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Identification and Molecular Characterization of Rat CXCR3: Receptor Expression and Interferon-Inducible Protein-10 Binding Are Increased in Focal Stroke

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ABSTRACT

We describe here the cloning and characterization of a rat homolog of the chemokine receptor CXCR3. The predicted amino acid sequence of rat CXCR3 contains 367 amino acid residues, sharing 96 and 87% amino acid sequence identity to the murine and human CXCR3, respectively. Among a large panel of chemokines tested, only interferon-inducible protein-10 (IP-10), interferon-γ-induced monokine, and interferon-inducible T cell α-chemoattractant demonstrated specific abilities to induce an intracellular calcium mobilization response in human embryonic kidney 293 cells transfected with rat CXCR3 expression vector. ^{125}I-IP-10 competition binding studies to the CXCR3-transfected human embryonic kidney 293 cells demonstrated that human IP-10 and interferon-inducible T cell α-chemoattractant are more potent ligands than human interferon-γ-induced monokine. Following our previous observation for the induced expression of IP-10 in focal stroke, we demonstrate here the time-dependent up-regulation of CXCR3 mRNA in the rat ischemic cortex after permanent occlusion of the middle cerebral artery. A significant increase in ^{125}I-IP-10-specific binding to ischemic cerebral cortical samples was obtained and paralleled the increase in CXCR3 mRNA expression. The changes in receptor expression and ligand binding correlate highly with known changes in leukocyte accumulation, and gliosis occurred after focal stroke. These data suggest that CXCR3/IP-10 may be a potential novel therapeutic target in focal stroke. In addition, the cloning of rat CXCR3 provides an important tool for the investigation of the pathophysiological role of CXCR3 in other rodent disease models.

Chemokines are small secreted proinflammatory molecules that chemoattract and activate specific leukocyte subpopulations in vitro and regulate leukocyte trafficking in vivo (Rollins, 1997; Luster, 1998). They are thought to initiate and maintain inflammatory responses and may play a role in the pathology of a number of inflammatory diseases, including ischemia/reperfusion injury. Chemokines are traditionally classified into subgroups [e.g., CXC (α) or CC (β)] based on the presence or absence of an amino acid between the first two cysteine residues. In general, CXC chemokines that contain a proximal "ELR" motif attract neutrophils, whereas non-"ELR"-containing CXC chemokines attract lymphocytes and CC chemokines attract mononuclear cells (Miller and Krangel, 1992; Baggiolini et al., 1994). Recently, additional classes of chemokines including C and CXXXC chemokines, and their diverse chemoattractive functions, have been described (Rollins, 1997; Luster, 1998; Ransohoff and Tani, 1998).

Chemokine receptors belong to the superfamily of G protein-coupled seven-transmembrane domain receptors (Premack and Schall, 1996). Based on ligand preference, chemokine receptors can be divided into separate classes, including C-, CC-, CXC-, and CX3C- chemokine receptors and orphan "chemokine-like" receptors. Chemokine receptors interact with their ligands in a specific, shared, or promiscuous fashion and/or with viral ligands (Premack and Schall, 1996). On activation with an appropriate ligand, the known chemokine receptors produce an increase in intracellular calcium (Premack and Schall, 1996), and this activity has been used to monitor receptor activation.

ABBREVIATIONS: IP-10, interferon-inducible protein-10; Mig, interferon-γ-induced monokine; I-Tac, interferon-inducible T cell α-chemoattractant; PCR, polymerase chain reaction; MCAO, occlusion of the middle cerebral artery; RACE, rapid amplification of cDNA ends; RT, reverse transcription; HEK, human embryonic kidney; Ct, threshold cycle; RANTES, regulated on activation normal T cell expressed and secreted; MIP, macrophage inflammatory protein; ENA, epithelial-derived neutrophil-activating peptide; PARC, pulmonary and activation-regulated chemokine; HCC, hemofiltrate C-C chemokine; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; GCP, granulocyte chemotactic protein; GRP, growth-related oncogen protein; PF, platelet factor; SDF, stromal cell-derived protein; CINC, cytokine-induced neutrophil chemoattractant; MCP, monocyte chemotactic protein; IL, interleukin.

Interferon-inducible protein-10 (IP-10) and interferon-γ-induced monokine (Mig), CXC chemokines, specifically activate the human CXCR3 (Loetscher et al., 1996). Both human CXCR3 and the murine ortholog of CXCR3 have been cloned and characterized (Loetscher et al., 1996; Soto et al., 1998). Recently, another CXC chemokine, interferon-inducible T cell α-chemoattractant (I-Tac), which activates CXCR3 (Cole et al., 1998), has been identified. In addition, a murine CC chemokine, 6Ckine, was reported to bind to the mouse CXCR3 (Soto et al., 1998), whereas human 6Ckine failed to interact with the human or mouse CXCR3 (Jenh et al., 1999). The expression of CXCR3 has been identified on activated T cells and natural killer cells (Loetscher et al., 1996; Qin et al., 1998) and in a proportion of circulating blood T cells and B cells (Qin et al., 1998). Although the primary role of ligands for CXCR3, whose production is stimulated by interferon-γ (IFN-γ), is recruitment of activated T cells (Rollins, 1997; Luster, 1998), IP-10 has also exhibited monocyte chemoattraction (Taub et al., 1993). In addition, IP-10 is involved in a wide range of cellular activities, including vascular smooth muscle cell migration and proliferation (Wang et al., 1996) and astrocyte chemoattraction (Wang et al., 1998).

Cerebral ischemia is associated with an intense inflammatory reaction that contributes to the secondary progression of ischemic brain injury (Kochanek and Hallenbeck, 1992; Feuerstein et al., 1998). After brain ischemia, significant polymorphonuclear cell infiltration and accumulation occur between 1 and 48 h and are followed by mononuclear cell infiltration between 2 and 15 days after ischemia (Kochanek and Hallenbeck, 1992; Clark et al., 1993; Garcia et al., 1994). This inflammatory reaction in the ischemic brain is driven by the de novo expression of inflammatory cytokines, chemokines, and adhesion molecules in the lesion (Feuerstein et al., 1998). Previously, we reported the induction of IP-10 mRNA and protein expression after focal stroke, which appears to play a pleiotropic role in prolonged leukocyte recruitment, as well as in astrocyte migration/activation after ischemic brain injury (Wang et al., 1998). This report further characterizes the pathophysiological role of IP-10 in ischemic disease processes, as well as the molecular cloning and characterization of rat CXCR3. Using a recently developed and very sensitive quantification method of real-time polymerase chain reaction (PCR) (Gibson et al., 1996; Heid et al., 1996), elevated expression of CXCR3 was noted in ischemic cortical samples after permanent occlusion of the middle cerebral artery (MCAO). Furthermore, a concomitant increase in ^{125}I-IP-10 binding was associated with the increased CXCR3 expression in the ischemic brain. These changes are correlated with increased leukocyte accumulation in the ischemic brain after focal stroke and suggest a role for IP-10/CXCR3 interaction in the recruitment of inflammatory cells into the brain in response to ischemic injury.

Experimental Procedures

Materials. The human chemokines IP-10, Mig, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MCP-2, eotaxin-2, ENA-78, PARC, 6Ckine, fractakine, HCC-1, MDC, TARC, I-309, and GCP-2 were purchased from R&D Systems (Minneapolis, MN). Human chemokines MCP-1, MCP-3, MCP-4, RANTES, MIP-5, GROα, GROβ, eotaxin-1, PF-4, lymphotactin, I-TECK, and I-Tac were obtained from PeproTech, Inc. (Rocky Hill, NJ). Human chemokines RANTES, IP-10, IL-8, eotaxin-1, and SDF-1 were produced by SmithKline Beecham (King of Prussia, PA). Rat chemokines CINC-2α and CINC-2β and mouse 6Ckine and mouse Mig were purchased from R&D Systems. Mouse IP-10 was purchased from PeproTech.

