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Chemokine inhibition in rat stab wound brain injury using antisense oligodeoxynucleotides

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Abstract

Traumatic injury to the central nervous system (CNS) results in the breakdown of the blood–brain barrier and recruitment of hematogenous cells at the site of injury. The role of chemokines in this process has been well recognized and they have been regarded as promising targets for development of anti-inflammatory therapies. The expression of monocyte chemoattractant protein (MCP-1), in particular, has been closely linked to macrophage infiltration following trauma in rat brain. In this study we determined whether inhibition of MCP-1 following stab wound injury would reduce macrophage infiltration. Stab wound injured Sprague–Dawley rats were infused with MCP-1 sense or antisense oligonucleotides using an Alzet miniosmotic pump (1 μl/h for 3 days). Three days following injury, widespread gliosis was observed in both groups of rats as judged by glial fibrillary acidic protein (GFAP) immunoreactivity. Immunohistochemistry showed significantly less staining for MCP-1 in antisense treated animals. In addition, the number of macrophages were reduced by 30% in the antisense compared to the sense treated animals ($P < 0.05$). These results demonstrate that modulation of MCP-1 expression in stab wound injury directly affects monocytic infiltration and provide a basis for MCP-1 inhibition as a therapeutic strategy for controlling inflammatory events of traumatic brain injury. © 1998 Elsevier Science Ireland Ltd.

Keywords: Blood–brain barrier; Chemokines; Inflammation; Macrophage; Trauma

Following a traumatic injury to the adult mammalian central nervous system (CNS), reactive gliosis occurs in the surrounding neural tissue as a result of proliferation and hypertrophy of glial cells [4]. In addition, the wound becomes filled with hematogenously derived monocytes, which migrate from the blood into the damaged neural tissue where they transform into macrophages. Chemokines have been shown to act as soluble signals in many inflammatory processes associated with human disease states and model systems [9,10,12]. Because chemokines appear relatively selective in their actions compared to many other inflammatory mediators, they have been regarded as prime targets for the development of therapeutic strategies. Beta chemokines detected in rat brain following traumatic injury are: monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein 1 beta (MIP-1β), and regulated on activation, normal T-cell expressed and secreted (RANTES) [1,7].

Potent chemotactic activity of MCP-1 for monocytes has been well established in several studies [13,16]. MCP-1 expression in rats/mice subjected to penetrating brain injury has been demonstrated, and a close correlation between monocyte infiltration and elevated levels of MCP-1 following CNS trauma has been established [1,10]. Furthermore, mononuclear infiltrates composed mainly of monocytes and macrophages have been detected in transgenic mice overexpressing MCP-1 in the brain [6]. In order to determine whether MCP-1 inhibition would influence the infiltration of monocytes/macrophages (monocytes) in the injured CNS, we infused MCP-1 antisense oligonucleotides in stab wound injured rat brain. Our results demonstrate a significant, but not complete inhibition in the monocyte count

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at the site of injury in the antisense compared to sense treated animals. Stab wound injury was characterized by studying astrogliosis and confirming the expression of select cytokines and growth factors in the injured brain [8].

Adult Sprague–Dawley rats (250–300 g) were housed in the animal care facilities at the PAVA Health Care System. The stab wound surgery was performed as described previously [7]. Briefly, animals were deeply anesthesized, shaved on the head and placed in a stereotactic apparatus for surgery. The lesion was made 3 mm to the right of and 3 mm behind the bregma for a depth of 4 mm from the brain surface in a parasagittal orientation. For continuous infusion of the injured area, each rat was implanted with a osmotic minipump-brain infusion assembly. The minipump (average infusion rate, 1.0 μl/h; Alzet model 1003D; Alza, Palo Alto, CA, USA) was filled with either vehicle alone (artificial CSF, control), sense or antisense oligonucleotide (oligo) solution (1.0 μg/μl). The brain infusion assembly consisted of a catheter tubing and a stainless steel cannula with two-depth adjustment spacers to obtain stereotaxically correct depth. The tubing-cannula assembly was also filled with the appropriate solution and joined to the pump. Following the stab wound, the cannula was inserted at the injury site and secured in position with dental cement. The osmotic pump was housed in a subcutaneous pocket in the midcapsular area of the back of the rat. Nine rats were used for sense/antisense treatment and four rats served as a control group.

