2.3.13 Normal copper metabolism and lowering copper to subnormal levels for therapeutic purposes

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Introduction

In this chapter, we will first provide a review of current knowledge about copper metabolism. Copper is an essential trace element, and the normal diet contains an average of about 1.0 mg. This is about 25% more than is required, and most of the excess is normally excreted by the liver into the bile for loss in the stool. Hence, the liver is important in regulating copper balance and other aspects of metabolism. Excellent progress has been made in understanding copper metabolism in the body, thanks in part to discoveries of the genes that cause two copper-related diseases, ATP7A for Menkes’ disease and ATP7B for Wilson’s disease (see Chapter 16.1). Progress has also been helped by the elucidation of the roles of copper chaperones, evolutionarily conserved genes whose protein products facilitate transfer of copper to target proteins or vesicles. In the second part of the chapter, a new area involving copper will be reviewed, that of the therapeutic use of lowering copper to subnormal levels to treat cancer and diseases of inflammation and fibrosis.

Copper metabolism and its role in health and disease

Introduction

The essentiality of copper in human health has been recognized for more than 70 years. Severe copper deficiency, whether genetic or acquired, can produce devastating disease and death. The toxic properties of copper were brought to the forefront of scientific attention when a disease described by Wilson in the early 1900s called ‘hepatolenticular degeneration’ was later discovered to be due to copper accumulation and toxicity [1]. The liver plays a prominent role in copper distribution to organs and regulates overall system homeostasis. Bile, not urine, for eventual loss in the stool, is the major excretory route for copper. Normal urine copper loss is 20–50 µg/day, whereas stool copper loss is in the order of 1.0 mg/day. Transport through the blood to the absorption surfaces of cells has yet to be clarified with certainty. Transport through the membrane and into cytosolic proteins, however, is becoming better understood [2]. The discovery of two structurally related membrane-bound Cu-ATPases, ATP7A and ATP7B, defective in Menkes’ and Wilson’s diseases, respectively, has provided insight into intracellular copper movement and control of its excretion from the cell. These discoveries have also provided unprecedented biochemical insights into diseases of copper metabolism and have formed the basis upon which much of the current theories of cellular copper movement and homeostasis rest.
2.3 METABOLISM

Chemical properties and copper toxicity

Because copper is a redox metal, unfettered copper is a potential oxidant (or reductant) of cellular proteins, lipids and nucleic acids. This property mandates copper to be in a bound form and not a free ion in blood, extracellular fluids or cytosol. Of its two major valencies, Cu(I) and Cu(II), Cu(I) behaves as a progenitor of free radicals. As a $3d^{10}$ metal ion, Cu(I) also has the potential to antagonize Zn(II). Protein-bound forms of copper are generally responsible for targeted transport to functional biomolecules. The metallothioneins, a family of sulphydryl-rich metal-binding proteins, limit the buildup of mobile copper in cells and work with excretory systems to maintain a steady-state level in the cytosol. Metallothionein synthesis de novo is prompted by sudden influxes of copper and other metals into the cell, which clearly demonstrates a genetic-level response system to prevent buildup of copper and other toxic metals in cells and tissues [3]. The protective effects of metallothionein are overwhelmed over time, however, when systems designed to transport copper into bile or release copper from the cell fail to function because of genetic mutations.

The role of the liver in copper homeostasis

The prevailing understanding is that copper absorption occurs mostly at the intestinal mucosa, and copper utilization culminates with the incorporation of Cu(I) and Cu(II) ions into cellular enzymes or storage proteins. The liver intervenes as a major hub for copper distribution to peripheral organs or excretion in the bile (Fig. 1). Dietary copper entering the liver from the portal circulation passes into the liver parenchymal cells after being handed off from binding sites primarily on serum albumin. Transport through the membranes will be discussed below. Upon entering the hepatocytes, copper is met by glutathione (GSH) and sequestered. Another series of proteins, the copper chaperones (Table 1), direct copper to intracellular enzymes or organelles. When bound to chaperones, copper is then positioned to be incorporated directly into either enzymes such as copper/zinc superoxide dismutase [4] or vesicles that represent intracellular compartments along the secretory pathway [5]. The intracellular compartments represent staging areas for incorporating copper into ceruloplasmin or into biliary canaliculi. For example, ATP7B, a Cu-ATPase that is defective in Wilson’s disease, is positioned strategically to receive copper from ATOX1 (formerly HAH1), a copper chaperone, and excrete copper from liver via the biliary canaliculi [6]. Precisely how ATP7B governs the movement of copper into the bile is unknown but, clearly, an impairment in that step shifts copper homeostasis towards a failure of biliary excretion and the amassing over time of large amounts of copper in the liver and the rest of the body. Thus, a fault in both copies of the ATP7B gene prevents biliary copper excretion and holo-ceruloplasmin biosynthesis, which are the hallmarks of Wilson’s disease.
**2 FUNCTIONS OF THE LIVER**

