

MIP-1 α and MCP-1 Induce Migration of Human Umbilical Cord Blood Cells in Models of Stroke

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Abstract: Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein (MIP-1 α) are implicated in monocyte infiltration into the central nervous system (CNS) under pathological conditions. We previously showed that *in vivo* human umbilical cord blood cells (HUCB) migrate toward brain injury after middle cerebral artery occlusion (MCAO). We hypothesized that MCP-1 and MIP-1 α may participate in the recruitment of HUCB towards the injury. Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO), and 24 hours later the production of MCP-1 and MIP-1 α in the brain was examined with immunohistochemistry, ELISA, and western blotting. The chemotactic effect of MCP-1 and MIP-1 α , and the expression of MCP-1 receptor CCR2 and MIP-1 α receptor CCR1, CCR5 on the surface of HUCB were also examined. MCP-1 and MIP-1 α expression were significantly increased in the ischemic hemisphere of brain, and significantly promoted HUCB cell migration compared to the contralateral side. This cell migration was neutralized with polyclonal antibodies against MCP-1 or MIP-1 α . Also chemokine receptors were constitutively expressed on the surface of HUCB cells. The data suggested that the increased chemokines in the ischemic area can bind cell surface receptors on HUCB, and induce cell infiltration of systemically delivered HUCB cells into the CNS *in vivo*.

Key Words: β chemokines, stroke, human umbilical cord blood, migration, Transplantation.

INTRODUCTION

Intravenous delivery of human umbilical cord blood cells (HUCB) decreases neural damage in rodents subjected to middle cerebral artery occlusion (MCAO), and provides better behavioral and neurological recovery in a rodent model of stroke compared to direct intracranial delivery (Willing *et al.*, 2003). While an additional advantage of the systemic route is the ease of delivery, how these cells migrate into the brain to produce their effects is not clear, since the brain is insulated by the blood brain barrier (BBB) and has immune privilege. One possibility is that BBB is disrupted after the ischemic insult and granulocytes, macrophages, and other inflammatory cells leak into the ischemic brain passively. A second possibility is that stroke up-regulated chemoattractants, which promote HUCB cell migration into the brain. One of the potential chemoattractants is monocyte chemoattractant protein-1 (MCP-1). MCP-1 has been detected in injured brain after stroke (Babock *et al.*, 2003), predominantly in astrocytes (Che *et al.*, 2001). Other studies found that hypoxia-ischemia increases MCP-1 expression in multiple cell types around the site of injury in neonatal rodent

brain (Ivacko *et al.*, 1997). Following MCP-1 expression, leukocytes could be found in the lesioned hippocampus. In addition, mutant mice with a CCR2 (MCP-1 receptor) deficit had neither T cells nor macrophage infiltration in the denervated hippocampus, suggesting a critical role for MCP-1 and its receptor CCR2 in leukocyte migration (Babock *et al.*, 2003).

Another potential chemoattractant implicated in the trafficking of lymphoid and mononuclear cell into the CNS is macrophage inflammatory protein (MIP-1 α). It is up regulated as early as 3 to 6 hr post stroke in the ipsilateral hemisphere to the stroke, whereas the level of MIP-1 α in the contralateral hemisphere was similar to control levels (Kim *et al.*, 1995a; Takami *et al.*, 1997). MIP-1 α mRNA was also found present in an immortalized microglia cell line, cortical astrocytes and monocytes in culture (Murphy *et al.*, 1995). *In vivo*, MIP-1 α expression was correlated with mononuclear cell infiltration (Glabinski *et al.*, 1998). Further, extracts from ischemic brain induced human bone marrow stromal cell migration in culture, and this cell migration could be blocked by an MIP-1 α antibody, suggesting a chemoattractant effect of MIP-1 α (Wang *et al.*, 2002).

In this study we examined whether MIP-1 α and MCP-1 expression increased in the damaged hemisphere after stroke and if the presence of these chemokines in extracts of stroke brain would induce HUCB migration in an *ex vivo* assay system.

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MATERIALS AND METHODS

Middle Cerebral Artery Occlusion (MCAO)

Twenty Sprague Dawley rats were randomly assigned to the MCAO or normal groups as previously described (Willing *et al.*, 2003). Briefly, animals were anesthetized with isoflurane (2-5% in 2 L/min O₂). The right common, external and internal carotid arteries were isolated and an embolus inserted retrogradely through the external carotid, into the internal carotid, past the base of the skull to the origin of the MCA (approximately 25mm from insertion). The filament was permanently anchored in place and the incision closed. All animals were euthanized 24h post surgery, and the brains were harvested. Some of the brains were fixed with 4% paraformaldehyde in PBS, and the others were immediately frozen in liquid nitrogen.

