MINIREVIEW

Copper Control as an Antiangiogenic Anticancer Therapy: Lessons from Treating Wilson's Disease

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The search for new anticopper drugs for Wilson's disease is culminating in two excellent new drugs: zinc for maintenance therapy and tetrathiomolybdate (TM) for initial therapy. Both are effective and nontoxic. TM is a very potent, fast-acting new anticopper drug and its properties may be useful well beyond Wilson's disease. Angiogenesis (new blood vessel growth) is required for tumor growth, and a sufficient level of copper appears to be required for angiogenesis. We hypothesize that there is a "window" to which the copper level can be reduced that inhibits angiogenesis in tumors, but does not interfere with vital cellular functions of copper. Using TM therapy, this approach has worked to slow or stabilize tumor growth in several animal tumor models, and preliminary results are also very encouraging in human patients with a variety of advanced and metastatic malignancies. A hypothesis is advanced that copper availability has played a fundamental role in growth regulation throughout evolution and that is the reason that so many angiogenic promoters appear to be dependent upon copper levels. [Exp Biol Med Vol. 226(7):665–673, 2001]

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The War Against Copper: Is It Almost Over or Is It Just Beginning?

In January 1997, the U.S. Food and Drug Administration approved zinc acetate (trade name Galzin) for the maintenance therapy of Wilson's disease, an inherited disease of copper toxicity that is fatal if untreated (1–4). Prior chelation-type drugs, penicillamine and trientine, used in treating this disease (5, 6), though effective in getting rid of copper via the urine, were toxic to some patients, who were then left "between the devil and the deep blue sea." Zinc therapy marked a watershed in the treatment of Wilson's disease because zinc is fully effective and essentially nontoxic. Being a substance natural to the body, there are no problems with drug-related autoimmune disorders that plagued the earlier therapies. Zinc's mechanism, partially blocking the absorption of copper (7), is superior, allowing a more physiological regulation of copper balance and freeing up the patient from restrictive "low copper diets."

Zinc is almost ideal for the maintenance therapy of Wilson's disease, but is too slow acting to be optimal for patients as they initially present with acute copper toxicity, particularly those patients presenting with neurological symptoms. Penicillamine is disastrous in a large proportion of these patients because it makes about one-half of the patients worse, probably by mobilizing hepatic copper and by temporarily elevating brain copper further. Many patients, about 25% of those treated, never recover to their pre-penicillamine baseline, and are often left with serious, permanent, penicillamine-induced disability (8). For these patients we have developed tetrathiomolybdate (TM), a compound with a long and interesting history, beginning with molybdenum-induced copper deficiency in ruminants in Australia and New Zealand (9–11). It was eventually discovered that the sulfur-rich rumen was converting the molybdenum to thiomolybdates, and thiomolybdates given to rats proved to be potent anticopper agents, whereas molybdenum itself given to nonruminants was ineffective (12–15). Of the thiomolybdates, the tetra-substituted compound TM is the most potent. We have used TM for the initial treatment of 63 neurologically presenting Wilson's disease

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patients with excellent results (16–18). TM acts in the gut by forming a tripartite complex with food copper and food protein, preventing copper absorption. TM absorbed into the blood forms a tripartite complex with albumin and blood copper, rendering such bound copper nontoxic. Neurological function is well preserved during an initial treatment period lasting 8 weeks—long enough to control copper toxicity. Then the patients are treated with zinc maintenance therapy and most slowly recover much of their neurological function over 1 or 2 years. As with zinc, TM is essentially nontoxic in these patients, with the only risk appearing to be overtreatment and the depletion of the bone marrow of copper, producing a reversible anemia (16).

With our therapeutic cupboard now well stocked with two new, effective, and nontoxic anticopper therapies, each designed to complement the other and allow a continuum of therapy in Wilson's disease, one might assume that the medical war against copper has been won, or is at least almost over. However, it turns out that there is yet another "front" to this war—that of reducing copper levels to inhibit angiogenesis as a therapy for cancer and other diseases of neovascularization. That battle is just beginning. It appears that the lessons we have learned from treating Wilson's disease with anticopper drugs will be very useful in this new war.

