Review

Cancer Research

Role of the Human High-Affinity Copper Transporter in Copper Homeostasis Regulation and Cisplatin Sensitivity in Cancer Chemotherapy

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Abstract

The high-affinity copper transporter (Ctr1; SCLC31A1) plays an important role in regulating copper homeostasis because copper is an essential micronutrient and copper deficiency is detrimental to many important cellular functions, but excess copper is toxic. Recent research has revealed that human copper homeostasis is tightly controlled by interregulatory circuitry involving copper, Sp1, and human (hCtr1). This circuitry uses Sp1 transcription factor as a copper sensor in modulating hCtr1 expression, which in turn controls cellular copper and Sp1 levels in a 3-way mutual regulatory loop. Posttranslational regulation of hCtr1 expression by copper stresses has also been described in the literature. Because hCtr1 can also transport platinum drugs, this finding underscores the important role of hCtr1 in platinum-drug sensitivity in cancer chemotherapy. Consistent with this notion is the finding that elevated hCtr1 expression was associated with favorable treatment outcomes in cisplatin-based cancer chemotherapy. Moreover, cultured cell studies showed that elevated hCtr1 expression can be induced by depleting cellular copper levels, resulting in enhanced cisplatin uptake and its cell-killing activity. A phase I clinical trial using a combination of trientine (a copper chelator) and carboplatin has been carried out with encouraging results. This review discusses new insights into the role of hCtr1 in regulating copper homeostasis and explains how modulating cellular copper availability could influence treatment efficacy in platinum-based cancer chemotherapy through hCtr1 regulation. *Cancer Res; 72(18); 4616–21. ©2012 AACR.*

Introduction

Platinum–based antitumor agents, including cisplatin (cDDP), carboplatin (CBDCA), and oxaliplatin (L-OHP), have been used for treating human solid tumors for more than 3 decades, especially testicular and ovarian cancers, for which high response rates are attainable. Drug resistance is an important obstacle to achieving maximal therapeutic efficacy of these drugs. Overcoming drug resistance is very important for improving treatment outcomes, because once drug resistance has developed, other effective treatment options are limited. No effective strategy for combating cDDP resistance in cancer chemotherapy is currently available.

Mechanisms of cDDP resistance are complex. Here, we focus on a drug transport–associated resistance. This resistance mechanism has gained substantial attention after the discovery that high-affinity copper transporter (Ctr1; hCtr1 for humans) is the major transporter for cDDP (1). In this review, we discuss some inconsistencies published in the literature about mechanisms of hCtr1 regulation in copper homeostasis maintenance that are relevant to platinum-drug sensitivity.

It has been well established that the primary target of antitumor platinum drugs is DNA, by the formation of intra- and interstranded DNA-drug adducts. Many previous reports have shown that cDDP cytotoxicity is correlated with the amount of DNA adducts, which is proportional to the total platinum content inside the cells. These findings underscore the importance of the transport system in platinum-drug cancer chemotherapy. These studies indicated that cDDP enters cells by means of passive diffusion, driven by concentration gradients or by the endocytotic pathway (see reviews in refs. 2, 3). The discovery of hCtr1 as a platinum-drug transporter is intriguing because the physiologic and chemical properties in copper and cDDP are different (controversy number 1). However, a recent study using the site-specific mutagenization approach showed that amino acid residues involved in hCtr1-mediated cDDP transport are generally also required for copper transport, although differences in transport kinetics (*Km* and V_{max} values) exist (4). There are also reports that hCtr1 copy numbers in genetically engineered cell lines were not proportionally correlated with rates of copper and cDDP uptake (5, 6), despite many studies showing that elevated expression of hCtr1 confers cDDP sensitivity (7, 8). However, these genetically engineered cell lines were generated by transfection with recombinant *hCtr1* cDNA. Whether

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all of the transfected *hCtr1* is functional is not known, in light of our new understanding of the hCtr1 regulation mechanism, which is constrained within the context of overall copper homeostasis regulation (see below).

The importance of Ctr1 in cDDP transport overall can be found in *Ctr1*-knockout mouse embryonic fibroblast $Ctr1^{-/-}$ cells. These *Ctr1*-null cells display only 30% to 35% of residual cDDP transport activity in reference to the $Ctr1^{+/+}$ counterpart (9). This study showed that hCtr1 can also transport CBDCA with reduced efficiency and that L-OHP is a poor substrate for hCtr1 (9).

