Mcp-1 And App Involvement Of Glial Differentiation And Migration Of Neuroprogenitor Cells

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MCP-1 AND APP INVOLVEMENT IN GLIAL DIFFERENTIATION AND MIGRATION OF NEUROPROGENITOR CELLS.

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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ABSTRACT

Neuroprogenitor cells are an important resource because of their potential to replace damaged cells in the brain caused by trauma and disease. It is of great importance to better understand which factors influence the differentiation and migration of these cells. Previously it has been reported that neuroprogenitor cells undergoing apoptotic stress have increased levels of Amyloid precursor protein (APP) and increased APP expression results in glial differentiation. APP activity was also shown to be required for staurosporine induced glial differentiation of neuroprogenitor cells. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is expressed during inflammatory. The binding of MCP-1 to its chemokine receptor induces expression of novel transcription factor MCP-1 induced protein (MCPIP). MCPIP expression subsequently leads to cell death. Previous studies have shown that pro apoptotic factors have the ability to induce neural differentiation. Therefore, we investigated if MCPIP expression leads to differentiation of NT2 neuroprogenitor cells. Results showed that MCPIP expression increased glial fibrillary acid protein expression and also caused distinct morphological changes, both indicative of glial differentiation. Similar results were observed with MCP-1 treatment. Interestingly, APP expression decreased in response to MCPIP. Instead, we found APP activity regulates expression of both MCP-1 and MCPIP. Furthermore, inhibition of either p38 MAPK or JAK signaling pathways significantly reduced APP’s effect on MCP-1 and MCPIP. These data demonstrate the role APP has in glial differentiation of NT2 cells through MCP-1/MCPIP signaling. It is possible that increased APP expression after CNS injury could play a
role in MCP-1 production, possibly promoting astrocyte activation at injured site. We next investigated the effect that MCP-1 has on NT2 cell migration. Studies have shown that when neuroprogenitor cells are transplanted into the brain they migrate towards damaged areas, suggesting that these areas express factors that recruit migrating cells. Generally, after neuronal injury there is a neuroinflammatory response that results in increased chemokine production. We demonstrate that MCP-1 significantly induces the migration of NT2 neuroprogenitor cells. Activation of intracellular cyclic adenosine monophosphate (cAMP) or protein kinase C with forskolin and phorbol 12-myristate 13-acetate (PMA), respectively, was able to completely abolish the MCP-1 induced migration. Contrarily, neither extracellular signal-regulated kinase or p38 mitogen activated protein kinase was required for NT2 cells to respond to MCP-1. Interestingly, APP’s ability to activate MCP-1 expression was shown to play a role in NT2 cell migration. We showed that NT2 cells expressing APP were capable of inducing migration of other neuroprogenitor cells. Utilizing a MCP-1 neutralizing antibody we discovered that APP induced migration was not caused solely by increased MCP-1 production. Interestingly, APP increased expression of several C-C chemokines: MCP-1, Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), and macrophage inflammatory protein alpha (MIP-1 alpha). This demonstrates the unique role APP has in regulating chemokine production, which directly affects cell migration. Taken together, this study provides us with a greater understanding of the mechanisms involved in both glial differentiation and migration of NT2 neuroprogenitor cells.
I would like to give special thanks to my parents for their motivation and support throughout my college career.
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PART 1. MCP-1 INVOLVEMENT IN GLIAL DIFFERENTIATION OF NT2 NEUROPROGENITOR CELLS THROUGH APP

Introduction

Chemokines are a family of conserved cytokines that have been shown to induce the migration of several leukocyte subpopulations into damaged tissues. In the adult brain, microglia and astrocytes are believed to be the main source of chemokine production (Hayashi et al. 1995; Karpus et al. 1995; Oh et al. 1999; Coughlan et al. 2000). The central nervous system (CNS) has been shown to produce chemokines in response to several inflammatory and disease conditions, including allergic encephalitis, Alzheimer’s disease, multiple sclerosis, ischemia-induced neurodegeneration, and trauma (Glabinski and Ransohoff 1999). Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C chemokine family, is a potent chemotactic factor for monocytes (Rollins 1997). A recent study showed that the binding of MCP-1 to its receptor, CCR2, induces the expression of a novel transcription factor, MCP-induced protein or MCPIP. MCPIP expression up regulates several pro apoptotic genes, which leads to cell death (Zhou et al. 2006). Other than their neuro inflammatory properties, chemokine function in the CNS is still not fully understood.

Amyloid precursor protein (APP) is a ubiquitous glycoprotein receptor that is involved in the pathogenesis of Alzheimer’s disease. APP undergoes enzymatic processing to produce fragments with various functions (De Strooper and Annaert 2000). One of the fragments, beta-amyloid peptide (AB), is a main constituent of senile plaques (De Strooper and Annaert 2000; Golde 2003) seen in Alzheimer’s disease.
While the physiological function of APP is not fully understood, its structure suggests that APP might function as a receptor via its G$_0$ binding domain or as a ligand via its soluble N-terminal domain (Yamatsuji et al. 1996). Previously, neural stem cells undergoing apoptotic stress showed an increase in expression of APP and the surrounding cells underwent glial differentiation (Marutle et al. 2007). APP expression is capable of inducing glial differentiation and that neural stem cells transplanted into APP transgenic mice differentiated predominantly into astrocytes (Kwak et al. 2006b). APP was also shown to be required in pro apoptotic factor staurosporine induced glial differentiation of neuroprogenitor cells (Kwak et al. 2006a). These results demonstrate that increased APP signaling under apoptotic conditions causes glial differentiation. In this study, we demonstrate that APP is capable of inducing expression of both MCP-1 and MCPIP. Increased levels of MCP-1 and MCPIP were able to activate glial differentiation in NT2 neuroprogenitor cells.
Materials and Methods

Cell Culture

The NTera-2/Clone D1 cells (NT2) were plated at a density of 5 X10^6 per 75cm^2 in tissue culture treated flask (Corning). Cell culture media was Dulbecco’s modified Eagle’s medium with F-12 (DMEM/F12, Invitrogen), supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologics). An incubation chamber was used to maintain a humidified atmosphere of 5% CO\textsubscript{2} and 37\textdegree C. NT2 cells were passed twice a week by 0.05% Trypsin/EDTA (Invitrogen) treatment.