Cloning of Rat CXCR3 cDNA. PCR primers (5′-CGAATTCATGTACCTTGAGGTTAGTGAACG-3′ for the forward and 5′-CGGATCCTTACAAGCCCAGGTAGGAGG-3′ for the reverse) were synthesized based on the published mouse CXCR3 sequence (Soto et al., 1998) and included an *Eco*RI or a *Bam*HI restriction enzyme site, respectively. Reversibly transcribed rat spleen cDNA [primed with oligo(dT)] was used as a template for PCR under standard reverse transcription (RT)-PCR conditions (Wang et al., 1996). The PCR fragment containing the entire coding region was subcloned into a pCR2.1 vector. The cDNA insert was released from the plasmid with *Eco*RI and *Bam*HI double digestions and further subcloned into a pCDN expression vector (Aiyar et al., 1994) under control of a CMV promoter. The resultant plasmid DNA was sequenced on both strands using universal and specific sequence primers.

To isolate the full-length cDNA, rapid amplification of cDNA 5′-ends (5′-RACE) and 3′-RACE approaches were applied using the RACE kit from Life Technologies (Gaithersburg, MD) and gene-specific RACE primers were synthesized. For 5′-RACE, a reverse primer (5′-AAGAGGAGGCTGTAGAGGACTGG-3′) was used for initial PCR, and a second primer (5′-AGGCAATGTCCGAGGCATC-3′) was used for the nested PCR. For 3′-RACE, a sense primer (5′-CGTAGCCAAGTCAGTCACCTCAG-3′) was followed by a nested primer (5′-CACGGAGAGAATCATCCTGG-3′) for the amplification of cDNAs reversibly transcribed from both spleen and rat ischemic cortical tissues. The nested PCR products were gel-purified, subcloned into pCR2.1, and sequenced. The cDNA sequence generated by 5′- and 3′-RACEs not only overlapped with some coding sequence but also extended 148 and 410 bp from the 5′- and 3′-untranslated sequences of rat CXCR3 mRNA, respectively. DNA sequencing revealed a single-base mismatch in both forward and reverse PCR primers used for the coding sequence cloning (i.e., the base 15 is C for rat and T for mouse in the forward primer and base 12 is T for rat and C for mouse in the reverse primer). Despite the difference in the nucleotide sequences, their coding sequences are identical. The full-length rat CXCR3 cDNA sequence was assembled from individual cDNA fragments and further confirmed by RT-PCR using primers located in the extreme 5′- and 3′-cDNA sequence. Sequencing analysis was performed using the DNASTAR software system (DNASTAR Inc., Madison, WI).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium with high glucose and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected with pCDN (empty vector) and pCDN-rCXCR3 in the presence or absence of a Gqi5 expression vector (Wilkie et al., 1991) using LipofectAMINE Plus according to the manufacturer's instructions (Life Technologies).

Calcium Mobilization. A microtiter plate-based Ca^{2+} mobilization fluorometric imaging plate reader assay (Molecular Devices, Sunnyvale, CA) (Schroeder and Neagle, 1996) was used for the functional characterization of HEK 293 cells transiently expressing rat CXCR3. The day after transfection, cells were plated in poly(D-lysine)-coated 96-well black/clear plates (Becton Dickinson, Bedford, MA). After 18 to 24 h, the medium was aspirated and replaced with 1 μM Fluo-3 acetoxymethyl ester (Molecular Probes, Eugene, OR) in Hanks' balanced salt solution with 10 mM HEPES, 200 μM $CaCl_2$, 0.1% BSA, and 2.5 mM probenecid. After a 1-h incubation (37°C, 5% CO_2), cells were washed three times with the same buffer without dye. The Fluo-3-loaded cells were exposed to varying concentrations of chemokines. After initiation of the assay, fluorescence was read every second for 1 min and then every 3 s for the following minute. Agonist was added at 10 s, and concentration-response curves were generated by calculating maximal fluorescent counts above background. The EC_{50} value is the concentration of agonist producing 50% of the maximal responses.

Radioligand Binding Assays. Receptor binding assays were performed with membranes of HEK 293 cells transiently expressing the human and rat CXCR3. Cells were cultured at 37°C in a humidified incubator under 5% CO_2, 95% air and were harvested by centrifugation at 600*g* for 10 min. Pellets containing 1×10^8 cells were frozen in liquid nitrogen, passed through three freeze/thaw cycles, and then resuspended in ice-cold 25 mM HEPES (pH 7.4), 1 mM EDTA containing protease inhibitors (100 μg/l bacitracin, 10 μM phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 10 μg/l soybean trypsin inhibitor) at a concentration of 1×10^8 cells/40 ml. The suspension was homogenized using a Dounce (glass/glass) homogenizer (20–25 strokes) and homogenized with three pulses of 10 s on a 3/4 setting (Polytron tissue homogenizer; Brinkmann Instruments, Westbury, NY). This suspension was centrifuged at 300*g* for 10 min. The pellet was discarded, and the supernatent fraction was centrifuged at 40,000*g* for 30 min at 4°C. The pellet was resuspended in homogenizing buffer using the Polytron and washed once. The membrane pellet was resuspended in assay buffer (50 mM HEPES, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1% BSA, pH 7.4) at a concentration of 1 to 4 mg protein/ml. Membranes (6×10^5 cell equivalents) were incubated with 0.15 nM ^{125}I-IP-10 (New England Nuclear Life Sciences; specific activity, 2200 Ci/mmol) in the absence or presence of unlabeled IP-10 (100 nM for nonspecific binding) for 90 min at room temperature in 96-well polystyrene enzyme immunoassayA/radioimmunoassay plates in a final volume of 100 μl. Binding was terminated by rapid filtration through GF/C filters that were presoaked with 0.5% polyethylenimine (Sigma Chemical Co., St. Louis, MO) for 1 h, using a 96-well tissue harvester (Unifilter-96 Harvester; Packard). Filters were washed 10 times with wash buffer (25 mM HEPES, 0.5 M NaCl) and air-dried; then, 50 μl of scintillation fluid (Micro Scint 20; Packard) was added to each filter. The amount of radioactivity bound to the filters was determined by liquid scintillation spectrometry.

For binding assay using rat brain tissues, rats were subjected to permanent MCAO for 6 or 24 h or 2 or 10 days, or sham surgery for 2 days using the procedure described later. Membranes were prepared by homogenizing rat brain segments in ice-cold hypotonic buffer (20 mM Tris-HCl, pH 7.4, 1.0 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 100 μg/ml bacitracin, 100 μM benzamidine, and 10 μM phenylmethylsulfonyl flouride) with a Brinkmann Polytron for 20 s on a setting of 4. Samples were then centrifuged at 40,000*g* for 30 min at 4°C and washed once using the same buffer. The pellet was resuspended in binding buffer containing 50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 0.1% BSA. at a concentration of 2 mg/ml. Binding and filtration were performed as described earlier for the receptor membrane assay using concentrations of 0.15 and 0.30 nM ^{125}I-IP-10.

Focal Brain Ischemia. Cerebral focal ischemia or sham surgery was carried out under stereotaxic control in male spontaneously hypertensive rats (Taconic Farms Inc., Germantown, NY), at 18 weeks of age and weighing 250 to 330 g, by permanent MCAO as described previously (Barone et al., 1992; Wang et al., 1998). Briefly, the middle cerebral artery was permanently occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein using electrocoagulation (Force 2 Electrosurgical Generator; Valley Lab Inc., Boulder, CO). In sham-operated rats, the dura was opened over the middle cerebral artery, but the artery was not occluded. Rats were overdosed with pentobarbital, and forebrains were removed at 1, 3, 6, and 12 h and 1, 2, 5, 10, and 15 days after permanent MCAO and at 12 h and 5 days after sham surgery. The ischemic cortex (i.e., the cortex ipsilateral to surgery) was dissected from the ipsilateral hemisphere; the contralateral (control) cortex was dissected from the nonischemic contralateral hemisphere of the same rat (Wang et al., 1998). The cortical samples were immediately frozen in liquid nitrogen and stored at −80°C.

Rats were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* [Dept. of Health, Education, and Welfare Publication no. NIH 85-23, revised 1985]. The work was conducted in a facility accredited by the Association for Assessment & Accreditation of Laboratory Animal Care (AAALAC International). Procedures using lab animals were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals.