The clinical relevance of hCtr1 expression and platinum-drug cancer chemotherapy was reported by Ishida and colleagues (10). These investigators analyzed an array-based hCtr1 expression data set consisting of 91 patients with ovarian cancer who had been treated with a platinum drug and a taxane and found a correlation between high hCtr1 expression levels and longer disease-free survival times. We analyzed an independent gene expression database published by Tothill and colleagues (11), derived from 285 serous and endometrioid tumors of the ovary, peritoneum, and fallopian tube, and found that patients with elevated hCtr1 expression in their tumors had more favorable outcomes after platinum-drug treatment than did those expressing low hCtr1 levels (12). It is important to emphasize that this correlation was at the hCtr1 mRNA expression level. A similar correlation was found in platinum-based therapy for lung cancer (13).

Importantly, recent studies have shown that levels of hCtr1 expression can be modulated by manipulating the cellular bioavailable copper pool (14–16). These findings have resulted in a phase I clinical trial in advanced ovarian cancer chemotherapy at The University of Texas MD Anderson Cancer Center that uses a copper chelator (trientine) as an enhancer for CBDCA treatment (17).

Key Finding: Copper Chelation Induces hCtr1 Expression and Chemosensitization to cDDP Treatment

The conceptual development of using copper chelators as chemosensitizers for platinum-drug treatment evolved from our previous study using cell lines overproducing glutathione (GSH), by transfection with recombinant DNA encoding the heavy subunit of γ-glutamylcysteine synthetase (γ-GCSh), a rate-limiting enzyme for *de novo* biosynthesis of GSH. These γ-GCSh/GSH–overproducing cells exhibited elevated hCtr1 expression and increased sensitivity to cDDP treatment. These transfected cell lines exhibited reduced cellular bioavailable copper content, as evidenced by the reduced activities of several copper-containing enzymes, because GSH is an abundant physiologic copper chelator (14). These phenotypes were reversed when the elevated GSH levels were reduced. This finding was contradictory to the vast amount of previously published results showing that induction of γ-GCSh/GSH expression was responsible for cDDP resistance, because GSH is an important cytoprotector (inconsistency number 2). However, it is important to note that all these reports of cDDP-resistant cells were published before the discovery that hCtr1 is a cDDP transporter and that recent results have shown that most, if not all, cDDP-resistant variants contain various degrees of reduced hCtr1 levels (7). Elevated expression of γ-GCSh/GSH levels in these cDDP-resistant cells could be explained as being due to the oxidative stress-induced mechanism during drug treatment, because GSH is an important ROS sensor and/or regulator. Consistent with this notion are the observations that elevated γ-GCSh/GSH expression can be found in cells treated with other prooxidants, including cytokines, antitumor agents, and carcinogens; but no enhanced cDDP resistance was associated with these treatments (see review in ref. 18 and references therein). Induction of hCtr1 expression by copper-lowering agents was further confirmed with the use of additional copper-lowering agents (15, 16).

The Role of hCtr1 in the Regulation of Copper Homeostasis in Cultured Cells

To understand how copper chelation induces hCtr1 expression, it is important to elucidate how mammalian copper homeostasis is regulated. Mechanisms of copper homeostasis maintenance are evolutionarily conserved from yeasts, fruit flies, and plants, to humans. Steady-state cellular copper levels are maintained by a balance among copper transporters (Ctr1), copper chaperones (Atox1 for intracellular distribution), copper storage (Ctr2), and copper exporters (ATP7A and ATP7B). All of these transporters can also transport cDDP (7). However, Ctr1 is an important regulator in response to environmental copper stresses.

We recently showed that copper deficiency induced by treating human cancer cells with copper chelators, or by expressing a dominant-negative *hCtr1* recombinant, upregulates *hCtr1* expression, whereas copper sufficiency achieved by treating cells with $CuSO_4$, or by overexpressing the wild-type *hCtr1* recombinant, downregulates endogenous *hCtr1* expression (4, 15, 16). The upregulation or downregulation of hCtr1 under these 2 different copper-stressed conditions is respectively, controlled by Sp1 binding to or dissociating from the GC boxes located at the *hCtr1* promoter. Not only hCtr1, but also Sp1 itself, is regulated by copper bioavailability (Fig. 1A). Thus, human copper homeostasis is regulated by the Cu-Sp1-hCtr1 interregulatory loop (Fig. 1B). This regulatory loop uses Sp1 oscillation in regulating hCtr1 expression to rebalance the cellular copper budget (15). The sensing mechanism of copper bioavailability by Sp1 lies on the zinc finger (ZF) domains located at the C terminus of Sp1. Copper stress–induced transcription regulation of *Ctr1* in other organisms also uses ZF motifs in the transcription regulators as sensors in response to copper inadequacies (19).

