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METALLOTHIONEIN AND THE TRACE MINERALS

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INTRODUCTION

Metallothionein and certain trace elements are inextricably linked. The protein occurs only in vivo with its complement of bound metals. Its occurrence in tissues is greatly influenced by changes in trace element status, and its detection and assay are often dependent on monitoring its metal component. Since the discovery of metallothionein as a renal cadmium-binding protein

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more than 30 years ago, our knowledge of its physicochemical properties and biosynthesis has grown considerably. No longer is it considered only as a protein that detoxifies cadmium and other toxic metals. Its antiquity, ubiquity, and inducibility by a wide range of stimuli, including zinc, copper, and "stress" factors to be discussed, suggest that it plays a vital role in the regulation of metabolic processes that utilize these essential trace elements.

Occurrence

Metallothionein has been isolated from a wide range of tissues, including liver, kidneys, pancreas, and intestine (54). A number of isoforms of the protein are present, with slightly different amino acid composition and charge, depending on the species. Indeed as immunologic techniques for its detection have improved, metallothionein has been found in most other tissues, including brain, thymus, bone marrow, and reproductive organs. Detection by subcellular fractionation indicates that metallothionein occurs principally in the cytosol, but immunocytochemical studies have consistently revealed its presence also in nuclei (72, 83). Appreciable amounts of metallothionein have been found in particulate fractions of copper-loaded livers, possibly in the lysosomes, as reported in Bedlington terriers (52) or in nuclei, as reported in fetal deer (60). Although metallothionein is mainly of intracellular origin, it also occurs in small amounts in extracellular fluids such as plasma, bile, and urine (46, 87, 88).

The concentration of the protein in tissues is highly variable and is influenced by many nutritional, physiologic, and developmental factors (13, 21). For example, concentrations are greatly decreased in tissues of zinc-deficient animals (16) and are increased after imposition of many types of stress or metal administration (21). They are generally elevated during fetal development (83) and vary dramatically among species. Hepatic concentrations in rats tend to be much lower than those in humans or farm animals.

Physicochemical Properties

The characteristic features of metallothionein are its low molecular weight, about 6500, and its unusual amino acid composition: Cysteine accounts for 30% of the residues and aromatic amino acids are absent. Sequence studies have shown that the distribution of the cysteine residues along the polypeptide chain is fixed, regardless of the source or isoform of the protein (55). There is a high proportion of cys-x-cys sequences. The other main feature of metallothionein is its high metal content, with 7 gram atoms of cadmium or zinc per mole or up to 12 gram atoms of copper per mole. This content is equivalent to one metal atom per three or two cysteine residues, respectively. The cadmium- and copper-induced metallothioneins usually also contain zinc as a secondary metal.

All the cysteine residues are involved in metal binding, with some sulphur atoms bridging between two metal atoms, and there are no disulphide bonds. A tetrahedral arrangement of four sulphur atoms surrounds each zinc atom; a trigonal arrangement surrounds the copper atoms. Superimposed on this distribution is the localization of the metals in two polynuclear clusters in two distinct domains, each containing half of the polypeptide chain (see 55). In (cadmium, zinc)-metallothioneins the *C*-terminal or α-domain contains four metal atoms, whereas the *N*-terminal or β-domain contains only three (106). In copper-metallothionein, each domain has the potential to bind six copper atoms. There are important differences in the preferential binding of metals in the two domains: copper binding occurs preferentially in the β-domain (75) and cadmium in the α-domain (106). These differences in stoichiometry and distribution of metal binding may hold some signficance in cell recognition systems, since they might permit distinction between different metalloforms of the protein.

There are also marked differences in the avidity of metals for the protein. Copper binds in its cuprous form more firmly than cadmium, which in turn binds more firmly than zinc. These differences in binding strength are relevant to the involvement of metallothionein in metal-metal interactions.

SYNTHESIS OF METALLOTHIONEIN

Induction by Metals

Synthesis of metallothionein is induced by parenteral administration of a wide range of metals including cadmium, zinc, copper, mercury, gold, and bismuth. That control of synthesis occurs at the transcription level is shown by the inhibitory effects of cycloheximide, actinomycin D, and puromycin on the process. This effect has been confirmed by measurement of mRNA levels using cell-free translation systems and by direct assay using cDNA probes. The rate of protein synthesis closely parallels the production of metallothionein mRNA (28, 61), and a high rate of transcription can be detected within one hour of stimulation by metals. The mRNA levels reach a maximum at about 6–8 h after exposure to an inducer, although the maximal levels of metallothionein occur after 1–2 days (82).

The relative induction capacity of different metals is inversely correlated with their softness parameters, which suggests that the induction process can sense their electronic configurations (53). Cadmium is a particularly potent inducer of metallothionein synthesis and has been studied extensively in a wide range of organisms and cell systems (102, 103).