Copper and Angiogenesis: Basic Studies

Scientific interest in copper and angiogenesis dates back a couple of decades (19–21). The rabbit cornea has been used in two ways to study the role of copper in angiogenesis. In the first method, material in the form of a pellet containing various substances is implanted in the cornea, and blood vessel growth (angiogenesis) is directly observed. The pellet itself does not stimulate angiogenesis, but when copper sulfate is contained in the pellet, strong angiogenesis occurs (19). The copper can also be supplied by the copper-containing molecule, ceruloplasmin, and produce angiogenesis (20). The apo-ceruloplasmin molecule, without copper, is not angiogenic (20). If prostaglandin E_1 (PGE_1) is in the pellet, it is strongly angiogenic, and copper accumulates in the newly vascularized region (21). In the second method using the rabbit cornea, the rabbit itself is made mildly copper deficient by a combination of penicillamine therapy and a low copper diet. Now, when PGE_1 is included in the pellet and implanted into the cornea, angiogenesis is greatly reduced compared with its effect in normal (noncopper deficient) rabbits (21).

The two types of experiments show different things. The first shows that copper and copper-containing molecules are angiogenic in this model. The second, however, is more interesting in the context of our topic. It shows that mild copper deficiency reduces angiogenesis produced by a known angiogenic stimulant.

Tumor Growth and Angiogenesis

Folkman pioneered and promulgated the concept that tumor growth is dependent upon angiogenesis (22–28). According to this concept, a tumor cell can multiply to a small cell mass of about 2 mm, with cells in the mass able to get nutrients by diffusion. However, to grow beyond this, a blood supply must be developed, a process called angiogenesis (also sometimes referred to as neovascularization). As the tumor continues to grow, angiogenesis must continue in order to supply nutrients to the tumor cells in the ever-expanding mass of the tumor.

In the normal human, angiogenesis is necessary and critically important during early embryonic and fetal growth and development. A certain amount of angiogenesis is required during childhood growth and development, although to less and less an extent as the individual matures. In adults, the only obvious requirements for angiogenesis are for wound healing and in the uterus during the menstrual cycle.

The absolute dependence of tumor growth on angiogenesis and the relative lack of a need for angiogenesis in adults has suggested that the need for angiogenesis might be an Achilles' heel of cancer, including advanced and metastatic cancer (22–30). This theory proposes that if therapies could be found that inhibit angiogenesis, they might be effective against further cancer growth while being nontoxic to normal tissue.

Angiogenesis in normal cells and tissues involves a complex interaction and balance between stimulators and inhibitors. Brem (29) lists 35 endogenous stimulators and 18 inhibitors. These would be available to the normal organism at various times and places according to the types of cells and tissues, developmental states, and the environmental situation. Most of the angiogenic promoters also appear to be available for recruitment by various types of tumors. Tumors can not only synthesize their own angiogenic promoters within their own cells, but they can also recruit host cells, which then become involved in the invasive process. These host cells can synthesize still other angiogenic promoters, thus assisting in the tumor growth and invasion process.

As a result, tumor angiogenesis is a very complex subject. The review by Beckner (30) lists nine positive tumor angiogenic factors (or promoters) made by inflammatory cells, 13 made by endothelial cells, 15 made by fibroblasts and/or occurring in the extracellular matrix, and 20 made by tumor cells. Of course, there is some overlap of factors appearing in more than one of these lists, but nonetheless, the total number of angiogenic promoters possibly involved in tumorogenesis is daunting. Further, there is a complex array of interactions among the various promoters, as illustrated in a table provided by Beckner (30). For example, vascular endothelial growth factor (VEGF) is shown as interacting with 16 other angiogenic promoters.

One question that can be asked is: Why all this redundancy in angiogenic promoters? One possible explanation is that normal growth and tissue repair, both dependent upon angiogenesis, has to occur in a large array of environments such as embryonic, fetal, and beyond life stages, in a variety

of tissues, in a variety of metabolic and oxygenation states, and in a variety of tissue injury conditions (septic, hypoxic, etc.). Thus, diversity in protecting angiogenesis and tissue growth may be generally critical to development and survival.