Posttranslational regulation mechanisms have also been reported for regulating hCtr1 by copper stresses. High copper levels induce trafficking of hCtr1 from the plasma membrane to the endosomal and/or lysosomal compartments, in which hCtr1 may (20) or may not (21) be degraded. cDDP-induced internalization of hCtr1 was also reported (22), but in another report, cDDP treatment did not internalize hCtr1, but rather induced hCtr1 stabilization through protein cross-linking (23).

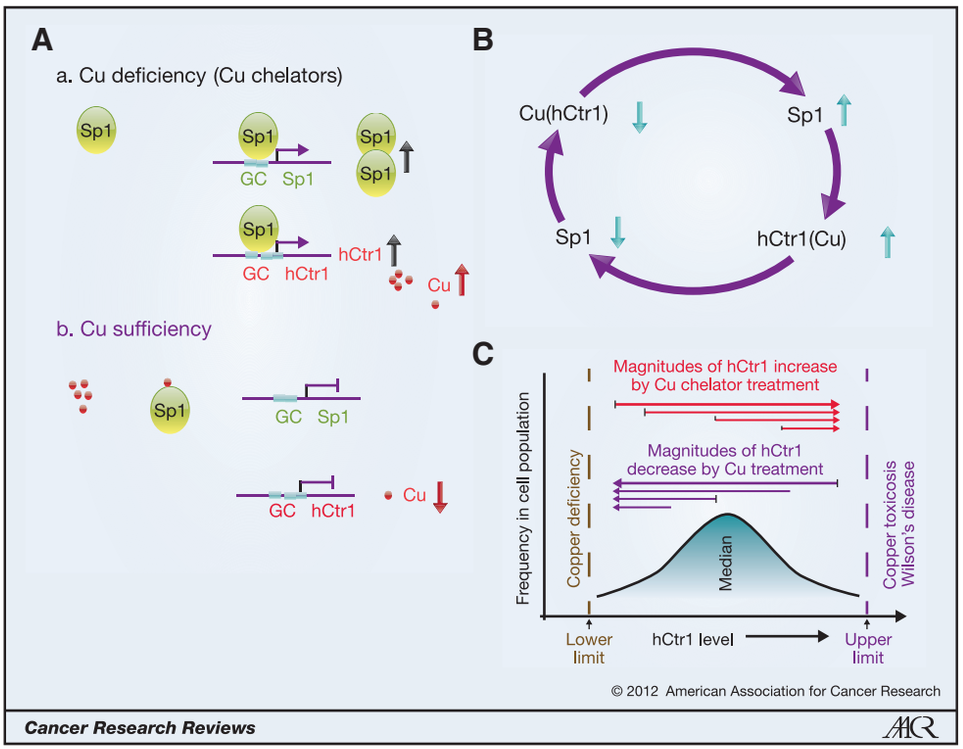

Figure 1. Regulation of hCtr1 and Sp1 expression by copper (Cu) bioavailability. A, upregulation of Sp1 and hCtr1 expression under copper deficiency by enhanced promoter binding of Sp1 to these genes (a); copper sufficiency prevents Sp1 binding to these promoters (b). B, the Cu-Sp1-hCtr1 interregulatory loop for copper homeostasis regulation. Reducing the copper levels (downward arrow), either by treating cells with copper chelators or by expressing the *hCtr1* dominant-negative recombinant, upregulates Sp1 and hCtr1 expression (upward arrows); likewise, increasing the copper levels, either by treating cells with $CuSO_4$ or by transfection with wild-type *hCtr1* cDNA, downregulates Sp1 and hCtr1 expression. C, magnitudes of hCtr1 regulation by copper bioavailabilities depend upon the intrinsic hCtr1 levels. Low hCtr1-expressing cells exhibit greater magnitudes of hCtr1 induction than do high hCtr1-expressing cells by copper chelator (red lines); likewise, greater reduction in hCtr1 induction was observed in high hCtr1-expressing cells by copper treatment than in low hCtr1-expressing cells (purple lines).

Although the source for this inconsistency (number 3) is not certain, it is most likely due to different anti-hCtr1 antibodies used in these studies, because Ctr1 is an evolutionarily conserved protein and a good anti-hCtr1 antibody has been difficult to obtain. Anti-hCtr1 antibodies prepared from different laboratories often showed different antigen recognition patterns by Western blotting. Even the "monomeric hCtr1" detected by these antibodies often showed different molecular masses. In addition, there is a report that copper bioavailability also regulates hCtr1 protein stability (24). Regardless, the mechanistic insight into these posttranslational regulation mechanisms requires further elucidation, particularly from the copper homeostasis point of view.

