Caveolin-1 Knockdown by Small Interfering RNA Suppresses Responses to the Chemokine Monocyte Chemoattractant Protein-1 by Human Astrocytes*

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Astrocytes regulate the integrity of the blood-brain barrier and influence inflammatory processes in the central nervous system. The pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1), which is both released by and stimulates astrocytes, is thought to play a crucial role in both these activities. Because astrocytes have been shown to possess caveolae, vesicular structures that participate in intracellular transport and signal transduction events, we reasoned that expression of the major structural protein of these organelles, caveolin-1, might feature critically in the cellular responses to MCP-1. To test this hypothesis, caveolin-1 level was “knocked down” in human astrocyte cultures by using a small interfering RNA approach. This method resulted in efficient (>90% loss) and specific knockdown of caveolin-1 expression while sparing glial fibrillary acidic protein as well as several other proteins involved in endocytosis. Astrocytes suffering caveolin-1 loss showed diminished ability to down-modulate and internalize the MCP-1 receptor (CCR2) in response to exposure to this chemokine and also demonstrated significantly reduced capacity to undergo chemotaxis and calcium flux when MCP-1-stimulated. The results highlight a potentially prominent role for caveoleae and/or caveolin-1 in mediating astrocyte responses to MCP-1, a feature that might significantly dictate the progression of inflammatory events at the blood-brain barrier.

Once considered largely the province of the immune system, the chemokine network, which is largely responsible for directing the migration of leukocytes out of the vasculature and into tissue recesses, is now generally recognized to also have a functional foothold in the central nervous system. Indeed, a panoply of chemokines and their cognate receptors is expressed within the central nervous system by all the parenchymal cell types (1, 2) as well as by endothelial cells of the brain microvasculature (3). Astrocytes, in particular, have garnered significant attention as the major central nervous system sources of chemokines in a variety of neuropathological states associated with inflammation, e.g., demyelinating diseases, viral encephalitides, ischemia, and trauma (4–10). Given that astrocytes extend foot processes onto the microvessels that constitute the blood-brain barrier (11), it may be logically construed that the release of chemokines by these cells is pivotal to the location and scope of central nervous system inflammatory processes (12).

Of the several chemokines evidenced to be expressed by astrocytes, monocyte chemoattractant protein-1 (MCP-1), recently designated CCL2 (13), has perhaps been the most vigorously investigated. In addition to these cells being able to express and release MCP-1 (14–21), they are also considered to be targets of this chemokine (22–27). This laboratory first described the existence of saturable, high affinity binding sites for MCP-1 on cultured human astrocytes and supported this finding by immunocytochemical detection of CCR2, the major recognized receptor for this chemokine (23). Other laboratories have since confirmed these findings in astrocytes from human (28, 29) and rat (30) sources.

Most recently we have extended our findings to demonstrate that the CCR2 expressed by astrocytes is, in fact, bona fide in nature. Somewhat surprisingly, astrocyte internalization of MCP-1/CCR2 was determined to occur at least partly via caveolae, flask-shaped membrane invaginations considered to possibly represent a specialized type of lipid raft (31, 32). To gauge caveolae participation, these organelles were disrupted by treatment with the cholesterol sequestering agent filipin, which prevented MCP-1-induced, down-modulation of cell-surface CCR2 expression. This observation of possible caveolae involvement differs from those noted by others, who described chemokine-induced receptor internalization to be achieved by the more commonly acknowledged clathrin-coated pit pathway (33–35). Insofar as caveolae and their major structural protein, caveolin-1, both of which are expressed by astrocytes (36, 37), have been implicated in regulating signal transduction events as well as subserving endocytosis and transcytosis (38), caveolin-1-bearing membrane domains may be critical in mediating one or more of the responses of astrocytes to stimulation by MCP-1.

To test this possibility and mindful of the prospect that filipin could potentially exert broad-ranging effects unrelated to caveolae, we reasoned that targeted knockdown of caveolin-1 might be critical in mediating one or more of the responses of astrocytes to stimulation by MCP-1.