Irrespective of the evolutionary reasons for angiogenic promoter redundancy, it makes a diverse array of factors potentially available to tumors to facilitate their growth, invasion, and metastatic processes. This diversity, in turn, potentially complicates the development of effective antiangiogenic therapies aimed at stopping tumor growth. Some key questions with respect to antiangiogenic therapy are asked in Table I.

If the answer to Question 1 is yes, it simplifies the task of a therapy designer because this single molecular mechanism can be targeted, with the expectation of a single agent having efficacy on a wide variety of tumors. VEGF probably comes closest to fulfilling this requirement because it is involved in angiogenesis in a wide array of tumor types and is the target of so many other angiogenic regulatory molecules, as discussed earlier.

Unfortunately, however, the answer to Question 2 also appears to be yes, at least partially. While some positive factors act primarily on VEGF, it appears that a fairly large number can act independently to stimulate angiogenesis in tumors (30). This probably means that a therapy aimed at inhibiting only VEGF will not be successful, or will be only partially successful. Some tumor types may have redundant systems, and other tumor types may develop resistance to VEGF inhibition by recruiting alternate angiogenic pathways.

So far the answer to Question 3 also seems to be yes, with a wide variety of angiogenic promoters isolated from various types of tumors (29, 30). Again, this complicates the therapeutic task, suggesting that different inhibitors may be required for different tumors.

It would, of course, be wonderful if the answer to Question 4 were to be yes because it would mean that a therapy designer would have the potential to shut down tumor angiogenesis with a single approach, much as would be the case if the answer to Question 1 were yes. So, is there a common regulatory element? We believe there is. It appears that a requirement for relatively high levels of copper for activity is a property shared by a large number of angiogenic promoters, which may allow a reduction in the body's copper levels as a therapeutic tool to shut down angiogenesis in many cancers. We will return to this exciting topic after briefly reviewing other antiangiogenic strategies tried so far.

Table I. Key Questions Relating to Angiogenic Promoters That Will Influence the Effectiveness of Antiangiogenic Anticancer Therapies and Strategies

1. Is there a single major angiogenic promoter on which tumor angiogenesis primarily depends? That is, do the other promoters primarily regulate a single promoter—a kind of final common pathway?
2. At the other extreme, are there multiple independently acting angiogenic promoters available to all or most tumors?
3. Is there great variation from tumor type to tumor type in the number and identity of more or less independently acting angiogenic promoters?
4. Is there a common denominator regulatory aspect shared by many angiogenic promoters that can be used as a therapeutic target to downregulate angiogenesis in general?

Antiangiogenic Anticancer Approaches (Noncopper Related)

Over the last decade, work has started in earnest to try to exploit this potential Achilles' heel of one of the world's worst scourges (31). As might be imagined from the number and diversity of angiogenic promoters, a large number of antiangiogenic approaches are being tried. Brem (29) has done a good job of summarizing and reviewing these approaches and has listed the therapeutic agents under clinical trial. Brem (29) lists eight therapeutic agents being studied as protease inhibitors, eight more under inhibitors of endothelial cell migration or proliferation, eight agents under antagonists of angiogenic growth factors, two that inhibit endothelial-specific integrin or survival signaling, six agents with individual mechanisms of action, and three agents that work through reducing copper levels (an area to be discussed in the next section).

Because of the key role of VEGF, several agents are aimed at interfering with its action (29). There is a VEGF antibody being studied by Genetech, and another agent is under study by Sugen and another by Novartis that blocks VEGF receptor signaling. Thalidomide is a drug used many years ago in Europe for sedation that turned out to have teratogenic side effects. It is being studied by Celgene because it blocks the activity of VEGF and possibly other angiogenic promoters. Interferon-α is undergoing trial as an inhibitor of VEGF production.

Two protein agents that are natural substances in the body and are angiogenic inhibitors have been studied by O'Reilly *et al.* (32, 33). One is angiostatin (32), a 38-kDa protein that inhibits angiogenesis in a variety of ways, including inhibition of endothelial cell proliferation. Angiostatin is an internal fragment of the plasminogen molecule and has demonstrated potent antitumor effects in animal models.