Taqman PCR. Total RNA was prepared by homogenizing the cortical tissues in an acid guanidinium thiocyanate solution and extracted with phenol and chloroform as previously described (Chomczynski and Sacchi, 1987). PCR primers and Taqman probes for CXCR3 (the present report) and rpL32 (X06483; Rajchel et al., 1988) were designed using a software program from Perkin-Elmer Applied Biosystems (Foster City, CA). The forward (1440–1459 bp) and reverse (1511–1531 bp) primers for rat CXCR3 are 5′-CCTGCCTCCGCTGTTTTAGA-3′ and 5′-CCTCTTCTCACACAGGGATGG-3′, respectively, and the probe (sense, 1462–1484 bp) is 5′-TAGTTGCCTGGAGCCCCACCACC-3′. The sequences for rpL32 are 5′-TGTCCTCTAAGAACCGAAAAGCC-3′ for the forward (314–336 bp), 5′-CGTTGGGATTGGTGACTCTGA-3′ for the reverse (365–385 bp) primers, and 5′-TCGTAGAAAGAGCAGCACAGCTGGCC-3′ for the probe (sense, 338–363 bp). The Taqman probe consists of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA).

A two-step RT-PCR was performed using Taqman Reverse Transcription Reagents and TaqMan Gold RT-PCR kit (Perkin-Elmer Applied Biosystems) according to the manufacturer's specification. RT reaction used 3 μg of total RNA in a total volume of 40 μl containing 1× Taqman ET buffer consisting of 5.5 mM $MgCl_2$, 500 μM concentration of each dNTP, 2.5 μM oligo $d(T)_{16}$ primers, 0.4 U/μl RNase Inhibitor, and MultiScribe Reverse Transcriptase. RT reaction was carried out at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

A thermal stable AmpliTaq Gold DNA Polymerase was used for the second-strand cDNA synthesis and DNA amplification. Real-time PCR was performed with 4 μl of RT products (300 ng of total RNA), 1× Taqman buffer A, 5.5 mM $MgCl_2$, 200 μM dATP/dCTP/dGTP, 400 μM dUTP, 200 nM primers (forward and reverse), 100 nM Taqman probe, 0.01 U/μl AmpErase, and 0.025 U/μl AmpliTaq Gold DNA Polymerase in a total volume of 50 μl. PCR was performed at 50°C for 2 min (for AmpErase UNG incubation to remove any uracil incorporated into the cDNA) and 95°C for 10 min (for AmpliTaq Gold activation) and then run for 40 cycles at 95°C for 15 s and 60°C for 1 min on the ABI PRISM 7700 Detection System.

To determine the absolute copy number of the target transcript, a cloned plasmid DNA for rat CXCR3 and rpL32 was serially diluted (every 5-fold) and used to produce a standard curve as described elsewhere (Wang et al., 2000). Each plasmid was run with at least triplicate samples, and the ΔRn (the ratio for the amount of reporter dye emission to the quenching dye emission) and threshold cycle (Ct) values were averaged from each reaction. Data were analyzed using a Sequence Detector V1.6 program (Perkin-Elmer Applied Biosystems). The Ct values of CXCR3 and rpL32 in the cortical tissues generated by real-time PCR were compared with the plasmid DNA standard to determine the copy number of their transcripts.

Statistical Analysis. Statistical comparisons were made by ANOVA (Fisher's protected least-squares difference), and values were considered to be significant at $P < .05$.

Results

Molecular Cloning and Characterization of Rat CXCR3 cDNA. To isolate the coding sequence of the rat CXCR3 cDNA, a PCR approach was used with a pair of primers synthesized according to the mouse CXCR3 cDNA sequence (Soto et al., 1998). A total 1104-bp coding sequence of rat CXCR3 was cloned and used for functional characterization.

The full-length cDNA of rat CXCR3 (Fig. 1) was obtained

by 5′- and 3′-RACE. It contains 1563 nucleotides, sharing 94 and 84% nucleotide sequence identity to mouse and human CXCR3 cDNA, respectively. The deduced amino acid sequence of rat CXCR3 shares 96 and 87% sequence identity (or 98 and 92% similarity) to its mouse and human orthologs, respectively (Fig. 2).

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AGATTACCCT TTGGAAGAGG CTGTGCTGAG CCGTGAAATC TTCACTTCCT CTGTTCACGG CAAGTTCCCA ACCACAAGTG     80

CCAAAGGCAG AGAAGCAGGC AGCACGAGAC CTGACCCCAG CAGCCACAGC CAGAGCAGCA GCCAAGCC  ATG TAC CTT    157
                                                                             M   Y   L

GAG GTC AGT GAA CGT CAA GTG CTA GAT GCC TCG GAC ATT GCC TTT CTT CTG GAA AAC AGC ACA TCT    223
 E   V   S   E   R   Q   V   L   D   A   S   D   I   A   F   L   L   E   N   S   T   S
                         10                                  20
CCC TAC GAT TAT GGG GAA AAC GAA AGC GAC TTC TCT GAC TCC CCG CCC TGC CCA CAG GAC TTC AGC    289
 P   Y   D   Y   G   E   N   E   S   D   F   S   D   S   P   P   C   P   Q   D   F   S
                     30                                  40
CTG AAC TTT GAC AGA ACC TTC CTG CCA GTC CTC TAC AGC CTC CTC TTT TTG CTG GGG CTG CTA GGC    373
 L   N   F   D   R   T   F   L   P   V   L   Y   S   L   L   F   L   L   G   L   L   G
             50                                  60
AAT GGG GCA GTG GCT GCT GTG CTA CTG AGC CAG CGC ACT GCC CTG AGC AGC ACA GAC ACC TTC CTG    421
 N   G   A   V   A   A   V   L   L   S   Q   R   T   A   L   S   S   T   D   T   F   L
 70                                  80                                  90
CTC CAC CTG GCT GTG GCC GAT GTA CTG CTG GTA CTA ACC CTC CCA CTG TGG GCA GTA GAT GCT GCT    487
 L   H   L   A   V   A   D   V   L   L   V   L   T   L   P   L   W   A   V   D   A   A
                             100                                 110
GCC CAG TGG GTT TTC GGC TCT GGT CTC TGC AAA GTG GCA GGT GCC CTG TTC AAC ATC AAC TTC TAC    553
 A   Q   W   V   F   G   S   G   L   C   K   V   A   G   A   L   F   N   I   N   F   Y
                         120                                 130
GCA GGG GCC TTC CTG CTG GCC TGT ATA AGC TTT GAC CGC TAC CTG AGC ATA GTG CAT GCC ACC CAG    619
 A   G   A   F   L   L   A   C   I   S   F   D   R   Y   L   S   I   V   H   A   T   Q
                     140                                 150
ATC TAC CGC AGG GAC CCC TGG GTA CGT GTA GCC CTC ACC TGC ATT GTT GTG TGG GGT CTC TGT GTG    685
 I   Y   R   R   D   P   W   V   R   V   A   L   T   C   I   V   V   W   G   L   C   V
             160                                 170
CTC TTT GCC CTC CCA GAT TTC ATC TTC CTG TCA GCC AGC CAT GAT CAG CGC CTC AAT GCC ACC CAT    751
 L   F   A   L   P   D   F   I   F   L   S   A   S   H   D   Q   R   L   N   A   T   H
 180                                 190                                 200
TGC CAG TAC AAC TTC CCA CAG GTG GGT CGG ACT GCT CTG CGT GTA CTG CAG CTA GTG GCT GGT TTC    817
 C   Q   Y   N   F   P   Q   V   G   R   T   A   L   R   V   L   Q   L   V   A   G   F
                             210                                 220
CTG ATG CCC CTT CTA GTC ATG GCC TAC TGC TAT GCC CAT ATC CTG GCT GTG CTG CTG GTC TCC AGA    883
 L   M   P   L   L   V   M   A   Y   C   Y   A   H   I   L   A   V   L   L   V   S   R
                         230                                 240
GGC CAG AGG CGC TTT CGA GCT ATG AGG CTA GTG GTG GTG GTG GTG GTG GCC TTT GCC GTC TGC TGG    949
 G   Q   R   R   F   R   A   M   R   L   V   V   V   V   V   V   A   F   A   V   C   W
                     250                                 260
ACC CCC TAT CAC CTG GTG GTG TTA GTG GAT ATC CTT ATG GAC GTG GGA GTT TTG GCC CGC AAC TGT   1015
 T   P   Y   H   L   V   V   L   V   D   I   L   M   D   V   G   V   L   A   R   N   C
             270                                 280
GGT CGA GAA AGC CAT GTG GAC GTA GCC AAG TCA GTC ACC TCA GGC ATG GGC TAC ATG CAC TGC TGC   1081
 G   R   E   S   H   V   D   V   A   K   S   V   T   S   G   M   G   Y   M   H   C   C
 290                                 300                                 310
CTC AAT CCA CTG CTC TAT GCC TTT GTG GGA GTG AAG TTC AAA GAA CAA ATG TGG ATG TTG CTC ATG   1147
 L   N   P   L   L   Y   A   F   V   G   V   K   F   K   E   Q   M   W   M   L   L   M
                             320                                 330
CGC CTG GGC CGC TCT GAC CAG AGA GGA CCG CAG CGG CAG CCT TCA TCT TCA CGG AGA GAA TCA TCC   1213
 R   L   G   R   S   D   Q   R   G   P   Q   R   Q   P   S   S   S   R   R   E   S   S
                         340                                 350
TGG TCT GAG ACA ACT GAG GCC TCC TAC TTG GGC TTG TAA    TTCAGGAATG CAACTGGAGC CTGTGCAACC   1282
 W   S   E   T   T   E   A   S   Y   L   G   L   *
                     360
CAAGTCCTAA CACACTCCAG GCTCTTCTCC TTGTAGTTGG GCTAGCTCTA ACTTACCCAT AACTTTGCTG CTGGGACTCA   1362