Transfection

Transfection of plasmid DNA was performed with Lipofectamine 2000 (Invitrogen). Transfections were performed on subconfluent NT2 cells in 6 well plates (Corning) or 35 mm glass bottom dishes (MatTek). Transfection efficiency was determined by observation of GFP expression under fluorescence microscope or immunoblot analysis with GFP antibody. AICD plasmid transfection efficiency was determined by co-transfection with GFP plasmid. Plasmids: pEGFP-C1 (Clonetech), pCDNA4/His-Max TOPO (Invitrogen), pCEP-APP-GFP, MCPIP-GFP (provided by Dr. Pappachan E. Kolattukudy, University of Central Florida), AICD pCDNA3 APP-C59 (provided by Dr. Fuyuki Kametani, Tokyo Institute of Psychiatry).

Small interference RNA

Small interference (siRNA) for human APP695 (Accession: A33292) was designed using Ambion software. Target sequence for APP695 (5’ATCTTTGGAACAGGAAGCAG3’). Preparation of siRNA was done using Silencer
Expression (Ambion). SiRNA cassette was then subcloned into pCDNA4/His-Max TOPO plasmid (Invitrogen).

Reagents and Antibodies

**Primary Antibodies:** rabbit anti-GFAP (Promega); mouse anti-APP (22C11) (Chemicon); mouse anti-GFP (Invitrogen); rabbit anti-Beta Actin (Cell Signaling); mouse anti-Cleaved PARP (Cell Signaling); mouse anti-ERK; mouse anti-ERK1/2 (pT202/pY204) (BD Biosciences); rabbit ant-STAT3 (BD Biosciences); rabbit anti-STAT3 (pY705) (Cell Signaling); mouse anti-p38 MAPK; mouse anti-p38 MAPK (pT180/pY182) (BD Biosciences). **Secondary antibodies:** anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Amersham); TRITC conjugated antibody (Jackson Immuno). **Reagents and Kinase Inhibitors:** Secreted type Amyloid Precursor protein alpha S9564-25UG (Sigma). Trypan blue (Sigma); JAK1 inhibitor (calbiochem); p38 MAPK inhibitor-SB203580 (Sigma); and ERK1/2 inhibitor-PD098059 (Sigma). Cells were pretreated with kinase inhibitors for 45 mins prior to plasmid transfection.

Immunoprecipitation and Immunoblot analysis

Protein samples were collected from cells lysed by ice cold lysis buffer consisting of 1% NP40, 150mM NaCl, 50mM Tris, pH 8.0 and protease inhibitor cocktail (Calbiochem). The protein concentration was measured using Bradford reagent (BioRad). For immunoprecipitation experiment cell lysates were immunoprecipitated with an antibody against ERK, STAT3, p38 MAPK using protein G-Sepharose (Amersham Bioscience). Then precipitates or cell lysates were heated at 90°C for 10 min in a sample loading buffer and separated on NuPAGE 4-12% Bis-Tris Gel (Invitrogen) for 60 mins at 200 V
and subsequently transferred to a PVDF membrane (30 V for 90 mins). Membranes were incubated in blocking solution (5% milk in PBS with 0.05% Tween 20 (PBS-T) (Invitrogen) for 2 hours at room temperature (RT). Membranes were probed with primary antibody in blocking solution overnight at 4°C. The membranes were washed three times for 5 mins with PBS-T and then incubated with horseradish peroxidase conjugated secondary antibodies in blocking solution for 2 hours at RT. After three washes with PBS-T protein bands were visualized with ECL plus chemilluminescence reagent (Amersham Bioscience). Immunoblot images were captured by KODAK Image Station 2000MM. Protein values were normalized to Beta Actin expression. Image analysis and data normalization was done using ImageJ 4.10 software (NIH).

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde 30 mins at RT, washed three times for 5 mins in PBS, then incubated in PBS-T for 1 hour at RT. Cells were then blocked with 3% Normal Donkey Serum in PBS-T (blocking solution). Next, samples were incubated with primary antibody diluted in blocking solution for 2 hours at RT. Cells were washed three times for 5 mins in PBS prior to adding secondary antibody, diluted in blocking solution. Secondary antibody incubation was done overnight at 4°C. Cells were then washed thoroughly and air-dried prior to adding Vectashield mounting media with DAPI (Vector).

**Microscopy**

Imaging was done using an inverted fluorescent microscope (Leica DMI 6000B) with differential interphase contrast (DIC). DAPI/Rhodamine/FITC filter cube (Chroma) was
utilized for fluorescent microscopy. Image analysis was done using Openlab Software 4.0.1 (Improvision).

**Quantitative RT-PCR**

Total RNA was extracted from the cells with Trizol reagent (Invitrogen). cDNA synthesis and RT-PCR reactions were performed using iQ SYBER Green Supermix (Biorad). RT-PCR conditions were pre denaturation for 2 mins at 94°C; 35 cycles of 94°C for 15 s: 55-60°C for 30 s; and 72°C for 30 s; and a post extension at 72°C for 2 mins. RT-PCR reaction was performed using iCycler (Biorad). Fold change was determined using the pfaffl method (Pfaffl 2001).

