In Vivo Inhibition of CC and CX$_3$C Chemokine-induced Leukocyte Infiltration and Attenuation of Glomerulonephritis in Wistar-Kyoto (WKY) Rats by vMIP-II

By Shizhong Chen,* Kevin B. Bacon,† Li Li,* Gabriela E. Garcia,* Yiyang Xia,* David Lo,* Darren A. Thompson,§ Michael A. Siani,§ Tadashi Yamamoto,| Jeffrey K. Harrison,¶ and Lili Feng*

From the *Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; †Neurocrine Biosciences, San Diego, California 92121; §Gryphon Sciences, South San Francisco, California 94908; the |Department of Pathology, Institute of Nephrology, Niigata University School of Medicine, Niigata 951, Japan; and the ¶Department of Pharmacology and Therapeutics, University of Florida, Gainesville, Florida 32610

Summary

Chemokines play a central role in immune and inflammatory responses. It has been observed recently that certain viruses have evolved molecular piracy and mimicry mechanisms by encoding and synthesizing proteins that interfere with the normal host defense response. One such viral protein, vMIP-II, encoded by human herpesvirus 8, has been identified with in vitro antagonistic activities against CC and CXC chemokine receptors. We report here that vMIP-II has additional antagonistic activity against CX$_3$CR1, the receptor for fractalkine. To investigate the potential therapeutic effect of this broad-spectrum chemokine antagonist, we studied the antiinflammatory activity of vMIP-II in a rat model of experimental glomerulonephritis induced by an antiglomerular basement membrane antibody. vMIP-II potently inhibited monocyte chemotactic protein 1–, macrophage inflammatory protein 1b–, RANTES (regulated on activation, normal T cell expressed and secreted)–, and fractalkine-induced chemotaxis of activated leukocytes isolated from nephritic glomeruli, significantly reduced leukocyte infiltration to the glomeruli, and markedly attenuated proteinuria. These results suggest that molecules encoded by some viruses may serve as useful templates for the development of antiinflammatory compounds.

Key words: vMIP-II • CX$_3$CR1 • chemokine • glomerulonephritis • inflammation

The recruitment and activation of leukocytes at sites of pathogenesis or injury is the hallmark of inflammation. Although physiological inflammation is required for host defense and wound repairs, reactions that are disproportionate to the magnitude of the immune challenges are at the core of most inflammatory and autoimmune diseases. It is increasingly evident that chemokines play a crucial role in these physiological and pathological processes (1), and have been regarded as rational targets for the development of antiinflammatory reagents (2). However, the approach of antichemokine therapy has been hampered by the pleiotropy and redundancy of the chemokine system. Not only are multiple chemokines with overlapping activities frequently induced in inflammatory diseases, but often many different chemokine receptors are expressed by the activated leukocytes. Consequently, it may be difficult to control inflammation with an agent designed to neutralize the activity of a single chemokine. Molecules that have the capacity to bind and antagonize multiple types of chemokine receptors may provide a rational approach to overcome difficulties associated with this potential redundancy. Recently, a chemokine analogue encoded by human herpesvirus 8 has been identified and termed vMIP-II (3–5). In vitro, vMIP-II competes with native chemokines in the binding of a number of human CC and CXC chemokine receptors and blocks the actions of these chemokines on human monocytes (Mo; reference 4). To investigate the in vivo antiinflammatory activity of this broad-spectrum chemokine antagonist, we used vMIP-II in a well-established kidney inflammatory disease model, anti–glomerular basement membrane antibody–induced experimental glomerulonephritis in Wistar-Kyoto (WKY) rats (6). We found that, at nanomolar concentrations, vMIP-II effectively attenuated leukocyte infiltration to the kidney and significantly reduced the ensuing renal injury in the treated rats.