GTGACAGCTC AGCATATCCA GGTCTCCTGA GAACCACTCT CAGCTCCAAG GACAGCAGCA CCTTTACTGA GCCATGCCCT   1442

GCCTCCGCTG TTTTAGAACT AGTTGCCTGG AGCCCCACCA CCCTTCTAAA TTAGCAGATA GCACTCAGCC ATCCCTGTGT   1522

GAGAAGAGGA AGAGACAAAT AGCACTGAAG GCCAGGCCTT GCTCAGCACT GAATGTGTTT ATGGTCTCCA ATATCTCAAC   1602

ATGTGCCCAA TTTTATGGCT AGAAACCCTG CTTAAACTTT CAATAAACAA GATAATGAGG AAAAAAAAAA AAAAAA       1678
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Fig. 1. Nucleotide sequence of rat CXCR3. The sequence of the rat CXCR3 cDNA was assembled from the initial PCR clone for the coding region and the 5- and 3′-RACE clones that overlap with the coding sequence and extend the 5′- and 3′-nontranslated sequences. The numbers on the rightmost column refer to the nucleotide positions. The predicted amino acid sequence is shown under the corresponding nucleotide sequence. The sequence of rat CXCR3 cDNA has been deposited in the GenBank (accession no. AF223642).

Functional Analysis of Cloned CXCR3 in HEK 293 Cells. The rat and human CXCR3 cDNAs were subcloned into a mammalian expression vector, and the resultant plasmids were transiently cotransfected into HEK 293 cells with or without the Gqi5 plasmid. The CXCR3 receptor transfected alone into HEK 293 cells responded, but this activity was enhanced by the cotransfection with promiscuous G proteins and G protein chimeras. Of these cotransfected G proteins, Gqi5 produced the best response, and thus its cotransfection data are illustrated, which are in agreement with previous reports (Offermanns and Simon, 1995; Coward et al., 1999). The transfectants were tested for intracellular calcium mobilization responses to a large collection of chemokines, including MCP-1, MCP-2, MCP-3, MCP-5, RANTES, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-5, IL-8, GROα, GROβ, CINC-2α, CINC-2β, NAP2, IP-10 (human and murine), Mig (human and murine), eotaxin-1, eotaxin-2, ENA-78, PF-4, lymphotactin, PARC, 6Ckine (human and murine), Fractakine, HCC-1, MDC, TARC, I-309, GCP-2, I-TECK, SDF-1, and I-Tac (human) using the 96-well fluorescent imaging plate reader. This technology allowed for the first time the characterization of both human and rat CXCR3 using the same sister plates of chemokines to assess their relative potencies. Of the chemokines tested, only IP-10 (human and murine), I-Tac, Mig (human and murine), and murine 6Ckine induced cytoplasmic calcium transient responses in the rat CXCR3-transfected HEK 293 cells, and the same ligands, except for murine 6Ckine, responded in the human CXCR3-transfected cells. The concentration-dependent calcium mobilization responses for IP-10 (human and murine), Mig (human and murine), 6Ckine (human and murine), and I-Tac in the rat and human CXCR3 transfectants are depicted in Fig. 3, A and B, respectively, and in Table 1. There was no response to any ligand in empty vector (pCDN) transfected or the untransfected parental cell line (data not shown).

The EC_{50} values obtained for human IP-10, murine IP-10, human Mig, murine Mig, I-Tac, murine 6Ckine, and human 6Ckine for the rat receptor were 2.8, 2.2, 21.1, 4.2, 13, >100, and ≫100 nM, and the values for the human receptor were 13.5, 24.1, 43.3, 9.3, 28.9, ≫100, and ≫100 nM, respectively (Fig. 3, A and B). The most notable difference between the rat and human CXCR3 was that the rat receptor responded to high concentrations of murine 6Ckine and human CXCR3 did not.

Cross-desensitization studies were performed using IP-10, I-Tac, and Mig with both rat and human CXCR3-transfected cells. After being maximally stimulated (330 nM), these ligands desensitized a second response to the same ligand. Maximal responses of IP-10 and I-Tac did not respond to a second stimulation with the other two ligands; but Mig was unable to completely desensitize the responses to either IP-10 or I-Tac (data not shown).

^{125}I-IP-10 binds to rat and human CXCR3-transfected HEK 293 cell membranes with high affinity. Competition binding studies were run with the same ligands used in the calcium functional assay. Competition by the ligands for ^{125}I-IP-10 binding, like the calcium responses, was similar for the transiently transfected rat and human CXCR3 (Fig. 4 and Table 1). Both human and murine IP-10 displaced with high affinity: IC_{50} values were 2.3 and 3.3 nM for rat CXCR3 and 2.4 and 4.1 nM for human CXCR3, respectively (Fig. 4, A and B). I-Tac displaces with IC_{50} values for rat and human CXCR3 of 40 and 145 nM, respectively. Murine Mig displaced with IC_{50} values of 480 and 880 nM for rat and human CXCR3, respectively, whereas human Mig did not displace up to 1 μM (Fig. 4, A and B).

Expression of CXCR3 mRNA in Rat Cortex after MCAO. Because a marked induction of IP-10 was demonstrated in the ischemic cortex after permanent MCAO (Wang et al., 1998), we investigated the expression of CXCR3 mRNA in the ischemic cortex tissues using the same animal model. Because the level of CXCR3 mRNA expression in cortex is too