**RT-PCR Primers**

GFAP (+) 5-AAGCAGTCTACCCACCTCAG-3,
(-)5-ATCCCTCCCAGCACCTCATC-3;

APP (+) 5-CTTGAGTAAACTTTGGGACATGGCGCTGC-3,
(-)5-GAACCCTACGAAGAAGCC-3;

Beta Actin (+) 5-GACAGGATGCAGAAGGAGAT-3,
(-) 5-TTGCTGATCCACATCTGCTG-3;

MCP-1 (+) 5-GCGAGCTATAGAAGAATCACC-3,
(-) 5-ATAAACAGGGGTGTCTGGGG-3;

MCPIP (+) 5-AACTGGAGAAGAAGAAGATCCTGG-3,
(-) 5-ATTGACGAAGGAGTACATGAGCAG-3
**Statistical analysis**

Experiments were done in triplicate and standard deviation was determined. A T-test analysis was performed to determine statistical significance of experimental group compared to control group.
Results

Cell death caused MCPIP expression

The transcription factor MCPIP has previously been shown to induce apoptosis in various cell lines (Hausmann et al. 1998; Zhou et al. 2006). To determine the effect MCPIP has on NT2 cells, we performed a cell death dye exclusion assay utilizing trypan blue. NT2 cells were transfected with either GFP (control) or MCPIP-GFP and analyzed 1, 3, and 5 days post transfection. Results showed that MCPIP expression caused a significant increase in cell death compared to control at all indicated time points (Figure 1). Day 5 showed the most significant increase in cell death, with MCPIP at 53.65% compared to 26.7% for GFP. As expected, plasmid transfection caused low levels of cell death as seen in GFP transfected cells. We also showed that MCPIP induced apoptosis in NT2 cell by immunoblot analysis with anti cleaved PARP antibody. These results demonstrate that MCPIP expression in NT2 neuroprogenitor cells induces apoptosis, as previously seen in other cell lines.
Figure 1 MCPIP increases cell death in NT2 cells

(A) Percent cell death after MCPIP-GFP or GFP (Control) transfection. Cells were harvested at 1, 3, 5 days post transfection. Cell death was detected by trypan blue. (B) Immunoblot analysis of GFP expression and PARP cleavage in NT2 cells transfected with MCPIP-GFP or GFP. Analyzed 5 days post transfection. Protein values were normalized to Beta Actin expression. GFP expression demonstrates equal transfection efficiency. Note: MCPIP-GFP was approximately 90kDa compared to GFP alone (27 kDa). * Indicates statistical significance (**P<0.01, ***P<0.001).
Induction of glial differentiation by MCPIP expression in NT2 cells

Previously, pro apoptotic factors have been shown to be involved neural differentiation (Kwak et al. 2006a). Therefore, we investigated if the pro apoptotic nature of MCPIP could induce differentiation in NT2 neuroprogenitor cells. The results showed that MCPIP caused a significant increase in GFAP mRNA, indicative of glial differentiation (Figure 2A). Immunoblot analysis with GFAP specific antibody confirmed these results (Figure 2B). Immunocytochemistry showed increased levels of GFAP expression, as well as distinct astrocyte morphology for MCPIP transfected cells (Figure 2C and 2D). Taken together, these results demonstrate that MCPIP expression is capable of inducing glial differentiation in NT2 cells.
Figure 2  Increased glial fibrillary acidic protein expression and morphological changes caused by MCPIP expression

(A) RT-PCR analysis of GFAP in NT2 cells transfected with MCPIP-GFP or GFP (control) 24 hours post transfection. Figure represents fold change vs. control.  (B) Immunoblot analysis of GFAP in cells transfected with MCPIP-GFP or GFP, analyzed 24 hours post transfection. Protein values were normalized to Beta Actin expression. * Indicates statistical significance (*P<0.05, ***P<0.001). Immunocytochemistry showing GFAP expression in NT2 cells transfected with GFP (C) or MCPIP-GFP (D). Analyzed 24 hours post transfection. Scale bar in panel represents 15 µm.
Induction of glial differentiation by MCP-1 treatment in NT2 cells

It is already shown that MCP-1 is responsible for activation of transcription factor MCPIP. Therefore, we tested MCP-1 for its ability to induce glial differentiation. The RT-PCR results showed that MCP-1 caused a significant increase in GFAP expression at 24 hours, especially for 100ng/ml and 300ng/ml concentrations (Figure 3A). Immunoblot analysis with anti GFAP antibody confirmed these results (Figure 3B). Increased GFAP expression and morphological changes indicative of astrocytic differentiation were also observed for MCP-1 treated cells (Figure 3C and 3D). These results demonstrate that MCP-1 activates glial differentiation in a similar manner as MCPIP.
Figure 3  Increased glial fibrillary acidic protein expression and morphological changes caused by MCP-1

(A) RT-PCR analysis of GFAP in NT2 cells treated with MCP-1 at indicated concentrations. Analyzed 24 hours post treatment. Figure represents fold change vs. control (untreated). (B) Immunoblot analysis of GFAP in cells treated with MCP-1 or untreated after 24 hours. Protein values were normalized to Beta Actin expression. * Indicates statistical significance (*P<0.05, **P<0.01). Immunocytochemistry showing GFAP expression in NT2 cells untreated (C) or treated with MCP-1 (100ng/ml) (D). Analyzed 24 hours after treatment. Scale bar in panel represents 15 μm.
MCPIP expression decreases amyloid precursor protein levels

Previously, staurosporine induced glial differentiation of NT2 cells was shown to require APP expression (Kwak et al. 2006a). Therefore, we expected that MCPIP expression might increase APP levels, and that APP activity could play a significant part in the differentiation process. Interestingly, results showed that MCPIP expression significantly decreased APP mRNA and protein levels in NT2 cells (Figure 4A and B). This data suggests that MCPIP induced glial differentiation does not require increased APP expression, as previously seen with staurosporine.
Figure 4  MCPIP decreases APP mRNA and protein levels

(A) RT-PCR analysis of APP expression in cells transfected with MCPIP-GFP or GFP (control). Analyzed 24 hours post transfection. (B) Immunoblot analysis of APP expression in NT2 cells transfected with MCPIP-GFP or GFP control. Analyzed 24 hours post transfection. Protein values were normalized to Beta Actin expression. Figure represents fold change vs. control. * Indicates statistical significance (*P<0.05).
Amyloid precursor protein induces expression of MCP-1 and MCPIP.

