Astrocyte Differentiation Selectively Upregulates CCL2/Monocyte Chemoattractant Protein-1 in Cultured Human Brain-Derived Progenitor Cells

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ABSTRACT
Chemokines (chemoattractant cytokines) and their receptors are present in the brain and may play roles in both neurodevelopment and neuropathology. Increased brain levels of monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, are found in patients with human immunodeficiency virus type 1 (HIV-1)-associated dementia and other acute and chronic neurologic diseases. Although the function of CCL2 in the brain is unclear, it is believed that upregulation of this chemokine during neuropathologic or neuroinflammatory conditions leads to recruitment of activated monocytes into the brain, where they differentiate into macrophages producing neurotoxic and inflammatory molecules. We recently showed that human fetal brain-derived progenitor cells are susceptible to HIV-1 and JC virus infection, and that differentiation toward an astrocyte phenotype increased virus production from these cells. In the current study, we found that in the absence of infection, progenitors produced moderate levels of CCL2 (5.6 ng per million cells). Astrocyte differentiation over 3 weeks increased CCL2 protein levels 30-fold in a biphasic manner, whereas neuronal differentiation decreased production 20-fold. Electromobility shift assays (EMSAs) demonstrated increased nuclear NF-κB levels within 2 h of initiating astrocyte differentiation, and inhibitors of NF-κB activation partially blocked the CCL2 increase in differentiating astrocytes. Transfection of progenitors with mutated CCL2 promoter/CAT reporter constructs showed that the distal promoter region, containing NF-κB and NF-I binding sites, is important for differentiation-induced CCL2 upregulation. Together these results suggest that the transcription factor NF-κB, and possibly NF-I, contribute to the upregulation of CCL2 chemokine production during the differentiation of human progenitor cells toward an astrocyte phenotype.

INTRODUCTION

Many acute and chronic neurological disorders, including progressive neurodegenerative diseases and viral encephalopathies, involve activation of glial cells and subsequent neuron loss. Activated microglia and/or reactive astrocytes may contribute to neuronal survival by producing trophic factors, removing cellular debris, and counteracting the neurotoxic effects of excess reactive oxygen species and glutamate. However, with chronic stimulation, such as a persistent viral infection or accumulation of protein aggregates, these same cells may exacerbate neuropathology by contributing to the chronic inflammatory response in the brain.

Increased brain levels of chemokines, chemotactic cytokines that activate and recruit cells to a site of injury, are associated with the pathogenesis of inflammatory neurodegenerative and autoimmune diseases, including multiple sclerosis (Ransohoff et al., 1996; Ransohoff and Tani, 1998), Alzheimer's disease (Johnstone et al., 1999; Lue et al., 2001; Sun et al., 2003), and stroke (Minami and Satoh, 2003; Campbell, 2004), as well as viral and bacterial infections of the central nervous system (Kielian, 2004), including human immunodeficiency virus (HIV) (Cinque et al., 1998; Conant et al., 1998; Kelder et al., 1998). More recently, some chemokines and their receptors have been identified as mediators of communication in both the developing brain and adult central nervous system. For example, stromal cell-derived factor-1 (SDF-1, or CXCL12) and fractalkine (CX3CL1) may be involved in the guidance of neuronal, glial, or progenitor cell migration, as well as modulation of Ca^{2+} mobilization and transport (Asensio and Campbell, 1999; Bajetto et al., 2001; Lazarini et al., 2003). However, the extent to which brain-derived chemokines and their receptors form a communication network under nonpathologic conditions remains unclear.

Within the context of neuropathology, much attention has been paid to MCP-1, now termed CCL2. This member of the CC or β class of chemokines is produced in

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response to tissue injury, and binds with nanomolar affinity to the chemokine receptor CCR2 expressed on activated monocytes and other leukocytes, as well as endothelial cells. The target cells respond with integrin activation, cytoskeletal reorganization, and directional migration, culminating in extravasation and accumulation near regions of high chemokine concentrations in tissue (Huang et al., 2000). Increased cerebrospinal fluid levels of CCL2 are associated with recruitment of monocytes into the brain, where these cells differentiate into activated macrophages and secrete numerous inflammatory and neurotoxic molecules. Although constitutive production of CCL2 in the brain has not been demonstrated, its receptor, CCR2, has been found to be constitutively expressed in human and rat brain (van der Meer et al., 2000; Banisadr et al., 2002), as well as on human fetal astrocytes (Andjelkovic et al., 2002), suggesting a physiologic role in the absence of neuropathology.

In this laboratory, we developed a human brain-derived multipotential cell culture model system, using serum-free media containing epidermal growth factor (EGF) and basic fibrinogen growth factor (bFGF), in which nestin$^+$ progenitor cells are maintained as monolayers of undifferentiated, actively proliferating cells. These cells can be differentiated into glial fibrillary acidic protein (GFAP)$^+$ astrocytes by changing the growth conditions to a serum-containing media; alternatively they can be differentiated into a β-III tubulin$^+$ neuronal population by changing to serum-free media containing BDNF and platelet-derived growth factor (PDGF) (Messam et al., 2003). These cells were used previously to determine that the level of transcription factor NF-1X in the different cell phenotypes determined the susceptibility to JCV infection (Messam et al., 2003). In addition, we showed that neural progenitor cells are permissive for HIV infection, and that differentiation of infected cells from a nestin$^+$, GFAP$^-$ neural progenitor state to a GFAP$^+$ astrocytic, but not neuronal phenotype, is associated with increased production of virus (Lawrence et al., 2004). This culture system provides an excellent model system to examine differences in molecular factors between different stages of neural cell development.

In the current study, we used the same cell system to examine differences in CCL2 production in neural progenitor cells, astrocytes, and neurons, and to address the mechanisms of CCL2 regulation these cells. We compared the kinetics of CCL2 induction through differentiation or cytokine stimulation, transfected cells with promoter-reporter constructs to determine which region of the promoter is critical for CCL2 upregulation, and investigated the role of NF-κB in CCL2 upregulation during astrocyte differentiation.

MATERIALS AND METHODS

Human Neural Progenitor Cell Culture

Human brain-derived progenitor cells from 8-week fetal brain samples were grown as monolayers on poly-D-lysine in serum-free neurobasal media supplemented with 0.5% bovine serum albumin (BSA; Sigma, St. Louis, MO), 2 mM glutamine, N2 (Invitrogen, Carlsbad, CA), Neural Survival Factor (Clonetics, Walkersville, MD), EGF (20 ng/ml) and bFGF (25 ng/ml) (Sigma), as described previously (Messam et al., 2003). Progenitor cell cultures grown at 10–40% confluence were at least 98% positive for nestin staining and did not express GFAP. Astrocyte differentiation was initiated by changing to Eagle's MEM supplemented with 10% fetal calf serum and 2 mM glutamine; after 3 weeks the cells were 100% immunoreactive for GFAP expression and showed reduced nestin expression. Neuronal differentiation (MAP-2$^+$, βIII-tubulin$^+$) involved changing the growth factors in the progenitor media to brain-derived neurotrophic factor (BDNF, 10 ng/ml) and platelet-derived growth factor (PDGF)-A/B (10 ng/ml) for 3 weeks. BDNF, PDGF, and the NFκB blockers caffeic acid phenethyl ester (CAPE) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma. Human recombinant tumor necrosis factor-α (TNF-α) was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant human bone morphogenetic protein (BMP)-2 and recombinant human ciliary neurotropic factor (CNTF) were from Research Diagnostics.

