CHAPTER V

DISCUSSION AND CONCLUSIONS

It is clear that too much iron is neurotoxic, although iron is essential for normal brain functions as a cofactor of many enzymes involved in neuronal oxidative metabolism, neurotransmitter and myelin syntheses. This is due to the ability of iron to catalyze the generation of free radicals (Dallman, 1986; Yagi *et al.*, 1992). Free iron can react with hydrogen peroxide and superoxide normally produced in biological systems to generate more potent hydroxyl radicals. These toxic molecules can induce lipid peroxidation, DNA strand breaks, and degradation of biomolecules (Halliwell *et al.*, 1984; Floyd, 1990). Therefore, free iron under normal physiological conditions is tightly regulated. Such regulatory mechanisms are involved a sequester of iron by iron binding proteins and low molecular weight molecules, such as transferrin, ferritin, citrate, and ATP or by iron-storage cells e.g. oligodendrocytes and microglia (Weaver *et al.*, 1989; Connor *et al.*, 1995; Cheepsunthorn *et al.*, 1998, Cheepsunthorn *et al.*, 2001c). In many brain diseases including Alzheimer, iron convincingly contributes to the process of neurodegeneration (Sayre, 1999; Sayre *et al.*, 1997; Smith *et al.*, 1997; Smith *et al.*, 1995; Floyd *et al.*, 1990; Hall, 1993; Halliwell *et al.*, 1992). As a result, the distributions of iron binding and iron transport proteins have been extensively examined in AD brain tissues (Burdo *et al.*, 2003; Connor *et al.*, 1992a; Connor *et al.*, 1992b). As one indicator for the disruption of brain iron metabolism in AD, it is important to note that activated microglia in the senile plaques contain high levels of iron. This can also be reflected by an increase in expression of intracellular iron storage protein ferritin (GrundkeIqbal *et al.*, 1990; Kaneko *et al.*, 1989; McGeer *et al.*, 1987; Ohgami *et al.*, 1991). These observations could suggest that the activity of plaque-associated microglia might be tightly connected to their iron requirements.

Therefore, modulation of intracellular iron levels may influence the involvement of microglia in the progression of this disease.

To investigate whether iron-rich activated microglia contribute to the formation of senile plaques as seen in AD brains, we have developed an *in vitro* model of iron loading in activated microglia using a rat microglial cell line HAPI (Cheepsunthorn *et al.*, 2001a). We demonstrated that the addition of iron (50 μg/ml) in the presence of LPS (1 μg/ml) to the medium was sufficient to generate iron-rich activated microglia as observed in the brains of patients with AD. With this model, we show that activity of brain MMPs is modified by intracellular iron status. First, the amount of MMP-9 secreted by activated microglia is increased, if the cells are loaded with iron. This finding is consistent with the observation that MMP-9 is localized predominantly in the amyloid plaques (Backstrom *et al.*, 1996). Our results further indicate that iron-rich activated microglia, in addition to brain endothelial cells (Asahina *et al.*, 2001), are the potential source of plasma MMP-9, which is elevated in patients with Alzheimer's disease (Lorenzl *et al.*, 2003). The role of MMP-9 in the catabolism of Aβ-peptides was also reported. Backstrom and colleagues (1996) demonstrated that active MMP-9 could process Aβ with a cleavage site being the Leu34-Met35. With this information in mind, one could speculate that an increased secretion of MMP-9 from iron-rich activated microglia associated with amyloid plaques could be beneficial. Because these iron-rich activated microglia might be actively removing the deposited Aβ, which was neurotoxic to neurons, particularly in the hippocampus (Cotman *et al.*, 1992). thus, the formation of senile plaques should be detained and the numbers of existing plaques in the affected brain should be diminished overtime. Disappointingly, it does not happen the way one would assume as evidenced in AD patients and in the transgenic models expressing mutant βAPP (Games *et al.*, 1995). This could be because a coexisting of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 within the plaques (Peress *et al.*, 1995), in addition to the disruption of microglial scavenger function. Here, we demonstrated for the first time that activated microglia when loaded with iron

significantly decreased their phagocytic activity. In fact, iron could be an essential component influencing the uptake of Aβ by activated microglia additional to a slow Aβ-degrading nature of this cell type (Paresce *et al.*, 1997).