Materials and Methods

Synthesis of vMIP-II and Fractalkine. Synthetic chemokines were generated by native chemical ligation of peptides synthesized by solid-phase methods on a peptide synthesizer (model 430A; Ap...
Product Biosystems, Inc., Foster City, CA; reference 7). The resulting chemokines were purified by reverse-phase HPLC and characterized by electrospray mass spectrometry. The purified synthetic chemokines were reconstituted to 0.1 mg/ml in PBS before use.

Induction, Treatment, and Analysis of Anti-GBM GN in WKY Rats. At day 0, male WKY rats (Charles River Laboratories, Wilmington, MA), 10–12 weeks of age and 200–220 g of body weight, were given one intravenous injection of anti-GBM antibody (7) at a dose of 25 μg/100 g body weight. These rats were then given two daily intravenous injections of PBS or vMIP-II (12.5 μg/injection/rat, 25 μg total per day) for a period of 6 d, starting from day 0. On days 3 and 5, 24-h rat urine excretion was collected. Different groups of rats were killed on days 4 and 6 to collect blood and kidney tissues. Proteinuria was assayed by the sulfosalicylic method (8). Urine and blood creatinine was determined using a creatinine diagnostic kit (Sigma Chemical Co., St. Louis, MO).

RNA Analysis. Glomeruli were prepared from rat kidneys as previously described (8). Total RNA was isolated from glomeruli using a one-step method (9). 5 μg of total RNA from each sample was used for R N ase protection assay, following a previously described protocol (10). R iboprobes for chemokines MIP-1α, MIP-1β, MCP-1, RANTES, and the housekeeping gene L32 are described elsewhere (Xia, Y., S. Chen, Y. Wang, G. Ku, C.B. Wilson, D. Lo, and L. Feng, manuscript in preparation).

Western blot analysis and ELISA. The protein levels of MIP-1β and RANTES in rat glomeruli were analyzed by Western blot analysis as described previously (8), with some modification. Isolated glomeruli from each rat were solubilized in 20 mM PBS containing 0.5% Triton X-100, 10 mM EGTA, 1 mM PM SF, and 10 μM leupeptin. After centrifugation, the supernatants were collected and enriched by binding to heparin Sepharose CL-6B beads (Pharmacia Biotech, Piscataway, NJ). The protein contents of the eluted lysates were determined by a BCA protein assay kit from Pierce (Rockford, IL). 100 μg of protein from each sample was electrophoresed in a NuPAGE gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane. The protein blot was first probed with anti–MIP-1α or anti-RANTES antibody and then with horseradish peroxidase–conjugated second antibody. Antibody binding was detected by the addition of chemiluminescent substrate (SuperSignal Kit; Pierce) and exposure to autoradiograph film (Wolf X-ray Corp., West Hempstead, NY). The protein level of MCP-1 in the glomerular lysate was quantified from nephritic glomeruli following the method of Cook et al. (11). The chemotaxis assay was performed as previously described (12). In brief, glomerular inflammatory leukocytes were resuspended at 2 × 10^6/ml in DMEM plus 10% heat-inactivated FCS. 25 μl of increasing concentrations of chemotactic agent was placed in the lower wells of a 48-well chemotaxis chamber (NeuroProbe, Cabin John, MD) and separated from 50 μl of cell suspension in the top wells by an 8- or 5-μm pore-size polyvinylpyrrolidone-free polycarbonate filter. After incubation at 37°C for 2 h, sedimented cells on the top surface of the filter were wiped off and migrated cells on the undersurface were fixed in methanol and stained using Diff-QuiTM. Results are expressed as mean ± SEM cell number per five high-power fields (×400) and are representative of n = 3 experiments performed in duplicate.