ELISA and RNase Protection Assay

Cell-free supernatants were collected from progenitors, differentiating astrocytes, or differentiating neurons and stored at −20°C until use. Cytokine and chemokine protein levels were determined by Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), according to manufacturer's protocol. In some experiments, the concentrations were normalized to cell number, determined by trypan blue exclusion. Ribonuclease protection assay (RPA) was used to quantitate mRNA expression levels of chemokines in progenitor and differentiating astrocyte populations. Total RNA was isolated from each culture using Qiagen RNAeasy mini kits following the manufacturer's recommended protocol (Qiagen, Valencia, CA). RPA was performed using the RiboQuant RNase Protection Assay hCK5 kit (BD Biosciences, San Diego, CA), which contained cDNA templates for chemokines lymphotactin (Ltn, or XCL1), RANTES (CCL5), IP-10 (CXCL10), MIP-1β (CCL4), MIP-1α (CCL3), MCP-1 (CCL2), IL-8 (CXCL8), and I-309 (CCL1). Templates for housekeeping gene products L32 and GAPDH were included as internal controls in both kits. Labeled antisense RNA probes were synthesized from these cDNA templates, using [α-^{32}P] UTP (Perkin-Elmer Sciences, Boston, MA) in an in vitro transcription reaction. Probes were then hybridized with 10 μg of total RNA from each condition. Probe synthesis, hybridization, and digestion with proteinase K and RNase were performed according to the manufacturer's protocol with modifications described previously (Seth et al., 2003). Samples were resolved on 5% denaturing acrylamide gels dried under vacuum at 80°C and exposed to autoradiographic film at −80°C for 6–24 h prior to devel-

opment. Blots were scanned and quantitated using NIH Image software (Scion Corporation, Frederick, MD) normalizing to the intensity of GAPDH in the same reaction.

Immunofluorescence

For colocalization of astrocyte and progenitor markers GFAP and nestin, respectively, cells were fixed with 50% methanol/50% acetone, blocked with 1% BSA, and permeabilized with 0.02% Triton X-100. Cells were then incubated with monoclonal mouse anti-human GFAP (1:200, Chemicon MAB360) and rabbit polyclonal antiserum against human nestin (1:200) (Messam et al., 2000) for 2 h in an humidified chamber. After washing, the secondary antibodies were added: FITC-conjugated goat anti-mouse IgG (1:100, Jackson ImmunoResearch, West Grove, PA; 15-095-146) and rhodamine Red-X-conjugated donkey anti-rabbit IgG (1:100, Jackson ImmunoResearch; 111-295-144). Control experiments confirmed a lack of cross-reactivity using this simultaneous staining protocol. Samples were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) to visualize nuclei. Fluorescent stained cultures were visualized using a Zeiss Axiovert 200M microscope with a 20× Plan Neofluar DIC II objective.

Nuclear Extract Preparation

Cells (2–5 × 10^6 cells per 162-mm^2 flask) were washed with cold Tris-buffered saline (TBS), scraped, and pelleted by centrifugation at 1,500*g* for 5 min at 4°C. The pellets were resuspended in 1 ml cold TBS, transferred into an Eppendorf tube and centrifuged again for 15 s. The cell pellets were resuspended in 400 μl of ice-cold buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol [DTT]) containing Protease Inhibitor Cocktail (Roche, Cat. 1 836 153) and incubated for 15 min to swell the cells. 25 μl of 10% Nonidet P-40 (NP-40) was added to the mixture followed by a brief vortex. The homogenates were centrifuged at 13,000 rpm, 4°C for 30 s, and supernatant fraction containing cytosolic proteins were collected and stored at −80°C. The nuclear pellets were resuspended in 50 μl ice-cold buffer C (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing Protease Inhibitor Cocktail. Samples were placed on a shaking platform for 15 min at 4°C, then centrifuged at 13,000 rpm for 5 min at 4°C. Supernatant fractions containing nuclear proteins were stored at −80°C. Protein concentrations were determined by the method of Bradford (1976).

Electrophoretic Mobility Shift Assay

Oligonucleotides with the nucleotide sequence of the intact NF-κB binding site (5′-TAC AAG GGA CTT TCC-3′) or a mutated version (5′-TAC AAG TTA CTT TAC-3′, mutated NF-κB residues are shown in boldface type) were synthesized by Life Technologies (Invitrogen). The oligonucleotides for both DNA strands of the authentic or mutated binding sites were annealed to form double-stranded structures. The annealed wildtype NF-κB probe was labeled with [γ-^{32}P] ATP for 2 h at 37°C, and purified by centrifugation through a Microspin G-25 column (Amersham Pharmacia Biotech, Cat. 27-5325-01) at 735*g* for 2 min. The labeled probe (>100,000 cpm; 0.2 pmol/μl) was incubated on ice for 30 min with 0.2 vol of 5× Reaction Buffer (50 mM Tris-HCl [pH 7.9], 250 mM NaCl, 25 mM $MgCl_2$, 2.5 mM EDTA, and 5 mM DTT), 10% glycerol, 0.1 μg/μl poly (dI-dC), and 7–10 μg of nuclear extract from either progenitor cells or differentiating astrocytes in the presence or absence of a 250-fold excess of either unlabeled wild-type oligonucleotide or unlabeled mutant oligonucleotide. The reaction mixture was electrophoresed on a 6% polyacrylamide-Tris-glycine gel either for 3 h at room temperature, 300 V, or overnight at 4°C, 125 V, with 1× TGE buffer (25 mM Tris, 190 mM glycine, and 1 mM EDTA). The gel was subsequently dried, and samples were visualized by autoradiography with Kodak BioMAX-AR/MS film.