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<tr>
<th>Organism</th>
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<tr>
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<tr>
<td>Human orthologue of ATX1 (formerly HAH1)</td>
<td>ATP7A, ATP7B</td>
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**Transporters**

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<td>Yeast</td>
<td>Membrane copper transport</td>
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<| Human orthologue of Ctr1p | Membrane copper transport | [7] |

**Transmembrane movement and the copper chaperones**

Gaining access to a cell requires movement across the membrane. hCTR1, a membrane copper transporter in humans (Table 1), has a counterpart in the yeast, Ctr1p, and it is through studies of the yeast protein that many of the properties of copper transporters in humans have become known [7]. Based on these studies, one can surmise that hCtr1 is selective only for Cu(I). This observation necessitates a copper reductase enzyme that changes Cu(II) to Cu(I) to coordinate with transmembrane movement. Precisely how the Cu(I) ions move through the hCTR1 portal is unknown, but recent data suggest that a mechanism involving endocytosis of the carrier is an option [8,9].

Once inside the cell, the metabolic fate of copper relies on copper chaperones, as introduced in the previous section. Chaperones are copper-binding proteins that perform two important functions: (i) sequestering copper to prevent free metal-catalysed oxidations; and (ii) recognizing and conducting copper transfer to intracellular proteins or vesicles. Ions generally rely on the mass action principle to drive the movement of freely diffusible forms. Because free copper concentration is negligible in a cell, it cannot drive reactions and, because of the demand of the many different proteins that require copper for function, intracellular movement relies on copper-binding transport proteins to target specific proteins [10]. A chaperone-bound copper in transit is able to exchange with a target protein, such as enzymes or other proteins, or a membrane-bound Cu-ATPase. Copper chaperones emulate the copper sites on the target protein, thus facilitating the transprotein transfer of copper from chaperone to target [11]. At the present time, several such chaperones are known (Table 1). CCS (copper chaperone for superoxide dismutase) delivers copper to a major antioxidant enzyme in the cell [12]. COX17 is required to move copper into the mitochondria for the binding and assembly of cytochrome c oxidase [13] and is essential in embryonic development [14]. ATOX1 mediates the entrance of copper into the secretory pathway through ATP7A and ATP7B, which drive an energy-dependent relocation of copper into vesicles [15].

**Cu-ATPases in absorption and cellular homeostasis**

There are two phases to intracellular copper transport and movement; these are soluble and vesicle associated. The soluble phase includes components that provide copper to cytosolic enzymes; the vesicle phase is believed to be part of the trans-Golgi network for excreting copper from cells. As noted above, two different but functionally similar Cu-ATPases, ATP7A and ATP7B (Table 2), provide the entrance. Wilson’s and Menkes’ diseases have roots in genetic impairments in ATP7B and ATP7A respectively. ATP7A is found in practically every cell tested, with the exception of adult hepatocytes [16]. ATP7B is prominent in liver, kidney and brain and plays less of a role in other organs [17]. Vesicles laden with ATP7A or ATP7B are mobile and transverse the space between the Golgi and cell surfaces to release copper as part of intestinal absorption or into the biliary canaliculi at the apical surface of hepatocytes. Vesicles that contain ATP7A provide copper to enzymes such as tyrosinase (pigmentation) [18], lysyl oxidase (connective tissue integrity) [19], dopamine-β-monooxygenase (neurotransmitter biosynthesis) [20] or peptidylglycine α-amidating monoxygenase (PAM, neuropeptide hormone biosynthesis; Table 3) [21]. Enhancing the concentration of extracellular copper instigates movement of vesicles towards the plasma membrane [22]. Movement and direction are a function of residues in the N-terminal, copper-binding domain of ATP7A. An exposed dileucine signal at the C-terminal is believed to control

