THE ROLE OF CERULOPLASMIN IN BRAIN IRON TRANSPORT

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THE HONG KONG
POLYTECHNIC UNIVERSITY

2000

DECLARATION

The work submitted in this thesis is the result of investigations carried out by the author. The material in this thesis has not been accepted in any substance for any degree, and is being concurrently submitted in candidature for any other degree.

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ACKNOWLEDGMENTS

The investigations described in this thesis were carried out in Laboratory of Iron Metabolism in the Department of Applied Biology and Chemical Technology (ABCT), The Hong Kong Polytechnic University.

There are many people I would like to thank. Without help of those people, this thesis could never be realized. I am deeply indebted to my Chief-supervisor, Dr. Z. M. Qian for his enormous helpful advice and valuable support. I am also indebted to my Co-supervisor, Professor Tang Pak Lai for his encouragement and guidance. Many thanks to Ms. To Yu, Dr. F. Y. D. Yao and Miss Ke Ya in our research group for their kind help and suggestions in writing this thesis. Thanks to Miss Mable Yau, Mr. C. H. Cheng and all the technicians in the Applied Biology Section of the Department of ABCT for their valuable technical assistance.

I am much indebted to the Departmental Research Committee of Department of ABCT and Research Committee of the Hong Kong Polytechnic University for giving me a Research Scholarship and supporting my study in this university.

I would like to thank Dr. S D Aust and Mr. Chris Reilly for providing the protocols for rat ceruloplasmin isolation and valuable suggestions, though we never met.

Moreover, thanks to Professor Y. M. Fung and Dr. Y. H. Tang for their encouragement and valuable advice in isolating rat ceruloplasmin. Also, thanks to the Su Zhou Medical School for generously giving us the glioma cell line BT325.

I would also like to thank Professor Evan Morgan who taught me the necessary experimental techniques and gave me his valuable advice. There are many people in his laboratory that I would like to thank for their valuable advice, encouragement and friendships. They are Dr. Debbie Trinder, Mr. Anthony Kicic, Mrs. Donna Savigni and especially thank to Miss Anita Chua for her tremendous support and being my best friend.

With all my heart, I would like to thank my best friends: Miss Christy Law, Miss Sandy Ng, Miss Karen Law, Miss Rebecca Tsang, Miss Joey Chan, Ms. Eva Yip, Miss Mavis Choi and Miss Priscilla Kwan. Thanks for always being there through thick or thin. There are also some people that I would never thank enough. They are Ms. Irene Leung and Ms. Or Ying Ying. Thanks for your understanding and always being sweet to me! I would also like to thank Dr. Victar Hsieh, Miss Laerke Thomasen and Dr. Ashraf Mohamed. Thanks for your invaluable support and friendships. Moreover, many thanks to Miss Christine Kwok, Miss Reiko Cheung, Mr. John Lum, Mr. Alex Chan, Miss Carrie Chau, Miss Edith Lai and Miss Lavina Sze. Thank you so much for bringing the joy to my study in this university.

Finally, I would like to thank my parents and sisters for their endless encouragement and support throughout my life. I love you all!

CONTENTS

Abstract of thesis entitled

The Role of Ceruloplasmin in Brain Iron Transport

Submitted by

Tsoi Ying King

For the degree of Master of Philosophy

at the Hong Kong Polytechnic University in December 1999.

ABSTRACT

This research project aims to study the role of Ceruloplasmin (CP) in brain iron transport by the use of transferrin-free iron ($^{59}Fe^{2+}$) and glioma cell line BT325. CP is a copper-binding protein suggested functioning in iron metabolism.

In this study, rat CP was isolated successfully by the modified protocol (Ryan et al. 1992). The isolated rat CP has an A_{610}/A_{280} ratio of 0.044 (indicates the purity is near 100%) and an oxidase activity of 239.8 units/ml (using N,N-dimethyl-p-phenylenediamine as substrate) (Chapter 3). The isolated rat CP was used for the investigation of the species-specificity of CP functions in brain iron transport. The iron stimulatory effect of CP was not species-specific, at least among the three CPs (human CP, rat CP and bovine CP) tested (Chapter 6). The Fe^{2+} uptake properties of the BT325 cells were also characterized (Chapter 4). The optimum pH was in the range of 5.5-6.0. The uptake increased with a rise in iron concentration (0-5 μM) and showed saturation, giving a V_{max} of 7.64 pmole Fe/mg protein and mean K_m of 0.05

μM. Also, three buffers (0.32 M sucrose, 0.155 M sodium chloride solutions and the maintenance medium of the cell line, RPMI 1640 medium) were used for buffer selection. At low iron concentration (1 μM Fe^{2+}), no significant difference in the iron uptake was observed. A 30-minute incubation time was chosen for subsequent experiment, as iron uptake was stable in this condition. In Chapter 5, the effect of CP on the iron flux of brain cells was investigated. CP did not aid in iron release in iron-loaded glioma cells. Although iron release was observed when apoTf (20 and 50 μg/ml) was present in the incubation mixture, the effect was not due to CP but apoTf. Therefore, it is concluded that CP did not involve in the iron release of this glioma cells. To further investigate the role of CP in iron flux, the effect of CP on iron uptake was studied (Chapter 7). By incubating the cells with (0-300 μg/ml) CP and 1 μM Fe^{2+} at 37°C for 30 minutes, iron uptake was increased significantly. The minimal effective CP concentration had to be more than 5 μg/ml and the maximal uptake was reached at 30 μg/ml. The results demonstrated that CP helped in iron uptake but not release, and it plays a role in brain iron metabolism. Since there are reports in which CP stimulated iron uptake was only observed in iron-deficient cells, the effect of CP on iron uptake of iron-deficient BT325 was studied (Chapter 8). Two groups of iron-deficient glioma cells were prepared by incubating them with 2 mM Fe^{3+} chelator desferoxamine mesylate (DFO) and 0.5 mM Fe^{2+} chelator bathophenanthroline disufonic acid (BP) for 16 hours in serum-free RPMI medium at 37°C. Before this preparation, the effect of these iron chelators on the growth of this cell line was investigated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) assay. It was showed that an incubation time

longer than one day with these two iron chelators inhibited the growth of these cells. The incubation of 0-30 μg/ml CP and 1 μM Fe^{2+} with both the iron-sufficient and iron-deficient cells for 30 minutes at 37°C demonstrated that there was no significant difference between the iron uptake of these two groups. These results are not consistent with recent findings that CP is able to stimulate iron uptake only in iron-deficient cells. This may be due to differences in experimental conditions and the use of cell type. In addition, the importance of CP ferroxidase activity on iron uptake of BT325 cells was supported by the use of ferroxidase-defective CPs, apoCP and heat-inactivated CP which did not stimulate iron uptake. Finally, CP-stimulated iron uptake was shown to be inhibited by both 10 μM divalent and trivalent cations (Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+} and Cr^{3+}). This raises the suggestion that these metals may be transported by the same mechanism which needs further investigation.

LISTS OF PUBLICATIONS

Qian ZM, Chan KM, **Tsoi YK**, Wang Q, Ke Y and Zhou XF (1998) Immunohistochemical analysis of CP expression in human glioma cell. RFBUSP 34(Supp.1):86(IX Biennial Meeting Internet.Soc.FRR., Sept., Sao Paulo, Brasil).

Qian ZM, Pu YM, Chen WF, To Y and **Tsoi YK**: Iron Metabolism and CNS Diseases; in Qian ZM (ed): Iron Metabolism – Fundamental and Clinical Aspects. Sciences Press, PRC, 2000, pp329-352.

Wang Q, Qian ZM, Pu YM, To Y and **Tsoi YK**: Iron Mesurement; in Qian ZM (ed): Iron Metabolism – Fundamental and Clinical Aspects. Sciences Press, PRC, 2000, pp 165-184.

Xiao DS, Xu B, Chen WF, Tong YH, Shen X, Chen RS, Wang GF, Zhou XF, Ke Y, **Tsoi YK**, To Y and Qian ZM (1999) Changes in plasma concentrations of nitric oxide and iron in long-term swimming exercise in rats. Physiological Research 48(supp 1):S139

Xiao DS, Xu MF, Chen WF, Ke Y, **Tsoi YK**, Shen X and Qian ZM (1999) Effects of long-term strenouous exercise on transferrin-free iron uptake by bone marrow cells in rats. Physiological Research 48(Supp 1):S139

Chapter 1

INTRODUCTION

1.1 INTRODUCTORY STATEMENT

This chapter provides a general introduction to the aim of this thesis. Why studying brain iron transport is important? What are the mechanisms involved in iron transport? Why ceruloplasmin was chosen in this study? Moreover, what are the recent findings on this issue are included in this introduction.

1.2 WHY IRON IS IMPORTANT?

Iron has been used since early times as a metal capable of restoring strength to people suffering from weakness. Physicians in Egypt, Greece and India used iron in their 'therapeutics'. In the 17^{th} century, Sydenham identified the value of iron in chlorosis, the 'green sickness' of iron-deficient anaemic girls.

Being present in every cell in the body and is the most abundant trace metal in the brain, iron serves as an essential component of numerous cellular enzymes. These include the cytochrome oxidases, a number of enzymes in the citric acid cycle,

ribonucleotide reductase (the rate-limiting step for DNA synthesis), and NADPH reductase (Yehuda and Youdim 1991).

With respect to neurological activity, the availability of iron is crucial for brain cell viability (Beard et al. 1993; Benjamin 1995, Hu and Connor 1996). Iron is involved in the function and synthesis of dopamine, serotonin, catecholamines, and possibly γ-aminobutyric acid (GABA) and myelin formation (Beard et al. 1993). It is the key component of the heme in cytochrome proteins, permitting mitochondrial electron transfer during cellular respiration. Because neurons are especially dependent upon aerobic metabolism (indeed, the brain has a higher rate of oxidative metabolism than any other organ), it can be argued that iron is likely to be especially critical in the brain (Connor and Benkovic 1992; Benjamin 1995). Moreover, iron is involved in synthesis and degradation of fatty acids and cholesterol and likely has an important role in both myelinogenesis and myelin maintenance (Connor and Benkovic 1992). Although iron is an essential nutrient, it is also a potent toxin (Benjamin 1995). Unregulated iron ("free" iron) is highly toxic and is a prime initiator of lipid peroxidative damage (Connor and Benkovic 1992). Iron in its ionic form can catalyze reactions which produce reactive oxygen species (such as the potent hydroxyl free radical) that cause DNA and protein damage as well as cell death by a process known as oxidative stress (Klomp and Gitlin 1996).

The interest in brain iron dates back at least to Spatz, who is credited with publishing the first hitochemical study documenting high iron concentration in the basal ganglia in 1922 (Yehuda and Youdim 1991; Benjamin 1995). It is found that the brain iron

concentration is high relative to other organs, suggesting substantial importance for brain function. Despite being absolutely essential, free ionic iron is very toxic when present in high concentration in or around cells. This paradox, especially the possibility that iron-catalyzed toxic free radicals might mediate neurodegenerative diseases has intrigued many neurochemists and neuropathologists to resolve. Not just they are interested, the unique mechanism of iron in self-regulation through translational control of iron-binding proteins has also catches the attention of molecular neurobiologists. And the neuroimmunologists are likely to become familiar with brain iron because of the element's critical influence upon immune cell activation. The paramagnetic nature of iron offers the opportunity to visualize it using magnetic resonance imaging (MRI) has also fascinated many neuroimaging experts (Hall et al. 1992; Benjamin 1995). With input from such a diversity of fields, elucidating iron's role in the brain has become a propitious and realistic goal (Benjamin 1995).

1.2.1 BRAIN IRON DEVELOPMENT & DISTRIBUTION

Neurons and glia require iron as do all cells for many aspects of their cell physiology (Hu and Connor 1996). Iron is distributed to different cell types in the brain in a heterogeneous fashion through the action of transferrin (Tf), transferrin receptors (TfRs), and the metabolic needs of those cells (Beard et al. 1993). And iron uptake into the brain is a continual process throughout life (Hu and Connor 1996). In human and rat brain iron increases with age and reaches its maximum values at the age of 30-40 years and 4-5 weeks postnatal respectively (Yehuda and Youdim 1991; Hu and

Connor 1996). These observations may due to the expression of Tf receptor (TfR) during development. Studies in rat brain showed that the TfR density was low at birth and increased about 4 times by postnatal day 18. The TfR level was then stabilized into adulthood (3 months of age) (Connor 1994).

The most notable aspect of iron in the brain is its highly characteristic localization (Yehuda and Youdim 1991; Hu and Connor 1996) and uneven distribution when compared to other metals (e.g. copper, zinc, magnesium and manganese). Hallgren and Sourander in 1958 have found the iron content of globus pallidus, red nucleus, putamen and substantia nigra are higher than the iron levels in the liver, the main site of iron metabolism in the body. In which, the concentration of iron in the whole brain is about one-fifth that of the liver (Hallgren and Sourander 1958). And in the basal ganglia iron is present at a concentration equal to that found in the liver (Hu and Connor 1996).

1.2.2 BRAIN IRON MEDIATED EFFECTS

Brain iron level is significant in several brain functions. On cerebral asymmetries, iron is found to potentiate the activity of the left hemisphere and subjects exhibited an increased level of verbal fluency with a decreased level of performance in non-verbal auditory tasks. Moreover, iron is required for normal function of the rat parietal association cortex (Ruiz et al. 1984).

The deposition of iron in localized brain areas may cause epileptic fits. This finding has been supported by direct injection of an aqueous solution of iron salts into the brain which causes experimental epilepsy (Willmore and Rubin 1981). It is also suggested that changes in brain iron level may be involved in schizophrenia (Casanova et al. 1992;Weiser et al. 1994). Iron is also involved in regulating thyroid functions (Rosenzweig and Volpe 1999), lipid metabolism and in some immunological functions such as fever. The potentially negative effects of iron deficiency on the immune system have been studied in humans and animals. The number of circulating T-cells is reduced in iron-deficient animals. However, the mode of action of iron was not clear (Yehuda and Youdim 1991).

1.2.3 BRAIN IRON & DISEASES

An imbalance in brain iron and hence a dysfunction in iron-related metabolism are suspected in some neurological disorders (Gerlach et al. 1994; Hu and Connor 1996; Lan and Jiang 1997).

Increased brain iron level is associated with Alzheimer's disease (AD), Parkinson's disease (PD), Hallervorden-Spatz syndrome (HVS), multiple sclerosis, Pick's disease, Huntington's chorea, Kaschin-Beck's disease, and tardive dyskinesia (Yehuda and Youdim 1991; Connor and Benkovic 1992; Hall et al. 1992; Hodgkins and Blair 1997).

On the other hand, individuals with iron deficiency are irritable and apathetic - their level of attention and exploratory behavior are reduced. They may also suffer from anorexia and a decrease in physical exercise. Moreover, insufficient iron levels during early postnatal development will result in mental and motor impairments which then persist into adulthood (Yehuda and Youdim 1991). Iron deficiency can alter neurotransmission involving dopamine, the major neurotransmitter of the extrapyramidal system of the brain. It seems that iron deficiency alters the function of a specific postsynaptic dopamine receptor. Iron may directly involved in the biochemical and functional aspects of D_2 receptors or indirectly involved via other chemical processes that serve to maintain the proper functioning or synthesis of these receptors (Connor 1994).

The high levels of iron deposited in the brain may readily induce reactive oxygen species to initiate lipid peroxidation of cellular membranes via Fenton type reactions. Thus, increase the oxidative stress in susceptible brain areas such as the frontal and temporal cortex, the key target areas for neuronal degeneration and senile plaque and neurofibrillary tangle formation (Hodgkins and Blair 1997).

How and why iron accumulates in the substantia nigra and globus pallidus in normal circumstances and why its concentration is further exaggerated in these regions in patients with neurodegenerative diseases is not known (Yehuda and Youdim 1991). Because of the current lack of understanding of iron regulation in the brain, it is difficult to ascertain whether changes in iron levels or in the histological distribution of iron reported in many of these diseases is in response to the disease state or part of

the pathogenesis. Thus, iron must be presented to the brain and transported to cells within the brain in a timely and well-regulated manner. Since the imbalance of brain iron can causes the above-mentioned neurological disorders, regulating the optimal level of iron in the brain seems to be a very important function (Yehuda and Youdim 1991). The importance of elucidating the role(s) of iron regulatory proteins in the maintenance of brain iron homeostasis is thus clear (Connor and Benkovic 1992).

1.2.4 IRON TRANSPORT MECHANISMS

1.2.4.1 Proteins Involved in Brain Iron Metabolism

In normal circumstances, iron does not cross the blood brain barrier (BBB) which is a barrier prevents macromolecules diffusing freely from plasma into cerebrospinal fluid (CSF) and leads to a much lower protein concentration in the latter (65 g/liter, cf. 0.25 g/liter) (Thomas et al. 1989).

Iron which is not immediately involved in metabolic activity in the brain is stored in an inactive form as ferritin. Ferritin has been reported to account for as much as one-third of the iron in the brain (Yehuda and Youdim 1991). It is present in almost all living organisms (Bacon and Tavill 1984). Eukaryotic ferritins have a molecular weight of 450 kDa and consists of different ratios of H and L chains (Juan and Aust 1998). The turnover of iron in the brain is significantly slower than in the liver. Thus, in nutritional iron deficiency it is serum iron binding capacity and the liver iron

stores which are depleted more rapidly and to a greater extent. Brain iron storage capacity remains rather constant (Yehuda and Youdim 1991).

The iron transport protein, transferrin (Tf) is responsible for delivering iron to the brain across the BBB via a specific receptors located on the brain microvasculature. It was first isolated and characterized in 1947 by Laurell and Ingelman. Since then, the functional importance of it has been well documented (Huebers and Finch 1987). Tfs are widely distributed in the physiological fluids of vertebrates and some invertebrates. Tfs belong to a family of related metal-binding transport glycoproteins with *in vivo* specificity for iron. Members of this family include serum Tf, lactoferrin, ovotransferrin (also known as conalbumin), and melanotransferrin (MTf; formerly known as tumor antigen p97). In human, the gene code for Tf is located at chromosome 3. This 80 kDa protein can bind two atoms of Fe^{2+} with high affinity (K_d = 10-23). The iron is complexed with Tf in the ferric form (Richardson and Ponka 1997). Iron reportedly enters the brain homogeneously and then redistributes, possibly via Tf, into subcortical regions, particularly the basal ganglia (Yehuda and Youdim 1991). Normally, the level of Tf transcript expression in the brain increases from birth to adulthood. The brain is the only organ in which a postnatal increase in Tf transcript is observed (Connor and Benkovic 1992).

1.2.4.2 Transferrin-dependent Iron Uptake

The best described process for iron uptake into mammalian cells is the Tf/Tf Receptor(TfR)-mediated pathway which was described in an excellent review in

Qian and Tang 1995. In this pathway, iron uptake was initiated with the binding of diferric transferrin to receptors on the cell membrane. Then the Tf-iron enters the cell by endocytosis of the receptor-Tf-iron complex. Iron is released from Tf within the acidic endocytic vesicles. The TfR complex is recycled to the cell surface, where apotransferrin (apoTf) dissociates from TfR. According to accumulated information the process of Tf endocytosis can be distinguished into 6 main steps: binding, internalization (endocytosis), acidification, dissociation and reduction, translocation, and cytosolic transfer of iron into intracellular compounds such as ferritin or haem (Qian and Tang 1995).

At cellular level, the regulation and management of iron is primarily by two proteins found in most cells throughout the body: the Tf receptor (TfR) and ferritin. By controlling the level of expression of these two proteins, the cell can determine the amount of iron acquired (proportional to the number of TfR) and sequestered (proportional to the cytoplasmic level of ferritin). The co-ordinated control of TfR and ferritin by cellular iron occurs at the post-transcriptional level and is mediated by cytoplasmic RNA binding proteins, known as the iron regulatory proteins (IRPs). The same elegant system that exists in systemic organs has been demonstrated in brain cells for regulating iron concentration and availability (Qian and Wang 1998).

The TfR is a disulfide-bonded dimer consisting of two identical 95 kDa molecular weight subunits i.e. total molecular weight of 180 kDa. It is a transmembrane glycoprotein that contains at least three N-asparagine linked oligosaccharides. Each receptor can bind one or possibly two molecules of Tf. Its gene located at

chromosome 3. TfRs have very high affinity for diferric Tf with an estimated association constant of 2-7 x 10^{-9} M. This allows even very low levels of iron-loaded Tf to be bound by its receptor (Trowbridge et al. 1984; Huebers and Finch 1987). The physiological significance of the TfR is suggested by its presence in virtually all cultured cells and by the fact that hypotranferrinemic animals develop severe hemochromatosis (Craven et al. 1987). However, it is highly possible that TfR expression is not the only factor determining iron uptake by brain cells, nor is Tf the only transporter of iron in the brain (Qian and Wang 1998). Although Tf is considered to be the iron transporter and responsible for its transport into the brain across cell membrane, Tf distribution does not coincide with the highly localized iron-rich brain regions described previously (Yehuda and Youdim 1991).

1.2.4.3 Transferrin-independent Iron Uptake

In normal individuals more than 99% of the iron in plasma is bound to Tf. A much smaller fraction, known as non-Tf-bound iron, circulates complexed to low molecular weight molecules such as ascorbate and certain amino acids (Wright et al. 1986). Non-Tf iron transport system was present on a wide variety of cells (Basset et al. 1986; Wright et al. 1986; Morgan 1988; Thorstenson 1988; Wright et al. 1988; Sturrock et al. 1990; Kaplan et al. 1991; Seligman et al. 1991; Randell et al. 1994; Neumannova et al. 1995; Qian et al. 1996; Trinder et al. 1996; Baker et al. 1998; Gutierrez et al. 1998a). Although incubation conditions are artificial, leading to debate on the nature of non-Tf-bound iron uptake, the existence of it is unquestioned because it clearly occurs in hypotransferrinemic mice (Craven et al. 1987) and

humans (McNamara et al. 1999), and plays a critical role in the pathophysiology of hereditary hemochromatosis and other iron-overloaded states (Fleming et al. 1999; Garrick et al. 1999). It has been suggested that non-Tf-bound iron enters the cells by different mechanisms, such as carrier-mediated uptake, simple diffusion and lipid peroxidation mediated iron uptake (Qian et al. 1996).

The function and mechanism of the Tf-independent mechanisms are not well understood (Qian and Tang 1995; Qian et al. 1996). Two pathways for non-Tf-bound iron uptake have been reported in rabbit reticulocytes. One is a high-affinity mechanism that is limited to reticulocytes, saturates at a low iron concentration, and is inhibited by metabolic inhibitors. The other is a low-affinity process that is found in both reticulocytes and erythrocytes, becomes more prominent at higher iron concentrations (Egyed 1988; Egyed 1991; Hodgson et al. 1995). There is increasing evidence that non-Tf-bound iron may be quantitatively more important in producing the iron loading and hepatic damage seen in hemochromatosis and other iron-overload diseases (Wright et al. 1988). Researchers have suggested that the Tf-independent pathway may clear potentially toxic, low molecular weight iron chelates (Attieh et al. 1999). Recently, two putative iron transporters, DMT1 (Divalent Metal Transporter) (Gruenheid et al. 1995) and SFT (Stimulator of Fe Transport) (Gutierrez et al. 1997), have been identified in mammalian cells.

1.2.5 RECENT FINDINGS ON OTHER IRON TRANSPORT PROTEINS

1.2.5.1 DMT1 (Divalent Metal Transporter)

Tf-independent iron transport mechanism is not well understood. Even though a number of iron-binding proteins have been identified, the roles for specific proteins in mediating iron absorption have not been definitively assigned. A recently identified putative proteins called DMT1 (Divalent Metal Transporter) (Gruenheid et al. 1995) is suggested to be another principle iron transport protein in higher organisms (Gruenheid et al. 1995; Lee et al. 1998).

Many investigators (Gruenheid et al. 1995; Gunshin et al. 1997; Fleming and Andrews 1998; Andrews 1999) studying iron transport now refer to *Nramp2* or DCT1 as DMT1. This new designation was used throughout this thesis, although the referenced literature uses DMT1 or DCT1. DMT1 is a member of the Natural Resistance Associated Macrophage Protein (*Nramp*) family (Gruenheid et al. 1995). The common features of this family include 12 predicted transmembrane domains and an extracytoplasmic loop with potential glycosylation sites.

Nramp1 was the first member of the family (Fleming and Andrews 1998). It was involved in the natural resistance to various antigenically and taxonomically unrelated microorganisms such as *Mycobacterium bovis*, *Salmonella typhimurium* and *Leshmania donovani* (Gruenheid et al. 1995). *Nramp1* is expressed primarily in macrophages and mutations at this locus cause susceptibility to infectious diseases (Gruenheid et al. 1999). The mechanism of action of *Nramp1* in host defense is unknown. Originally, it was hypothesized to be involved in nitrate and nitrite

transport (Vidal et al. 1993). To date this hypothesis has not been validated experimentally, and it is unlikely to be correct. Recently it has been shown that *Nramp1* expressed in transfected COS cells altered cellular iron balance and promoted the release of accumulated iron. These observations led to the speculation that *Nramp1* confers resistance to pathogens by depletion of iron or another metal from endosomes. This inhibits the growth of organisms dependent on the metal(s), perhaps because of a need for the detoxifying metalloenzyme superoxide dismutase. Although no coding sequence mutations have been found, *Nramp1* has also been implicated in mycobacterial resistance in human proteins (Fleming and Andrews 1998). The human *NRAMP1* cDNA encodes an open reading frame of 550 amino acid residues and a calculated molecular weight of mass of 59 880 (Kishi and Tabuchi 1997).

DMT1 was first identified in 1995 on the basis of its homology to *Nramp1* (Gruenheid et al. 1995). There is nearly complete homology between transmembrane domains 4 of *Nramp1* and DMT1. Strikingly, the arg^{185} mutation in DMT1 affects the amino acid immediately C-terminal to that altered by the asp^{169} mutation in *Nramp1*. Taken together, these findings suggest that transmembrane domain 4 is functionally important in *Nramp* proteins (Fleming and Andrews 1998). Unlike *Nramp1* which is expressed exclusively in reticuloendothelial macrophages, DMT1 has a much broader range of tissue expression. Immunoblotting experiments indicate that DMT1 is present in a number of cell types, including hemopoietic precursors, and is coexpressed with *Nramp1* in primary macrophages and macrophage cell lines (Gruenheid et al. 1999). DMT1 is expressed as a 90-100 kDa integral membrane

protein extensively modified by glycosylation (>40% of molecular mass) (Gruenheid et al. 1999).

The role of this transmembrane protein in iron transport was suggested by the observation of two murine animals. Mutations in DMT1 have been shown to be associated with microcytic anemia in *mk* mice and defective iron transport in *Belgrade* rats (*b* rat) (Lee et al. 1998).

Mice with the *mk* mutation (*mk* mice) have defects in both the import of iron into intestinal epithelial cells and the import of iron into red cells. As a result, they have a combination of low total body iron stores and iron deficiency anemia that is not correctable by parenteral administration of iron. The known components of the Tf cycle appear to be intact in *mk* mice (Fleming and Andrews 1998).

b rats have an iron transport defect that is similar to that in *mk* mice. Studies using radioactive iron bound by iodine 125-labeled Tf have shown that diferric Tf is taken up into *b* reticulocytes, but the iron is poorly retained, and much is inappropriately recycled to the extracellular space along with Tf. In addition to the erythroid abnormality, *b* rats have impaired intestinal absorption iron as well as diminished acquisition of iron by other cell types. Overall, the functional abnormalities suggest that there is a defect in a membrane carrier of iron that is common to many tissues, including the erythron and the intestine (Fleming and Andrews 1998).

Both *mk* mice and *b* rat have a gly185arg missense mutation in DMT1 suggested that DMT1 function is particularly important for intestinal iron absorption and erythroid iron utilization (Fleming and Andrews 1998; Andrews et al. 1999a).

In 1997, the full length cDNA for human DMT1 was isolated and characterized by screening an oligo-dT primed brain cerebral cortex cDNA library with human *Nramp1* homologue. The Nucleotide sequence analysis reveals that the cDNA has 4142 bp in length which coded for a protein of 561 amino acid residues with a molecular weight mass of 61 456. Predicted amino acid sequence analysis of the DMT1 molecule indicates that *Nramp1* and DMT1 sequences share 64% identical residues overall, whereas only 21% at the NH_2-terminal cytoplasmic domain, where the *Nramp1* molecule was associated with microtubules, was found to be identical. This suggests that the NH_2-terminal region of theDMT1 molecule may have particular function, different from that of the *Nramp1* molecule (Kishi and Tabuchi 1997).

Screening of three human cDNA libraries (an intestine GT10 library, an ovary lambda DR2 library and a thymus GT10 library) (Lee et al. 1998) and the Northern bolt analyses of transgenic mice with dietary iron deficiency (Fleming et al. 1999) revealed that there were primarily two splice forms of DMT1. One splice form containing Iron Responsive Element (IRE) called DMT1(IRE) form, was homologous to the rat DMT1 cDNA in that it contained one iron-responsive element in the 2.2 kb 3' untranslated region. The other splice form of DMT1, designated DMT1(non-IRE) form, replaced the C-terminal 18 amino acids of the IRE form with

a novel 25 amino acids and had a new 3' untranslated region that did not contain a classical IRE (Lee et al. 1998).

The expression of DMT1 in intestinal cells has been shown to be regulated in response to iron status. Rats that are made iron deficient have markedly increased levels of DMT1 mRNA detectable by Northern blotting of intestinal epithelial cells (Fleming et al. 1999). In addition, transient overexpression of the wild type but not G185R DMT1 in HEK293T cells results in a robust stimulation of cellular ^{55}Fe uptake (Gruenheid et al. 1999). The basis of this regulation has not yet been fully defined. The putative promoter of the human DMT1 gene contains several potential metal response elements, suggesting that there may be transcriptional regulation in response to metal levels (Andrews 1999).

By using an *Xenopus* oocyte expression method, Gunshin et al. 1997 showed that DMT1 has unusually broad substrate range that includes Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}. DMT1 mediates active transport that is proton-coupled and depends on the cell membrane potential. Moreover, it functions optimally at low pH (approximately pH 6) (Gunshin et al. 1997). The discovery of DMT1 protein co-localizes with Tf in specific endosomes confirms that DMT1 is the endosomal iron transporter (Su et al. 1998).

Taken together, these results indicate that DMT1 plays a key role in iron metabolism by transporting free Fe^{2+} across the endosomal membrane and into the cytoplasm. Moreover, the ubiquitous expression of its mRNA suggests that it may be involved in

iron metabolism in other tissues as well (Fleet 1998; Fleming and Andrews 1998; Gruenheid et al. 1999). To date, no mutations in DMT1 have been reported in humans. The specific function of DMT1 remains to be defined (Andrews et al. 1999b) have raised several outstanding questions about *Nramp* proteins need to be answered. Is the IRE in DMT1 important in the control of iron absorption in response to fluctuations in body iron stores? Which tissues express the form of DMT1 mRNA that contains the IRE? What purpose does the non-IRE form of DMT1 serve? What role does DMT1 play in tissues other than intestine and erythroid precursors? Is it important in iron transport in the CNS, liver, or kidney? What role does *Nramp1* play? Is it involved in reticuloendothelial cell recycling of iron from effete erythrocytes? Is DMT1 expression or function altered in patients with hereditary hemochromatosis? The identification of the metal transport function of *Nramp* proteins has not only helped to elucidate iron homeostasis, it has also provided new insight not mechanism of host resistance (Fleming and Andrews 1998).

