Lipocalin-2 Is a Chemokine Inducer in the Central Nervous System

ROLE OF CHEMOKINE LIGAND 10 (CXCL10) IN LIPOCALIN-2-INDUCED CELL MIGRATION

Received for publication, August 31, 2011, and in revised form, October 20, 2011. Published, JBC Papers in Press, October 26, 2011, DOI 10.1074/jbc.M111.299248

Shinrye Lee, Jong-Heon Kim, Jae-Hong Kim, Jung-Wan Seo, Hyung-Soo Han, Won-Ha Lee, Kiyoshi Mori, Kazuwa Nakao, Jonathan Barasch, and Kyoungho Suk

From the Departments of *Pharmacology and ‡Physiology, Brain Science & Engineering Institute, Cell and Matrix Research Institute, and the §School of Life Sciences and Biotechnology, Kyungpook National University School of Medicine, Daegu 700-422, Korea, the †Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan, and the **Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10027

Background: LCN2 has been implicated in cell morphology and migration.

Results: LCN2 promotes cell migration through up-regulation of chemokines (CXCL10) in brain both in vitro and in vivo.

Conclusion: LCN2 is a chemokine inducer in the CNS and may accelerate cell migration under inflammatory conditions in an autocrine or paracrine manner.

Significance: LCN2 could be targeted to therapeutically modulate glial responses in various neuroinflammatory disease conditions.

The secreted protein lipocalin-2 (LCN2) has been implicated in diverse cellular processes, including cell morphology and migration. Little is known, however, about the role of LCN2 in the CNS. Here, we show that LCN2 promotes cell migration through up-regulation of chemokines in brain. Studies using cultured glial cells, microvascular endothelial cells, and neuronal cells suggest that LCN2 may act as a chemokine inducer on the multiple cell types in the CNS. In particular, up-regulation of CXCL10 by JAK2/STAT3 and IKK/NF-kB pathways in astrocytes played a pivotal role in LCN2-induced cell migration. The cell migration-promoting activity of LCN2 in the CNS was verified in vivo using mouse models. The expression of LCN2 was notably increased in brain following LPS injection or focal injury. Mice lacking LCN2 showed the impaired migration of astrocytes to injury sites with a reduced CXCL10 expression in the neuroinflammation or injury models. Thus, the LCN2 proteins, secreted under inflammatory conditions, may amplify neuroinflammation by inducing CNS cells to secrete chemokines such as CXCL10, which recruit additional inflammatory cells.

Lipocalin 2 (LCN2)2 is a small hydrophobic molecule-binding protein that is also called 24p3. LCN2 plays an important role in diverse cellular processes, such as cell death/survival (1–4), cell migration/invasion (5, 6), cell differentiation (7, 8), iron delivery (1, 7, 9, 10), inflammation (11), and insulin resistance (12). There is, however, a debate on the role of LCN2 in cell migration. Previously, LCN2 facilitated gastrointestinal mucosal regeneration by promoting cell migration (13). Recently, LCN2 was also associated with an increased cytokine secretion and migratory activity of endometrial cancer (14). In ovarian cancer, however, LCN2 expression blocked epithelial to mesenchymal transition, one of the hallmarks of invasive neoplasia (15). LCN2 reduced adhesion/invasion partly by suppressing FAK activation in pancreas carcinoma cells (16). In contrast to numerous publications on LCN2 in various peripheral tissues, little is known about the role of LCN2 in the CNS. Recently, we have reported that the LCN2 protein is secreted by microglia and astrocytes in the CNS and promotes morphological changes and cell migration in an autocrine manner (17, 18). However, it is not clearly understood how LCN2 induces reactive astrocytosis or how LCN2 orchestrates cell migration.

In the CNS, chemokines are generally found under both physiological and pathological conditions, such as development, synaptic transmission, homeostasis, injury, and disease-associated neuroinflammation (19–21). CNS chemokines can be classified, according to their function, into neuromodulatory and inflammatory chemokines. Neuromodulatory chemokines have a neurotransmitter/neuromodulatory role in the brain, with characteristics such as neuronal expression, colocalization with classical or peptide neurotransmitters, pre- and post-synaptic receptor localization, and electrophysiological effects (22). Chemokine (CXC motif) ligand 12 (CXCL12)/SDF-1, chemokine (CX3C motif) ligand 1/fractalkine, and chemokine (CXC motif) ligand 1 (CXCL1) ⁄ GROα are a few examples of neuromodulatory chemokines. The major role of inflammatory protein; PIAS3, protein inhibitor of activated STAT3; CCL, chemokine (CC motif) ligand; dpi, days post-injury; icv, intracerebroventricular.
**LCN2 as a CNS Chemokine Inducer**

Chemokines are the recruitment of effector cells to inflammation sites (23). The various types of inflammatory chemokines include chemokine (CC motif) ligand 2 (CCL2)/MCP-1, chemokine (CC motif) ligand 5 (CCL5)/RANTES, chemokine (CXC motif) ligand 2 (CXCL2)/IL-8, and chemokine (CXC motif) ligand 10 (CXCL10)/IP-10. CXCL10, which is also known as IP-10 (IFN-γ-inducible protein of 10 kDa) (24, 25), acts via the CXCR3 receptor and is expressed in both glia and neuronal cells in the CNS (26, 27). The expression of CXCL10 is often triggered by inflammatory mediators, such as LPS (28), IFN-γ (24), or microbial toxins (29). Increased CXCL10 levels were considered to be critical for the increased migration of inflammatory cells into the CNS (30, 31). Many different CNS cells have been identified as sources of chemokines, including microglia, astrocytes, neuronal cells, and endothelial cells (27, 32). However, the existence of regulatory mechanisms of inflammatory chemokine expression in the brain is far from clear.

In the present study, we present evidence that LCN2 up-regulates chemokine expression in multiple cell types in the CNS, thereby providing a molecular basis of LCN2-induced cell migration that has been previously observed in the CNS, as well as peripheral tissues. In particular, astrocyte-derived CXCL10 plays a central role in LCN2-induced cell migration. Additionally, our results indicate that JAK2/STAT3 and NF-κB pathways partly mediate LCN2 up-regulation of CXCL10 and glial fibrillary acidic protein (GFAP) expression in astrocytes. Using mouse model, we confirmed that LCN2 influences CNS cell migration in vivo. Our results revealed a strong induction of LCN2 in the mouse brain following LPS injection or injury. Notably, there was a pronounced reduction in astrocyte migration and GFAP/CXCL10 expression in brain of LCN2-deficient (LCN2−/−) mice. The in vitro and in vivo data point to a key role for LCN2-CXCL10 axis in cell migration and reactive astrogliosis following brain inflammation or injury.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cells**—The following chemicals were obtained from Sigma: LPS from *Escherichia coli* 0111:B4 prepared by phenolic extraction and gel filtration chromatography, phorbol 12-myristate 13-acetate, ATP, pyrrolidine dithiocarbamate, and polymyxin B. JAK2 inhibitor (E)-N-benzyl-2-cyano-3-(3,4-dihydroxyphenyl)acrylamide α-cyano-(3,4-dihydroxy)-N-benzylcinnamamide tyrophiost B42; AG490), JAK1 inhibitor (trans-3,3’,4,5’-tetrahydroxystilbene; piceatannol), and IKK-2 inhibitor (SC-514) were purchased from Calbiochem (La Jolla, CA). The recombinant human TNF-α, mouse IFN-γ, and NOS murine melanoma cell-derived mouse LCN2 proteins were purchased from R & D Systems (Minneapolis, MN). The bacterially expressed recombinant mouse LCN2 protein was prepared, as previously described (18). In brief, the recombinant mouse LCN2 protein was expressed as a GST fusion protein in the BL21 strain of *E. coli*, which does not synthesize siderophore. The protein was purified by using glutathione-Sepharose 4B beads (GE Healthcare). All other chemicals, unless otherwise stated, were obtained from Sigma. The transformed mouse cerebral endothelial cell line, bEnd.3 (CRL-2299; ATCC, Manassas, VA) (33), was grown in DMEM supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). C6 rat glioma cells were maintained in DMEM supplemented with 5% heat-inactivated FBS (Invitrogen) and gentamicin (50 μg/ml). The mouse primary astrocyte and microglia cultures were prepared from the brains of 0–3-day-old ICR mice (Samtako Co., Osan, Korea), as previously described (18). The purity levels of astrocyte and microglia cultures were >96% and >95% as determined by GFAP and isocitrate B4 staining, respectively. Primary cultures of dissociated cerebral cortical neurons were prepared from embryonic day 20 ICR mice, as described previously (34, 35). Briefly, mouse embryos were decapitated, and the brains were rapidly removed and placed in a culture dish with cold PBS. The corti- ces were isolated and then transferred to a culture dish containing 0.25% trypsin-EDTA (Invitrogen) in PBS for 30 min at 37 °C. After two washes in serum-free neurobasal medium (Invitrogen), the cortical tissues were mechanically dissociated with a gentle pipetting. Dissociated cortical cells were seeded onto 6-well plates coated with poly-α-lysine (Falcon; BD Biosciences), using serum-free neurobasal medium containing 10% FBS, 0.5 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, N2 supplement (Invitrogen), and a B27 supplement (Invitrogen). The cells were maintained by changing the media every 2–3 days and were grown at 37 °C in a 5% CO2 humidified atmosphere. The purity of the neuronal cultures was determined by immunocytochemical staining, using an antibody against a neuron-specific marker, microtubule-associated protein 2 (Promega, Madison, WI). Animals used in the current research were acquired and cared for in accordance with guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the institutional review board of the Kyungpook National University School of Medicine.