The other molecule, endostatin (33), has received a great deal of public attention. It is a 20-kDa C-terminal fragment of collagen XVIII. It is an antiangiogenic molecule that inhibits endothelial cell proliferation and has also proven to be a potent antitumor agent in animal models. Both angiostatin and endostatin are protein molecules and therefore cannot be given orally.

From the examples cited here, it should be clear that the approaches being taken by investigators are quite diverse. While some encouraging clinical results are beginning to

emerge, it is too early to say which of these approaches, if any, will yield useful anticancer therapies.

Copper Control as an Antiangiogenic Anticancer Therapy: Animal Studies

The first steps in testing new ideas for cancer therapy usually involve looking at effects of the relevant agent in cell cultures of tumor cells and then using the agent or drug in animal model studies. In this case, cell culture studies are inappropriate because there is no expected direct toxicity of antiangiogenic agents on tumor cells in culture. However, animal model studies are quite appropriate and have strongly supported the antiangiogenic concept of cancer therapy, including the use of copper reduction as an antiangiogenic strategy.

Brem and coworkers (34, 35) were the first to examine the effect of copper limitation on tumor growth in animals. They made rabbits and rats partially copper deficient by using penicillamine and a low copper diet. They then implanted brain tumors into the brains of these and control animals. The results were very impressive in one aspect and very disappointing in another.

First, as to the positive results, the brain tumors in the copper-deficient animals were markedly smaller than in the control animals (34). The surface of the brains over the implants in the control animals showed a plethora of new vascularization, whereas this was not present in the copper-deficient animals. Cut sections through the tumors and surrounding normal brain tissue revealed marked invasion of blood vessels from the tumor into the normal brain tissue in control animals, and this was completely missing in copper-deficient animals (35).

The disappointing results were a lack of survival advantage in the copper-deficient animals (34). The treated animals died at approximately the same time after tumor transplantation as the control animals. It appears that cerebral edema and brain stem herniation, the likely cause of death in most of the animals, was approximately equal. The bottom line was that while the proof of the principle that relative copper deficiency inhibited tumor angiogenesis and reduced tumor growth was supported, the therapy seemed to have no overall benefit. Another negative aspect of the study was that metastases to lung from tumor implanted in the thigh was not affected by a copper-deficient state (34).

Reading this literature and having developed an extremely potent and safe anticopper therapy in TM (as discussed in the first section of this paper), I decided to reexamine the question of anticopper antiangiogenesis therapy in cancer. I carried out a mouse sarcoma tumor model study using MCA205 cells injected subcutaneously, and I obtained positive results with TM treatment with a significantly reduced rate of tumor growth (Brewer GJ, unpublished data). With TM therapy, a tripartite complex of albumin, copper, and TM is formed in the blood, which clears slowly and makes serum copper an inaccurate reflector of body copper status. I, therefore, used plasma ceruloplasmin as a surrogate marker of copper status and it worked well. Ceruloplasmin is a copper-containing plasma protein synthesized by the liver and released into the blood according to copper availability in the liver (36).

However, this tumor mouse model was not ideal, and I joined forces with an oncologist, Dr. Sofia Merajver. Dr. Merajver designed an elegant study using the HER2-neu transgenic mouse model in which control mice develop mammary cancer during the first year of life. Treated animals were given TM daily by gastric gavage. Over a period of 270 days, 18 of 22 control mice developed obvious mammary cancer, while all 15 of the TM-treated mice were free of visible tumor, although small clusters of malignant cells were present on microscopic examination (37). Release of a few of the treated mice from TM therapy caused their tumors to grow into detectable masses. TM treatment of a few of the control mice allowed tumor growth stabilization in mice whose tumors could be followed humanely for a long enough period of observation.

These results were very exciting to us because they suggested not only tumor growth stabilization with TM therapy, but also potential prophylaxis in patients with high genetic risks of developing cancer.