This Cu-Sp1-hCtr1 interregulatory mechanism suggests the following scenarios in copper homeostasis control: This regulatory mechanism is dynamic: changes in 1 component would feed forward to affect levels of the other 2, resulting in a new equilibrium. This dynamic regulation mechanism may explain why the magnitudes of hCtr1 and Sp1 regulated by copper stresses are small (most often within 2-fold) in most cell settings and may often be disregarded as being within normal experimental variations. This scenario underscores the importance of using qualitatively and quantitatively reliable measurement methods, such as isoform-specific *hCtr* probes in the RNase protection assay (15, 16). This regulation loop is tightly controlled, indicating that there are maximal and minimal ranges in which each of these components can swing. This scenario suggests that the magnitudes of hCtr1 regulation depend on the intrinsic (basal) hCtr1 levels and that cells with high levels of hCtr1 expression would have greater magnitudes of hCtr1 downregulation than would those expressing reduced levels of hCtr1 when exposed to copper. Likewise, cells expressing reduced levels of hCtr1 would have a greater amplitude of hCtr1 upregulation by copper chelators than would those expressing higher levels (Fig. 1C). This second scenario is particularly relevant to platinum-based chemotherapy, given the observations that tumor cells expressing reduced levels of hCtr1 are often associated with cDDP resistance because of reduced cDDP transport capacity (7, 8). Greater induction of hCtr1 expression by copper chelators would have greater platinum transport for cell killing; this has been shown in 3 pairs of cDDP-resistant cell lines (12). In cancer chemotherapy, initial treatment using platinum drugs would eradicate the high hCtr1-expressing population and spare cells intrinsically resistant to cDDP. The ability to resensitize these intrinsic platinum-resistant cells by copper chelators would improve the treatment efficacy of platinum drugs in cancer chemotherapy.

Issues Relevant to the Clinical Use of Copper-Lowering Agents as cDDP Enhancers

The phase I pilot study done at MD Anderson Cancer Center to evaluate the concept of using copper chelators for improving the effectiveness of platinum drugs involved 5 patients with platinum-resistant high-grade epithelial ovarian cancers. These patients were treated with trientiene and CBDCA. Two patients experienced severe adverse events that included myelosuppression, especially anemia requiring transfusion. Dose-limiting toxicity was not observed within the first 28 days. After 2 cycles of therapy, partial remission was achieved in 1 patient (10+ months), stable disease in 3 patients (2, 3.5+, and 5 months, respectively), and progressive disease in 1 patient. Better tumor responses were associated with greater decreases in copper levels using the surrogate biomarker serum ceruloplasmin (17). This study provides the first-in-human preliminary data showing that platinum resistance in tumors could be, at least partially, overcome in some patients through the use of a copper-lowering agent. Further study using a larger patient population with improved strategies (see below) is warranted. In the meantime, several relevant issues are discussed below.

First, copper-lowering agents such as trientine and *D*-penicillamine have been used for more than 4 decades for treating copper toxicosis in Wilson disease, a genetic disorder caused by defects in *ATP7B*. Another copper-lowering agent, tetrathiomolybdate (TM), has been in clinical trials as an antitumor agent. Copper is a cofactor required for several angiogenic mediators, including VEGF, basic fibroblast growth factor, interleukin-1 (IL-1), and IL-8 (25). It has been shown that many patients with malignancies of the breast, colon, lung, prostate, and brain display elevated copper contents in their serum and tumors (25). Copper-lowering agents have been used in monotherapy by targeting the angiogenic growth mechanisms of the tumors (26). Combining copper-lowering agents and platinum drugs in cancer chemotherapy may have additive antitumor effects (and may also have additive cytotoxicities; see below). This strategy is essentially a new use of old drugs and, therefore if proven, would be a low-cost treatment option for patients with cancer.

Second, although the trientine/CBDCA protocol is targeting platinum-resistant tumors with reduced hCtr1 expression, mechanisms of cDDP resistance are multifactorial, and reduced hCtr1 alone is not the only reason for drug resistance. Other factors, including elevated ATP7A/ATP7B transporters (27) and hCtr2 (28), may also affect the treatment efficacy. To maximize the effect of copper chelators, determination of biomarkers associated with various cDDP resistance mechanisms should be very helpful to stratify patients who may benefit most from the treatment.