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<td>ATP7B</td>
<td>Causes Wilson’s disease</td>
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<td>Murr1</td>
<td>Causes canine copper toxicosis in Bedlington terriers</td>
<td>[25]</td>
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<td>XIAP</td>
<td>None known – interacts with Murr1</td>
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localization of vesicles to the trans-Golgi network [23]. Connecting ATP7A and ATP7B, two functionally similar proteins, with two dissimilar diseases has given invaluable insight into pathways of copper movement and homeostasis. Identifying ATP7B with Wilson’s disease is a clear indication that ATP7B (i) incorporates copper into ceruloplasmin for excretion into the plasma and (ii) delivers copper to the apical environment of the liver cell. Identifying ATP7A with Menkes’ disease tags this Cu-ATPase as an indispensable factor in the absorption of copper across the intestine as well as movement across the blood–brain barrier [24] and into other cells such as the kidney. Moreover, connecting ATP7A-laden vesicles with copper incorporation into apo-tyrosinase, apo-lysyl oxidase and apo-PAM gives a molecular understanding of the phenotypes that are seen in experimental copper deficiencies and inborn genetic diseases, such as Menkes’ disease, and age-related impairments in copper metabolism.

**Future directions**

Several recent discoveries could have an important impact on our understanding of copper metabolism and disease. Murr1 (Table 2) is the name given to a factor that is defective in canines that amass liver copper [25]. The discovery of Murr1 has resolved a mystery as to why Bedlington terriers and some other canine breeds have a Wilson-like copper toxicosis yet, unlike Wilson’s disease, have a perfectly functional ATP7B. Connecting ATP7A with Menkes’ disease tags this Cu-ATPase as an indispensable factor in the absorption of copper across the intestine as well as movement across the blood–brain barrier [24] and into other cells such as the kidney. Moreover, connecting ATP7A-laden vesicles with copper incorporation into apo-tyrosinase, apo-lysyl oxidase and apo-PAM gives a molecular understanding of the phenotypes that are seen in experimental copper deficiencies and inborn genetic diseases, such as Menkes’ disease, and age-related impairments in copper metabolism.

**Lowering copper to subnormal levels for therapeutic purposes**

**Introduction**

In the past, lowering copper levels for therapeutic purposes was used primarily for the treatment of Wilson’s disease, a disease of
copper accumulation and copper toxicity. Wilson’s disease is covered in Chapter 16.1, and here therefore, we are not referring to Wilson’s disease, but to a series of disease conditions that now appear to be potentially treatable by lowering copper levels. Copper levels are essentially normal in these diseases to begin with, but are lowered by therapy to a midrange, not so low as to cause clinical copper deficiency, but low enough to inhibit certain processes involved in specific diseases (for reviews see [31–33]). These processes are angiogenesis, fibrosis and inflammation, and the diseases are cancer, diseases of excessive fibrosis and diseases of excessive inflammation.

Copper and angiogenesis

It is now well established that angiogenesis is required for cancer growth and progression [34,35], and that antiangiogenic therapies are a valid approach to cancer treatment [36]. The essential role of copper in angiogenesis is not so well known, but nonetheless the research in this area dates back two decades [37,38]. It was shown that mild copper deficiency in rabbits, produced by penicillamine and a low copper diet, reduced the angiogenic response in the cornea to known angiogenic stimulants [38]. Tumours explanted to brains of rats and rabbits made copper deficient by this same approach grew more slowly and failed to show vascular invasion of normal tissue, compared with tumours explanted into control rabbits [39,40].

Most of the antiangiogenic, anticancer work that has been done using the lowered copper level approach, subsequent to the work mentioned above, has been done using the anticooper drug, tetrahexomolybdate (TM), originally introduced for the treatment of Wilson’s disease [41]. This drug has also been used for the antifibrotic and anti-inflammatory studies discussed later in the chapter. The only significant toxicity of copper-lowering therapy with TM in these diseases is overtreatment. The mild copper deficiency produced from overtreatment causes mild bone marrow suppression with anaemia and/or leucopenia, easily corrected by decreasing the dose of TM.

Lowering copper levels with TM for cancer therapy

Preclinical studies

Numerous preclinical studies have established the efficacy of TM in preventing the growth of various cancers in mice. One study used transgenic mice (Her-2/neu) genetically programmed to develop mammary cancer during the first year of life [42]. TM given daily by oral gavage beginning on day 100 completely prevented the development of visible mammary tumours, while most of the controls had developed gross tumours by day 270 \( (P < 0.01) \). The study was stopped, and a few TM-treated mice were autopsied. Histological sections of the breasts in these mice revealed small, avascular clusters of tumour cells. A few TM-treated mice were followed after stopping TM, and they all developed gross mammary tumours.