Perhaps, an increase in inflammatory response in the brains of patients with AD could be mediated by iron-rich environment and the activation of microglia as demonstrated by our studies. For instance, an increase in collagenase activity of MMP-9 secreted from iron loaded activated microglia may contribute to the blood–brain barrier dysfunction in AD brain through the degradation of type IV collagen, which forms the important component of capillary basal lamina in brain tissue. Then, plasma extravasation into brain parenchyma could occur following by glial activation and a cascade of cytokine production leading to CNS damage. Among other inflammatory cytokines, interleukin (IL)-1β is a key mediator in the pathogenesis of acute and chronic inflammation in the CNS, including in AD (Rothwell *et al.*, 1997; Tringali *et al.*, 2000). Brain IL-1β is mainly produced by activated microglia as a precursor protein (Giulian *et al.*, 1986). These precursor IL-1β, particularly secreted from activated microglia in the vicinity of amyloid plaques, can be processed by MMP-9 into biologically active form (Schonbeck *et al.*, 1998; Chauvet *et al.*, 2001). Therefore, it is possible that enhanced MMP-9 activity in iron-rich activated microglia could enhance the role of IL-1β in AD inflammation too. Elevated levels of mature IL-1β could promote local inflammatory response at least by upregulating the expression of cell adhesion molecules such as ICAM-1 and V-CAM-1 on brain endothelial cells and astrocytes. Because the induction of these adhesion molecules on brain cells play a significant role in the extravasation, migration, and activation of lymphocytes in the CNS (Hery *et al.*, 1995; McCarron *et al.*, 1993). The presence of TIMP-1 in amyloid plaques may prolong the activity of IL-1β by preventing the degradation of active IL-1β mediated by MMP-9 and MMP-1 (Peress *et al.*, 1995, Ito *et al.*, 1996). In addition, IL-1β was previously reported to play a role in regulating the synthesis of heparan sulfate proteoglycan known to tightly associate with deposited Aβ. Therefore, elevated

levels of IL-1β may be important for the aggregation of Aβ peptides within the brains of AD patients (Mrak *et al.*, 1996; Sheng *et al.*, 1996; Donahue *et al.*, 1999). These findings, taken together, further implicate iron-rich activated microglia as key players in the formation of amyloid plaques and neurotoxicity induced by Aβ peptides.

Secondly, we demonstrate that iron-rich environment diminishes the secretion of MMP-2 in an *in vitro* model of fibroblasts. This finding suggests that iron accumulation in the brain, as a result of the disruption of normal brain iron metabolism previously reported for AD, could affect the amount of MMP-2 secretion from brain cells such as neurons and astrocytes (Rosenberg *et al.*, 1996; Clark *et al.*, 1997; Gasche *et al.*, 1999; Heo *et al.*, 1999; Deb *et al.*, 2003). Several studies have suggested the involvement of MMP-2 in AD pathogenesis. For example, it has been reported that MMP-2 possesses α-secretase activity, which is necessary for the production of a soluble extracellular domain of APP (Miyasaki *et al.*, 1993). Interestingly, it has been reported that MMP-2 is also capable of degrading plaque-derived Aβ (Roher *et al.*, 1994). Therefore, a decreased MMP-2 activity may contribute to the deposition of amyloid plaques as well as Aβ-induced neuro-degeneration in the brains of patients with AD.