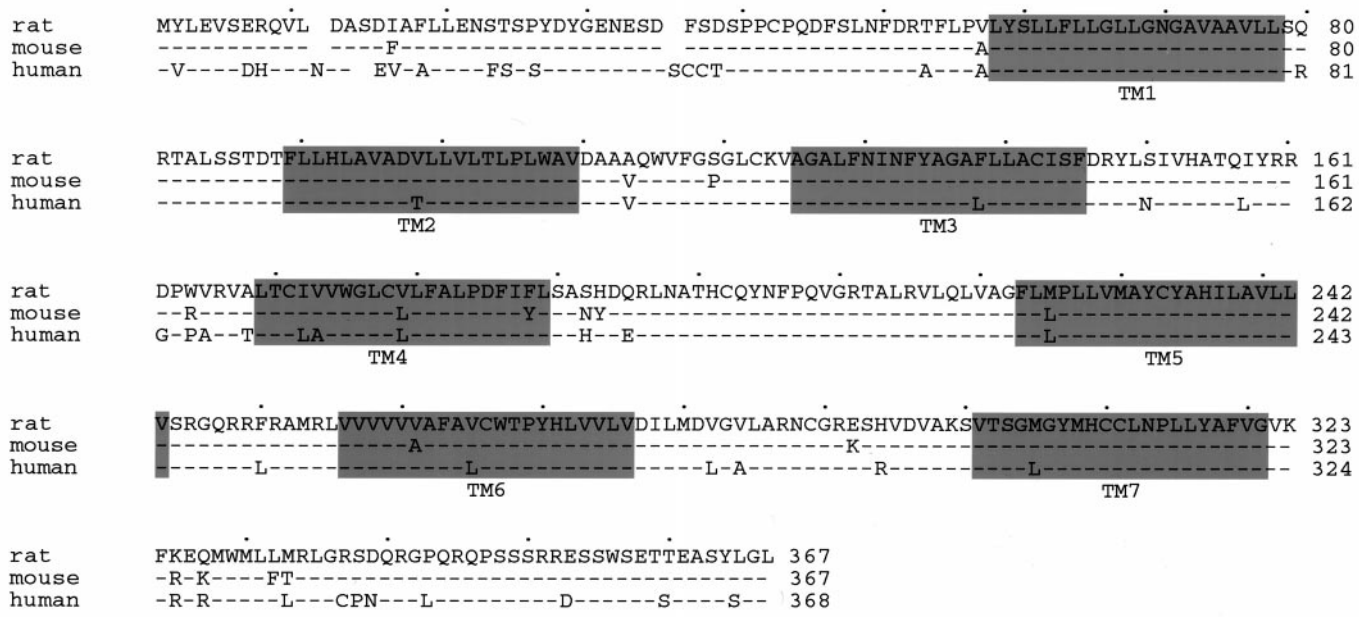

Fig. 2. Alignment of the predicted amino acid sequence for rat CXCR3 with its human and murine arthologs. Mouse (accession no. AF045146, Soto et. al., 1998) and human CXCR3 (accession number X95876; Loetscher et al., 1996) sequences were previously reported. Identical amino acid sequences are shown in a dashed line, and their differences are indicated. There are a total of 367 amino acids in rat and mouse CXCR3 compared with 378 in human CXCR3.

low to be detected by Northern analysis, a recently developed sensitive technique of real-time PCR (Gibson et al., 1996; Heid et al., 1996) was applied. For quantification purposes, we applied the cloned plasmid DNA for rat CXCR3 and rpL32 (a housekeeping control) as the real-time amplification standard to determine the absolute copy number of the template (Wang et al., 2000). A representative real-time PCR for the determination of CXCR3 standard is illustrated in Fig. 5, where the cloned CXCR3 plasmid DNA was in a dilution series of 1.65 to 7.24 log molecule (or 16.4 fg to 64 pg) copies as the template. The amplification data were normalized with the rpL32 gene, which is known to be consistently expressed in ischemic brain tissue (Wang et al., 1998).

Quantitative data ($n = 4$) for CXCR3 mRNA expression in cortical samples from MCAO or sham-operated animals are illustrated in Fig. 6. Low levels of CXCR3 mRNA (<10 copies of mRNA/ng RNA or μg tissue) were observed in sham-operated cortex and in contralateral cortex. The expression of

TABLE 1

Receptor binding affinity and calcium mobilizing potency of chemokines for the rat CXCR3 and human CXCR3

Values presented are the EC_{50} (mean ± S.E.) for chemokine-induced calcium mobilization in HEK 293 rCXCR3 and HEK 293 hCXCR3 cells from six concentration-response curves generated in two independent experiments. Binding values are the IC_{50} values (mean ± S.E.) for three experiments run with duplicate samples for each concentration of chemokine.

Chemokine	EC_{50}		IC_{50}	
	rCXCR3, Ca^{2+}	hCXCR3, Ca^{2+}	rCXCR3, Bind	hCXCR3, Bind
			nM	
hIP-10	2.8 ± 0.3	13.5 ± 3.1	2.3 ± 0.1	2.4 ± 0.4
mIP-10	2.2 ± 0.4	24.1 ± 2.7	3.3 ± 0.6	4.1 ± 0.7
hMig	21.1 ± 1.7	43.3 ± 4.9	>1000	>1000
mMig	4.2 ± 0.2	9.3 ± 2.1	480 ± 47	880 ± 68
I-Tac	13.0 ± 1.9	28.9 ± 3.6	40 ± 11	145 ± 18
m6Ckine	>100	≫100	>1000	>1000
h6Ckine	≫100	≫100	>1000	>1000

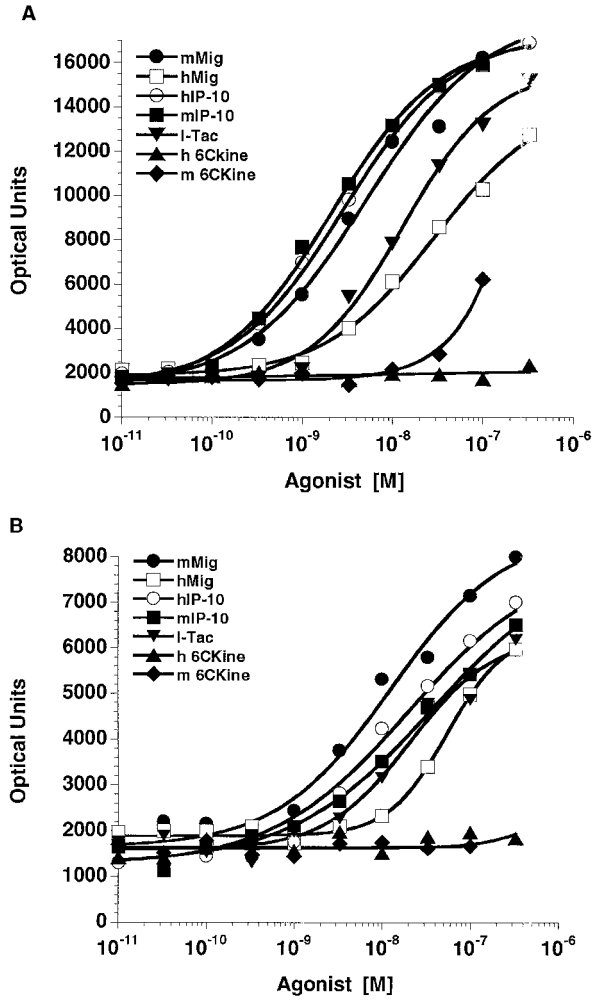

Fig. 3. Concentration-dependent effect of CXCR3 ligands on rat and human CXCR3-transfected HEK 293 cells. HEK 293 cells were transiently cotransfected the rat (A) or human (B) CXCR3 expression vector (or empty control vector pCDN; data not shown) with a Gqi5 expression vector. The tranfectants were assayed for intracellular calcium mobilization in response to various concentration of recombinant human IP-10 (○), murine IP-10 (■), human Mig (□), murine Mig (●), human I-Tac (▼), human 6Ckine (▲), and murine 6Ckine (♦). Values presented are the mean of six concentration-response curves (three run on separate plates in two individual assays).