1.2.5.2 SFT (Stimulator of Fe Transport)

Another protein called SFT (Stimulator of Fe Transport) was suggested to be another transmembrane iron transporter. It was first identified in 1997 by functional expression cloning in *Xenopus oocytes*. The SFT-mediated transport has properties defined for Tf-independent iron uptake and its co-localization with late Tf-cycle endosomes indicate it may also participate in intracellular iron membrane transport (Gutierrez et al. 1997). SFT gene is located in human chromosome 10q21 and DNA sequence analysis revealed that it lacks known metal-regulated transcriptional or

translational control elements (Gutierrez et al. 1998b). SFT has six transmembrane-spanning segments and an REXXE motif resembling domains involved in iron binding by ferritin and in iron uptake mediated by the yeast transporter Ftr1. The N- and C-terminal domains were both intracellularly disposed. The REXXE motif may play an important role in regulating SFT activity through interaction with intracellular iron (Yu and Wessling-Resnick 1998).

Recent data obtained from HeLa cells showed that SFT was able to stimulate uptake of both Tf- and non-Tf-bound iron uptake. The assimilation of non-Tf-bound iron by these cells stably expressing SFT was time- and temperature-dependent. Moreover, cellular copper and iron status were found to influence SFT function. Copper-depleted HeLa cells failed to express SFT to stimulate iron uptake above basal levels (Yu and Wessling-Resnick 1998). In iron-depleted rodent BHK cells, SFT transport and iron-binding functions were impaired (Yu and Wessling-Resnick 1998). Furthermore, HeLa cells exposed to high levels of iron down-regulate SFT expression in a time-dependent and reversible fashion. These observations suggest that homeostatic regulation of SFT expression not only ensures that sufficient levels of iron are maintained but also limits excessive assimilation to prevent potentially harmful effects of this toxic metal (Yu et al. 1998).

The iron uptake stimulated by SFT has some different properties from those of DMT1 and is not pH dependent (Andrews et al. 1999b). Since iron trafficking may be too important a function to rely on a single pathway for either form of movement,

and DMT1 and SFT may overlap functionally (Garrick et al. 1999). Their precise function(s) required further investigation.

The recent studies on Melanotransferrin (MTf) (Jefferies et al. 1996), Lactoferrin receptor (LfR) (Faucheux et al. 1995) and Ceruloplasmin (CP) (Klomp et al. 1996) also have greatly improved our knowledge of iron transport in the brain (Qian and Wang 1998). In this research project, the role of CP in brain iron transport was investigated.

1.3 CERULOPLASMIN (CP)

1.3.1 WHY STUDY CP?

Despite recognition that iron is important for normal neurological function, the proteins involved in maintaining iron homeostasis within the brain have received little attention until the past 5 years. Until then, studies aimed at determining both general and cellular control of iron in the brain have increased (Connor and Benkovic 1992). Among those researches, most of them are concerning about the iron **uptake** mechanism and rarely concern about the **release** of iron from brain cells. Kaplan had stated that "One of the major unresolved issues in iron metabolism is the mechanism by which iron is released from cells" (Kaplan 1996). Ceruloplasmin (CP) has been suggested functioning in **promoting iron efflux** from cells in the central nervous

system (CNS) (Klomp and Giltin 1996; Harris et al. 1995). Thus, in this research project, the role of the CP in brain iron transport was investigated.

CP was first isolated by Holmberg and Laurell (1948) from pig serum as an abundant copper-containing protein that they termed ceruloplasmin or "the sky-blue protein." Now it is also known as the iron(II):oxygen oxidoreductase owing to its ferroxidase activity (Arnaud et al. 1998).

Being the only known multicopper oxidase in animals (Saenko et al. 1994; Logan 1996; Miyajima et al. 1996). This blue serum α2-glycoprotein is mainly synthesized in hepatocytes and secreted as a holoprotein with 6 atoms of copper incorporated during biosynthesis (Harris et al. 1995, DiSilvestro 1988, Percival and Harris 1990). About 10% of circulating CP occurs as the apoprotein, but it is unclear of this is secreted from the liver without copper or results from a low rate of copper exchange in the plasma and extracellular fluids (Percival and Harris 1990). The normal plasma concentration in an adult man is about 300 μg/ml (Manolis and Cox 1980; Lamb and Leake 1994). In rat, it represents less than 1% of total serum protein (Ryan et al. 1992). The availability of intracellular copper may be rate-limiting for CP secretion (Gitlin et al. 1992). In plasma, copper occurs predominantly bound tightly to CP. Estimates range from 60% to greater than 90% of the serum copper is bound this way (Miyajima et al. 1996).

1.3.2 STRUCTURE OF CP

CP has been studied in a variety of species including human (Ryden 1972; Noyer et al. 1980; Farver et al. 1999), pig (Ryden 1972), rabbit (Ryden 1972; Morell et al. 1968; Mainero et al. 1996), horse (Ryden 1972; Madda et al. 1987), rat (Manolis and Cox 1980; Weiner and Cousins 1983; Ryan et al. 1992), sheep (Calabrese et al. 1988b), bovine (Calabrese et al. 1981), chicken (Starcher and Hill 1966; Machonkin et al. 1999), goose (Hilewica-Grabska et al. 1988) and from reptilian, turtle (Giovanni et al. 1990). The human CP has an isoelectric point of about 4.4 and an absorption peak at 605 nm (Shreffler et al. 1967). The reported molecular weights of them are very similar which in the range of about 100-135 kDa (Table 1). With advanced technology, the molecular weight of human CP was confirmed to be 132 kDa (Takahashi et al. 1984).

The human CP is a single polypeptide chain of 1046 residues and 4 asparagine-linked oligosaccharide chains. Its gene has been mapped to chromosome 8 in humans and chromosome 2 in rats. CPs in general are less complex structurally than the oligomeric blue oxidases found in plants and fungi. Unlike the latter, CP is polyfunctional (Saenko et al. 1994) and is unique in being able to oxidize both organic and inorganic substrates (Gitlin 1992).

In 1976, Ryden and Bjork first postulated that the copper atoms in the protein are distributed as two type 1 or 'blue' coppers, one type 2 ('non-blue'), one type 3 (binuclear) center. A unique type 4 (mononuclear) Cu^{+} has also been postulated to fulfill the total complement.

Table 1. The relative molecular weight of CP from different species.

Source	Relative molecular weight	References:
Human	160 000 110 000 134 000 135 000 130 000 132 000	Kasper and Deutsch 1963; Ryden 1972; Ryden and Bjork 1976; Noyer et al. 1980; Arnaud et al. 1988; Takahashi et al. 1984;
rat	124 000 131 000 126 000	Manolis and Cox 1980; Weiner and Cousins 1983; Ryan et al. 1992;
Pig	102 000	Ryden 1972;
Horse	106 000 115 000	Ryden 1972; Madda et al. 1987
Goose	121 300	Hilewica-Grabska et al. 1988
Chicken	133 000 140 000	Stevens et al. 1984 Calabrese et al. 1988a
Bovine	125 000	Calabrese et al. 1981
Rabbit	106 000 125 000	Ryden 1972; Mainero et al. 1996;
Sheep	130 000	Calabrese et al. 1988b
Turtle (reptilian)	Not reported	Giovanni et al. 1990

Type 1 copper is responsible for the unusually strong electronic absorption around 600nm and is paramagnetic. Type 2 copper is essentially silent in the optical spectrum, but contributes to the EPR spectrum with a lineshape quite typical of regularly coordinated tetragonal complexes (Musci et al. 1993). Moreover, it is shielded from the aqueous environment (Saenko et al. 1994). Type 3 copper consists of a pair of metal ions, antiferro-magnetically coupled to give an EPR-silent species. It absorbs in the near-UV region of the electronic spectrum, giving a shoulder around 330nm (Musci et al. 1993). The X-ray structure of human serum CP has been solved at a resolution of 3.1 Å. The structure reveals that the molecule is comprised of six plastocyanin type domains arranged in a triangular array. There are six copper atoms: three form a trinuclear cluster sited at the interface of domains 1 and 6, and there are three mononuclear sites in domains 2, 4 and 6. Each of the mononuclear coppers is coordinated to cysteine and two histidine residues, and those in domains 4 and 6 also coordinate to a methionine residue. In domain 2, the methionine is replaced by a leucine residue which may form van der Waals type contacts with the copper. The trinuclear centre and the mononuclear copper in domain 6 form a cluster essentially the same as that found in ascorbate oxidase, strongly suggesting an oxidase role for CP in the plasma (Zaitseva et al. 1996; Musci et al. 1999).

1.3.3 CP SYNTHESIS AND ITS RECEPTORS

While most plasma CP derives from hepatic synthesis, this protein is also produced in other tissues including brain (Mollgard et al. 1988; Aldred et al. 1995; Loeffler et al. 1996), heart (Linder and Moor 1977), uterus (Thomas and Schreiber 1989),

placenta, yolk sac (Aldred et al. 1987), mammary gland (Jaeger et al. 1991), lung (Fleming and Gitlin 1990; Fleming et al. 1991), testis (Skinner and Griswold 1983), and synovium (Dixon et al. 1988). In which the lung is the predominant site of extrahepatic CP gene expression during fetal development in the rat (Fleming et al. 1991). In non-hepatic adult tissue, CP mRNA has been found principally in organs of reproduction (Skinner and Griswold in 1983; Aldred et al. 1987; Thomas and Schreiber 1989).

1.3.3.1 CP Synthesis

Copper is incorporated into newly synthesized CP is proceeded before CP secretion. The copper incorporation process results in an apparent conformational change in the CP molecule which does not affect the secretory rate of the protein. Moreover, this process is independent of N-linked carbohydrate addition. (Sato and Gitlin 1991). In a pulse-chase study of murine CP biosynthesis and secretion in glial cell cultures. CP was first synthesized as a precursor polypeptide of 130 kDa apparent molecular mass which was converted to a mature peptide of 132 kDa after 15 minutes. Secretion of the 132 kDa species was almost completed after 2 hours of chase, although some (<5%) of the 130 kDa band remained intracellular after 8 hours (Klomp et al. 1996). The expression of CP mRNA in fetal rat brain was detected between 14.5 and 20.5 days of gestation, and the levels of CP mRNA in newborn rat were higher than that of the adult rats (Thomas et al. 1989).

1.3.3.2 Glycosylphosphatidylinositol (GPI)-anchored CP

There are two forms of CP. One is the secreted form of nonanchored soluble CP (Loeffler et al. 1996; Klomp and Gitlin 1996) as mentioned above. The other one is the glycosylphosphatidylinositol (GPI)-anchored form of CP (Patel and David 1997; Salzer et al. 1998; Fortna et al. 1999). The reported molecular weights of the GPI-anchored CP in astrocytes and Schwann cells, and Sertoli cells were 140 kDa (Salzer et al. 1998) and 135 kDa (Fortna et al. 1999) respectively. This form of CP is not seen on hepatocytes and cells of the choroid plexus, both of which are known to secrete CP.

The GPI-anchored form of CP found on astrocytes which has ferroxidase activity may regulate iron transport in and out of neurons and glia in the CNS, and may help in limiting lipid peroxidation in a tissue that is highly susceptible to oxidative injury (Patel and David 1997). Apart from these, the finding that Schwann cells also express the GPI-anchored form of CP suggests that this GPI may play a role in axonal-glial interactions (Salzer et al.1998). Since the serum CP does not cross the BBB and the levels of the secreted form of CP in the CSF is normally very low (1 μg/ml) (Del Principe et al. 1989), the GPI-anchored form of CP on astrocytes is likely to be the major of this molecule in the CNS (Patel and David 1997).

Like the brain, testis also posses a blood barrier acting as nutritional gatekeepers. Thus, the GPI-anchored CP may be the dominant form expressed by Sertoli cells and that Sertoli cell detergent-insoluble glycolipid-enriched membrane microdomains

which is enriched by GPI-anchored CP may play a role in iron metabolism within the seminiferous tubule (Fortna et al. 1999).

It is not yet clear whether the CPs detected in the conditioned media of those reported nonanchored form of CP in astrocytes (Klomp and Gitlin 1996) and Sertoli cells (Skinner and Griswold in 1983) are actually the secreted type or are the GPI-anchored form of CP which were released from the cell surface due to cleavage of the GPI-anchor (Salzer et al.1998). Further investigation is need.

Factors reported to cause changes in the synthesis of CP are copper, hormones and leucocytic endogenous mediator (Weiner and Cousin 1983). Copper does not affect the rate of synthesis or secretion of apoceruloplasmin (apoCP) (Nakamura et al. 1995), but failure to incorporate copper during biosynthesis results in an unstable protein lacking oxidase activity (Percival and Harris 1990; Gitlin et al. 1992). In addition, the presence of copper in CP was reported to increase its longevity in the serum of animals (Weiner and Cousin 1983).

1.3.3.3 CP Receptors

CP receptors were also found on the cell membrane. CP receptors were first characterized in the membrane of chick heart and aorta (Stevens et al. 1984). Since then, CP receptors were also found in rat liver endothelial cells (Kataoka and Tavassoli 1984; Tavassoli 1985), human erythrocytes (Barnes and Frieden 1984), lymphocytes, monocytes and granulocytes (Kataoka and Tavasssoli 1985), human

fibroblasts (Sasina et al. 1998) and hemin-induced K562 cells (Percival and Harris 1988). The data suggests that these organs have the capacity to make this protein (Stevens et al. 1991). The reported molecular weight of CP receptors isolated from human erythrocytes is 60 kDa (Barnes and Frieden 1984). CP receptors were suggested to be a receptor of internalizing type and its density on the cell surface depends on CP availability in the culture medium and is regulated by the negative feedback mechanism (Saina et al. 1998). Moreover, the internalization of CP receptor is temperature sensitive. At 4°C, only surface binding occurred. Internalization was observed at 37°C (Kataoka and Tavassoli 1985; Tavassoli et al. 1986; Sasina et al. 1998).

CP binds with high affinity to membranes of various cells. In most cases, saturation of membrane receptors is reached with nonamolar quantities of the CP, which in effect is below the level normally found in plasma (Kataoka and Tavassoli 1985; Percival and Harris 1988). The mechanisms of CP recognition by these specific receptors and their intervention in copper transfer have not been fully elucidated. However, such an interaction leads to a cross-membrane transport of copper has been shown. The interaction probably requires Cu^{2+}, since specific binding is lost when the CP is treated with ascorbate. In K562 cells, when binding is carried out at 37°C, part of bound ^{67}Cu penetrates the cell. The fraction penetrating is proportional to the CP concentration in the incubation medium. However, CP protein does not penetrate the cells (Percival and Harris 1988). Similar result was demonstrated in Chinese hamster ovary (CHO) cells (Orena et al. 1986). Thus, how copper ions move across membrane barriers such as those present in brain, intestine, and placenta is unknown

but seems clearly to depend on the presence of CP (Saenko et al. 1994). In another study using human red blood cells, CP was found to be bound on the membranes, but does not penetrate through it into the red blood cells (Arnaud et al. 1998).

However, CP protein has been reported to be internalized during its encounter with certain cells in culture medium such as liver endothelium (Kataoka and Tavassoli 1984; Tavassoli 1985; Tavassoli et al. 1986) and leukocytes (Kataoka and Tavassoli 1985). For K562 cells (Percival and Harris 1988) and CHO cells (Orena et al. 1986), no endocytosis of CP was observed. Therefore, it may not be the principal mode of copper delivery to these tissues. The apparent contradiction does not reflect two different mechanisms rather two different receptors. Kupffer cells, for example, cannot take up gold-labeled sheep CP unless liver endothelial cells are also present. Liver endothelium has the capacity to desialylate CP and internalize the modified molecule via an asialoglycoprotien receptor. The uptake of a partially degraded CP molecule by this receptor, therefore, may be the basis of the endocytosis reactions attributed to such cells (Kataoka and Tavassoli 1984; Tavassoli 1985). Endocytosis by this mechanism, however, is not likely to be related to a copper transport function (Saenko et al. 1994). The finding that CP receptor numbers are down regulated by iron loading provides further evidence of a link between iron and CP (Crowe and Morgan 1996).

1.3.4 FUNCTIONS OF CP

The precise physiological function of CP remains controversial, although many workers tend to consider the protein multifunctional (Percival and Harris 1990; Saenko et al. 1994). At least four main functions have been attributed to CP, those of copper transport, ferroxidase activity, amine oxidase activity and as an antioxidant in the prevention of the formation of free radicals in serum (Zaitseva et al. 1996). The following are summary of physiological functions attributed to CP :

1) the ferroxidase activity is considered pivotal in mobilizing iron for transport via the protein Tf (Saenko et al. 1994);
2) its ferroxidase activity also eliminates free iron from the plasma, thereby protecting blood and membrane lipids from peroxidative damage. This is supported by its ability to protect phospholipids in vascular walls and erythrocyte membranes from peroxidation as well as DNA from scission. Thus, acting for antioxidant defense (Saenko et al. 1994). Moreover, it can partially protects rat heart against myocardial injury induced by oxygen free radicals (Chahine et al. 1991);
3) as the major copper-containing component of plasma, CP is a donor of copper to extrahepatic tissues;
4) its amine oxidase activity has the potential to control levels of biogenic amines in plasma, cerebral, spinal, and interstitial fluids;
5) with sequence homology to blood clotting factors V and VIII and an ability to bind to platelets, CP may partake in blood clotting or its regulation (Saenko et al. 1994; Pan et al. 1995);
6) as a deaminase;

7) having superoxide dismutase-like activities (Goldstein et al. 1979; Aldred et al. 1987), it can inhibits superoxide-induced lipid peroxidation, but apparently does not possess superoxide dismutase activity (Gutteridge 1983; Weiner and Cousin 1983);
8) as an acute phase protein in the inflammatory response (Aldred et al. 1987; Thomas and Schreiber 1989; Fleming et al. 1991; Saenko et al. 1994);
9) for tissue angiogenesis (Gitlin et al. 1992) and
10) as an endogenous neuronal depolarizing factor (Wang et al. 1995).

Many of the projected functions of CP are related to the six copper atoms bound to the peptide chain. Suggested that Cu centers in CP have been the focus of much of the research on this protein. Also, it is postulated that the limiting step in a CP-catalyzed reaction is a function of the rapidity of reduction of the 'blue' copper center and not the affinity of the substrate for its binding site on the protein (Zaitseva et al. 1996).

For the catalytic activity of CP towards CNS amines, such as dopamines, adrenaline, and serotonin, have suggested CP regulated their fluid levels. However, a role for CP in the metabolism of these substances has not been established. Moreover, since the pH optimum for CP-catalyzed oxidation of adrenaline, *p*-phenylendiamine, catechol, and dimethy-*p*-phenylendiamine, is close to 5 (Ryan et al. 1992), and a higher pH results in a sharp decline in activity, it is unlikely that CP regulates these amines *in vivo* (Saenko et al. 1994). However, other researchers have suggested CP could have significant physiological impact to the brain cells. They based on the reactions at

which CP produces (DHI)-melanin from 5,6-dihydroxyindole and THP-melanin from tetrahydropapaveroline at pH 7.4 (Rosei et al. 1998).

CP has long been suggested as an *in vivo* antioxidant (Gutteridge 1978). The antioxidant function of CP has been demonstrated in endothelial cells and endogenous neutrophil, and endothelial cell proteins (Krsek-Staples and Webster 1993). CP was a much more effective peroxyl radical scavenger than superoxide dismutase, desferroxamine and bovine serum albumin (Atanasiu et al. 1998).

CP's antioxidant effect may involve:

a. donating copper to tissues for the synthesis of copper containing antioxidant enzymes;
b. directly scavenging superoxide anions; or
c. oxidatively incorporating Fe^{2+} into Tf or serum ferritin, thus rendering the iron redox inert.

The last function is of particular importance because CP can oxidize the ferrous complexes back to the less reactive ferric state (Gutteridge 1991). Moreover, it effectively catalyzes the oxidation of Fe^{2+} while directly reducing molecular oxygen to water, thus preventing the formation of partially reduced species of oxygen ($O_2^{\cdot}$, OH, H_2O_2), which are potentially deleterious to biomolecules (Ryan et al. 1992). However, the findings that heat-denatured CP was also able to inhibit some Fe^{2+} dependent radical formation lead to the argument that the effective chain-breaking antioxidant effect of CP is independent of its catalytic ferroxidase activity (Atanasiu

et al. 1998). Other researchers have pointed out that CP 'denaturation' does not always imply "inactivation", as far as the oxidase activity is concerned (Calabrese et al. 1988b). Thus, the ferroxidase activity is still important for CP's antioxidant effect.

Being an antioxidant CP can inhibit lipid peroxidation induced by iron and copper ions (Olson and Holtzman 1980; Gutteridge 1983; Saenko et al. 1994). In the latter, protection is attributed to non-specific copper-binding sites on the CP molecule (Gutteridge 1983). CP inhibits only those lipid peroxidations that are induced by a chemical redox system (Olson and Holtzman 1980; Gutteridge 1983; Miura et al. 1993; Saenko et al. 1994). No antioxidant effect of CP is observed when free radicals are formed in the absence of redox processes, e.g., upon illumination with UV-light (Al-Timimi and Dormandy 1977; Saenko et al. 1994). This antioxidant property of CP may be of particular importance in protecting the brain from active oxygen radicals damage. Since the brain contains a large amount of polyunsaturated fatty acids and a very high metabolic rate, it is very susceptible to free radicals attack (Beard et al. 1993).

During a variety of disease and stress states, human serum CP levels increase. Therefore, plasma CP has been a diagnostic marker for assessing the prevalence of brief inflammations that often accompany certain cancers, brain tumors (Casaril et al. 1989; Varela et al. 1997), rheumatoid arthritis, tuberculosis, hypoxia, psoriasis, biliary cirrhosis and other chronic liver diseases. It is also noted that CP level will rise during pregnancy (Wolf 1982; Saenko et al. 1994, DiSilvestro et al. 1988).

CP is extremely susceptible to oxidative modification *in vitro* and this may add to the susceptibility toward proteolytic attacks (Arnaud et al. 1988; Calabrese et al. 1988b). Such susceptibility has been a major obstacle in the study of the physicochemical characteristics of the protein. It is well known that once purified, CP easily undergoes conformational changes that lead to irreversible modifications of its spectroscopic properties (Musci et al. 1990).

1.3.5 CP FERROXIDASE ACTIVITY & IRON TRANSPORT

A unique feature of CP is its ability to oxidize Fe^{2+} to Fe^{3+} (Kawanami et al. 1996). This CP ferroxidase activity was first demonstrated by Osaki et al. in 1966. It has been stated that iron is the best substrate for CP (Ryan et al. 1992). As a substrate, Fe^{2+} has the lowest apparent K_m and the highest V_{max} of any of CP's multiple substrates. At neutral pH, CP increases the rate of the nonenzymatic oxidation of serum Fe^{2+} in humans by 10- to 100-fold. It catalyzes the oxidation of 4 atoms of Fe^{2+} with the concomitant production of water from molecular oxygen. This prevents the free radical formation which occurs during spontaneous ferrous oxidation [eqt. 1] (de Silva and Aust 1992).

$$4Fe^{2+} + O_2 + 4H^+ \xrightarrow{CP} 4Fe^{3+} + 2H_2O \text{ (eqt. 1)}$$

The oxidation of ferrous iron to ferric iron proceeded 10 to 20 times more rapidly in the presence of CP than in its absence (Ryan et al. 1992; Kawanami et al. 1996;

Logan 1996), suggesting a role for CP in ferric iron uptake by Tf. It was the first attempt to link iron metabolism with the biological functions of copper. The hypothesis offered a clear rationale as to how one trace metal, copper was able to regulate the homeostasis of a second, iron, a biochemical cooperation observed some 30 years earlier but still defying explanation (Harris 1995; Harris 1999). Consistent with this concept, the anemia that develops in copper-deficient animals is unresponsive to iron but is correctable by CP administration (Harris et al. 1995). Another possible of CP may be the loading of iron into ferritin, an activity that would classify CP as an iron detoxifying agent (Boyer and Schori 1983). Ryan and co-workers have found CP effectively inhibited net iron release from ferritin (via iron reincorporation into ferritin) and thus effectively inhibited ferritin-dependent lipid peroxidation (Ryan et al. 1992). This research group had proposed that the mechanism by which iron is placed into ferritin is catalyzed by CP and found that ferritin plus CP is an effective 'antioxidant'. Iron in ferritin loaded by CP behaves similarly to iron loading into ferritin is maximum at 1:1 molar ratio of CP to ferritin, that CP forms a complex with ferritin, and that the maximum amount of iron loaded into ferritin (~2500 atoms) is the same as the total amount of iron that can be found in ferritin *in vivo* (Ryan et al. 1992; de Silva and Aust 1992). Lipid peroxidation can occur during oxidation of ferrous iron by CP (Aust 1995). In saline solution at physiological pH, iron presented to apoferritin was not incorporated into the core in the absence of CP (Kawanami et al. 1996).

When Tf is present, it binds the ferric product and thus protects it from subsequent reduction. The presence of both CP and Tf in the plasma normally provides

considerable antioxidant protection by preventing iron induced free radical formation (Saenko et al. 1994; Logan 1996). Human cells store iron in the ferric state in ferritin and haemosiderin. When iron is needed by the body, the ferric iron is reduced and moved to the outside of the cells. But whether or not this is so, CP catalyzed oxidation of the exported ferrous iron is still required because spontaneous ferrous oxidation cannot provide a sufficiently large supply of ferric iron for binding to Tf and subsequent distribution to the body (Logan 1996).

Such a mechanism predicts that oxidation of Fe^{2+} to Fe^{3+} precedes ferric ion attachment to Tf, the ferric iron-transport protein. Additionally, CP can also catalyze the incorporation of Fe^{3+} into ferritin, the iron storage protein (Saenko et al. 1994). Some researchers found that ferritin does not accumulate iron oxidized by CP (Treffry et al. 1995). However, recent studies showed that iron loading is occurred in the presence of CP. The entire BC loop of H chain of ferritin might be crucial for its interaction with CP for iron loading, as it increases CP ferroxidase activity and the rate of iron loading into ferritin (Juan and Aust 1998; Juan and Aust 1999).

Thus, by catalyzing the oxidation of Fe^{2+}, CP promotes both of the incorporation of iron into Tf and ferritin. A specific ability of CP to stimulate iron mobilization from the stores in the liver of copper-deficient animals may be related to its powerful ferroxidase activity and links CP with iron metabolism. At the same time, the ferroxidase activity removes 'free' or unbound Fe^{2+} and Fe^{3+} from caches in cell membranes or serum, preventing their participation in free-radical generating reactions (Fig. 1).

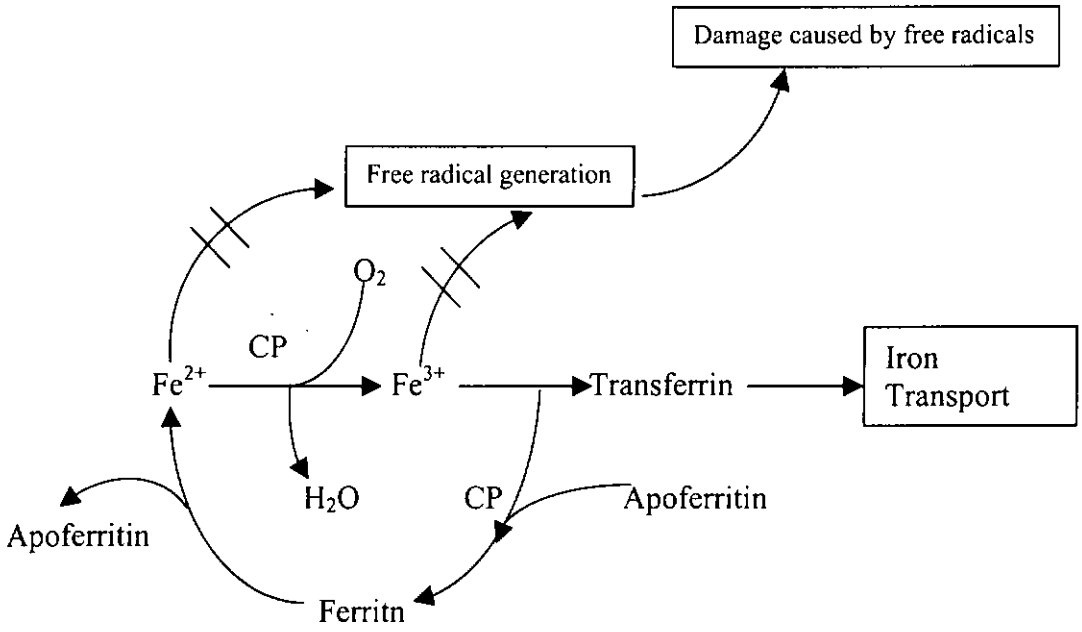

Fig. 1. Role of CP ferroxidase activity in iron transport and in prevention of free radical processes involving Fe^{2+} or Fe^{3+}. CP catalyzed oxidation of Fe^{2+} to Fe^{3+} in the presence of O_2 (which undergoes four-electron reduction to H_2O without oxygen radicals released) This enables Fe^{3+} to be attached to Tf or to ferritin. CP removes unbound Fe^{2+} and Fe^{3+} from cell membranes or serum thus preventing their participation in free-radical generating reactions (This figure is reproduced from Saenko et al. 1994).

Thus, the 'free iron' concentrations in the plasma are related to the copper-binding properties and ferroxidase activities of CP. Because copper atoms in CP have no access to O_2^- through diffusion, CP must protect cells from this the progenitor of free radical reactions through other mechanisms. As mentioned above, CP is capable of increasing the rate of incorporation of Fe into Tf and ferritin, thereby effectively eliminating unbound Fe in plasma as fast as it forms. This putative protective role for the ferroxidase activity in antioxidant action of CP has been confirmed by a number of laboratories and extends from membrane lipids to DNA (Saenko et al. 1994).

1.3.6 CP'S SIGNIFICANCE IN BRAIN IRON REGULATION

Despite years of investigation, the functional role of CP has not been clarified. A direct role in copper transport is unlikely. It is because kinetic data indicates that the copper is turned over at the same rate as the protein (Holtzman and Gaumnitz 1970b) and plasma CP does not readily exchange its copper atoms with unbound copper owing to its center's distortion which poses an energy barrier to the binding of copper ions (Saenko et al. 1994).

A link of CP and iron metabolism has been suggested by many researchers (Lee et al. 1968; Barnes and Frieden 1984; Saenko et al. 1994; Harris 1995; Crowe and Morgan 1996; Klomp and Gitlin 1996). In copper-deficient swine the copper deficiency results in a disorder of iron storage and that CP administration could correct this situation (Lee et al. 1968). This hypothesis is strengthened by recent studies in yeast identifying a CP homologue (*FET3*) that functions as an essential copper oxidase. *FET3* is able to catalyze the conversion of Fe^{2+} to Fe^{3+} necessary for high-affinity iron transport to take place (Askwith et al. 1994; De Silva et al. 1995; Stearman et al. 1996).

The synthesis of CP in the CNS (Klomp and Gitlin 1996; Patel and David 1997; Salzer et al.1998) and the discovery of aceruloplasminemia (Harris et al. 1995; Kawanami et al. 1996; Klomp and Gitlin 1996; Logan 1996; Miyajima et al. 1996;

Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998) suggest a previously unrecognized role for CP in brain iron metabolism.