Copper Control as an Antiangiogenic Anticancer Therapy: Clinical Studies

With these dramatic preclinical results, and already having TM in the human in Wilson's disease patients, we quickly moved to a pilot clinical study. We recently reported on the first 18 metastatic cancer patients treated with TM (38). Phase 1 objectives of learning about dose, management of the drug, and possible toxicity were achieved. We established that ceruloplasmin levels in the blood also worked well as a surrogate marker of copper status in the human, as it had in the mouse.

To be evaluable for efficacy, patients have to stay on therapy long enough to have their ceruloplasmin levels in the target range of 5 to 15 mg/dl (normal is 20–35 mg/dl) for at least 60 days. The reason for this is that stability is not expected until significant copper depletion occurs, including copper depletion from the tumor, and there is good evidence that tumors sequester extra copper (39–42). If the observation period is shorter than 60 days, there probably has not been adequate opportunity for disease stabilization to occur. In the original publication (38), six of the 18 patients were evaluable for efficacy. Five achieved disease stabilization, including one with regression of lung metastases, and the sixth had stable disease except for progression at one site. The length of disease stability, or freedom from progression (FFP) ranged from 120 to 413 days. For comparison, FFP in the usual phase 1 study averages about 60 days. The only side effect of TM was rare overtreatment resulting in anemia, which required temporary cessation of drug (38).

This phase 1 study has been ongoing and a long-term follow-up is being reported (43 [Merajver, et al., unpub-

lished]). A total of 40 patients have been entered by now (including the original 18), and 18 of these are evaluable by our criteria. All 18 have achieved disease stabilization for longer than 2 months, with the average being 9.5 months, two as long as 30 months, with six patients still on study. Eleven different types of malignancies are represented by these 18 patients.

It must be admitted that this clinical trial is uncontrolled, and final conclusions cannot be drawn until large numbers of patients with specific cancers are studied under controlled conditions. However, my tentative interpretation of these results is that they show reasonable evidence of efficacy. While it is true that occasional patients with certain types of advanced metastatic cancer will have periods of disease stability, this type of spontaneous stability for a lengthy period is rare with many of the cancers in our study. And lengthy periods of disease stabilization in our study are relatively common, not just the exception. For example, if one selects 6 months of disease stabilization as somewhat unusual spontaneous occurrences with the malignancies under study, nine of our 18 evaluable patients achieved this period of stabilization or better, with two who have not yet achieved it still on study. Clearly, there is very strong, but anecdotal, evidence of efficacy in two patients, one with metastatic breast cancer and one with metastatic chondrosarcoma, who have had stable disease while on TM therapy for over 2.5 years (43).

An important question is: Will TM (and its associated clinical copper deficiency) be effective therapy in all metastatic solid tumors? We have evidence that it is effective against many in both the clinical and preclinical studies, as shown in Table II, but will there be exceptions? Part of the answer will probably come from whether some tumors use angiogenic promoters that do not depend upon copper, or whether they use promoters that have a high enough affinity for copper to obtain copper under TM treatment conditions. If VEGF is as critical to tumor angiogenesis as now seems likely, and if VEGF is truly copper-dependent for activity (see next section), these situations may not be common. Another question is whether bone metastases can obtain nutrients from bone marrow and not depend upon neovascularization.

Another important question in contemplating possible clinical use of TM therapy is whether there will be side effects from prolonged mild copper deficiency. A normal level of plasma ceruloplasmin, *per se*, does not seem physiologically important because most patients with Wilson's disease have a low ceruloplasmin level, and once their copper toxicity is treated, they seem to bear no clinical consequences from the continued low ceruloplasmin level in their blood (1–4). However, our TM therapy in cancer, almost by definition, is lowering the level of available copper in various organs because it is that lowered level that causes the liver to synthesize and release less ceruloplasmin into the blood (36)—the measure we are using to regulate our TM dose. So far, we see no toxicity related to long-term treatment as long as we keep ceruloplasmin levels above about 5.0 mg/dl (38, 43). This is the approximate cutoff point where the bone marrow begins to be unduly depleted and where cellular synthesis decreases. However, it is very important to determine any long-term consequences of mild copper deficiency. The National Cancer Institute RAID program is now conducting an evaluation of this question, as well as studying TM toxicity in general.