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** The abbreviations used are: MCP-1, monocyte chemoattractant protein-1; siRNA, small interfering RNA; dsRNA, disordered RNA; PBS, phosphate-buffered saline; HBSS, Hank’s balanced salt solution; DMEM, Dulbecco’s modified Eagle’s medium; GFAP, glial fibrillary acidic protein; rh, recombinant human.
to caveolae, we chose to use a small interfering RNA (siRNA) approach to transiently and specifically knockdown expression of caveolin-1 in cultured, human astrocytes. Findings indicate that ablation of caveolin-1 expression results in significantly diminished ability of MCP-1 to induce down-modulation of MCP-1 surface binding as well as promote chemotaxis and calcium flux in these cells. These observations suggest that caveolae and/or caveolin-1 might have a considerably broader role in chemokine activities than previously anticipated.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals**—All reagents were purchased from Sigma unless specified otherwise.

**Primary Human Astrocyte Culture**—Human fetal astrocytes were derived brain tissue obtained from second-trimester aborted fetuses, the gestational ages of which ranged from 19 to 22 weeks. Informed consent was obtained from all participants who, at the time of elective termination of pregnancy, indicated no risk factors for human immuno-deficiency virus-1 infection. Astrocytes cultures were prepared as previously described (24). Briefly, cerebral tissue was thoroughly washed with phosphate-buffered saline (PBS), separated from meninges, and minced into small blocks. The minced tissue was then incubated in Hanks’ balanced salt solution (HBSS; Invitrogen) containing 0.25% trypsin and 0.04% EDTA at 37 °C for 30 min. During and after enzyme treatment the tissue was mildly triturated to produce a single cell suspension, and the dissociated cells were plated at a density of 2 × 10^5 in 12 ml in T75 flasks. Cultures were maintained up to 2 weeks in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal calf serum with 1% antibiotic/antimycotic (Invitrogen) in a humidified atmosphere (5% CO₂) at 37 °C. Then cultures were shaken at 200 rpm for 2 h at 4 °C to dislodge weakly adherent microglia. Cultures were then shaken for an additional 18 h at 37 °C to remove the neuronal population. The remaining enriched astrocyte population was further purified by trypsinization and replating.

Cell purity was determined by immunocytochemistry using a monoclonal antibody to glial fibrillary acidic protein (GFAP, Sigma) antibody, and cultures were assessed to ~99% astrocytes (GFAP-positive). Culture medium was changed twice a week.

**Estimation of Caveolin-1 Protein Half-life**—Astrocytes (1 × 10^5) were seeded onto 12-well tissue culture plates (BD Biosciences). After 24 h the cells were incubated with 10 μg/ml cycloheximide for 0, 1, 3, 5, 12, 15, and 24 h. After these respective times, astrocytes were harvested and lysed with radioimmunoprecipitation assay lysis buffer (10 mM Tris, pH 7.6, 0.14 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). To account for the effects of cycloheximide on cell growth, astrocytes plated in parallel wells were counted, and volumes of cell cultures (extracts containing equal number of cells) were separated by 12% SDS-polyacrylamide gel electrophoresis and subject to Western blotting with antibody to caveolin-1.

**Caveolin-1 T7 siRNA Synthesis**—T7 siRNA was designed according to the methods described by Donze and Didear (39). The sequence (GATCGACGTGTCACCGCC) was selected as the target region and corresponds to nucleotides 422–440 uniquely present in the human caveolin-1 coding region (GenBank™ accession number NM 001753). To generate the siRNA, the following DNA oligonucleotides were synthesized by Invitrogen: 5′-TAAATACGACTCATATAG-3′ (T7 promoter); 5′-TCGCGGTTGACCAGGTCGATCTATAGTGAGTCGTATTA-3′ (sense); 5′-GAGATGACCTTACAGTTCAGCTATAGTGAGTATTA-3′ (antisense).

The same nucleotides used to generate the siRNA probe were used in scrambled sequence to generate a disordered siRNA (dsRNA). This served as a control for nonspecific effects due to transfection of duplex RNA. Specifically, the sequences of the control oligonucleotides employed were GCGCGGGCTTACAGTTCAGCTATAGTGAGTATTA-3′ (sense) and 5′-TCGCGGTTGATACACCCCTTTAAGTGGACGCTCAGTATT-A-3′ (antisense). For *in vitro* transcription 38-nucleotide DNA template oligonucleotides were designed to produce 19-nucleotide siRNA, utilizing T7 RNA polymerase as described (39–41). Briefly, one molar of T7 promoter and sense or antisense oligonucleotides were mixed and annealed in 50 μl of T4 DNA Ligase buffer (1 mM Tris, pH 8.0, 1 mM EDTA) at 95 °C for 5 min, then cooled slowly to room temperature. In *in vivo* transcription was performed using the AmpliScribe™ T7 High Yield transcription kit (Epicerent Technologies, Madison, WI). One μg of annealed DNA template was incubated in a 20 μl of transcription reaction overnight at 37 °C. After that, 1 unit of RNase-free DNase I (Epicerent Technologies) was added at 37 °C for 15 min. For generation of siRNA duplexes, sense and antisense siRNA strands were mixed and heated at 95 °C for 5 min, then cooled slowly to room temperature. The duplex siRNA was further purified by ethanol precipitation. After centrifugation, the resulting pellet was rinsed with 70% ethanol, air-dried, resuspended in RNase-free water, and stored at −20 °C. The concentration of siRNA was estimated by elution at 260 nm.

