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A Requirement for Copper in Angiogenesis

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Although two decades have passed since copper was shown to stimulate blood vessel formation in the avascular cornea of rabbits, only recently have clinical trials established that Cu privation by diet or by Cu chelators diminishes a tumor's ability to mount an angiogenic response. These data have shed new light on the functional role of Cu in microvessel development and, of equal importance, stimulated new nutritional models of cancer therapeutic intervention.

Key words: copper metabolism, copper function, blood vessel formation, tumors, cancer

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Introduction

If tumors are to grow and thrive, they must develop a blood supply. Judah Folkman recognized this fact in a 1971 hypothesis that stated "every increment in tumor growth requires an increment in capillary growth."[1] The hypothesis provoked research into neovascularization or the mechanism by which tumor cells elicit new blood vessel growth from the surrounding tissue. Specific angiogenic factors that comprise the network of components that respond to tumor cell stimuli were identified and, in time, a sequence of cellular events was hypothesized. Such events include the activation of factors that mediate migration, mitosis, and differentiation of endothelial cells, and the reshaping of matrix proteins into the familiar tubular structure of capillary anatomy, each of which has a rather specific requirement for Cu.

Copper as a Neovascular Agent

In a series of pioneering studies, MacAuslan and Gole induced intraocular vascularization in rats by putting micromolar amounts of $CuSO_4$ into anterior chamber implants.[2] The outward show of blood vessels streaming from the implant was interpreted to result from the migration of a specific subset of endothelial cells in response to an unknown angiogenic stimulus. Cu was thought at first to be acting as a chemotaxic agent.[3] Later, however, Hannan and MacAuslan showed that Cu evoked the synthesis of fibronectin in cultures of bovine endothelial cells,[4] suggesting that effects of Cu were more likely to be internal. Fibronectin deposits on the surface of endothelial cells were deemed important for tracking and forming an adherent endothelium. Micromolar amounts of Cu (10^{-6} M) thus appeared to control endothelial cell migration and angiogenesis. Later Cu was found to stimulate microvessel formation in the avascular cornea of rabbits, thus dismissing species specificity or uniqueness as a factor in the response. Feeding rabbits diets deficient in Cu suppressed blood vessels appearance,[5] however, which firmly established Cu as one of the causative agents. Cu was connected with cancer cells in a study that showed mammary adenocarcinomas had a higher percentage of Cu-positive cells; this finding led Fuchs and de Lustig to postulate a correlation between Cu deposits and angiogenic and metastatic ability.[6] Early studies also showed that proinflammatory compounds, such as prostaglandin E-1 (PGE-1) and interleukins (IL), stimulated blood vessel formation in animal models. Leukocytes in a stressful or Cu-dependent manner were postulated to release matrix-destroying collagenase, which is believed to assist the movement of pre-existing endothelial cells from a confined connective tissue environment.[7]

Copper and Growth Factors

The effects of Cu on neovascularization and metastasis were not pursued forcefully because early research into angiogenesis focused more on growth factors and cell-signaling agents in the response. Fibroblast growth factor (FGF), an extracellular mitogen, was shown to induce endothelial cell migration and proliferation. Vascular endothelial growth factor (VEGF), synaptotagamin, and S100A13 were likewise shown to have a role. In time, a basic protein with exceptional in vivo angiogenic activity was isolated from tumor cells.[8] The protein, named angiogenin, was the first tumor-derived angiogenic factor. Cu was given only sporadic interest until the demonstration that a 48-hour exposure to 500 μM Cu in

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serum-free medium doubled the number of human endothelial cells in culture. The Cu supplementation was selective for endothelial cells and showed little or no enhancement of fibroblasts or arterial smooth muscle cells. Neither Zn nor Fe at the same concentration as Cu caused a rise in cell numbers, the former two metals in fact manifesting a suppression in endothelial cell growth.[9] Such results elevated Cu to the status of a growth factor for endothelial cells that could be on a par with FGF-1 in growth-stimulating efficacy. This raised the question as to whether Cu acted alone or manifested its effect through contact with known angiogenic factors. A direct effect was ruled less likely when it was shown that Cu added to recombinant FGF-1 resulted in the formation of an inactive dimer,[10] possibly through the oxidation of sensitive cysteine groups in the protein. An indirect effect, however, has not been ruled out and is currently a major focus of research in angiogenesis.

Table 1 lists a series of adhesive and growth-promoting factors whose angiogenic action has been shown to depend on Cu. The protein fibronectin is mentioned because Cu has been shown to stabilize the 3-D architecture or "mat" of this adhesive protein.[11] At physiologic concentrations, Cu reportedly induces the synthesis of vascular endothelial growth factor, which promotes angiogenesis in a healing wound.[21] Both FGF-1 and IL-1β are released from endothelial cells in response to Cu. Significantly, both factors lack a signal sequence and hence a means to enter the endoplasmic reticulum–Golgi secretory pathway for release. Without a signal sequence neither protein can be secreted. Cu apparently causes the proteins to complex with S100A13, which has a signal sequence and, as a complex, exit the cell by a nonconventional export system.[20] In the extracellular medium, FGF-1 is free to bind to its receptor on endothelial cells and trigger a mitotic response. SPARC (osteonectin or BM40), a transiently expressed matrix-binding protein, has been projected to mediate the movement of Cu for the angiogenic response. Both the intact SPARC protein and the smaller peptide fragments with Cu-binding motifs were shown to stimulate the appearance of endothelial cords in vitro and an angiogenic response in vivo.[19] Whether SPARC brings Cu into the export system or functions as a "chaperone" for Cu has not been determined.