Transient Transfection of Plasmid DNA Constructs

For CCL2 promoter transfection experiments, we used the expression plasmid pMCP-1(3.5)CAT in which the 3.5-kb 5′ untranslated region of the CCL2 gene was linked to chloramphenicol acetyltransferase (CAT) reporter gene (Li and Kolattukudy, 1994), as well as deletion mutants containing only the proximal CCL2 promoter: pMCP-1 (−349)CAT, pMCP-1(−292)CAT, pMCP-1(−213)CAT, and pMCP-1(−147)CAT (Zhou et al., 1998). One day prior to transfection, progenitor, astrocyte or neuron cultures were seeded at 70–80% confluence in poly-D-lysine-coated 12-well plates (300,000 cells per well). Cells were treated for 5–6 h at 37°C with 2 μg pNL4-3 DNA and 1.5 μg Lipofectamine 2000 (Invitrogen) per well, diluted in a final volume of 1 ml OptiMem media (Invitrogen) per well, following the manufacturer's recommended protocol. Immediately following transfection, media was removed and replaced with 2 ml of fresh media. Control cultures in each experiment were transfected with pCAT-Basic. Using a construct for GFP expression under the control of a CMV promoter, transfection efficiency was approximately 40% for both progenitor and astrocyte populations, consistent with our previously reported findings (Lawrence et al., 2004), with less than 10% cell death compared with untransfected controls. Cell lysates were obtained and assayed using a CAT ELISA kit (Roche, Indianapolis, IN), according to the manufacturer's recommended protocol; results were adjusted based on the protein concentration in the lysates, measured by the Non-Interfering Protein Assay kit (Geno Technology, St. Louis, MO).

RESULTS

CCL2 Expression in Human Neural Progenitor Cells, Astrocytes, and Neurons

In five preliminary experiments we found consistently large differences in extracellular CCL2 production between human brain-derived progenitor cells, progenitor-derived astrocytes (20- to 30-fold greater), and progenitor-derived neurons (near the threshold of detection). Inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ)-stimulated CCL2 production from all three cell types. To characterize the phenotypic differences in chemokine production from these cell phenotypes, we collected supernatant samples as well as total RNA from cells maintained as progenitors or shifted to astrocyte differentiating media, over a period of 3 weeks, the time we have established as sufficient for differentiation (Messam et al., 2003). For each cell type, media was changed in all wells 24 h before collection, and cell counts were performed on representative wells at the time of collection, in order to control for differences in cell density. Because of the requirement of standardizing the cell culture density to maintain the appropriate phenotypic characteristics, the cells were replated as needed to avoid overcrowding. To avoid any transient replating effects on CCL2 production, samples were not collected less than 72 h after replating.

Supernatant samples were analyzed by ELISA for the levels of pro-inflammatory chemokines CCL2 (MCP-1), CCL7 (MCP-3), CCL3 (MIP-1α), CCL5 (RANTES), and CXCL-10 (IP-10), as well as cytokines TNF-α, IFN-γ, and IL-1β, which are known to induce chemokine production. Of these, CCL2 was the only product detected. As shown in Figure 1, levels of CCL2 were low but physiologically significant in progenitors, an average of 5.4 ng/10^6 cells/day. After 3 days of differentiation toward a neuronal phenotype, CCL2 levels decreased by 66%, and after 14 days, neuronal CCL2 production was consistently near 0.3 ng/10^6 cells. This result was intriguing given that PDGF, a component of the neuronal media, is known to upregulate CCL2 production. In contrast, there was a 10-fold increase in CCL2 production within 24 h of starting astrocyte differentiation. After a plateau during the first week, CCL2 levels increased further during the second and third weeks of astrocyte differentiation, to levels approximately 30 times higher than progenitor cells grown in parallel. This biphasic pattern of increased CCL2 production was consistent over several experiments.

To determine whether the increase in CCL2 occurred at the transcriptional level, similar experiments were performed on a larger scale and total RNA was isolated from either progenitors or differentiating astrocytes at various time points during differentiation. After the indicated number of days, total RNA was isolated and 10 μg samples were used to measure the expression levels of eight chemokines using the CK5 Riboquant RNase protection assay system (BD Biosciences). Only CCL2 was detected in progenitors and at any point during differentiation, including in mature astrocytes differentiated for 21 days (Fig. 2A). Quantification of band density, normalized to GAPDH density (Fig. 2B), showed a nearly 4-fold increase in CCL2 transcription after 1 day of astrocyte differentiation relative to progenitors. Consistent with ELISA results, CCL2 mRNA levels in 3-day and 5-day differentiating astrocytes were decreased to less than 2-fold above that in progenitors, but increased again at 8 days and beyond to between 4-fold and 5-fold over progenitor levels. The additional bands that appeared in the control RNA and differentiating astrocyte lanes most likely reflect RNA degradation products. Among these, two particularly strong bands migrated near the same location as would be expected for CXCL8 and CCL1; however, we did not detect protein expression by ELISA.

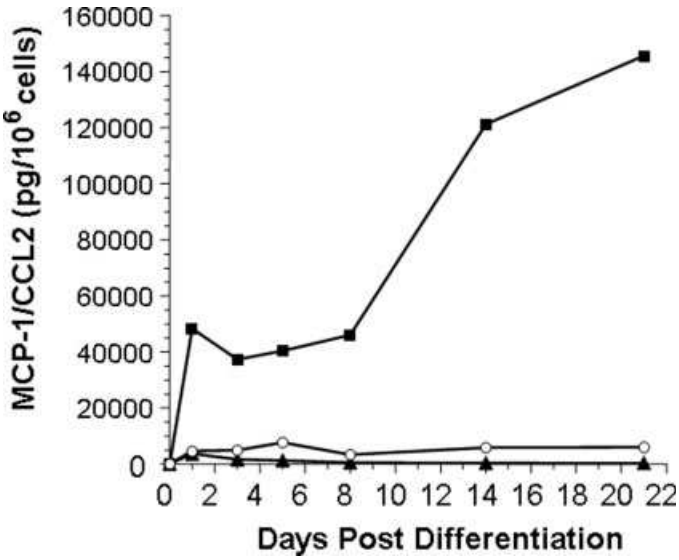
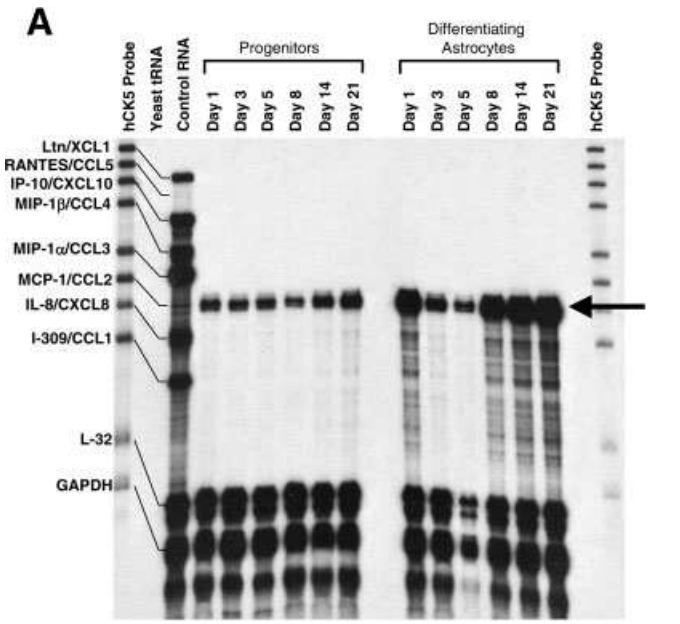


Fig. 1. Changes in CCL2 production during progenitor differentiation to astrocytes and neurons. Progenitor cells were seeded in poly-D-lysine coated 12-well plates (100K cells/well). On day 0, media in all wells was changed either to fresh progenitor media (bFGF+EGF) (○), astrocyte media (EMEM+10% serum) (■), or neuronal media (BDNF+CNTF) (▲). Supernatant samples were collected at various times post-differentiation, up to day 21, and immediately frozen and stored at −20°C. Data shown are means of duplicate CCL2 protein levels ± standard deviation, determined by ELISA, and representative of three experiments.