**Transfection of siRNA**—Astrocytes were seeded onto 6-well tissue culture plates at 60–80% confluency and in the absence of antibiotic ~18 h before transfection. Just before the transfection protocol, culture medium was removed, and the cells were washed once with PBS and then transfected with siRNA (0.2 μg/well) using Oligofectamine (2 μl/well) (Opti-MEM 1 medium; both from Invitrogen). An equal volume fresh media containing 20% serum was added. A second transfection was performed 24 h after the initial transfection, following an identical protocol. After an additional period of 24 h (i.e., 48 h after the first transfection) cells were utilized for analysis or experimentation.

**Immunocytochemistry**—For immunocytochemical verification of caveolin-1 knockdown, astrocytes (2 × 10^5) were plated on poly-lysine-coated Falcon Culture Slides (BD Biosciences). After allowing 24 h for adherence/growth, cells were transfected with siRNA as described above. After the transfection protocol astrocytes were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and then incubated for 1 h at room temperature containing 5% fetal bovine serum (FBS) at 0.2% Tween 20 at 4 °C overnight to reduce nonspecific antibody binding. To detect phosphorylated caveolin-1, astrocytes were plated and processed in the same manner but were not subjected to transfection. After the blocking step cells were incubated with either rabbit polyclonal anti-caveolin-1 (1:2000 dilution; Transduction Laboratories, San Diego, CA), mouse monoclonal phospho-caveolin-1 (Tyr-14) antibody (1:100 dilution; Transduction Laboratories), mouse monoclonal anti-GFP (1:200 dilution; Sigma), or mouse monoclonal anti-clathrin (1:200 dilution; Transduction Laboratories). Next, cells were washed with PBS followed by incubation with the following, respective secondary antibodies, each at 1:2000 dilution for 1 h at room temperature: fluorescein isothiocyanate-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA); rhodamine-conjugated goat anti-mouse IgG (Roche Applied Science); 7-amino-4-methylcoumarin-3-acetic acid-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). All primary and secondary antibodies were diluted in blocking buffer. After staining with secondary antibodies, samples were washed again with PBS and then mounted in Vectorshield mounting medium (Vector Laboratories). Controls for the immunostaining procedure were prepared similarly but with the omission of primary antibody. Samples were viewed and photographed under an Olympus IX70 microscope (20×/0.4 NA objective, Olympus America Inc., Melville, NY) equipped with epifluorescence optics and a Hamamatsu ORCA-ER camera.

**Immunoblot Analysis**—Whole cell lysates were prepared and separated by SDS-PAGE followed by transfer onto nitrocellulose membranes (Bio-Rad). Nitrocellulose membranes were blocked in 1% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 for 1 h, and membranes were washed in Tris-buffered saline containing 0.1% Tween 20 for 15 min. For generation of the respective proteins: 6% (clathrin and EE1), 7.5% (adaptins-α and -β), 10% (GFAP and flotillins 1 and 2), or 12% (caveolin-1, phospho-caveolin-1). Nonspecific protein binding was blocked by the incubation of the membranes in 5% nonfat milk (Bio-Rad). Immunodetection was performed with primary antibodies to GFAP (1:1000 dilution, Sigma), clathrin (1:10,000 dilution, Transduction Laboratories), clathrin heavy chain (1:200), and -2 (1:5000 dilution, Transduction Laboratories), clathrin, adaptins-α and -β, and EE1 (all at 1:1000 dilution and all from Transduction Laboratories), and anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Cappel, Aurora, Ohio). Blots were developed with an enhanced chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity; Pierce) according to the manufacturer’s instructions. A digitized image of each blot film was obtained using a flatbed scanner. Relative quantification of proteins was performed by determining the mean pixel intensity associated with individual bands (of constant pixel size) on scanned
autoradiographs using Adobe PhotoShop 6.0 software. To obtain a mean pixel intensity value corresponding solely to specific protein immunoreactivity, a background noise value (of pixel size equal to that used to outline protein bands and selected from an irrelevant area of the autoradiograph) was subtracted from each protein value. Corrected mean pixel intensity values were typically graphed as relative protein amount along the ordinate, except in the case of caveolin-1 \( t_{1/2} \) determination, where values were expressed as percent caveolin-1 immunoreactivity present at time 0.