Contrary to our initial hypothesis, MCPIP expression decreased APP activity. It is possible that APP may act upstream of MCPIP and APP might be able to regulate MCPIP activity. Therefore, we investigated if APP expression is capable of increasing MCPIP and MCP-1. The results showed that 24 hours post APP transfection both MCP-1 and MCPIP expression significantly increased (Figure 5). Gene silencing of APP by siRNA significantly suppressed normal levels of both MCP-1 and MCPIP mRNA expression in NT2 cells (Figure 5). These results suggest that MCP-1 and MCPIP expression is effectively regulated by APP signaling.
Figure 5  APP expression regulates MCP-1 and MCPIP mRNA

RT-PCR analysis of APP, MCP-1, and MCPIP in NT2 cells transfected with APP-GFP, siAPP, or GFP (control). Analyzed 24 hours post transfection. Figure represents fold change vs. control. * Indicates statistical significance (*P<0.05, **P<0.01, ***P<0.001).
Secreted type APP alpha and APP intracellular domain effect on MCP-1 and MCPIP

We next tested whether the processed forms of APP, secreted type APP alpha (sAPP\textsubscript{\(\alpha\)}) and APP intracellular domain (AICD), have an effect on MCP-1 and MCPIP expression. We treated NT2 cells with sAPP\textsubscript{\(\alpha\)} at 25, 50, and 100nM concentrations and measured MCP-1 and MCPIP expression by RT-PCR. Results showed that sAPP\textsubscript{\(\alpha\)} significantly increased MCP-1, but not to the same extent as transfected full length APP (Figure 6A). Interestingly, MCPIP expression did not increase in response to either sAPP\textsubscript{\(\alpha\)} or AICD. Increasing the concentration of sAPP\textsubscript{\(\alpha\)} did not elevate MCP-1 levels any further, demonstrating its effect was not dose dependent (Figure 6A). Similar results were observed after NT2 cells were transfected with AICD plasmid (Figure 6B). In conclusion, these results show that both processed forms of APP, sAPP\textsubscript{\(\alpha\)} and AICD, are capable of increasing expression of MCP-1, but not MCPIP.
Figure 6  Secreted APP alpha and APP intracellular domain (AICD) effect on MCP-1 and MCPIP

(A) RT-PCR analysis of MCP-1 and MCPIP in NT2 cells treated with secreted type APP alpha (25, 50, 100nM) or transfected with AICD (B).  RT-PCR analysis of MCP-1 and MCPIP in NT2 cells transfected with AICD or GFP (control). Figure represents fold change vs. control (GFP). Analyzed 24 hours post treatment/transfection. Indicates statistical significance (*P<0.05, **P<0.01)
Amyloid precursor protein’s effect on MCP-1 and MCPIP expression relies on p38 MAPK and JAK/STAT signaling

We have shown that APP can increase MCP-1 and MCPIP expression. Next, we examined which signal transduction pathways are involved in this process. In order to determine what mechanism APP uses in its induction of MCP-1 and MCPIP we investigated the p38 mitogen-activated protein kinase (MAPK), extracellular signal-related protein kinase (ERK1/2), and Janus activated kinase (JAK) signal transduction pathways. NT2 cells were pretreated with p38 (SB203580), ERK1/2 (PD098059), and JAK1 inhibitors for 45 minutes at 25uM, 50uM, and 250nM, respectfully. Samples were then transfected with APP-GFP or GFP and analyzed 24 hours post transfection. Results showed that the p38 MAPK and JAK specific inhibitors caused a significant decrease in both MCP-1 and MCPIP (Figure 7A). The ERK1/2 inhibitor, PD098059, had no effect on either MCP-1 or MCPIP activity. Similar results were observed using increased concentrations of ERK1/2 inhibitor (data not shown). In addition, we also investigated the phosphorylated state of the specific proteins involved in these pathways. Results showed that APP expression increased phosphorylation of both p38 and STAT3 (Figure 7B). There was no increase in phosphorylation of ERK1/2 proteins, confirming the results obtained using PD098059. These results indicate that APP relies on activation of both p38 MAPK and JAK/STAT3 signaling pathways to induce MCP-1 and MCPIP expression.
Figure 7  APP’s effect on MCP-1 and MCPIP expression relies on p38 MAPK and JAK/STAT signaling

(A) RT-PCR analysis of MCP-1 and MCPIP in NT2 cells transfected with APP. NT2 cells were pretreated for 45 mins with 50uM of ERK1/2 inhibitor (PD098059), 25uM of p38 MAPK inhibitor (SB203580), 250nM of JAK1 inhibitor, or untreated. NT2 cells were then transfected with APP-GFP or GFP (control). Figure represents fold change over control (GFP). Analyzed 24 hours post transfection. (B) Immunoblot analysis of Phosphorylated ERK (pT202/pY204), p38 MAPK (pT180/pY182), and STAT3 (pY705). Prior to immunoblot analysis cell lysates were immunoprecipitated with anti ERK1/2, p38 MAPK or anti STAT3, respectively. Basal levels of phosphorylation was observed for controls, as expected. * Indicates statistical significance (*P<0.05, **P<0.01, ***P<0.001).
Discussion

Our study characterizes the involvement APP and chemokine MCP-1 have in glial differentiation of NT2 neuroprogenitor cells. NT2 cells have the ability to develop into both mature neurons and astrocytes, making them an attractive in vitro model system for analyzing neural differentiation (Bani-Yaghoub et al. 1999; Kwak et al. 2006a). It has been reported that apoptosis was closely associated with neural differentiation (Oppenheim 1991) and that pro apoptotic factors could induce glial differentiation in NT2 cells (Kwak et al. 2006a). Therefore, we investigated the effect transcription factor MCPIP has on cell death and differentiation in NT2 cells. Results showed that MCPIP expression not only induced cell death, but also promoted increased expression of GFAP and astrocyte cell morphology, indicative of glial differentiation. Similar results were seen with MCP-1 treatment, particularly at higher concentrations. Lower concentrations of MCP-1 were unable to induce expression of GFAP or cause any morphological changes. This suggests that low concentrations of MCP-1 may not be sufficient enough to activate MCPIP to the level needed to induce glial differentiation.