Although CCL2 was the only chemokine detected in progenitors, differentiating astrocytes, or mature astrocytes, we found that these populations had the capacity to produce other chemokines. TNF-α stimulation of both progenitors and astrocytes further increased CCL2 production, and also induced production of CCL5 and CXCL8 (data not shown). Table 1 shows that TNF-α (10 ng/ml) increased progenitor levels of CCL2 30-fold within 4 h, and by 24 h, TNF-α-stimulated progenitors had produced 50-fold more CCL2 than unstimulated cells. Although the stimulation of CCL2 was 5-fold to 10-fold greater with TNF-α stimulation compared with differentiation, in both cases the increased production was detected within 4 h, and the rate of accumulation was linear (data not shown). Because we found very low



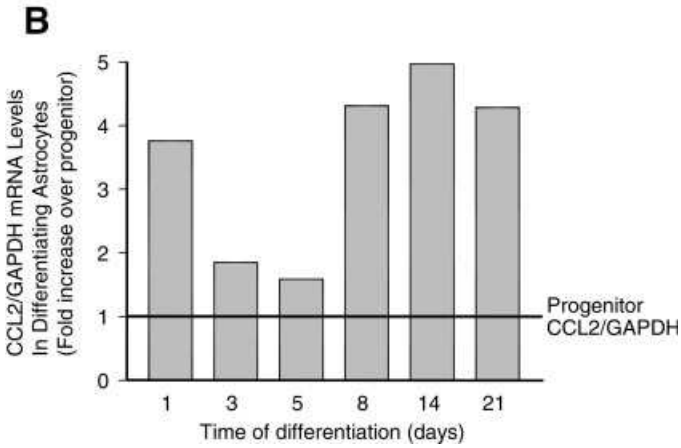


Fig. 2. Changes in chemokine mRNA expression during progenitor-to-astrocyte differentiation. **A:** RNase protection assay for chemokine expression in progenitors or differentiating astrocytes at 1, 3, 5, 8, 14, or 21 following the shift to astrocyte media. Each lane contains 10 μg total RNA. Arrow indicates band containing undigested CCL2 probe bound to RNA from both progenitor and differentiating astrocyte samples. **B:** Quantification of autoradiography bands using NIH Image software. CCL2-to-GAPDH intensity ratio for each time point of differentiating astrocytes was normalized to the average intensity ratio of progenitors. Representative of two experiments.

or undetectable levels of the CCL2-inducing cytokines TNF-α, IFN-γ, and IL-1β at any point before, during or after differentiation, and previously we found that no CCR2 was expressed on either progenitors, astrocytes or neurons (Lawrence et al., 2004), it is unlikely that autocrine stimulation contributed to the increased CCL2 levels in the differentiating astrocytes.

To determine whether TNF-α treatment stimulated differentiation, we compared immunofluorescent staining for the progenitor marker nestin and the astrocyte marker GFAP in progenitor cells, progenitors exposed to TNF-α, and differentiating astrocytes. Progenitor cells

*TABLE 1. TNF-α Stimulation of CCL-2 Production by Progenitor Cells**

	Hours	
Post stimulation	Progenitors	Progenitors + TNF α
4	411 ± 59	12,420 ± 1,207
8	669 ± 69	36,802 ± 2,441
16	1,305 ± 95	82,697 ± 5,421
24	2,177 ± 181	125,198 ± 25,176

*Progenitor cells were seeded in 12-well plates at a density of 2×10^5 cells/well; the following day, media was replaced and in some wells recombinant human TNF-α was added (10 ng/ml). Cell-free supernatant samples were taken at the time points indicated and analyzed by ELISA for the presence of CCL2. Data shown are mean ± SD of accumulated chemokine production (pg/10^6 cells) from triplicate samples. Representative of three experiments.

seeded 1–2 days earlier at a density of 100,000 cells per well (400 cells per mm^2) were switched to fresh progenitor media with or without TNF-α (50 ng/ml), or astrocyte media. After 2 days, cells were fixed in methanol/acetone (1:1) and stained for immunofluorescent detection of nestin (red) and GFAP (green) as described in Materials and Methods. Compared with cells maintained in progenitor media (Fig. 3A), progenitors exposed to astrocyte differentiation media for 2 days (Fig. 3C) showed increased GFAP expression colocalized with nestin, as well as phenotypic changes consistent with a change to an astrocyte phenotype, but progenitors treated with TNF-α for 2 days (Fig. 3B) showed no such increase in GFAP expression and could not be distinguished from untreated progenitor cells.

Nuclear NF-κB Binding Protein in Differentiating Astrocytes

Since NF-κB is known to mediate TNF-α effects including chemokine production, and because NF-κB binding to the distal promoter region is critical for TNF-α stimulation of CCL2 (Ping et al., 1999), we investigated whether the differentiation-induced upregulation of CCL2 occurred through a similar mechanism. To test whether NF-κB activation was associated with the early stages of progenitor-to-astrocyte differentiation in our cell cultures, we performed competitive electrophoretic mobility shift assays (EMSAs) using radiolabeled oligonucleotides containing the consensus sequence for NF-κB as described in Materials and Methods. Media on progenitor cell cultures was either maintained or changed to astrocyte differentiation media. After specific time points between 2 h and 21 days, nuclear proteins were extracted and competitive gel shift experiments were performed.

A faint gel-shifted band was detected in nuclear protein fractions from progenitor cells incubated with the NF-κB probe (Fig. 4, lane 1); specificity was confirmed by the elimination of this band with the addition of excess cold (unlabeled) oligonucleotide added (lane 2) but not mutant oligonucleotide (lane 3). A much stronger NF-κB specific band was detected from cells that had been exposed to astrocyte differentiation media for 2 h (Fig. 4, lanes 4–6). By 6 h, the band intensity was