**Measurement of Calcium Flux**—Measurement of calcium flux was performed as previously detailed (24). In short, astrocytes were plated on Delta T dishes (0.15-mm thickness, black-slide; Biotache Inc., Butler, PA) and transfected with siRNA as described above. At 24 h after transfection the cells were loaded with 2 \( \mu \)M Fura Red and 2 \( \mu \)M Fluo-3 (Molecular Probes, Eugene, OR) in loading buffer (HBSS with 0.1% bovine serum albumin, 20 mM Hepes, pH 7.4) and incubated at 37 °C in 5% CO\(_2\) for 45 min. After this period the cells were washed twice and incubated in a fresh loading buffer for another 30 min at 37 °C. Scanning confocal imaging was performed with a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) and 63\( \times \)1.25 NA oil objective after adding recombinant human (rh) MCP-1 (Peprotech Inc, Rocky Hill, NJ) directly to the cells. Samples were excited with an argon laser at 488 nm, and the emitted fluorescence was detected with green (Fluo-3, 515 nm) or red (Fura Red, 610 nm) filters. Ratiometric images were obtained using MetaMorph version 4.6 software (Universal Imaging Corp., Downingtown, PA).

**Chemotaxis Assay**—Cell migration was performed using 96-well microchemotaxis filter chambers (pore size, 12 \( \mu \)m; Neuro Probe, Inc. Bethesda, MA) that were coated with 50 \( \mu \)g/ml poly-lysine and air-dried before experimentation. Astrocytes transfected with siRNA were trypsinized, washed, and allowed to recover surface protein expression by incubation in DMEM containing 10% fetal bovine serum for 90 min at 37 °C in 5% CO\(_2\). After that cells were washed twice with PBS and resuspended in serum-free DMEM containing 1% bovine serum albumin, and 50 \( \mu \)l of suspension (2 \( \times \)10\(^5\) cells) was added to the upper well of the chemotaxis chamber. A volume of 30 \( \mu \)l containing varied concentrations of rhMCP-1 was placed in the lower chamber to induce chemotaxis, and the entire chemotaxis chamber was placed for 12 h at 37 °C in 5% CO\(_2\). After chemotaxis was complete the upper surface of the filter was thoroughly swiped with a cotton swab to remove the surface-bound, nonmigrating cells. This swiping process is critical to accurately resolve and quantify those cells that have actually migrated into the filter from ones only adherent to the filter surface. After swiping filters were fixed and stained with Diff-Quick (Dade Diagnostics, Aguada, PR), and the number of migrating cells was counted under a light microscope at 40× magnification. A chemotactic index for each condition was calculated as number of cells migrating in response to MCP-1/number of cells migrating in the absence of MCP-1.

**MCP-1 Binding Assay**—MCP-1 binding to astrocytes was performed essentially as described previously (23, 24). Briefly, cells were first reacted with biotinylated-rhMCP-1; MCP-1 Fluorokine kit, R&D Systems, Minneapolis, MN) at 50 nm for 1 h at 4 °C. The cells were incubated with fluorescein-conjugated avidin (R&D Systems) for an additional 30 min at 4 °C. All samples were viewed with a Zeiss LSM 510 microscope using an Fluar 40×/1.3 NA oil objective, and images obtained were processed with Adobe Photoshop 6.0. Quantification of MCP-1 binding was accomplished in the manner previously detailed (23, 24). In short, we recorded mean pixel intensity values from a total of 10 randomly chosen areas (512 \( \times \) 512 pixels) containing at least 10 cells each that were generated from triplicate samples for each condition assayed. Mean pixel intensity values were obtained using Adobe Photoshop 6.0 and averaged for the respective conditions. To obtain values specifically due to MCP-1 binding background noise was negated from each sample by subtracting fluorescence values obtained using an irrelevant protein, biotinylated soybean trypsin inhibitor (negative control), in place of labeled MCP-1. During this procedure constant conditions of aperture, pinhole, detector gain, amplitude offset, amplitude gain, brightness, and contrast were maintained. The adjusted mean pixel intensities were graphed as relative MCP-1 binding