Neural Cell Hypoxia Culture

Neurons, astrocytes and microglia were isolated from fetal rat brain at embryonic day (E) 17 as described (Mackay, 2001; Rothenberg *et al.*, 1999), and grown separately in culture to confluency. Before hypoxia, the gas-tight chamber (Hornung *et al.*, 2000; Wang *et al.*, 2002) was flushed with 5% CO₂ and 95% N₂ and the plates washed with low glucose color free DMEM medium. For the hypoxia group, medium was changed to 1 ml hypoxia pre-treated, low glucose, color free DMEM and cultures placed in the gas-tight chamber. The cultures were exposed to hypoxia (5% CO₂ and 95%N₂) at 37°C for 2 hours (Lombardi *et al.*, 2003). The control group was treated with low glucose color free DMEM under normoxic conditions for 2 hour. The media were harvested, and cells prepared for western blotting.

Immunohistochemistry Staining

MIP-1 α (or MCP-1) Double Labeling

The fixed brains were sectioned on a cryostat at 30 μ m thick. The sections were blocked with 10% goat serum, 0.3% Triton X100 in PBS for 1 hour, and then incubated with primary antibody cocktail at 4°C overnight. The cocktail consisted of goat anti MIP-1 α (Santa Cruz; 1:100) or rabbit anti MCP-1 (Novus; 1:100) with either mouse anti OX-42 (CD11b/c) (Abcam; 1:100), mouse anti TuJ1 (Chemicon; 1:400) or chicken anti GFAP (Chemicon; 1:100). After washing, the sections were incubated with a secondary antibody cocktail of rhodamine-conjugated goat anti-rabbit IgG (Molecular Probes; 1:500) and FITC-conjugated goat anti-mouse IgG (Molecular Probes; 1:500). The sections were mounted and examined with a confocal microscope.

Chemokine Receptor Immunolabeling

HUCB smears were fixed with 4% paraformaldehyde, washed and then air dried. The slides were stained with primary and secondary antibody as described above. The primary antibodies used were goat anti-human CCR5 (Capralogics Inc, 1:200), rabbit anti- human CCR1, and mouse anti-human CCR2 (Abcam Inc, 1:200). Secondary antibodies were FITC-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG (Molecular Probes; 1:500) (Molecular Probes; 1:500).

Western Blotting

The brains were cut sagittally into stroke side (ipsilateral to MCAO) and non-stroke side (contralateral to MCAO) and the tissue lysed. An equivalent amount of protein was loaded on SDS-12% polyacrylamide gel and transferred to nitrocellulose paper. The membranes were immunoblotted with anti-rat MIP-1 α (Chemicon) followed by horseradish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed using enhanced chemiluminescence dye (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) and autoradiographed. Neural cells from culture were treated similarly.

MCP-1 ELISA Assay

The brains were cut sagittally into stroked side and non-stroked side. Tissues were weighed, placed in clear DMEM (Gibco, 150mg/ml), homogenized and centrifuged at 2000g for 20 minutes. The supernatants were collected, filtered and adjusted to the same protein concentration. The ELISA assay was performed according to manufacturer's protocol (Amersham Bioscience), and the concentration of chemokine was determined on a plate reader at absorbance of 450nm and 550nm.

Cell Migration Assay

The standard (chemokine protein) or tissue extract samples (300 μ L) with or without MCP-1 or MIP-1 α antibodies (1:100) were pipetted into the bottom wells of a 96-well plate. Freshly thawed HUCB cells were directly pipetted into the top well at a concentration of 100,000 cells per 60 μ L. The migration chamber was incubated at 37°C with 5% CO₂ from 4 hours to 24 hours. The top well plate was then removed and the bottom plate was centrifuged, and 200 μ l media removed. The number of migrated cells was determined with Cell Titer-Glo Luminescent Cell Viability Assay, (Promega) according to the manufacturer's protocol. The plate was read in a plate reader. The migration assays were performed twice. Each sample, control and standard was performed in triplicate.