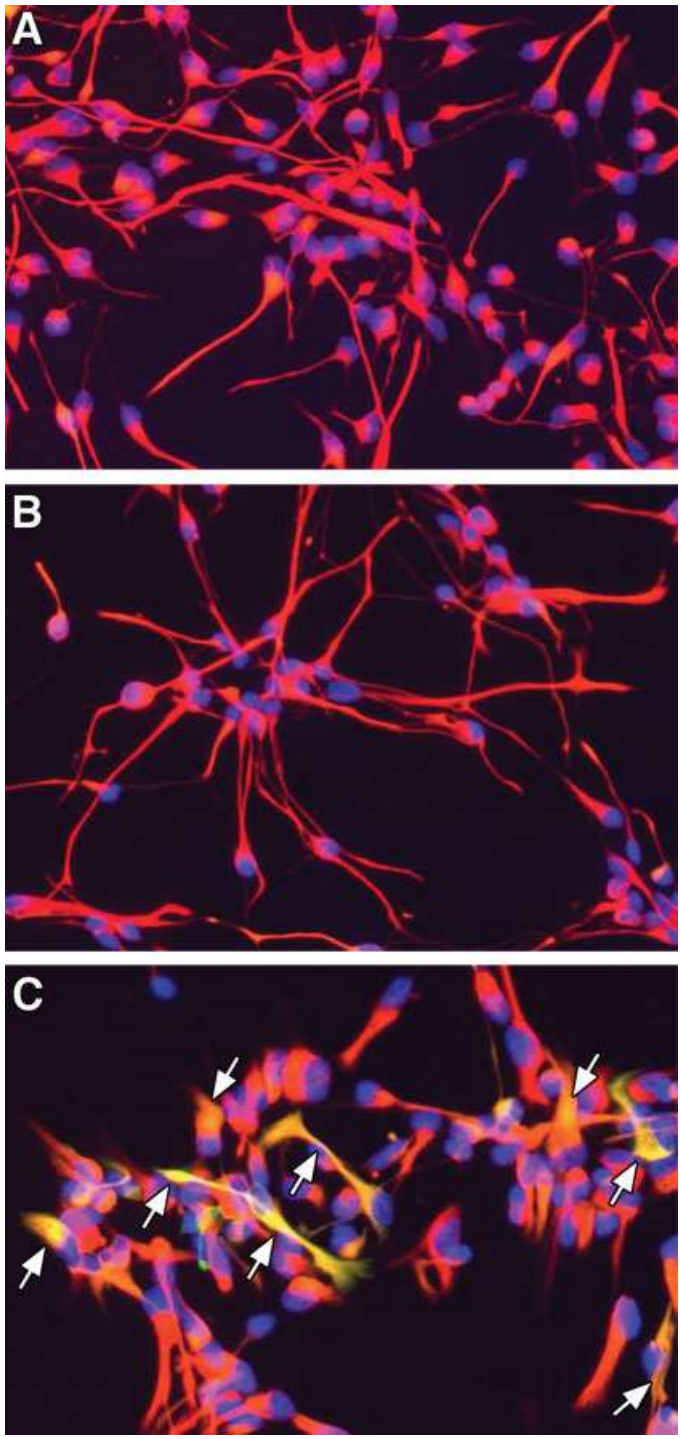


Fig. 3. Nestin and GFAP expression in differentiating astrocytes and TNF-α stimulated progenitor cells. Progenitor cells grown on poly-D-lysine-coated glass cover slips were either (**A**) maintained as progenitors, (**B**) exposed to TNF-α (50 ng/ml), or (**C**) switched to astrocyte differentiation media for 48 h. Green, GFAP; red, nestin; blue, DAPI. Arrows indicate colocalization of GFAP and nestin (yellow). Representative of two experiments.

diminished but still stronger than in progenitor cells (Fig. 4, lanes 7–9). At later time points throughout 3 days, very little NF-κB was detected, but at 5, 14 or 21 days of astrocyte differentiation, NF-κB binding was again strong (Fig. 4, lanes 10–12), consistent with the secondary rise in CCL2 production shown in Figures 1 and 2.

NFκB Inhibition and CCL2 in Differentiating Astrocytes

To determine whether NF-κB activation was required for differentiation-induced CCL2 upregulation, we used TPCK, a serine protease inhibitor that blocks NF-κB activation by preventing IκB degradation, or CAPE, an anti-oxidant that selectively inhibits NF-κB translocation. Progenitor cells were seeded in poly-D-lysine-coated 12-well plates (100K cells/well). The following day, media in all wells was changed either to fresh progenitor media (bFGF+EGF) or astrocyte media (EMEM + 10% serum), in the absence or presence of CAPE (1 μg/ml) or TPCK (10 μM). These doses inhibited TNF-α stimulation of CCL2 by 70–80% and did not affect cell viability by trypan blue exclusion, whereas higher doses were toxic to progenitor cells. In Figure 5, the results from experiments 1 and 2 showed that after 24 h, the differentiation-induced CCL2 production was inhibited by blocking NF-κB activation, but only by 30–40% with TPCK and 40–55% with CAPE. In the differentiating population, toxicity was observed with higher doses of the NF-κB inhibitors, so we could not determine whether more complete inhibition was possible. However, since the drugs were more effective at inhibiting the much greater CCL2 stimulation by TNF-α, it is likely that NF-κB acts in concert with another transcription factor to mediate the differentiation-induced increase in CCL2 production.

We considered that the increase in CCL2 following the change to astrocyte media could be explained simply by the addition of 10% serum to the cells rather than by differentiation. To induce differentiation in the absence of serum, we added bone morphogenic protein (BMP, 80 ng/ml) and ciliary neurotrophic factor (CNTF, 50 ng/ml) to the progenitor media, resulting in increased GFAP expression and morphologic changes indicative of astrocyte differentiation (data not shown). After 24 h of treatment with CNTF and BMP, we observed a 4.8-fold increase in CCL2 production in the absence of serum (Fig. 5, experiment 3). Addition of CAPE to cells stimulated with CNTF and BMP in the absence of serum resulted in cell death, which we did not observe when added to cells differentiating in serum. It is likely that some factor in serum provided some protection against CAPE toxicity at that dose. However, we again found a partial but statistically significant block by the NF-κB inhibitor TPCK, resulting in CCL2 levels that were still 3.3-fold higher than in progenitor cultures (Fig. 5). This finding, in addition to the lack of induction of other chemokines and cytokines in differentiating astrocytes and the downregulation of CCL2 in differentiating neurons, suggests that the selective stimulation of CCL2 production is at least partially dependent on NF-κB activation during differentiation.