Of course, it will be important to determine whether antiangiogenesis therapy with TM interacts favorably with other modalities of cancer treatment. We have a positive mouse model study of lung cancer in which radiation therapy and TM therapy combined to have a greater effect than either alone (44). So far, we only have anecdotal (but encouraging) clinical evidence that interactions with other anticancer modalities will be positive. Additional studies to answer this question have been initiated.

Finally, if TM does prove safe, would it prove effective as a preventive in patients at high risk for getting cancer, such as those with cancer predisposing genotypes, as suggested by our findings with the HER2-neu transgenic mouse study (37)? And will it be useful in other noncancer diseases of neovascularization such as diabetic retinopathy, rheumatoid arthritis, and psoriasis?

TABLE II. Tumor Types Stabilized by Copper Reduction Therapy

Clinical studies			Animal studies			
Malignancy	Drug	References	Malignancy	Species	Drug	References
Angiosarcoma[a]	TM	38, 43	MCA 205 sarcoma	Mouse	TM	Brewer G, unpublished data
Melanoma[a]	TM	38, 43	HER2/neu mammary carcinoma	Mouse	TM	37
Renal cell carcinoma[a]	TM	38, 43	9L Gliosarcoma	Rat	Penicillamine	34
Breast carcinoma[a]	TM	38, 43	VX2 Carcinoma	Rabbit	Penicillamine	34
Hemiangioendothelioma[a]	TM	38, 43	Lewis lung high metastatic carcinoma	Mouse	TM	44
Chondrosarcoma[a]	TM	38, 43				

[a] These tumors were stabilized for at least 6 months.

TABLE III. Conceptual View of the Effects of Three Levels of Copper in the Body, and Three Related Hypotheses

Body status of copper	Ceruloplasmin levels	Angiogenic promoters can be activated	Pathological angiogenesis can occur	Cellular housekeeping requirements for Cu
Normal range	20–35	Yes	Yes	Are met
Mid range (chemical-mild Cu deficiency)	5–15	No	No	Are met
Low range (clinical Cu deficiency)	0–5	No	No	Are compromised

Hypothesis 1. There is a window of relative copper deficiency in which angiogenesis is inhibited, but cellular housekeeping requirements for copper are not compromised.
Hypothesis 2. One or more angiogenic promoters, key to angiogenesis in a variety of cancer types, require copper for activity.
Hypothesis 3. The angiogenic activity of one or more of the copper-dependent angiogenic promoters of Hypothesis 2 is sensitive to copper levels in the "mid-range," as above.

Copper Control as an Antiangiogenic Anticancer Therapy: Possible Mechanisms of Action

Both our preclinical and clinical studies support Hypothesis 1 of Table III, which suggests that there is a "window" of relative copper deficiency, with plasma ceruloplasmin values between 5 and 15 mg/dl (in the human), in which angiogenesis is interrupted, resulting in stable disease in several metastatic cancer types without apparent toxicity. We view the results so far as very encouraging, but as suggested in the beginning of this paper, this phase of the war against copper may be just beginning. There are numerous important questions to answer, some of which we have already posed, such as the breadth, degree, and the length of efficacy, toxicity, interaction with other therapeutic modalities, and possible use in prophylaxis. But an extremely important underlying question, because the answer will affect the answers of so many of the other questions, is: How does lowering copper levels work as an antiangiogenic therapy?

What is the mechanism of action? A rather general (and unsatisfying) answer to this is that tumors of many types sequester copper (39–42), and *ipso facto*, copper must be important for the well being of tumors. However, a more satisfactory answer may be emerging as the molecules involved in promotion of angiogenesis are increasingly identified and studied. VEGF, as we have already discussed, is a key factor, perhaps a dominant factor, in promoting angiogenesis in tumors, and it interacts with numerous other factors that promote angiogenesis in many different ways (30, 45–49). It is clear that VEGF binds copper with a high affinity, because it can be purified by using a copper-affinity column (50). This suggests that copper might be required for VEGF angiogenic activity, and there is one claim to this effect (29).