RESULTS

Presence of MIP-1 α and MCP-1 in the Stroked Brain

Both MCP-1 and MIP-1 α were found in the brain 24 hour after MCAO using immunolabeling. No positive staining was found in the contralateral hemisphere. Double immunohistochemistry staining revealed that both MCP-1 and MIP-1 α were found in neurons (Fig. (1)), astrocytes (Fig. (2)), and some microglia (Fig. (3)). MIP-1 α expression was verified with western blotting (Fig. (4A)) from extracts of stroked brain. Further, in enriched cultures, MIP-1 α was expressed in cultured astrocytes and neurons. After hypoxia, MIP-1 α increased in neurons compared to neurons only exposed to normoxia conditions (Fig. (4B)). MCP-1 expression was verified with ELISA. It was significantly increased on the stroked side of the brain compared to the contralateral side after stroke ($p < 0.05$) (Fig. (5A)). In enriched cultures, hypoxia induced MCP-1 expression particularly in microglia and astrocytes ($p < 0.05$) (Fig. (5B)).

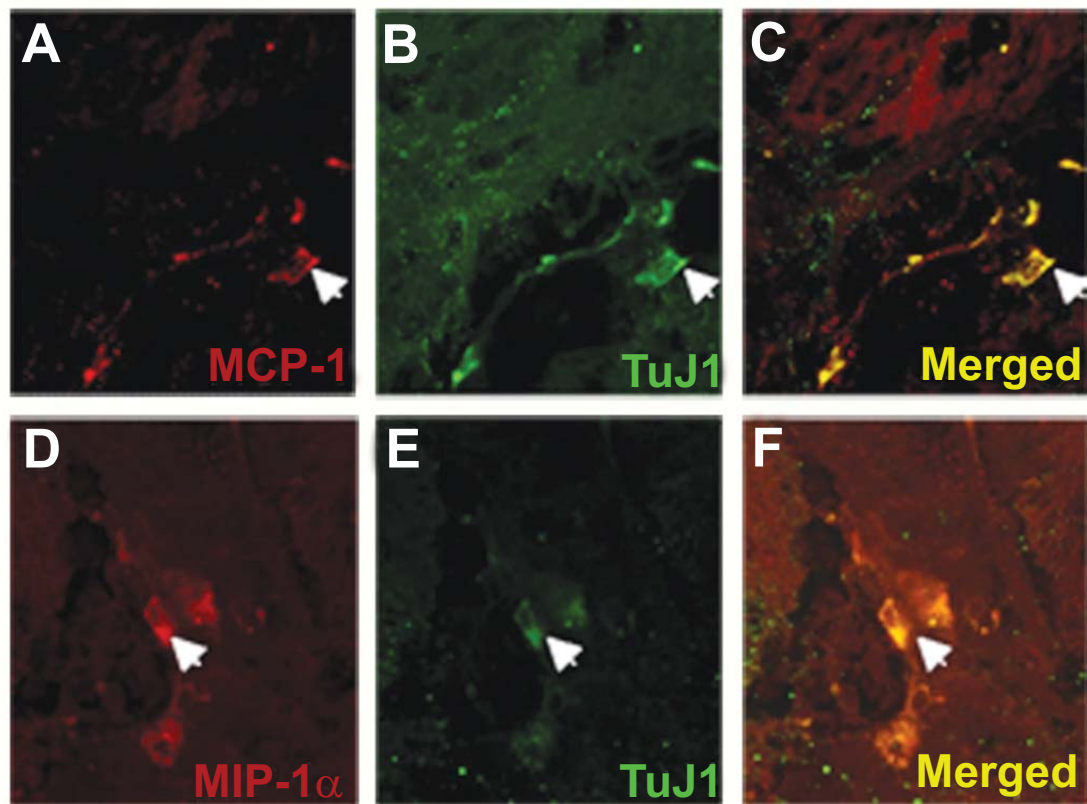


Fig. (1). MCP-1 and MIP-1 α expression in neurons after stroke. Stroked rat brain was immunolabelled with MCP-1 (A, red) and then labeled with an antibody to TuJ1 to identify immature neurons (B, green). (C) MCP-1 was found in neurons on the ipsilateral (stroked) side of the brain. (D) MIP-1 α (red), (E) TuJ1 and (F) MIP-1 α positive neurons (TuJ1, yellow, arrows).