**DNA Microarray Analysis**—Mouse primary astrocyte cultures were treated with the recombinant LCN2 protein (10 μg/ml) or left untreated for 8 h. Total RNA was isolated and labeled with either cyanine 3- or cyanine 5-conjugated dCTP (Amersham Biosciences) by a reverse transcription reaction, using reverse transcriptase SuperScript II (Invitrogen). The labeled cDNAs were mixed, placed on an Agilent mouse whole oligonucleotide chip (G4122A; Agilent Technologies, Santa Clara, CA), and covered by a hybridization chamber. Hybridized slides were scanned with the Axon Instruments GenePix 4000B scanner, and the scanned images were analyzed with the software programs GenePix Pro 5.1 (Axon, Union City, CA) and GeneSpring 7.2 (Sillicongetics, Redwood City, CA). A complete description of the DNA microarray platform and results is available under Gene Expression Omnibus accession number GSE15667.

**Traditional Reverse Transcription-PCR and Real Time PCR**—Total RNA was extracted from cells and adult mouse tissues by using the TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. Reverse transcription was conducted using Superscript II (Invitrogen) and oligo(dT) primer. PCR amplification, using specific primer sets, was carried out at a 55–60 °C annealing temperature for 20–30 cycles. The PCR was performed by using a DNA Engine Tetrad Peltier Thermal Cycler (MJ Research, Waltham, MA). For the analysis of PCR products, 10 μl
of each PCR was electrophoresed on 1% agarose gel and detected under UV light. β-Actin was used as an internal control. The real time PCR was performed using one-step SYBR® PrimeScript™ RT-PCR kit (Perfect Real Time; Takara Bio Inc., Tokyo, Japan) according to the manufacturer’s instructions, followed by detection using the ABI Prism® 7000 sequence detection system (Applied Biosystems, Foster City, CA). The $2^{-ΔΔCT}$ method was used to calculate relative changes in gene expression determined by real time PCR experiments (36). Nucleotide sequences of the primers were based on published cDNA sequences (Table 1).

**Table 1**

DNA sequences of the primers used for traditional and real time RT-PCR

| Mouse cDNAs | RT-PCR type | Primer sequences | GenBank™ accession number | Amplicon size |
|-------------|-------------|-----------------|--------------------------|---------------|
| ccl4        | Traditional | Forward, 5'-GCC CTC TCT CTC TCT TCT TCT-3' | NM_013652 | 196 |
|             |             | Reverse, 5'-GTT TCG TCG TCT TCT TCT TCT-3' |             |     |
| ccl20       | Traditional | Forward, 5'-GGA AGG ACA ACC CTT TCT-3' | NM_018960 | 177 |
|             |             | Reverse, 5'-AGG ACA CTT TCT TCT TCT-3' |             |     |
| ccl21       | Traditional | Forward, 5'-TGC GAC TGC TGC TGC TGC TGC-3' | NM_009140 | 303 |
|             |             | Reverse, 5'-CTC AGG CTT TCT TCT TCT-3' |             |     |
| ccl10       | Traditional | Forward, 5'-AGG AGA AAG ACA ACC CTT TCT-3' | NM_013174 | 186 |
|             |             | Reverse, 5'-AGG AGA AAG ACA ACC CTT TCT-3' |             |     |
| il-6        | Traditional | Forward, 5'-GAA AGA GCA GCC CAA ACC CTT TCT-3' | NM_031168 | 159 |
|             |             | Reverse, 5'-GAA AGA GCA GCC CAA ACC CTT TCT-3' |             |     |
| cox2        | Traditional | Forward, 5'-ACA CAC TAC TAC TAC TAC TAC-3' | NM_019097 | 497 |
|             |             | Reverse, 5'-ACA CAC TAC TAC TAC TAC TAC-3' |             |     |
| inos        | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_018812 | 158 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| pias3       | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_008491 | 363 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| lcn2        | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_021551 | 157 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| z4p3R       | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_00180488 | 308 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| megalin      | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_009910 | 180 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| cxxcr3       | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_007393 | 287 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |
| gfp          | Traditional | Forward, 5'-TGC TGC TGC TGC TGC TGC TGC-3' | NM_008084 | 171 |
|             |             | Reverse, 5'-TGC TGC TGC TGC TGC TGC TGC-3' |             |     |

**Cell Migration Assays**—Cell migration was determined by using a 48-well Boyden chamber (NeuroProbe, Gaithersburg, MD), according to the manufacturer’s instructions. ACM, the recombinant CXCL10 protein, or anti-CXCL10 antibody was placed into base wells separated from the top wells by polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size; 25 × 80 mm; NeuroProbe). The cells were harvested by trypsinization, resuspended in DMEM, and added to the upper chamber at a density of 1 × 10⁴ cells/well. The cells were incubated at 37 °C under 5% CO₂ for 12 to 72 h. Zigmond-Hirsch checkerboard analysis (38) was performed in triplicate to distinguish between concentration-dependent cell migration (chemotaxis) and random migration (chemokinesis). ACM and the recombinant CXCL10 protein of varying concentrations were added to the upper and/or lower wells of the Boyden chambers for the checkerboard analysis. For the checkerboard analysis, trypsinized cells were added to the upper chamber at a density of 2 × 10⁴ cells/well and incubated for 48 h. At the end of the incubation, nonmigrating cells on the inner side of the membrane were removed with a cotton swab. Migrated cells on the underside of the membrane were fixed with methanol for 10 min and stained with Mayer’s hematoxylin (Dakocytomation, Glostrup, Denmark) for 20 min. Photomicrographs of five randomly chosen fields were taken (Olympus CK2, Tokyo, Japan) (original magnification, ×100), and the cells were enumerated to calculate the average number of cells that had migrated. All of the migrated cells were counted, and the results were presented as the means ± S.D. of triplicate results. The *in vitro* scratch wound healing assay was performed as previously described (39). In brief, a scratch wound was created by using a
TABLE 2
Partial list of genes that were up or down-regulated by LCN2 as determined by DNA microarray analysis of astrocytes