Competitive Binding Assays. A filtration protocol was used for equilibrium binding of 125l-labeled fractalkine. 5 × 10^6 cells were incubated with 0.2 nM 125I-labeled fractalkine in the presence of unlabeled fractalkine or vMIP-II in the following buffer for 2 h at 22°C: 25 mM Hepes, 80 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, and 0.5% BSA, adjusted to pH 7.4. The reactions were aspirated onto polyethylenimine–treated GF/C filters (Packard, Meriden, CT) using a 96-well cell harvester (Packard). The filters were washed twice in 25 mM Hepes (pH 7.4), 500 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, and counted on a Packard Top Counter. The resulting data was analyzed using GraphPad Prism™ software (GraphPad Software, Sorrento Valley, CA).

Histopathology. Kidney tissue samples were fixed in 10% neutralized buffered formalin (NBF) or methanol-Carnoy (Methacarn) fixative solution, and were embedded in paraffin. For light microscopy examination, 5-μm paraffin sections of NBF-fixed tissues were stained with periodic acid-Schiff reagent. The number of crescentic glomeruli per 100 glomeruli of each rat was calculated and expressed as a percentage. For staining of CD8+ and ED1+ infiltrates, 5-μm paraffin sections of methacarn-fixed tissues were dehydrated and microwave-heated in 10 mM of sodium citrate (pH 6.0) at 800 watts for 10 min. The slides were reacted with mAb MRC-OX8 against rat CD8 (Pharmingen) or mAb ED-1 against rat macrophages (M-622 Chemicon, Temecula, CA), and goat anti–mouse secondary antibody. Antibody binding was detected by an alkaline phosphatase antialkaline phosphate kit and developed with a New Fuchsin substrate (DAKO Corp., Carpinteria, CA). Positively stained cells per 100 glomeruli of each rat were counted and expressed per glomerular cross-section.

Results and Discussion

Anti-GBM GN in WKY rats is characterized by an accumulation of CD8+ cells and ED1+ Mφ/MØs in the glomeruli (6), and CC chemokines are likely to play an important role in this form of renal inflammation. We first examined the expression of CC chemokines MCP-1, MIP-1α, MIP-1β, and RANTES in normal and nephritic glomeruli of WKY rats. Normal glomeruli had very little mRNA and protein expression of these chemokines (Fig. 1). Intravenous injection of anti-GBM antibody induced a profound mRNA expression of MCP-1, MIP-1β, and RANTES in the glomeruli (Fig. 1a). The induction was prominent 3 d after the antibody injection, persisted through day 7, and started to subside by day 9. Compared with MCP-1, MIP-1β, and RANTES, the induction of MIP-1α mRNA expression was not as significant. Western blot analysis of MIP-1β and RANTES protein (Fig. 1b) and ELISA analysis of MCP-1 protein (Fig. 1c) confirmed that the protein levels of these CC chemokines correlated with their mRNA levels in the glomeruli. In addition to CC chemokines, we found that the CX3C chemokine, fractalkine, was also induced in the glomeruli of anti-GBM GN of WKY rats.

The expression of multiple chemokines coincides temporally with the influx of CD8+ cells and ED1+ Mφ/MØs into the glomeruli of anti-GBM GN of WKY rats. In vitro, MCP-1, MIP-1β, RANTES, and fractalkine all induced...
strong migratory responses in cells prepared from the glomeruli of WKY rats 3 d after the anti-GBM antibody injection (Fig. 2 a). The antagonistic activity of vMIP-II against these chemokines was investigated. vMIP-II efficiently inhibited chemotactic activities of MCP-1, MIP-1α, and RANTES mRNA expression. Each lane represents a single rat sampled. Probes contain polylinker regions and are longer than the protected bands. Rat ribosomal L32 gene was used as a housekeeping gene. (b) Western blot analysis of MIP-1β and RANTES protein expression. Each lane represents samples pooled from three rats. (c) ELISA analysis of MCP-1 protein level. Each data point represents samples pooled from three rats and is expressed as mean ± SD.