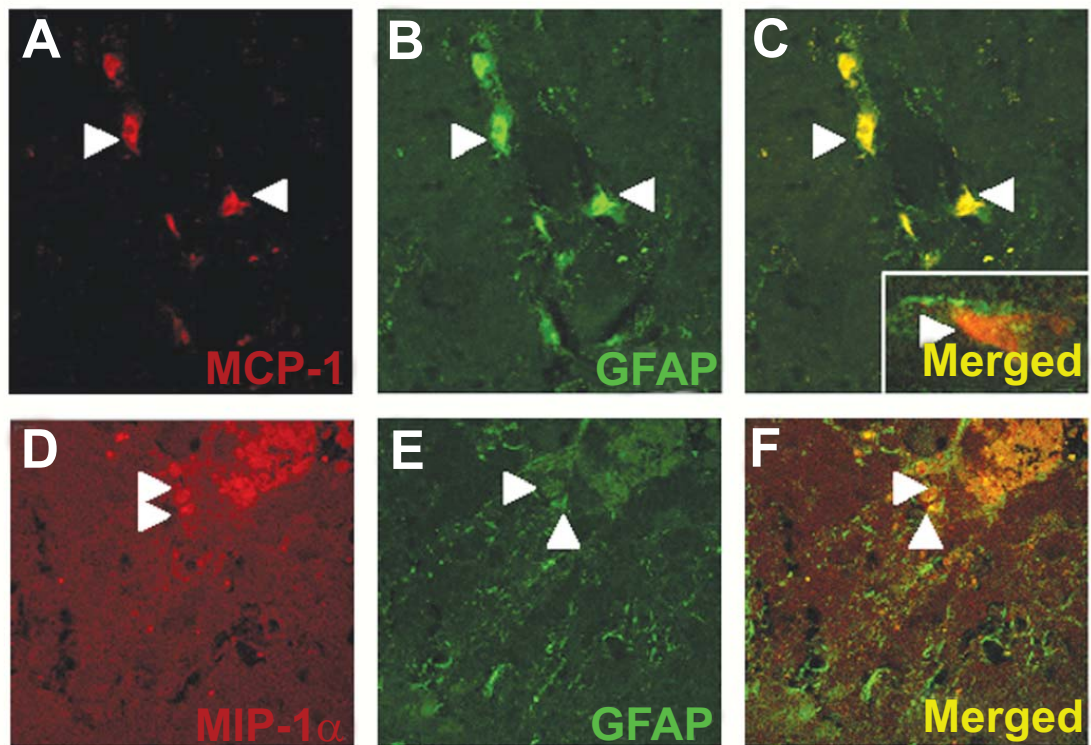


Fig. (2). MCP-1 and MIP-1 α expression in astrocytes after stroke. Stroked rat brain was immunolabelled with MCP-1 (A, C, red) or MIP-1 α (D, F, red), and then double labeled with antibody to GFAP (astrocytes, B, E, green). Both MCP-1 and MIP-1 α could be found in astrocytes of the infarcted area (merged C, F). Inset in C showing that not all GFAP+ astrocytes expressed MCP-1.