Fibroblastic growth factors (FGF), acidic and basic (synonymous with FGF1 and FGF2) are also angiogenic promoters and also bind copper with a high affinity (51, 52). Angiotropin, a monocyte-derived angiogenic factor, contains copper within the molecule (53). The binding of angiogenin, a potent angiogenic factor, to endothelial cells appears to be modulated by copper (54), although this has been challenged (55). SPARC (secreted protein, acidic and rich in cysteine) is a source of copper-binding peptides that stimulate angiogenesis (56). Ceruloplasmin, heparin, and the tripeptide Gly-His-Lys (also referred to as liver growth factor) are angiogenic when complexed to copper (57).

The above citations list nine angiogenic substances that are reported to bind or contain copper, and they support a preliminary hypothesis (Hypothesis 2 of Table III) that copper is critically involved in the action of one or more of them, and this provides a mechanism for the therapeutic efficacy of a lowered copper level. Admittedly, final proof of the hypothesis that lowered copper levels reduce angiogenesis through reducing the angiogenic stimulating activity of one or more of the listed factors is not in, but for the sake of further discussion I will make the assumption that this hypothesis will be proven correct.

Hard upon its heels, then, must come Hypothesis 3 of Table III, which suggests that one or more key angiogenic promoters is sensitive to activation by copper in the "mid-range" of body copper status. That is, well before cellular enzymes requiring copper are affected, one or more of these factors becomes less active. In this sense, the ability of the liver to put copper into ceruloplasmin and secrete it into the blood seems to be a reflection of the copper availability required for the hypothesized copper-dependent angiogenic promoter(s).

In regard to copper concentrations, many investigators using *in vitro* experiments appear to be unaware of the very low concentrations of ionic or free copper that exists *in vivo*, even extracellularly. While serum copper in the human is about 100 μg/dl, or about 1.5 μM, 90% of that copper is in ceruloplasmin and is therefore not readily available to act on angiogenic promoters. Even the remaining 10% is bound to protein and other molecules. Intracellularly, ionic copper is kept so low (in yeast, less than one atom per cell!) that some processes use copper chaperones to move copper from one molecule to another (58, 59). If some of the angiogenic promoters require copper chaperones, it may be the relative affinity of the chaperone for copper that will determine the activation of the angiogenic promoter.

Why Copper? Is Copper a Basic Regulator of Growth Control?

If we accept the hypothesis that copper is a key regulator of angiogenesis, it leads to another important question: Why is it that copper is filling this role, and not iron, zinc, calcium, magnesium, etc., which are all cations important in physiologic and biochemical functions? Angiogenesis is in many ways almost synonymous with growth, so the question can be restated: During evolution, has copper played a fundamental role in regulating growth? It is well established that copper has very important functions in a key respiratory enzyme (cytochrome oxidase), in many other important enzymes, such as superoxide dismutase and various oxidases and oxygenases, in transcription factors, and in many other important systems. But so do iron, zinc, and other metals. Of course, copper has unique molecular properties that cannot be replaced in specific proteins with other metals, but the question being considered here is why should copper have been singled out by evolution, if it has been, for a special role in regulating growth and angiogenesis?

I hypothesize that the key is variability in the environmental availability of copper relative to other cations, which given that the organism has an absolute requirement for copper for survival, determines whether a primitive organism should also grow or remain in a relatively quiescent state.

To sustain this hypothesis, there must be variability in the distribution, or at least the bioavailability, of copper around the world. Is there such evidence? First, there is some evidence that copper content of soils in various parts of the world is quite variable (60–63) and the same is not true for other essential trace metals except, occasionally, zinc (61). There is even better evidence for geographic variation in bioavailability of copper (64–69). One excellent kind of evidence is that grazing animals such as sheep, which obtain their minerals from grass that obtains its minerals from soil (in the days before mineral supplementation), were not infrequently affected by copper deficiency diseases (65–69). This indicates that their particular pastures were deficient in copper. To my knowledge, this kind of deficiency in grazing animals has not been generally described for other metals such as zinc and iron.