| Description | Gene symbol | GenBank™ accession number | Fold change |
|-------------|-------------|----------------------------|-------------|
| Increased expression | CCL20 | NM_016960 | 6.50077 |
| Immunoresponse gene 1 | IRG1 | XM_127883 | 5.09345 |
| Interleukin 6 | IL-6 | NM_031168 | 4.77762 |
| Chemokine (CC motif) ligand 4 | CCL4 | NM_013652 | 3.90144 |
| Chemokine (CXC motif) ligand 2 | CXCL2 | NM_009140 | 3.58657 |
| Chemokine (CXC motif) ligand 1 | CXCL1 | NM_008176 | 3.55145 |
| Nitric-oxide synthase 2, inducible, macrophage | Nos2 | NM_010927 | 3.51580 |
| Chemokine (CC motif) ligand 3 | CCL3 | NM_011337 | 3.28548 |
| Chemokine (CC motif) ligand 5 | CCL5 | NM_013653 | 2.54231 |
| Chemokine (CXC motif) ligand 5 | CXCL5 | NM_009141 | 2.05752 |
| Chemokine (CC motif) ligand 7 | CCL7 | NM_013654 | 2.34586 |
| Chemokine (CXC motif) ligand 10 | CXCL10 | NM_021274 | 2.19630 |
| Chemokine (CXC motif) ligand 9 | CXCL9 | NM_008599 | 2.19380 |
| Prostaglandin-endoperoxide synthase 2 | PTX3 | NM_008967 | 2.36835 |
| Toll-like receptor 2 | COX-2 | NM_011198 | 1.97592 |
| UBE2G2 | ARHGEF10 | NM_172751 | 2.60634 |
| Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25 | SLC25A25 | NM_146118 | 2.66600 |
| Nuclear receptor-binding protein | NRBP | NM_147201 | 0.23797 |
| Cell cycle progression 1 | CCNG1 | NM_028181 | 0.24613 |
| RAB31, member RAS oncogene family | RAB31 | NM_133685 | 0.24669 |
| Ubiquitin-conjugating enzyme E2G 2 | UBE2G2 | NM_019803 | 0.24804 |
| TBC1 domain family, member 14 | TBC1D14 | NM_133910 | 0.25170 |
| DEAF1/D/H (Asp-Glu-Ala-(Asp/His)) box polypeptide 3, X-linked | DDX3X | NM_010028 | 0.25526 |
| Eukaryotic translation elongation factor 2 | EF2 | NM_007907 | 0.26671 |
| Rho guanine nucleotide exchange factor 10 | ARHGEF10 | NM_172751 | 0.26643 |
| Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25 | SLC25A25 | NM_146118 | 0.26660 |
| Zinc finger, UBR1 type 1 | ZUBR1 | XM_01479455 | 0.26705 |
| Similar to RAN-binding protein 5 | LOC436227 | XM_621068 | 0.26737 |
| Guanine nucleotide binding protein, α12 | GNAt | NM_010302 | 0.26968 |
| Ets variant gene 5 | ETV5 | NM_023794 | 0.27306 |
| AFG3 (ATPase family gene 3)-like 2 (yeast) | AFG1L2 | NM_027130 | 0.27435 |
| HECT, UBA, and WW domain-containing 1 | HUBWE1 | NM_021528 | 0.27821 |
| Cytoplasmic linker 2 | CYLN2 | NM_009990 | 0.27926 |

10-μl pipette tip on confluent cell monolayers in 24-well culture plates and placed into DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C under 5% CO2 during migration of monolayer into the cleared wound area. The wound area was observed by microscopy (Olympus CK2) (magnification, ×100). Relative cell migration distance was determined by measuring the wound width and subtracting this from the initial value as previously described: cell migration distance = initial wound width at day 0 − wound width at the day of measurement (40). Three nonoverlapping fields were selected and examined in each well (three wells/experimental group). The results were presented as a fold increase of migration distance.

Morphological Analysis of Astrocytes, Microglia, and Neuron Cells—The morphological analysis of astrocytes or neuron cells was performed by using fluorescence microscopy (Olympus BX50). The cells were blocked with 1% BSA in PBS-Tween 20 for 10 min and incubated in PBS containing 3% BSA and mouse anti-GFAP antibody (1:30 dilution; Biogenex, San Ramon, CA) or mouse anti-microtubule-associated protein 2 antibody (1:600 dilution; Promega). After two washes in PBS-Tween 20, the cells were incubated with anti-mouse IgG–FITC-conjugated secondary antibody (BD Biosciences). Astrocyte processes were quantified as previously described, with a slight modification (18, 41, 42). The average process length was based on the longest process for each cell from a minimum of five randomly chosen microscopic fields containing at least 100 cells. Neuronal processes were quantified as previously described, but with a slight modification (43). In brief, the total number of neuronal processes longer than one cell body diameter was counted. The number of neuronal processes was determined from a minimum of five randomly chosen microscopic fields containing at least 100 cells. The morphological analysis of microglia was performed by using phase contrast microscopy following peroxidase-labeled isoelectric B4 staining (1:500 dilution; Sigma) (17). Demarification of microglia was quantified as previously described with a slight modification (17, 44). The percentage of ramified cells was determined from a minimum of five randomly chosen fields containing at least 100 cells.

Western Blot Analysis—Astrocyte cultures or adult mouse tissues were lysed in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration in cell lysates was determined by using a Bio-Rad protein assay kit. An equal amount of protein from each sample was separated by 12% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies (rabbit polyclonal anti-phospho-STAT3 at Ser727/Tyr705 and anti-total STAT3 antibodies (Cell Signaling Technology, Beverly, MA); mouse monoclonal anti-GFAP antibody (Biogenex); goat polyclonal anti-mouse LCN2 antibody (R & D Systems); and monoclonal anti-α-tubulin clone B-5–
1-2 mouse ascites fluid (Sigma) and HRP-conjugated secondary antibodies (anti-goat, anti-rabbit, and anti-mouse IgG; Amersham Biosciences), followed by ECL detection (Amersham Biosciences).

Nuclear Extraction and EMSA—Nuclear extracts were prepared from astrocyte cultures, and the electrophoretic mobility shift assay was conducted as described previously (45). Nuclear extracts (5ug/ml) were mixed with double-stranded NF-κB oligonucleotide (5’-GAT CCC AAC GGC AGG GGA-3’; Promega), which was end-labeled with [γ-32P]dATP using T4 polynucleotide kinase. Labeled nucleic acids were purified using a mini Quick Spin column (Roche Applied Science). The binding reactions were performed at 37 °C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly(dI-dC), and 1 mM DTT. For the supershift assay, antibody against p65 subunit of NF-κB (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was coincubated with nuclear extracts in the reaction mixture for 30 min at 4 °C before adding the radiolabeled probe. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 180 V in 0.5 Tris-boric acid-EDTA buffer. The gels were vacuum-dried for 1 h at 80 °C and exposed to x-ray film at −70 °C for 24 h.

Assessment of Cytotoxicity by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide Assay—Cells (5 × 10^4 cells in 200 μl/well) were seeded in 96-well plates and treated with various stimuli for the specific time periods. After treatment, the medium was removed and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (0.5 mg/ml) was added, followed by incubation at 37 °C for 2 h in a CO_2 incubator. After insoluble crystals were completely dissolved in Me_2SO, absorbance at 570 nm was measured by using a microplate reader (Anthos Labtec Instruments, Wals, Austria).

Nitrite Quantification—Astrocyte cultures were treated with stimuli in 96-well plates, and then NO_2^- in culture media was measured to assess NO production levels by the Griess reaction as described previously (18). Fifty microliters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2% phosphoric acid) in 96-well plates and incubated at 25 °C for 10 min. The

---

**FIGURE 1. Induction of chemokine gene expression by LCN2 in astrocytes, microglia, endothelial cells, and neuron cells.** Astrocytes (A and E), microglia (B and E), bEnd.3 endothelial cells (C), and neuron cells (D) were treated with the recombinant LCN2 protein (10 μg/ml) for 8 h, and the total RNA was isolated for traditional RT-PCR or real time PCR. The cells were also treated for 8 h with LPS (100 ng/ml), TNF-α (10 ng/ml), or LPS (100 ng/ml) plus IFN-γ (50 units/ml) for comparison purposes. The mRNA levels of chemokines (CCL4, CCL20, CXCL2, and CXCL10) and other inflammatory genes (IL-6, COX-2, iNOS, and PIAS3) were determined by traditional RT-PCR (A–D) or real time PCR (E). β-Actin or GAPDH was used as an internal control. The results are one representative of more than three independent experiments (A–D) or means ± S.D. (n = 3) (E).
LCN2 as a CNS Chemokine Inducer

absorbance at 540 nm was measured with a microplate reader (Anthos Labtec Instruments). NaNO₂ was used as the standard to calculate NO toxicity.

Mouse Breeding and Maintenance—LCN2-deficient mice were a gift from Dr. Shizuo Akira (Osaka University, Japan). LCN2 wild-type (LCN2+/+) and LCN2-deficient (LCN2−/−) mice were back-crossed for eight to ten generations onto the C57BL/6 background to generate homozygote and heterozygote animals as described previously (9, 46). Genotype was confirmed by PCR analysis of genomic DNA (46). The C57BL/6 mice that were used for breeding were purchased from the Samtako Co. LCN2−/− mice were age- and sex-matched with C57BL/6 controls.