As mentioned before, CP is also produced in brain (Zahs et al. 1993; Aldred et al. 1995; Loeffler et al. 1996; Patel and David 1997; Mollgard et al. 1988; Salzer et al. 1998). The importance of CP synthesis in this interfacing tissue extends beyond a copper transport role. It could be related to a paracrine system for copper movement (Aldred et al. 1987; Saenko et al. 1994). Moreover, the synthesis of Tf in these tissues, together with the catalytic activity of CP in the oxidation of iron prior to binding to Tf, may be of significance in the transport of iron across compartment barrier. The ability of CP to oxidize serotonin and the catecholamines, adrenaline, noradrenaline, dopamine, and dopa, may be of importance in other extracellular compartments, especially the CSF (Aldred et al. 1987). Since under ordinary circumstances CP does not cross the BBB, it seems likely that such a role would be mediated by CNS expression of CP (Arnaud et al. 1998; Harris et al. 1996).

The genetic characterization of aceruloplasminemia provides initial insight into the specific role of CP in iron metabolism in the brain. Aceruloplasminemia is a newly recognized autosomal recessive disorder of iron metabolism due to mutations in the CP gene (Harris et al. 1995; Gitlin 1998). Patients with aceruloplasminemia do not synthesize CP, and they exhibit systemic siderosis, neural and retinal degeneration and diabetes. No defect in copper accumulation, but show excessive iron deposition in selected tissues indicated that **CP plays little role in copper transport but has an essential role in iron metabolism**. Tissue injury is associated with excessive iron

accumulation most probably related to the generation of toxic oxygen radicals (Miyajima et al. 1996). The mechanisms of systemic iron accumulation in aceruloplasminemia are presumed to result from rapid ferrous iron uptake in the liver, pancreas, and other tissues due to the loss of CP ferroxidase activity. This implies that the oxidation of iron for subsequent binding to Tf is an essential function of CP (Klomp and Gitlin 1996; Harris et al. 1995). Another finding that supports the hypothesis that CP plays a role in brain iron metabolism is that patients with hereditary CP deficiency have striking features of the neuropathological changes in affected areas such as neuronal cell loss and iron deposition in basal ganglia. These are very similar to patients with HVS (Kawanami et al. 1996), PD (Boll et al. 1999) and other neurodegenerative disorders (Loeffler et al. 1996). The mechanisms of iron accumulation in the retina and brain are unknown (Klomp and Gitlin 1996; Harris et al. 1995). Since Fe^{3+} is the only iron form that can bind to apoTf and enter the bloodstream, CP deficiency would decrease recycling of iron from cell to plasma, resulting in cellular iron deposition (Kawanami et al. 1996).

Further lines of evidence supporting the role of CP in CNS iron metabolism include the change of CP concentration and ferroxidase activity in patients with neurodegenerative disorders. In the CSF of PD patients, the CP ferroxidase activity is reduced and this change may be related to the development of PD (Boll et al. 1999). However, other researchers have found that CP level is increased in the CSF of AD patients and suggested the concentration of CP is not high enough to contribute to CSF antioxidant defense (Loeffler et al. 1994).

Study in normal aging persons and AD patients showed that there was loss in excess of one-third of the CP in both gray and white matter from superior temporal gyrus in AD brains. In normal aging control group, CP level increased in the gray matter but remained at the same level in the white matter. However, in AD patients, gray matter CP levels failed to increase with age and the level of CP in white matter actually decreased. The researchers suggested that the reduction in CP concentration could cause decreased in cellular metabolic processes such as the electron transport system and a decrease in the ability of the brain to protect itself from oxidative damage. Moreover, they hypothesized that brain iron transport is diminished in AD brain (Connor et al. 1993). A more recent study showed AD and age-matched controls have similar levels of CP that is confined to neurons but CP is significantly increase within the neuropil of AD patients. These suggested the neuronal induction of CP is feeble in AD, even while there is an increase in tissue CP. Therefore, a failure of neuronal CP to respond to iron may be an important factor that leads to an accumulation of redox-active iron in neurons in AD (Castellani et al. 1999).

Increased brain CP concentrations in these disorders may indicate a localized acute phase-type response and/or a compensatory increase to oxidative stress (Loeffler et al. 1996) and possibly alteration in brain iron homeostasis (Connor et al. 1993; Castellani et al. 1999). Since the CP levels were increased in some, but not in all regions of the brain cells, further study in determining the significance of CP in brain iron metabolism is required. Furthermore, past studies based on either immunological or enzymatic determinations, have pointed out that only minor quantitative changes of CP occur upon aging in both normal human and human with dementia (Lyngbye

and Kroll 1971; Molaschi et al. 1996). It is also suggested that during aging CP is subjected to oxidative modifications which are likely to be the source of conformational changes around the copper sites leading to an intramolecular electron rearrangement among the various copper sites (Musci et al. 1993). Thus, it is important to investigate whether the causes of disorders in these neurodegenerative diseases are due to the quantitative change of CP concentration and/or the qualitative change of CP ferroxidase activity due to oxidative modifications or disturbed expression.

1.3.7 CONCLUSION

All these pieces of information imply that CP may function in brain iron metabolism. What exactly is the role of CP in iron metabolism? There are two different hypotheses. One of them is the traditional hypothesis which suggested that CP functions in iron release from cells (Lee et al. 1968; Osaki and Johnson 1969; Roeser et al. 1970; Osaki et al. 1971; Frieden and Hsieh 1976; Harris 1995; Kaplan 1996; Klomp et al. 1996; Young et al. 1997; Harris et al. 1999; Richardson 1999; Vulpe et al. 1999). Based on the study in perfused mammalian liver, Osaki et al. 1971 proposed that the amount of iron mobilized is not dependent on either the ferroxidase or apoTf concentration. It is the ferroxidase activity that results in the substantial elimination of Fe^{2+}, generating a maximum concentration gradient from the iron stores to the capillary system. Thus, promoting a rapid iron efflux from the iron storage cells of the perfused liver (Osaki and Johnson 1969; Roeser et al. 1970; Osaki et al. 1971). It is possible that local CP production in these cells facilitates this

process. Together with the latest finding of another CP homologue, hephaestin which is suggested for iron egress from intestinal enteroxytes into the circulation (Vulpe et al. 1999). It is thus suggested that in the absence of CP, iron would be unable to move into the brain extracellular fluid and CSF for eventual excretion causing iron accumulation in certain areas of the brain such as the basal ganglia (Klomp et al. 1996).

The other hypothesis was evolved recently. It is suggested that CP cannot regulate iron release (Logan 1996) and CP functions in iron uptake of cells (Mukhopadhyay et al. 1998; Attieh et al. 1999). Logan made his statement based on the fact that CP is normally present in excess which allows iron turnover to increase when extra iron is required (by up to 5 times normal in pigs). Study in cultured HepG2 and K562 cells showed that CP ferroxidase activity stimulates cellular iron uptake (Mukhopadhyay et al. 1998; Attieh et al. 1999), and in K562 cells, it is stimulated by a trivalent cation-specific transport mechanism in cultured cells (Attieh et al. 1999). Apart from this, another a CP homologue called *FET3* identified in yeast which functions as an essential copper oxidase necessary for high-affinity iron transport (Askwith et al. 1994; De Silva et al. 1995; Stearman et al. 1996) also strengthened this hypothesis.

These two hypotheses suggest that further analysis of the function of CP within the CNS may be of value in elucidating the mechanisms of neuronal loss in aceruloplasminemia and perhaps in other neurodegenerative disorders where abnormalities in iron metabolism have been demonstrated (Klomp et al. 1996).

1.4 OBJECTIVES

In this research study, the role of CP in brain iron transport was investigated by using the glioma cell line BT325. Histological studies indicate that maintenance of iron homeostasis in the brain is the responsibility of neuroglia and possibly the choroid plexus (Connor and Benkovic 1992). CP either in secretion form or the GPI-anchored form has been demonstrated in the glial cell, astrocytes (Zahs et al. 1993; Loeffler et al. 1996; Klomp and Gitlin 1996; Patel and David 1997; Salzer et al. 1998). Thus, it is suggested that glial cell-specific CP gene expression is essential for iron homeostasis and neuronal survival in the human CNS (Klomp and Gitlin 1996). Much of our present knowledge of glial cell function stems from studies of glioma cell lines, both rodent (C6, C6 polyploid and TR33B) and human (1321N1, 138MG, D384, R-111, T67, Tp-276MG, Tp-301MG, Tp-483MG, Tp-378MG, U-118MG, U-251MG, U-373MG, U-787MG, U-1242MG, and UC-11MG) (Brismar 1995). In this study, the glioma cell line BT325 is a human glioblastoma multiforme cell line established from a glioma on the frontal lobe of a senior man (Shao et al. 1988). (For details please refer to Materials and Methods.) Elevated serum CP levels in these cells have been reported (Seitz and Wechsler 1987; Manjula et al. 1992).

The non-Tf bound iron uptake of cells was studied instead of Tf-bound iron in this research project. It is because of the unique function of CP which oxidizes Fe^{2+} to Fe^{3+}. This reaction was predicted to be preceded before ferric ions attach to Tf (Fig. 1) (Saenko et al. 1994). Recently, a study in HepG2 cells showed that CP did not

affect Fe uptake from Tf or nitrilotriacetic acid (NTA) (Richardson 1999). The followings are the objectives of this research project.

(1) To isolate rat CP from rat serum by a modified protocol for the investigation of the species-specificity of CP function (in Chapter 6), and discussed about the many different methods employed in the purification/isolation of this protein from different species.

(2) To characterize the non-Tf-bound iron uptake properties of the glioma cell line BT325. The results obtained will add to the knowledge on this cell line and for subsequent experimental condition selections.

(3) To investigate the effect of CP on iron flux of brain cells. Whether CP functions in iron release or uptake was studied.

(4) To investigate the species-specificity of CP in stimulating iron uptake.

(5) To find out the effect of CP on iron uptake of cells with different iron status (iron-sufficient and iron-deficient)

(6) To examine the effect of divalent and trivalent ions on the iron uptake stimulatory effect of CP.

(7) To investigate the importance of CP ferroxidase activity.

The findings obtained will advance our knowledge of the functions of CP in brain iron transport which may have broad implications for understanding the mechanisms of neuronal injury in a number of human neurodegenerative diseases.

Chapter 2

MATERIALS, APPARATUS AND METHODS

2.1 MATERIALS

2.1.1 SOURCE OF REAGENTS AND BIOCHEMICALS

Acrylamide	Sigma Chemical Co., MO, USA
ε-amino-n-caproic acid	Calbiochem Ltd., CA, USA
Apotransferrin	Sigma Chemical Co., MO, USA
2-b-mercaptoethanol	Sigma Chemical Co., MO, USA
Bathophenanthroline-disulfonic acid	Sigma Chemical Co., MO, USA
Bovine ceruloplasmin	Sigma Chemical Co., MO, USA
Bromophenol blue	Sigma Chemical Co., MO, USA
Coomassie blue R-250	Sigma Chemical Co., MO, USA
DEAE Sepharose Fast Flow	Pharmacia Biotech Inc., Sweden
Deferoxamine mesylate	Sigma Chemical Co., MO, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT)	Sigma Chemical Co., MO, USA
Fetal bovine serum (qualified, heat inactivated)	Gibco BRL, USA
Human ceruloplasmin	Sigma Chemical Co., MO, USA
N-2-hydroxyethylpiperazine-2-ethanesulfonic acid (Hepes)	Sigma Chemical Co., MO, USA

2-mercaptoethanol	Gibco BRL, USA
Penicillin-streptomycin	Gibco BRL, USA
1,4-piperazinediethanesulphonic acid (Pipes)	Sigma Chemical Co., MO, USA
p-phenylenediamine	Gibco BRL, USA
Pronase	Calbiochem, La Jolla, CA
Protein Assay Kit	Sigma Chemical Co., MO, USA
RPMI Medium 1640	Gibco BRL, USA
Rat serum	Sigma Chemical Co., MO, USA
SigmaMarker (High Molecular Weight Range MW 36,000-205,000)	Sigma Chemical Co., MO, USA
Sodium dodecyl sulfate	Sigma Chemical Co., MO, USA
Sodium sulfide	Sigma Chemical Co., MO, USA
Superdex 200 prep grade	Pharmacia Biotech Inc., Sweden
Temed	Bio-Rad Laboratories Inc., USA
Trypan blue solution	Fluka, Buchs, Switzerland
Trypsin(2.5%, 10X)	Gibco BRL, USA

2.1.2 RADIOCHEMICAL

Radioisotope $^{59}FeCl_3$ with specific activity of 0.16 mCi/ml (28.8 μg Fe/ml in 0.1 M HCl) was obtained from Radiochemical Center, Amersham International Inc., Buckinghemshire, England, UK.

2.1.3 GLIOMA CELL LINE BT325

The BT325 is a generous gift from the Su Zhou Medical School. It is a cell line of human glioblastoma multiforme which has been established from a glioma on the frontal lobe of a senior man. This cell line was designated BT325 and has been maintained in culture for over four years. They have the adherent property in normal culturing condition. Various morphologic features, globose, bipolar, stellate and multinuclear giant cells have been observed from the culture. Doubling time was approximately 36.5 hours. The karyotype was shown hypotetraploid and had one marker chromosome in most cells. Cell cycle phase and relative DNA content in cells were measured from 9^{th} to 57^{th} passage by FCM (Flow Cytometry) and PLM (Percent Labeled Mitoses) curves. Cells inoculated into nude mice subcutaneously grew into tumors similar to the original tumor histologically. Microtublues and bundles of filaments can be observed by electron microscope. Cells are positively stained by antiserum to glial fibrillary acidic protein. Results of PTAH (Phosphotungistic acid haematoxylin) stained and Foot's stain proved these cells were malignant glioma. The rate of vitality of those frozen cells around 77% showed the growth and character of the cell line was stable (Shao et al. 1988).

2.1.4 SOLUTIONS

2.1.4.1 Double Distilled Water

Water which had been distilled and deionized was further treated by passing it through a Fi-streem water purification system (England).

2.1.4.2 Phosphate Buffered Saline (PBS)

This was prepared by diluting one part of sodium phosphate buffer to 50 parts 0.15 M NaCl. The pH was adjusted to 7.4.

All solutions were prepared with double distilled water (ddH_2O). The solutions and media used in cell culture were sterilized by passing through a 0.22 μM Millipore membrane (Millipore Asia Ltd., Hong Kong).

2.2 APPARATUS

2.2.1 TOOLS FOR CELL CULTURE

Cells were plated on 75 cm^2 culture flasks, 6-well plates and 96-well plates purchased from Iwaki Glass Co., Ltd., NY, USA.

2.2.2 LAMINAR FLOW CABINET

All cell culture work was performed inside a Nuair Laminar Flow Cabinet, model Nu425-400E (Nuair, Inc., USA) in the animal cell culture room at the Department of

Applied Biology and Chemical Technology of the Hong Kong Polytechnic University. Cells were incubated in a CO_2 incubator, model TC2323 (Shel LAB).

2.2.3 AUTOCLAVE

All the tools for cell culture were cleaned with ddH_2O and then sterilized by autoclave them at 121°C, 15 PSI for 20 minutes before use (Model HA-30, Japan).

2.2.4 MICROSCOPE

Observation of culture was made by using a Nikon inverted microscope (Diaphot-TMD, Japan).

2.2.5 INCUBATOR

A water bath (JB4, Grant instruments, UK) was used for all incubation during the iron uptake and cell culture experiment. These were generally performed at 37°±0.5°C.

2.2.6 TOOLS FOR RAT CERULOPLASMIN ISOLATION

A peristaltic pump, model 500, a XK 16/20 column, a XK 26/100 column, a packing reservoir RK 16/26 were purchased from Pharmacia Biotech Inc., Sweden.

Centricon Plus-80 centrifugal filter devices with high flow biomax membrane 50,000 NMWL were purchased from Amicon Ltd., USA. A fraction collector, Retriever 500 (ISCO, Inc., USA) was used to collect the samples from rat ceruloplasmin isolation.

2.2.7 pH METER

pH value of all solutions were measured with an Orion research, Model 701 digital pH meter, in conjunction with Beckman Calibration buffers.

2.2.8 MICROTITER PLATE READER

An automatic Microtiter Plate Reader, model 450 (Bio-Rad Laboratories Inc., USA) was used to measure the absorbance values at 570 nm in the MTT Assay.

2.2.9 ELECTROPHORESIS CELL

Soidum dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run with a Mini-protein II Electrophoresis Cell (Bio-Rad Laboratories Inc., USA).

2.2.10 IMAGING DENSITOMETER

An imaging densitometer, Model GS-670 (Bio-Rad Technology Ltd., USA) was used to scan the gel slab after electrophoresis.

2.2.11 CENTRIFUGES

A bench top centrifuge, model 2010 (Kubota Corporation, Japan) was used in cell culture preparation and a Beckman Model J2-21 Centrifuge (Beckman Instruments, Inc., USA) was used in iron uptake experiment.

2.2.12 RADIOACTIVITY MEASUREMENTS

The activity of the γ-emitters, 59Fe was measured using a three channel γ-scintillation counter (Packard 5003 COBRA Q, USA).

2.3 GENERAL METHODS

2.3.1 RAT CP ISOLATION

2.3.1.1 Buffers Preparation

Buffer A: 25 mM phosphate buffer (PB), pH 6.8, containing 20 mM ε-aminocaporic acid (ACA)

Buffer B: 100 mM PB, pH 6.8, containing 20 mM ACA

Buffer C: 30% v/v 200 mM PB, pH 6.8 containing 20 mM ACA added to 100 mM PB, pH 6.8 containing 20 mM ACA

Buffer D: 45% v/v 200 mM PB, pH 6.8 containing 20 mM ACA added to 100 mM PB, pH 6.8 containing 20 mM ACA

Buffer E: 200 mM PB, pH 6.8 containing 20 mM ACA

Buffer F: 25 mM sodium acetate, pH 5.6 with 100 mM NaCl and 20 mM ACA

2.3.1.2 Procedures

Rat CP was purified by the method described by Ryan et al. in 1992 with little modification. All the purification procedures were carried out at 4°C and the chromatographic columns were wrapped in foil to prevent damage from light.

1. The DEAE-Sepharose Fast Flow anion exchange column (1.5 x 15 cm) was equilibrated with 150-200 ml of buffer B (1 ml/min) until the A_{280} nm of the eluent approaches 0.
2. After the equilibration, the 20 ml of rat serum diluted with 40 ml of buffer A was loaded to the column.
3. The stepwise elution gradient was begun with washing the column with 100 ml of buffer C and then with 100 ml of buffer D. CP retention was evidenced by the appearance of a blue band at the top of the column.
4. Then the CP was eluted with buffer E until the blue band disappeared. The A_{610}/A_{280} ratio of the eluent collected was measured. Tubes showing blue coloration and having A_{610}/A_{280} ratio above 0.025 were collected for further purification.
5. The eluent from these tubes were pooled and concentrated by using an Amicon concentrator (Centricon Plus-80 Centrifugal Filter Devices with high flow biomax membrane 50,000 NMWL). It was centrifuged at 4000 rpm for 40

minutes at 4°C, then at 1200 rpm for 3 minutes at 4°C (Beckman Model J2-21 Centrifuge).

6. The final volume of the solution was approximately 4 ml. This concentrated eluent was then loaded onto a Superdex 200 prep grade gel filtration column (2.5 x 100 cm). This column was equilibrated with buffer F.
7. The concentrated sample was eluted with buffer F at 0.3 ml/min. Fractions with A_{610}/A_{280} ratio of about 0.045 were collected.
8. The protein concentration was measured with protein assay kit (Sigma Chemical Co., MO, USA).

2.3.2 PREPARATION OF ApoCP AND HEAT-INACTIVATED CP

ApoCP and heat-inactivated CP were used for the studying of the effect of ferroxidase activity of CP on iron-uptake. They were prepared by the following methods:

2.3.2.1 Preparation of ApoCP

9.5 ml of human CP (10 mg/ml) was dialyzed against 2 L of 10 mM *N,N*-diethyldithiocarbamate (DDC) for 24 h in the presence of reducing 1.0 mM Na_2S (favouring the copper depletion) pH 7.2 at 4°C (the solution will be previously de-aerated). Then the copper-depleted CP will be dialyzed for another 24 hours against

10 mM potassium phosphate buffer. The resultant brown precipitate, Cu^{2+}-DDC will be removed by centrifugation at 50 000 g for 40 minutes (Beckman Model J2-21 Centrifuge). The blue-color will be lost, clearly indicating the alteration of the copper center (Morpurgo et al. 1987; Wang et al. 1995).

2.3.2.2 Preparation of Heat-inactivated CP

Heat-inactivated CP will be obtained by heating it at 75°C for 2 min. The overall protein conformation will be altered by this treatment with the loss of the integrity of blue copper center of the enzyme activity. However, this process does not remove copper from CP (Wang et al. 1995).

2.3.3 ENZYMATIC ASSAY OF CP

The method was provided by Sigma Chemical Co., MO, USA. The principle is that when N,N-dimethyl-p-phenylenediamine was oxidized by CP, a colored oxidized product is formed. The experimental conditions: 37°C, pH = 5.5, A550nm and light path of 1 cm. The enzymatic activity of CP was measured by continuous spectrophotometric rate determination.

2.3.3.1 Reagents Preparation

Reagent A: 200 mM sodium acetate buffer, pH 5.5 at 37°C (100 ml). It was

prepared in deionized water using sodium acetate, trihydrate and adjusted to pH 5.5 at 37°C with 1 M acetic acid.

Reagent B: 153 mM N,N-dimethyl-p-phenylenediamine solution (DPD) (10 ml). It was freshly prepared in deionized water using N,N-dimethyl-phenylenediamine, monohydrochloride and stored on ice. It was kept from light.

Reagent C: 100 mM sodium chloride solution (enzyme diluent) (100 ml). It was prepared in deionized water using sodium chloride.

Reagent D: rCP solution. The isolated solution was diluted with cold buffer.

2.3.3.2 Procedure

The following reagents (in milliliters) were pipetted into suitable cuvettes:

	Test	Blank
Reagent A (Buffer)	2.00	2.00
Deionized water	0.80	0.80
Reagent C (Enzyme diluent)	-----	0.10
Reagent D (rCP)	0.10	-----

The reagents were mixed by inversion and equilibrated to 37°C. The A550 nm was monitored until constant, using a suitably thermostatted spectrophotometer. Then reagent B (in milliliter) was added:

	Test	Blank
Reagent B (DPD)	0.10	0.10

The reagents were immediately mixed by inversion and the increase in A550 nm was recorded for approximately 5 minutes. The A550 nm/minute was obtained using the maximum linear rate for both the Test and Blank.

2.3.3.3 Calculations

$$\text{Units/ml enzyme} = \frac{(\text{A550nm/min Test} - \text{A550 nm/min Blank})(3)(df)}{(0.01)(7)(.1)}$$

3 = Total volume (in milliliters) of assay

df = Dilution factor

= Change in absorbance at 550 nm (unit definition)

7 = conversion factor to published unit definition of a 7 ml reaction volume

3 = volume (in milliliters) of enzyme used

2.3.3.4 Unit Definition

One unit is arbitrarily defined as the amount of "oxidase" which will cause a A550 nm of 0.01 per minute using N,N-dimethyl-p-phenylenediamine as substrate at pH 5.5 and 37°C, in a 7 ml reaction volume.

2.3.3.5 Notes

1. Do not use HCl for adjusting the pH. The chloride ion is an inhibitor of human CP and the concentration must be kept constant. The NaCl is necessary for diluting the enzyme solution.

2. The solution is stable for approximately 2-3 hours. A fresh solution should be prepared if an increase in absorbance is seen in the Blank.

2.3.4 SDS-PAGE

2.3.4.1 Materials

1. Human CP, rat CP, bovine CP and standard protein marker.
2. 0.1% Coomassie Blue R-250
3. 10% acetic acid
4. 40% methanol

2.3.4.2 Procedure

The SDS-PAGE (0.75 mm thick) was carried out by a method described by (Laemmli 1970). The samples were analyzed under reducing conditions. The power used was 200 volts and the run time was approximately 45 minutes. After running, the gel was fixed and stained with 0.1% Coomassie Blue R-250 in 40% (v/v) methanol and 10% acetic acid at room temperature for 30-40 minutes. The stained gel was destained with 40% methanol in 10% acetic acid for 1 to 3 hours until the

background was clear. Then the gel was scanned by an imaging densitometer for record.

2.3.5 CELL CULTURE

The glioma cell line BT325 was maintained in 75 cm^2 culture flasks. The medium (RPMI 1640 containing 10% FBS) was changed every 3 days. Subculture was prepared by removing the medium and incubating it with 3 ml of 0.25% trypsin in each flask. The flasks were placed in the incubator at 37°C for about 15 minutes until the cells detached. Then the cells were transferred to a 15 ml centrifuge tube containing 3-5 ml of fresh medium and centrifuged at 3000 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was triturated in 2 ml of fresh medium. The cell number was counted by trypan blue exclusion under the microscope, and the required number of cells was plated in the flasks (for maintenance) and 6-well plates/96-well plates (for experiments). All the apparatus and medium used for cell culture was sterilized before use. Frozen stocks of the cells were also prepared by the subculture method, except that the fresh medium used was at 4°C and 10% DMSO was used as a preservative (Shao et al. 1988).

2.3.5.1 Preparation of iron-sufficient and iron-deficient cells

For the preparation of iron-deficient cells, the glioma cells were incubated in 6-well plates with 2 mM DFO and 0.5 mM BP in 2 ml of serum-free RPMI medium overnight at 37°C before each experiment. For the preparation of iron-sufficient

cells, the cells were simply incubated with 2 ml serum-free RPMI medium overnight at 37°C. All the media (with or without iron chelators) were filter sterilized before being added to the 6-well plates.

2.3.6 MTT ASSAY

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) assay was used to investigate the effect of the iron chelators, desferoxamine mesylate (DFO) and bathophenanthroline disufonic acid (BP) on the growth of the cell line. This is one of the most frequently used methods for measuring cell proliferation and neural cytotoxicity. It is widely believed that MTT is reduced by active mitochondria in living cells (Denizot and Lang 1986; Liu et al. 1997). Thus, increases in A570 nm obtained in the MTT assay indicate increases in the number of viable cells. It was performed in 96-well microtiter plates. In this study, MTT assay was performed according to (Liu et al. 1997).

2.3.6.1 Materials

1. MTT solution: 5 mg/ml of MTT solution was prepared by dissolving 50 mg MTT in 10 ml of 0.9% NaCl and warmed at 60°C to dissolve.
2. Solubilization solution: 5% iso-butyl alcohol, 10% HCl and 10% sodium dodecyl sulfate was added.

2.3.6.2 Procedure

1. BT325 cells of 90 µl (approximately 10^4 cells) per well, were plated in RPMI 1640 medium at 37°C.
2. After 4 hours, 10 µl of chemicals with the planned concentrations were added to the wells.
3. The cells were incubated with the 100 µM, 500 µM, 1 mM and 2 mM of DFO and BP for the planned periods of time (1-4 days).
4. For the control, no chemical was added.
5. For the blank, only plain medium was added, i.e. no cells and chemicals.
6. After the incubation, 20 µl of 5 mg/ml MTT was added to each well.
7. 4 hours later, 100 µl of a solubilization solution was added.
8. Absorbance values at 570 nm were determined the next day with an automatic microtiter plate reader (Bio-Rad Laboratories Inc.), using 655 nm as the reference wavelength.

2.3.7 PREPARATION OF Tf-FREE METAL SALTS SOLUTION

The solutions of Tf-free metal salts were prepared immediately before use. Firstly, the non-radioactive salts ($FeCl_2$, $CuCl_2$, $MgCl_2$, $ZnCl_2$, $CaCl_2$, $AlCl_3$ and $CrCl_3$) were dissolved in 0.32 M sucrose solution to give a final concentration of 2 mM. Then

aliquots of these metal ion solution were mixed with 1 μM $^{59}Fe^{2+}$ solution to give a final concentration of 10 μM metal ions.

2.3.8 PREPARATION OF RADIOACTIVE IRON ($^{59}Fe^{2+}$) SOLUTION

2.3.8.1 Materials

1. $^{59}FeCl_3$: 0.366 mM in 0.1 M HCl
2. $^{56}FeSO_4 \cdot 7H_2O$: 15 mg + 25 ml of 0.1 M HCl
3. 0.32 M Sucrose solution: 21.907 g sucrose + 0.27704 g Pipes + 200 ml of ddH_2O

2.3.8.1 Procedure

The $^{59}Fe^{2+}$ solution was prepared by a method described by (Morgan 1988).

1. Stock iron solution was prepared by mixing $^{59}FeCl_3$ (28.8 μg Fe/ml in 0.1 M HCl) with $^{56}FeSO_4$ (2 mM in 0.1 M HCl) in a molar ratio of 1:10.
2. 50-fold molar excess of 2-mercaptoethanol and 0.32 M sucrose were added to give an iron concentration of 62.5 μM.
3. Varying amounts of this solution were added to the cell monolayer to give the required iron concentration in the incubation.
4. A concentration of 1.0 μM was used except where indictated.

5. The radioactive iron solution was prepared immediately before use and was used within 1 week.

2.3.9 MEASUREMENT OF THE Tf-FREE ($^{59}Fe^{2+}$) IRON TRANSPORT

Transferrin-free iron ($^{59}Fe^{2+}$) transport by the cultured cells was measured by a method described by (Trinder et al. 1996). BT325 cells were grown in 6-well plates and were ready for experiments until they reached about 80% confluence.

1. The culture medium was decanted and the cells were washed with 37°C Hanks' buffer (pH 7.4) in order to remove dead cells and tissue debris.
2. The cells were then preincubated twice with 2 ml RPMI 1640 medium (serum-free) for 15 mintutes at 37°C. This was done to remove fetal bovine serum and endogenous transferrin (Kaplan et al. 1991; Trinder et al. 1996).
3. Then the cells were washed with 37°C Hanks' buffer (pH 7.4) for three times. $^{59}Fe^{2+}$ of the corresponding concentrations with a final volume of 1 ml was added to the cells for 30 minutes at 37°C.
4. After this incubation, the medium was decanted and the cells were washed with 4°C Hanks' buffer for four times to stop the reaction.
5. 1 ml of ice-cold pronase (1 mg/ml) was added to the cells for 60 minutes.
6. In the measurement of the effect of pH, the cells were transferred to centrifuge tubes and spun at 4000 rpm for 15 minutes to separate membrane bound and

internalized fractions for verification of iron internalization. Total iron uptake was measured in the other experiments with the cells directly transferred to counting tubes for measurement of their radioactivities with a γ-counter.

7. The protein content was measured by a protein assay kit (Sigma Chemical Co., MO, USA). The results were expressed as pmol Fe/mg protein.

For the measurement of iron release, cells were pre-loaded with iron by incubation with 1 ml of 1 μM $^{59}Fe^{2+}$ in 0.32 M sucrose solution buffered with 4 mM Pipes for 1 hour.

2.3.10 MEASUREMENT OF THE EFFECT OF CP ON Tf-FREE ($^{59}Fe^{2+}$) IRON TRANSPORT

Please refer to the corresponding chapters for details.

2.4 ANALYTICAL METHODS

2.4.1 CELL COUNT

BT325 cells in RPMI 1640 medium were counted by staining with 0.2% trypan blue solution before plating. 10 μl of stain was added to 90 μl medium with mixing. Viable cells were distinguished from dead cell by their transparent property under the microscope and were counted. Dead cells would take up the stain and blue granules of cellular cytoplasm were observed.

2.4.2 STATISTICS

Statistical analyses of the data were performed using Microsoft Excel software. The means and S.E.M. values were calculated from standard equations. The student's *t* test was used to compare the results obtained with different groups of experimental data. Differences were considered statistically significant when the probability, *P*, was less than 0.05.