Sense (5′CTT CTG GGC CTG TTG TTC AC3′) and antisense (5′GGG ACG CCT GCT GCT GGT GAT TC3′) oligos were constructed to target the coding region of the MCP-1 gene and synthesized from Operon Technologies, Alameda, CA, USA. The oligos were dissolved in an artificial cerebrospinal fluid (CSF) solution (Na 150 mM, K 3mM, Ca 1.4 mM, Mg 0.8 mM, P 1 mM, Cl 155 mM) at a concentration of 1.0 μg/μl.

Animals were sacrificed for immunohistochemistry 3 days following surgery as previously described [7]. All immunohistochemical stainings were performed using the Elite Vectastain ABC Kit (ABC Vector Laboratories, Burlingame, CA, USA) as described previously [7]. The glial fibrillary acidic protein (GFAP) antibody was prepared in our laboratory and has been described previously [4]. The monocyte/macrophage marker (MAB 1435; Chemicon International, Temecula, CA, USA) and rabbit anti-human MCP-1 (R&D Systems, Minneapolis, MN, USA) were used at a dilution of 1:500 and 1:1000, respectively. Sections were also stained with the appropriate normal sera for control comparison. Staining with the normal sera appeared as a uniform light background. The light micrographs shown are representative of the stainings observed. At least three sections were stained for each group. The extent of the lesioned brain area in control and sense/antisense oligo-treated animals was measured by an Olympus microscope equipped with a Sony video camera/monitor using a VAS II image analysis and measurement system (Mideo Systems, Huntington Beach, CA, USA). For quantitating the degree of infiltration, immunopositive cells in MAB1435 stained sections were counted blind in 10 randomly selected areas spanning a standard lesion area in each slide. Data are the mean ± SEM ($n = 9$ for sense/antisense and $n = 4$ for control). Statistical significance was assessed by the Student's t-test. A value of $P < 0.05$ was accepted as significant.

In coronal sections, the stab wound lesions from control and sense/antisense oligo-treated animals appeared as necrotic tissue extending from the cortex through the corpus callosum into the hippocampus. The damaged tissue contained numerous erythrocytes. The tissue appeared grossly normal elsewhere. To establish that the extent of injury for all groups was comparable, quantitative measurements were made using a VAS II image analysis and measurement system. The depth of injury was measured from the surface below and the breadth was measured as the widest area of necrotic tissue observed in the section. The extent of injury was computed in arbitrary units (a.u). The depth of injury for the sense and antisense treated groups was 400 ± 64 and 402 ± 52 (mean ± SEM), respectively. The breath of injury for the two groups ranged from 200 to 276 a.u. These data suggest that no differences were observed between the sense and antisense oligo-treated animals in the parameters measured. Intense astrogliosis was detected surrounding the injured area at 3 days post-injury. Hypertrophic astrocytes with thick processes stained heavily for their intermediate filament protein GFAP were observed (data not shown). The extent of gliosis appeared comparable between all groups of animals.

The time course for MCP-1 expression following mechanical injury in rat CNS has been previously reported [1]. MCP-1 staining is seen as early as 6 h after injury. By 24 h, immunoreactivity can be localized to macrophages,