The dynamics of metallothionein synthesis in relation to zinc and copper metabolism have been comprehensively reviewed (21). Generally, a close relationship exists between zinc status and the levels of metallothionein in

tissues. Compared with cadmium and zinc, copper is a relatively poor inducer of hepatic metallothionein synthesis in mice at dose levels that approximate the induction threshold (13). At higher doses, however, there is little difference between copper and zinc in their ability to stimulate metallothionein production (13).

Several metallothionein genes have been identified in the mammalian species so far examined. Only four of the nine human metallothionein genes, which are all clustered on chromosome 16, are functional. The *cis*-acting or regulatory elements for gene expression are positioned upstream from the 5' untranslated region of the metallothionein gene. Promotion is initiated through the DNA interaction of metals or other inducing agents, which are coupled to binding molecules such as proteins (*trans*-acting factors). The precise or approximate location of the promoter sequences for different inducers has been identified by analyzing point mutations in the 5' flanking region (43). At least four distinct metal regulatory elements are involved in the promotion of mammalian metallothionein gene transcription by metals such as cadmium, zinc, and copper. A basal promoter and regulatory elements for glucocorticoids, interferon, and endotoxin-related factors have also been identified. The functionality of these sequences has been assessed by transgene studies in which a metal regulatory element is inserted in the promoter region of a foreign gene and the expression of that gene by metals is measured (24, 51, 68). Fine mapping of one of the mouse metallothionein-I metal regulatory elements has shown that a highly conserved heptanucleotide core of the 17–base pair promoter sequence plays a crucial role in the induction response (24).

Although the *trans*-acting elements for metals remain unidentified, the ability of metals with low softness parameters, for example cadmium, to promote gene transcription at low concentrations suggests that binding to the *trans*-acting factor is very stable. Much of the toxicity and detoxification of these metals is effected through interaction with thiol ligands, which would support the suggestion that –SH or –SeH groups may be involved in the induction process (53). A specific protein complex with the DNA of one of the mouse metal regulatory elements has been detected following the addition of zinc to HeLa cell nuclear extracts (105). In another study (90), complementary oligonucleotides of this metal regulatory element were used to identify a nuclear protein with a high affinity for the relevant promoter region.

Any of the functional metal regulatory elements will confer heavy metal responsiveness, although all are required for maximum gene expression. The regulatory elements promote transcription with varying degrees of efficiency, but there is no evidence of discrimination for different metal inducers. The degree of metallothionein synthesis varies widely according to the inducer and the gene. For example, the sheep fibroblast metallothionein-Ia gene is three

times more responsive to cadmium than to copper, whereas the difference is 21-fold in the metallothionein-Ic gene (84). The human metallothionein-Ig gene has at least four metal regulatory elements that are not cell-type specific, and yet cadmium, copper, and zinc induce its expression in hapatoma-derived but not in lymphoblastoid cell lines (33). In mice the metallothionein-I genes are more heavily methylated in testes than in the liver, which may account for the slower or inefficient induction and thus the greater metal sensitivity of metallothionein in the testes (8). Interestingly, the basal level of metallothionein mRNA in rat testes is very high compared with that in the liver and other tissues (91). However, X-irradiation of mice does not increase metallothionein mRNA levels in testes, although levels in other tissues are elevated.

Induction by Stress Factors

Such a large number of nonmetal factors also induce metallothionein synthesis that the question arises as to whether they stimulate synthesis independently and directly or their influence is translated by a smaller number of cellular mediators. The synthesis of metallothionein by all these agents is closely linked to the metabolism of zinc, and many of the agents characteristically increase hepatic zinc at the expense of plasma zinc. It could be argued that all known inducers, including heavy metals, are stress factors and may elicit a general stress response in addition to any specific effect. For example, cadmium induces metallothionein synthesis directly through specific gene promoters but may also induce a prooxidant state in cells (79), which could indirectly stimulate the production of natural antioxidants such as metallothionein (see below). Many of the stress inducers also raise circulating levels of glucocorticoids, which stimulate metallothionein synthesis in the liver and to a lesser extent in the heart, kidney, skeletal muscle, and spleen of the mouse (41). Glucocorticoids were therefore thought to mediate some of the effects of the stress factors on metallothionein synthesis. However, administration of adrenocorticotropic hormone to rats suppresses the increase in hepatic metallothionein levels caused by restraint stress (46). Furthermore, adrenalectomy or treatment with a glucocorticoid receptor blocker increases basal and stress-induced matallothionein levels (46). These changes in hepatic metallothionein levels may be due to modulation of the synthesis of the protein or to glucocorticoid-induced changes in its secretion or degradation.

Other steroid hormones, such as estrogens and progesterone, can also induce metallothionein synthesis (55), and studies using human cell lines have shown that the progesterone receptors bind to the same metallothionein gene regulatory elements as do the glucocorticoid receptors (92).