Chapter 3

RAT CERULOPLASMIN ISOLATION (A MINI-PREPARATION)

3.1 ABSTRACT

Ceruloplasmin (CP) is reported to be a multifunctional protein which may also take part in the iron metabolism. For a more thorough understanding of the biological role of this protein, it must be isolated with proper protocol. Since there is no commercially available rat CP (rCP), it has to be isolated from rat serum in the laboratory. This part of experiment describes how rCP was isolated by a modified method described by (Ryan et al. 1992) and discusses about the many different methods employed in the purification/isolation of CP from different species. In this mini-preparation, rCP was successfully isolated with A_{610}/A_{280} ratio of 0.044 (indicates the purity is near 100%) and a N,N-dimethyl-p-phenylenediamine oxidase activity of 239.8 units/ml. SDS-polyacrylamide-gel electrophoresis result showed 2 bands of apparent molecular weights of 136 kDa and 116 kDa of the isolated rat CP correspond to the intact CP and proteolytic fragments formed during the purification and/or storage respectively. The isolated rCP was used for the subsequent experiment.

Keywords:

Ceruloplasmin isolation	Rat serum	*p*-phenylenediamine
Ion-exchange chromatography	Gel filtration	Purification/isolation

3.2 INTRODUCTION

Ceruloplasmin (CP) is a blue serum α2-glycoprotein which is mainly synthesized in hepatocytes and secreted as a holoprotein with 6 atoms of copper incorporated during biosynthesis (DiSilvestro et al. 1988; Percival and Harris 1990; Harris et al. 1995). The human protein is a single polypeptide chain of 1046 residues and 4 asparagine-linked oligosaccharide chains with an overall mass of 132 kDa. Its gene has been mapped to chromosome 8 in humans and chromosome 2 in rats (Saenko et al. 1994). About 10% of circulating CP appear as apoprotein, but it is unclear if this is secreted from the liver without copper or present as a result from a low rate of copper exchange in the plasma and extracellular fluids (Percival and Harris 1990). It is the only known multicopper oxidase in animals (Saenko et al. 1994; Logan 1996; Miyajima et al. 1996). In general, it is structurally less complex than the oligomeric blue oxidases found in plants and fungi. Unlike the latter, CP is polyfunctional (Saenko et al. 1994). At least four main functions have been attributed to CP, those of copper transport, ferroxidase activity, amine oxidase activity and as an antioxidant in the prevention of the formation of free radicals in serum (Zaitseva et al. 1996). CP has also been suggested in promoting iron efflux from cells in the CNS system

(Harris et al. 1995; Klomp and Gitlin 1996). But recently, there are contradictory findings on this function of CP (Mukhopadhyay et al. 1998; Attieh et al. 1999).

In this part of research, a mini-isolation of rat CP (rCP) will be performed by a modified method described by Ryan and co-worker (1992). This part of experiment described how rCP was isolated and discussed about the many different methods employed in the purification/isolation of CP from different species.

3.3 MATERIALS AND METHODS

3.3.1 MATERIALS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO USA). Rat serum was purchased from Gibco BRL (Life Technologies, Inc., USA). Centricon Plus-80 centrifugal filter devices with high flow biomax membrane 50,000 NMWL were obtained from Amicon (Bedford, MA, USA). ε-amino-n-caproic acid was purchased from Calbiochem (CA, USA). Column XK 16/20, Column XK 26/100, packing reservoir RK 16/26, Pump-500, and all the chromatography media (DEAE Sepharose Fast Flow, Superdex 200 prep grade) were purchased from Pharmacia Biotech Inc. (Uppsala, Sweden). Fraction collector Retriever 500 was purchased from ISCO Inc. (Lincoln, Nebraska, USA). Standard 3.5 ml disposable polystyrene cuvettes and Mini-protean II Electrophoresis Cell were obtained from Bio-Rad Laboratories Inc. (California, USA). Centrifuge was from

Beckman Instruments, Inc. (Fullerton, CA USA). Quartz cuvette (SAF-10UVS) was from Cathoden Ltd. (Cambridge, UK).

3.3.2 METHODS

3.3.2.1 Rat CP isolation

3.3.2.1.1 Buffers Preparation

Please refer to Chapter 2.

3.3.2.1.2 Procedure

rCP was purified by the method described by Ryan et al. in 1992 with little modification. All the purification procedures were carried out at 4°C and the chromatographic columns were wrapped in foil to prevent damage from light. The DEAE-Sepharose Fast Flow anion exchange column (1.5 x 15 cm) was equilibrated with 150-200 ml of buffer B (1 ml/min) until the A_{280} nm of the eluent approaches 0. After the equilibration, the 20 ml of rat serum diluted with 40 ml of buffer A was loaded to the column. The stepwise elution gradient was begun with washing the column with 100 ml of buffer C and then with 100 ml of buffer D. CP retention was evidenced by the appearance of a blue band at the top of the column. Then the CP was eluted with buffer E until the blue band disappeared. The A_{610}/A_{280} ratio of the eluent collected was measured.

Tubes showing blue coloration and having A_{610}/A_{280} ratio above 0.025 were collected for further purification. The eluent from these tubes were pooled and concentrated by using an Amicon concentrator (Centricon Plus-80 Centrifugal Filter Devices with high flow biomax membrane 50,000 NMWL). It was centrifuged at 4000 rpm for 40 minutes at 4°C, then at 1200 rpm for 3 minutes at 4°C (Beckman Model J2-21 Centrifuge). The final volume of the solution was about 4 ml. This concentrated eluent was then loaded onto a Superdex 200 prep grade gel filtration column (2.5 x 100 cm). This column was equilibrated with buffer F. The concentrated sample was eluted with buffer F at 0.3 ml/min. Fractions with A_{610}/A_{280} ratio of about 0.045 were collected. The protein concentration was measured with protein assay kit (Sigma Chemical Co., MO, USA). SDS-polyacrylamide gel electrophoresis was performed by the method of (Laemmli 1970) and rCP enzymatic assay was performed as described in methodology.

3.3.2.2 Enzymatic assay of rCP

Please refer to Chapter 2.

3.3.2.3 SDS-PAGE

Please refer to Chapter 2.

3.4 RESULTS

3.4.1 CHROMATOGRAPHY ON DEAE-SEPHAROSE FAST FLOW ANION EXCHANGE COLUMN

After running through the DEAE-Sepharose Fast Flow anion exchange column, the A_{610}/A_{280} ratio of the eluents were measured (Fig.2). For pure CP the A_{610}/A_{280} ratio should be about 0.045 but none of the eluents collected was close to this value which means further purification is required. Therefore, the blue fractions of tube number 19-31 which have A_{610}/A_{280} ratio above 0.025 were pooled for the next purification step.

3.4.2 CHROMATOGRAPHY ON SUPERDEX 200 PREP GRADE GEL FILTRATION COLUMN

The pooled eluents were concentrated by using an Amicon concentrator to about 4 ml. Then it was loaded onto the Superdex 200 prep grade gel filtration column, fractions of tube number 36-42 were pooled and the A_{610}/A_{280} ratio 0.044 (indicates the purity is near 100%).

3.4.3 SDS-PAGE RESULT

An electrophoretic analysis was also performed to assess the purity of this final product. As shown in the SDS-PAGE gel (Fig.4), the isolated rCP gave a band of apparent molecular weights of 136 kDa. This 136 kDa band was most probably the intact rCP which has been reported to have close to that molecular weight. In addition, there was a minor band with apparent molecular weight of 116 kDa. This corresponds to one of the proteolytic fragments formed during purification and/or storage. There should be other bands corresponding to the remaining proteolytic fragments from rCP but they were poorly stained, thus hard to identify their apparent

molecular weight. Similar value of proteolytic fragment of 116 kDa was also reported (Ryan et al. 1992).

3.4.4 RAT CP ENZYMATIC ASSAY RESULT

The enzyme assay conducted (Fig. 5) shown that the rCP solution has an oxidase activity of 239.8 units/ml. Therefore, in this part of research study, rCP was successfully purified from rat serum.

3.5 DISCUSSION

The rCP purification described here is of preparative scale which aims to provide enough protein for the later experiments. The original method was described by Ryan and his colleagues (Ryan et al. 1992) with the use of Fast Protein Liquid Chromatography (FPLC). Instead of using the FPLC system, this purification was carried out with the use of a peristaltic pump. The yield of the rCP cannot be compared with the original method, as there is no data for comparison. But the A_{610}/A_{280} ratio is similar, 0.044 in this case as compared to their reported 0.045. From SDS-PAGE gel (Fig.4), the isolated rCP gave a band of apparent molecular weights of 136 kDa. This 136 kDa band was most probably the intact rCP which has the similar reported molecular weight. Also, there was a minor band with apparent molecular weight of 116 kDa. This is corresponding to one of the proteolytic fragments formed during purification. There should be other bands corresponding to the remaining proteolytic fragments from rCP but they were poorly stained, thus hard

to identify their apparent molecular weight. Similar value of proteolytic fragment of 116 kDa was also reported from the study of Ryan et al. 1992. The specific fragmentation of the rCP molecule may due to trace amounts of proteases, which seem to originate from blood plasma (Moshkov et al. 1979).

Since its first isolation by Holmberg and Laurell in 1948 from human and porcine serum (Arnaud et. al 1998), there are reports of CP isolated from other source of sera and by various methods. CP isolated from human (Ryden 1972; Noyer et al. 1980; Farver et al. 1999;), pig (Ryden 1972), rabbit (Ryden 1972; Morell et al. 1968; Mainero et al. 1996), horse (Ryden 1972; Madda et al. 1987), rat (Manolis and Cox 1980; Weiner and Cousins 1983; Ryan et al. 1992), sheep (Calabrese et al. 1988b), bovine (Calabrese et al. 1981), chicken (Starcher and Hill 1966; Machonkin et al. 1999), goose (Hilewica-Grabska et al. 1988) and from reptilian, turtle (Giovanni et al. 1990) serum have been studied.

The source of starting material is usually whole serum (or placental serum) or fractions thereof, such as the α-globulin fraction. Various large-scale procedures involve precipitation with ammonium sulfate, acetone, ethanol, or caprylic acid, along with ion-exchange and gel-filtration chromatography (Noyer et al. 1980). The gel filtration steps are believed to remove fragments and major contaminants present in the CP-containing fractions (Manolis and Cox 1980).

Many of the methods employed the use of an ion exchange column and then gel filtration. Although most of the isolation protocols are quite similar, sometimes CP

from different species needs different isolation protocol due to the different nature of CP (Starcher and Hill 1966). For example in the preparation of human CP, a major contaminant called C3 (third component of complement) appears after DEAE-Sephadex chromatography which will convert to C3b during storage. On the other hand, rCP apparently carry different charges and a higher ionic strength is required to elute it from the DEAE-column. Moreover, its mobility is more cathodal on electrophoresis in agarose gel than human CP (Manolis and Cox 1980).

CP is extremely susceptible to limited proteolysis which makes it difficult to prevent degradation during purification. In order to minimize the from the purification process, many different purification procedures of CP have been reported in an effort to accomplish the preparation of it in the shortest possible time and under mild conditions (Arnaud et al. 1988; Calabrese et al. 1988b).

These give rise to various modifications such as the use of derivatized Sepharose (Mondovi 1988), addition of protease inhibitors e.g. ε-amino caproic acid (Noyer et al. 1980; Weiner and Cousins 1983; Mondovi 1988; Ryan et al. 1992), precipitation step such as the polyethylene glycol (PEG) precipitation (Noyer et al. 1980), acetate precipitation step (Calabrese et al. 1981) and the ethanol-chloroform precipitation. The later is believed to denature the apo-CP making it unsuitable for studying intact protein (Manolis and Cox 1980). For the acetate precipitation step, it was used for removal of decolorized and poorly active CP molecules (Calabrese et al. 1981). Also, special care has to be taken when dealing with CP from different species. Human CP was reported to be more susceptible to proteolytic attack than rCP (Ryan et al. 1992).

During and after the purification, it is important to monitor the amount and purity of the isolated CP solution. Measurement of CP levels mainly relies on methods that involve quantification of oxidase activity (Weiner and Cousins 1983) and A_{610}/A_{280} ratio.

Many of the projected functions of CP are related to the 6-7 Cu atoms bound to the peptide chain (Saenko et al. 1994). And the type 1 copper of this protein is responsible for the unusually strong electronic absorption around 600 nm (Musci et al. 1993). By making use of this property, CP can be measured by the A_{610}/A_{280} ratio. It is a spectrophotometric indication of the ratio of copper atoms to protein which is consistent with an intact copper-protein complex (Lamb and Leake 1994). The absorption at 610 nm measures only CP fully complexed with copper (or at least with both type I copper atoms) (Noyer et al. 1980). On the other hand, the CP concentration can also be calculated by the equation, $A_{610}{}^{1mM} = 10.9$ (Treffry et al. 1995).

While for the oxidase assaying method, the most well known one was discovered by Holmberg and Laurell in 1951 They observed that *p*-phenylenediamine (PPD) is particularly useful as a substrate for measuring the oxidase activity of CP because the purple oxidation product of PPD can be determined directly by spectrophotometry. The basic principle of the reaction is that at pH 5.4, CP catalyzes the oxidation of PPD to yield a colored product, which is believed to correspond either to Bandrowski's base or to Wuerster's red. The rate of formation of the colored oxidative product is proportional to the concentration of serum CP if a correction is

made for nonenzymatic oxidation of PPD. Therefore, simultaneous assays are carried out with and without sodium azide, which inhibits the enzymatic oxidation of PPD. The difference between the results of the two assays is proportional to the CP concentration. The PPD-oxidase is subject to a lag phase, owing to oxidation of serum ascorbic acid. To avoid this potential source of error, timing of the reaction is delayed until after the lag phase. In general, the PPD-oxidase methods for measurement of serum CP are more sensitive than measurements of the absorbance change of CP at 610 nm before and after decolorization with ascorbic acid or cyanide, or CP by immunodiffusion or enzymatic oxidation of Fe^{2+} (Sunderman and Nomoto 1970). This technique is also used for assessing patient's CP level in serum. Apart from these methods, electrophoretic analysis is frequently used in combination.

It is also important to note that there are many influencing parameters in the CP isolation such as the choice of pH and buffers. There are different optimal pH for CP isolated from different species. Hence, special care is needed to minimize the their influences to the results. The optimal pH for PPD-oxidase activity in human serum is 5.45, and that of the rat is 5.2. Furthermore, the choice of buffer will affect the PPD-oxidase activity measured (Ryan et al. 1992). In the presence of phosphate buffer, the PPD-oxidase activity of rat serum was markedly inhibited but it was only slightly inhibited for human serum. Moreover, bicarbonate ion was found to inhibit oxidase activity of frog CP but does not inhibit the activity of human CP (Sunderman and Nomoto 1970). In one study, rCP was found to contain PPD activity and about one-third of the ferroxidase activity found with human CP. In another investigation,

however, rCP was found to contain more PPD activity and approximately 500-fold less ferroxidase activity than its human counterpart (Ryan et al. 1992).

Even when CP was successfully purified, special handling and storage procedures are still required. Though there are researchers suggested that the proteolysis would be insignificant during a brief period in storage and one week purification time (Manolis and Cox 1980), most research workers find it hard to purify and store this protein without degradation. It is known that a major obstacle in the study of the physicochemical characteristics of the protein is that once it was purified, it easily undergoes conformational changes that lead to irreversible modifications of its spectroscopic properties, in particular those detected by EPR spectroscopy (Musci et al. 1993). In the case of human CP, when it was stored at 4°C, a 0.2% solution of CP in 0.015 M phosphate buffer, pH 6.9, containing 0.1M NaCl, had its A_{610}/A_{280} ratio reduced by approximately 10% in a month, and some colorless precipitate appeared. When frozen in liquid nitrogen and stored at -20°C, a similar solution lost its blue color completely after 2 weeks storage (Noyer et al. 1980). For rCP isolated by the method described by Manolis and Cox 1980, the A_{610}/A_{280} ratio of their freshly prepared CP decreased from 0.051 to 0.045 in a week at 4°C, possibly indicating loss of bound copper. A similar value (0.050 to 0.052) was also reported by Holtzman and Gaumnitz in 1970a.

3.6 CONCLUSION

In this study, rCP was successfully isolated with A_{610}/A_{280} ratio of 0.044 (indicates the purity is near 100%) and a N,N-dimethyl-p-phenylenediamine oxidase activity of 239.8 units/ml. SDS-polyacrylamide-gel electrophoresis gave 2 bands of apparent molecular weights of 136 kDa and 116 kDa of the isolated rat CP correspond to the intact CP and proteolytic fragments formed during the purification and/or storage respectively. The isolated rCP was used for the subsequent experiment. Like Starcher and Hill in 1966 said, "There is no one single method that can be applied in isolation different CPs from varies sources. Modifications are often required depends on ones' own needs." The same applies to the storage of this protein (Noyer et. al 1980). Special precautions have to be taken during the purification and storage procedure to minimize the lost of intact and active CP.

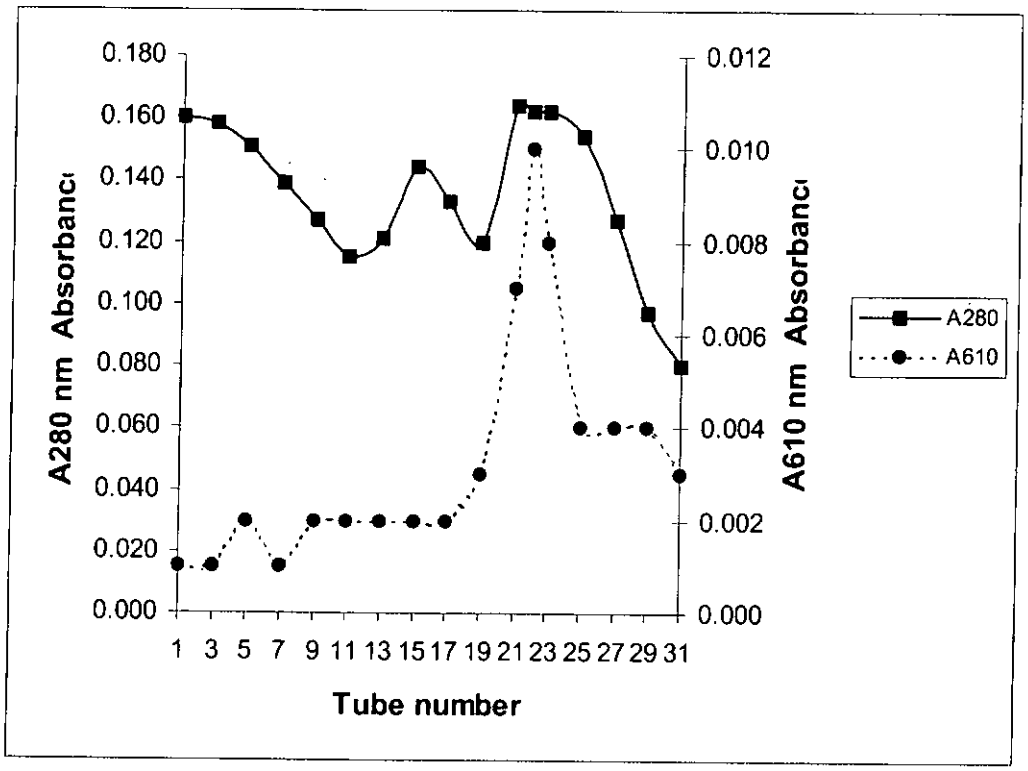

Fig. 2. Chromatography on DEAE-Sepharose Fast Flow of the rat serum. Column (1.5 x 15 cm), 4°C, phosphate buffer (pH 6.8) with 20 mM ε-aminocaporic acid elute in a stepwise gradient from 100 mM to 200 mM. Flow rate was 1 ml/min. [Starting from tube no. 1-15 (Buffer C); from tube no. 15-21 (Buffer D); from tube no. 21-21 (Buffer E)]

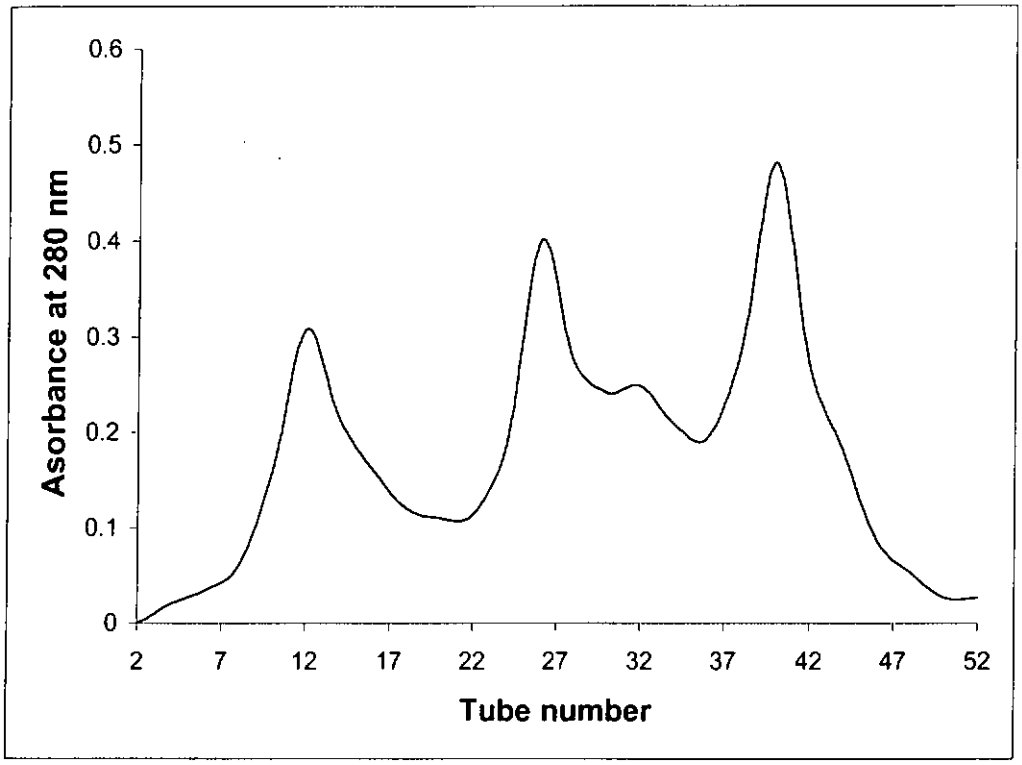

Fig. 3. Chromatography on Superdex 200 prep grade gel filtration column (2.5 x 100 cm) of the pooled blue fractions derived from chromatography on DEAE-Sepharose Fast Flow (Fig.2). 4°C, 25 mM sodium acetate (pH 5.6) with 100 mM NaCl and 20 mM ε-aminocaporic acid. Flow rate 0.3 ml/minute.

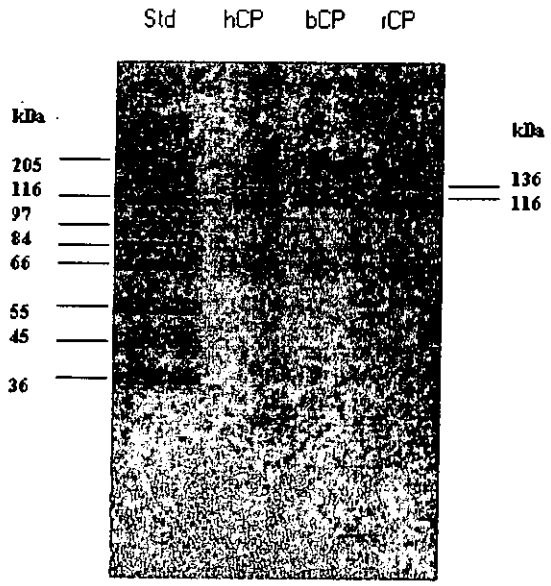

Fig. 4. Coomassie Blue staining of a 10% SDS-polyacrylamide gel. Abbreviations used: Std, standards [the molecular-weight markers myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (97, 000), fructose-6-phosphate kinase (84,000), bovine albumin (66, 000), glutamic dehydrogenase (55, 000), ovalbumin (45, 000), glyceraldehyde-3-phosphate dehydrogenase (36, 000); hCP, human CP; bCP, bovine CP; rCP, rat CP.

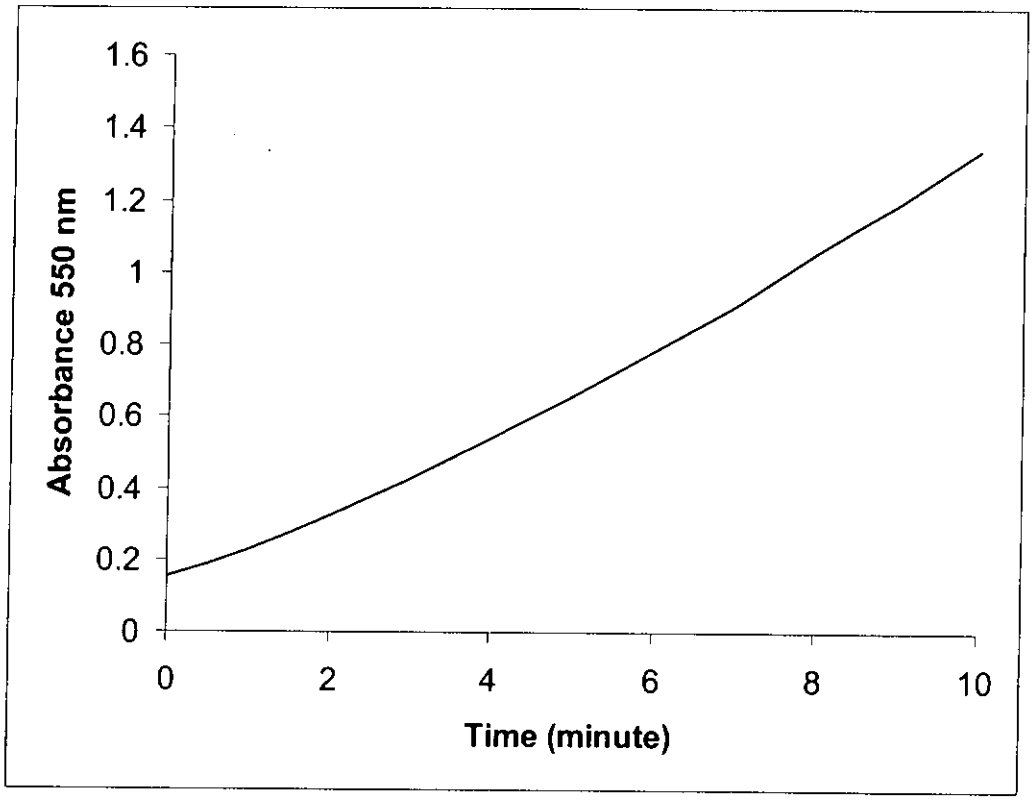

Fig. 5. The rCP oxidase assay curve.

Chapter 4

CHARACTERIZATION OF NON-TRANSFERRIN IRON (Fe^{2+}) UPTAKE BY A GLIOMA CELL LINE (BT325)

4.1 ABSTRACT

In this part of research, the characteristics of ferrous iron, Fe^{2+}uptake in the glioma cell line (BT325) was investigated. The non-Tf bound iron uptake of cells was studied instead of Tf-bound iron in this research project. It is because of the unique function of CP which oxidizes Fe^{2+} to Fe^{3+}. This reaction was predicted to be preceded before ferric ions attach to Tf (Fig. 1) (Saenko et al. 1994). The optimum pH was found to be in the range of 5.5-6.0. Iron internalization was observed suggested non-transferrin iron transport took place in these cells. The uptake increased with a rise in iron concentration (0-5 μM) and showed saturation, giving a V_{max} of 7.64 pmole Fe/ mg protein and mean K_m of 0.05 μM. A 30-minute incubation time was chosen for the subsequent experiments in this study, since iron

uptake was stable in 20-30 minute incubation time. Moreover, this incubation time had been documented in other study as the standard incubation time for studying iron transport in cultured cells. In addition, three buffers were used for this investigation. They were sucrose, sodium chloride solutions and the maintenance medium of the cell line, RPMI 1640 medium. At a lower iron concentration (1 μM Fe^{2+}), the three buffers did not give any significant difference in the iron uptake of the cells. The results obtained here will add to the knowledge on this cell line for subsequent experimental condition selections. Moreover, it was the first report on the characteristics of Fe^{2+} uptake of this cell line.

The results obtained here support that non-Tf iron transport took place in this cell line. Moreover, it was the first report of the Fe^{2+} uptake characteristics of this cell line.

Keywords:

Iron uptake (ferrous iron, Fe^{2+})	Glioma cell line	Buffer
Time	Concentration	

4.2 INTRODUCTION

Iron is present in every cell of the body and is the most abundant trace metal in the brain (Yehuda and Youdim 1991). It serves as an essential component of numerous

cellular enzymes. These include the cytochrome oxidases, a number of enzymes in the citric acid cycle, ribonucleotide reductase (the rate-limiting step for DNA synthesis), and NADPH reductase.

With respect to neurological activity, the availability of iron is essential for brain cell viability (Beard et al. 1993; Benjamin 1995; Hu and Connor 1996). Iron is involved in the synthesis and function of dopamine, serotonin, catecholamines, and possibly γ-aminobutyric acid (GABA) and myelin formation (Beard et al. 1993). It is the key component of the heme in cytochrome proteins, permitting mitochondrial electron transfer during cellular respiration. Because neurons are especially dependent upon aerobic metabolism (indeed, the brain has a higher rate of oxidative metabolism than any other organ), it can be argued that iron is likely to be crucially important in the brain (Connor and Benkovic 1992; Benjamin 1995). In addition, iron is involved in the synthesis and degradation of fatty acids and cholesterol and likely plays an important role in both myelinogenesis and myelin maintenance (Connor and Benkovic 1992). Although an essential nutrient, iron is also a potent toxin (Benjamin 1995). Unregulated iron ("free" iron) is highly toxic and is a prime initiator of lipid peroxidative damage (Connor and Benkovic 1992). Iron in its ionic form can catalyze reactions producing reactive oxygen species (such as the potent hydroxyl free radical), which cause DNA and protein damages as well as cell death by a process known as oxidative stress (Klomp and Gitlin 1996).

An imbalance in brain iron and hence a dysfunction in iron-related metabolism are suspected in some neurological disorders (Hu and Connor 1996). Increased brain iron

level is detected to be associated with AD, PD, HVS, multiple sclerosis, Pick's disease, Huntington's chorea, Kaschin-Beck disease and tardive dyskinesia (Yehuda and Youdim 1991; Connor and Benkovic 1992; Hodgkins and Blair 1997).

Glia cells require iron as in other cells for many aspects of their cell physiology (Hu and Connor 1996). In this part of research, the characteristics of Fe^{2+}uptake in the glioma cell line (BT325) was investigated. BT325 is originated from human brain glioma. It is a cell line of a human glioblastoma multiforme (Shao et al. 1988) The results obtained here will add information on this cell line and for subsequent experimental condition selection. Moreover, it was the first report of the Fe^{2+} uptake characteristics of this cell line.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St. Louis, USA). 6-well plates and 75 cm^2 culture flasks were from Iwaki Glass Co., Ltd. (NY, USA). Fetal bovine serum (qualified, heat inactivated), trypsin(2.5%, 10X), penicillin-streptomycin and RPMI Medium 1640 were purchased from Gibco BRL (Life Technologies, Inc., USA) The radioisotope $^{59}FeCl_3$ (100 μCi) was purchased from Amersham International (Buckinghemshire, England, UK). The glioma cell line BT325 was a generous gift from Su Zhou Medical School.

4.3.2 METHODS

4.3.2.1 Cell preparation

Please refer to Chapter 2.

4.3.2.2 Preparation of radioactive iron ($^{59}Fe^{2+}$) solution

Please refer to Chapter 2.

4.3.2.3 Measurement of the Tf-free ($^{59}Fe^{2+}$) iron transport

Please refer to Chapter 2.