**Phosphorylation of Caveolin-1**—Astrocytes (1 \( \times \)10\(^5\)) were seeded in growth media onto 12-well plates. After attachment overnight cells were serum-starved for 12 h and then preincubated with 100 \( \mu \)g/ml orthovanadate in serum-free DMEM for 20 min. After this treatment, astrocytes were rinsed with DMEM and incubated with MCP-1 (10 nmol/10 min in serum-free DMEM. Cells were then either harvested in radiomimprecipitation lysis buffer for Western blotting or fixed for immunocytochemistry.

**Statistics**—Differences in protein expression and biologic activity between control astrocytes and those subject to siRNA-mediated caveolin-1 knockdown were assessed by Student’s t test. \( p < 0.05 \) was considered significant.

**RESULTS**

To first gauge the possible sensitivity of astrocytes to caveolin-1 knockdown by siRNA, a rough approximation of the half-life \( t_{1/2} \) of this protein was determined. The rationale for such determination was that, if caveolin-1 had too long a turnover time, this would preclude the action of the siRNA, which only has a transient existence once transfected. Fig. 1 shows by Western blotting that the level of caveolin-1 declined nearly 50% between 12 and 18 h after inhibition of protein synthesis. Reasoning that such turnover could potentially allow for siRNA-mediated knockdown of caveolin-1, we proceeded to evaluate this approach.

Transfection of astrocytes with siRNA directed at a specific caveolin-1 mRNA sequence resulted in significant and specific suppression of caveolin-1 protein, as judged by Western blotting (Fig. 2). At 24 h post-transfection the level of caveolin-1 had decreased by −20% of the control value and further declined to about 10% of control by 48 h post-transfection. This response was clearly due to specific action by the caveolin-1-
directed siRNA, as transfection with a dsiRNA of identical nucleotide length failed to alter the caveolin-1 protein level. However, despite significantly affecting caveolin-1, transfection with caveolin-1 siRNA produced little to no change in flotillins 1 and 2, other proteins associated with caveolae (42), or in GFAP. Because altered GFAP is considered a marker for astrocyte activation (43), it may be reasoned that the siRNA-mediated knockdown of caveolin-1 does not generally perturb astrocyte function.

Also left relatively unchanged by the action of caveolin-1 siRNA were several other proteins, e.g. clathrin, adaptins-α and -β, and EEA1, all of which are selectively associated with clathrin-coated vesicles (44). That siRNA-mediated knockdown of caveolin-1 could occur in the absence of any significant alteration in the expression of proteins critical to the clathrin-mediated endocytic pathway is particularly noteworthy, as it strongly suggests that this approach causes relatively exclusive interference with caveolin-1 expression and, hence, should only affect caveola-mediated membrane events.

Immunofluorescent confirmation of the effect of astrocyte transfection with caveolin-1 siRNA is shown in Fig. 3. Cells transfected with siRNA, but not the dsiRNA sequence, show a clear loss in staining intensity when compared with control cells (only subject to transfection conditions). Moreover, the effect appears universal among the cells, as it is associated with a clear loss in staining intensity.

**FIG. 2.** Specific knockdown of caveolin-1 expression in cultured human astrocytes by siRNA. Astrocytes, cultured on 6-well plates, were subject to transfection with either caveolin-1 siRNA, a dsiRNA of scrambled nucleotide sequence, or just transfection reagent without RNA (control). At 24 or 48 h post-transfection astrocytes were extracted in radioimmune precipitation assay buffer, and samples containing representative numbers of cells were assayed by Western blotting. A, Western blots of caveolae-associated proteins and GFAP. B, relative quantification of the blots in A. C, Western blots of proteins associated with clathrin-coated pits. D, relative quantification of blots in C. All blots shown are representative of three experiments.