Figure 1. Analysis of CC chemokine expression in the glomeruli of anti-GBM GN in WKY rats. GN was induced in WKY rats by intravenous injection of anti-GBM antibody on day 0. On days 0, 3, 5, 7, and 9, the rats were killed and glomerular samples were prepared for analysis. (a) RNase protection analysis of MCP-1, MIP-1α, MIP-1β, and RANTES mRNA expression. Rat ribosomal L32 gene was used as a housekeeping gene. (b) Western blot analysis of MIP-1α and RANTES protein expression. Each lane represents samples pooled from three rats. (c) ELISA analysis of MCP-1 protein level. Each data point represents samples pooled from three rats and is expressed as mean ± SD.

Figure 2. vMIP-II inhibition of chemotactic activity of CC and CX3C chemokines and displacement of 125I-labeled fractalkine in a competitive binding assay. (a) Chemotaxis of inflammatory leukocytes from the glomeruli of WKY rats with anti-GBM GN. The chemotactic responses expressed as numbers of migratory cells per five high-power fields (N umb/5 hpf) of the glomerular infiltrates to different concentrations of MCP-1, MIP-1α, RANTES, and fractalkine with the presence of varying amounts of vMIP-II (●, 0 nM; ■, 3 nM; ▲, 10 nM; ●, 30 nM) were shown. (b) Competitive binding assay of 125I-labeled fractalkine. 125I-labeled fractalkine binding to the glomerular cells from WKY rat with anti-GBM GN was performed with the presence of varying amounts of fractalkine or vMIP-II. Our findings indicate that vMIP-II is a CX3CR1 antagonist, and extend the spectrum of chemokine antagonism of vMIP-II to include that of the CX3C chemokine.

The in vivo activity of vMIP-II was investigated next. WKY rats with anti-GBM GN were treated with vMIP-II or with PBS as a control. In the control group, anti-GBM GN led to a prominent glomerular and periglomerular accumulation of CD8+ cells and ED1+ Mf/Mos (Fig. 3, a and c). This infiltration was significantly attenuated by vMIP-II treatment (Fig. 3, b and d). Consequently, the severe glomerular hypercellularity and crescentic formation characteristic of anti-GBM GN (Fig. 3 e) were markedly reduced in the vMIP-II treatment group (Fig. 3 f). Quantitative study indicated that the glomerular accumulation of CD8+ and ED1+ infiltrates and frequency of crescentic glomeruli in the vMIP-II treatment group were <50% of those in the control rats (P <0.001, student's t test; Fig. 4). As a result of the attenuation of inflammatory lesions in the kidney, normal renal function was largely maintained in anti-GBM GN WKY rats treated with vMIP-II. 24-h urinary protein of the vMIP-
Figure 3. Photomicrographs (original magnification, ×400) of the glomeruli from WKY rats with anti-GBM GN that were treated with PBS or with vMIP-II. (a–d). Kidney sections of PBS- (a and c) or vMIP-II–treated rats (b and d) immunohistochemistry stained for CD8+ cells (a and b) or ED1+ Mφs (c and d). Sections were sampled on day 4 after anti-GBM antibody injection. (e–f) Periodic acid-Schiff staining of kidney section of PBS- (e) or vMIP-II–treated rats (f). Sections were sampled on day 6 after anti-GBM antibody injection.
II-treated group was mild, being less than one-third that of the control group (P < 0.001; Fig. 5 a), and the serum creatinine levels in the experimental group were also significantly lower than the control group (P < 0.001; Fig. 5 b).