4.4 RESULTS

4.4.1 EFFECT OF pH

In the initial experiments, the effect of pH of the incubation media on the Fe^{2+} uptake was investigated using BT325 cells incubated with 1 μM Fe^{2+}. The uptake of total Fe^{2+} into the cells was influenced by the pH of the incubation medium, optimum uptake occurring at pH 5.5-6.0 (Fig. 6). The uptake of Fe^{2+} at 6.0 was significantly higher than that of at pH 7.4 ($P < 0.001$). The same effect was also observed in membrane bound and internalized iron uptake. Moreover, from pH 5.5-6.8 the

internalized iron uptake was significantly higher than that of membrane bound iron ($P < 0.05$). This suggested that iron internalization took place in these cells.

4.4.2 EFFECT OF IRON CONCENTRATION

The total Fe^{2+} uptake of the BT325 cells increased with an increase in iron concentration. There was no significant difference between the iron uptake from 2 to 5 μM ($P < 0.01$). The V_{max} value calculated from the plateau levels of uptake rate in Fig.7 was 7.64 pmole Fe/ mg protein. The mean K_m value was 0.05 μM.

4.4.3 TIME COURSE OF Fe^{2+} UPTAKE

For the selection of incubation time for the subsequent experiments in this study, a time course of Fe^{2+} uptake of BT325 cells was investigated. As from Fig. 8, it was observed that the total iron uptake of BT325 cells increased significantly from 1 minute until 20 minutes ($P < 0.05$). There was no significant difference in iron uptake from 0-60 seconds ($P > 0.05$) and from 20-30 minutes ($P > 0.05$). Thus, in the following experiment, a 30-minute incubation time was used. This incubation time has been used in other studies as a standard incubation time (Richardson and Ponka 1994; Baker et al. 1998).

4.4.4 EFFECT OF BUFFERS

In this experiment, three buffers (RPMI 1640, 0.32 M Sucrose and 0.155 NaCl) were chosen to illustrate the effect of buffer on Fe^{2+} uptake. Among the three of them, the uptake of Fe^{2+} was significantly higher in both sucrose ($P < 0.05$) and NaCl ($P < 0.05$) solution than in the RPMI 1640 medium. And there is no noticeable difference between the iron uptake of sucrose and NaCl solution ($P > 0.05$). However, at 1 μM Fe^{2+} difference in the iron uptake of BT325 cells from the three buffers ($P > 0.05$) was minimal.

4.5 DISCUSSION

Ferrous ion, Fe^{2+}, is important for the maximal *in vitro* activity of a number of enzymes including several dioxygenases and hydroxylases, aconitase and ribonucleotide reductase (Lambeth et al. 1982). In this part of research, the characteristics of Fe^{2+}uptake in the glioma cell line BT325 was investigated.

In the initial experiments, the effect of pH of the incubation media on the ferrous iron uptake was investigated. The uptake of Fe^{2+} was influenced by the pH of the incubation medium, optimum uptake occurring at pH 5.5-6.0 (Fig. 6). The uptake of Fe^{2+} at 6.0 was significantly higher than that of at pH 7.4 ($P < 0.001$). Moreover, iron internalization was observed.

In another study, the optimum pH for Fe^{2+} uptake in rabbit reticulocytes was 6.5 (Morgan 1988; Qian et al. 1996). These results contrast markedly with the effects of pH on Tf-Fe uptake which was maximal at about pH 8.0 (Morgan 1988). At lower pH, the Tf-Fe bonds are protonated and there is strong evidence to indicate that Fe is released from Tf via a decrease in pH in the endosome (Richardson and Ponka 1997). Therefore, a higher pH value is needed for Tf-Fe remains at its binding state before taking up by the cells.

A pH of 5.8 was chosen for subsequent studies of Fe^{2+} uptake since a maximal or near-maximal rate was observed at this pH. Moreover, lower pH could reduce the rate of oxidation of Fe^{2+}. Lambeth et al. 1982 found that the rate of the oxidation of Fe^{2+} was remarkably sensitive to the pH and composition of the solution. At pH 7.4, the rates of autoxidation of Fe^{2+} were most rapid (happens within minutes) in several buffers with low affinities for metals. Yet, the stability increased as the pH decreased and more than 90% of Fe^{2+} remained after 1 hour at pH 6.0.

The results also showed that non-transferrin (non-Tf) iron uptake took place in this cell line. Although mammalian cells use the serum iron-binding protein, Tf as their major iron source, non-Tf iron transport has been demonstrated in a wide variety of cells (Wright et al. 1986; Wright et al. 1988; Thorstenson 1988; Sturrock et al. 1990; Kaplan et al. 1991; Neumannova et al. 1995; Olakanmi et al. 1997). Non-Tf-bound iron enters the cells by different mechanisms, such as carrier-mediated uptake, simple diffusion and lipid peroxidation mediated iron uptake. The function of the Tf-independent mechanisms is not well understood (Qian and Tang 1995; Qian et al.

1996), but there is increasing evidence that non-Tf-bound iron may be quantitatively more important in producing the iron loading and hepatic damage seen in hemochromatosis and other iron-overload diseases (Wright et al. 1988). A suggested role for the Tf-independent pathway was to clear potentially toxic, low molecular weight iron chelates (Attieh et al. 1999). Recently, two putative iron transporters, DMT1 (Divalent Metal Transporter) (Gruenheid et al. 1995) and SFT (Stimulator of Fe Transport) (Yu and Wessling-Resnick 1998), have been identified in mammalian cells which need further investigation.

When the cells were incubated with increasing concentration Fe^{2+}(Fig. 7), the total iron uptake increased with it and shown saturation at concentration 2 to 5 μM ($P < 0.01$). The V_{max} value was 7.64 pmole Fe/ mg protein and the mean K_m value was 0.05 μM. No comparison of these values can be made at the present time, as there are no reports about the Tf-free ($^{59}Fe^{2+}$) iron uptake of other glioma cell line. In the selection of incubation time for the subsequent experiments in this study, a time course experiment was conducted and a 30-minute incubation time was chosen. This incubation time has been used in other studies as a standard incubation time (Richardson and Ponka 1994; Baker et al. 1998).

The effect of buffers on the ferrous iron uptake of this cell was also investigated. Three buffers (RPMI 1640, 0.32 M Sucrose and 0.155 NaCl) were chosen. The RPMI 1640 medium was used in maintaining the cell line. The idea of using phosphate buffer for this experiment was eliminated. It was suggested that the rate of autoxidation was increased by phosphate, citrate, EDTA, and pyrophosphate. This

led to the generalization that chelators with oxygen donors, which preferentially bound Fe^{3+}, promoted the autoxidation of Fe^{2+}. However, chelators containing strong nitrogen donors preferentially bound and thus stabilized Fe^{2+} (Lambeth et al. 1982).

Among the three buffers, the uptake of Fe^{2+} was significantly higher in both sucrose ($P < 0.05$) and NaCl ($P < 0.05$) solution than in the RPMI 1640 medium. And there is no significant difference between the iron uptake of sucrose and NaCl solution ($P > 0.05$). Moreover, at 1 μM Fe^{2+} there was no significant difference in the iron uptake of BT325 cells from the three buffers ($P > 0.05$). The reason why the uptake is lower in RPMI 1640 medium may due to the presence of bicarbonate ions in it. Ferrous iron was found to have limited solubility in bicarbonate buffers (Wright et al. 1988). In the case of sucrose and NaCl solution, the finding was contrary to the one carried out for Fe^{2+} uptake in rabbit reticulocytes. It was reported that in reticulocytes the uptake of Fe^{2+}, from NaCl solution was much lesser than that from sucrose solution. As the NaCl concentration was raised, Fe^{2+} uptake decreased. The inhibition of Fe^{2+} uptake was in a non-competitive manner. The effects of NaCl on Fe^{2+} uptake did not appear to be specific for either Na^+ or Cl^-. KCl, NaI, Na_2SO_4 and sodium phosphate also inhibited Fe^{2+} uptake at the similar degree as NaCl, while $CaCl_2$ and $MgCl_2$ demonstrated somewhat greater inhibition. This effect was suggested to be due to the ionic strength of the solution. It was possibly due to the ions' divalent nature or to ion specific interactions with cell surface components (Morgan 1988). It is also true for Mn^{2+} uptake which was greatest when the cells were incubated in sucrose than in KCl (Chua et al. 1996).

The uptake of Fe^{2+} in glucose solution was a little lower than that in sucrose or mannitol, but there was almost no difference between the rates of Fe^{2+} uptake in sucrose and mannitol (Qian et al. 1996). For the sucrose solution, the form of iron transported into the cells was not in the form of an iron-reducing agent or a Fe-sucrose complex. It might be ionic iron, probably Fe^{2+} since this was the form of iron which was used in most experiments efficiently transported. In the case of Tf-free-iron uptake of reticulocytes, the use of isotonic sucrose solution (pH 6.4-6.5 and 1 μM Fe) was considered a standard condition for the measurement of Fe^{2+} uptake. This is because of it has ability to form weak complexes with iron which are stable up to pH values above neutrality plus its inability to pass through cell membranes. Based on the above-mentioned information, sucrose was chosen for this experiment.

4.6 CONCLUSION

In conclusion, the optimum pH found for the glioma cell BT325, was in the range of 5.5-6.0. Moreover, internalization of iron was observed i.e. non-Tf iron uptake took place in this cell line. The uptake increased with increasing iron concentration and shown saturation giving a V_{max} of 7.64 pmole Fe/ mg protein and mean K_m of 0.05 μM. A 30-minute standard incubation time was chosen, since iron uptake was stable in 20-30 minute incubation time. Additionally, sucrose was chosen as the standard buffer as explained in the discussion. The results obtained here support that non-Tf iron transport took place in this cell line. Moreover, it was the first report of the Fe^{2+} uptake characteristics of this cell line.

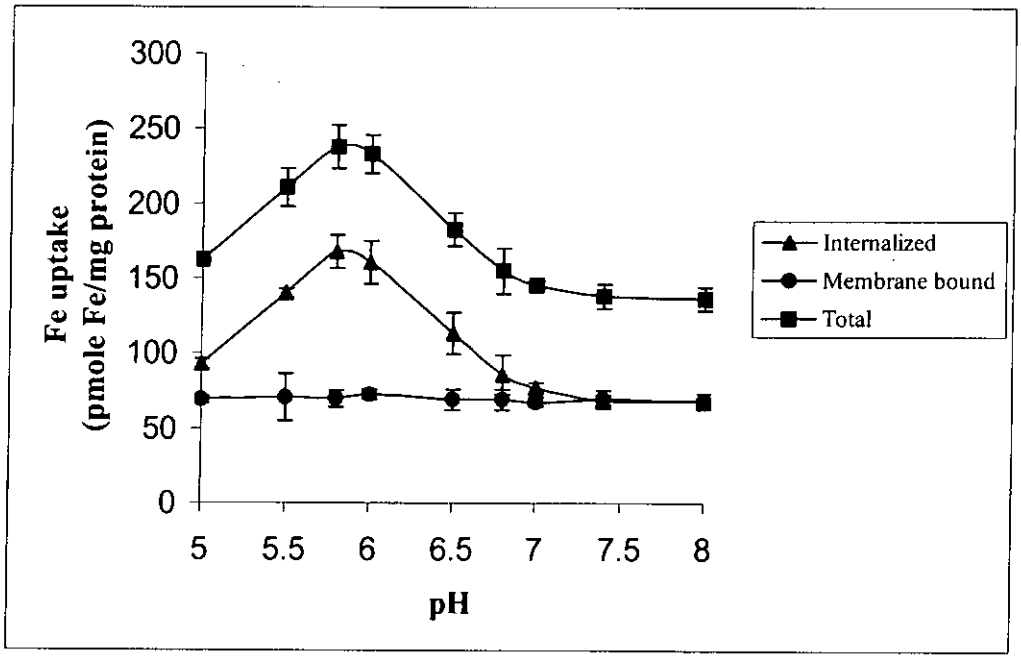

Fig. 6. The effect of pH on $^{59}Fe^{2+}$ uptake of BT325 cells. The cells were incubated with RPMI 1640 medium at varying pH with 1 μM $^{59}Fe^{2+}$ at 37°C for 30 minutes. Radioactivity in the cells was measured with γ-counter. Each point is the mean±S.E.M. of three experiments.

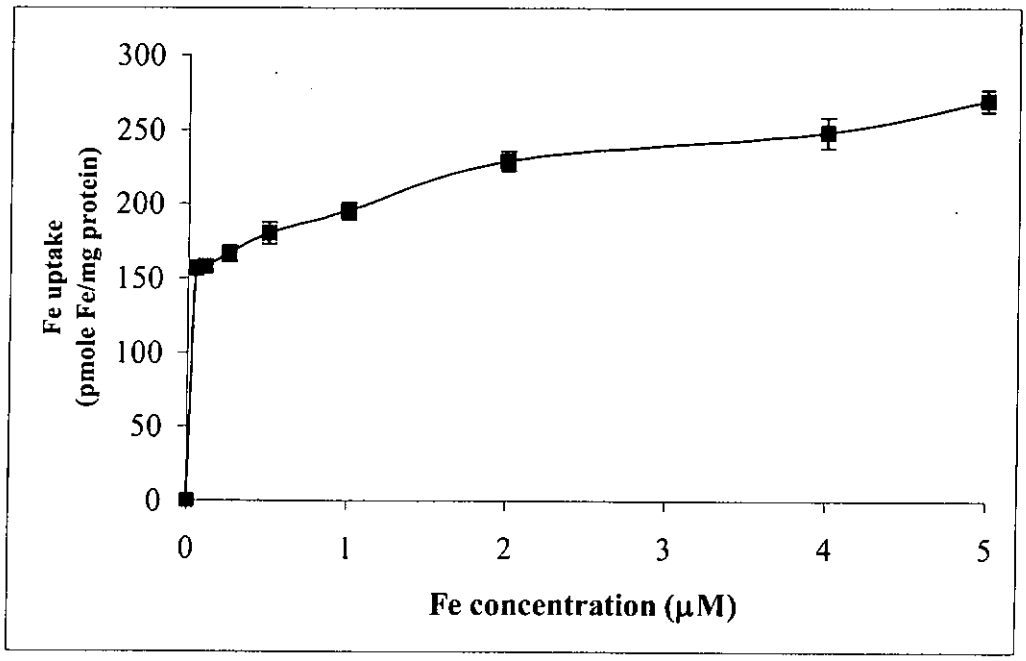

Fig. 7. The effect of iron concentration on the $^{59}Fe^{2+}$ uptake of BT325 cells. The cells were incubated with varying concentration (1-5 μM) of $^{59}Fe^{2+}$ in RPMI 1640 medium pH 5.8 at 37°C for 30 minutes. Radioactivity in the cells was measured with γ-counter. Each point is the mean±S.E.M. of three experiments

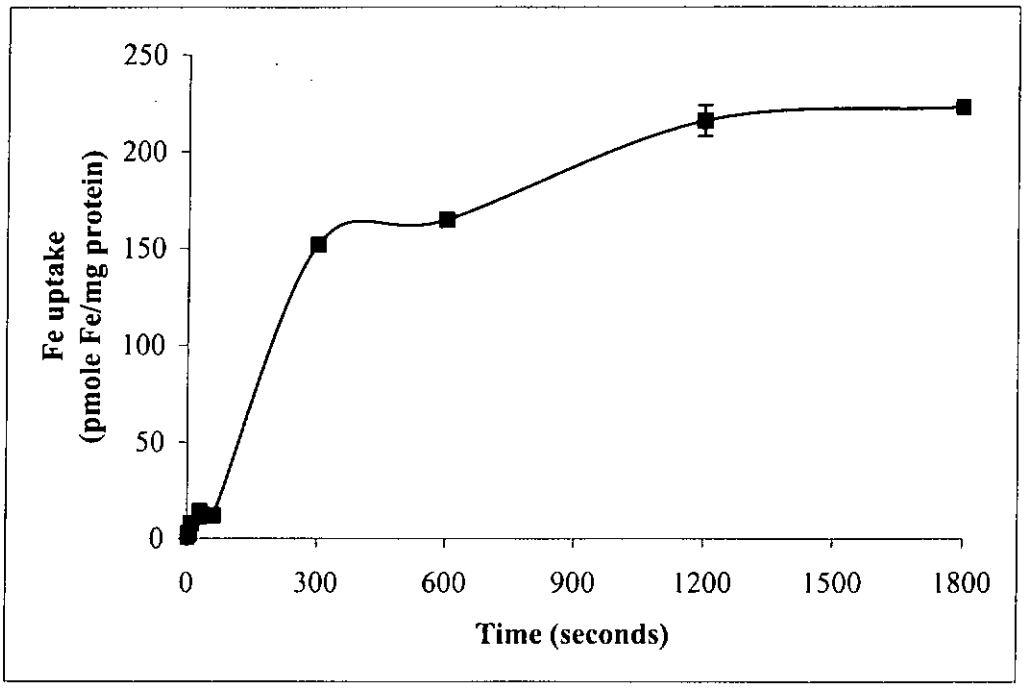

Fig. 8. Time course of Fe^{2+} uptake of BT325 cells. The cells were incubated with 1 μM $^{59}Fe^{2+}$ in RPMI 1640 medium, pH 5.8 for varying period of time (0-30 minutes) at 37°C. Radioactivity in the cells was measured with γ-counter. Each point is the mean±S.E.M. of three experiments.

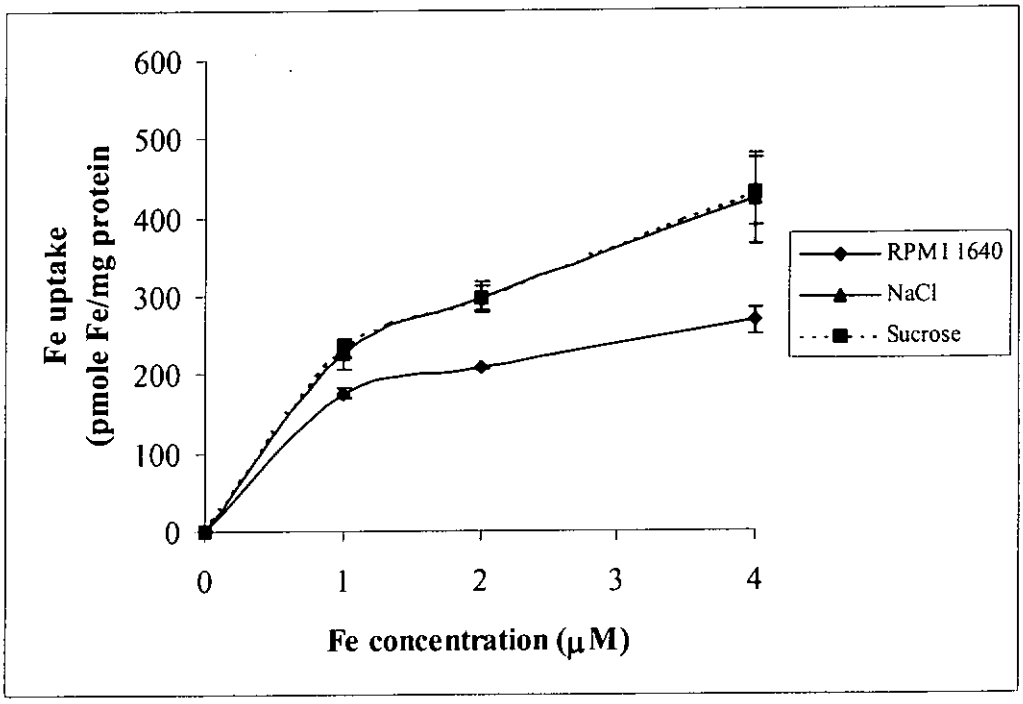

Fig. 9. Effect of different buffers on the Fe^{2+} uptake by BT325 cells. The cells were incubated with varying concentration of Fe^{2+} in RPMI 1640 medium buffered to pH 5.8, 0.155M NaCl buffered to pH 5.8 by 4 mM Pipes and 0.32 M sucrose buffered to pH 5.8 by 4 mM Pipes, for 30 minutes at 37°C. Radioactivity in the cells was measured with γ-counter. Each point is the mean±S.E.M. of three experiments.

Chapter 5

EFFECT OF CERULOPLASMIN ON IRON FLUX IN BRAIN CELLS

5.1 ABSTRACT

Ceruloplasmin (CP) has been suggested to function in iron release of cells. However, recent studies in two mammalian cells showed that CP stimulated iron uptake. These findings reveal an essential role for CP in human iron metabolism. This experiment aims to investigate the effect of CP on the iron flux of brain cells (glioma cell line BT325). The results showed that CP did not enhance iron release of the cells. Although in the presence of apoTf (20 and 50 μg/ml) iron release was observed when, the effect was not due to CP but apoTf. Therefore, it is concluded that CP did not involve in the iron release of this glioma cells. To further investigate the role of CP in iron flux, the effect of CP on iron uptake was studied. By incubating the cells with (0-300 μg/ml) CP and 1 μM Fe^{2+} at 37°C for 30 minutes, iron uptake was increased significantly. The minimal effective CP concentration had to be more than 5 μg/ml and the maximal uptake was reached at 30 μg/ml. The results obtained in this study showed that CP helped in iron uptake but not release and it plays a role in

brain iron metabolism. It was the first report on the effect of CP on Fe^{2+} uptake of brain cells.

Keywords:

Ceruloplasmin (CP) Ferrous iron (Fe^{2+}) Glioma cells

Apotransferrin

5.2 INTRODUCTION

CP is a blue serum α2-glycoprotein which binds more than 90% of copper in the plasma (DiSilvestro et al. 1988; Percival and Harris 1990; Harris et al. 1995; Miyajma et al. 1996). The precise physiological function of CP remains controversial, although many workers tend to consider the protein multifunctional (Percival and Harris 1990; Saenko et al. 1994). At least four main functions have been attributed to CP, those of copper transport, ferroxidase activity, amine oxidase activity and as an antioxidant in the prevention of the formation of free radicals in serum (Zaitseva et al. 1996).

CP has also been suggested in promoting iron efflux from cells in the CNS (Harris et al. 1995; Klomp and Gitlin 1996) and in liver cells (Young et al. 1997). The decreased expression or the absence of expression of this protein may induce iron accumulation in the brain cells in aceruloplasminemia and perhaps in other neurodegenerative diseases where abnormalities in iron metabolism have been

documented (Harris et al. 1995; Kawanami et al. 1996; Klomp and Gitlin 1996; Logan 1996; Miyajma et al. 1996; Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998). In copper-deficient swine, the copper deficiency results in a disorder of iron storage and that CP administration could correct this situation (Harris et al. 1995).

Recently, there are contradictory findings in which CP was shown to enhance iron uptake in mammalian cells (Mukhopadhyay et al. 1998; Attieh et al. 1999). The importance of CP in iron metabolism is further supported by the studies on a CP homologue (*FET3*) in yeast that functions as an essential copper oxidase and mediates ferrous iron uptake (Askwith et al. 1994).

Histological studies indicate that maintenance of iron homeostasis in the brain is the responsibility of neuroglia and possibly the choroid plexus (Connor and Benkovic 1992). And it has been suggested that glial cell-specific CP gene expression is essential for iron homeostasis and neuronal survival in the human CNS (Klomp and Gitlin 1996). BT325, a glioma cell was thus used in this study to investigate the role of CP in brain iron.

5.3 MATERIALS AND METHODS

5.3.1 MATERIALS

6-well plates and 75 cm^2 culture flask were from Iwaki Glass Co., Ltd. (NY, USA), pronase was from Calbiochem (CA, USA). Fetal Bovine Serum (qualified, heat inactivated), trypsin(2.5%, 10X), penicillin-streptomycin and RPMI Medium 1640

were purchased from Gibco BRL (Life Technologies, Inc., USA) The radioisotope $^{59}FeCl_3$ (100 μCi) was purchased from Amersham International (Buckinghemshire, England, UK). Human ceruloplasmin and apotransferrin were from Sigma Chemical Co. (St. Louis, USA). Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St. Louis, USA). The glioma cell line BT325 was a generous gift from Su Zhou Medical School.

5.3.2 METHODS

5.3.2.1 Cell preparation

Please refer to Chapter 2.

5.3.2.2 Preparation of radioactive iron ($^{59}Fe^{2+}$) solution

Please refer to Chapter 2.

5.3.2.3 Measurement of the Tf-free ($^{59}Fe^{2+}$) iron transport

For measurement of Tf-free iron ($^{59}Fe^{2+}$) transport by the cultured cells, cells cultured in 6-well plates reached about 80% confluence was used. The culture medium was decanted and the cells were washed with 37°C Hanks' buffer (pH 7.4) in order to remove dead cells and debris. Then cells were preincubated with 2 ml RPMI 1640 medium (serum-free) twice for 15 minutes at 37°C to remove fetal bovine serum and endogenous transferrin (Kaplan et al. 1991; Trinder et al. 1996).

For the measurement of iron release, cells were pre-loaded with iron by incubation with 1 ml of 1 μM $^{59}Fe^{2+}$ in 0.32 M sucrose solution buffered with 4 mM Pipes for 1 hour. Then the medium was decanted and the cells were washed with 37°C Hanks' buffer (pH 7.4) for three times. 1 ml of 1 μM $^{59}Fe^{2+}$ in 0.32 M sucrose solution buffered with 4 mM Pipes with the corresponding CP concentration was added. After this incubation, the medium was decanted and the cells were washed with 4°C Hanks' buffer for four times to stop the reaction. 1 ml of ice-cold pronase (1 mg/ml) was added to the cells and left to stand for 60 minutes. For measuring the effect of CP on iron release, the cells were transferred to centrifuge tubes and spun at 4000 rpm for 15 minutes to separate membrane bound and internalized fractions. In other experiments, the cells were directly transferred to the centrifuge tubes. Their radioactivities were counted with a three channel γ-scintillation counter (Packard 5003 COBRA Q). In some sections of this study, total radioactivity of the samples was measured directly with the γ-counter without being separated into membrane bound and internalized fractions. The protein content was measured by a protein assay kit Sigma Chemical Co. (St. Louis, USA). The results were expressed as pmol Fe/mg protein.

For the measurement of iron uptake, procedure was the same except that the step of preloading iron into the cells was omitted.

5.4 RESULTS

5.4.1 EFFECT OF CP ON IRON RELEASE

Iron release was measured from the BT325 cells preloaded with iron in increasing concentration of CP (0-300 μg/ml, no CP for the control). In which the 300 μg/ml of CP is the physiological CP concentration in a healthy human adult (Lamb and Leake 1994). No significant change was observed in the iron release of the cells ($P > 0.05$).

5.4.2 EFFECT OF APOTRANSFERRIN ON IRON RELEASE

It has been suggested that the effect of CP on iron release was greatest in the presence of apoTf (Young et al. 1997). In order to find out whether this is also true for the brain cell, the effect of apoTf in iron release was first determined (Fig. 11). When the iron-loaded cells were incubated with increasing concentration of apoTf (0-60 μg/ml), the amount of iron uptake decreased significantly ($P < 0.001$). The maximal effect was observed at the concentration of 30 μg/ml which has about 60% drop in iron uptake as compared with the control group (no CP and apoTf).

5.4.3 EFFECT OF CP AND APOTRANSFERRIN ON IRON RELEASE

The combining effect of apoTf and CP on iron release was studied. The release of iron from the CP group (0-30 μg/ml and no apoTf) was insignificant ($P > 0.05$) when

compared to the control group (no CP and apoTf). While for the apoTf groups (20 μg/ml and 50 μg/ml), iron release was significant ($P < 0.001$). This finding is the same as in the previous experiment (Fig. 11). However, the effect of CP in iron release was not enhanced by the addition of apoTf. There was no significant change in the iron release found within the apoTf groups with increasing CP concentrations ($P > 0.05$). The results presented here were in contrast to the traditional view of CP functioning in iron release. Experiments were therefore performed to determine whether the effect of CP on this brain cell was similar to the findings recently reported in hepatocytes (Mukhopadhyay et al. 1998) and erythroleukemic cells (Attieh et al. 1999), which suggest that CP plays a role in iron uptake.

5.4.4 EFFECT OF CP ON IRON UPTAKE

To study the effect of CP on iron uptake, the preloading of iron into the brain cells was omitted from the procedure. In Fig. 13, the cells were incubated with CP of 0-300 μg/ml. The iron uptake increased significantly with increasing CP concentration ($P < 0.001$). The maximum effect occurred with 30 μg/ml of CP. Another experiment was thus performed with lower CP concentration (0-50 μg/ml) (Fig. 14). To affect iron uptake, the minimum CP concentration had to be more than 5 μg/ml. And once again, the maximum effect was observed in 30 μg/ml ($P < 0.001$). The findings of section of the study showed that CP plays a role in the iron uptake of brain cell.

5.5 DISCUSSION

The discovery of aceruloplasminemia (an autosomal recessive disorder of iron metabolism due to mutations in the CP gene) and other hereditary CP deficiency diseases (Harris et al. 1995; Kawanami et al. 1996; Klomp and Gitlin 1996; Logan 1996; Miyajma et al. 1996; Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998) have suggest a previously unrecognized role CP plays in brain iron metabolism.

The decreased expression or the absence of expression of this protein may induce iron deposition in basal ganglia and in the red and dentate nuclei. The iron deposition in these areas are markedly increased in neurodegenerative disorders such as PD (Okamoto et al. 1996; Miyajima et al. 1996; Takahashi et al. 1996). Therefore, it was suggested that CP functions in promoting iron efflux from cells in the CNS (Harris et al. 1995; Klomp and Gitlin 1996). However, recent research findings demonstrated that instead of enhancing iron release, CP enhanced iron uptake in mammalian cells (Mukhopadhyay et al. 1998; Attieh et al. 1999). The CP's role in iron uptake was further supported by the studies on a CP homologue (*FET3*) in yeast, which was said to function as an essential copper oxidase and mediate ferrous iron uptake (Askwith et al. 1994). Consistent with this concept, the anemia that developed in copper-deficient animals was unresponsive to iron, yet correctable by CP administration (Harris et al. 1995).

To find out the role of CP in brain iron transport, a glioma cell, BT325, was used in this study. As mentioned earlier, histological studies have indicated that neuroglia is responsible for maintaining the iron homeostasis in brain. And it has been suggested

that glial cell-specific CP gene expression is also essential for iron homeostasis and neuronal survival in the human CNS (Klomp and Gitlin 1996). Moreover, the recent findings of a glycosyl-phosphatidylinositol-anchored form of CP in glial cells, which is likely to be the major form of CP in the CNS may reflect an important involvement in iron transport (Patel and David 1997; Salzer et al. 1998).

The results in this study indicated that CP did not enhance the iron release of this glioma cells (Fig.10). This finding was in contrast to the traditional hypothesis that supposed CP might involve in iron efflux. In view of this negative result, a second part of experiment was performed.

A unique, yet but controversial feature of CP is its ability to oxidize Fe^{2+} to Fe^{3+} (Kawanami et al. 1996). It has been stated that iron is the best substrate for CP (Ryan et al. 1992). It catalyzes the oxidation of 4 atoms of Fe^{2+} with the concomitant production of water from molecular oxygen. This prevents the free radical formation which occurs during spontaneous ferrous oxidation (Osaki and Johnson 1969). When apoTf is present, it binds the ferric product and thus protects it from subsequent reduction. The presence of both CP and apoTf in the plasma normally provides considerable antioxidant protection by preventing iron induced free radical formation (Saenko et al. 1994; Logan 1996). The oxidation of ferrous iron to ferric iron proceeded 10 to 20 times more rapidly in the presence of CP than in its absence (Ryan et al. 1992; Kawanami et al. 1996; Logan 1996). This suggests a role for CP in ferric iron uptake by Tf (Harris 1995). In 1997, Young and his colleagues suggested that the effect of CP on iron release was greatest in the presence of apoTf in liver

cells. In order to find out its validity in the case of brain cells, the effect of CP and apoTf in iron release had to be determined.