Catecholamines also induce metallothionein synthesis in rats (11), but there is some controversy as to whether they mediate the effects of the various stress factors. Adrenergic blockade in male rats decreased liver metallothio-

nein induction by sham adrenalectomy (10) and exogenously administered catecholamines (12), but in a subsequent study using female rats the administration of adrenoceptor blockers was ineffective (47). These results are not consistent with the relative paucity of β-receptors in male rat liver. Cyclic AMP may mediate the induction of metallothionein synthesis by epinephrine and glucagon (12, 21), because analogs of this second messenger also increase hepatic metallothionein levels at the expense of plasma zinc (26). Assay of metallothionein-I mRNA in primary rat hepatocyte cultures indicates that an analog of cyclic AMP promotes metallothionein gene transcription directly; the additive effect of dexamethasone and the cyclic AMP analog suggests that the two regulatory pathways are independent (74).

Infection

Bacterial infection also induces a marked increase in hepatic metallothionein levels and a decrease in serum zinc concentrations (94), both characteristic of an acute-phase response. Administration of bacterial endotoxin elicits many of the effects observed with the pathogen itself: rats injected with endotoxin show reduced serum zinc levels only 3 h after treatment and greatly elevated hepatic metallothionein after 18 h (1). Hepatic metallothionein mRNA levels in Syrian hamsters increase fourfold, 6 h after administration of endotoxin (30).

Several mechanisms have been suggested for the induction of metallothionein synthesis during infection. Interleukin-1, which is produced and released from monocytes and activated macrophages in response to infection, stimulates the synthesis of metallothionein and uptake of zinc by the liver of male (22) and pregnant female (49) rats. This finding suggests that glucocorticoids mediate some of the cytokine-stimulated induction of metallothionein, since interleukin-1 causes the release of these hormones via stimulation of ACTH release (49). However, studies with transgenic mice carrying metallothionein-thymidine kinase fusion genes have shown that the endotoxin-related promoter site for metallothionein gene transcription is independent of the site used by glucocorticoids and metals (27).

An alternative explanation for the induction of metallothionein by infection postulates the release of a macrophage-derived heat-stable protein factor, distinct from all other known inducers of metallothionein, including interleukin-1 (50). This factor, which is produced by macrophages in response to endotoxin exposure, stimulates metallothionein synthesis and zinc accumulation by Chang liver cells in culture (36). Moreover, the products obtained from human peripheral mononuclear cells and murine spleen cells stimulated by endotoxin or concanavalin A induce metallothionein synthesis in the human B cell line RPMI 1788 (78), whereas direct exposure of the cell line to endotoxin has no effect (1, 78). Glucagon also stimulates metallothio-

nein synthesis and may be a common mediator for interleukin-1 and endotoxin (93). In contrast to endotoxin, however, exogenous glucagon administration does not reduce serum zinc concentration.

In addition to interleukin-1, the lymphokine interferon can promote the transcription of metallothionein-II genes in human T98G neuroblastoma cells (34, 35). When Type I interferon-inducing agents, Newcastle disease virus, and the double-stranded nucleotide polyinosinic acid-polycytidilic acid were administered to low and high interferon-producing strains of mice (7), hepatic levels of metallothionein increased in both strains by both treatments, but serum interferon levels, although increased, did not correlate with those of metallothionein. The conclusion was that both interferon-inducing agents induce metallothionein by a mechanism distinct from interferon.

DEGRADATION OF METALLOTHIONEIN

In contrast to the wealth of knowledge about the biosynthesis of metallothionein, remarkably little is known of the mechanisms controlling its degradation or disappearance from tissues. Indeed the uncertainty about the fate of metals after their incorporation into metallothionein has contributed greatly to the uncertainty about the function of this protein. Pulse-labeling experiments in which rats were injected with various inducing metals and labeled amino acids indicate that the degradation rate is determined by the nature of the metal bound to the protein. Thus the half-lives of cadmium-, zinc-, and copper-induced metallothioneins are 80 (44), 20 (31), and 17 h (14), respectively, in the livers of zinc-adequate rats. Lower values are obtained in zinc-deficient animals (14, 44). Slight differences are also observed in the half-lives of the two principal isoforms of the protein; metallothionein-I has a shorter half-life than metallothionein-II (4). Similar effects of metal binding on degradation have been reported for metallothionein in Chinese hamster ovary cell lines; the half-lives of the cadmium-, zinc-, and gold-induced proteins are 24, 10, and 0.75 h, respectively (67).