We have previously demonstrated APP’s involvement in glial differentiation of neural stem cells and that APP expression was required in staurosporine induced astrocytic differentiation in NT2 cells. Staurosporine treated cells underwent apoptosis, resulting in increased expression of APP. This increase in APP was responsible for induction of glial differentiation (Kwak et al. 2006a). Therefore, we investigated if APP has a similar role in MCPIP induced cell death and glial differentiation. Unexpectedly,
we found that MCPIP expression significantly reduced APP levels. This suggests that increased APP expression is not responsible for inducing glial differentiation, as previously seen with staurosporine treatment. Instead, we found that APP activity could directly regulate expression of both MCP-1 and MCPIP. Secreted type APPα or AICD were also able to significantly increase MCP-1 expression, but not to the extent that transfected APP obtained. Interestingly, both processed forms of APP had no effect on MCPIP activity. Given that MCP-1 is required to induce MCPIP activity, the increase resulting from sAPPα and AICD might not be sufficient to reach the threshold needed to induce MCPIP. This suggests that sAPPα and AICD are not solely responsible for MCP-1 and MCPIP activation. It is possible that their combined effect is needed or amyloid beta (Aβ), the other processed form of APP, is required to fully activate MCPIP activity. Further investigation is necessary to better understand the effect APP processing has on MCP-1/MCPIP expression. Previously, APP expression has been shown to increase in response to cell death (Lesort et al. 1997; Kinoshita et al. 2002; Nishimura et al. 2003). The increased APP expression has been attributed to its anti-apoptotic abilities, where it functioned as a neuroprotective factor (Lesort et al. 1997; Kinoshita et al. 2002). Activation of chemokine MCP-1 can be part of APP’s neuroprotective function, where it helps facilitate neuroinflammation and astrocyte activation at site of injury.

Through use of signaling pathway inhibitors, we investigated the mechanism by which APP regulated MCP-1 and MCPIP expression. Results showed that ERK1/2 activity, previously shown to be required for APP expression, was not required for APP’s
activation of MCP-1 and MCPIP (Liu et al. 2003; Kwak et al. 2006a). Interestingly, both JAK and p38 MAPK inhibitors were able to suppress APP function. Increased phosphorylation of both p38 MAPK and STAT3 was also observed, further confirming the results. It has been reported that JAK/STAT signaling was involved in regulating differentiation of cerebral cortical precursor cells (Bonni et al. 1997; Rajan and McKay 1998). STAT3 was shown to mediate astrocyte differentiation in the developing fetal mouse brain (Yanagisawa et al. 1999). In addition, the p38 MAPK pathway was shown to be required for MCP-1 production (Wong et al. 2005) and APP mediated anti-apoptotic signaling (Burton et al. 2002). Overall, both signaling pathways appear to play a specialized role in this APP/MCP-1 induced glial differentiation of NT2 neuroprogenitor cells.

Astrocytes have been identified as the primary source of MCP-1 in the brain in response to multiple sclerosis, ischemic brain injury, and brain trauma (Glabinski and Ransohoff 1999). Increased expression of MCP-1 is present in senile plaques, microglia, and microvessels in Alzheimer's disease (Ishizuka et al. 1997; Grammas and Ovase 2001). It was shown that mice over expressing both APP and MCP-1 had microglia accumulation in the brain (Sadowski et al. 2004). It is possible that MCP-1 is involved in the pathogenesis of several neuropathies by the recruitment of activated monocytes and/or microglia into the CNS, where they produced neurotoxic and inflammatory molecules. Although it has been shown that MCP-1 mediates inflammation in the CNS, it is still unknown if MCP-1 has alternative cellular functions in the brain (Ransohoff and Tani 1998; Cardona et al. 2003). Activation of glial
differentiation could be another function of MCP-1 during neuroinflammation. It is already established that astrocytes play an important role in injury repair and are the main source of MCP-1 production (Glabinski and Ransohoff 1999; Sofroniew 2005). Therefore, it is possible that MCP-1 induced glial differentiation is a repair mechanism that increases production of astrocytes, facilitating rapid recovery. Unfortunately, astrocyte activation after injury can also have negative effects. Increased astrocyte production can result in excess inflammation, toxic edema, cytotoxicin production, and formation of glial scar, preventing effective repair (Glabinski and Ransohoff 1999; Sofroniew 2005). It is essential to examine the connection between neuroinflammation and astrocyte activation and the role they might have in neuronal repair and protection.

In conclusion, we propose that elevated APP levels after CNS injury can result in increased MCP-1 production. This increase in MCP-1 can lead to astrocytic activation, eventually resulting in gliosis, causing excess damage. Further investigation is necessary to determine if this mechanism is responsible for the excess gliosis present in several neurodegenerative disorders. This information can help provide insight on the factors that regulate neural differentiation in the adult brain, particularly for use in stem cell therapy.
PART 2. APP INDUCED MIGRATION OF NT2 NEUROPROGENITOR CELLS INVOLVING MCP-1

Introduction

The use of neural stem cells (NSCs) to help repair damaged areas in the brain has been a subject of great interest. These cells have the ability to differentiate and replace damaged tissue in the central nervous system (CNS) and are an attractive candidate for therapy of neurodegenerative diseases (Gage 2000, 2002). The two main sources of these stem cells are the subventricular zone around the lateral ventricles and the subgranular zone of the dentate gyrus (Gage 2000; Parati et al. 2004). Unfortunately, clinical use of these stem cells is limited due to ethical concerns and technical difficulties in collecting sufficient amounts of these cells. Instead, alternative sources of human neurons are being investigated. NT2 neuroprogenitor cells, which are derived from a human teratocarcinoma, are an attractive alternative. NT2 neuroprogenitor cells are a transfectable cell line, which has the ability to proliferate in culture and differentiate into pure neurons (Andrews et al. 1984; Pleasure and Lee 1993). Once transplanted, these cells were able to survive, extend processes, and form functional synapses allowing them to fully integrate within the host (Kleppner et al. 1995; Trojanowski et al. 1997; Ferrari et al. 2000). A better understanding of how these cells migrate and eventually incorporate into the CNS is of much importance.