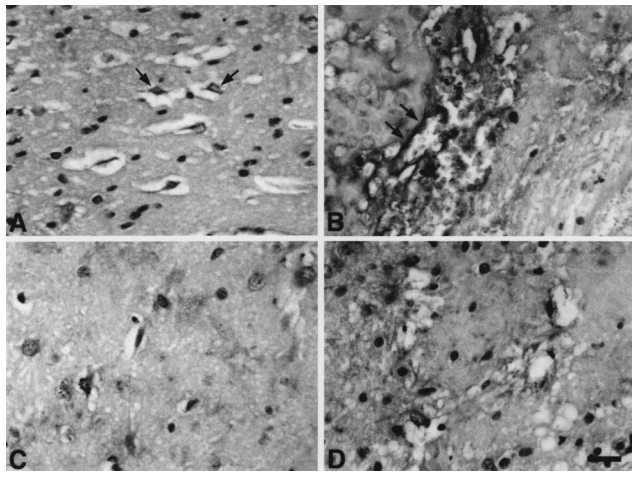

Fig. 1. Immunoreactivity for MCP-1 in sense (A,B) and antisense (C, D) oligonucleotides infused stab wound injured rat brains. The staining for MCP-1 in sense oligonucleotide treated brains is seen in a few cells (A, arrows) and is predominantly in the diffused form (B, arrows). Both cellular (C) and diffused (D) stainings are considerably reduced in antisense oligonucletide treated brains. Scale bar, 20 μm.

endothelial cells and glial cells and is also observed in the form of diffused reactivity suggestive of protein bound to the extracellular matrix. By 72 h, diffused staining is evident, however, specific cellular staining is somewhat diminished. To confirm that antisense oligo treatment resulted in decreased MCP-1 expression, sections were stained for MCP-1 immunoreactivity. As shown in Fig. 1A,B, in sense oligo-treated animals, MCP-1 reactivity at 72 h after lesioning was noted in a few cells (A) and was predominantly in the form of diffused staining (B). Similar staining pattern was observed in brain sections from the control animals (data not shown). In contrast, both cellular (Fig. 1C) and diffused staining (Fig. 1D) were considerably diminished after antisense oligo-treatment.

Sections of the stab wound injured rat brains were also immunostained with a marker specific for the monocyte-macrophage cells. The mouse anti-rat monocytes /macrophages antibody (MAB 1435) recognizes a single chain glycoprotein that is expressed on the lysosomal membrane of myeloid cells. The antigen is expressed by majority of the tissue macrophages and is weakly expressed by granulocytes. Strongly immunopositive cells were seen in the lesioned area in sections from sense oligo-treated animals. These cells appeared to be densely packed at the site of injury (Fig. 2A). Similar staining was observed in sections from vehicle treated animals (data not shown). In sections from chronically antisense oligo infused brains, fewer immunoreacitve cells were observed at the site of injury. Some of the cells also appeared to show weak immunostaining (Fig. 2B). Quantitative analysis of the immunopositive cells was performed to determine if MCP-1 antisense oligo treatment resulted in decreased number of monocytes at the site of injury. Cells showing strong MAB 1435 staining were counted in ten randomly selected fields spanning the entire area of injury from vehicle and sense/antisense oligo-treated animals. The cell counts ranged from 300 to 460 for the vehicle, 250 to 600 for the sense, and 100 to 500 for antisense oligo-treated group. As shown in Table 1, cell counts from vehicle and sense oligo-treated groups were comparable. However, cell counts from antisense oligo-treated animals showed a 30% decrease as compared to sense oligo-treated animals ($P < 0.05$).

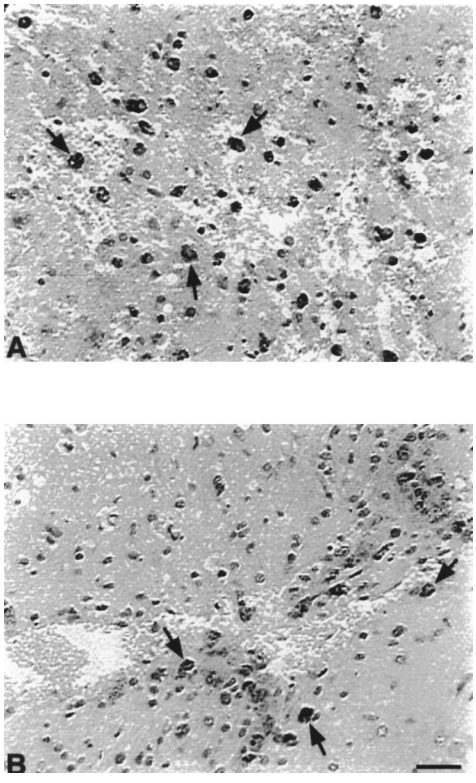

Fig. 2. Immunostaining for monocytes/macrophages in MCP-1 antisense oligonucleotides infused stab wound injured rat brain using the anti-monocyte macrophage antibody marker MAB 1435. Many monocytes (arrows) are observed at the site of injury in the sense oligonucleotide (A) compared to the antisense oligonucleotide (B) treated brains. Scale bar, 33 μm.