**FIG. 3.** Immunocytochemical detection of caveolin-1 knockdown in human astrocyte cultures. Astrocytes were plated on chamber slides, subject to transfection with caveolin-1 siRNA, dsiRNA of scrambled nucleotide sequence, or just transfection reagent without RNA (control) and then analyzed by immunocytochemistry 48 h later. Scale bar = 80 μm.
with the near entire population of astrocytes and not just a subset. Though the density of caveolae, per se, was not determined here, several prior reports have indicated that manifestation of these organelles in a variety of cell types is dependent upon caveolin-1 expression (45, 46). Thus, given the near 90% ablation of caveolin-1 expression, it is reasonable to assume that caveolae as well as caveolin-1 were significantly reduced in siRNA-treated astrocytes.

Based on the collective evidence highlighting the specificity and efficiency of the siRNA approach to greatly diminish caveolin-1 expression, experiments were conducted to evaluate the effects of caveolin-1 loss on astrocyte responses to MCP-1 challenge. To begin with, we investigated the consequence of caveolin-1 knockdown on ligand-induced down-modulation of MCP-1 binding. Binding of MCP-1 and other chemokines at 37 °C results in down-modulation of their cognate receptors through internalization and recycling (47), a process indicated by transient loss and then recovery of cell-surface binding sites. In a previous report from this laboratory it was shown that prior exposure of astrocytes to unlabeled MCP-1 at 37 °C caused reduction in subsequent binding of a labeled derivative of this chemokine at 4 °C (24). The degree to which surface binding was lost depended upon the time of pre-exposure to unlabeled ligand (2 h produced a maximal decline) and could be partially reversed by first treating the astrocytes with filipin, a compound known to disrupt caveolae. Furthermore, both MCP-1 and its cognate receptor, CCR2, could be visualized in the interior of astrocytes after exposure of these cells to chemokine at 37 °C. These results prompted the interpretation that MCP-1 induced internalization of CCR2 by astrocytes and that this process was partially mediated by caveolae. However, bearing in mind the possibility that filipin could exert unrecognized, deleterious effects not specific to caveolae, these ex-
Astrocytes subjected to caveolin-1 knockdown exhibited overt suppression in other responses to MCP-1 as well. As indicated in Fig. 5, control astrocytes demonstrated a calcium flux in response to this chemokine, a phenomenon previously reported for human astrocytes by this laboratory (24) and for murine astrocytes by others (27). However, after transfection with caveolin-1 siRNA, cells exhibited a significantly muted calcium transient. Such was not the case after dsiRNA transfection, signifying that the reduction in calcium flux was associated with the loss of caveolin-1 and not due merely to the transfection process.

In addition to interfering with receptor internalization and calcium flux, siRNA-mediated loss of caveolin-1 also antagonized the ability of MCP-1 to stimulate astrocyte chemotaxis. Previous work from this laboratory (24) and others (22, 27) showed that human and murine astrocytes, respectively, can induce a chemotactic response. In agreement with these prior reports, Fig. 6 reveals that control astrocytes migrate toward a gradient of MCP-1, with peak chemotaxis occurring in response to an applied chemokine concentration of 10 nM. This concentration approximates the $K_d$ of MCP-1 binding to human astrocytes (23), and the bell-shapes curve of the response is typical of chemotactic factors. Compared with control cells, astrocytes transfected with caveolin-1 siRNA exhibit a significantly lessened chemotaxis at 10 nM MCP-1 and failed to display a bell-shaped curve within the concentration range tested.

Last, to derive additional indication that caveolin-1 might be involved in astrocyte responses to MCP-1, the ability of this chemokine to stimulate tyrosine phosphorylation of caveolin-1 was explored. Reports from several laboratories describe phosphorylation of caveolin-1 on tyrosine 14 after both ligand- and non-ligand-mediated events (48–50), with phosphorylation purported to alter both caveolin-1 localization within the plasma membrane (51) and the morphological state of caveolae (52) as well as to be required for endocytosis through the caveolar pathway (53). Fig. 7 shows that MCP-1 also induces this event in astrocytes. Western blotting indicates that the extent of tyrosine 14-phosphorylated caveolin-1 (phospho-caveolin-1) increased nearly 100% after stimulation of astrocytes with MCP-1 for 10 min. Immunocytochemical detection of phospho-caveolin-1 confirmed the chemokine-induced phosphorylation response. Although phospho-caveolin-1-positive cells could be observed in astrocytes not receiving chemokine treatment, MCP-1 exposure decidedly heightened the degree of staining and clearly delimited the fine astrocytic processes (not seen in unstimulated controls).