In this study, we demonstrated by assessing a number of disease parameters that vMIP-II has anti-inflammatory activity in anti-GBM GN in WKY rats. vMIP-II treatment attenuates leukocyte infiltration in the kidney, suppressed the onset of inflammation, and protected the kidney from inflammatory injury. The protection was not due to simple interference in the binding of rabbit anti-GBM antibody to rat kidneys. Immunofluorescent staining revealed rabbit IgG binding along the capillary walls of glomeruli in a linear pattern, with no discernible difference in the intensity between the control and experimental groups (data not shown). The attenuation of leukocyte infiltration cannot be attributed to a depletion of CD8+ cells or Møs by vMIP-II treatment. Flow cytometry profiles of blood CD8+ cells and ED1+ Mø were indistinguishable between the vMIP-II- and PBS-treated rats (data not shown). Consistent with its in vitro activity, the anti-inflammatory activity of vMIP-II is probably a direct result of its interference with the chemotactic recruitment of leukocytes into the kidney. Kledal et al. found that vMIP-II binds to human chemokine receptors CCR1, CCR2, CCR3, CCR5, and CXCR4, and antagonizes the action of MCP-1α, MIP-1β, and RANTES on freshly prepared human Møs, and they suggested that vMIP-II may help to prevent leukocyte recruitment in response to viral infection (4). Extending these findings, we showed that vMIP-II inhibited the chemotactic activity of rat chemokines MCP-1, MIP-1β, RANTES, and fractalkine on activated leukocytes isolated from nephritic glomeruli of WKY rats with anti-GBM GN. In particular, ours is the first report of the antagonistic activity of vMIP-II against fractalkine receptor. MCP-1, MIP-1β, RANTES, and fractalkine were dramatically induced in the nephritic glomeruli of WKY rats with anti-GBM GN (Fig.1). As a broad-spectrum chemokine antagonist, vMIP-II could interfere with the activities of these chemokines in vivo, and thus prevent lymphocyte and Mø recruitment into the diseased kidney. In addition to leukocyte recruitment, MCP-1 has recently been found to mediate direct effects upon resident renal cells and to play a critical role in crescent formation and deposition of type I collagen in a murine crescentic nephritis model (16). It is possible that vMIP-II can interfere with the MCP-1 effect on resident renal cells and help to improve the renal function in inflammatory GN.

Bacon et al. reported that RANTES could directly activate T cells and induce proliferation (17), an effect that seems to be mediated through a receptor different from the G protein-coupled chemokine receptors. It remains to be determined whether vMIP-II can inhibit the T cell activation function of RANTES as well.

Extensive efforts have been expended in the search and development of antichemokine therapeutic agents (18–20), and this in turn has contributed to the understanding of chemokine functions. In this respect, antichemokine and antichemokine receptor antibodies have constituted a major part of the validation of the critical role of chemokines in inflammatory diseases (21). On the other hand, for therapeutic interventions, antichemokine antibodies or reagents specific for a single ligand may not be effective. The bulk of data suggest that more than one chemokine is responsible for the recruitment of any individual cell type in inflammatory diseases, and the key chemokine may vary from disease to disease. Moreover, the key chemokine may vary with the progression of an inflammatory disease. Thus, the antagonism of one particular chemokine is prone to complications. Fujinaka et al. (22) have recently shown that MCP-1 plays an important role in the anti-GBM GN in WKY rats, and that the injection of anti-MCP-1 mAb significantly suppresses Mo/Mø infiltration and reduces proteinuria during the early phase of GN. However, the same treatment is ineffective during the later stages of GN, and the authors suggested that other chemotactic factors may be important during later stages of the disease. Agents with broad-spectrum antagonism to chemokines are therefore more desirable for inflammatory disease interventions. Viruses have coevolved with the host defense system and must constantly develop countermeasures to interfere with the physiological function of the immune system (23, 24). The rapidity of the viral genetic cycle coupled with the life-and-death selection imposed by the hostile host has optimized the molecular mimicry mechanism. Although det-
rimental to the physiological functions of the host immune system, viral antagonists thus evolved may be powerful agents for treating pathological conditions and valuable prototypes for rational design of chemokine antagonists. The application of vMIP-II may be just one example of our “mimicry of viral mimicry.”

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Address correspondence to Lili Feng, Department of Immunology, IMM 5, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Phone: 619-784-8262; Fax: 619-784-8558; E-mail: llifimm@scripps.edu

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