In this study, apoTf alone could enhance iron release and the effect is concentration-dependent (Fig. 11). The maximal effect observed was at 30 μg/ml which had caused about 60% drop in iron uptake as compared with the control group (no apoTf). When apoTf and CP were added together, there was also a significant release in iron ($P < 0.001$). But the effect was lesser when apoTf was used alone. Therefore, even when CP and apoTf were used together with exhibited iron release, there was no further enhancement of iron release due to the presence of CP. Besides, the effect may be due only to apoTf as the effect was more significant without the presence of CP. As for the suggestion made by Young et al. in 1997 which claimed that CP's effect on iron release was greatest with the presence of apoTf, the same explanation may apply. It is because the effect was detected only with the addition of a large amount of apoTf (500 μg/ml apoTf vs. 32 μg/ml CP). Other researchers also reported that the addition of apoTf or nitrilotriacetic acid to the medium as Fe^{3+} acceptors did not show enhancement of iron release and suggested that the difference may due to experimental conditions (Attieh et al. 1999).

The effect of CP on iron uptake was thus investigated since there were reports in which CP was shown to increase the iron uptake of mammalian cells (Mukhopadhyay et al. 1998; Attieh et al. 1999) and yeast (Askwith et al. 1994). In Fig. 13, the iron uptake increased with increasing CP concentration ($P < 0.001$), which is considered extremely significant. The maximum effect occurred when CP

was about 30 μg/ml. Another experiment was thus performed with lower CP concentration (0-50 μg/ml) (Fig. 14) which gave a minimum CP concentration of more than 5 μg/ml for stimulating of iron uptake. And once again, the maximum effect was observed at 30 μg/ml ($P < 0.001$). This finding showed that CP functions in the iron uptake of brain cells. The reason why CP stimulated iron uptake take may due to its ability to oxidize Fe^{2+} to Fe^{3+} (Kawanami et al. 1996), therefore increase the substrate available for a trivalent transporter as suggested by Attieh et al. 1999. Interestingly, in all cases in which CP was reported to promote iron uptake, the concentration used was much lower than the CP concentration (~300 μg/ml) in a healthy human adult (Lamb and Leake 1994). For HepG2 cells, a human hepatocellular carcinoma line, the maximal increase was observed at 10 to 30 μg/ml. (Mukhopadhyay et al. 1998) While for K526, a human erythroleukemic cell line, the maximal uptake was at about 30 μg/ml (Attieh et al. 1999). It has to note that the values obtained for both cells were in their iron-deficient state. As suggested by Mukhopadhyay et al. 1998, this observation may account for the apparently normal iron metabolism in patients with Wilson's disease who have lower than normal levels of plasma CP and for iron deficiency in patients with levels less than 5% of normal.

5.6 CONCLUSION

In conclusion, the results showed that CP did not enhance iron release of the cells. Although in the presence of apoTf (20 and 50 μg/ml) iron release was observed when, the effect was not due to CP but apoTf. By incubating the cells with (0-300 μg/ml) CP and 1 μM Fe^{2+} at 37°C for 30 minutes, significant increase in iron uptake

was observed. The minimal effective CP concentration had to be more than 5 μg/ml and the maximal uptake was reached at 30 μg/ml. The results obtained in this study showed that CP helped in iron uptake but not release and it plays a role in brain iron metabolism. It was the first report on the effect of CP on Fe^{2+} uptake of brain cells.

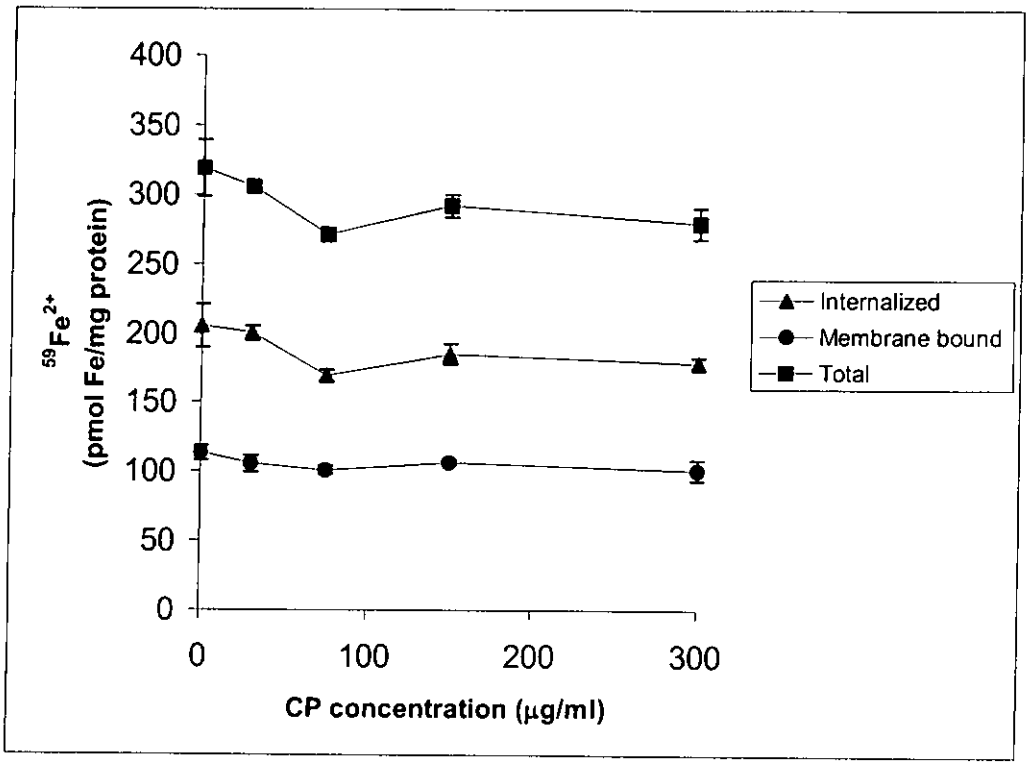

Fig. 10. The effect of CP on iron release of BT325 cells. The cells were preloaded with iron $^{59}Fe^{2+}$ by incubation with 1 μM $^{59}Fe^{2+}$ for an hour at 37°C. And then incubated with CP (0-300 μg/ml) for 30 minutes. The membrane bound, internalized and total radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

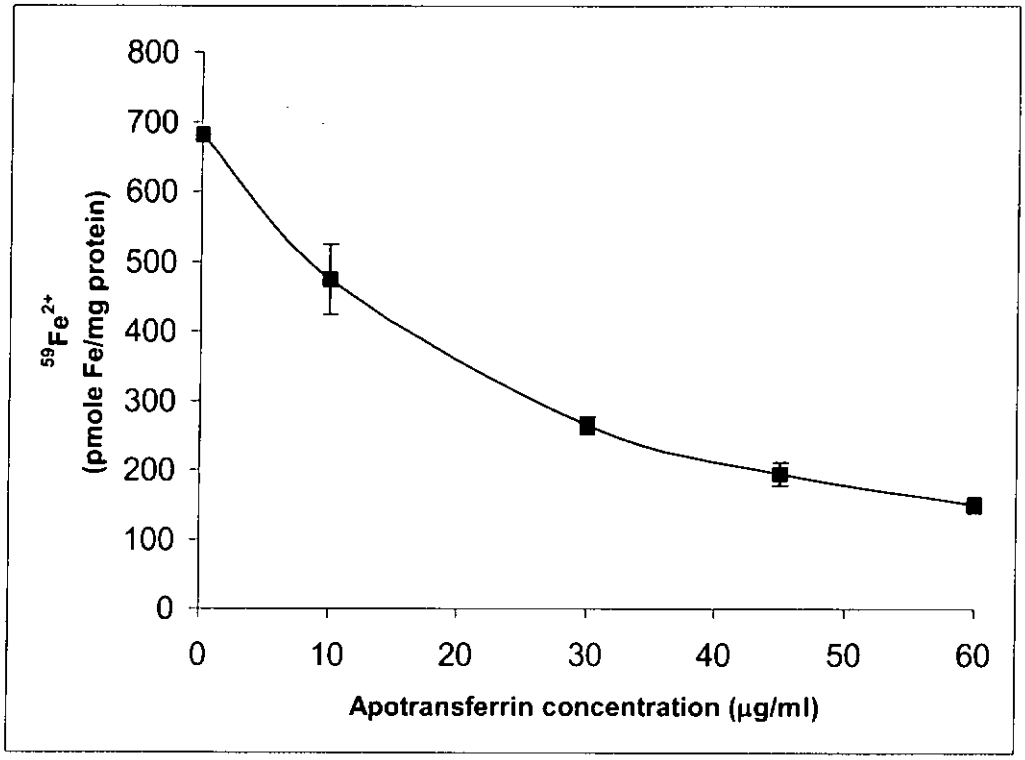

Fig. 11. The effect of ApoTf on iron release of BT325 cells. The cells were preloaded with iron $^{59}Fe^{2+}$ by incubation with 1 μM $^{59}Fe^{2+}$ for an hour at 37°C. And then incubated with apoTf (0-60 μg/ml) for 30 minutes. The total radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

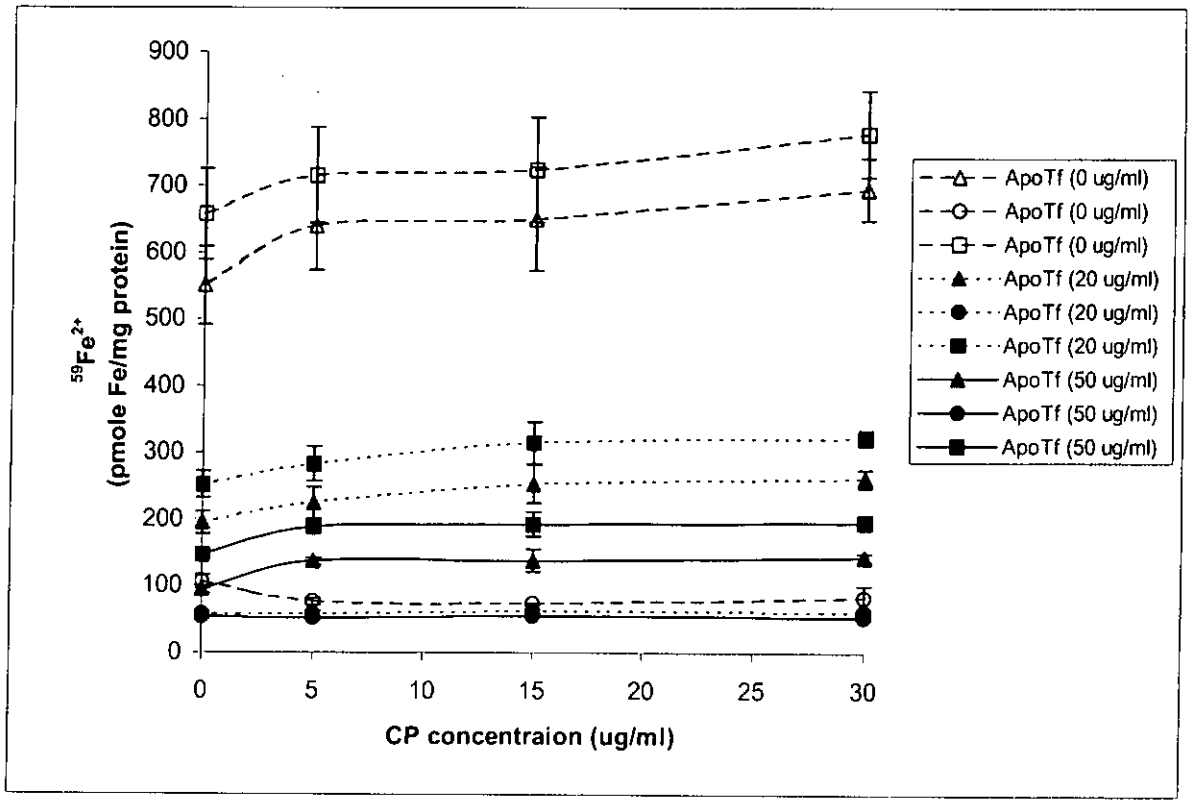

Fig. 12. The effect of CP and apoTf on iron release of BT325 cells. The cells were preloaded with iron $^{59}Fe^{2+}$ by incubation with 1 μM $^{59}Fe^{2+}$ for an hour at 37°C. And then incubated with varying concentration of apoTf (0,20 and 50 μg/ml) and CP (0, 5, 15 and 30 μg/ml) for 30 minutes. The internalized, membrane bound and total radioactivities of the cells were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments. [Symbols used: internalized (▲),membrane bound (●) and total (■).]

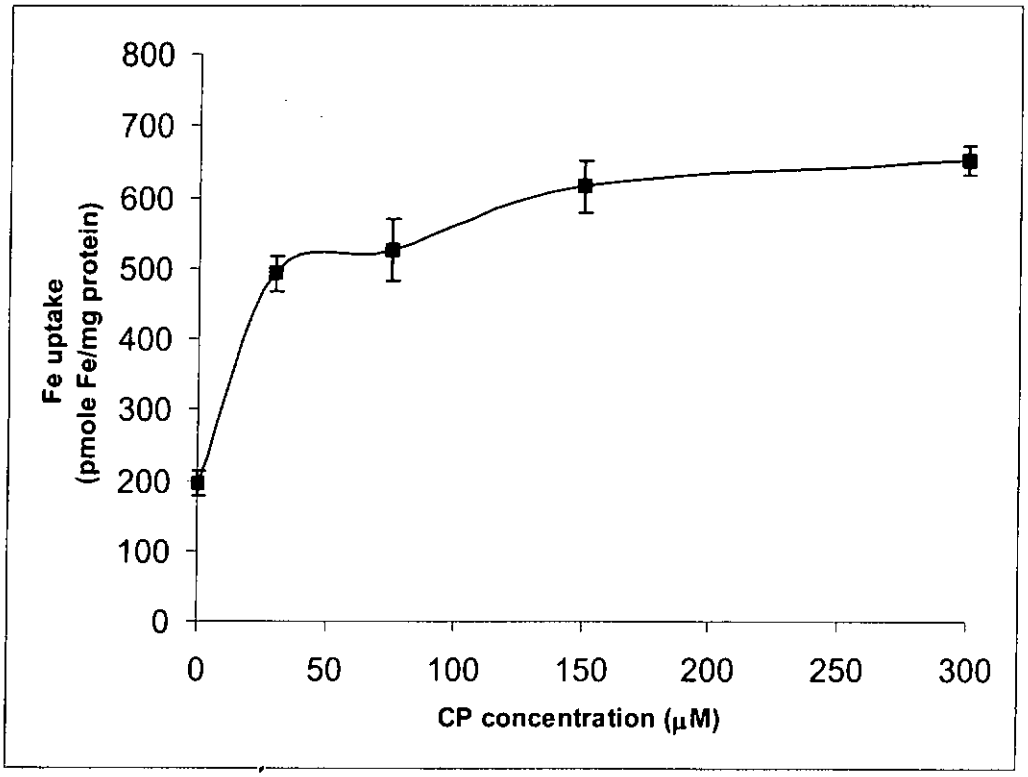

Fig. 13. The effect of CP on iron uptake of BT325 cells. The cells were incubated with 1 μM $^{59}Fe^{2+}$ and CP (0-300 μg/ml) for 30 minutes at 37°C. The total radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

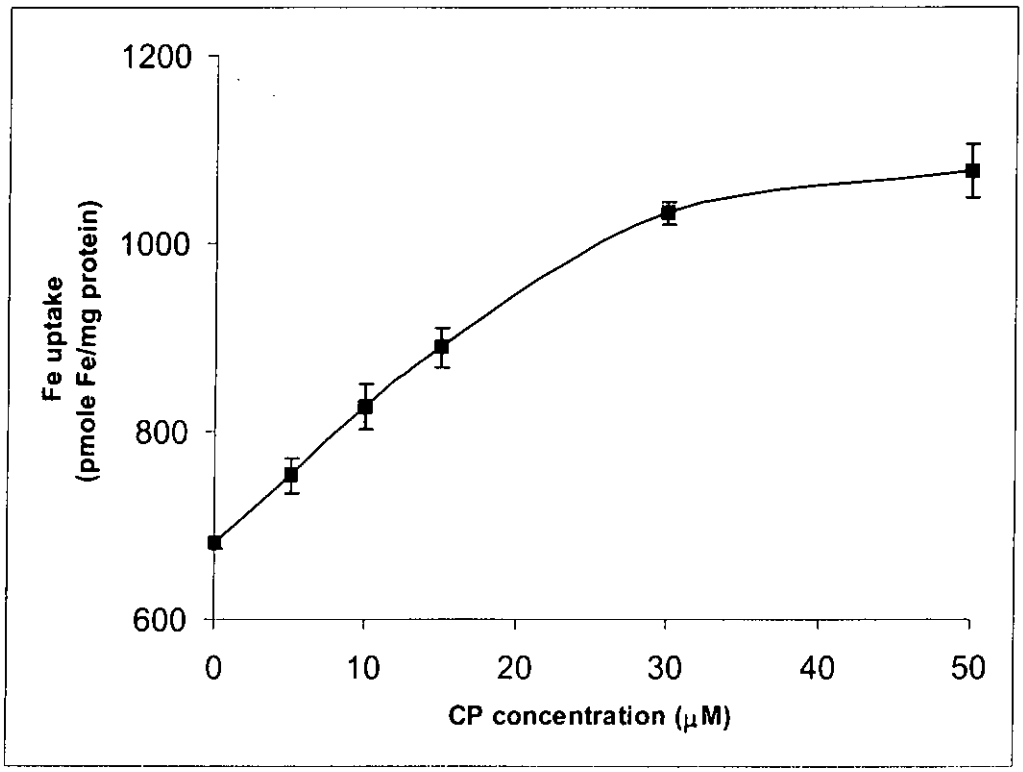

Fig. 14. The effect of CP on iron uptake of BT325 cells. The cells were incubated with 1 μM $^{59}Fe^{2+}$ and CP (0-50 μg/ml) for 30 minutes at 37°C. The total radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

Chapter 6

THE EFFECT OF HUMAN, RAT AND BOVINE CERULOPLASMINS IN STIMULATING IRON UPTAKE IN BRAIN CELLS.

6.1 ABSTRACT

Ceruloplasmin (CP) is a blue serum α-glycoprotein which has been suggested functioning in iron metabolism in the CNS. In the previous chapter, human CP (hCP) was found to stimulate iron uptake by glioma cells. This experiment aims to find out if this effect is species-specific by comparing the effect of hCP, rat CP (rCP) and bovine (bCP) in stimulating the iron uptake by the human glioma cell line BT325. The cells were incubated with 1 μM $^{59}Fe^{2+}$ and 150 μg/ml of different CPs (hCP, rCP and bCP) for 30 minutes at 37°C. The results showed that all the CPs used can stimulate iron uptake by the brain cells.

Keywords:

Ceruloplasmin (CP) Ferrous iron (Fe^{2+}) Glioma cells

Species

6.2 INTRODUCTION

Ceruloplasmin (CP) is the only known multicopper oxidase in animals (Saenko et al. 1994). It is a blue serum α-glycoprotein mainly synthesized in hepatocytes and secreted as a holoprotein with 6 atoms of copper incorporated during biosynthesis (DiSilvestro et al. 1988; Percival and Harris 1990; Harris et al. 1995).

Since its first isolation by Holmberg and Laurell in 1948 from human and porcine serum (Arnaud et. al 1998), CP has been isolated from numerous species including human (Ryden 1972; Noyer et al. 1980; Farver et al. 1999;), pig (Ryden 1972), rabbit (Ryden 1972; Morell et al. 1968; Mainero et al. 1996), horse (Ryden 1972), rat (Manolis and Cox 1980; Weiner and Cousins 1983; Ryan et al. 1992), sheep (Calabrese et al. 1988b), bovine (Calabrese et al. 1981), chicken (Starcher and Hill 1966; Calabrese et al. 1988a; Machonkin et al. 1999), goose (Hilewica-Grabska et al. 1988) and from reptilian, turtle (Giovanni et al. 1990).

In the recent findings, CP was suggested in promoting iron efflux from cells in the CNS (Harris et al. 1995; Klomp and Gitlin 1996) and in liver cells (Young et al. 1997). The decreased or absence of expression of this protein may induce iron acccumulation in the brain cells in patients with aceruloplasminemia and perhaps

with other neurodegenerative diseases (Harris et al. 1995; Kawanami et al. 1996; Logan 1996; Miyajima et al. 1996; Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998). Reports that contradictory to this hypothesis are also published. CP was shown to enhance iron uptake in human hepatocellular carcinoma line (Mukhopadhyay et al. 1998) and human erythroleukemic cell line (Attieh et al. 1999). Moreover, a CP homologue in yeast known as *FET3* was found to mediate high affinity iron uptake (Askwith et al. 1994). In this study, CP was also found to enhance iron uptake in human glioma cells BT325 (Chapter 5).

This experiment aims to compare the effect of CP obtained from three different species in stimulating iron uptake in the glioma cell line BT325. The CPs used were from human, rat and bovine.

Human CP (hCP) has a molecular weight of 132 kDa. As analyzed by protein sequencing techniques and later by cDNA sequencing, the polypeptide chain has 1046 amino acid residues (molecular weight of 120 085) and attachment sites for four glucosamine-based oligosaccharides (Messerschmidt and Huber 1990). There are six copper atoms in hCP. Three of them form a trinuclear cluster sited at the interface of domains 1 and 6 and the other three are mononuclear sites in domains 2, 4 and 6 (Zaitseva et al. 1996).

Rat CP (rCP) has a molecular weight of 124 kDa (Manolis and Cox 1980). Its amino acid sequence showed 93% homology to the hCP and contains a 19-amino acid leader peptide plus 1040 amino acids of mature protein (Fleming and Gitlin 1990).

Other differences in the amino acid sequence of the rCP as compared with hCP included an increase in methionine and cystine/cysteine, and a decrease in histidine, tyrosine and tryptophan. rCP is a more acidic protein than human CP, as reflected in its reduced concentration of lysine (Manolis and Cox 1980). Moreover, rCP is more resistant to plasmin-mediated proteolysis than was hCP and has a much broader pH profile than hCP. Although both hCP and rCP were cleaved initially to products with apparent molecular weights of 116 kDa and 20 kDa, rCP was resistant to further proteolysis, whereas hCP was cleaved to smaller fragments (apparent 116kDa and 20 kDa, and 67 kDa and 50 kDa from the 116 kDa precursor). By the analysis of the N-terminal amino acid sequence of both CPs, it was found that hCP contains a proteolytically susceptible arginine residue which rCP contains a five-amino-acid deletion at this same proteolytic site (Ryan et al. 1992).

Bovine CP (bCP) has a molecular weight of 125 kDa and contains six atoms of copper per molecule (Calabrese et al. 1981; Dooley et al. 1981). Three copper atoms were detectable by E.P.R. spectral analysis with Type 2/Type 1 ratio of 1:3. The amino acid composition of bCP is very similar to that of hCP, but significantly different from that of rCP. The most apparent differences are more lysine and less *S*-containing amino acid and leucine in the bCP. There is strong resemblance between bCP and hCP. Both proteins show pH-dependent changes in the E.P.R. spectrum. This behavior has not been found in other CPs, and seems to be associated with lower stability towards denaturation and degradation process (Calabrese et al. 1981).

6.3 MATERIALS AND METHODS

6.3.1 MATERIALS

6-well plates and 75 cm^2 culture flask were from Iwaki Glass Co., Ltd. (NY, USA), pronase was from Calbiochem (CA, USA). Fetal Bovine Serum (qualified, heat inactivated), trypsin(2.5%, 10X), penicillin-streptomycin and RPMI Medium 1640 were purchased from Gibco BRL (Life Technologies, Inc., USA) The radioisotope $^{59}FeCl_3$ (100 μCi) was purchased from Amersham International (Buckinghemshire, England, UK). Human and bovine ceruloplasmins were from Sigma Chemical Co. (St. Louis, USA). Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St. Louis, USA). The glioma cell line BT325 was a generous gift from Su Zhou Medical School.

6.3.2 METHODS

6.3.2.1 Cell preparation

Please refer to Chapter 2.

6.3.2.2 Preparation of CPs

The human and bovine CPs were purchased from the Sigma Chemical Co., (MO, USA). The rat CP was prepared by modified method of Ryan et al. 1992 described in Chapter 3.

6.3.2.3 Preparation of radioactive iron ($^{59}Fe^{2+}$) solution

Please refer to Chapter 2.

6.3.2.4 Measurement of the transferrin-free ($^{59}Fe^{2+}$) iron transport

To measure of Tf-free iron ($^{59}Fe^{2+}$) transport by the cultured cells, cells were grown in 6-well plates until they reached about 80% confluence. The culture medium was decanted and the cells were washed with 37°C Hanks' buffer (pH 7.4) in order to remove dead cells and tissue debris. The cells were then preincubated twice with 2 ml RPMI 1640 medium (serum-free) for 15 minutes at 37°C. This was done to remove fetal bovine serum and endogenous transferrin (Kaplan et al. 1991; Trinder et al. 1996).

The preincubated cells were then washed three times with 37°C Hanks' buffer (pH 7.4). 150 μg/ml of CPs from human, bovine and rat in 1 ml of 1 μM $^{59}Fe^{2+}$ were added into the corresponding wells. The 1 μM $^{59}Fe^{2+}$ was prepared in 0.32 M sucrose solution buffered with 4 mM 1,4-piperazinediethanesulphonic acid (Pipes) at pH 5.8. After this incubation, the medium was decanted and the cells were washed with 4°C Hanks' buffer four times to stop the reaction. 1 ml of ice-cold pronase (1 mg/ml) was added to the cells and left to stand for 60 minutes on ice. For verification of iron internalization, the cells were transferred to centrifuge tubes and spun at 4000 rpm for 15 minutes to separate membrane bound and internalized fractions. Their radioactivities were counted with a Packard 5003 three channel γ-scintillation counter

(COBRA Q). The protein content was measured using a protein assay kit from Sigma Chemical Co. (MO, USA). The results were then expressed as pmol Fe/mg protein.

6.4 RESULTS

6.4.1 THE EFFECT OF hCP, rCP AND bCP IN STIMULATING IRON UPTAKE IN BT325 CELLS.

As shown in Fig. 15, hCP, rCP and bCP all could stimulate iron uptake in the glioma cells ($P < 0.001$). Moreover, iron uptake in the internalized fractions of the cells was significantly greater ($P < 0.001$) than the membrane bound fractions suggesting iron was mostly taking up by the cells.

6.5 DISCUSSION

In Chapter 5, it was shown that hCP stimulates iron uptake in glioma cells. This experiment aims to study the effect of CP obtained from three different species in stimulating iron uptake in this glioma cell line, thus to find out if this effect is species-specific to the cells. The results showed that hCP, rCP and bCP all could stimulate iron uptake in the brain cells.

These results were not as expected, since species specificity for CP binding has been observed in other study. hCP was found to bind very weakly to the chick heart and aortic membranes (Stevens et al. 1984). Moreover, based on the findings that rCP

has less ferroxidase activity than does hCP ferroxidase activity (Roeser et al. 1970; Ryan et al. 1992), it was predicted that the stimulatory effect of hCP is much stronger than that of rCP. One explanation for this discrepancy was the use of different experimental design. The comparison of rCP and hCP ferroxidase activity conducted by Ryan et al. 1992 was carried out with histidine:$FeCl_2$ and at pH 6.5. In this experiment, a different iron substrate, $^{59}Fe^{2+}$ was used at pH 5.8. In their study, it was also found that rCP maintained appreciable activity at acid pH, where the hCP had little activity (Ryan et al. 1992). Since a lower pH value was used in this study, the stimulatory effect of hCP may be lowered. For bCP, no comparative study on the strength of its ferroxidase activity with that of hCP and rCP has been reported. So, it can be concluded that bCP exhibited similar iron uptake stimulating effect as hCP and rCP under this experimental condition.

6.6 CONCLUSION

This study showed that hCP, rCP and bCP could stimulate iron uptake in the human glioma cell line BT325 and the degree of stimulation among these proteins was insignificant. Thus, the stimulation does not necessary be species-specific for these cells.

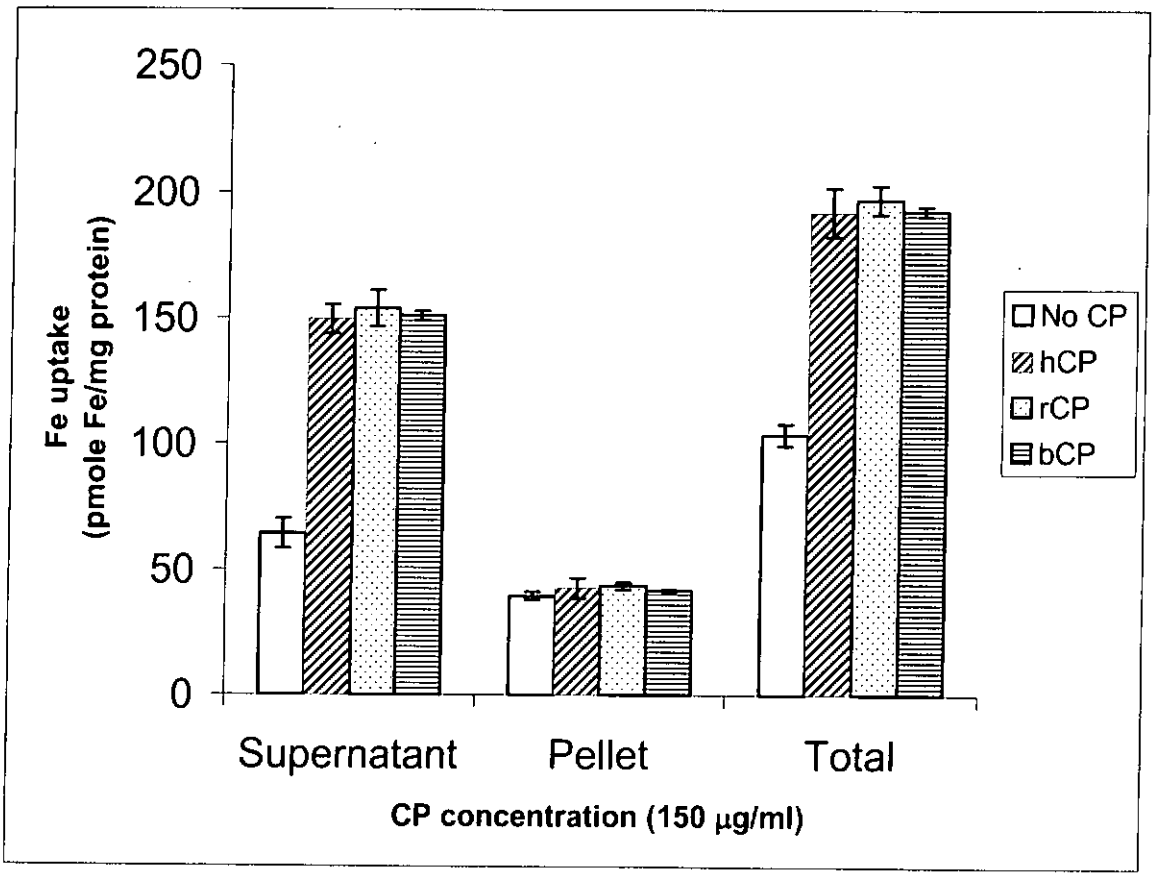

Fig. 15. The effect of CPs from different species to stimulate iron uptake in BT325 cells. The cells were incubated with 150 µg/ml hCP, rCP and bCP and 1 µM $^{59}Fe^{2+}$ for 30 minutes at 37°C. Each point is the mean±S.E.M. of three experiments.