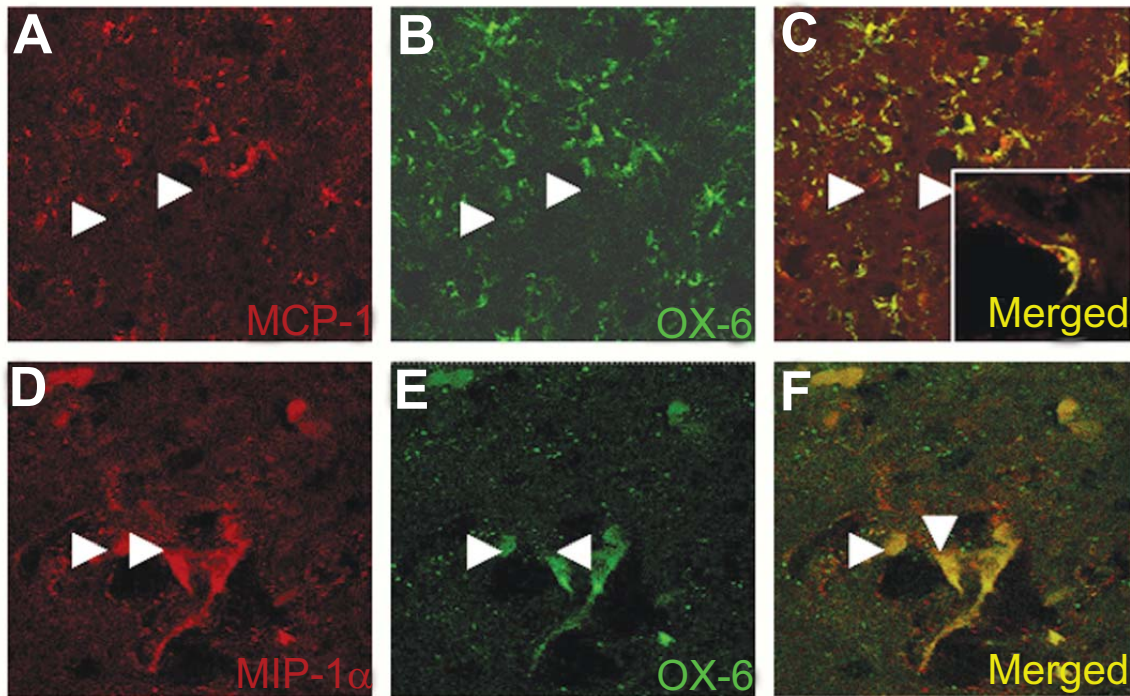


Fig. (3). MCP-1 and MIP-1 α expression in microglia after stroke. Stroked rat brain was immunolabelled with MCP-1 (A, C, red) or MIP-1 α (D, F, red), and then double labeled with antibody to OX-6 (microglia, B, E, green). Both MCP-1 and MIP-1 α could be found in microglia of the infarcted area (merged C, F).

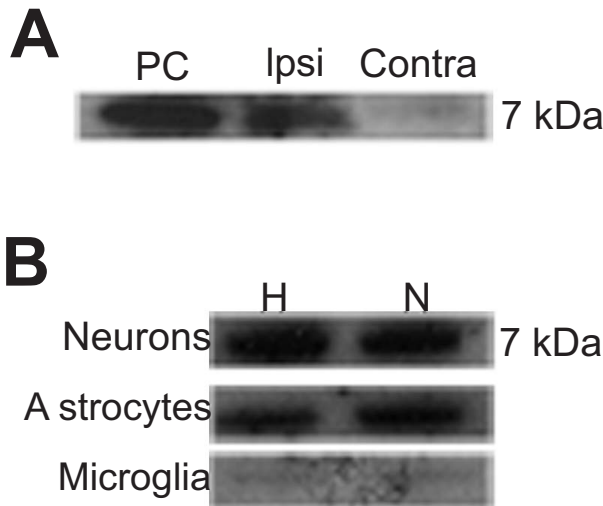


Fig. (4). Western blot analysis of MIP-1 α protein expression in the brain after MCAO. (A) MIP-1 α was present within the ipsilateral (Ipsi, stroked) hemisphere and not on the contralateral side of the brain (Contra). PC = positive control. (B) MIP-1 α was expressed in enriched cultures of neurons, astrocytes and microglia under hypoxic (H) and normoxic (N) conditions.

Chemotactic Effect of MCP-1 and MIP-1 α on HUCB Cell Migration

MIP-1 α receptor CCR1 and CCR5 as well as MCP-1 receptor CCR2 were all expressed on the cell surface of HUCB cells (Fig. (6)) suggesting that HUCB cells could respond to expression of these chemokines. Indeed, MIP-1 α induced migration *in vitro*, especially at the lowest dose, 30 ng/ml ($p < 0.05$; Fig. (7A)). HUCB cell migration to MCP-1 reached its maximum at a concentration 600 ng/ml although

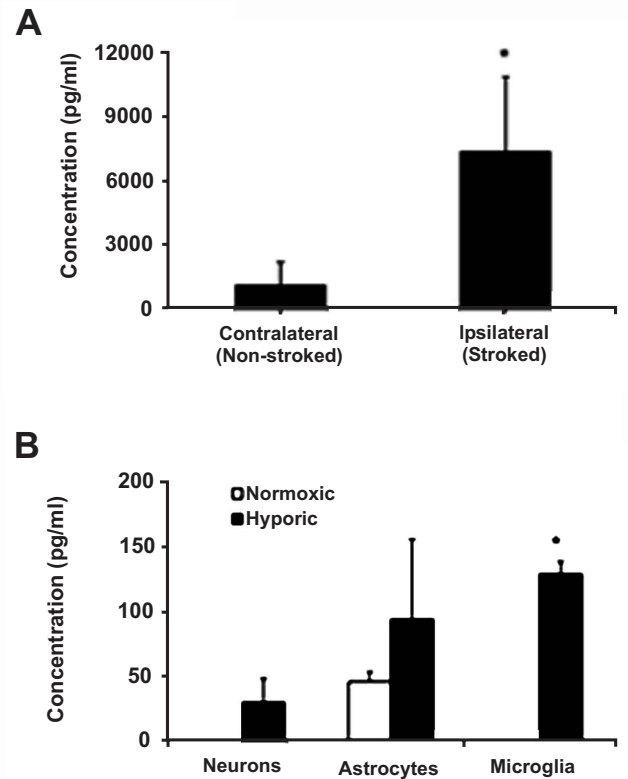


Fig. (5). Protein expression in MCP-1 brain after MCAO tissue and in enriched neural cell cultures. (A) MCP-1 expression increased on the stroked side of the brain as determined with ELISA. (B) After enriched cultures were exposed to hypoxia, MCP-1 concentration in the media of microglia and astrocyte cultures was significantly higher than in neuronal medium (*, $p < 0.05$).