Incubation of metallothionein in vitro with lysosomal and other proteolytic enzymes has confirmed that rat liver metallothionein-I is more readily degraded than metallothionein-II and that zinc-metallothionein degrades more rapidly than cadmium-metallothionein (64). These results are consistent with the concept that the ease of removal of metal from metallothionein determines the rate of degradation. Removal of metal changes the conformation of the protein from a highly ordered state to a random chain configuration and renders the peptide bonds more accessible to proteolytic enzymes. Even partial removal of metal, such as selective removal of zinc from the β-domain of (cadmium, zinc)-metallothionein, renders the protein susceptible to attack by neutral proteases and yields the cadmium-containing α-fragment as a

stable product (106). Conversely, removal of zinc from (copper, zinc)-metallothionein leads to the production of the copper-containing β-fragment upon treatment with subtilisin (75). In both cases the holoprotein is resistant to proteolysis by subtilisin or other neutral proteases.

That metal removal is a rate-limiting step in the degradation of metallothionein is further indicated by the similarities in the apparent degradation rate and the rate of removal of metal from the protein (14, 31). However, these results could be an inevitable consequence of the analytic techniques used if the apoprotein exhibits abnormal chromatographic behavior because of aggregation phenomena. Nevertheless, the rate of degradation of metallothionein in monolayer cultures of rat hepatocytes (17) and in HeLa cells (58) is inversely proportional to cellular zinc content and is increased when the cells are grown in zinc-deficient medium. Under these conditions, loss of zinc from the protein is likely to increase its rate of degradation. Also significant is that the half-life of zinc-metallothionein in regenerating rat liver, where zinc demand is high, is shorter than that in liver from zinc-injected rats (81).

Different conclusions have been reached in studies on the kinetics of the degradation and removal of zinc from ^{35}S-labeled zinc-metallothionein in cultured Ehrlich ascites tumor cells (59). The rate constant for loss of zinc was at least 0.6 h^{-1}, whereas the rate constant for biodegradation of ^{35}S-labeled protein was 0.07–0.014 h^{-1} in control cells and 0.12–0.18 h^{-1} in cells treated with Chelex. The conclusion was that biodegradation of metallothionein protein cannot account for the rate of loss of zinc from the protein and that loss of zinc may also occur by metal-ligand exchange processes.

Very little is known about the site of metallothionein degradation, although a common assumption is that degradation occurs in the lysosome, since this organelle plays a general role in the degradation of other proteins and lysosomal enzymes can degrade metallothionein in vitro (64). However, recent studies on the effects of chloroquine, a lysosomotrophic amine, and of a neutral protease inhibitor indicate that both lysosomal and nonlysosomal compartments are involved in the degradation of zinc-metallothionein in hepatocytes (17).

Although studies in vivo and in vitro are both in agreement on the susceptibility of cadmium- and zinc-metallothioneins to proteolysis, this is not the case with copper-metallothionein. This metalloform of the protein is readily degraded in vivo but is resistant to attack by lysosomal enzymes in vitro (64). The stability in vitro is consistent with the greater strength of the copper-thiol bond and the concept that ease of removal of metal determines the rate of degradation.

One possibility is that the disappearance of copper-metallothionein from the cytosol in vivo is associated not with its degradation but with its uptake by lysosomes, where it may accumulate in an aggregated form, as in the liver of Bedlington terriers (52). The half-life of particulate copper-metallothionein in

rat liver is only 25 h, however, which indicates rapid turnover (64). The particulate copper-metallothionein found in fetal liver is assumed to be present also in lysosomes, but fractionation of fetal deer liver before and after selective disruption of lysosomal membranes with digitonin did not support this view (60). The protein appeared to be present in nuclei, which is consistent with the immunolocalization studies carried out with other species (72, 73, 83).

Another reason for the discrepancy between the results in vivo and in vitro may be that degradation in vivo is preceded by modification of the protein, possibly through the action of oxygen free radicals. Such treatment releases zinc from (copper, zinc)-matallothionein in vitro and renders the protein susceptible to degradation (5; H. Cunningham and I. Bremner, unpublished observations). Alternatively, the disappearance of copper-metallothionein from the liver in vivo could be due to its secretion into the bile or plasma. Several components that react with antibodies to metallothionein have been detected in the bile of copper-injected rats (87). These correspond in chromatographic behavior to monomeric copper-metallothionein and to aggregated and degraded forms of the protein. The amount of immunoreactive material excreted in the bile accounts for only 1–2% of the metallothionein lost from the liver, however.

Metallothionein is also present in the plasma of rats, albeit in relatively low concentrations. Concentrations increase in animals subjected to inflammatory or physical stress or dosed with metals (46, 88). The liver seems to be the main source of extracellular metallothionein, as plasma and liver metallothionein concentrations are often (but not invariably) correlated. Approximately 10% of the hepatic copper-metallothionein in copper-loaded rats may be secreted into plasma, but whether this occurs by active secretion or leakage from cells is unknown (15). Hidalgo et al (46) suggest that glucocorticoids have a permissive role in mobilizing metallothionein from tissues to serum. However, proper assessment of the contribution of secretion to the turnover of metallothionein requires determination of its flux rate through the plasma pool.

