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Induction, Regulation, Degradation, and Biological Significance of Mammalian Metallothioneins

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ABSTRACT: MTs are small cysteine-rich metal-binding proteins found in many species and, although there are differences between them, it is of note that they have a great deal of sequence and structural homology. Mammalian MTs are 61 or 62 amino acid polypeptides containing 20 conserved cysteine residues that underpin the binding of metals. The existence of MT across species is indicative of its biological demand, while the conservation of cysteines indicates that these are undoubtedly central to the function of this protein. Four MT isoforms have been found so far, MT-1, MT-2, MT-3, and MT-4, but these also have subtypes with 17 MT genes identified in man, of which 10 are known to be functional. Different cells express different MT isoforms with varying levels of expression perhaps as a result of the different function of each isoform. Even different metals induce and bind to MTs to different extents. Over 40 years of research into MT have yielded much information on this protein, but have failed to assign to it a definitive biological role. The fact that multiple MT isoforms exist, and the great variety of substances and agents that act as inducers, further complicates the search for the biological role of MTs. This article reviews the current knowledge on the biochemistry, induction, regulation, and degradation of this protein in mammals, with a particular emphasis on human MTs. It also considers the possible biological roles of this protein, which include participation in cell proliferation and apoptosis, homeostasis of essential metals, cellular free radical scavenging, and metal detoxification.

I. METALLOTHIONEIN

Metallothionein (MT) was first identified as a cadmium-binding protein in equine kidney in 1957[1] and was subsequently purified and characterised by Kägi and Vallee.[2,3] MT is characterized by its low molecular weight (6 to 7kDa), high metal content, characteristic amino acid composition — high content of conserved cysteine residues and absence of aromatic amino acids - spectroscopic features indicating tetrahedral-thiolate complexes and metal thiolate clusters.[4] MT is a ubiquitous metal binding protein with strong affinity for group Ib and IIb transition metals. MT is a major zinc binding intracellular thiol and in many cases represents the single most abundant intracellular protein thiol. However, despite the accumulation of detailed information on both the biochemical and molecular aspects of MT structure and expression, its biological role is still not clearly understood more than 40 years after its discovery. MT is thought to be involved in the homeostasis of essential metals[5-10] and metal detoxification,[6,11-16] as originally suggested by Piscator in 1964,[17,18] although it also appears to act as a potent free radical scavenger.[19-21]

II. DEFINITION AND CLASSIFICATION OF MT

MTs were originally classified according to the definition that they should be "polypeptides resembling equine renal metallothionein in several of their features".[22] MTs were initially divided into three classes according to their structural characteristics. Class I MTs are defined as polypeptides with a high degree of cysteine conservation compared to those in equine kidney. Class II MTs are polypeptides with less well conserved cysteine residues, only distantly related to equine MT. Class III MTs are defined as atypical, nontranslationally synthesized metal thiolate polypeptides.[22,23] This classification has now been superseded by a complex but more stringent evolutionary classification into families, subfamilies, subgroups and isoforms.[24]

There is considerable sequence homology amongst MTs from different species. For example, in mammals 56% of all MT residues are conserved. These comprise all 20 cysteine residues and most of the lysines, serines and arginines.[25] These conserved residues are thought to play a role in metal binding.[26] Lysines may be involved in the detoxification function of MT[27,28] and those in the alpha domain appear to be important in maintaining the conformational integrity of the protein[28] by interaction with the metal thiolate clusters.[29] Studies using mutant MTs suggest that the conserved serines in the MT sequence could play a role in the stability of metal-binding ligands.[30]

There is also similarity across phyla. MT in *Neurospora crassa* contains only 25 amino acids and while it is significantly smaller than mammalian MT, its primary structure is very similar to the amino-terminal half of mammalian MTs.[31] Thus, the class I MTs display divergent evolution, since the differences in amino acid sequence increase with increasing taxonomic distance. In contrast, the existence of the cys-x-cys and cys-cys sequences in the otherwise unrelated class I and class II MTs is evidence for convergent evolution probably as a consequence of metal complexation requirements.[25]

III. BIOCHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF MT

Mammalian MTs are single chain polypeptides of 61 to 68 amino acid residues with an N-terminal acetylmethionine and often alanine at the carboxyl terminus. Mammalian MTs contain 20 cysteine residues, which are central to the binding of metals. In fact, all MTs have characteristic cys-x-cys, cys-x-y-cys, and cys-cys sequences, where x and y are non-cysteine amino acids. The stoichiometry is such that there are 7 bivalent ions for every 20 cysteines, which form metal thiolate complexes, thereby enabling the MT to bind between 7-10 g atoms of metal/mol MT in a two domain structure.[32] The intramolecular metal linkages stabilise the protein secondary structure and hence, loss of metal causes structural changes, rendering the polypeptide chain vulnerable to proteolysis.[33] Stability is also influenced by the nature of the metals bound to MT. In mammals, predominantly Zn^{2+}, but sometimes also Cu^{+} are bound *in vivo* under physiological conditions, but several other less abundant transition metals, such as Cd^{2+}, Bi^{3+}, Pt^{2+}, Ag^{+}, and Hg^{2+} also bind avidly to MT either *in vitro*, or following their administration to animals.[11] It would appear however that *in vivo*, pure forms of metal-metallothionein complexes do not occur since there is a rapid redistribution of the metals to generate mixed-metal proteins as demonstrated for Zn- and Cd-metallothioneins.[34,35] Interindividual and tissue variations in the metal composition of MT are thought to relate to differences in metal exposure.[32] The binding affinity of different metals for MT varies considerably and has the following order: $Zn^{2+} < Pb^{2+} < Cd^{2+} < Cu^{+} < Ag^{+} = Hg^{2+} = Bi^{3+}$, thereby making zinc readily displaceable by other metal ions.[32]