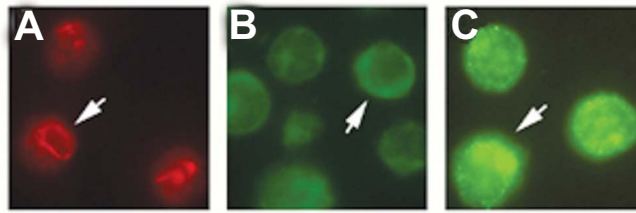


Fig. (6). MIP-1 α receptor CCR1 (A), CCR5 (B) and MCP-1 receptor CCR2 (C) were present on the surface of HUCB cells ($\times 100$).

this was not significantly different from the lower doses. In contrast, the 400 ng/ml concentration was significantly different from the 30 ng/ml, 50 ng/ml and 100 ng/ml doses, respectively (* $p < 0.05$; Fig. (7B)).

Effect of Brain Tissue Extract on HUCB Cell Migration

Tissue extract from the stroked brain induced more HUCB cells to migrate across a membrane than extract of the non-stroked side did ($p < 0.05$; Fig. (8A & 8B)). Antibodies to both MIP-1 α (Fig. (8A)) and MCP-1 (Fig. (8B)) antibody depressed migration toward non-stroked values ($p < 0.05$).

DISCUSSION

MIP-1 α was observed on the ipsilateral injured side of the brain 24 hours after MCAO as determined with immunohistochemistry and western blotting. Our results are consistent with previous studies that reported MIP-1 α expression as early as 1 hour after onset of the MCAO and which peaked at 4-6 hour post surgery in the injured hemispheres (Takami *et al.*, 1997; Kim *et al.*, 1995). MIP-1 α has also been found to increase after other brain injuries such as ischemia (Takami *et al.*, 1997), stab wound (Ghirnikar *et al.*, 1996), hypoxia, olfactory target ablation (Thomas *et al.*, 2002).

The majority of MIP-1 α in the body is produced by circulating monocytes. In the CNS, the inducible MIP-1 α could exist in astrocytes, microglia, endothelial cells or neurons. Controversy exists, with some groups finding MIP-1 α in the astrocytes (Kim *et al.*, 1995b; Miyamoto and Kim, 1999) while others report that it is only produced by Mac-1 mRNA-positive cells including microglia/macrophages (Takami *et al.*, 1997). Babcock *et al.* (2003) observed that the MIP-1 α was expressed by glial cells and could direct leukocytes to the CNS after axonal injury (Che *et al.*, 2001). Other investigators indicated MIP-1 α was expressed by neuronal cells in culture (Miyamoto and Kim, 1999). Immunostaining shows that MIP-1 α can be in all three neural cells, but western blotting shows MIP-1 α was mainly produced by astrocytes and neurons. The inconsistency between these studies most likely reflects differences between animal models and cell culture techniques.

Similar to MIP-1 α , MCP-1 was also found in the ischemic brain around the infarct. ELISA assay revealed that the amount of MCP-1 on the stroked side was 7 fold greater than the contralateral side. MCP-1 was produced by neurons, astrocytes and microglial cells. This is consistent with previous work showing that MCP-1 was detected as early as 3 to 6 hr post stroke in the ipsilateral hemisphere (Babcock *et al.*, 2003) and peaked at 2-3 days post MCAO (Che *et al.*, 2001). When we examined cultures enriched for neurons, astrocytes or microglia, MCP-1 was mainly expressed in microglia and astrocytes after hypoxia. Using immunohistochemistry double staining, Che *et al.* (2001) found the majority of MCP-1 positive cells were astrocytes. This is consistent with work in the cortico-striatal slice (Norberg *et al.*, 1999).

However, what leads to MCP-1 and MIP-1 α upregulation after stroke is not known. Recent studies revealed that the production of MCP-1 and MIP-1 α was regulated by pro- and anti-inflammatory cytokines induced by the ischemic insult.