Research shows that when neuroprogenitors are transplanted into the brain, they migrate towards areas of brain damage (Fricker et al. 1999; Arvidsson et al. 2002; Iwai et al. 2003; Glass et al. 2005). This suggests there are certain factors associated with
damaged areas in the brain and these factors recruit migrating cells. Brain damage, including trauma, infection, ischemia, and neurodegeneration, generally results in a neuroinflammatory response, leading to activation of astrocytes and microglia. This neuroinflammatory response leads to the upregulation of cytokines and chemokines (Huang et al. 2000).

Chemokines are a family of conserved cytokines that have been shown to induce the migration of several leukocyte subpopulations into damaged tissues. Microglia and astrocytes are believed to be the main sources of chemokine production in the adult brain (Glabinski and Ransohoff 1999; Oh et al. 1999; Coughlan et al. 2000). The CNS has been shown to produce chemokines in response to several inflammatory and disease conditions, including allergic encephalitis, Alzheimer’s disease, multiple sclerosis, ischemia-induced neurodegeneration, and trauma (Glabinski and Ransohoff 1999).

Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C chemokine family, is a potent chemotactic factor for monocytes (Rollins 1997). MCP-1 has also been shown to be an effective chemoattractant to a variety of other cell types (Marra et al. 1999; Cambien et al. 2001a; Widera et al. 2004; Yan et al. 2007). It is proposed that MCP-1 is involved in the pathogenesis of several neuropathies by recruitment of activated monocytes and/or microglia, where they produce neurotoxic and inflammatory molecules.

Previously, MCP-1’s effect on NT2 neuroprogenitor cell migration has not been investigated. In this study we report MCP-1’s ability to induce migration of NT2
neuroprogenitor cells, as well as the intracellular signaling involved in the process. We also discover the novel role Amyloid precursor protein has in chemokine production and cell migration.
Materials and Methods

Cell Culture
The NTera-2/Clone D1 cells, NT2 (ATCC) were plated at a density of 5 X10^6 per 75cm^2 in tissue culture treated flask (Corning). Cell culture media was Dulbecco’s modified Eagle’s medium with F-12 (DMEM/F12, Invitrogen), supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologics). An incubation chamber was used to maintain a humidified atmosphere of 5% CO_2 and 37°C. NT2 cells were passed twice a week by 0.05% Trypsin/EDTA (Invitrogen) treatment. In order to avoid any inconsistencies in cellular behavior, all NT2 cells used in these experiments were at passage number 5.

Transfection
Transient transfection of plasmid DNA was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol on subconfluent NT2 cells in 24 well plates (Falcon) or 6 well plates (Corning). Plasmids: pCEP-APP-GFP (kindly provided by Dr. Beth Ostaszewski, Harvard Medical School), pEGFP-C1 (Clonetech).

Migration Assay
NT2 cell migration was analyzed using FluoroBlok migration insert system with 8 micrometer pores (BD Biosciences). Cells were pretreated with 10uM Cell tracker dye (Molecular probes) for 15 min prior to migration assay. Cells were washed with phosphate buffered saline (PBS), trypsinized and plated in migration inserts at a density of 150,000 cells/cm^2. Migration inserts were then placed in a 24 well plate (Falcon) with specific chemoattractant in media or media alone (control). Cells were incubated with
chemoattractant for up to 5 hours. Measurement of fluorescent intensity, which correlates to cell migration, was carried out using a Bio-Tek microplate reader (excitation 480nm and emission 528nm).

**APP Induced Migration Assay**

NT2 cells were plated in a 24 well plate (Falcon) at a density of 150,000 cells/cm\(^2\) and transfected with GFP or APP-GFP, treated with secreted APP alpha (150nM), or left untreated (control). 24 hours later, migration inserts containing NT2 cells (pre treated with Cell tracker dye, 10uM) at a density of 150,000 cells/cm\(^2\) were placed in wells containing transfected/treated NT2 cells (chemoattractant). Migration inserts were incubated with transfected/treated or control cells for 3 hours. After incubation migration inserts were removed and placed in new well containing media only. Measurement of fluorescent intensity was carried out using microplate reader (Bio-Tek).

**Quantitative RT PCR**

Total RNA was extracted from the cells in 6 well plate with Trizol reagent (Invitrogen). cDNA synthesis and RT-PCR reactions were performed using iQ SYBER Green Supermix (Biorad). RT-PCR conditions were pre denaturation for 2 mins at 94°C; 35 cycles of 94°C for 15 s: 55-60°C for 30 s; and 72°C for 30 s; and a post extension at 72°C for 2 mins. RT-PCR reaction was performed using iCycler (Biorad). Fold change was determined using the pfaffl method (Pfaffl 2001).

**RT-PCR Primer Sequences:**

MCP-1 (+)5'-GCGAGCTATAGAAGAATCACC-3',

(-)5'-ATAAAACAGGGTGTCTGGGG-3;
MCPIP (+) 5-AACTGGAGAAGAAGAAGATCCTGG-3,
(-) 5-ATTGACGAAGGAGTACATGAGCAG-3;
MIP-1 Alpha (+) 5-TGCATCACTTGCTGCTGACACG-3
(-) 5-CAACCAGTCCATAGAAGAGG-3
MIP-1 Beta (+) 5-CCAAACCAAAAAGAAGCAAGC-3
(-) 5-AGAAACAGTGACAGTGACC-3
RANTES (+) 5-CTTTGTCACCCGAAAGAACC-3
(-) 5-GTTTCATCATGTTGGCCAGG-3
Beta Actin (+) 5-GACAGGATGCAGAAGGAGAT-3,
(-) 5-TTGCTGATCCACATCTGCTG-3;

Reagents and Antibodies
Recombinant human monocyte chemotactic protein-1 (ABR). Phorbol 12-Myristate 13-acetate (PMA), 250nM (Sigma). Forskolin, 75uM (Calbiochem). SB203580, 25uM (Sigma). PD098059, 50uM (Sigma). Secreted amyloid precursor protein alpha (sAPPalpha), 150nM (Sigma). Rabbit polyclonal anti-MCP-1 (Abcam) was used to neutralize MCP-1 at a concentration of 10ug/ml. Anti Rabbit IgG antibody (Amersham).