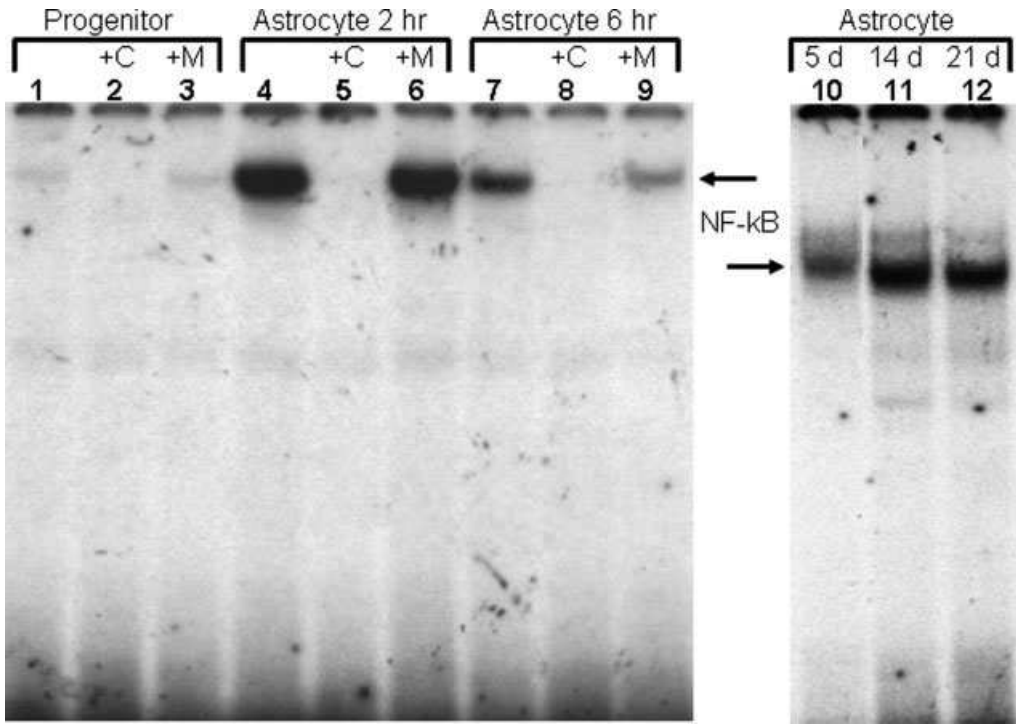


Fig. 4. Electrophoretic mobility shift assay for nuclear protein levels of NF-κB. An annealed and [^{32}P]-labeled oligonucleotide probe containing an NF-κB consensus sequence was incubated with nuclear protein from progenitors (**lanes 1–3**) and cells exposed to astrocyte differentiation media for 2 h (**lanes 4–6**), 6 h (**lanes 7–9**), 5 days (**lane 10**), 14 days (**lane 11**), or 21 days (**lane 12**). Binding specificity was confirmed competition with a 250-fold excess of unlabeled homologous oligonucleotide (+C, **lanes 2, 5, 8**) or unlabeled mutant oligonucleotide (+M, **lanes 3, 6, 9**). Representative of three experiments.

CCL2 Promoter Activation During Progenitor-to-Astrocyte Differentiation

To determine which regions of the CCL2 promoter are necessary for the response to astrocyte differentiation, we transiently transfected progenitor cells with plasmid constructs containing either the full or proximal-only regions of the human CCL2 promoter linked to the CAT reporter gene (Fig. 6). Zhou and colleagues (1998) used these constructs previously and determined that a specific region of the proximal promoter was critical for IFN-γ-mediated CCL2 stimulation, whereas similar studies using the murine promoter/CAT reporter constructs and DNA footprinting found that NFκB and NF-I binding to the distal promoter region was important for TNF-α stimulation of CCL2 (Ping et al., 1996; Kumar and Boss, 2000). Progenitor cells were transfected with the CAT reporter constructs containing either full promoter, or one of the proximal promoter fragments (−349 or −147). Control cells were transfected with pCAT-Basic plasmid DNA. On the following day all media was changed, with some samples switched to astrocyte media to begin differentiation, and after an additional 2 days, lysates were collected and assayed for CAT activity. Figure 7A shows that in differentiating cells, full promoter activity was 6-fold greater than in progenitors. However, there was only a 2-fold increase in the activity of the proximal promoter regions. Baseline CAT activity was below the level of detection in control cells transfected with the pCAT-basic plasmid.

In a similar experiment, cells that had been differentiating in astrocyte media for 3 or 7 days were transfected with the same set of CCL2 promoter or control constructs. Lysates were collected 3 days after transfection. CAT activity with the full CCL2 promoter was 2-fold greater in the cells transfected at day 7 of differentiation compared with day 3 (Fig. 7B). Thus, the activity of the full promoter was correlated with the time the cells had been differentiating along the astrocyte pathway and with the increase in CCL2 protein production over time. Again, the activity level of the proximal promoter (−349) was much less than the full promoter in the differentiating cells (Fig. 7B), but still there was an increase in the proximal promoter activity during differentiation. Full and proximal promoter activity levels were similar in fully differentiated astrocytes (data not shown). Together these findings suggest that the distal region is important for CCL2 promoter activation at the early and later stages of astrocyte differentiation, but there may also be a modest involvement of the proximal promoter region.

Additional preliminary experiments using deletion mutants of the murine CCL2 promoter (Ping et al., 1996) suggested that, like TNF-α, differentiation stimulation of CCL2 involved both the NFκ and NF-I binding sites within the distal promoter region (data not shown). Earlier we determined that levels of NF-I, particularly NF-IX, were higher in astrocytes compared with progenitors in this cell culture system (Messam et al., 2003). Overexpression of NF-I family members A, B, C, or X by transfection (Monaco et al., 2001; Messam et al., 2003) resulted in modest 10–25% increases in CCL2 production in both progenitors or differentiating astrocytes, whereas transfection of these factors into fully differentiated astrocytes did not affect CCL2 levels (data not shown). These initial findings suggest that although NF-I

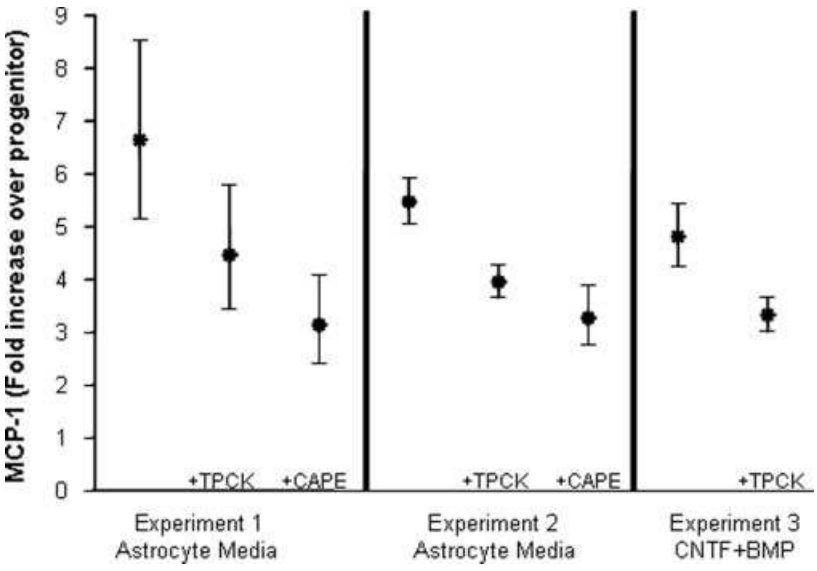


Fig. 5. Effect of NF-κB inhibition on differentiation-induced CCL2 production. ELISA determination of CCL2 protein levels from first 24 h of astrocyte differentiation, in absence or presence of 10 μM TPCK or 1 μg/ml CAPE, relative to undifferentiated progenitor cells. Experiments 1 and 2 were designed identically, with triplicate samples and using astrocyte media (10% serum) to induce differentiation. In Experiment 3, astrocyte differentiation was initiated without serum, but with bone morphogenic protein (BMP, 80 ng/ml) and ciliary neurotrophic factor (CNTF, 50 ng/ml) replacing other growth factors in normal progenitor media. CAPE was not included due to toxicity in the absence of serum. Data shown are mean CCL2 levels in differentiating cells divided by progenitor control levels; error bars indicate 95% confidence intervals calculated using normalizing logarithmic transformations.