Cortical Stab Wound Injury Model—Cortical stab wound injury was performed as described previously (47). The mice were anesthetized with enflurane and placed in a stereotaxic instrument. A midline incision was made through the scalp, and the skin was retracted laterally. The hole was drilled over the right cerebral hemisphere, exposing the dura. A 30-gauge needle was inserted into the cortical site coordinated as 0.5 mm anterior to bregma, −3 mm lateral to bregma, and 1 mm below the skull and left in place for 5 min. After the needle was removed, the mice were allowed to recover and returned to their cages at 2 days post-injury (dpi). For immunohistochemical analysis, the animals were anesthetized by ether and transcardially perfused with 4% paraformaldehyde in PBS at 2 dpi. The brains were postfixed and cryo-protected by 30% sucrose in PBS. The fixed brains were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo, Japan) for frozen section and then cut into 12-μm-thick coronal sections. After PBS washing, the sections were permeabilized in 0.1% Triton X-100 and blocked with 1% BSA and 5% normal donkey serum. The sections were incubated with mouse monoclonal anti-GFAP antibody (1:50 dilution; BioGenex) at 4 °C overnight. The sections were then incubated with secondary antibodies (donkey FITC-conjugated anti-rabbit IgG antibody (1:200 dilution; Jackson Immunoresearch Laboratories, West Grove, PA)). Data acquisition and immunohistological intensity measurement of GFAP staining was performed with a National Institutes of Health Image program. Composite images of stained section were Fast Fourier transform band-pass filtered to eliminate low frequency drifts (~20 pixels = 50 mm) and high frequency noises (~1 pixel = 2.5 mm). The image was binary thresholded at 50% of the background level, and then the particles were converted to a subthreshold image area with the size less than 300 and larger than 20 pixels, which was judged as GFAP-positive cells. The range (20–300 pixels) was obtained from the analyzed size of GFAP-positive cells from six sections for each experimental group. To count the GFAP-positive cells, five squares (200 μm × 200 μm) were placed to peri-region of injection in the subthreshold image of the six independent sections. The cells in the five squares were counted and statistically analyzed.

Mouse Models of Neuroinflammation—Either intracortical or intracerebroventricular injection of LPS was done to induce brain inflammation in mice. LCN2+/+ or LCN2−/− mice received an intracortical injection of 1 μl (5 μg) of LPS. Briefly, animals (body weight of about 30 g) were placed in a stereotaxic instrument (Stoelting, Wood Dale, IL) and given an intracortical injection of LPS using a 5-μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted into the cortical site (coordinate 0.5 mm anterior to bregma, −3 mm lateral to bregma, and 1 mm below the skull) under brief enflurane anesthesia. Intracerebroventricular injection was delivered as previously described (48). After brief enflurane anesthesia, the mice were placed in a stereotaxic instrument and given an injection at the site coordinated as 0.2 mm anterior to bregma, −1 mm lateral, and 2.5 mm depth from the skull using a 5-μl Hamilton microsyringe fitted with a 26-gauge needle. The injection volume was 3 μl (15 μg) of LPS. Flow rate of injection was 0.1 μl/min. At 2 dpi, for immunohistochemical analysis, the animals were anesthetized and transcardially perfused with 4% paraformaldehyde. The brains were postfixed and cryo-protected. The fixed brains were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo, Japan) for frozen section and then cut into 12-μm-thick coronal sections. After PBS washing, the sections were permeablized in 0.1% Triton X-100 and blocked with 1% BSA and 5% normal donkey serum. The sections were incubated with rabbit polyclonal anti-LCN2 antibody (1:50 dilution; Santa Cruz), or mouse monoclonal anti-GFAP antibody (1:500 dilution; BioGenex) at 4 °C overnight. The sections were then incubated with secondary antibodies (donkey FITC-conjugated anti-rabbit IgG antibody (1:200 dilution; Jackson Immunoresearch Laboratories, West Grove, PA)) or donkey CyTM3-conjugated anti-mouse IgG antibody (1:200 dilution; Anthos Labtec Instruments). NaNO₂ was used as the standard to calculate NO toxicity.

FIGURE 2. An increase of CXCL10 expression by LCN2 in astrocytes. Astrocytes were incubated with the recombinant LCN2 protein (10 μg/ml) or LPS (100 ng/ml) plus IFN-γ (50 units/ml) for 24 h. The amounts of CXCL10 protein in the culture media were measured by specific ELISA (A). The results are means ± S.D. (n = 3), *p < 0.05 compared with the untreated control. The stable overexpression or knockdown of lcn2 expression was achieved by transfection with sense or antisense lcn2 cDNA in C6 rat glioma cells. The increased or decreased lcn2 expression in the stable transfectants (S3, lcn2 sense transfectant; AS7, lcn2 antisense transfectant) compared with cells transfected with an empty vector (V2) was confirmed by RT-PCR (B). Changes in the CXCL10 mRNA levels in the stable transfectants were also assessed by RT-PCR (C). β-Actin was used as an internal control. The results are one representative of more than three independent experiments.
FIGURE 3. Astrocyte-derived CXCL10 promoted the migration of astrocytes. Astrocytes (1 × 10⁴ cells/upper well) were exposed to LCN2 (10 μg/ml)-stimulated ACM or the recombinant CXCL10 protein (10 ng/ml) in the presence or absence of CXCL10 neutralizing antibody (CXCL10 Ab; 10 ng/ml) as indicated. ACM-None, untreated ACM; ACM-LCN2, LCN2-treated ACM (see “Experimental Procedures” for the preparation of ACM). After treatment for the indicated time periods, either wound healing assay (A) or the Boyden chamber assay (B) was performed to evaluate cell migration. A representative microscopic image for each condition was shown (magnification, ×100) (upper). The quantification of cell migration was done by either measuring the degree of wound closure (wound healing assay) or enumerating the migrated cells (Boyden chamber assay) as described under “Experimental Procedures” (lower). The results are means ± S.D. (n = 3). *, p < 0.05 compared with ACM-None at the same time point; **, p < 0.05 compared with ACM-LCN2 at the same time point; #, p < 0.05 compared with the untreated control (None) at the same time point. For the checkerboard analysis, migration of astrocytes (2 × 10⁴ cells/upper well) in response to the indicated concentrations of ACM-LCN2 (C) and the recombinant CXCL10 protein (D) placed in upper and/or lower well was determined using the Boyden chamber assay. The quantification of cell migration was done by enumerating the migrated cells after 48 h as described under “Experimental Procedures.” The results are the means ± S.D. (n = 3). *, p < 0.05 between the treatments indicated.

TABLE 3
Checkerboard analysis for promigratory effects of ACM-LCN2 in astrocytes
ACM-LCN2 was added to the upper and/or lower wells of the Boyden chambers for the checkerboard analysis. After cells were incubated at 37 °C under 5% CO₂ for 48 h, cell migration was assessed as described under “Experimental Procedures.” NT, not tested.

| ACM-LCN2 (%) in lower well | 0      | 1      | 10     | 100    |
|---------------------------|--------|--------|--------|--------|
| ACM-LCN2 (%) in upper well| 0      | 1      | 10     | 100    |
| 0                         | 64.2 ± 4.2 | 62.2 ± 5.4 | 63.1 ± 5.3 | 62.4 ± 3.8 |
| 1                         | 114.4 ± 5.1 | NT     | NT     | 101.2 ± 4.6 |
| 10                        | 189.1 ± 3.6 | NT     | NT     | 175.7 ± 4.5 |
| 100                       | 275.3 ± 2.6 | 266.2 ± 6.1 | 265.8 ± 4.4 | 248.1 ± 5.1 |

Jackson Immunoresearch Laboratories). The counter stain was performed using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). To count the GFAP-positive cells, five 100-μm × 100-μm squares in each brain section were randomly chosen (Olympus BX51) (original magnification, ×400–1000). The same position in the five independent brain sections was visualized. The cells in the five squares were counted and statistically analyzed. The number of GFAP-positive cells/mm² was calculated according to the size of the specific brain area. The results were...
**LCN2 as a CNS Chemokine Inducer**

**TABLE 4**
Checkerboard analysis for promigratory effects of recombinant CXCL10 protein in astrocytes

The recombinant CXCL10 protein was added to the upper and/or lower wells of the Boyden chambers for the checkerboard analysis. After cells were incubated at 37 °C under 5% CO2 for 48 h, cell migration was assessed as described under “Experimental Procedures.” NT, not tested.

| CXCL10 (ng/ml) in lower well | 0 | 1 | 10 | 100 |
|------------------------------|---|---|----|-----|
| 0                            | 67.3 ± 4.6 | 66.7 ± 5.2 | 65.2 ± 3.4 | 66.1 ± 3.6 |
| 1                            | 126.4 ± 6.2 | NT | NT | 108.8 ± 3.4 |
| 10                           | 198.9 ± 4.8 | NT | NT | 181.1 ± 4.8 |
| 100                          | 294.3 ± 6.3 | 293.6 ± 5.2 | 291.1 ± 4.6 | 271.3 ± 3.5 |

Presented as the means ± S.D. (n = 3). Quantification of GFAP-positive cells was done using the ImageJ program (National Institutes of Health Image).