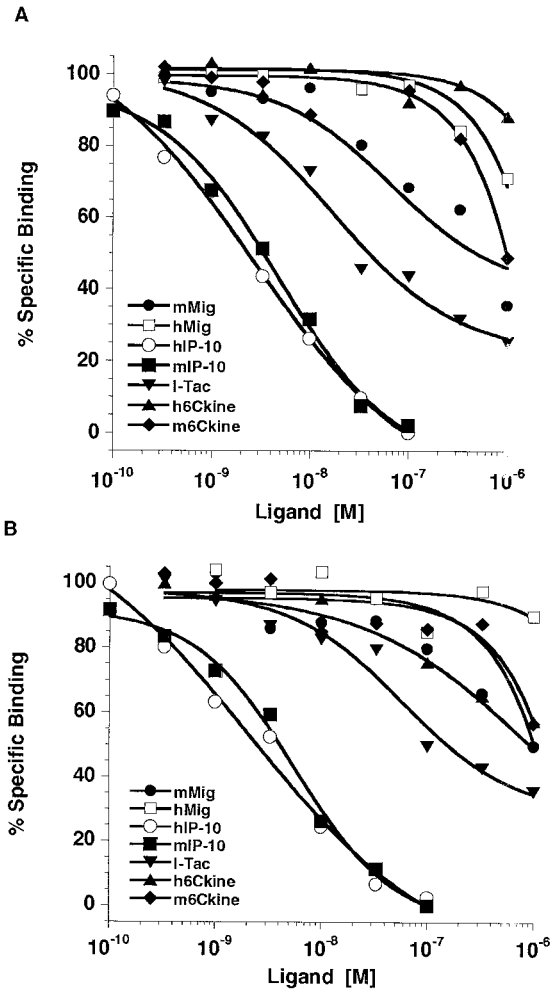

Fig. 4. Concentration-dependent effect of CXCR3 ligands on ^{125}I-IP-10 binding to rat and human CXCR3-transfected HEK 293 cell membranes. HEK 293 cells were transiently cotransfected the rat (A) or human (B) CXCR3 expression vector with a Gqi5 expression vector. The transfectants were used to prepare membranes for the ^{125}I-IP-10 binding assay. The specific ^{125}I-IP-10 binding was displaced with varying concentrations of recombinant human IP-10 (○), murine IP-10 (■), human Mig (□), murine Mig (●), human I-Tac (▼), human 6Ckine (▲), and murine 6Ckine (♦). Data shown are a representative experiment of three run with duplicate samples for each concentration.

CXCR3 mRNA was slightly induced in the ipsilateral (ischemic) cortex at 12 h after MCAO (15 copies per μg tissue) but significantly induced at 2 days (34 $\pm$ 3 copies/μg tissue, or 3-fold increase over sham-operated controls; $P < .05$, $n = 4$) and 5 days (43 $\pm$ 15 copies, or 4-fold increase; $P < .01$) after ischemic injury. The elevated expression of CXCR3 mRNA was sustained for up to 15 days (2-fold increase over controls) after brain ischemia. The expression of the house-keeping gene (rpL32) mRNA in the cortical samples is illustrated in Fig. 6 (bottom).

Binding of ^{125}I-IP-10 to Rat Cortical Membranes after Focal Brain Ischemia. To further explore a potential role of induced expression of IP-10 and CXCR3 in focal stroke, the specific binding of ^{125}I-IP-10 was performed using cortical samples at 6 and 24 h and 2 and 10 days after permanent MCAO. Figure 7 depicts the ^{125}I-IP-10 binding data (0.19–0.24 nM) on cortical membranes at these time points after ischemia or after sham surgery. Although specific IP-10 binding was observed in all of the samples, a significant increase in the ischemic cortical samples was observed from 24 h (1.8-fold increase over controls; $n = 4$, $P < .05$) to 10 days (2.3-fold increase; $n = 4$, $P < .01$).

Discussion

Our present study reports the molecular cloning and characterization of a rat ortholog of the chemokine receptor CXCR3. The deduced amino acid sequence of rat CXCR3 is highly conserved among species, sharing 96 and 87% sequence identity (or 98 and 92% similarity) to the murine and human CXCR3, respectively. The high conservation of CXCR3 sequence may determine the nature of its specific interactions to IP-10, I-Tac, Mig, and murine 6Ckine but not the other chemokines tested. On the other hand, it is interesting to note differences in the affinity of the rat CXCR3 interaction with its ligands compared with human and murine CXCR3. As shown here, human and murine IP-10 and murine Mig are more potent ligands than human Mig for inducing intracellular calcium mobilization and for receptor

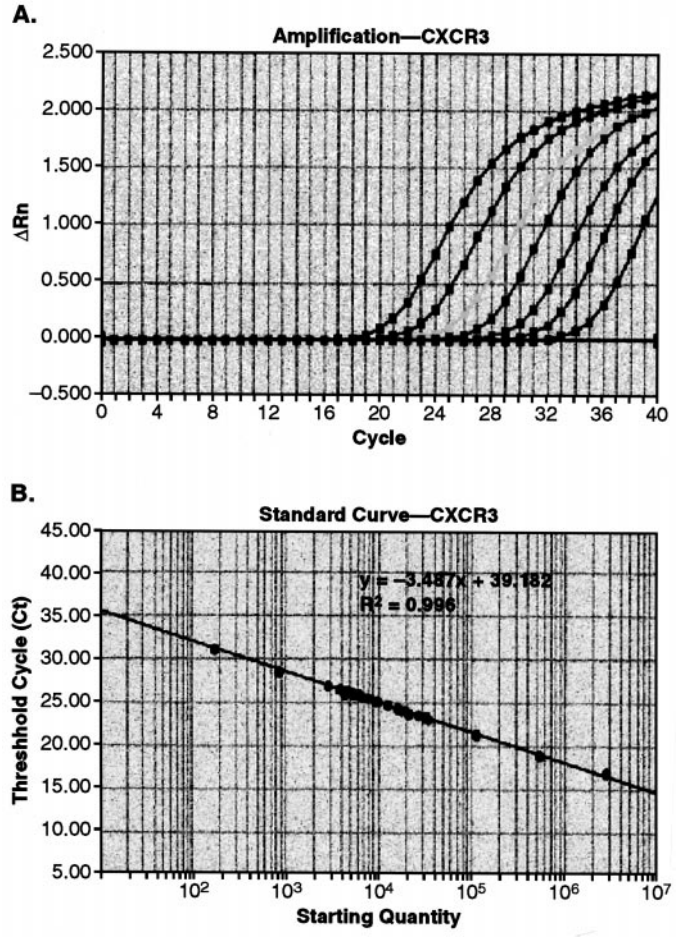

Fig. 5. Real-time PCR of CXCR3 standard. Amplification plots (A) show the accumulation of rat CXCR3 cDNA in real-time PCR detected by the ABI PRISM 7700 Sequence Detector. Five-fold serial dilutions of the plasmid DNA were used as the templates, ranging from 1.65 to 7.24 log molecules. For each dilution, the ΔRn (the ratio for the amount of reporter dye emission to the quenching dye emission) is plotted against the cycle number. Ct is the fractional cycle number at which ΔRn crosses some fixed threshold baseline. B, the input DNA templates (log copy numbers) against the Ct values. All of our testing samples (reversibly transcribed from 400 ng of total cellular RNA) are within the standard range (B, 10^3 to 10^5 molecules).

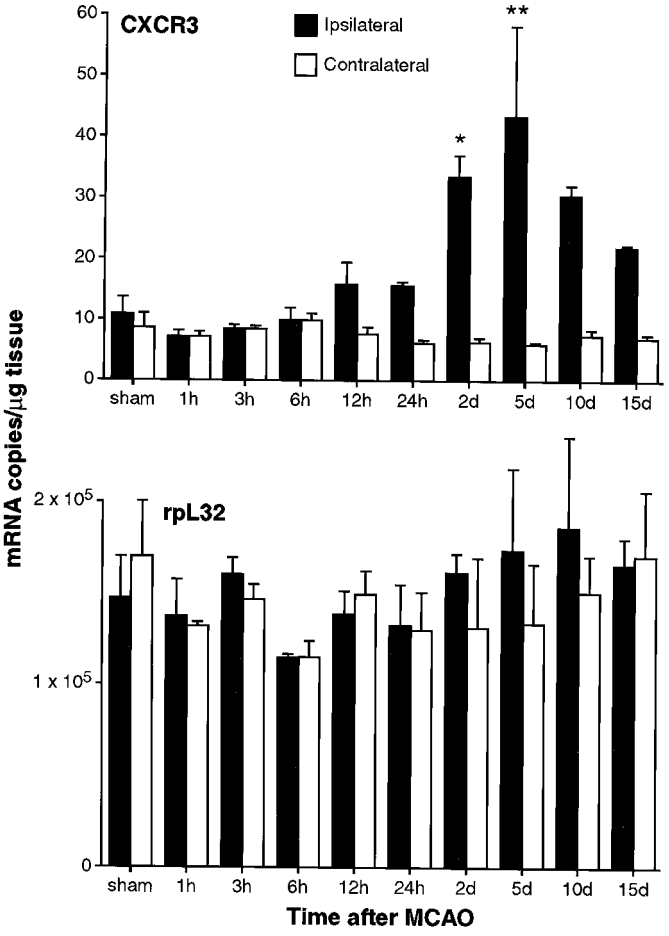

Fig. 6. Temporal expression of CXCR3 in rat cortical samples after permanent MCAO. Total cellular RNA was isolated from ipsilateral and contralateral cortices of rats subjected to sham surgery (5 days) or after 1, 3, 6, 12, and 24 h, and 2, 5, 10, and 15 days of MCAO. RT and PCR were carried out using Taqman quantification. Data were analyzed based on Ct values of each sample and normalized with rpL32. The absolute copy numbers of CXCR3 were determined according to the standard. Quantitative data ($n = 4$) for CXCR3 mRNA expression after MCAO was displayed graphically (top). The rpL32 data ($n = 4$) were determined based on the Ct value and reflect its unnormalized levels in each sample (bottom). *$P < .05$, **$P < .01$, compared with sham-operated animals.