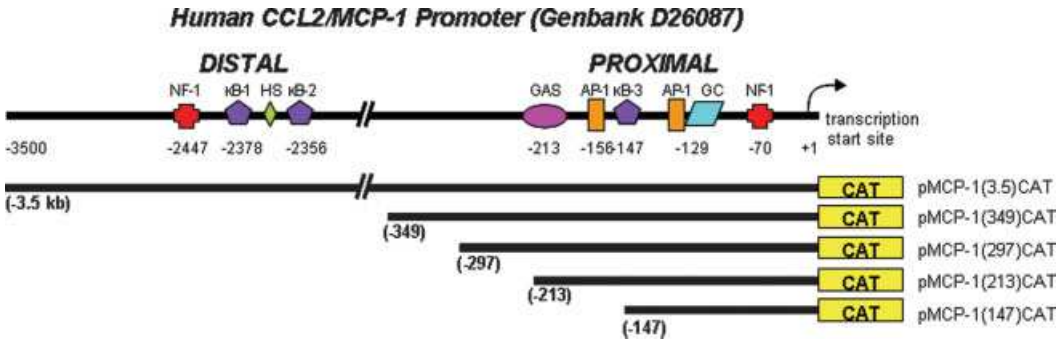


Fig. 6. CCL2 promoter-CAT reporter DNA constructs. Full and proximal-only deletion mutants of the human CCL2 5′ flanking region inserted into a pCAT-Basic plasmid (Zhou et al., 1998) used for transfection studies.

alone is not able to substantially modulate CCL2 expression, it may contribute to its regulation during the differentiation process. Experiments are in progress to clarify the roles of specific members of the NFκB and NF-I transcription factor families in CCL2 regulation during progenitor-to-astrocyte differentiation in this system.

DISCUSSION

Using a human brain-derived cell culture system that we described previously (Messam et al., 2003), we found that multipotential neural progenitor cells constitutively produced moderate levels of the inflammatory chemokine CCL2. Differentiation of these cells toward a neuronal phenotype inhibited CCL2 production 20-fold, whereas initiation of astrocyte differentiation resulted in a biphasic upregulation of CCL2: a rapid 10-fold increase of mRNA and protein within 24 h, followed by a plateau, and a secondary upregulation after 8 days, ultimately resulting in 30-fold higher levels in differentiated astrocytes compared with progenitors. Differentiation selectively increased CCL2 expression, whereas TNF-α induced expression of the chemokines CCL5 and CXCL8 and further stimulated CCL2 production.

The CCL2 upregulation we observed in differentiating astrocytes could not be explained by the addition of serum in the astrocyte media. Initiating differentiation by substituting the growth factors BMP and CNTF to the serum-free progenitor media produced a similar rise in CCL2 levels within 24 h, and this increase was blocked to the same degree by inhibiting NF-κB activation. It is possible that the presence of PDGF in serum could have stimulated CCL2 production, as this growth factor stimulates the CCL2 gene (Freter et al., 1996; Bogdanov et al., 1998). However, adding PDGF and BDNF to progenitor cells, which initiated differentiation toward a neuronal phenotype, resulted in a 20-fold suppression of CCL2 production. In addition, it is unlikely that the high levels of CCR2 production in differentiating astrocytes resulted from autocrine or paracrine augmentation. Although there is evidence for CCR2 expression on human fetal astrocytes (Andjelkovic et al., 2002), we previously we found mRNA and protein expression of CXCR4 but no other CC or CXC receptors on astrocytes, neurons, and progenitors in this cell system (Lawrence et al., 2004). These findings suggest that the phenotype of these brain-derived cells, rather than extracellular factors, regulates the production of CCL2.

TNF-α stimulation is known to activate NF-κB p50 and p65 translocation in human fetal astrocytes (Atwood et al., 1994), and to stimulate CCL2 production in fibroblasts in a NF-κB-dependent manner (Ueda et al., 1994;

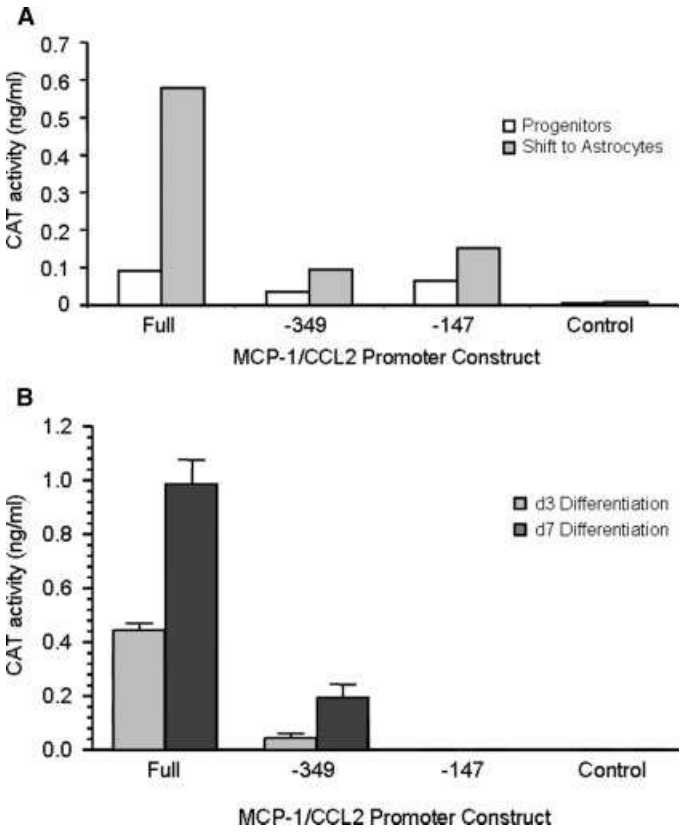


Fig. 7. Full vs. proximal human CCL2 promoter activity in progenitors and differentiating astrocytes. **A:** CAT reporter levels for full or partial CCL2 promoters in progenitor cells or in differentiating astrocytes. All cells were transfected as progenitors, then after 1 day, media in some wells was changed to begin astrocyte differentiation. Lysates were collected 72 h after transfection. Data shown are mean CAT activity from triplicate samples normalized to equivalent protein levels. Representative of five experiments. **B:** Same as A, but at the time of transfection cells had been differentiating for either 3 days or 7 days in astrocyte media. Representative of two experiments.