Statistical Analysis—All of the data were presented as the means ± S.D. from three or more independent experiments, unless stated otherwise. Statistical comparisons between different treatments were done by either a Student’s t test or one-way analysis of variance with Dunnett’s multiple-comparison tests by using the SPSS version 18.0K program (SPSS Inc., Chicago, IL). Differences with a value of p < 0.05 were considered to be statistically significant.

**RESULTS**

**LCN2 Up-regulates Chemokine Expression in the CNS**—We have previously demonstrated that LCN2 plays a central role in reactive astrogliosis (18). To gain a deeper understanding of how LCN2 participates in the phenotypic transformation of astrocytes, we have analyzed the global gene expression profile of LCN2-stimulated astrocytes by DNA microarray analysis (Table 2). A complete list of genes with differential expression is available in the Gene Expression Omnibus under accession number GSM392157. Chemokines constituted the major group of genes up-regulated by LCN2. Among 40 genes that showed more than a 2-fold increase, 10 genes (25%) belonged to the chemokine family. Traditional RT-PCR or real time PCR analysis was conducted for individual chemokine genes to validate the microarray results (Fig. 1). The up-regulation of CXCL2/GRO-β and CXCL10/IP-10 was confirmed by RT-PCR in astrocytes treated with LCN2 (Fig. 1A). The induction of other inflammatory genes, such as IL-6, COX-2, and iNOS, was also confirmed (Fig. 1A). The cells were also treated with LPS, LPS/IFN-γ, or TNF-α for comparison purposes. Different stimulatory conditions were used for the different CNS cell types because of their specific stimulatory requirements. LCN2 induced the down-regulation of 475 genes with greater than a 2-fold change, among which a decrease in the protein inhibitor of activated STAT3 (PIAS3) expression was confirmed (Fig. 1A). Up-regulated or down-regulated genes with greater than 1.5- or 3.5-fold changes, respectively, are listed in Table 2. In the next set of experiments, we explored the possibility that LCN2 may up-regulate the chemokine gene expression in other cell types that are present in the CNS. Most chemokines analyzed also showed the increased expression in LCN2-treated microglia, endothelial cells, and neuronal cells (Fig. 1, B–D). Expression of some of the chemokines/cytokines was also analyzed by real time PCR, which gave a similar result (Fig. 1E). The results suggest that LCN2 may act as a general chemokine inducer in the CNS. LCN2 concentration used in the current study is based on the previous reports. We and others have shown that LCN2 protein concentrations in the blood of normal mice are ~100 ng/ml (or 4 nM). LCN2 is one of the most highly induced molecules in inflammatory disorders, whose concentrations reach 30 μg/ml in the blood and 40 μg/ml in the urine (10, 49). Locally, even higher levels may be achieved in pathologic conditions. The recombinant GST protein, which was purified in the process of LCN2 preparation (18), was used as a control. The GST protein did not up-regulate chemokine expression (data not shown). The constitutive or inducible expression of lcn2 and its receptors has been either previously demonstrated for microglia (17) and astrocytes (18) or shown in...
this study for endothelial cells and neuronal cells (supplemental Fig. S1, A–D). The results suggest that LCN2 may directly act on these cell types to up-regulate chemokine expression.

Recently, two cellular receptors for LCN2 have been identified. Megalin, a member of the low density lipoprotein receptor family, has been shown to bind human LCN2 with high affinity and to mediate its endocytosis (50). Brain type organic cation transporter (24p3R) is another cell surface receptor for mouse lcn2, which has been shown to mediate apoptosis or iron uptake (1). LCN2 may act on these receptors to initiate its activity.

Chemokines Mediate LCN2-induced Cell Migration—Based on the RT-PCR analysis of chemokines, CXCL10 showed the most significant change in the mRNA expression levels in LCN2-stimulated astrocytes. LCN2 enhancement of CXCL10 expression at protein levels was confirmed by ELISA (Fig. 2, A and B). It has been previously shown that CXCL10 protein concentrations in the astrocytes are 300–1000 pg/ml (51), which is consistent with the current results. LCN2 induction of CXCL10 expression in astrocytes was further confirmed by the overexpression or knockdown of lcn2 expression in the C6 glioma cell line (Fig. 2, B and C). C6 glial cells with an increased or decreased expression of lcn2 were generated by stable transfection with sense or antisense lcn2 cDNA (18). Levels of lcn2 expression were correlated with those of CXCL10, supporting the hypothesis that endogenous LCN2 up-regulates CXCL10 expression in astrocytes. Moreover, the LCN2-up-regulated CXCL10 expression was consistently found in microglia, endothelial cells, and neuronal cells, as well as in astrocytes (Fig. 1). Of several chemokines, CXCL10 expression was most strongly up-regulated by LCN2 in all of these cell types (Fig. 1). These results prompted us to hypothesize that chemokines, such as CXCL10, may mediate LCN2-induced cell migration. LCN2 is an autocrine mediator of reactive astrocytosis (18). Thus, LCN2-up-regulated chemokines may also be responsible for the morphological features of reactive astrocytosis phenotype. This hypothesis was tested by evaluating the effect of LCN2-treated ACM on the CNS cell migration and morphology. The cell migration assay, such as in vitro wound healing assay (Fig. 3A) and the Boyden chamber assay (Fig. 3B), revealed that LCN2-treated ACM (ACM-LCN2) augmented astrocyte migration, which was inhibited or mimicked by CXCL10 neutralizing antibody, respectively (Fig. 3, A and B). In the wound healing assay, astrocytes treated with ACM-LCN2 or the recombinant CXCL10 protein became confluent at 72 h. Checkerboard analysis (Fig. 3, C and D, and Tables 3 and 4) was performed with different dilutions of ACM-LCN2 and the recombinant CXCL10 protein to determine whether the ACM-LCN2 and the recombinant CXCL10 protein cause chemotaxis (directed movement) or chemokinesis (random movement) of the astrocytes. Maximal migration occurred when high concentrations of the ACM-LCN2 and the recombinant CXCL10 protein were placed in the lower chamber, and no significant increase in migration was observed when equal concentrations of the ACM-LCN2 and the recombinant CXCL10 protein were placed on both sides of the chamber, thereby indicating that the ACM-LCN2 and the recombinant CXCL10 protein were strongly chemotactic to the astrocytes (Fig. 3, C and D, and Tables 3 and 4). The GST protein used as a control was without effect (data not shown). The LCN2 regulation of CXCL10 or PIAS3 expression in astrocytes was con-
LCN2 as a CNS Chemokine Inducer

Figure 6. The effect of LCN2-treated ACM on the morphology of astrocytes and microglia. ACM was prepared after the treatment of primary astrocytes with LCN2 (10 μg/ml) for 24 h. The addition of LCN2-treated ACM (ACM-LCN2) induced morphological changes in primary astrocytes and primary microglia cultures after 24 h (A). Primary astrocytes were stained with GFAP antibody (original magnification, ×400), followed by the incubation with anti-mouse IgG-FITC-conjugated secondary antibody. Primary microglia were stained with the peroxidase-labeled isoelectin B4 (original magnification, ×100), followed by incubation with diaminobenzidine tetrahydrochloride. Scale bars, 25 μm. The length of the longest process in each astrocyte or the percentage of ramified microglia was assessed by examining several randomly chosen microscopic fields (B). The results are the means ± S.D. (n = 3). *, p < 0.05; compared with the untreated ACM control (ACM-None).