binding in rat CXCR3-transfected HEK 293 cells (Figs. 3 and 4). Human 6Ckine failed to activate this rat receptor, whereas murine 6Ckine showed a modest response at concentrations of 100 nM. In contrast to the rat receptor (i.e., human IP-10 > I-Tac > human Mig > murine 6Ckine), the order of ligand potency to induce intracellular calcium mobilization functional responses in human CXCR3-transfected cells is I-Tac > Mig > IP-10 (Loetscher et al., 1996; Cole et al., 1998). Data presented in this report demonstrate that IP-10, I-Tac, and Mig all have similar potencies at the human CXCR3 and that murine Mig may be the most potent ligand for this receptor. Our data are also consistent with the previous report (Jenh et al., 1999) that no activity was observed in human CXCR3 in response to human or murine 6Ckine. The potencies for the chemokines for the rat and human CXCR3 in this study should be directly comparable because the receptors were transfected identically into the HEK 293 cells and the same sister plate of chemokines were tested against both receptors. The ligand potency order in mouse is also different (i.e., Mig > IP-10 > 6Ckine; Soto et al., 1998). Taken together, these data clearly suggest CXCR3 in different species has some shared but distinct biochemical and pharmacological/biological features for its interaction with ligands.

These species differences in biochemical and pharmacological features of CXCR3 may be explained based on available data. First, sequences are different among species for both ligands and receptors and their cross-species interactions. As described in the present report, only an 87% amino acid sequence identity is observed for human and rat CXCR3. Among the CXCR3 ligands, only the arthologs for IP-10 have been reported in all three species. Rat IP-10 is known to share 97 and 94% amino acid sequence similarity to murine and human IP-10, respectively (Wang et al., 1996). Therefore, the high affinity of human and murine IP-10 for rat and human CXCR3 may reflect the highly conserved sequences in this ligand-receptor pair across the two species. Although it is still too early to conclude that the low affinity for murine 6Ckine exhibited by rat CXCR3 is due to a cross-species difference, previous reports show that a specific interaction can be demonstrated between murine 6Ckine and mouse CXCR3 (Soto et al., 1998) but not between human 6Ckine and human/mouse CXCR3 (Jenh et al., 1999). It is not clear why human Mig induces a strong calcium response (although with lower potency than IP-10 and I-Tac) but demonstrates such low affinity to displace the ^{125}I-IP-10 binding to both rat and human CXCR3. Additional studies are in progress with labeled I-Tac and human Mig to better understand this observation. Second, the cell lines used for the transfection and functional assays may contribute to the discrepant results. Loetscher et al. (1996) transfected human CXCR3 in mouse preB cells (300-19), human promyelocytic cells (GM-1), and human acute T cell leukemia cells (Jurkat), and the transfectants were used for functional assays. HEK 293 cells were used for rat (the present work), mouse (Soto et al., 1998), and human (Cole et al., 1998) CXCR3 transfection. However, because the data for human CXCR3 transfactants in various cell lines are comparable (Loetscher et al., 1996; Cole et al., 1998), this cell line possibility could be excluded. In addition, other factors, such as the source of chemokines and the levels of G-proteins expressed in the cells, may contribute to the discrepant results. To this end, we obtained chemokines (especially the CXCR3 ligands, IP-10, Mig, I-Tac, and 6Ckine) from various manufacturers and tested/confirmed their biological activities. Moreover, because many receptors are not functionally coupled to Ca^{2+}/IP_3 in HEK 293 cells in the absence of a "promiscuous" G protein or a G protein chimera (Offermanns and Simon, 1995), we transfected CXCR3 into the cells in the absence or presence of Gα15, Gα16, Gqi5, Gqo5, and Gqs5 expression vectors and determined that the best calcium mobilization response was in the presence of Gqi5 (Wilkie et al., 1991; Schroeder and Neagle, 1996). Therefore, our present study was performed by cotransfecting CXCR3 with Gqi5 expression vectors in HEK 293 cells.

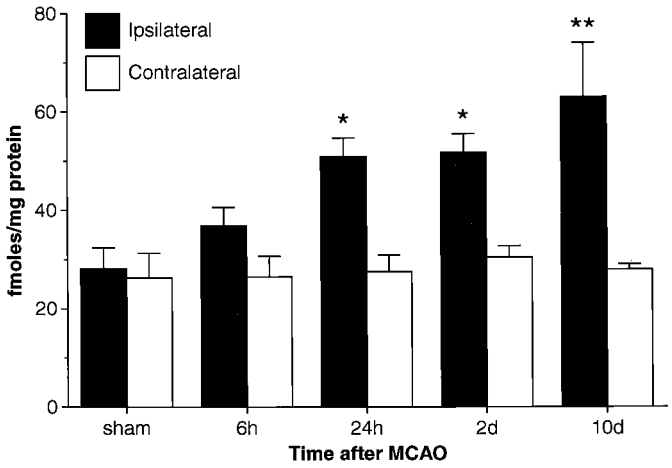

Fig. 7. The binding assay of ^{125}I-IP-10 to its specific receptor in ischemic and nonischemic cortex. Cellular membranes were prepared from ischemic and nonischemic brain tissues from 6 and 24 h and 2 and 10 days after MCAO or from sham-operated animals (2 days). The specific binding for ^{125}I-IP-10 was determined by subtracting the background signals.

The induction profile of CXCR3 mRNA after focal stroke in rat is interesting. The message began to increase as early as 12 h but did not reach significance until 2 and 5 days after MCAO. In contrast, IP-10 mRNA was significantly induced as early as 3 h and reached a peak expression at 6 h after MCAO; the second wave induction of IP-10 mRNA was observed much later at 10 and 15 days (Wang et al., 1998). Clearly, the mRNA expression profiles of the ligand and receptor are not the same. In addition, the specific binding to CXCR3 (to a significant level at 24 h after stroke) preceded the significantly elevated level of the mRNA (2 days). It is possible that the high basal level of CXCR3 mRNA expression in the brain (Fig. 6) may be responsible to the early increase in the specific CXCR3 binding activity and thus may react to the first wave of IP-10 induction after ischemic insult before the induced expression of the receptor, whereas the second wave of IP-10 induction correlated with the increased expression of CXCR3 and enhanced binding activity after ischemic brain injury.

The cellular sources of CXCR3 expression in normal and ischemic brain have not been investigated. Other studies indicated that activated T cells are likely the preferential cellular source for CXCR3 expression (Loetscher et al., 1996; Qin et al., 1998). Immunohistochemical studies of various inflammatory tissues (including rheumatoid arthritis, chronic vaginitis, and ulcerative colitis) revealed that virtually all T cells within the lesions, particularly in perivascular regions, expressed CXCR3 (Qin et al., 1998). Similar CXCR3

expression is localized to T cells of perivascular inflammatory infiltrate in the central nervous system of patients with multiple sclerosis (Sorensen et al., 1999). In addition to massive infiltration of leukocytes in the ischemic brain (Kochanek and Hallenbeck, 1992; Clark et al., 1993; Garcia et al., 1994), T cells were also detected in ischemic brain lesions by using T cell-specific markers (Schroeter et al., 1994; Jander et al., 1995). $CD5^+$ T cells were detected on the pial surface at day 1 and with increasing numbers at the edges of infarcts at days 3 and 7 after brain MCAO in rats, along with a significant number of $CD5^-/CD8^+$ natural killer cells in the lesion (Schroeter et al., 1994). A similar profile of T cell infiltration into the ischemic lesion was reported for the photochemically induced focal ischemia in rat (Jander et al., 1995). These data suggest that the infiltrated/accumulated T cells and natural killer cells in the ischemic brain tissue may be the cellular sources contributing to the induced expression of CXCR3 after stroke. Therefore, an antagonist of CXCR3 may decrease the extent of inflammatory cell infiltration/accumulation after ischemic injury.