Another study used xenografts of SUM149 inflammatory breast cancer cells in mice, and TM markedly inhibited tumour growth and caused reduced microvessel density compared with controls [42]. In another study, Dunning prostate cancer cells were injected into nude mice [43]. TM treatment retarded tumour growth. Combination therapy with TM and the PHSCN peptide sequence, a competitive inhibitor of a fibronectin sequence, improved survival and the number of metastatic lesions.

In a lung cancer model study in mice, Lewis lung high metastatic cancer cells were injected into the upper leg [44]. Radiation therapy significantly slowed tumour growth, TM therapy also significantly slowed tumour growth, and the combination was additive in slowing tumour growth. The effect of TM in squamous cell carcinoma cells was evaluated in another mouse study and showed dramatic inhibition of tumour growth [45]. A combination study with doxorubicin against the SUM149 inflammatory breast cancer cells injected into nude mice was reported [46]. TM or doxorubicin alone significantly inhibited tumour growth, while the combination completely prevented tumour growth. Apoptosis of tumour cells was enhanced in all treatment groups, but especially in the combination therapy group.

A study of TM therapy was carried out in dogs affected with a variety of advanced and metastatic cancers [47]. Nine dogs in this 13-dog study were evaluable. Three of the nine had relatively prolonged periods of disease stabilization, and a fourth dog, with metastatic osteosarcoma, had a relatively prolonged partial remission.

Clinical studies

A phase I/II study of 42 patients with advanced and metastatic cancer was carried out [48]. Eighteen patients were evaluable. Freedom from progression averaged 11 months, much longer than the 1–2 months expected in this type of patient if they were on no treatment. Quality of life was stabilized after steady deterioration in the period prior to treatment. Particularly positive results were seen in a few patients. One patient, with metastatic chondrosarcoma, has survived 5 years with what appears to be a complete remission. Using a blood flow-sensitive ultrasound technique, it was possible to show drastic blood flow reduction in a metastasis as a result of TM therapy in a patient with metastatic renal cancer.

Nine phase II trials of TM therapy in specific advanced cancers have been initiated. One, on renal cancer, has been completed and published [49]. Four out of nine evaluable patients had at least 6 months of disease stabilization, but results overall were not strongly positive. Preliminary results in other trials, such as mesothelioma and hepatocellular cancer, are more encouraging.

Mechanism of action

The anticancer mechanism of action almost certainly involves reducing copper availability. This conclusion is based in part on in vitro studies in which TM inhibition of endothelial cell tube
formation, under the influence of fibroblastic growth factor, was abrogated by copper supplementation [42]. Second, anticancer effects have been obtained with two different anticopper drugs, TM as reviewed above and penicillamine as studied by Brem et al. [39,40]. These two drugs both lower copper availability, but through completely different mechanisms of action.

A further conclusion is that the anticancer mechanism of action of lowering copper levels is almost certainly due to anti-angiogenic effects. In vitro studies of TM action have shown inhibition of numerous angiogenic cytokines (vascular endothelial growth factor, fibroblastic growth factor, interleukin-6, nuclear factor kappa B (NF-kB), and interleukin-8) [42]. In vivo studies during TM therapy have also shown inhibition of angiogenic cytokines, including NF-kB [50]. Both clinical and preclinical studies have shown inhibition of blood flow or decreased microvessel density as a result of TM therapy [48]. Numerous angiogenic cytokines have been shown to bind copper or to be copper dependent [33].

Lowering copper levels with TM for antifibrotic therapy

The pathway for fibrosis involves activation of transforming growth factor beta (TGF-β), a cytokine that activates connective tissue growth factor (CTGF), which in turn activates numerous genes involved in fibrosis (see Section 6, Cirrhosis). Excessive activation of this pathway is believed to cause fibrotic disease, such as pulmonary fibrosis, cirrhosis of various types, interstitial renal fibrosis (the endstage of many types of kidney disease) and scleroderma, to name but a few [51]. It has been hypothesized that this pathway is copper dependent, based upon one of the activators of TGF-β being SPARC (secreted protein acidic and rich in cysteine), known to be copper dependent, and the high cysteine content of CTGF, often an indicator of copper binding [33].

The copper dependence of fibrosis was tested in the bleomycin mouse model of pulmonary fibrosis. TM therapy almost completely prevented the pulmonary fibrosis from bleomycin [52]. TGF-β protein levels were elevated in the lungs of bleomycin-treated animals, and this elevation was almost completely prevented by TM [53]. Protection by TM against fibrotic disease was also tested in the carbon tetrachloride mouse model of cirrhosis [54]. After 12 weeks of carbon tetrachloride injections, untreated mice developed severe fibrosis. Fibrosis from carbon tetrachloride injections was prevented by TM therapy. Serum TGF-β levels were elevated by carbon tetrachloride, and this elevation was almost completely prevented by TM. The protection of TM against excessive fibrosis seems to be independent of any effect of TM on preventing inflammation [52–54].