Conflicting results have been obtained on the secretion of metallothionein from cultured cells. Surprisingly only 1% of total cellular metallothionein was secreted into the medium when rat hepatocytes were maintained in monolayer cultures (17). Significant secretion was recorded, however, when hepatocytes were cultured in medium containing insulin and hepatocyte growth factor (99).

FUNCTIONS OF METALLOTHIONEIN

In considering the functions of metallothionein, one must remember that it is present in varying amounts in most tissues, it binds several essential and

nonessential metals, and its synthesis is induced by these metals and also by physical or inflammatory stress. Moreover it is a highly conserved protein in evolutionary terms. Therefore, the protein either performs one basic role common to all these conditions or has a multiplicity of functions, depending on the particular circumstance. The consensus view appears to favor the latter option, since metallothionein has been reported to act in the detoxification of heavy metals, in the homeostatic control of zinc and copper absorption and metabolism, as a metal-transfer protein, as a metal-storage protein, in the regulation of cell differentiation, as a sulphur-storage protein, as a free-radical scavenger, and as an acute-phase protein. Despite the unusual structure of metallothionein and its ability to bind metals selectively in its two domains, that any protein could be so versatile seems unlikely.

Detoxification of Heavy Metals

Not surprisingly, particular attention has focused on the involvement of metallothionein in metal metabolism. Metallothionein was first discovered as a cadmium-binding protein, and speculation began soon afterward as to its role in the detoxification of cadmium and other heavy metals. Much evidence supports such a role, as the cytotoxic effects of cadmium become apparent only when its binding to metallothionein is saturated (103). Furthermore, prior induction of metallothionein synthesis by treatment with zinc has a protective effect against cadmium toxicity (25), and cell lines that show increased cadmium tolerance often have elevated metallothionein contents (29). However, detoxification of heavy metals is probably an adventitious rather than a primary role of metallothionein, and it may reflect the chemical similarities between cadmium and zinc. In evolutionary terms, metallothionein was more likely produced to participate in the metabolism of essential rather than nonessential elements.

Control of Zinc and Copper Absorption

The presence of metallothionein in most if not all cell types suggests that it plays a general role in the handling of metals. Nevertheless, metallothionein may be specifically involved in the control of the intestinal absorption of zinc and copper, since an inverse relationship exists between the efficiency of zinc absorption in the rat and the binding of the metal to intestinal metallothionein (21, 66, 85). In zinc-deficient animals, in which the metal is absorbed with high efficiency, little metallothionein is present in the intestinal mucosa to limit transfer of zinc into the plasma. In contrast, in zinc-loaded animals, where zinc absorption is low, the metal is apparently incorporated into intestinal metallothionein, thus limiting its transfer into the plasma. This hypothesis, however, was originally based on experiments in which rats

received a large parenteral dose of zinc and which therefore may have limited physiologic relevance.

Zinc loading increases the size of the endogenous mucosal zinc pool, and failure to take this increase into account can lead to a false impression of changes in the efficiency of zinc absorption (96). Moreover, no clear inverse relationship exists between mucosal metallothionein concentrations and zinc absorption in mice (32, 96). Indeed metallothionein has been suggested to play a facilitating role in zinc absorption. Recent studies involving intestinal perfusion of zinc-adequate and zinc-deficient rats and of fasted animals conclude, however, that the rate of zinc absorption is indeed inversely related to intestinal metallothionein levels, but the portion of mucosal ^{65}zinc available for absorption is directly related to intestinal metallothionein (48). The mucosal zinc buffer role of metallothionein and the opposing view of a facilitating role in zinc absorption may therefore not be mutually exclusive views of a function in the intestine.

Little change in mucosal metallothionein concentration was found over a range of zinc intakes in rats, among which changes in zinc absorption have been reported (42). Only with very high zinc intakes were there reports of major increases in metallothionein levels; in such circumstances one cannot exclude the possibility that the protein is involved in the control of zinc excretion, an idea consistent with aspects of the immunocytochemical localization of the protein in the gut (76). Furthermore, when rats were given diets with 1, 6, or 36 mg copper and 5, 30, and 180 mg zinc/kg, a significant increase in metallothionein mRNA levels was recorded only in animals given 180 mg zinc and 1 mg copper/kg (9). This finding is consistent with the associated increase in the amount of copper and zinc bound in intestinal metallothionein (80) and indicates that metallothionein gene expression was highest in that group. However, the treatments had no effect on the absorption of either metal (80). Zinc appears better able to bind to factors that interact with the metallothionein promoter and stimulate transcription when copper intakes are low. The importance of metallothionein in regulating zinc absorption over a physiologic range of intakes remains unclear, but from a teleologic point of view, a system designed to regulate zinc absorption would be unlikely to depend on increased synthesis of a protein that binds copper more avidly and instead inhibits its absorption (see below).