Statistical analysis
Experiments were done in triplicate and standard deviation was determined. A T-test analysis was performed to determine statistical significance (P < 0.05) of experimental group compared to control group.
Results

Effect of MCP-1 on NT2 cell migration

Several studies have shown MCP-1’s ability to act as a potent chemoattractant to various types of cells (Rollins 1997; Cambien et al. 2001b; Widera et al. 2004; Yan et al. 2007). We investigated the effect MCP-1 has as a chemoattractant towards NT2 neuroprogenitor cells. Cells were exposed to increasing concentrations of MCP-1 (10, 20, 50, 100, 200 ng/ml) or plain media (control) for 2 to 5 hours. Results show that MCP-1 acts as a strong chemoattractant in a dose and time dependent manner, with 200ng/ml of MCP-1 for 5 hours causing the most significant effect (Figure 8). Lower concentrations of MCP-1 (10ng/ml) were still sufficient to induce migration. These results demonstrate that MCP-1 can induce migration of NT2 cells and confirms previous studies claiming MCP-1’s chemotactic ability influences many cell types.
Figure 8  MCP-1 increases NT2 cell migration in a dose dependent manner.

NT2 cell migration was induced with increasing concentrations of MCP-1 and compared to untreated controls from 2 - 5 hours. Prior to migration assay NT2 cells were treated with cell tracker dye (10uM). Cell migration was measured using fluorescent plate reader. Results are shown as relative fluorescent units (RFU). All experiments were done in triplicate. Error bars represent standard deviation. *Indicates statistical significance compared to control (*P<0.05).
Effect of pharmacologic inhibitors on NT2 cell migration towards MCP-1.

To investigate the role of various signaling pathways on MCP-1 induced NT2 cell migration, we studied the effect of several pharmacologic inhibitors on MCP-1’s chemotactic activity. NT2 cells were pretreated with inhibitors for 30 mins at the indicated concentrations prior to exposure to MCP-1. Our results showed that Forskolin, which is an activator of intracellular cAMP, significantly inhibited MCP-1 induced migration (Figure 9). Similarly, PMA, which is a protein kinase C (PKC) activator, also significantly inhibited migration towards MCP-1. In contrast, inhibition with PD098059 and SB203580 did not affect migration, indicating activation of ERK/MAPK (mitogen-activated protein kinase) and p38 MAPK do not appear to be required for effective response MCP-1. Pretreatment with PD098059 and SB203580 for longer periods of time or higher concentrations did not have an effect (data not shown). This suggests that activation of ERK/MAPK and p38 MAPK are not required for chemotactic response to MCP-1. Interestingly, PKC and cAMP activation are both able to significantly attenuate the chemotactic response to MCP-1.
Figure 9  Effect of pharmacologic agents on MCP-1 induced NT2 cell migration

NT2 cells were pretreated 30 mins with following agents: PD09059 (50uM), SB203580 (25uM), Forskolin (75uM), PMA (250nM), or left untreated (MCP-1 alone). Cells were then tested for their ability to respond to MCP-1 (100ng/ml) after 3 hours of incubation. Cell migration was measured using fluorescent plate reader. Results are shown as relative fluorescent units (RFU). All experiments were done in triplicate. Error bars represent standard deviation. *Indicates statistical significance compared to control (*P<0.05).
Chemotactic activity of APP transfected NT2 cells.

Previously, we have shown that NT2 cells transfected with Amyloid precursor protein (APP) showed an increase in MCP-1 expression (Vrotsos 2007). Therefore, we predict that APP transfected NT2 cells, which express MCP-1, could potentially act as a chemoattractant to other NT2 cells. To test this hypothesis we transfected NT2 cells with APP and 24 hours later measured their ability to act as a chemoattractant for untreated NT2 cells. Figure 10 shows that APP transfected cells are capable of inducing migration compared to control (GFP transfected). Similarly, secreted type APP alpha (sAPPα) treated cells were also shown to induce NT2 cell migration. Taken together, these data suggest that cells expressing increased levels of APP could act as a chemoattractant to migrating cells.
Figure 10  Amyloid precursor protein induces NT2 cell migration.

NT2 cells were transfected with full length APP or treated with secreted APP alpha (150nM) and tested for their ability to induce NT2 cell migration. NT2 cells were incubated for 3 hours in the presence of transfected APP, transfected GFP, secreted APP alpha, MCP-1 (100ng/ml), or untreated (Control) NT2 cells, as described in Materials and Methods. Cell migration was measured using fluorescent plate reader. Results are shown as relative fluorescent units (RFU). All experiments were done in triplicate. Error bars represent standard deviation. *Indicates statistical significance compared to control (*P<0.05).
Effect of MCP-1 neutralization on APP induced migration.