Results were obtained for microglia or neuronal cultures (Fig. 5, if not all, cell migration-promoting activity of LCN2. Similar to the chemokines up-regulated by LCN2, may account for most, and cell migration. The results indicate that CXCL10, among the chemokines in LCN2 proteins expressed in bacteria and mammalian cells, have the same effects on the chemokine expression and cell migration. The results indicate that CXCL10, among the chemokines up-regulated by LCN2, may account for most, if not all, cell migration-promoting activity of LCN2. Similar results were obtained for microglia or neuronal cultures (Fig. 5, A and B), indicating that LCN2-up-regulated chemokines promote the migration of both glia and neurons. The expression of the CXCL10 receptor, CXCR3, was detected in primary astrocytes, primary microglia, or primary cortical neuron cultures (supplemental Fig. S1E), supporting an important role for CXCL10/CXCR3 in CNS cell migration. ACM-None, ACM-LCN2, CXCL10 protein, or mLCN2 protein did not significantly affect cell proliferation or viability of primary astrocytes, microglia, or neuron cultures as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays (data not shown), indicating that the data in Figs. 3–5 are independent of the effects on cell proliferation or cell death. Effects of ACM prepared after the LCN2 treatment on cell morphology were next determined. ACM-LCN2 also induced morphological changes in glia (Fig. 6) and neurons (data not shown): the cellular process extension was observed for astrocytes and neurons, whereas amoeboid transformation of microglia was induced. As far as astrocytes are concerned, the morphological change reflects phenotypic alteration during reactive astrogliosis. This further supports that LCN2 induces reactive astrogliosis and CNS cell migration via the secretion of chemokines, such as CXCL10.

STAT3 and NF-κB Mediate LCN2-up-regulated CXCL10 Expression in Astrocytes—We next examined signaling pathways that led to CXCL10 up-regulation in LCN2-exposed astrocytes. Based on the results of the microarray analysis that showed the down-regulation of PIAS3 by LCN2, we tested the involvement of the STAT3 signaling in the LCN2-up-regulated CXCL10 expression. LCN2 alone induced phosphorylation of STAT3 at serine 727 and tyrosine 705 (Fig. 7A). The LCN2-induced STAT3 phosphorylation at the two amino acid residues showed slightly different time kinetics (Fig. 7B). LCN2 also enhanced STAT3 phosphorylation induced by phorbol 12-myristate 13-acetate, ATP, and IFN-γ. The total levels of STAT3 proteins were not affected under this condition. AG490 (JAK2/STAT3-specific inhibitor), but not piceatannol (JAK1/STAT1-specific inhibitor), significantly attenuated LCN2-induced CXCL10 protein production as determined by ELISA (Fig. 7C). In contrast, LPS/IFN-γ-induced CXCL10 production was suppressed by both AG490 and piceatannol, indicating the importance of both JAK2/STAT3 and JAK1/STAT1 pathways under this condition, which was used for comparison purposes. The level of LCN2-induced CXCL10 production was comparable with that of LPS-induced CXCL10 and was not abolished by polymyxin B treatment, ruling out the possibility of LPS contamination in the process of recombinant LCN2 preparation (supplemental Fig. S2, A and C). mLCN2 of mammalian origin was also used to further exclude the possibility of LPS contamination (Fig. 4). Polymyxin B, AG490, and piceatannol did not affect cell viability at the concentrations used in the current study (supplemental Fig. S2, A, D, and E). Taken together, these results indicate that LCN2 up-regulates CXCL10 production partly through the JAK2/STAT3 signaling pathway in astrocytes. The LCN2 activation of the JAK2/STAT3 pathway may be facilitated by either the down-regulation of inhibitory PIAS3 expression or the up-regulation of IL-6 expression; this is based on the microarray analysis showing that LCN2 down-regulates PIAS3 and up-regulates IL-6 expression (Fig. 1A). Previously, PIAS3 proteins had blocked the DNA binding activity of STAT3 and inhibited STAT3-mediated gene activation (52), whereas IL-6 is known to stimulate the STAT3 pathway that is also a critical component of reactive astrogliosis (53). In the mean time, LCN2-induced GFAP expression was also diminished by AG490 (Fig. 7D), indicating that JAK2/STAT3 signaling may also be involved in LCN2-induced GFAP expression and the process extension of astrocytes during reactive astrogliosis. Because NF-κB is a canonical pathway that is involved in numerous inflammatory gene expression, the effect of LCN2 on NF-κB signaling was also determined. LCN2 strongly induced NF-κB activation (Fig. 7E). Additionally, LCN2-induced NO production was reduced by pyrrolidine dithiocarbamate (NF-κB inhibitor), further supporting that LCN2 induces astrocyte NO production and activation through...
NF-κB (Fig. 7F). NO may be involved in the amplification of LCN2-induced GFAP expression, because NO had previously enhanced GFAP expression in astrocytes (18, 54). Studies using pharmacological inhibitors, such as pyrrolidine dithiocarbamate (PTDC, NF-κB-specific inhibitor; 0–10 μM) or piceatannol (JAK1/STAT1-specific inhibitor, 50 μM; 0–10 μM) for 30 min prior to their treatment with the recombinant LCN2 protein (10 μg/ml) or LPS (100 ng/ml) plus IFN-γ (50 units/ml) for 24 h. The concentration of nitrite in the culture media was measured by the Griess reagent. The results are the means ± S.D. (n = 3), *p < 0.001 compared with the treatment without inhibitors. Astrocytes were pretreated with AG490 for 30 min prior to the treatment with the recombinant LCN2 protein (10 μg/ml) for 24 h. The expression of GFAP protein levels was assessed by Western blot analysis, respectively (D). After astrocytes were treated with the recombinant LCN2 protein (10 μg/ml) or LPS (100 ng/ml) plus IFN-γ (50 units/ml) for 1 h, an EMSA analysis of the nuclear extracts was conducted by using a 32P-labeled NF-κB oligonucleotide probe (E). Binding specificity was determined by the supershift assay using antibody against p65 (p65 Ab) or its coincubation with an unlabeled probe containing the NF-κB binding sequence (cold probe) to compete with the labeled oligonucleotide. The results are one representative of more than three independent experiments. Primary astrocytes were pretreated with pyrrolidine dithiocarbamate (PTDC, NF-κB-specific inhibitor; 0–10 μM) for 30 min prior to their treatment with the recombinant LCN2 protein (10 μg/ml) or LPS (100 ng/ml) plus IFN-γ (50 units/ml) for 24 h (F). The concentration of nitrite in the culture media was measured by the Griess reagent. The results are the means ± S.D. (n = 3), *p < 0.001 compared with the treatment without inhibitors. Astrocytes were pretreated with SC-514 (IKK-specific inhibitor, 10 μM) for 30 min prior to the treatment with the recombinant LCN2 protein (10 μg/ml) for 8–24 h. The expression of GFAP at mRNA or protein levels after 8 or 24 h was then assessed by RT-PCR or Western blot analysis, respectively (G).

**Functional Analysis of LCN2 in Vivo Using Mouse Brain Injury and Neuroinflammation Models**—To investigate the role of LCN2 in CNS cell migration in vivo, we used mouse models of brain injury and inflammation. We first investigated the expression of LCN2 in adult mouse tissues from LCN2 wild-type (LCN2+/+), LCN2 heterozygous (LCN2+/-), and LCN2-deficient (LCN2-/-) mice. As described previously (12, 55, 56), the LCN2 expression was detected in brain, lung, liver, and kidney of LCN2+/- mice, but not LCN2-/- mice; the expression level was dependent on the gene dosage (supplemental Fig. S3). We next investigated the role of LCN2 in the well-established models of brain injury and inflammation (cortical stab wound injury model as well as intracortical and intracerebroventricular (icv) injection of LPS). Adult mice (LCN2+/- and LCN2-/-) received cortical stab wound injuries. The animals were then perfused, and brain tissue was prepared for
LCN2 as a CNS Chemokine Inducer