It is also interesting to note that the elevated expression of CXCR3 mRNA correlates with the accumulation of macrophages in the ischemic lesion in the same animal model (Clark et al., 1993; Barone et al., 1995), suggesting that ischemia-induced expression of IP-10 and CXCR3 may contribute to leukocyte infiltration/accumulation after brain injury, as has been demonstrated previously in vitro (Taub et al., 1993). In addition, the similar profiles of CXCR3 expression/binding activity and gliosis, as well as the chemotactic property of IP-10 on astroglia (Wang et al., 1998), could not exclude a possible role of IP-10/CXCR3 in gliosis after ischemic brain injury.

In conclusion, the present study describes the cloning and characterization of the rat ortholog of CXCR3 and demonstrates its distinct biochemical, pharmacological, and biological properties. The temporal expression of CXCR3 mRNA, along with its specific ligand IP-10, and the increased IP-10 binding activity that occurs after stroke suggest that this ligand and receptor are actively involved in the inflammatory reaction, and possibly gliosis, after ischemic brain injury and that CXCR3 antagonists may therefore provide a novel therapeutic opportunity in ischemic stroke.

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References

Aiyar N, Baker E, Wu HL, Nambi P, Edwards RM, Trill JJ, Ellis C and Bergsma DJ (1994) Human AT1 receptor is a single copy gene: Characterization in a stable cell line. *Mol Cell Biochem* **131:**75–86.

Baggiolini M, Dewald B and Moser B (1994) Interleukin-8 and related chemotactic cytokines–CXC and CC chemokines. *Adv Immunol* **55:**97–179.

Barone FC, Hillegass LM, Tzimas MN, Schmidt DB, Foley JJ, White RF, Price WJ, Feuerstein GZ, Clark RK, Griswold DE and Sarau HM (1995) Time-related changes in myeloperoxidase activity and leukotriene B_4 receptor binding reflect leukocyte influx in cerebral focal stroke. *Mol Chem Neuropathol* **24:**13–30.

Barone FC, Price WJ, White RF, Willette RN and Feuerstein GZ (1992) Genetic hypertension and increased susceptibility to cerebral ischemia. *Neurosci Biobehav Rev* **16:**219–233.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:**156–159.

Clark RK, Lee EV, Fish CJ, White RF, Price WJ, Jonak ZL, Feuerstein GZ and Barone FC (1993) Development of tissue damage, inflammation and resolution following stroke: An immunohistochemical and quantitative planimetric study. *Brain Res Bull* **31:**565–572.

Cole KE, Strick CA, Paradis TJ, Ogborne KT and Loetscher M (1998) Interferon-inducible T cell alpha chemoattractant (I-TAC): A novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* **187:**2009–2021.

Coward P, Chan SDH, Wada HG, Humphries GM and Conklin BR (1999) Chimeric G proteins allow a high-throughput signalling assay of G_i-coupled receptors. *Anal Biochem* **270:**242–248.

Feuerstein GZ, Wang XK and Barone FC (1998) Inflammatory mediators and brain injury: The role of cytokines and chemokines in stroke and CNS diseases, in *Cerebrovascular Diseases* (Ginsberg MD and Bogousslavsky J eds) pp 507–531, Blackwell Science, Cambridge, MA.

Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S and del Zoppo GJ (1994) Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). *Am J Pathol* **144:**188–199.

Gibson UE, Heid CA and Williams PM (1996) A novel method for real time quantitative RT-PCR. *Genome Res* **6:**995–1001.

Heid CA, Stevens J, Livak KJ and Williams PM (1996) Real time quantitative PCR. *Genome Res* **6:**986–994.

Jander S, Kraemer M, Schroeter M, Witte OW and Stoll G (1995) Lymphocytic infiltration and expression of intracellular adhesion molecule-1 in photochemically induced ischemia in rat cortex. *J Cereb Blood Flow Metab* **15:**42–51.

Jenh CH, Cox MA, Kaminski H, Zhang M, Byrnes H, Fine J, Lundell D, Chou CC, Narula SK and Zavodny PJ (1999) Species specificity of the CC chemokine 6Ckine signaling through the CXC chemokine receptor CXCR3: Human 6Ckine is not a ligand for the human or mouse CXCR3 receptors. *J Immunol* **162:**3765–3769.

Kochanek PM and Hallenbeck JM (1992) Polymorphonuclear leukocytes and monocytes/macrophages in the pathologenesis of cerebral ischemia and stroke. *Stroke* **23:**1367–1379.

Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggioloni M and Moser B (1996) Chemokine receptor specific for IP-10 and Mig: Structure, function, and expression in activated T-lymphocytes. *J Exp Med* **184:**963–969.

Luster AD (1998) Chemokines-chemotactic cytokines that mediate inflammation. *N Engl J Med* **338:**436–445.

Miller MD and Krangel MS (1992) Biology and biochemistry of the chemokines: A family of chemotactic and inflammatory cytokines. *Crit Rev Immunol* **12:**17–46.

Offermanns S and Simon MI (1995) $G\alpha_{15}$ and $G\alpha_{16}$ couple a wide variety of receptors to phospholipase C. *J Biol Chem* **270:**15175–15180.

Premack BA and Schall TJ (1996) Chemokine receptors: Gateways to inflammation and infection. *Nat Med* **2:**1174–1178.

Qin SX, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B and Mackay CR (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* **101:**746–754.

Rajchel A, Chan YL and Wool IG (1988) The primary structure of rat ribosomal protein L32. *Nucleic Acids Res* **16:**2347.

Ransohoff RM and Tani M (1998) Do chemokines mediate leukocyte recruitment in post-traumatic CNS inflammation? *Trends Neurosci* **21:**154–159.

Rollins BJ (1997) Chemokines. *Blood* **90:**909–928.

Schroeder KS and Neagle BD (1996) FLIPR: A new instrument for accurate, high throughput optical screening. *J Biomol Screening* **1:**75–80.

Schroeter M, Jander S, Witte OW and Stoll G (1994) Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. *J Neuroimmunol* **55:**195–203.

Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin SX, Rottman J, Sellebjerg F, Strieter RM, Frederiksen JL and Ransohoff RM (1999) Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* **103:**807–815.

Soto H, Wang W, Strieter RM, Copeland NG, Gilbert DJ, Jenkins NA, Hedrick J and Zlotnik A (1998) The CC chemokine 6ckine binds the CXC chemokine receptor CXCR3. *Proc Natl Acad Sci USA* **95:**8205–8210.

Taub DD, Lloyd RA, Conlon K, Wang JM, Ortaldo RJ, Harada A, Matsushima K, Kelvin JD and Oppenheim JJ (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* **177:**1809–1814.

Wang XK, Ellison JA, Siren AL, Lysko PG, Yue TL, Barone FC, Shatzman A and Feuerstein GZ (1998) Prolonged expression of interferon-inducible protein-10 in ischemic cortex after permanent occlusion of the middle cerebral artery in rat. *J Neurochem* **71:**1194–1204.

Wang XK, Li X, Currie RW, Willette RN, Barone FC and Feuerstein GZ (2000) Application of real-time polymerase chain reaction to quantitate induced expression of interleukin-1β mRNA in ischemic brain tolerance. *J Neurosci Res* **59:**238–246.

Wang XK, Yue TL, Ohlstein EH, Sung CP and Feuerstein GZ (1996) Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response. *J Biol Chem* **271:**24286–24293.

Wilkie TM, Scherle PA, Strathmann MP, Slepak VZ and Simon MI (1991) Characterization of G-protein alpha subunits in the Gq class: Expression in murine tissues and in stromal and hematopoietic lines. *Proc Natl Acad Sci USA* **88:**10049–10053.

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