**DISCUSSION**

In this report we demonstrated the use of siRNA to knockdown expression of caveolin-1 to assess the role of this protein in mediating astrocyte responses the chemokine MCP-1. The gene silencing effect was both efficient and highly specific, resulting in an ~90% reduction in caveolin-1 protein level while apparently not affecting the expression of numerous other proteins also involved in endocytic events or housekeeping functions. Furthermore, the reduced expression of caveolin-1 correlated with a diminished ability of cultured astrocytes to down-modulate the binding of MCP-1 as well as calcium flux and undergo chemotaxis in response to this chemokine. It was additionally shown that MCP-1 stimulation of astrocytes caused heightened phosphorylation of caveolin-1, underscoring the involvement of this protein in MCP-1-evoked responses in these cells.

Although caveolin-1 knockout mice have been generated (45,
mitigating factors. This is a particularly important assessment, not only because the action of siRNA is transient it is doubtful that the results clearly depict the utility of this method in producing near complete ablation of caveolin-1 while sparing other proteins whose mRNA transcripts bear regions of homology to caveolin-1 message. In further support of the siRNA approach to resolve aspects of endocytosis, Motely et al. (57) most recently employed this method to selectively deplete cells of clathrin and the adaptor protein AP-2, which recruits clathrin onto the plasma membrane.

The inhibition of ligand-induced down-modulation of MCP-1 binding to astrocytes in caveolin-1 siRNA-treated cells corroborates previous results from this laboratory of experiments employing filipin to preferentially disrupt caveolae (24). As with the case using filipin, siRNA-mediated knockdown of caveolin-1 expression reversed the loss of labeled MCP-1 binding that results from pre-exposure of astrocytes to unlabeled chemokine, again consistent with the interpretation that caveolae are involved in CCR2 internalization and the down-modulation process. Quantitatively, the effect of siRNA treatment was somewhat greater than that which was observed with filipin in that it produced a near 75% recovery of the surface binding activity expressed by astrocytes not pre-exposed to unlabeled MCP-1. The previously reported effect of filipin, on the other hand, was to restore MCP-1 binding activity to ~60% that detected in cells not subject to MCP-1 pre-exposure. Such a modest discrepancy may reflect the heightened efficiency and specificity of the siRNA approach. The near coincident effects of filipin treatment and caveolin-1 knockdown observed here parallel that recently described for ligand-induced alterations in epidermal growth factor and platelet-derived growth factor receptor activities (56) and underscore a likely role for caveolae in the MCP-1-induced down-modulation process.

The decrease in MCP-1-induced calcium flux observed in caveolin-1 siRNA-treated astrocytes is consistent with recent reports that caveolin-1 and/or caveolae play a significant role in regulating calcium entry (58–60). In further accord with our results Drab et al. (45) report that small arteries from caveolin-1-deficient mice displayed a weaker calcium-dependent contractile response to several vasoactive agonists as well as attenuation in calcium-related myogenic tone of cerebral arteries.

To the best of our knowledge neither caveolae nor caveolin-1 have yet been linked to chemotaxis, so the finding that caveolin-1 knockdown significantly mitigated astrocyte chemotaxis in response to MCP-1 is particularly novel. However, this result is in accord with other reports that chemotaxis is inhibited by methyl-β-cyclodextrin (61) and amphotericin B (62), two compounds that, like filipin, sequester cholesterol and thereby, among other effects, severely disrupt caveolae integrity and function. Inasmuch as the effect of caveolin-1 siRNA appeared to be highly restrictive to caveolin-1 protein and not astrocyte morphology nor growth rate (data not shown) was perceptibly altered by siRNA treatment, it is unlikely that the suppression of either chemotactic response or calcium flux stemmed solely or largely from a permissive toxic effect. Instead, it would seem that caveolin-1 critically participates in the chemotactic and calcium responses of astrocytes to MCP-1.

Although calcium mobilization has yet to be causally associated with chemotaxis, in MCP-1-treated cells, the fact that caveolin-1 knockdown dually affected calcium flux and chemotaxis by astrocytes in response to MCP-1 is in accord with the recent finding that human monocyte chemotaxis to MCP-1 requires involvement of protein kinase Cδ (63), a protein kinase C isotype that is calcium-dependent. A similar correlative suppression of MCP-1-induced calcium mobilization and chemotaxis in human monocytes has also been recently observed after treatment of these cells with the sympathomimetic amine dobutamine (64), thus possibly indicating a functional causality between these processes after MCP-1 exposure to varied cell types.
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