Chapter 7

EFFECT OF CERULOPLASMIN ON IRON UPTAKE OF IRON-DEFICIENT BRAIN CELLS

7.1 ABSTRACT

The aim of this experiment is to compare the effects of ceruloplasmin (CP) in stimulating iron uptake in iron-sufficient and iron-deficient glioma cells. Two groups of iron-deficient glioma cells were prepared by incubating them with the 2 mM Fe^{3+} chelator desferoxamine mesylate (DFO) and 0.5 mM Fe^{2+} chelator bathophenanthroline disufonic acid (BP) for 16 hours in serum-free RPMI medium at 37°C. Before this preparation, the effect of these two iron chelators on the growth of this cell line was investigated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) assay. It was showed that an incubation time longer than one day with these two iron chelators inhibited the growth of these cells. The use of 1000-2000 μM DFO did not significantly inhibit the cells, therefore 2 mM DFO was used in the preparation. For BP, the use of 100-2000 μM of it showed increasing inhibition on the growth of the cells. Thus, a lower concentration (0.5 mM) of BP was chosen to prepare the iron-deficient cells. These results also showed

that iron is essential for the growth of these cells. Using 0-30 μg/ml of CP incubated with 1 μM Fe^{2+} for 30 minutes at 37°C for both the iron-sufficient and iron-deficient cells showed that there was no significant difference between the iron uptake of these two groups. These results are not consistent with recent findings that CP is able to stimulate iron uptake only in iron-deficient cells. This may be due to differences in experimental conditions and the use of cell type.

CP was able to stimulate iron uptake in both groups of cells and there was no significant difference between these two groups. These results are not consistent with recent findings that CP is able to stimulate iron uptake only in iron-deficient cells. This may be due to differences in experimental conditions.

Keywords:

Ceruloplasmin (CP)	Ferrous iron (Fe^{2+})	Glioma cells
Iron deficiency	MTT assay	Iron metabolism

7.2 INTRODUCTION

Iron is the most abundant trace metal in the brain (Yehuda and Youdim 1991). Its availability is essential for brain cell viability (Beard et al 1993; Benjamin 1995; Hu and Connor 1996). It is involved in the synthesis and function of dopamine, serotonin and catecholamines, and possibly in γ-aminobutyric acid (GABA) and myelin formation (Beard et al. 1993). It is also the key component of the heme in cytochrome proteins, permitting mitochondrial electron transfer during cellular

respiration. Because neurons are especially dependent on aerobic metabolism (indeed, the brain has a higher rate of oxidative metabolism than any other organ), it can be argued that iron is likely to be especially critical in the brain (Benjamin 1995; Connor and Benkovic 1992).

Although an essential nutrient, iron is also a potent toxin (Beard et al 1993). Unregulated iron ("free" iron) is highly toxic and is a prime initiator of lipid peroxidative damage (Connor and Benkovic 1992). Iron in its ionic form can catalyze reactions which produce reactive oxygen species (such as the potent hydroxyl free radical) that cause DNA and protein damage as well as cell death by a process known as oxidative stress (Dawson and Dawson 1996; Klomp and Gitlin 1996). An imbalance in brain iron and hence a dysfunction in iron-related metabolism are suspected in some neurological disorders (Hu and Connor 1996). Increased brain iron level is associated with AD, PD, HVS, multiple sclerosis, Pick's disease, Huntington's chorea, Kaschin-Beck's disease, and tardive dyskinesia (Yehuda and Youdim 1991; Connor and Benkovic 1992; Hall et al. 1992; Hodgkins and Blair 1997). An increased level of iron has been reported in psychotic patients. On the other hand, individuals with iron deficiency are irritable and apathetic - their level of attention and exploratory behavior are reduced. They may also suffer from anorexia and a decrease in physical exercise (Yehuda and Youdim 1991). Moreover, insufficient iron levels during early postnatal development will result in mental and motor impairments which then persist into adulthood.

Because of the current lack of understanding of iron regulation in the brain, it is difficult to ascertain whether changes in iron levels or in the histological distribution of iron reported in many of these diseases is in response to the disease state or part of the pathogenesis. Still, iron must be presented to the brain and transported to cells within the brain in a timely and well-regulated manner (Conner and Benkovic 1992).

The Tf-dependent pathway has been considered the main route for iron transport. This involves receptor-mediated endocytosis of Tf. Apart from this, it has been suggested that non-Tf-bound iron enters the cells by different mechanisms, such as carrier-mediated uptake, simple diffusion and lipid peroxidation mediated iron uptake. The function of the Tf-independent mechanisms is not well understood (Qian and Tang 1995; Qian et al. 1996), but there is increasing evidence that non-Tf-bound iron may be quantitatively more important in producing the iron loading and hepatic damage seen in hemochromatosis and other iron-overload diseases (Wright et al. 1988). Researchers have suggested that the Tf-independent pathway may clear potentially toxic, low molecular weight iron chelates (Attieh et al. 1999). Recently, two putative iron transporters, DMT1 (Divalent Metal Transporter) (Gruenheid et al. 1995) and SFT (Stimulator of Fe Transport) (Yu and Wessling-Resnick 1998), have been identified in mammalian cells.

Genetic studies in yeast have identified a number of factors involved in the trans-membrane transport of iron. One of the factors which mediates high affinity iron uptake known as *FET3* is a homologue of the mammalian protein ceruloplasmin (CP) (Askwith et al. 1994). Aceruloplasminemia and other hereditary CP deficiency

diseases have now been identified and linked to abnormalities in iron metabolism (Harris et al. 1995; Kawanami et al. 1996; Klomp and Gitlin 1996; Logan 1996; Miyajima et al. 1996; Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998). A role for CP in iron metabolism has been suggested (Harris et al. 1995; Klomp and Gitlin 1996; Young et al. 1997; Gitlin 1998; Mukhopadhyay et al. 1998; Attieh et al. 1999).

CP is a blue serum α2-glycoprotein which binds more than 90% of copper in the plasma (DiSilvestro et al. 1988; Percival and Harris 1990; Harris et al. 1995; Miyajima et al. 1996). The precise physiological function of CP remains controversial, although many workers tend to consider the protein multifunctional. (Percival and Harris 1990; Saenko et al. 1994) At least four main functions have been attributed to CP, those of copper transport, ferroxidase activity, amine oxidase activity and as an antioxidant inhibiting the formation of free radicals in serum (Zaitseva et al. 1996). It has been suggested that the ferroxidase activity of this protein is involved in iron metabolism (Saenko et al. 1994). There are reports that CP stimulates iron uptake in mammalian cells and that is more effective in iron-deficient cells (Mukhopadhyay et al. 1998; Attieh et al. 1999).

In this study, the effect of CP on the iron uptake of iron-sufficient and iron-deficient brain cells was investigated. Two iron chelators were used to prepare the iron-deficient cells, desferoxamine mesylate (DFO) and bathophenanthroline disufonic acid (BP). The iron-sufficient cells were simply grown in normal medium, i.e. untreated. BP is a specific Fe^{2+} chelator to which the cell is impermeable. It is able

to inhibit DNA synthesis when cell growth is initiated with growth factors. That effect is not mediated by inhibition of the ribonucleotide reductase. The site in the cell from which iron is extracted remains to be established. A possible focus for BP inhibition of cell growth is the plasma membrane redox system, but how BP inhibits the plasma membrane redox system and cell proliferation is unknown. It is possibly through iron extraction rather than by chelating extracellular iron (Alcain et al. 1994). On the other hand, DFO is the most potent permeable Fe^{3+} chelator and a far more specific ferric complexing agent than EDTA, diethylenetriamine penta-acetic acid (DTPA) or Tf (stability constants: 10^{31}, 10^{25}, 10^{29} and 10^{27} respectively). It shows no significant affinity for ferrous ions (stability constant: $\sim 10^{10}$). It removes iron from ferritin and hemosiderin and continues to do so until its maximum theoretical binding capacity is attained. However, in the case of Tf, DFO takes up only some 10-15% of the iron from totally saturated Tf. There is no demonstrable exchange of iron from ferrioxamine to Tf, but DFO cannot withdraw iron from the porphyrin system (Keberle 1964; Alcain et al. 1994). It combines with Fe^{3+} in a 1:1 molar ratio, but the precise cellular location of DFO chelatable iron is unknown (Miyajima et al. 1997). The addition of DFO to culture media can inhibit cell proliferation by interfering with ribonucleotide synthesis (Alcain et al. 1994). *In vivo*, it chelates exclusively with non-heme iron. Its two main clinical uses are the chelation and removal of free serum iron following acute iron intoxication and the removal of excess iron stores in idiopathic haemochromatosis or in transfusional haemosiderosis (Evans and Shepherd 1975). In certain experiments, the addition of DFO has been used to prevent trace iron from interfering with the reaction (Lovstad 1996). Before the

preparation of iron-deficient cells, the effects of these two iron chelators on the growth of this cell line was investigated by MTT assay.

7.3 MATERIALS AND METHODS

7.3.1 MATERIALS

The 6-well plates, 96-well plates and 75 cm^2 culture flasks used in these experiments were purchased from Iwaki Glass Co., Ltd. (NY, USA). The pronase was from Calbiochem (CA, USA). The fetal bovine serum (qualified, heat inactivated), trypsin(2.5%, 10X), penicillin-streptomycin and RPMI Medium 1640 were purchased from Gibco BRL (Life Technologies, Inc., USA). The radioisotope $^{59}FeCl_3$ (100 μCi) was purchased from Amersham International Inc. (Buckinghemshire, England, UK). Human ceruloplasmin was purchased from Sigma Chemical Co. (MO, USA). Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (MO, USA). The glioma cell line BT325 was a generous gift from Su Zhou Medical School.

7.3.2 METHODS

7.3.2.1 Cell preparation

Please refer to Chapter 2.

7.3.2.2 MTT assay

Please refer to Chapter 2.

7.3.2.3 Preparation of iron-sufficient and iron-deficient cells

Please refer to Chapter 2.

7.3.2.4 Preparation of radioactive iron ($^{59}Fe^{2+}$) solution

Please refer to Chapter 2.

7.3.2.5 Measurement of the transferrin-free ($^{59}Fe^{2+}$) iron transport

Please refer to Chapter 2. The procedure is the same except the use of cells include iron-sufficient and iron-deficient BT325 cells.

7.4 RESULTS

7.4.1 EFFECT OF IRON CHELATORS ON THE GROWTH OF BT325 CELLS

The Fe^{3+} chelator DFO and Fe^{2+} chelator BP were used to prepare iron-deficient cells in this experiment. Before the preparation, their effect on the growth of the glioma cells was investigated using MTT assay.

As shown in Fig. 16, A570nm absorbance decreased significantly ($P < 0.001$) with increasing incubation time for both chelators used. There was no significant change ($P > 0.05$) in the A570nm absorbance of cells incubated with 1000 and 2000 μM DFO as compared with the control. However, for the cells incubated with BP, the A570nm absorbance decreased significantly ($P < 0.001$) as the concentration of BP increased.

These results indicate that the iron chelators cannot be incubated with the cells for more than one day and that a lower BP concentration should be used, otherwise the viability of these cells will be decreased. Therefore, in the next experiment 2000 μM DFO and 500 μM BP were used to prepare iron-deficient cells, incubating the cells with these chemicals for only one day.

7.4.2 EFFECT OF CP ON IRON UPTAKE OF IRON-SUFFICIENT AND IRON-DEFICIENT BRAIN CELLS

In this experiment, the effect of CP on iron uptake by iron-sufficient and iron-deficient brain cells was investigated (Fig. 17). For the iron-sufficient cells, total iron uptake increased significantly ($P < 0.01$) with increasing CP concentration, as in chapter 5. For the iron-deficient cells (the DFO-treated group), total iron uptake did

not increase significantly with the addition of 15 μg/ml CP ($P > 0.05$). However, a significant increase in total iron uptake was observed when 30 μg/ml of CP was used ($P< 0.05$). For another group of iron-deficient cells (the BP-treated group), similar results were found. Significant increase in total iron uptake was observed only when 30 μg/ml was used ($P < 0.01$). Thus, the minimum concentration of CP to stimulate iron uptake by either iron-deficient group was 30 μg/ml. For the iron-sufficient cells, as determined in the previous experiment, the minimum effective concentration was 5 μg/ml. For the internalized iron, changes were the same as those reported for total iron. On the other hand, there was no significant change in membrane bound iron uptake with increasing CP concentration for any group ($P > 0.05$).

The findings of this part of the study show that CP has a role in iron uptake of for both iron-sufficient and iron-deficient cells. There was no significant difference in iron uptake between iron-sufficient and iron-deficient cells ($P > 0.05$).

7.5 DISCUSSION

The results show that CP increases $^{59}Fe^{2+}$ uptake by brain cells *in vitro*. This result is consistent with studies conducted using a human hepatocellular carcinoma cell line (Mukhopadhyay et al. 1998) and a human erythroleukemic cell line (Attieh et al. 1999), except that they found CP to be effective only in iron-deficient cells

Before studying CP's effect on iron uptake by iron-deficient cells, the effect of these two iron chelators on cell growth was studied by MTT assay (Liu et al. 1997). As

showed in Fig. 16, the cell growth was inhibited by longer incubation time with the chemicals, and the inhibitory effect of BP is greater than that of DFO. Therefore, 2000 μM of DFO and a lower concentration of BP (500 μM) were used. Moreover, a shorter incubation time (1 day) was used in the preparation of iron-deficient cells. This is because a longer incubation time causes cell death by inhibiting DNA synthesis (Alcain et al. 1994) and induces irreversible effects on cell proliferation (Tabor and Kim 1991; Renton and Jeitner 1996). This part of the experiment also showed that iron is essential for cell growth.

The results of the study of CP and iron uptake by iron-sufficient and iron-deficient brain cells (Fig. 17) showed that CP increases iron uptake in both iron-sufficient and iron-deficient cells. A higher CP concentration was required to stimulate iron uptake in iron-deficient cells (30 μg/ml) than in iron-sufficient cells (5 μg/ml). When both type of cells were incubated at a high CP concentration (30 μg/ml), there is no significant difference in the iron uptake of these two types of cells. This is consistent with the finding that the anemia which develops in copper-deficient animals is unresponsive to iron but is correctable by administrating CP (Harris et al. 1995). However, this result contradicts reports that CP is only effective in stimulating iron uptake in iron-deficient cells. It suggests that a factor required for uptake is induced or activated by iron deficiency (Mukhopadhyay et al. 1998; Attieh et al. 1999). The difference may be due to experimental conditions. In this study, two changes of serum-free medium were used to incubate the cells, each for 15 minutes. This served to remove fetal bovine serum and endogenous transferrin (Kaplan et al. 1991; Trinder et al. 1996) before the addition of CP. In the experiments of Mukhopadhyay's and

Attieh's groups, the conditioned medium was retained. The removal of the conditioned medium before measuring iron uptake inhibited about 60 to 70% of the increase in iron-deficient cells (Mukhopadhyay et al. 1998). Another possibility is the use of different cell type. In this experiment glioma cells was used while in their experiments human heptocellular carcinoma cell line (Mukhopadhyay et al. 1998) and human erythroleukemic cell line (Attieh et al. 1999) were used.

7.6 CONCLUSION

By using the MTT assay, the effect of those two iron chelators, DFO and BP on the growth of this cell line was showed to inhibit cell growth with higher concentration and longer incubation time. These suggested that iron is essential for the growth of these cells. The use of 0-30 μg/ml of CP incubated with 1 μM Fe^{2+} for 30 minutes at 37°C for both the iron-sufficient and iron-deficient cells showed that there was no significant difference between the iron uptake of these two groups. These results are not consistent with recent findings that CP is able to stimulate iron uptake only in iron-deficient cells. This may be due to differences in experimental conditions and perhaps the use of cell type.

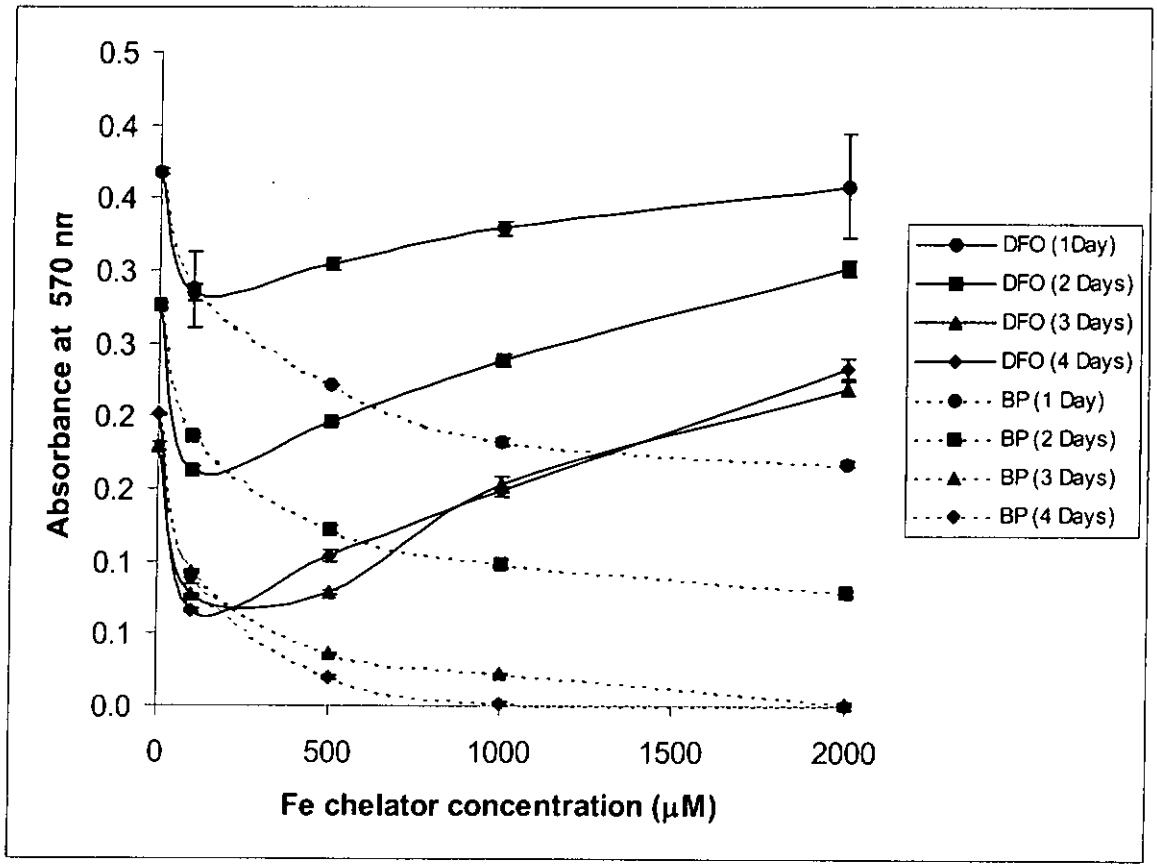

Fig. 16. The effect of iron chelators on the growth of BT325 cells. The cells were incubated with different iron chelators (DFO and BP) at different concentrations (0, 100, 500, 1000 and 2000 μM) for the indicated period of time (1-4 days). Each point is the mean±S.E.M. of four experiments.

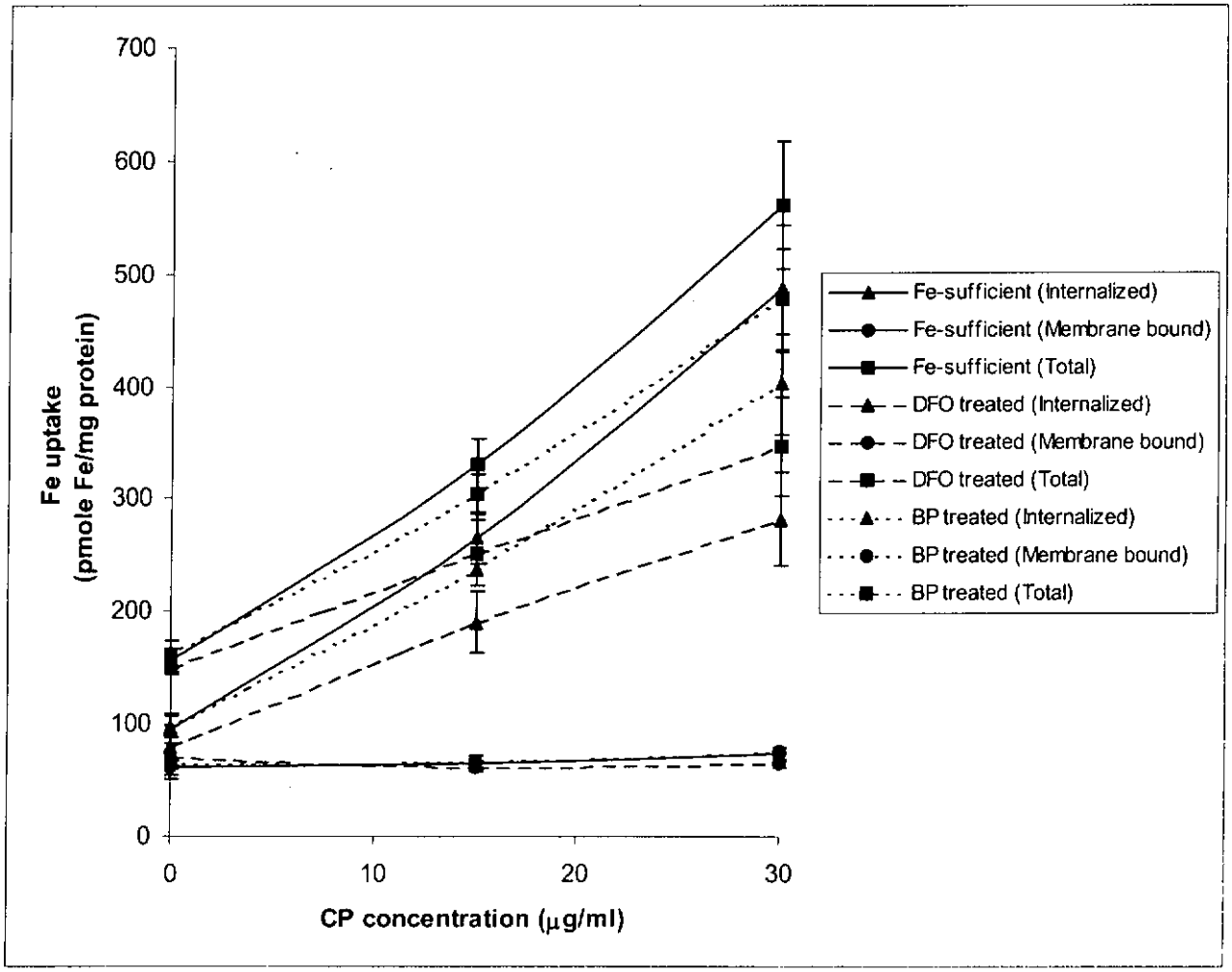

Fig. 17. The effect of CP on iron uptake by iron-sufficient and iron-deficient brain cells. The cells were incubated with 1 μM $^{59}Fe^{2+}$ and CP (0-30 μg/ml) for 30 minutes at 37°C. The internalized, membrane bound and total radioactivities of the cells were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

Chapter 8

EFFECT OF CERULOPLASMIN FERROXIDASE ACTIVITY AND METAL IONS ON CERULOPLASMIN-STIMULATED IRON UPTAKE IN BRAIN CELLS

8.1 ABSTRACT

In this study, the importance of ceruloplasmin (CP) ferroxidase activity on iron uptake of the glioma cell line BT325 was investigated. The use of ferroxidase-defective CPs, apoCP and heat-inactivated CP did not stimulate iron uptake illustrating that the ferroxidase activity plays an essential role in the iron uptake process. Moreover, CP-stimulated iron uptake was inhibited by both divalent and trivalent cations (Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+} and Cr^{3+}). This raises the suggestion that these metals may be transported by the same mechanism which needs further investigation.

Keywords:

Ceruloplasmin (CP)	Ferrous iron (Fe^{2+})	Glioma cells
Divalent cations	Trivalent cations	Ferroxidase activity

8.2 INTRODUCTION

Ceruloplasmin (CP) is a protein with controversial physiological functions. It was discovered in 1948 as a blue serum α2-glycoprotein. It binds more than 90% of copper in the plasma (DiSilvestro et al. 1988; Percival and Harris 1990; Harris et al. 1995; Miyajima et al. 1996). At least four main functions have been attributed to CP, those of copper transport, ferroxidase activity, amine oxidase activity and as an antioxidant in the prevention of the formation of free radicals in serum (Zaitseva et al. 1996).

CP has also been suggested in promoting iron efflux from cells in the CNS (Harris et al. 1995; Klomp and Gitlin 1996) and in liver cells (Young et al. 1997). The decreased or absence of expression of this protein may induce iron accumulation in the brain cells in aceruloplasminemia and perhaps in other neurodegenerative diseases where abnormalities in iron metabolism have been documented (Harris et al. 1995; Kawanami et al. 1996; Klomp and Gitlin 1996; Logan 1996; Miyajma et al. 1996; Okamoto et al. 1996; Takahashi et al. 1996; Gitlin 1998). In aceruloplasminemic mice, the disruption of CP gene resulted in abundant iron stores within reticuloendothelial cells and hepatocytes. These finding reveal an essential physiologic role for CP in determining the rate of iron efflux from cells with mobilizable iron store (Harris et al. 1999). Recently, a transmembrane-bound CP

homologue hephaestin in mouse was suggested as a multicopper ferroxidase necessary for iron efflux from intestinal enterocytes into the circulation. This further suggest that CP is an important link between copper and iron metabolism in mammals (Vulpe et al. 1999)

However, reports contradict to the traditional hypothesis that CP functions in iron release of cells have also been published. CP was shown to enhance iron uptake in certain mammalian cells (Mukhopadhyay et al. 1998; Attieh et al. 1999). Genetic studies in yeast have identified a CP homologue known as *FET3* which mediates high affinity iron uptake (Askwith et al. 1994). The results obtained in this study also showed that CP could stimulate iron uptake in glioma cells.

To further investigate the effect of CP in stimulating iron uptake in this glioma cell line. The importance of CP ferroxidase activity and the effect of multiple cations on CP-stimulated iron uptake were studied.

Reduction or lost in CP ferroxidase activity in which oxidizes Fe^{2+} to Fe^{3+} (Kawanami et al. 1996) has been suggested as the cause of several iron-related neurodegenerative diseases. It was postulated that reduced oxidative activity of CP in plasma might be either a cause or a consequence of AD (Snaedal et al. 1998). In the CSF of PD patients, the CP ferroxidase activity was also reduced (Boll et al. 1999). In this study ferroxidase-defective CPs, apoCP and heat-inactivated CP were used to find out the importance of ferroxidase activity in iron uptake.

Multiple divalent and trivalent cations (Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+} and Cr^{3+}) were also used for the investigation of their effect on CP-stimulated iron uptake. Among the cations, Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+} and Al^{3+} are common multivalent cations present in the biological systems (Reyes et al. 1994).

In the CNS, copper can prevent axonal swelling and degeneration of nerve cells (Hartmann and Evenson 1992). And serum copper tends to increase in elderly, even if its binding protein CP does not vary (Molaschi et al. 1996).

For the other divalent metal, Mg^{2+}, deficiency of it is an important etiological factor in the pathogenesis of AD (Hartmann and Evenson 1992). Binding of Mg^{2+} and Ca^{2+} to human and sheep CP had been reported. The Kd for calcium was found to be 1.4 mM and the Kd for magnesium to bind the human and sheep CP were 0.3 and 0.7 mM respectively (Musci et al. 1996a). It was suggested that Mg^{2+} and ATP may act as physiological modulators *in vivo* by triggering a complex pathway for CP acquires the native conformation after copper entry (Musci et al. 1996b).

Zinc concentration decreases with advancing age, primarily because of reduced food intake and absorption. Moreover, certain diseases that impair zinc absorption and/or increase its excretion are frequently found in the elderly. Zinc deficiency could be involved in the pathophysiology of AD (Molaschi et al. 1996). Besides, a genetically based progress inability of neurons to incorporate zinc ions into DNA and RNA enzymes may play a role in this disease (Hartmann and Evenson 1992). Study in primary biliary cirrhosis (PBC) patients showed increased CP activity and copper

content in the serum. Autopsy analysis of the livers indicated reduced level in the Fe and Zn levels. The reduction of Fe and Zn contents in the liver of the PBC patients indicated the possible relationship of CP to Fe and Zn metabolism as well as Cu metabolism (Sogawa et al. 1994). In rat, moderately high Zn in diet reduces plasma copper, but did not lower the plasma CP activity (Panemangalore and Bebe 1996). However, other researchers have found that CP activity was lower in zinc-supplemented sheep (Saylor and Leach 1980).

Ca deficiency may be an etiological factor in neurodegenerative diseases. Monkeys fed with a low calcium diet for more than 40 months shown that the motor neurons of the spinal cord have chromatolysis, accumulations of phosphorylated neurofilaments, axonal spheroids and inclusions, all of which are compatible with early amyotrophic lateral sclerosis (Hartmann and Evenson 1992).

For the trivalent cation Cr^{3+}, the trivalent state of it is commonly encounter in biological system. Absorbed chromium circulates as free Cr^{3+}, as Cr^{3+} bound to Tf or other plasma proteins, or as complexes (Ducros 1992). According to spectrophotometric titration of each individual amino acid located in the iron binding site of Tf, the tyrosine might be the most suitable ligand for the binding of chromium to Tf (Ani and Moshtaghie 1992). The resulting product is a stable complex with Tf. Together with the fact that Cr^{3+} binds tightly to iron sites in hen's egg conalbumin (Chiswell et al. 1998). It raises the suggestion that chromium may compete competitively with iron in binding to apo-Tf, and influence iron metabolism and its

related biochemical parameters (Ani and Moshtaghie 1992; Chiswell et al. 1998). Hence, Cr^{3+} is more likely to affect Tf-iron uptake.

For the Al^{3+}, it has been implicated as playing a toxic role in the pathologic lesions of AD (Abreo et al. 1999). One of the reasons may due to that it does not accumulate in ferritin *in vivo* which implies that cells are unable to detoxify Al by the same mechanism as that available for iron (Dedman et al. 1992). The only Al oxidation state in biological system is 3+ (Martin 1992). In the blood plasma, citrate is the main small molecule carrier (Martin 1986; Martin 1992; Ohman and Martin 1994) and Tf is the main protein carrier of Al^{3+} (Martin et al. 1987; Martin 1992). Tf can mediate cellular uptake of these elements via cell surface Tf receptors. It is suggested that Al-Tf down-regulates Tf receptors on oligodendrocytes and can limit Fe and Mn uptake through this mechanism (Golub et al. 1996). It was reported that Al-loaded neuroblastoma cells showed increased rates of ^{59}Fe and ^{125}I-Tf uptake and total cellular Fe content. These results suggested that the accumulation of Al in these cells resulted in increased iron uptake, inhibition of cell growth, and expression of neurofibrillary tangle protein, partially mimicking the pathological hallmarks of AD (Abreo et al. 1999). It is proposed that Al, when bound to Tf, inhibited iron uptake partly by down-regulating Tf-receptor expression and partly by interfering with intracellular release of iron from Tf (McGregor et al. 1990).