We have shown that NT2 cells expressing APP are capable of acting as an effective chemoattractant. We next tested if increased MCP-1 production is solely responsible for this chemotactic response or if another factor is involved. To investigate this, we utilized a MCP-1 neutralizing antibody and tested if it is capable of reducing cell migration. Results showed that MCP-1 neutralizing antibody significantly reduced APP induced NT2 cell migration (Figure 11). However, migration was only reduced 56% compared to control (IgG antibody), and was not completely abolished. Increased concentrations of MCP-1 neutralizing antibody were used but did not reduce migration any further. These data suggest that APP induced migration is not only caused solely by MCP-1, but relies on other chemotactic factors.
Figure 11  MCP-1 neutralizing antibody reduces APP induced migration

MCP-1 antibody (10ug/ml) was used to neutralize MCP-1 protein produced by APP transfected NT2 cells. Anti Rabbit IgG antibody (10ug/ml) was used as a control. Migration was measured in the absence or presence of neutralizing MCP-1 antibody. Cell migration was measured using fluorescent plate reader. Results are shown as relative fluorescent units (RFU). All experiments were done in triplicate. Error bars represent standard deviation. *Indicates statistical significance compared to control (IgG) (*P<0.05).
Amyloid precursor protein effect on chemokine expression

We next wanted to examine if APP is capable of activating other C-C chemokines besides MCP-1. Quantitative RT-PCR was done to measure any changes in the gene expression of MCP-1, MIP-1 alpha, MIP-1 beta, and RANTES after APP transfection. Results showed that NT2 cells transfected with APP caused a significant increase in MCP-1, MIP-1 alpha, and RANTES (Figure 12). Interestingly, MIP-1 Beta had no change in gene expression in response to APP activity. This demonstrates that APP activity induces expression of several chemokines.
Figure 12  Amyloid precursor protein effect on CC chemokine mRNA activity

RT-PCR analysis of MCP-1, MIP-1 Alpha, MIP-1 Beta, and RANTES in NT2 cells transfected with APP-GFP or GFP (control). Samples were analyzed 24 hours post transfection. Figure represents fold change in mRNA expression over control. All experiments were done in triplicate. Error bars represent standard deviation. *Indicates statistical significance compared to control (IgG). * Indicates statistical significance compared to control (*P<0.05).
Discussion

Several studies have been done utilizing NT2 neuroprogenitor cells for transplantation. The transplanted cells were shown to effectively migrate and incorporate with host tissue (Kleppner et al. 1995; Trojanowski et al. 1997; Ferrari et al. 2000). However, it is still unclear what factors are responsible for regulating NT2 cell migration and the intracellular signaling mechanism involved.

This study provides the first evidence that MCP-1 is capable of inducing migration of NT2 neuroprogenitor cells. Presently, the concentration of MCP-1 in the adult brain, particularly after injury, is not known. It was reported that the concentration of MCP-1 in human brain tissue from control and alcoholic patients was 90 and 212 pg/mg, respectively (He and Crews 2008). This demonstrates that altered conditions in the brain can have a dramatic effect on MCP-1 levels. It is possible that the local concentration of MCP-1, especially after injury, may be even higher. Previous in vitro studies examining MCP-1 induced migration used a concentration range of 0-200 ng/ml in human monocytes (Fine et al., 2001) and 0-250 ng/ml in hepatic stellate cells (Marra et al., 1999). Thus, we adopted a similar MCP-1 concentration range (10-200 ng/ml) for this investigation. Overall, these results suggest that MCP-1, which increases in response to brain injury and trauma, is likely a major factor causing neuroprogenitor cells to migrate to damaged regions of the brain (Glabinski et al. 1996) (Minami and Satoh 2003).

Several studies have been done investigating the intracellular signaling pathways involved in chemokine induced migration (Nick et al. 1997; Yen et al. 1997; Ashida et al.
2001; Fine et al. 2001). However, intracellular signaling in neuroprogenitor cells after exposure to MCP-1 or other chemotactic factors has never been examined. Our results showed that ERK/MAPK inhibitor (PD098059) and p38 MAPK inhibitor (SB203580) have no effect on MCP-1 mediated chemotaxis. These results are similar to previous studies where they demonstrate a minimal role for these kinases in leukocyte migration (Knall et al. 1997; Nick et al. 1997; Zu et al. 1998; Fine et al. 2001).

Conversely, activation of either intracellular cAMP or protein kinase C (PKC) with forskolin and PMA, respectively, completely abolished MCP-1 induced migration. These results are in accord with an earlier study demonstrating activation of PKC and cAMP desensitize PMNs response to several chemokines (Fine et al. 2001). It has also been reported that cAMP inhibits cell migration by preventing lamellipodia formation in mouse fibroblast cells (Chen et al. 2008). Interestingly, another study showed that PKC is capable of phosphorylating specific adenylyl cyclase isoenzymes, resulting in increased levels of intracellular cAMP (Zimmermann and Taussig 1996; Marjamaki et al. 1997). Therefore, it is likely that treatment with PMA, not only activates PKC, but also elevates cAMP levels. Further investigation is required to better understand the roles PKC and cAMP have in chemotactic inhibition of neuroprogenitor cells.

Amyloid precursor protein (APP) is a ubiquitous glycoprotein receptor that is involved in pathogenesis of Alzheimer’s disease. Previously, APP expression was shown to increase in response to cell death (Nishimura et al. 2003), where it is thought to serve a neuroprotective function (Lesort et al. 1997; Kinoshita et al. 2002).
studies report that traumatic brain injury can induce expression of APP (Pierce et al. 1996; Murakami et al. 1998; Ciallella et al. 2002).

We demonstrate that NT2 cells transfected with APP act as a chemoattractant to other NT2 cells. Since this effect was partially blocked by a MCP-1 neutralizing antibody, MCP-1 is involved in the mechanism of APP function, at least in part. However, use of MCP-1 neutralizing antibody could not completely abolish APP effect, indicating that this APP induced migration is not caused solely by increased MCP-1 production.

Although we found increased expression of RANTES and MIP-1 alpha, which are reported to be chemoattractants to monocytes (Fine et al. 2001), they may not affect NT2 cell migration. Thus, further investigations are necessary to find additional factors, which are involved in the APP induced migration.

Taken together, these data demonstrate the novel role APP has in both chemokine production and cell migration. It is possible that microenvironments in the CNS with increased levels of APP can have a significant effect on cell migration in that region. This information is beneficial because it provides new insight into the factors that influence NT2 neuroprogenitor cell migration. Further investigation of this mechanism would prove useful in future transplantation therapies by allowing better control of migrating cells and their incorporation into host tissues.
References