So if copper is variably available in the environment, did primitive organisms require copper, and if so, did they develop strategies to cope with this variability? In answer to the first question, yes, they require copper. Yeast, for example, has multiple vital copper-dependent enzymes (70). The essentiality of copper for yeast physiology is in keeping with an elaborate system in yeast to try to ensure adequate cellular levels of copper. In a low copper environment, there is derepression of a series of genes whose products enhance copper uptake (70–75). Two genes provide high affinity plasma membrane copper permeases (71, 72). Another gene codes for a metalloreductase that elaborates a reductant into the extracellular environment to mobilize copper (73). The copper regulatory enzymes and systems in yeast seem to be more complex and numerous than for other cations, with the possible exception of iron, and homologues of these same yeast genes seem to usually be present in mammals (58, 59).

Thus, yeast has evolved as if it has had to deal with low copper availability and it has the tools to acquire copper in a low copper environment. Now, the really important question for us is: If copper availability is too low, does it affect the growth (division) of yeast? Certainly in yeast, copper is required for growth, and yeast appear to be more sensitive to relative copper depletion than relative depletion of other cations in terms of growth. Yeast shift from aerobic metabolism to fermentation in the absence of copper, which is less able to sustain high rates of cell division. So the answer to our question (Does copper availability in yeast affect growth?) appears to be "yes."

Some recent findings in the organism *Podospora anserina* add further support for a role for copper as a primitive regulator of growth (76–78). *P. anserina* is a filamentous fungus with a limited lifespan. Mutation in the gene grisea results in reduced growth, along with other phenotypic changes. Grisea codes for a transcription factor, and this transcription factor requires copper for activity. In other words, adequate levels of copper, acting through this transcription factor, are required to maintain a normal growth rate.

Thus, it seems feasible that adequate availability of copper has been a primitive signal that growth should occur. If we assume that certain copper enzymes such as cytochrome oxidase were an absolute requirement for health and growth, relative copper availability as a growth signal would make sense, particularly if copper availability was somewhat variable. Perhaps other essential metals were much less variable in the environment of early organisms.

If a copper requirement for growth was fixed in primitive organisms in early evolution, copper modulation of growth could very well have remained in higher organisms, even if availability of copper became less variable. Moving up to mammals, copper deficiency is a potent teratogen (79, 80), probably more so than deficiency of other metals, and development of a normal mammalian fetus requires normal growth, which requires normal angiogenesis. Although the data are piecemeal at present, it seems possible that throughout evolution copper has played a fundamental role in regulating growth, and in higher organisms, perhaps part of that regulation has been retained by copper regulation of growth factors and angiogenesis.

My Current Interpretation of the Meaning of These Various Studies

First, I believe copper will be shown to be a fundamental regulator of growth throughout evolution. There is just too much evidence of its requirement for activity of angiogenic promoters and growth factors to ignore. These data, together with piecemeal (but emerging) data from lower organisms, and the sensitivity of the fetus to birth defects

from copper deficiency, indicate, at least to me, that copper plays a fundamental and general role in regulating growth.

Second, I believe there will be two separate and distinct levels of copper effects in mammals, holdovers from the early evolution discussed above. The higher level allows angiogenic promotion and growth, as well as cellular health. The lower level does not allow angiogenic promotion and growth, but does allow cellular health. This is in parallel with simple organisms: ample copper equals growth and health and low copper allows the cells to live, but not grow. Also in parallel is the fetus: ample copper equals growth and health and low copper allows the fetus to live, but not to develop properly.

Third, I believe that it is highly likely that lowering copper to levels in between the high and low levels described above, the therapeutic "window" discussed in the paper, will offer a safe and effective way to stabilize the growth of a wide variety of advanced and metastatic cancers. Given the evidence that some of the same angiogenic and growth factors are elevated in lymphomas and leukemias, this therapeutic approach may extend beyond solid tumors.

The correctness of my interpretations will determine the eventual usefulness of this approach to cancer therapy and other diseases of neovascularization. Based on encouraging results so far, it may be reasonable to conclude that the war on copper is just beginning.

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