Ping et al., 1999). In our progenitor cells, EN+MSAa showed that, like TNF-α stimulation, astrocyte differentiation increased nuclear NF-κB levels within 2 h; these levels were also increased between 8 and 21 days of differentiation. Compared with TNF-α stimulation of CCL2, NF-κB inhibitors were less effective during differentiation, suggesting that another transcription factor may be involved. TNF-α exposure and the subsequent NF-κB activation did not induce GFAP expression or morphological changes in the progenitor cells, providing evidence that the signaling pathways of differentiation and TNF-α stimulation are distinct.

The CCL2 upregulation in differentiating astrocytes largely involved the distal region of the promoter, which contains NF-κB and NF-I binding sites, as demonstrated by transfection studies with the highly homologous human CCL2 promoter-CAT reporter constructs. These constructs were used previously to show that only the proximal promoter region is needed to mediate IFN-γ stimulation of CCL2 in human astrocytes (Zhou et al., 1998), and that distal region is largely involved in CCL2 upregulation by TNF-α (Ping et al., 1996). NF-I binding to Site A of the distal promoter was shown to modulate the TNF-α effect (Kumar and Boss, 2000). It may be that in differentiating cells, CCL2 is regulated by a combination of specific NF-κB, NF-I, and/or other transcription factor family members. Previously we found differences in NF-I levels, particularly NF-IX, between progenitor cells, progenitor-derived astrocytes and progenitor-derived neurons (Messam et al., 2003). The level of NF-IX expression determined the susceptibility to JC virus infection in neural progenitor cells, progenitor-derived astrocytes and progenitor-derived neurons (Messam et al., 2003), as well as hematopoietic progenitor cells and progenitor-derived macrophages (Monaco et al., 2001). Ongoing studies in our laboratory are addressing the role of specific NF-I and NFκB family members in activation of the CCL2 promoter.

It is unclear why CCL2 was selectively produced by progenitors and differentiating astrocytes, whereas TNF-α induced expression of CCL2, CCL5, and CXCL8 in these cells. The same three chemokines are induced by TNF-α in human corneal keratinocytes, but only CXCL8 is produced by TNF-α-stimulated human corneal epithelial cells (Cubitt et al., 1993; Tran et al., 1996; Cubitt et al., 1997). Optimal CXCL8 stimulation by TNF-α seems to require synergistic cooperation between NF-κB and C/EBP binding (Roebuck, 1999), and promoter mutagenesis studies showed that NF-κB p65 homodimers and C/EBP transcription factors interacted with the CXCL8 promoter but not the CCL2 promoter in the corneal epithelial cells (Ritchie et al., 2004). In primary human astrocytes, cytokine stimulation of CCL5 expression was mediated by NF-κB p50 and p65 (Li et al., 2001) or a combination of NF-κB and C/EBP (Kim et al., 2004). It is possible that astrocyte differentiation results in a complex regulation of NF-κB and NF-I binding to primarily the distal CCL2 promoter. Constitutive production of CCL2 by progenitors may be regulated by the very low levels of NF-κB we observed in our nuclear extract samples, or by other factors that bind to the proximal promoter.

Numerous studies suggest that astrocytes are a significant source of CCL2 in the context of neuropathology. In vivo, astrocytes have been identified as a primary source of CCL2 in the brain response to HIV-1 associated dementia (Conant et al., 1998), simian immunodeficiency virus encephalitis (Zink et al., 2001), multiple sclerosis (McManus et al., 1998; Van Der Voorn et al., 1999; Mahad and Ransohoff, 2003), experimental autoimmune encephalitis (Huang et al., 2000), ischemic brain injury (Minami and Satoh, 2003), brain trauma (Glabinski et al., 1996), and axonal injury (Babcock et al., 2003). CCL2 production by cultured human astrocytes was found to be upregulated by HIV-1 Tat protein (Abraham et al., 2005; Conant et al., 1998; Weiss et al., 1999) and amyloid-β (Johnstone et al., 1999; Prat et al., 2000; Szczepanik et al., 2001; Smits et al., 2002).

Brain-derived CCL2 has been implicated in neuropathology by the recruitment of activated and/or infected monocytes into the CNS. In the context of HIV-1 infec-

tion, several studies have shown a specific upregulation of CCL2 in the CSF of patients with HIV-1 associated dementia (Conant et al., 1998; Kelder et al., 1998), as well as in cultured astrocytes and microglia exposed to the HIV-1 regulatory protein Tat (Conant et al., 1998; Cota et al., 2000; McManus et al., 2000). Zink et al. (2001) found that a high CSF-to-serum ratio of CCL2 predicted neurologic disease in macaques infected with a neurovirulent strain of SIV, suggesting that this chemokine could be a marker for neurologic disease. In addition, a high-secretor allele of CCL2 is associated with an elevated risk of dementia in HIV-1-infected individuals (Gonzalez et al., 2002). However, the CCL2 response may also be protective. A recent study showed that CCL2 protected neurons and astrocytes against glutamate- and HIV-1 tat-mediated toxicity (Eugenin et al., 2003), suggesting that this chemokine may play a critical role in the balance between neuroprotective and damaging inflammatory responses in the brain.

Independent of neuropathology, there is growing evidence that in the developing brain, chemokines and their receptors are expressed and may have a role in directing cell migration, trophic support, proliferation and/or differentiation (Asensio and Campbell, 1999; Bajetto et al., 2001). CCL2, in particular, has been shown to induce migration of neural stem cells (Widera et al., 2004), and the chemokine receptor CCR2, the major receptor for CCL2, was found to be expressed in adult rat and human brain (Banisadr et al., 2002; van der Meer et al., 2000) and by human fetal astrocytes in vitro (Andjelkovic et al., 2002). However, the exact role of CCL2 in normal brain function or neurodevelopment is not yet clear. In the adult brain, chemokines are not as easily detected, but their receptors have been identified. For example, the receptors CXCR4, CCR2, CCR5 and CX3CR1 were recently identified on neural progenitor cells isolated from the adult rat brain (Ji et al., 2004), suggesting a role in adult neurogenesis and/or repair processes. Constitutive chemokine production may play a role in regulating inflammatory responses (Asensio and Campbell, 1999) and/or allowing immune surveillance to occur by increasing permeability of the blood-brain barrier (Stamatovic et al., 2005).

As there is evidence for astrocyte heterogeneity in vivo, an intriguing question is whether certain subpopulations of astrocytes are involved in specific pathogenic and physiologic functions, including CCL2 production. In our cell culture model system, we isolated progenitor cells which were similar in morphology and nestin expression; progenitor-derived astrocytes were all GFAP positive and uniform in phenotype and morphology, based on a number of physiological, immunohistochemical, and flow cytometric analyses. While these cells may have the ability to alter phenotype depending on the anatomical location and/or response to external signals, the in vitro phenotype appears more homogeneous due to their derivation. In the studies presented here, we have used this relatively uniform population to study the molecular changes associated with CCL2 regulation during the process of human astrocyte differentiation. Further studies of promoter activation using this system and alternative cell populations could elucidate molecular factors that regulate CCL2 synthesis and its role in development and repair, and may identify therapeutic targets for modulating protective or inflammatory effects during neuropathogenesis.

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