Recently, gene expression of iron-rich activated microglia was analyzed using DNA microarray technology. We found that among the genes whose expression was influenced by cellular iron status were MMP-10 and MMP-1 (Cheepsunthorn *et al.*, 2001b). Therefore, in this study we included a RT-PCR assay to validate the results obtained by microarray. As expected, we found that iron in an iron rich environment induces cellular iron loading and further decreases the expression of MMP-10. Interestingly, we didn't observe such effect of iron on the expression of the MMP-1, but it was clearly shown that iron enhances the secretion of MMP-1 from activated microglia. This finding was in agreement with a report that levels of MMP-1 are elevated in AD brains (Leake *et al.*, 2000). However, very little was known about the

role of MMP-10 in the pathogenesis of AD. Nguyen and colleagues (1993) have reported that substrates of MMP-10 include collagen, gelatin, and proteoglycans. The later are components of the senile plaques and have been reported to play a role in persistence of plaques in the hippocampus of AD brain (Castillo *et al.*, 1997; Snow *et al.*, 1994; Young *et al.*, 1989). Therefore, a decrease in the expression of MMP-10 from iron-rich activated microglia associated with the plaques may contribute to Aβ deposition. Subsequently, aggregated Aβ peptides exert their neurotoxic effect by stimulating the production of cytokines, chemokines, and reactive oxygen species (Rossi *et al.*, 1996; Hu *et al.*, 1998; Johnstone *et al.*, 1999). Furthermore, increased secretion of MMP-1 from iron-rich activated microglia could disrupt the blood-brain barrier, which is common in AD (Kalaria, 1996).

In summary, our studies demonstrated that iron-rich environment causes intracellular iron loading in activated microglia. As a result, iron loaded cells become "rusting" as indicated by decreasing phagocytic activity. On the other hand, iron loaded cells are more "rousing" to neuroinflammation, at least, through the regulatory mechanisms involved MMP9, -2, -10, and-1. Such effect of iron may contribute to blood-brain barrier dysfunction, senile plaque formation and neurodegeneration in the brains of patients with Alzheimer's disease. Thus, lowering iron availability to activated microglia in combination with the administration of antioxidant and free radical scavengers could provide therapeutic inroads to fight against Alzheimer's disease.

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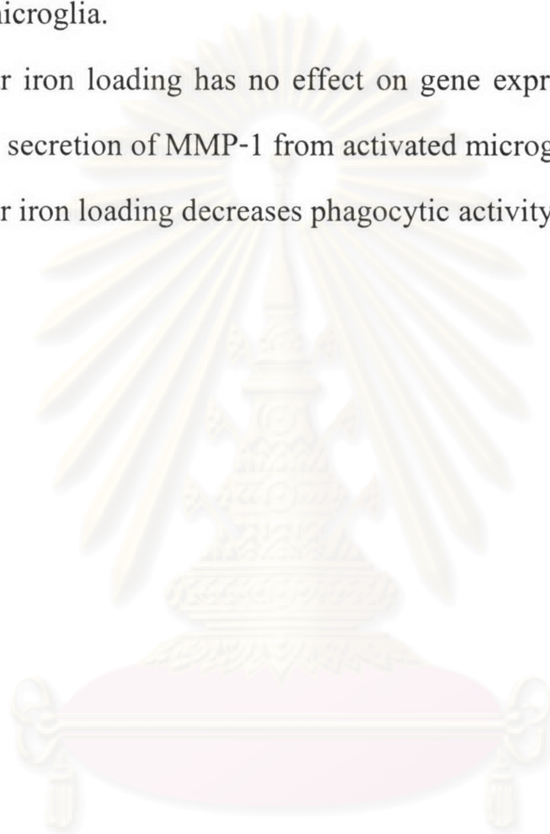
CONCLUSIONS

1. The addition of iron into the medium was sufficient to induce intracellular accumulation of iron in activated microglia.
2. Iron rich activated microglia increased the secretion of MMP-9.
3. Intracellular iron loading downregulates gene expression of MMP-10 in activated microglia.
4. Intracellular iron loading has no effect on gene expression of MMP-1, but it induces the secretion of MMP-1 from activated microglia.
5. Intracellular iron loading decreases phagocytic activity of activated microglia



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