immunohistochemical studies. The stab wound injury increased the expression of GFAP in cortex at 2 dpi, indicating the astrocyte migration toward the stab injury site (Fig. 8A). LCN2−/− mice showed a reduced astrocyte migration in response to cortical stab wound injury, when compared with LCN2+/+ mice (Fig. 8A). After the stab wound injury, the expression of LCN2 and CXCL10 was also investigated by quantitative real time PCR. The stab wound injury significantly increased the expression of lcn2 and CXCL10 mRNA at injury site compared with uninjured control mice (Fig. 8B). The expression of lcn2 and CXCL10 was negligible in the cortex of LCN2−/− mice after the focal injury in the cortex (Fig. 8B). These results further support the important role of LCN2 in the regulation of CXCL10 expression. In the next set of experiments, LCN2+/+ and LCN2−/− received intracortical or intracerebroventricular injection of LPS. The animals were then perfused, and brain tissue was prepared for immunohistochemical studies. LCN2 expression was found in the cortex and hippocampus, and the expression was completely absent in LCN2−/− mice as anticipated (Fig. 9, A and B), indicating a crucial role for LCN2 in the regulation of CXCL10 expression. LPS-induced up-regulation of CXCL10 mRNA expression showed a similar pattern (Fig. 9A). In addition, there was a decrease in both the number and intensity of GFAP-positive cells in the hippocampus of LCN2−/− mice compared with LCN2+/+ mice after icv injection of LPS (Fig. 9B). LCN2−/− mice displayed very little staining for LCN2 or GFAP, suggesting a reduced astrocyte migration and neuroinflammation in response to LPS injection. No significant difference in GFAP staining was found in the hippocampus of LCN2−/− mice between saline and LPS injection (data not shown). The increase in GFAP immunoreactivity was mainly detected in the hippocampus following icv injection of LPS in both LCN2+/+ and LCN2−/− mice. Quantification of the data revealed 55 and 37% decreases in the number of GFAP-positive cells in the cortex and hippocampus of LCN2−/− mice, respectively, when compared with LCN2+/+ mice after LPS injection (Fig. 9, A and B). In LCN2−/− mice, LPS-induced up-regulation of CXCL10 or GFAP mRNA expression in hippocampal tissue was insignificant, consistent with reduced neuroinflammation (Fig. 9B). Additionally, double labeling using antibodies against GFAP and LCN2 in hippocampus of the LCN2+/+ mice after icv injection of LPS indicated that LCN2 is expressed in the majority of astrocytes (Fig. 9C). Taken together, these results indicate that inflammation or injury-triggered astrocyte migration and GFAP/CXCL10 expression are significantly decreased in
LCN2\textsuperscript{−/−} mouse brain, supporting the pivotal role of LCN2 in reactive astrocytosis and brain inflammation in vivo.

**DISCUSSION**

In this study, we report that LCN2 up-regulates chemokine expression in the CNS cells and that these chemokines mediate LCN2-induced cell migration. LCN2 has been previously implicated in cell migration in a variety of different tissue types. LCN2 has been associated with an increased migratory activity of breast cancer (5, 57) and other cell types (6, 13, 58), which is consistent with the stimulating role of LCN2 in astrocyte migration, as demonstrated in our previous (18) and current studies. Thus, chemokines seem to mediate the cell migration-promoting activity of LCN2, at least in the CNS.

Chemokines in the brain have been shown to exert neuro-modulatory activity and to be involved in the migration of a broad spectrum of cells, including astrocytes, microglia, and neurons (32). For instance, astrocyte migration has been demonstrated, in vitro, in response to CCL2, CXCL10, and CXCL12 (30, 31, 59). Although the expression of chemokines is commonly induced by inflammatory cytokines and pathogens (60), less is known about other molecular mediators that induce chemokine expression in the CNS. The expression of chemokines is regulated by many stimulatory and inhibitory factors, such as Toll-like receptor-3, UDP, TGF-β, 17β-estradiol, and HIV-1 glycoprotein 120 in the CNS (19−21, 61−65). Now, our results indicate that chemokine expression is highly up-regulated by LCN2 and that these chemokines promote the migra-
tion of astrocytes, microglia, and neurons. Although the important role of CXCL10 in LCN2-induced cell migration has been demonstrated in this study, other chemokines and nonchemokines may also participate in LCN2-induced CNS cell migration. Moreover, cross-talk between chemokine and nonchemokine pathways may also be potentially important in controlling brain cell migration. Neuronal guidance molecules such as netrins, ephrins, semaphorins, slits, and the chemokine SDF have been previously implicated in regulating neuronal migration (66–74). The netrin, slit, semaphorin, and ephrin pathways are versatile, because these guidance cues have been implicated in a wide variety of axon guidance or targeting events. The functions of these guidance molecules are not restricted to axon migrations. Netrins and slits influence neuronal as well as mesodermal cell migrations (75–77); semaphorins mediate bone and heart morphogenesis (78); and ephrins direct neural crest migration and angiogenesis (79). Furthermore, semaphorins and slits affect the growth of retinal ganglion cell axons (80, 81).

We have previously shown that LCN2 plays a central role in reactive astrocytosis. LCN2 induced GFAP expression and cell migration in astrocytes (18). One of the hallmarks of reactive astrocytosis is an increase in the number of astrocytes. A local increase in the cell number may be due to either increased cell proliferation or recruitment of cells. The enhanced expression of chemokines and chemokine receptors has been described under various pathological conditions, such as multiple sclerosis, HIV infection, ischemia, and neoplasm (82–84). Reactive astrocytes express various chemokines and their receptors, which induce cell migration and other cellular reactions that are generally involved in reactive astrocytosis. Thus, chemokines may mediate the cell migration-promoting activity of LCN2 during reactive astrocytosis. Reactive astrocytosis is also accompanied by morphological changes. The involvement of LCN2 in the phenotypic change of cell morphology has been previously reported. LCN2 induced a process extension in astrocytes (18). LCN2 was an endogenous epithelial inducer (7) and stimulated the epithelial phenotype of transformed cells (85, 86). LCN2 also promoted tubulogenesis by regulating epithelial morphogenesis (87). LCN2-induced chemokines may participate in the modification of cell morphology. Previously, CCL5/RANTES, one of the inflammatory chemokines secreted by astrocytes, was critical for promoting neurite outgrowth and migration in cortical neurons. Therefore, chemokines secreted by activated astrocytes and other CNS cells may mediate distinct morphological changes in addition to cell migration (Figs. 3–6).

The JAK-STAT signaling pathway is known to be involved in hematopoiesis (88), immune responses (89), cellular homeostasis (90), gliogenesis (91), and reactive astrocytosis (53, 92). In the CNS, STAT3 is expressed by astrocytes, neurons, and other glial cell types (93), and the activation of STAT3 by phosphorylation markedly increases after CNS insults (94, 95). Here, we showed that the LCN2 induction of CXCL10 expression in

FIGURE 10. Schematic diagram depicting the promotion of CNS cell migration by LCN2-induced CXCL10 (A) and the possible pathway through which LCN2 induces astrocyte migration and morphological changes (B). A, LCN2 up-regulates CXCL10 expression in the multiple cell types in the CNS, such as astrocytes, microglia, neurons, and endothelial cells. Astrocyte-derived CXCL10 acts in a paracrine or autocrine manner to promote cell migration in the inflammatory scene. CXCL10 secreted by other cell types may play a similar role. The lcn2 receptor and CXCL10 receptor (CXCR3) are widely expressed in glia, endothelial cells, and neurons. B, LCN2 up-regulates CXCL10 and GFAP expression in reactive astrocytes through JAK2/STAT3 and NF-κB pathways. Although LCN2-up-regulated CXCL10 promotes cell migration, GFAP induction may lead to morphological changes observed in reactive astrocytosis. Based on the microarray analysis, LCN2 induces the up-regulation of IL-6 and down-regulation of Pias3, thereby facilitating the STAT3 pathway (dotted line). NO production, downstream of the NF-κB, may cooperate with the STAT3 pathway to induce GFAP expression. NO has been shown to induce GFAP expression in astrocytes (bold dotted line) (54). Moreover, IL-6 previously induced GFAP expression through the STAT3 pathway (96). Other pathways may also participate in astrocyte migration and morphological change under the current conditions.
astrocytes was partly mediated by the JAK2/STAT3 pathway, which was in accordance with a microarray analysis showing the down-regulation of the PIA53 by LCN2 (Fig. 1A and Table 2). Previously, CAMP-induced autocrine IL-6 enhanced the GFAP expression through STAT3. IL-6 was important for STAT3 activation and subsequent GFAP expression (96). Consistently, in this study, LCN2 also increased IL-6 expression in astrocytes (Fig. 1, A and E, and Table 2). The JAK2/STAT3 pathway was also involved in LCN2-induced GFAP expression. Thus, the JAK2/STAT3 signaling pathway appears to play a pivotal role in the LCN2 action in astrocytes. Our results indicated NF-κB as an additional signaling pathway that regulates the LCN2 actions (Fig. 7).