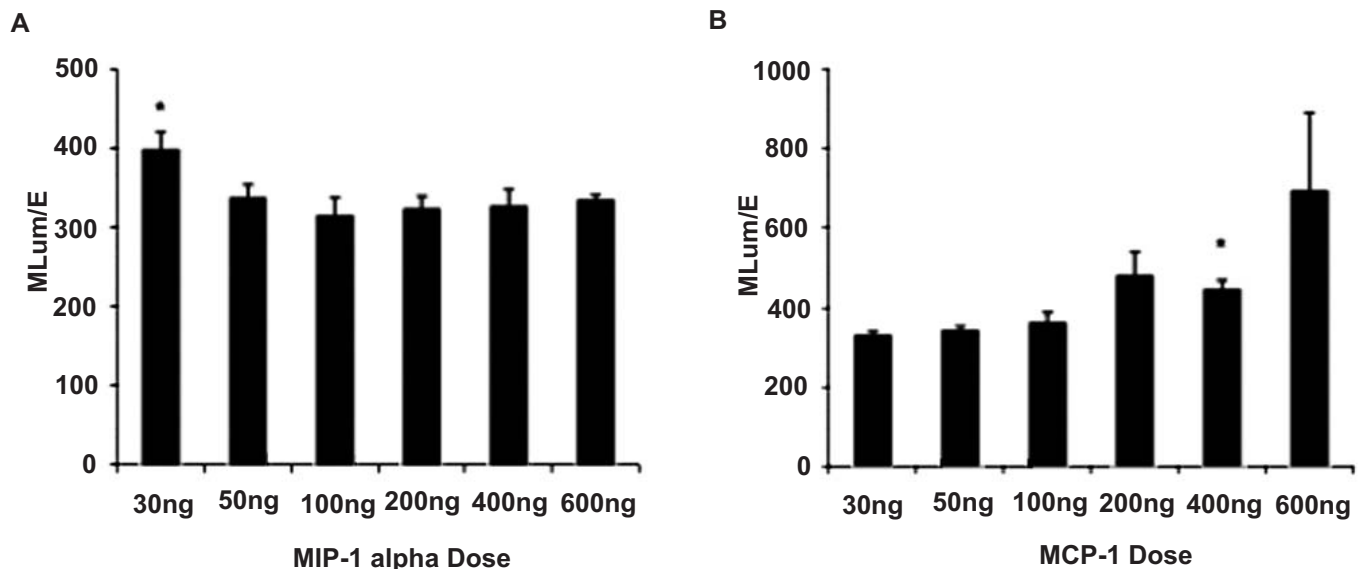


Fig. (7). Chemokines dose dependently induced HUCB cell migration. (A) MIP-1 α at a dose of 30 ng/ml produced optimal HUCB migration after 12 hour incubation, compared to all other MIP-1 α concentrations. (B) HUCB cell migration reached its maximum at a concentration of 600 ng/ml. The 400 ng/ml concentration was significantly different from the 30 ng/ml, 50 ng/ml and 100 ng/ml doses, respectively (* $p < 0.05$).