Lowering copper levels with TM for anti-inflammatory therapy

It was noted in the bleomycin studies that TM therapy inhibited tumour necrosis factor (TNF)α messenger levels in the lungs of bleomycin-treated animals at 7 days compared with bleomycin-treated controls [53]. This led to a series of four studies evaluating TM therapy in inflammation models. In all four cases, TM therapy strongly inhibited the inflammatory responses [54,55]. These models are concanavalin A-induced hepatitis, paracetamol (acetaminophen)-induced hepatitis, Adriamycin-induced cardiac inflammation and injury, and lupus adenopathy in the lpr mouse model. In most of these models, concomitant with suppression by TM of markers of inflammation and injury, inhibition of serum TNFα and/or another inflammatory cytokine, interleukin-1-beta (IL-1β), was shown when toxin- or disease-challenged animals were compared with the same animals receiving TM therapy. Inhibition of these cytokines seems to reduce inflammation, as shown by the efficacy of TNFα antibodies in various inflammatory diseases [56–59]. The mechanism by which TM inhibits these cytokines is not known. One possibility is that suppression of IL-2 release by activated T lymphocytes, which has been shown to result from TM therapy, results in less activation of inflammatory cells and therefore less release of TNFα and IL-1β by these cells.

Comment on prospects for antifibrotic and anti-inflammatory therapy with TM

Only clinical trials will determine whether copper-lowering therapy with TM will have clinical efficacy in fibrotic and inflammatory diseases is clinical trials. This approach is further advanced with fibrotic than with inflammatory diseases. Clinical trials are either under way, or under active planning, for idiopathic pulmonary fibrosis, primary biliary cirrhosis and scleroderma. There is essentially no effective treatment for fibrotic diseases such as these. Regarding inflammatory diseases, much of modern medicine deals with the discomfort and additional injury produced by inflammation in a large variety of diseases, many of them autoimmune. Therapeutic agents, such as steroids, non-steroidal anti-inflammatory drugs (NSAIDs), chemotherapeutic drugs and antibodies against specific inflammatory proteins (such as TNFα), have some efficacy. However, efficacy is only partial, and myriads of patients have continuing discomfort and tissue injury in spite of the best treatment. Time will tell whether copper-lowering therapy with TM can be of value against these diseases.

Summary

In this chapter we have first reviewed and summarized the exciting progress in understanding normal copper metabolism. The discovery that genetic defects in ATP7A and ATP7B cause Menkes’ and Wilson’s diseases, respectively, has not only provided a molecular basis for these diseases, but has also allowed an increasingly detailed understanding of copper transport and utilization in the body. The discoveries of numerous chaperones for moving copper between specific molecules has not only provided insight into mechanisms of copper movement, but also
underscores two principles about copper. One is that free copper is very toxic and simple diffusion and molecular uptake is not permissible. The second is the extreme evolutionary conservatism of copper-handling molecules, which go back in evolution at least as far as yeast.

Second, we have reviewed a new area for the use of anticopper drugs (summarized in Table 4). For a very long time, anticopper drugs have had one primary clinical use, the treatment of copper toxicity from excessive accumulation of copper such as in Wilson disease, canine copper toxicosis or copper-poisoned sheep. Here, we introduce the concept that lowering copper availability to a midrange can have therapeutic efficacy in a wide array of diseases.

### References


2.3.14 Trace elements and the liver

Brent A. Neuschwander-Tetri

Life would not be possible without a large number of ‘trace’ elements, each serving critical roles in metabolism and function (Table 1). This chapter reviews the function and relevant biology of trace elements with respect to the liver. Because copper has a special role as a trace element with respect to normal liver function and liver disease, it is covered in more detail in Chapter 2.3.13 and is not discussed further here.

Trace elements are necessary for normal function and are therefore associated with morbidity deficiencies states. They are also commonly toxic when present in excess, and this chapter will touch briefly on toxicity as it relates to the essential trace elements. Actual values for liver, plasma and total body content of the trace elements that are used to assess deficiency states and toxicity are catalogued in other resources and are not reviewed here [1–4]. Although such values are important in forensic medicine and epidemiological studies, they are rarely relevant to the routine practice of clinical medicine [5].