The absence of a direct effect of dietary copper on intestinal levels of metallothionein mRNA confirms that copper is not an effective inducer of metallothionein synthesis in the gut. The protein is unlikely to play any part in the homeostatic regulation of copper absorption in response to changes in copper supply (42). Nevertheless, an inverse relationship between copper absorption and intestinal metallothionein levels can occur as in rats subjected to tumor implantation or dosed with estrogens (19). The decreased efficiency

of copper absorption in brindled mice is also associated with increased intestinal metallothionein levels (23). Furthermore, the antagonistic effect of zinc on copper absorption has been attributed to zinc-induced synthesis of metallothionein, followed by increased and preferential binding of copper to the protein (42). Copper incorporated into mucosal metallothionein is assumed not be be transported into the plasma but eliminated on desquamation of the intestinal cells. However, no detailed investigation has ever been carried out of the fate of mucosal metallothionein. Because the primary defect in the brindled mice, as in patients with Menkes's disease, appears to be the efflux of copper from cells, the elevated gut levels of metallothionein probably reflect the increase in intracellular copper concentrations with resultant stimulation of metallothionein gene transcription, rather than any involvement in homeostatic regulation of copper absorption.

Hepatic Zinc and Copper Metabolism

The liver is generally regarded as one of the main sites of metallothionein synthesis in the body. This conclusion, however, is based largely on the results of experiments of limited physiologic relevance that involve administration of large parenteral doses of metals. When rats are given diets with normal levels of zinc and copper, only minor changes occur in liver metallothionein and metallothionein mRNA content, despite the fact that renal concentrations increase in response to dietary supplements of the metals (9).

The failure of modest dietary zinc supplements to induce hepatic metallothionein synthesis in the rat probably reflects the absence of any significant increase in tissue zinc content. Other studies have found that as rat-liver zinc concentrations increase above a threshold concentration of approximately 30 μg/g wet weight, most of the additional zinc is bound to metallothionein (13). Conversely, liver metallothionein and metallothionein mRNA concentrations in zinc-deficient rats rapidly decrease to nondetectable levels (16, 62).

Concentrations of liver metallothionein are greatly increased in fetal and neonatal animals (104). In most species, but not the bovine or the guinea pig, the protein binds zinc as the predominant metal (6). Fetal and neonatal concentrations are reduced if the maternal rats are given a zinc-deficient diet during pregnancy and lactation, but maternal copper and iron deficiencies have no effect (37, 38, 69). These results suggest that zinc is the main inducer of hepatic metallothionein synthesis during fetal development, although there may also be some hormonal stimulus. There has been speculation that the protein acts as a storage reserve for zinc, but the amount of metal deposited in this form meets only a small proportion of subsequent demands for zinc.

Another possibility is that the high concentrations of metallothionein are related to the demands for zinc during the processes of cell differentiation and

proliferation, since this element plays a key role in the control of gene expression (104). The finding that partial hepatectomy also results in increased metallothionein synthesis supports this view (81), but Webb has argued that this increase is merely a stress response (104). Moreover, if metallothionein is needed to sustain cell differentiation during development, why are elevated concentrations found only in the liver?

Although hepatic metallothionein concentrations are low in adult rats, this is not true for all species. A high proportion of the zinc in human liver can be in this form (18), and in pigs 80% of the copper is bound to metallothionein in both the cytosolic and particulate fractions of the liver (63). The binding of liver copper to metallothionein is complex: it is affected by species, age, route of administration of copper, and zinc status (13).

Although copper injection induced hepatic synthesis of metallothionein in the rat, dietary supplementation with copper does so only after liver copper levels exceed a threshold value of approximately 500 μg/g (15). A critical concentration of free or labile copper may be required before an interaction with the metal regulatory elements in the promoter region of the gene can occur. Copper, therefore, is not a particularly effective inducer of metallothionein synthesis in rat liver, and its binding to the protein may often depend on prior induction of its synthesis by some other agent, possibly zinc. Copper readily displaces zinc from metallothionein, both in vitro and in vivo. Although the metal complement of the isolated protein is always assumed to be the same as that within the cell, some redistribution of metals after homogenization of the tissue cannot be excluded.

Metallothionein does not appear to play an obligatory role in hepatic copper metabolism because this metabolism is normal even when no copper-metallothionein is present, as occurs in zinc-deficient animals. Metallothionein may act as a storage reserve for copper, and indeed it has been suggested that the copper-metallothionein present in fetal liver is required to meet copper demands during the rapid postnatal growth phase. However, the accumulation of copper during this period probably reflects the immaturity of biliary excretion mechanisms and the production of metallothionein is likely a consequence of the increased intracellular concentrations of copper. The main role of metallothionein in copper metabolism is therefore most likely to be in the cellular detoxification of the metal. Thus, the cytotoxicity of copper is reduced in the liver of species, such as the pig and dog, in which most of the hepatic copper is bound to metallothionein (52, 63). In species such as the sheep, in which only a small proportion of the copper is bound in this way, the hepatotoxic effects of copper are relatively great (63). This effect is consistent with the postulated role of the related copper-thionein in yeasts, in which removal and insertion of the copper-thionein gene respectively decreases and increases their tolerance of copper (98).