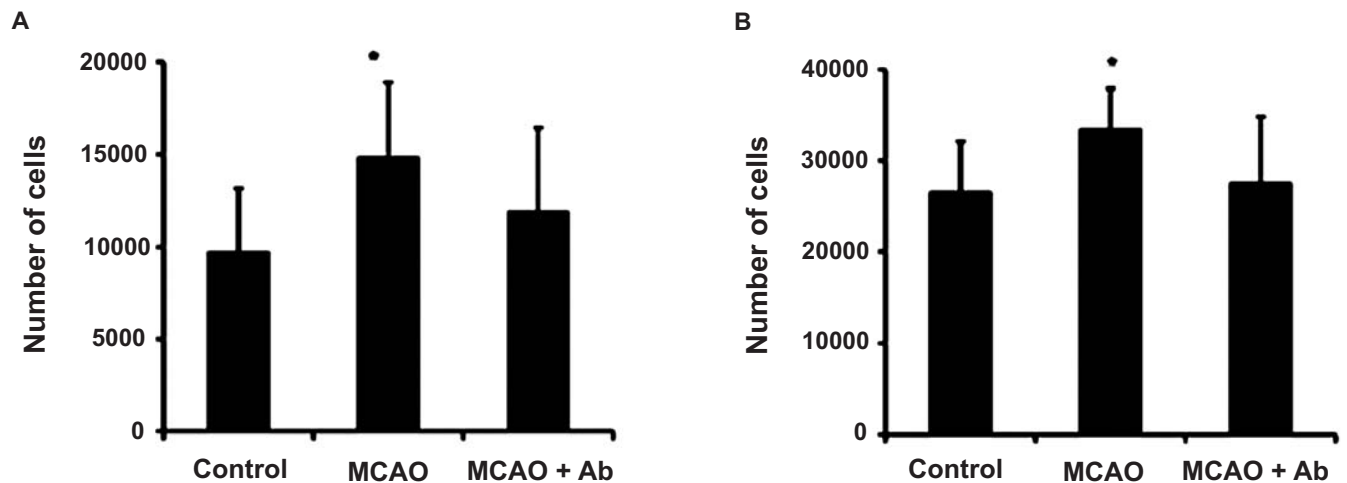


Fig. (8). Effect of MIP-1 α and MCP-1 on HUCB migration to brain tissue extract. (A) Extracts from the stroked side of the brain attracted more HUCB cells compared to the non stroked side, and this chemotactic effect was significantly depressed by MIP-1 α antibody; (B) MCP-1 antibody also depressed the migration of HUCB to ischemic brain extract. (* $p < 0.05$).

Interleukin (IL)-1 β production increased shortly after the onset of the stroke (Minami *et al.*, 1991); following that, MCP-1 and MIP-1 α production increased. Further, dexamethasone and IL-10 (both anti-inflammatory) significantly reduced MCP-1 expression. However, in knockout mice where interleukin-1 converting enzyme (ICE), a cysteine protease that cleaves inactive pro-IL-1 β to generate mature IL-1 β , was removed, MCP-1 expression was similar to wild type animals when animals underwent severe hypoxia. With a less severe hypoxic incident, ICE $^{-/-}$ did attenuate MCP-1 expression compared to wild type animals (Xu *et al.*, 2001). The inductive MCP-1 expression is regulated by ligands that trigger nuclear factor kappa B (NF-kappa B) DNA binding. High doses of IL-6 treatment remained without effect (Thibeault *et al.*, 2001). The early expression of IL-1 β could activate the NF-k β promoter to induce expression. The functional NF-k β then binds the promoter of MIP-1 α , and induces MIP-1 α production. When IL-1 β expression is blocked, MIP-1 α expression is inhibited (Guo *et al.*, 2004).

The production of MCP-1 and MIP-1 α could in turn elicit blood cell migration into the brain. In a stab wound model, MIP-1 α induced T cell and neutrophil infiltration into the brain 3 days after surgery; monocytes/macrophages were present in the injured area 12 days post injury (Ghirnikar *et al.*, 1996). In a human blood-brain-barrier model, MIP-1 α expression increased shortly after amyloid-beta stimulation, and was followed by monocyte migration from the blood side to the brain side (Fiala *et al.*, 1998). Further, in MIP-1 α knock out animals, there is a decrease in CD8 α^{-} dendritic cell migration into the CNS after infection with mouse hepatitis virus (MHV) (Trifilo and Lane, 2004). In addition, both MCP-1 and MIP-1 α are involved in inflammatory cell recruitment in other tissues (Cook *et al.*, 1995; Domachowski *et al.*, 2000) after infection. MCP-1 and MIP-1 α increase in hippocampus after entorhinodentate lesions prior to T cell and macrophage migration into the denervated hippocampus. When CCR2 (MCP-1 receptor) knockout animals were used, the cell migration was quenched, demonstrating a critical role for the CCR2 ligand MCP-1/CCL2 in leukocyte migra-

tion. Cellular infiltration was not altered by a mutation to CCR5 (a receptor of MIP-1 α and RANTES/CCL5) (Che *et al.*, 2001). The deletion of MCP-1 may also result in up-regulation of other cytokines (Ferreira *et al.*, 2005), suggesting a key role of MCP-1 in a cytokine network.

These findings suggested that MCP-1 and MIP-1 α may be responsible for HUCB cell infiltration into the CNS after intravenous administration in stroke animals. However, it can be argued that a large enough difference in species homology could result in no HUCB response to rodent chemokine signals. This is unlikely since our interface migration system did induce HUCB migration, and exhibited a dose effect. This could be due to the conservation of both MCP-1 and MIP-1 α ligands and their receptors across species (Nibbs *et al.*, 1998; Rutledge *et al.*, 1995). In addition, we found constitutive expression of MCP-1 and MIP-1 α receptors on HUCB.

In conclusion, we hypothesize that HUCB migration after ischemia begins with IL-1 β secretion at the injury site, which initiates MCP-1 and MIP-1 α secretion in activated astrocytes, microglia and some neurons. The accumulated MCP-1 and MIP-1 α form a concentration gradient and when combined with the receptor at the surface of HUCB cells, induce migration of systemically administered HUCB cells.

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