8.3 MATERIALS AND METHODS

8.3.1 MATERIALS

6-well plates and 75 cm^2 culture flask were from Iwaki Glass Co., Ltd. (NY, USA), pronase was from Calbiochem (CA, USA). Fetal Bovine Serum (qualified, heat inactivated), trypsin(2.5%, 10X), penicillin-streptomycin and RPMI Medium 1640 were purchased from Gibco BRL (Life Technologies, Inc., USA) The radioisotope $^{59}FeCl_3$ (100 μCi) was purchased from Amersham International (Buckinghemshire, England, UK). Human ceruloplasmin was from Sigma Chemical Co. (St. Louis, USA). Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St. Louis, USA). The glioma cell line BT325 was a generous gift from Su Zhou Medical School.

8.3.2 METHODS

8.3.2.1 Cell preparation

Please refer to Chapter 2.

8.3.2.2 Preparation of radioactive iron ($^{59}Fe^{2+}$) solution

Please refer to Chapter 2.

8.3.2.3 Preparation of apoCP and heat-inactivated CP

Please refer to Chapter 2.

8.3.2.4 Preparation of Tf-free metal salts solution

Please refer to Chapter 2.

8.3.2.5 Measurement of the Tf-free ($^{59}Fe^{2+}$) iron transport

To measure of transferrin-free iron ($^{59}Fe^{2+}$) transport by the cultured cells, cells were grown in 6-well plates until they reached about 80% confluence. The culture medium was decanted and the cells were washed with 37°C Hanks' buffer (pH 7.4) in order to remove dead cells and tissue debris. The cells were then preincubated twice with 2 ml RPMI 1640 medium (serum-free) for 15 minutes at 37°C. This was done to remove fetal bovine serum and endogenous transferrin (Kaplan et al. 1991; Trinder et al. 1996).

To study the effect of divalent and trivalent cations on CP-stimulated iron uptake. 1 ml of 10 μM metal ion salts in 1 μM $^{59}Fe^{2+}$ (control group i.e. no CP) and 1 ml of 10 μM metal ion salts in 1 μM $^{59}Fe^{2+}$ mixed with 30 μg/ml CP were added to the corresponding wells for 30 minutes at 37°C.

For the measurement of the effect of different species' CPs on iron uptake. Human, rat and bovine CP (150 μg/ml) were added to the incubation mixture for 30 minutes at 37°C.

For the measurement of the effect of ferroxidase activity of CP on iron uptake. Untreated CP, apoCP and heat-inactivated CP (0, 30, 150 and 300 μg/ml) were added to the incubation mixture for 30 minutes at 37°C.

After this incubation, the medium was decanted and the cells were washed with 4°C Hanks' buffer four times to stop the reaction. 1 ml of ice-cold pronase (1 mg/ml) was added to the cells and left to stand for 60 minutes on ice. For verification of iron internalization, the cells were transferred to centrifuge tubes and spun at 4000 rpm for 15 minutes to separate membrane bound and internalized fractions. Their radioactivities were counted with a Packard 5003 three channel γ-scintillation counter (COBRA Q). The protein content was measured using a protein assay kit from Sigma Chemical Co. (MO, USA). The results were then expressed as pmol Fe/mg protein.

8.4 RESULTS

8.4.1 EFFECT OF CP FERROXIDASE ACTIVITY ON IRON UPTAKE

The effect of CP ferroxidase activity on iron uptake was investigated. Three types of CP were used, untreated CP, apoCP (copper-removed CP) and heat-inactivated CP (CP with altered protein conformation, lost of the blue copper centerintegrity and enzyme activity, but the copper ions were retained) (Wang et al. 1995). In Fig. 18, CP-stimulated iron uptake was completely diminished in glioma cells incubated with the two ferroxidase-defective forms of CP, apoCP and heat-inactivated CP (P >

0.05). In the untreated CP, the iron uptake was increased significantly ($P < 0.01$). The results showed that the ferroxidase activity of CP is crucial in stimulating iron uptake of the glioma cell.

8.4.2 EFFECT OF METAL IONS ON CP-STIMULATED IRON UPTAKE

As shown in Fig. 19, the addition of these cations (10-times the molar concentration of $^{59}Fe^{2+}$) significantly inhibited both non-CP-stimulated and CP-stimulated iron uptake of the brain cells ($P < 0.001$). The degree of inhibition of iron uptake into the glial cell was 53%, 64%, 77%, 77%, 78%, 82% and 84% for Al^{3+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cr^{3+} and Cu^{2+} respectively.

8.5 DISCUSSION

The importance of CP ferroxidase activity in stimulating iron uptake by the brain cells was supported by the use of two forms of ferroxidase-defective CPs, apoCP and heat-inactivated CP (Fig. 18).

About 10% of circulating CP appear as apoprotein, but it is unclear if this is secreted from the liver without copper or present as a result from a low rate of copper exchange in the plasma and extracellular fluids (Percival and Harris 1990). For the apoCP, its ferroxidase activity was lost upon the removal of copper ions. In fact,

many of the projected functions of CP are related to the 6-7 Cu atoms bound to the peptide chain (Ryan et al. 1992). It was reported that a 21-year-old man with copper deficiency whose serum copper did not increase despite the administration of large doses of intravenous copper sulfate. His CP level as measured by an antibody technique was normal and his CP amino acid sequence was also normal. However, the CP oxidase activity was very low suggesting a defect in hepatic processing copper for incorporation into CP. In normal subjects Cu is rapidly incorporated into CP, thereby reducing the amount available for renal excretion (Buchman et al. 1994; Osaki and Johnson 1969). And the copper deficiency in rat resulted in a near-complete loss of serum CP oxidase activity (Gitlin et al. 1992). Additionally, the Long-Evans Cinnamon (LEC) mutant rat which possesses a mutation causing the deficiency in serum CP activity has normal coding for both of the CP gene and mRNA. And the serum CP level is normal. The metabolic labeling of hepatocytes with ^{64}Cu showed that copper incorporation into CP was deficient in the LEC rat causing deficiency in serum CP activity (Yamada et al. 1993). It was also postulated that the limiting step in a CP-catalyzed reaction is a function of the rapidity of reduction of the 'blue' copper (Ryan et al. 1992). However, it is unlikely that CP has a direct role in copper transport to the cells. As indicated by kinetic data, copper is turned over at the same rate as the protein (Harris et al. 1995) and plasma CP does not readily exchange its copper atoms with unbound copper owing to its center's distortion which poses an energy barrier to the binding of copper ions (Saenko et al. 1994). Moreover, studies from aceruloplasminemia patients showed that in the absence of CP no defect in copper accumulation was shown, rather excessive iron deposition in selected tissues was found. This indicated that CP plays little role in

copper transport but has an essential role in iron metabolism (Roeser et al 1970; Miyajima et al. 1996).

Under physiological condition, copper does not affect the rate of synthesis or the secretion of apoCP, but failure to incorporate copper during biosynthesis results in an unstable protein lacking oxidase activity. The oxidase activity is a unique feature of CP (Percival and Harris 1990), in which iron is known as the best substrate for it (Ryan et al. 1992). As shown in this study, the heat-inactivated CP which has Cu retains in the protein but lost in ferroxidase activity could not stimulate iron uptake in the brain cells. Thus, the ferroxidase activity is necessary for CP-stimulated iron uptake. Other researchers using ferroxidase-defective CPs prepared in different ways have produced the same results. They used apoCP which was prepared by removal of copper by complexing with cyanide under reducing conditions and the ammonium tetrathiomolybdate-treated CP in which the thiomolybdate bind copper and irreversibly inhibit CP ferroxidase activity. Both of the ferroxidase-defective CPs did not stimulate iron influx into the iron-deficient K562 cells (Atteih et al. 1999). The importance of ferroxidase activity in CP is further supported by the study in copper-deficient swine. Though the administration of inorganic copper could induce increases in plasma iron, it only happened after CP appeared in the circulation. When CP was administrated to these animals, plasma iron increased immediately and continued to rise at a rate proportional to the logarithm of the CP dose. In addition, it was necessary to give 100-150 μg/kg of inorganic copper in order to have a similar effect as the administration of 0.6-1.2 μg/kg of CP copper. The plasma iron response to this relatively high dose of inorganic copper was delayed as compared with the

response to CP, and the delay was associated with an increase in plasma CP of an order that would in itself simulate iron outflow. Thus, it appears that copper exerts its effect on plasma iron only by making CP synthesis possible (Lee et al, 1968; Roeser et al. 1970). As suggested by Attieh et al. 1999, ferroxidase activity is necessary for CP-stimulated iron uptake may due to that Fe^{3+} product is transported into the cell by a trivalent cation transporter. Moreover, the results also suggest that CP increased iron uptake by increasing available substrate (by formation of Fe^{3+}), not by activation of the transporter itself.

The reduction in both non-CP-stimulated and CP-stimulated iron uptake by the addition of divalent and trivalent cations in this study suggested that these metal ions may be transported by the same mechanism (Fig. 19). In terms of inhibitory effectiveness the metals were in the order of Cu^{2+}, $Cr^{3+} > Mg^{2+}$, Ca^{2+}, $Zn^{2+} > Fe^{2+} > Al^{3+}$.

For the case of non-CP-stimulated iron uptake, the inhibition by Ca^{2+}, Zn^{2+}, Fe^{2+} had been reported in erythrocytes (Chua et al. 1996; Savigni and Morgan 1998), reticulocytes (Morgan 1988) and in hepatocytoes (Baker et al. 1998). And their effect on reticulocytes is of a competitive type (Morgan 1988). Moreover, study about metal ions to compete for iron present in the iron chelators showed Cu^{2+} and Al^{3+} had the greatest competition against iron, while Mg^{2+} and Ca^{2+} had little or no effect and Zn^{2+} had an intermediate effect (Sheppard and Kontoghiorghes 1993).

Among the metal used, Cu^{2+} demonstrated the greatest inhibitory effect on both CP-stimulated and non-CP-stimulated Fe^{2+} uptake. Similar observations have been made in other cells under non-CP stimulating iron uptake condition. In cultured HeLa cells, Cu^{2+} could partially blocked the uptake of non-Tf iron in a dose-dependent manner, while the Tf-bound iron uptake was unaffected (Sturrock et al. 1990). And in perfused rat liver, Cu^{2+} could block the uptake of non-Tf iron (Wright et al. 1986).

However, the findings in this study were different from that reported by Attieh et al. 1999 using iron-deficient K562 cells. They found that even in the presence of 70-fold molar excess of the divalent cations (Ca^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+}), these metals did not substantially compete for CP-stimulated ^{55}Fe uptake. And in isolated rat hepatocytes, Cu^{2+} showed limited ability to inhibit iron uptake (Thorstenson1988). These discrepancies may due to different experimental setup. In fact, another researchers have stated that the effect of Cu^{2+} and Zn^{2+} to induce changes in transport, or to compete with iron for accumulation by the Tf-independent uptake system, was critically dependent on the composition of the media in which the cells were incubated. Cells incubated in a simple physiological salt solution (Hank's Balanced Salt Solution/4-(2-hydroxyethyl)-1-piperazineethanesulphoic acid) containing Cu^{2+} or Zn^{2+} showed an increase in the activity of the Tf-independent iron transport system. The ability of Cu^{2+} to inhibit non-Tf iron transport was affected by the presence of reductants. In an amino acid-containing medium Cu^{2+} did not interact with the Tf-independent transport system: it neither increased the activity of transport system nor blocked ^{59}Fe-NTA uptake by the transport system (Kaplan et al. 1991).

Was the Cu^{2+} ion competed for CP for copper transport into the cells and thus reduced iron uptake by the cells? As discussed previously, it is unlikely that CP has a direct role in copper transport to the cells (Harris et al. 1995). Rather, the result may due to non-specific binding to Cu-binding sites on the CP molecule which is believed to inhibit lipid peroxidation induced by copper ions (Olson and Holtzman 1980; Saenko et al. 1994).

As for Zn^{2+}, the result obtained in this research is consistent with the findings of Sturrock et al. 1990 in which Zn^{2+} and other divalent metals (Cd^{2+} and Mn^{2+}) were able to inhibit the uptake of non-Tf iron. Furthermore, Wright et al. 1986 in which Zn^{2+} reversibly inhibited ferrous iron uptake and the inhibition by Zn^{2+} was competitive in perfused rat liver.

The Tf-independent iron uptake system exhibits a Ca^{2+} dependence, being severally reduced in the absence of extracellular Ca^{2+} (Kaplan et al. 1991). Ca^{2+} enhanced iron internalization was reported for Fe-ascorbate uptake by suspensions of freshly isolated hepatocyte (Nilsen 1991) and for the uptake of Fe-citrate and other Fe complexes by HeLa cells (Sturrock et al. 1990). However, in this study, the presence of Ca^{2+} did not enhance the iron uptake but reduced it. This can be explained by differences in experimental protocols. In the present study, the Ca^{2+} was added to the incubation mixture with the $^{59}Fe^{2+}$ at the same time. While in the study conducted by Kaplan et al. 1991, the cells which showed 2-3-fold increase in non-Tf iron transport activity were previously been exposed to supraphysiological levels of Ca^{2+} for a period of time (30 minutes). Direct measurement of intracellular Ca^{2+} levels using the

chromophore Indo 1 demonstrated that incubation of cells under such condition resulted in an increase in intracellular free Ca^{2+}. Thus, the effect of increased extracellular Ca^{2+} in stimulating non-Tf-bound iron uptake may result from increased intracellular Ca^{2+} (Kaplan et al. 1991). Apart from this, in another study using iron-citrate, iron internalization by hepatocytes was enhanced by the addition of $CaCl_2$ to the incubation medium, up to a concentration of 1.5 mM but was inhibited by higher Ca^{2+} concentrations and by Zn^{2+} and Co^{2+}. This suggests that the transporter has a higher affinity for Fe than for other metals (Baker et al. 1998). The use of Ca and Zn^{2+} concentration in this study may be high enough to inhibit both non-CP and CP-stimulated iron uptake in the brain cells.

The trivalent cations, Cr^{3+} and Al^{3+} were also found to inhibit both non-CP-stimulated and CP-stimulated iron uptake in this study. *In vivo* study also showed that iron uptake was reduced significantly in the presence of chromium (Ani and Moshtaghie 1992). In addition, $AlCl_3$ at high concentration can cause an increase in iron uptake by an unknown, possibly non-specific, mechanism (McGregor et al. 1990). In this study, non-Tf iron ($^{59}Fe^{2+}$) was used and the addition of Al^{3+}, like Cr^{3+} inhibited the CP-stimulated iron uptake. In another study by Attieh et al. 1999, the same finding was found in K562 cells which Cr^{3+} and Al^{3+} inhibited CP-stimulated iron uptake. They further addressed that the inhibition by these metals was in a concentration-dependent manner and the effect was not due to inhibition of CP ferroxidase activity. It is likely that these ions may block iron uptake by competing with iron for a binding site on a non-specific trivalent cation-specific transporter. Moreover, CP did not increase ^{51}Cr uptake, thus providing evidence that CP does not

directly activate the transporter. Since divalent cations were also able to inhibit CP-stimulated iron uptake in this glioma cells, it cannot be concluded that CP ferroxidase activity stimulates cellular iron uptake by a trivalent cation-specific transport mechanism. This is mainly due to differences between the use of protocols. For examples, the use of cells (K562 cells vs. BT325 cells), the use of iron (Fe-NTA vs. $^{59}Fe^{2+}$) and the use of incubation temperature (25°C vs. 37°C).

Since trivalent metal ions such as Al^{3+} (100 μM) had been reported to inhibit CP ferroxidase activity (Huber and Frieden 1970), was the inhibitory effect due to inhibition of CP ferroxidase activity by these metal ions in this study? Attieh et al. 1999 has shown that $Al^{3+}$and Cr^{3+} at 10 μM (the same concentration used in this study) did not inhibit CP ferroxidase activity. This raises the suggestion that these metals are transported by the same unknown mechanism.

8.6 CONCLUSION

The results showed that the ferroxidase activity of CP was crucial in stimulating iron uptake of the glioma cell. Moreover, the uptake could be inhibited by both divalent and trivalent cations used in this study suggested they might be transported by the same mechanism. However, what mechanism involved is unknown.

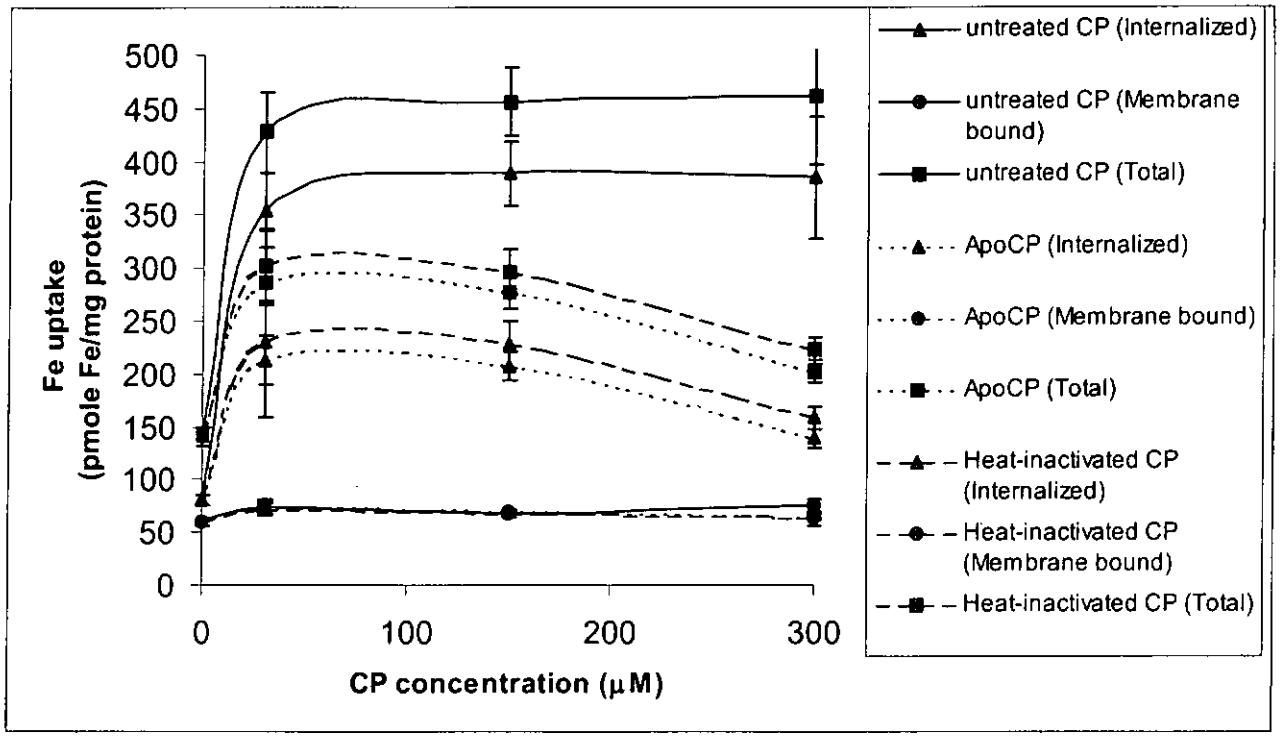

Fig. 18. The role of ferroixdase activity in CP-stimulated iron uptake. The glioma cells were incubated with untreated CP, apoCp, and heat-inactivated CP (0, 30, 150 and 300 μg/ml) and 1 μM of $^{59}Fe^{2+}$ for 30 minutes at 37°C. The radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

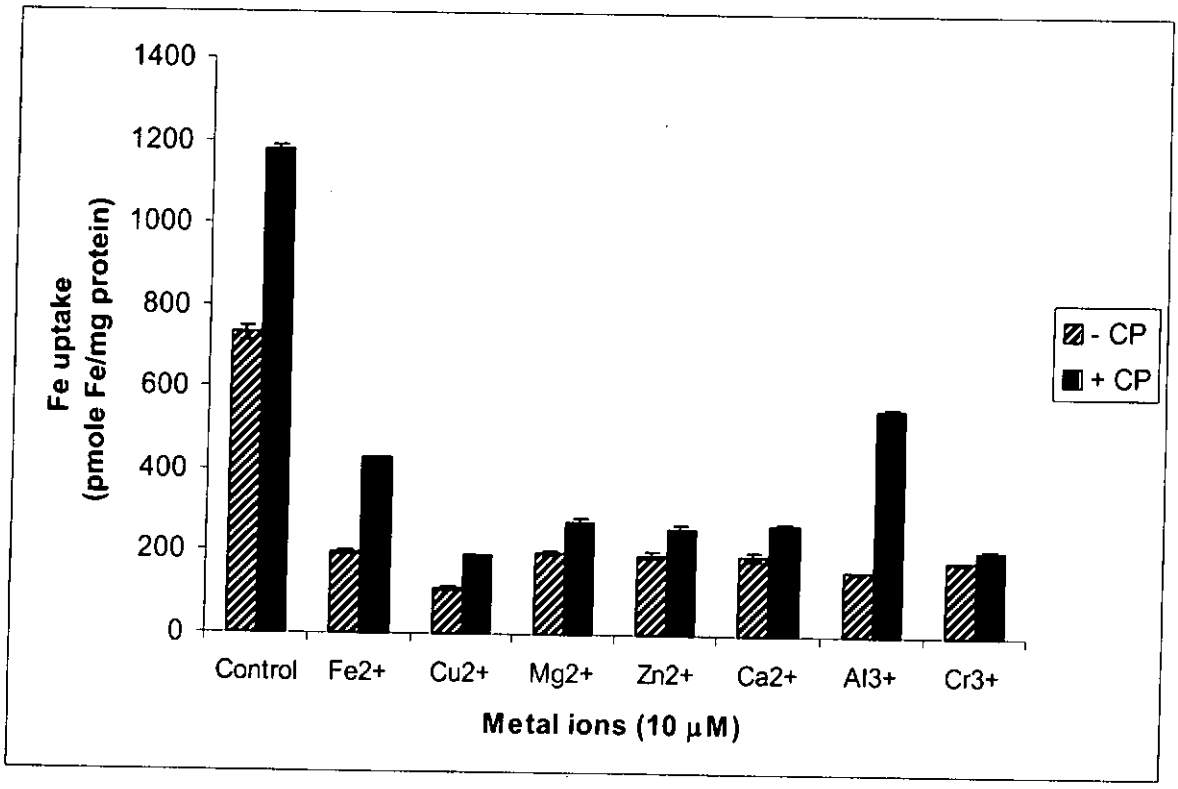

Fig.19. The effect of divalent and trivalent cations on CP-stimulated iron uptake. The brain cells were incubated with 1 μM $^{59}Fe^{2+}$ at 37°C for 30 minutes in the presence (solid bars) or absence (hatched bars) of 30 μg/ml CP, and with 10 μM chloride salts of Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+} and Cr^{3+}. The radioactivities were counted with a γ-counter. Each point is the mean±S.E.M. of three experiments.

Chapter 9

GENERAL DISCUSSION

Neurodegenerative diseases which result in dementia are amongst the most prevalent and devastating illnesses in the population (Harris et al. 1996). From the World Health Organization web site (http://www.who.int), the estimated cases of dementia in the population aged 60 and above are approximately 29 million which are projected to skyrocket to up to 80 million in a few decades from now. Dementia is strongly associated with abandonment, poverty and institutionalization, all of which carry dramatic consequences and concrete burdens for the individuals affected, their families and their communities. An imbalance in brain iron and hence a dysfunction in iron-related metabolism are suspected in some neurological disorders such as PD and AD (Hall et al. 1992; Gerlach et al. 1994; Hu and Connor 1996; Lan and Jiang 1997). Thus, a full elucidation of brain iron metabolism such as what proteins are involved in its regulation is required.

The aim of this research project is to study the role of Ceruloplasmin (CP) in brain iron transport. CP is a protein with controversial functions in the iron metabolism. There are two contradictory hypotheses regarding its functions: the **iron release** (Lee et al. 1968; Osaki and Johnson 1969; Roeser et al. 1970; Osaki et al. 1971; Frieden and Hsieh 1976; Harris 1995; Kaplan 1996; Klomp et al. 1996; Young et al. 1997; Harris et al. 1999; Richardson 1999; Vulpe et al. 1999) versus the **iron uptake**

(Mukhopadhyay et al. 1998; Attieh et al. 1999; Askwith et al. 1994; De Silva et al. 1995; Stearman et al. 1996) functions.

The non-Tf bound iron uptake of cells was studied instead of Tf-bound iron in this research project. It is because of the unique function of CP which oxidizes Fe^{2+} to Fe^{3+}. This reaction was predicted to be preceded before ferric ions attach to Tf (Fig. 1) (Saenko et al. 1994).

In this study, it was found that CP stimulated iron uptake in brain cells instead of iron release. The effect was independent of the iron status (iron-sufficient or iron-deficient) of the cells. These results are in consistent with the findings in HepG2 cells (a human hepatocellular carcinoma line exhibiting Tf-independent and –dependent iron uptake) (Mukhopadhyay et al. 1998) and K562 cells (a human erythroleukemic cell line) (Attieh et al. 1999) except that they have found CP stimulated iron uptake only occurs in iron-deficient cells. Divalent and trivalent cations (Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+} and Cr^{3+}) were shown to inhibit the iron uptake stimulated by CP suggested that these metals may be transported by the same mechanism. In the case of K562 cells, only trivalent cations were able to inhibit the uptake. This lead to the suggestion that CP ferroxidase activity is responsible for stimulating cellular iron uptake in these cells by a trivalent cation-specific transport mechanism (Attieh et al. 1999). The discrepancies may due to differences in the use of cell type and the experimental setup such as the use of incubation temperature. In this study used 37°C versus the nonphysiological temperature 25°C (Mukhopadhyay et al. 1998; Attieh et al. 1999). In another report where iron release was observed in HepG2 cells. The

researcher demonstrated that ^{59}Fe release was dependent on the incubation time, the cell line, and the incubation temperature (Richardson 1999). Furthermore, iron is not likely to exist *in vivo* as those substrates used in those experiments (e.g. ferrous ammonium sulfate or histidine complex).

The use of ferroxidase-defective CPs showed that the ferroxidase activity is essential for stimulation of iron uptake. This result supports the finding in (Atteih et al. 1999). Reduction or lost in CP ferroxidase activity in which oxidizes Fe^{2+} to Fe^{3+} (Kawanami et al. 1996) has been suggested as the cause of several iron-related neurodegenerative diseases (Snaedal et al. 1998; Boll et al. 1999). Some studies have pointed out that only minor quantitative changes of CP occur upon aging in both normal human and human with dementia (Lyngbye and Kroll 1971; Molaschi et al. 1996). Moreover, in AD patients there was loss in excess of one-third of the CP in both gray and white matter from superior temporal gyrus suggested failure in increasing the CP levels in this area Connor et al. 1993). Together with the finding that CP ferroxidase activity is reduced in the CSF of PD patients (Boll et al. 1999). CP ferroxidase is likely to play a crucial role in brain iron metabolism and/or in antioxidant activity. Is the cause of iron disruption in neurodegenerative disorders due to the loss of CP ferroxidase activity? Is this activity important for iron metabolism or for antioxidant effect or both? If there is a loss in CP ferroxidase, is it due to reduction in CP concentration or the disruption in gene expression like those patients with aceruloplasminemia or oxidative modifications upon aging (Musci et al. 1993)? These questions required further investigation.

Another observation made in this study was that the iron stimulatory effect of CP is not species-specific, at least among hCP, rCP and bCP, even though the amino acid sequences (Manolis and Cox 1980 Calabrese et al. 1981; Dooley et al. 1981; Messerschmidt and Huber 1990) and ferroxidase activity levels (Roeser et al. 1970; Ryan et al. 1992) are different in these CPs. However, further proofs of this hypothesis is required like the use of CPs obtained from a boarder ranges of species. In addition, it may worth studying the differences in the effect of CP produced from different tissues. The comparison of the mRNA expression from fetal brain and liver injected into *Xenopus oocytes* showed that a few proteins (Tf and CP) were secreted when liver mRNA was injected, but not when brain mRNA was injected. This suggests that there may be an important difference in the structure and/or processing of these proteins in the brain which may reflect a function different from that associated with them when they originate from the liver (Mollgard et al. 1988). Furthermore, as suggested by Ryan et al 1992 "A comparison of *in vitro* data generated for CP-related studies must be viewed critically before conclusions can be drawn from *in vivo* experimentation on the function and significance of human CP."

In conclusion, the above-mentioned observations suggest that elucidation of the specific role of CP in brain iron metabolism may have broad implications for understanding the mechanisms of neuronal injury in a number of human neurodegenerative diseases (Harris et al. 1996; Takahashi et al. 1996). This may provide a useful framework to advance our knowledge of the pathogenesis of neurodegenerative disease and may permit the development of novel therapeutic approaches to prevent or ameliorate neuronal injury in a variety of human neurologic

diseases associated with oxidative injury (Klomp and Gitlin 1996). CP's role in iron metabolism was noticed only for about 30 years. A lot of questions remain to be answered. For examples, what is the role of GPI-anchored CP which is only found in certain types of cells (Patel and David 1997; Salzer et al. 1998; Fortna et al. 1999). Is the GPI-anchored CP a carrier itself? Are there any iron transporter involved in the process such as DMT1 which can also transport Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}ion (Gunshin et al. 1997)? Moreover, it is important to know should CP fail, can the functions such as copper transport, iron mobilization and antioxidant functions still be performed *in vivo*? Is CP a backup or a front line system? The challenge, therefore, is to determine whether the functions of CP as an antioxidant, ferroxidase, or copper transporter should be labeled essential or supplemental (Saenko et al. 1994).

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ACA:	ε-Aminocaporic Acid
AD:	Alzheimer's Disease
ApoCP:	Apoceruloplasmin
b rat:	*Belgrade* rats
BBB:	Blood Brain Barrier
bCP:	Bovine ceruloplasmin
BP:	Bathophenanthroline disufonic acid
CHO:	Chinese Hamster Ovary
CNS:	Central Nervous System
CP:	Ceruloplasmin
CSF:	Cerebrospinal Fluid
Da:	Dalton
DDC:	*N,N*-Diethyldithiocarbamate
ddH_2O:	Double distilled water
DFO:	Desferoxamine mesylate
DMT1:	Divalent Metal Transporter (previously known as Nramp2 or DCT1)
DPD:	N,N-Dimethyl-p-phenylenediamine
DTPA:	Diethylenetriamine penta-acetic acid
EDTA:	Ethylenediaminetetraacetic acid
FCM:	Flow Cytometry
Fe^{2+}:	Ferrous ion

Fe^{3+}:	Ferric ion
FPLC:	Fast Protein Liquid Chromatography
GABA:	γ-Aminobutyric acid
GPI-anchor:	Glycosylphosphatidylinositol-anchor
hCP:	Human ceruloplasmin
Hepes:	N-2-Hydroxyethylpiperazine-2-ethanesulfonic acid
HVS:	Hallervorden-Spatz syndrome
IRE:	Iron Responsive Element
IRPs:	Iron Regulatory Proteins
LEC rat:	Long-Evans Cinnamon rat
LfR:	Lactoferrin Receptor
mk mice:	Mice with the *mk* mutation
MRI:	Magnetic Resonance Imaging
MTf:	Melanotransferrin
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide
Nramp:	Natural Resistance Associated Macrophage Protein
PB:	Phosphate Buffer
PBC:	Primary Biliary Cirrhosis
PBS:	Phosphate Buffered Saline
PD:	Parkinson's Disease
Pipes:	1,4-Piperazinediethanesulphonic acid
PLM:	Percent Labeled Mitoses
PPD:	*p*-Phenylenediamine

PTAH:	Phosphotungistic acid haematoxylin
rCP:	Rat ceruloplasmin
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFT:	Stimulator of Fe Transport
Tf:	Transferrin
TfR:	Transferrin receptors

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