Metallothionein as a Metal-Transfer Protein

A major problem in elucidating the role of metallothionein is that little is known of the fate of metals incorporated into the protein. As indicated above, synthesis of metallothionein enables organisms to adapt to changes in intracellular concentrations of zinc and copper and prevents adverse reactions with enzymes, membranes, or other molecules. It may also provide a buffering capacity that maintains intracellular steady-state kinetics for copper and zinc and ensures a supply of these metals for other metabolic functions. Direct in vitro exchange reactions between zinc-metallothionein and the apo-form of zinc-dependent enzymes have been reported (101), although this process is sometimes less efficient than incorporation of free zinc ions. Nevertheless the kinetics of zinc transfer from metallothionein, at least in Ehrlich cells, suggest there are rate-limiting ligand-substitution processes that do not involve degradation of the protein (59). Similar claims have been made for transfer of copper from metallothionein to apo-enzymes, but this process depends, at least in vitro, on prior oxidation of the copper (40).

Metallothionein as an Acute-Phase Protein

Synthesis of metallothionein by increased gene transcription provides an effective homeostatic regulatory mechanism to adapt to increases in intracellular metal concentrations. Less readily explained is the increased accumulation of metallothionein that occurs in the liver and, to a lesser extent, in bone, thymus, and other tissues in animals subjected to different types of stress. These stresses include restriction of food intake, bacterial infection, and inflammatory and physical stress such as restraint or exposure to high or low temperatures. The increased synthesis of metallothionein is accompanied by increased tissue zinc concentrations, and the protein occurs with zinc as the predominant bound metal. However, the increased zinc uptake is the consequence, not the cause, of the induction of metallothionein synthesis (57). This induction appears to be mediated by glucagon, glucocorticoids, and cytokines such as interleukins, tumor necrosis factor, and other as yet unidentified factors.

The hypozincemia that usually occurs in stressed animals appears to be a consequence of the induction of hepatic metallothionein synthesis and zinc is apparently removed from the circulation to occupy the vacant binding sites on the protein (22).

Synthesis of metallothionein under these circumstances can be regarded as part of an acute-phase response and, like synthesis of other acute-phase proteins, is probably designed to impart stress resistance or tolerance to cells and to maintain homeostasis. The benefit to the animal may arise from the influx of zinc into the cell, as this metal plays key roles in DNA metabolism and gene expression. Alternatively, it may arise from the ability of metallothionein to act as a free-radical scavenger.

Metallothionein as a Free-Radical Scavenger

The possible role of metallothionein as a free-radical scavenger has recently attracted considerable attention (13, 56). Aerobic radiolysis of an aqueous solution of metallothionein induced metal ion loss and thiolate oxidation. Damage by hydroxyl radicals appears to involve the metal-thiolate clusters and can be reversed by exposure to low pH and addition of glutathione and metals (100). Oxidation of thiols in vivo is probably limited to the disulphide level, and regeneration by glutathione may be possible. Metallothionein is reported to be much more effective than glutathione in preventing degradation of DNA by hydroxyl radicals (2).

Biological evidence of a free-radical scavenging role for metallothionein is based largely on electron spin resonance studies or changes in lipid peroxidation. For example, the concomitant increase in lipid peroxidation and metallothionein levels in rat liver after food and water deprivation and their further enhancement in the presence of dimethyl sulfoxide suggest a possible link between these factors (45). The enhancement of hepatic metallothionein caused by the deprivation stress was less marked, however, when the diet contained supplements of the antioxidant vitamin E, which suggests possible competition between metallothionein and vitamin E for free-radical scavenging. The resistance to oxidative stress of cadmium-tolerant Chinese hamster cells has been attributed to their high metallothionein content (65). Protection against chemical-induced free-radical damage in rat hepatocyte cultures was obtained by addition of zinc to the culture medium. Because the addition of zinc also increased cellular metallothionein levels, this protein could be one of the mediating factors scavenging free radicals and reducing membrane damage (20). The cardiac toxicity of the antitumor agent adriamycin is thought to result from extensive lipid peroxidation and can be reduced by pretreatment with heavy metals that increase heart metallothionein levels. Because this increase is positively correlated with the survival rate of mice after injection of adriamycin and negatively correlated with the degree of lipid peroxidation, an antioxidant role for metallothionein has been proposed (89).