Third, the effectiveness of copper-lowering agents in enhancing hCtr1 expression *in vivo* is a critical issue. A previous study of rats fed a copper-deficient diet failed to show increased Ctr1 mRNA levels in livers and small intestines, despite a substantial loss in copper levels in these organs (~69% to 89% reduction), as correspondingly compared with those in animals fed a copper-adequate diet (29). A cervical tumor model developed in transgenic HPV16/E2 mice showed elevated mCtr1 protein levels as compared with those in the normal cervix. TM treatments did not show further induction of mCtr1 expression in the tumor lesions of transgenic mice, nor was it seen in the cervix of the wild-type animals (10). In contrast, increased mCtr1 expression was found in several organs (kidney, duodenum, and brain) in copper-deficient, postnatal day-16 mice fed copper-deficient diets (30). Aside from the technical issues related to Ctr1 measurement as mentioned and because different tissue sources were used in these studies, this inconsistency (number 4) may reflect the complexity of *in vivo* Ctr1 regulation mechanisms. Further investigations are needed to address the following important issues: (i) The *in vivo* Ctr1-regulation mechanism by copper deprivation may be more stringent than the *in vitro* system using cultured cells; (ii) Elevated Ctr1 expression may be an intrinsic mechanism associated with tumor development; this may explain why elevated copper levels were observed in many human malignancies (25) and in the HPV16/E2 cervical tumors (10). Moreover, failure to further induce tumoric mCtr1 expression in the HPV16/E2 tumors by TM may be explained by the fact that these tumors already express elevated levels of mCtr1 (Fig. 1C); and (iii) the effectiveness of copper-lowering agents in enhancing cDDP sensitivity may be tissue specific: tissues expressing high levels of hCtr1 may be less sensitive to copper chelation-induced Ctr1 expression than those expressing reduced hCtr1 levels. A better understanding of hCtr1 expression in response to copper-chelation treatment in different settings may eventually lead to the use of hCtr1 inducibility as a predictor for the treatment outcome of platinum-drug therapy.

Fourth, drug-induced toxicity is also an important issue. Both copper chelators and cDDP are redox-active compounds and cause oxidative damage to cells. Combination therapy may exacerbate toxicity, resulting in a myriad of pathophysiologic consequences. In cancer chemotherapy, although cDDP is known to cause toxicities in many organs, including the kidney, nerve, ear, and bone marrow, CBDCA, which is known as the second-generation antitumor platinum drug, displays a great deal fewer toxicities in these organs, except bone marrow. Copper chelators are also known to cause bone marrow damage associated with anemia and/or leukopenia and worsen neurologic symptoms in Wilson disease (26). Accordingly, bone morrow seems to be the most likely target for the adverse events associated with copper chelator/CBDCA combination therapy. This event, indeed, occurred in our preliminary trientine/CBDCA trial, which involved only 5 patients with advanced ovarian cancer (17).

Clinical experience shows that the adverse effects inflicted by CBDCA and by copper-chelator therapy could be overcome when treatments were discontinued, indicating that the treatment-induced adverse events may be manageable. Consequently, we offer some suggestions: (i) modify the treatment schedule to sequential administration of a copper chelator first to induce hCtr1 expression, followed by CBDCA treatment to optimize the antitumor efficacy; (ii) carefully design a treatment holiday to minimize the cytotoxicities; and (iii) critically evaluate the combinations between CBDCA and various copper-lowering agents. These strategies may eventually

improve the overall therapeutic index for the use of copper-chelator and CBDCA combinations.

And fifth, recent studies have shown that cancer-initiating cells in human malignancies are resistant to cDDP (31). Although these cDDP-resistant cells may be in small populations and present in special niches, they are highly tumorigenic in the immunocompromised mice. Currently, we know very little about cDDP-resistance mechanisms associated with this tumor population. This drug-resistant population may be the most difficult target for complete elimination by chemotherapy. The role of hCtr1 in cDDP resistance in these cancer-initiating cells remains to be investigated.

Concluding Remarks and Perspectives

The discovery that Ctr1 can also transport platinum drugs has bridged the gap between 2 seemingly unrelated research fields: copper physiology and platinum-based cancer chemotherapy. Studies of the molecular basis of copper homeostasis regulated by copper-stressed conditions have resulted in ongoing clinical studies that use copper-lowering agents to enhance platinum-treatment efficacy for cancer chemotherapy. Although results await future large randomized studies, this treatment strategy is particularly attractive because it targets cDDP-resistant tumors, which are known to be difficult. Although both copper-lowering agents and platinum drugs have been extensively studied for a long time, much remains to be learned. Importantly, more investigations are needed to critically address many relevant issues mentioned in this article. Research aimed at a better understanding of how the platinum-drug transport capacity can be enhanced through hCtr1 upregulation and how the unwanted cytotoxicity can be simultaneously minimized may eventually facilitate the development of an effective copper-chelation strategy to combat the drug-resistance problem in platinum-based cancer chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.T. Kuo, S. Fu, N. Savaraj, H.H.W. Chen
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