The critical role of LCN2 in CNS cell migration and reactive astrogliosis in vivo was demonstrated in mouse brain injury or inflammation models. Injection of LPS into specific regions of rodent brain results in the activation of glial cells and inflammatory responses typically found in neuroinflammatory and neurodegenerative diseases (97–99). The LPS injection model has been extensively used to analyze the cellular and molecular mechanisms underlying inflammatory responses in the CNS (99, 100). In the current study, either cortical/ivc injection of LPS or stab wound insult was performed to induce brain inflammation or injury. The injection of LPS or stab wound injury increased the expression of GFAP and CXCL10 in the brain. Importantly, the induction of GFAP and CXCL10 was markedly reduced in the brain of LCN2−/− mice, suggesting that GFAP and CXCL10 expression is regulated by LCN2. The LPS-triggered expression of LCN2 and GFAP was colocalized, which was consistent with previous studies demonstrating that LCN2 was induced in primary astrocyte cultures under inflammatory conditions in vitro (18).

In summary, we present evidence that inflammatory chemokines are up-regulated in LCN2-treated astrocytes, microglia, endothelial cells, and neuronal cells. Chemokines, CXCL10 in particular, secreted from LCN2-treated astrocytes promoted the migration of microglia and neurons, as well as astrocytes themselves (see Fig. 10 for schematic diagram). The JAK2/STAT3 and IKK/NF-κB pathways were involved in the LCN2 induction of CXCL10 secretion and possibly other phenotypic changes associated with reactive astrogliosis. More importantly, mice lacking LCN2 showed an impaired astrocyte migration and a reduced expression of GFAP and CXCL10 following LPS exposure or stab wound injury. These results clearly establish an essential role for LCN2 as a protein required for the reactive astrogliosis and cell migration in the CNS. Thus, we propose that LCN2 is a chemokine inducer in the CNS and may accelerate cell migration under inflammatory conditions in an autocrine or paracrine manner. Lastly, our data suggest that LCN2 could be targeted to therapeutically modulate glial responses in various neuroinflammatory disease conditions.

Acknowledgment—We thank Dr. Shizuo Akira (Osaka University, Osaka, Japan) for generously providing the LCN2-deficient mice.

REFERENCES
1. Devireddy, L. R., Gazin, C., Zhu, X., and Green, M. R. (2005) Cell 123, 1293–1305
2. Nelson, A. M., Zhao, W., Gilliland, K. L., Zaenglein, A. L., Liu, W., and Thiboutot, D. M. (2008) J. Clin. Invest. 118, 1468–1478
3. Tong, Z., Wu, X., Ovcharenko, D., Zhu, J., Chen, C. S., and Kehrer, J. P. (2005) Biochem. J. 391, 441–448
4. Kehrer, J. P. (2010) Cell Biol. Toxicol. 26, 83–89
5. Yang, J., Bielenberg, D. R., Rodig, S. J., Doiron, R., Clifton, M. C., Kung, A. L., Strong, R. K., Zurakowski, D., and Moses, M. A. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 3913–3918
6. Bauer, M., Eickhoff, J. C., Gould, M. N., Mundhenke, C., and Searle, A. (2008) Breast Cancer Res. Treat. 108, 389–397
7. Yang, J., Goetz, D., Li, J. Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R., and Barasch, J. (2002) Mol. Cell 10, 1045–1056
8. Bolignano, D., Donato, V., Coppolino, G., Campo, S., Buemi, A., Lucanu, A., and Buemi, M. (2008) Am. J. Kidney Dis. 52, 595–605
9. Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., and Aderem, A. (2004) Nature 432, 917–921
10. Mori, K., Lee, H. B., Rapoport, D., Drexler, I. R., Foster, K., Yang, J., Schmidt-Ott, K. M., Chen, X., Li, J. Y., Weiss, S., Mishra, J., Cheema, F. H., Markowitz, G., Suganami, T., Sawai, K., Mukoyama, M., Kunis, C., D’Agati, V., Devarajan, P., and Barasch, J. (2005) J. Clin. Invest. 115, 610–621
11. Liu, Q., and Nilsen-Hamilton, M. (1995) J. Biol. Chem. 270, 22565–22570
12. Yan, Q. W., Yang, Q., Mody, N., Graham, T. E., Hsu, C. H., Xu, Z., Houstis, N. E., Kahn, B. B., and Rosen, E. D. (2007) Diabetes 56, 2533–2540
13. Playford, R. J., Belo, A., Poulson, R., Fitzgerald, A. J., Harris, K., Pawlucky, I., Ryon, J., Darby, T., Nilsen-Hamilton, M., Ghosh, S., and March-bank, T. (2006) Gastroenterology 131, 809–817
14. Lin, H. H., Liao, C. J., Lee, Y. C., Hu, K. H., Meng, H. W., and Chu, S. T. (2011) Int. J. Biol. Sci. 7, 74–86
15. Lim, R., Ahmed, N., Borregaard, N., Riley, C., Wafai, R., Thompson, E. W., Quin, M. A., and Rice, G. E. (2007) Int. J. Cancer 120, 2426–2434
16. Tong, Z., Kunnunakkara, A. B., Wang, H., Matsu, Y., Diagaradjan, P., Harikumar, K. B., Ramachandran, V., Sun, B., Chakraborty, A., Bresalier, R. S., Logsdon, C., Aggarwal, B. B., Krishnan, S., and Guha, S. (2008) Cancer Res. 68, 6100–6108
17. Lee, S., Lee, J., Kim, S., Park, J. Y., Lee, W. H., Mori, K., Kim, S. H., Kim, I. K., and Suk, K. (2007) J. Immunol. 179, 3231–3241
18. Lee, S., Park, J. Y., Lee, W. H., Kim, H., Park, H. C., Mori, K., and Suk, K. (2009) J. Neurosci. 29, 234–249
19. Charo, I. F., and Ransohoff, R. M. (2006) Engl. J. Med. 354, 610–621
20. Ubogu, E. E., Cossoy, M. B., and Ransohoff, R. M. (2006) Trends Pharmacol. Sci. 27, 48–55
21. Bertolini, C., Ragginozio, D., Gross, C., Limatola, C., and Eusebi, F. (2006) Neuropharmacology 51, 816–821
22. Rostène, W., Kitabgi, P., and Pasadaniantz, S. M. (2007) Nat. Rev. Neurosci. 8, 895–903
23. Salustro, F., Mackay, C. R., and Lanzavecchia, A. (2000) Annu. Rev. Immunol. 18, 593–620
24. Vanguri, P. (1995) J. Neuroimmunol. 56, 35–43
25. Luster, A. D., Unkeless, J. C., and Ravetch, J. V. (1985) Nature 315, 672–676
26. Majumder, S., Zhou, L. Z., Chaturvedi, P., Babcock, G., Aras, S., and Ransohoff, R. M. (1998) J. Neurosci. Res. 54, 169–180
27. Hesselgesser, J., and Horuk, R. (1999) J. Neurovirol. 5, 13–26
28. Sun, D., Hu, X., Liu, X., Whitaker, J. N., and Walker, W. S. (1997) J. Neurosci. Res. 48, 192–200
29. Fisher, S. N., Vanguri, P., Shin, H. S., and Shin, M. L. (1995) Brain Behav. Immun. 9, 331–346
30. Biber, K., Dijkstra, L., Trebst, C., de Groot, C. J., Ransohoff, R. M., and Boddeke, H. W. (2002) Neuroscience 112, 487–497
31. Odemis, V., Moepps, B., Gierschik, P., and Engele, J. (2002) J. Biol. Chem. 277, 39801–39808
32. Ambrosini, E., and Aloisi, F. (2004) Neurochem. Res. 29, 1017–1038
33. Montesano, R., Pepper, M. S., Möhle-Steinlein, U., Risau, W., Wagner, E. F., and Orci, L. (1999) Cell 92, 435–445
34. Enokido, Y., Akaneya, Y., Niinobe, M., Mikoshiba, K., and Hatanaka, H.