In the present study we used an antisense approach to selectively downregulate MCP-1 expression and study its effect on the recruitment of monocytes in stab wound injured rat brain. We show decreased monocytes at the lesion site in antisense oligo-treated animals (30%, $P < 0.05$). To our knowledge, this report is the first to demonstrate MCP-1 inhibition using antisense approach in rat brain trauma. Inhibition of MCP-1 using specific neutralizing anti-MCP1 antibodies has been shown to suppress inflammation in a range of pathological conditions [3,5, 11,17].

Our findings directly demonstrate that inhibition of MCP-1 expression results in reduced monocytic infiltration at the site of injury following trauma. The observation that sense oligo treatment did not result in decreased monocyte cell count supports the specificity of the antisense treatment. Furthermore, decreased MCP-1 immunoreactivity was also observed in brain sections from antisense oligo-treated animals compared to sense oligo-treated animals. The variability in stab wound injury from one animal to another was accounted for by comparing sections with similar lesioned areas.

To maintain effective concentrations of the antisense oligos, we chronically infused them directly into the lesion via osmotic minipumps for up to 3 days. Infusion was initiated immediately after injury as previous studies have shown that MCP-1 expression increases within hours after injury [1]. Although most studies use phosphorothioate oligodeoxynucleotides, the present study did not use chemically modified oligos. Continuous exposure of endogenous cells to MCP-1 antisense oligos at the site of injury may have been sufficient to decrease MCP-1 expression. In addition, unmodified oligonucleotides have shown to be effective in organs which have a very low nuclease content such as the CNS and the cerebrospinal fluid [2].

A 30% reduction in the number of MAB 1435 positive cells was seen at the site of injury. Perhaps increasing the amount of antisense oligos, complexing them with liposomes, or extending the time period for delivery would

Table 1

Effect of MCP-1 antisense oligonucleotides on macrophage infiltration in stab wound injured rat brain

Treatment	Cell count ± SEM
Vehicle alone ($n = 4$)	383 ± 37
Sense oligonucleotide treatment ($n = 9$)	416 ± 52
Antisense oligonucleotide treatment ($n = 9$)	292 ± 50*

Monocytes/macrophages were immunostained using the anti-monocyte macrophage antibody marker MAB 1435 and counted per standard lesion area. The number of macrophages following antisense treatment were reduced by 30% (*) compared to sense treated animals ($P < 0.05$, Student's t-test).

result in further reduction. A complete reduction in monocytes may not be possible as other chemokines expressed following brain trauma may play a role in the recruitment of inflammatory cells at the site of lesion. We have previously demonstrated the expression of two beta chemokines (MIP-1β, RANTES) in stab wound injured rat brain [7]. The effect of MCP-1 inhibition on the expression of these chemokines is not known. It is likely that they may exhibit greater increases due to compensatory mechanisms. To what extent these factors would induce monocytic infiltration in the absence of MCP-1 can only be determined in MCP-1 knockout animals. Perhaps a complete reduction in the hematogenous cell population may not be beneficial following injury. Limited macrophage infiltration after injury may aid in clearing debris and contributing neurite enhancing factors. Macrophages have been shown to produce factors that promote wound healing [15].

The present data point to the feasibility of using the antisense approach in inhibiting MCP-1 expression in vivo to modulate monocytic infiltration. Other strategies to target chemokines have included the use of anti-chemokine antibodies, chemokine antagonists, and gene therapy [14]. Experiments to determine whether decreased cellular invasion is effective in diminishing inflammation and secondary tissue damage following traumatic brain injury are currently under investigation. As more knowledge regarding chemokine structure and its interaction with specific receptors is obtained, their potential as therapeutic targets to control inflammation can be further exploited.

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