Table 1. Proangiogenic Mediators that Rely on Cu for Expression or Function

Mediator	Reference
Fibronectin	Ahmed et al.[11]
Collagenase	Lin and Chen[7]
Gangliosides	Gullino[12]
Prostaglandin E-1	Ziche et al.[5]
Heparin	Alessandri et al.[13]
Angiogenin	Soncin et al.[14]
S100A13	Mandinov et al.[15]
FGF-1 (acidic)	Landriscina et al.[16]
FGF-2 (basic)	Pan et al.[17]
FGF receptor-1	Patstone and Maher[18]
SPARC	Lane et al.[19]
Synaptotagamin	Prudovsky et al.[20]
Vascular endothelial growth factor	Sen et al.[21]
Tumor necrosis factor-α	Pan et al.[17]
Ceruloplasmin	Raju et al.[22]
IL-1α	Mandinov et al.[15]
IL-6	Pan et al.[17]
IL-8	Moriguchi et al.[23]
Nuclear factor-kB	Pan et al.[17]

FGF = fibroblast growth factor, SPARC = osteonectin/BM40, IL = interleukin.

Copper Chelators and Tumor Growth

Will regulating Cu through the diet or by applying Cu-specific chelators block cancerous tumor growth and enhance the survival times of animals and humans? In essence, how critical is Cu in fulfilling a complete angiogenic response? Early experiments established the feasibility of using agents that bind Cu to control tumor growth. Using either the avascular cornea model[5] or measuring proliferation of corneal blood vessels,[24] it was shown that D-penicillamine given daily by intravenous injections significantly inhibited rabbits from responding to neovascular stimuli. In one of the first therapeutic trials, Brem et al. found that a combination of dietary Cu deficiency and D-penicillamine shrunk tumors implanted into the brain of rabbits and stopped the infiltrative spread of a highly invasive 9L gliosarcoma in Fischer 344 rats. For reasons not yet clear, however, the treatment failed to inhibit growth and vascularization of tumors of the thigh muscle.[25] Trientine, another Cu chelator, suppressed tumor development and angiogenesis in liver cells,[23,26] presumably by inhibiting the synthesis or release of IL-8, a potent angiogenic cytokine.[23] A 7- to 10-day Cu deficiency alone failed to arrest the growth or alter the vascular density of a chondrosarcoma implanted into the cremaster muscle of rats, which led Schuschke et al. to conclude that Cu's effects may depend on the type of tumor, the host tissue, and the conditions of Cu depletion.[27]

Clinical Trials with Tetrathiomolybdate

Tetrathiomolybdate (MoS_4^{2-}, TM) is a potent metal chelator that binds Cu to proteins such as serum albumin, forming a complex that is only sparingly taken up by cells. Experiments with rats have shown that TM removes Cu in the liver in different ways depending on the

dose.[28] In time, Cu-TM complexes are excreted from the system more in the bile than in the plasma. TM has been used with good success to treat the liver Cu toxicosis associated with Wilson disease.[29] Past successes with D-pencillamine and trientine laid down a strong rationale for testing TM in a similar role as an anti-Cu/anti-angiogenic agent. According to Dr. George Brewer, a clinician who developed TM treatment for Wilson patients, and Dr. Sofia Marejvar, an oncologist at the University of Michigan Cancer Center, TM's action is rapid with good potency and a very good index of safety.[29] Preclinical studies had shown that C3H*/HeJ* mice implanted with head and neck squamous cell carcinomas and who drank water containing 50 mg of TM had carcinomas reduced to one-fifth the original size and 28% lower body Cu content. Tumors in rats that drank water without TM were unaffected.[30] In two animal models of breast cancer, tumor development in cancer-prone HER2*/neu* transgenic mice was eliminated by feeding 0.75 mg of TM per day. Mice fed only water developed tumors after 130 days (Figure 1). Among the factors suppressed by TM was nuclear factor kappa B (NF-kB), a transcription factor that has been linked to stress responses and that may regulate the expression of proangiogenic factors.[17]

Currently human trials are underway in Michigan testing three different dose levels of TM (90, 105, and 120 mg/day) administered to 18 human subjects with metastatic tumors.[31] Copper status of the subjects is being monitored by measuring the serum ceruloplasmin with the goal of reducing ceruloplasmin to 20% of its baseline value while keeping the hematocrit above 80%.

