On the basis of some of the known molecular and cellular neuropathological manifestations of prion diseases, we reasoned that chemokines may be important pathogenic factors. The findings in the present study showed that there is pronounced activation of cerebral RANTES (one of the CC chemokine family) gene product expression in scrapie compared to control mice. The increase was seen predominantly in reactive astrocytes.

The tissue specificity of cytokine gene response in scrapie, even though the scrapie agent is known the replicate in the periphery, particularly in the spleen (Gajdusek, 1977), suggests the existence of a highly localized cerebral host response to the infection. This view is supported further by the observation that expression of the murine acute-phase response gene homologue, EB22/5.3, was increased in the brain but not in the peripheral organs of scrapie-infected mice (Campbell et al., 1994). T-lymphocyte recruitment and microglia activation after i.c. inoculation of scrapie occurred long before onset of clinical disease (Betmouni et al., 1996; Betmouni and Perry, 1999; Lewicki et al., 2003). These reports did not show marked inflammatory cell recruitment to the brain late in the disease. Furthermore, T cells obtained from the periphery and the CNS failed to display detectable effector functions in vitro, such as cytotoxicity or expression or release of T helper cell type 1 (Th1) cytokines. Further evidence that this disease does not elicit a significant immune response is highlighted by the observation that scrapie-infected immuno-deficient mice show a similar disease course to that of their immunologically competent littermates (McFarlin et al., 1971). Therefore, we conclude that resident central nervous system cells are primarily responsible for the increased chemokine gene expression. Sources of RANTES in scrapie were reactive astrocytes, the most abundant cell type within the CNS, which are triggered to
release chemokines and cytokines after stimulation with bacterial products, components of the clotting cascade, products of activated T lymphocytes, β-amyloid, and interactions with activated leukocytes (Sun et al., 1997; Barnes et al., 1998; Johnstone et al., 1999; Dorf et al., 2000; Lee et al., 2000).

The relationship between the accumulation of PrPSc and the activation of chemokine gene expression in scrapie was not examined in the present study. However, from previous studies of hamsters (Bolton et al., 1991; Jendroska et al., 1991) and mice (Diedrich et al., 1991), it is known that the prion protein accumulates prior to the development of astrocytosis, indicating that PrPSc may be causally involved in the development of neuropathology. Whether PrPSc directly activates astrocytes remains unknown. But microglial array studies have shown CJD specific upregulation of many inflammatory cytokine linked transcripts (Baker and Manuelidis, 2003). In addition, using microarray technology changes in RANTES and in components of interferon pathways have been reported for whole brain in a TSE model (Baker et al., 2004). In any case, accumulation of PrPSc is also likely to precede activation of RANTES gene expression in scrapie, and the possibility that the prion protein is responsible for this activation should be considered.

It is interesting that there was upregulation of CCR1, CCR3, and CCR5 in hippocampus but not in whole brain. There were also some increases of these CCRs transcripts in thalamus of infected mice, but not in cortex and cerebellum (data not shown). Increased RANTES was also consistently found in the hippocampus from scrapie-positive mice. The selective anatomical distributions of RANTES as well as CCRs transcripts may be related to scrapie strain or the specific cell types and the microenvironment in these regions. For vacuolation, the major controlling influence on the pattern of changes was scrapie strain. Vacuolation was particularly intense in the mid- and forebrain regions of ME7-infected mice (Fraser and Dickinson, 1973; Fraser, 1979). Scrapie infection causes increased numbers of GFAP-positive astrocytes in the hippocampus area as well as in other brain areas. RANTES and CCRs transcripts were closely associated with GFAP-positive astrocytes in the hippocampus, whereas other brain regions with GFAP-positive astrocytes did not have RANTES and CCRs transcripts in our study; this indicates that astrocytes are not the only factor in the processes leading to the expression of these genes. The functions, cell types and microenvironment of the region need to be considered as well.

The hippocampus is a useful model to study interactions between groups of neurons as the connections between cells are well characterized. In this context, it may be important that there is an increase of chemokine receptors as well as RANTES in hippocampus of scrapie-infected mice. RANTES specifically modulates astrocyte receptors, upregulating intercellular adhesion molecule 1 (ICAM-1) and downregulating CX3CR1 expression (Luo et al., 2002). The downregulation of CX3CR1 expression in RANTES-stimulated astrocytes has potential implications for astrocyte–neuron interactions (Bush et al., 1999). During the advanced stages of neurodegeneration in the prion model, Hughes et al. (2002) demonstrated an increase in both fractalkine and its receptor in astrocytes and microglia of the CA1 region. In surviving neurons, expression of fractalkine appears altered relative to normal neurons. Although the molecular mechanisms that control astrocyte interactions with neurons have not been illuminated in this study, neurons express fractalkine, a cell membrane-tethered ligand for CX3CR1. The interactions of glial cells with neurons may be stabilized by fractalkine–CX3CR1 interactions. Any alteration in CX3CR1 expression may therefore facilitate breakdown of cellular interactions leading to increased susceptibility of neurons to damage in CNS diseases including the prion diseases.

In this study, to determine whether chemokine could be involved in pathogenic processes and/or reparative responses in prion diseases, we examined the expression of RANTES and its receptors in ME7 scrapie-infected mouse brains. Although the in vivo significance of increased expression of RANTES and of its receptors on astrocytes within the hippocampus remains to be clarified, it is possible that these changes could contribute to both neurodegeneration and local inflammatory response found in the CNS of prion diseases.

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References