The measurement of free-radical scavenging in biological systems is, by necessity, indirect, and evidence for this role for metallothionein is still tentative. Indeed, (copper, zinc)-metallothionein actually stimulated the lipid peroxidation (5) initiated by xanthine and xanthine oxidase in incubations of rat-liver microsomes. The resulting aggregation of the protein may have exposed copper in a form capable of initiating lipid peroxidation. Moreover, if metallothionein is an important antioxidant, its presence at high levels might be expected to inhibit further synthesis in response to the generation of free radicals. However, preinduction of hepatic metallothionein by zinc treatment of rats did not result in a lower metallothionein response to immobilization stress (45). Evidence also indicates that metallothionein synthesis in the newborn rat liver is independent of glutathione levels (39).

DIAGNOSTIC VALUE OF METALLOTHIONEIN ASSAYS

Analogies have frequently been drawn between the roles of ferritin and metallothionein in the control of iron and zinc metabolism, respectively. Concentrations of the proteins in tissues tend to reflect those of the metals, and small amounts of the proteins are secreted into the plasma. Assay of serum ferritin is used to assess iron stores and diagnose subclinical iron deficiency. Assay of metallothionein might provide similar information on zinc status, although it is not a specific zinc-binding protein (69, 88). Indeed, urinary metallothionein excretion has been used to assess cadmium exposure in humans (77). Plasma metallothionein concentrations are increased in copper-loaded animals in proportion to the increase in liver metallothionein content and could in theory be used to diagnose copper toxicosis (15). Nevertheless, zinc status is a major determinant of hepatic metallothionein concentrations, and zinc deficiency is possibly the only condition that reduces them.

Plasma metallothionein levels are reduced to nondetectable levels in rats given a zinc-deficient diet, whereas they are increased in animals subjected to certain types of stress (46, 88). Because the occurrence of stress-induced hypozincemia is one of the main reasons low plasma zinc levels cannot be used for unequivocal diagnosis of zinc deficiency, assay of plasma metallothionein could aid interpretation of this standard diagnostic test. If plasma concentrations of both zinc and metallothionein were low, zinc deficiency would be confirmed. Unfortunately, if zinc deficiency occurs in association with stress or infection, intermediate values for plasma metallothionein can be obtained (88). A similar problem occurs if urinary metallothionein concentrations are measured to obtain information on zinc status (16). These concentrations are decreased in zinc deficiency and increased after treatment with endotoxin, but intermediate values occur when the treatments are combined.

Metallothionein also occurs in blood cells at concentrations that, at least in young rats, considerably exceed those found in plasma (16). These concentrations decrease in a dose-dependent manner as dietary zinc concentrations are reduced and, moreover, are not greatly affected by stress factors; they, therefore, could provide a more specific and sensitive index of zinc status than that provided by plasma metallothionein levels (16). Density gradient centrifugation of rat blood cells shows, however, that metallothionein is not evenly distributed but is present mainly in the lightest and youngest cells, including the reticulocytes (70). This finding is consistent with studies on the maturation of rat reticulocytes in vitro that show that the protein has a half-life of only 6 h (N. Noble and I. Bremner, unpublished observations). It also explains why only low concentrations of metallothionein are found in the eldest erythrocytes from control and zinc-injected rats (70).

An important consequence of these findings is that any condition that affects erythropoietic activity and reticulocyte counts is also likely to affect blood cell metallothionein concentrations. Thus concentrations are increased in rats with hemolytic (70) or iron-deficiency (86) anemia, whereas they are decreased in food-restricted (86) and severely protein-deficient (71) rats. Similarly in cadmium-treated mice, erythropoietin administration increases and transfusion decreases erythrocyte cadmium-metallothionein levels (97). There is no indication that these treatments affect the specific synthesis of metallothionein; they simply modify the production of short-lived cells with a high metallothionein content. Any attempt to use assay of metallothionein to assess zinc status must take note of this fact.

The findings on the occurrence of metallothionein in reticulocytes of young rats does not necessarily apply to humans, since reticulocyte counts in these animals are much higher than those in humans. Fractionation on Percoll gradients of blood cells from humans has confirmed that the lightest cells have more metallothionein than the densest cells, but the differences are much less than those found in rats (F. Branca and I. Bremner, unpublished results). However, unlike the situation in rats, in which insignificant amounts of the protein occur in white blood cells, approximately 15% of the metallothionein in human blood cells is present in mononuclear cells. Leucocytes can synthesize metallothionein when cultured in cadmium-supplemented medium or, if they are stimulated by phytohemagglutinin, in zinc-supplemented medium (3, 95). Measurement of metallothionein in these cells may also provide information on trace metal status.

However, the plethora of conditions that induce metallothionein synthesis, from changes in metal status to acute-phase reactions, render the interpretation of measurements in isolation difficult.

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