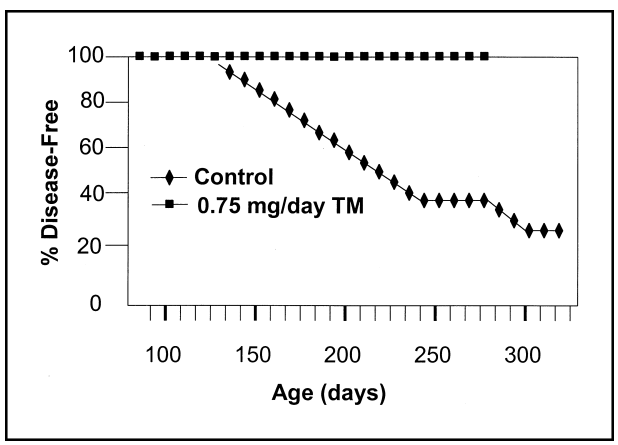

Figure 1. Inhibition of overt tumor development in Her2/*neu* transgenic mice. Mouse mammary tumor virus-Her2/*neu* transgenic mice develop tumors spontaneously. At 100 days, the mice were randomly assigned to two groups; one group was gavaged daily with water and the second with water containing 0.75 mg tetrathiomolybdate (TM). Palpable tumors and a disease-free survival curve were determined by weekly assessments. Adapted from reference 17.

Once stabilized, the patient can remain on the TM for 90 days or longer. After one year, five of the six patients that received TM and whose Cu level was reduced to 20% showed no growth in their tumors. The sixth patient with multiple tumors showed growth in only one. In what has been termed a Phase II trial,[32] 15 patients with advanced kidney cancer were given doses of TM to keep plasma ceruloplasmin within the target range (5–15 mg/dL). Assessments of tumor development were performed every 12 weeks. All patients responded to the TM by lowering serum Cu. Of 13 patients who were assessed, four had stable disease for at least 6 months during the depletion. A decrease in vascularity appeared to correlate with necrosis of a tumor mass. In this small cohort, serum levels of proangiogenic factors interleukins (IL)-6, -8, vascular endothelial growth factor, and basic fibroblast growth factor (bFGF) may correlate with Cu depletion but not with disease stability.

These initial studies have important implications. First, they suggest that total removal of Cu is not necessary to block angiogenesis. In essence, vital Cu-dependent functions could be undeterred. Second, these studies imply that TM may be an effective treatment for some cancers, but perhaps works best in combination with other antiangiogenic factors. Third, they show that angiogenic factors operate maximally when physiologic levels of Cu are adequate or at the high end of the scale. The latter presents a paradox, since Cu levels in serum of cancer patients are known to be elevated in response to cancerous tumor growth. One must therefore consider that elevating serum Cu may be a "cause that favors" rather than "a response against" further tumor development. More research will be needed to establish a cause-and-effect relationship between serum and tissue Cu levels in cancer patients, if indeed one does exist.

Conclusion

In its role as a cofactor, inducer, or binding ligand, Cu is an essential participant of an angiogenic response. Although its mechanism is not understood, Cu appears to exert multiple effects on angiogenesis and does not seem to be targeted to any one specific factor or stage. Rather, as indicated by the list of factors in Table 1, it may be safe to assume until further data reveals otherwise that Cu is a pleiotrophic agent capable of affecting numerous components of the angiogenic response system. One function that was not mentioned in this review is the well-established role of Cu in major artery formation. Indeed, one could say that the newly discovered responsibility of Cu in capillary development complements its well-known role in the synthesis of the collagen and elastin matrix of major arteries. In the latter, Cu is a cofactor for lysyl oxidase, the enzyme that catalyzes the oxidation of select lysine residues in soluble precursor

proteins that give large blood vessels their resilience and toughness.[33] A severe Cu deficiency in rapidly growing chicks and pigs lowers lysyl oxidase activity, ultimately resulting in aortic aneurysm and blood vessel rupture. With microvessel development now added to the list of functions, Cu may rightly be considered one of the most important nutrients in vascular system development.

Studies reviewed in this report have revealed that at physiologic levels Cu is able to activate proangiogenic cytokines. A small diminution of Cu has a telling effect on tumor vascularity while apparently having little or no effect on other cell functions. The rationale for using chelation therapy is to lower the Cu concentration in organs by blocking its uptake into cells, especially cells of the endothelium. Clinical studies are showing that it is possible to suppress blood vessel development while allowing vital functions of Cu to go on. It is perhaps too soon to know if a mild Cu deficiency benefits patients who are at genetic risk of developing tumors, but this is a rationale that should be studied more closely especially since Cu levels can be controlled through the diet. Investigators should also focus on why a variety of tumors have a propensity to accumulate Cu in intra- or perinuclear locations of the cell.[6] More attention must be directed at genes and proteins that govern Cu uptake, especially since cancerous transformations result in cells acquiring a capacity to sequester Cu substantially over normal cells.[34] Finally, we need to understand why in two studies striated muscle tumors appeared to resist a Cu-based therapy to stop tumor growth. The anomaly may reveal Cu response systems that are present in other cells but missing or non-functional in muscle cells.

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