IV. MAMMALIAN MT ISOFORMS

All mammalian species so far examined have multiple MT genes coding for a family of isoforms or isometallothioneins. In mammals, MTs fall into at least four subgroups, namely MT-1, MT-2, MT-3, and MT-4. The MT-1 and MT-2 isoforms, which differ by only a single negative charge, are the most widely expressed isoforms in different tis-

sues[25,31] and are the best studied. Within the main MT-1 and MT-2 charge-forms, isoforms of similar charge but distinct structure have been observed.[36-42] These isoforms are designated by a lower case letter, e.g., MT-1a, and they vary in number depending on the species.

Human MT genes are clustered at a single locus on chromosome 16 (16q13)[43,44] and at least 10 of the 17 genes so far identified, are functional. These encode multiple isoforms of MT-1 and, usually, a single isoform of MT-2.[45] Only single genes for MT-3 and MT-4 have been detected. The expression and the gene sequences of the functional genes have been reported for MT-1a,[46] MT-1b,[47] MT-1e and MT-1f,[48] MT-1g,[49] MT-1h and MT-1x,[45] MT-2a,[50] MT-3,[51] and MT-4.[52] The non-functional MT genes are MT-1c and MT-1d,[46] MT-1i, MT-1j, MT-1k and MT-1l,[45] and MT-2b.[53] A putative gene named MT-0 was identified from its cDNA sequence and found to be expressed in foetal liver[54] and also in monocytes.[55] However, gene sequencing revealed that MT-0 is in fact the same as MT-1h,[45] which is also expressed in brain.[56] MT-3 is preferentially expressed in the brain,[51] although recently it has also been detected in the maternal decidium, reproductive tissues, tongue, stomach, and heart as well as the kidney.[57-60] The MT-4 gene from mouse and human has been cloned and the expression of the gene appears to be limited to squamous epithelial cells.[52]

In pig, ten MT isoforms have been so far identified. Seven of them belong to the MT-1, two belong to the MT-2 and one MT-3 families,[61,62] whereas there are at least four functional genes (MT-1a, MT1b, MT-1c, and MT-2) and a pseudogene in sheep.[63] This diversity in MTs contrasts with that of other species such as rodents, in which only four functional MT genes are known. For example, murine MT genes are located on chromosome 8 and encode the isoforms: MT-1, MT-2, MT-3 and MT-4.[51,52] Metallothioneins may also show heterogeneity due to posttranslational modifications and MT from Zn and Cd-treated rats contains significant amounts of unacetylated MT-2.[64,65]

Constitutive levels of MT expression can be found in most cells, although these vary with cell type.[66] In man, basal levels of expression for the MT-2a gene appear to be higher than the MT-1.[44,61,62] Richards and co-workers found that human MT-2a was expressed about five times more efficiently than MT-1a in HeLa cells, osteosarcoma TK cells and human skin fibroblasts; all the MT-1 isoforms together made up 50% of the total human MT protein. This suggests that MT-1 isoforms are poorly expressed,[46] and the difference between MT-1 and MT-2 forms has been attributed to the enhancer activity in the MT-2a promoter.[67] Furthermore, differential expression of the human MT isoforms can be detected in various cell types.[46,67] For example, basal levels of MT-1b were detected in human hepatoblastoma HepG2 cells, hepatocarcinoma Hep3B2 cells and two renal carcinoma cell lines (A1212 and A498) but not in HeLa cells or in human skin fibroblasts.[47]

V. INDUCTION AND REGULATION OF MT

A range of different stimuli can induce MT. These include metals, hormones, cytokines, a variety of other chemicals, inflammation, and stress.[5] The most extensively studied of these inducers are metals and glucocorticoids, both being efficient inducers of MT. However, both these entities display species differences in isoform induction. In mice, both metals and glucocorticoids equally induce MT-1 and MT-2;[68] in man, metals induce all the MT

isoforms whereas glucocorticoids only induce MT-2a and MT-1e.[69] Thus, human MT isoforms are regulated independently of each other whereas in mouse the MT-1 and MT-2 isoforms are co-ordinately regulated.[68,70] With the exception of glucocorticoids, only metals have been shown thus far capable of inducing the human MT-1 isoforms. This confirms the apparent simplicity of the human MT-1 promoter region compared with that of MT-2a, which contains several enhancer regions.[69] Furthermore, the induction of different isoforms appears to be metal dependent as are the rates, extent and time course of MT mRNA transcript accumulation.[67,71] Recently, considerable progress has been made in elucidating the mechanism by which metals induce MT and the way in which expression of this protein is regulated, although the latter is not yet fully understood.[72] The regulation of the MT genes has been the subjects of extensive review elswhere.[66,69,72-83]

A. Transcriptional Control

Metal-induced MT synthesis is thought to be mediated via short *cis*-acting DNA sequences or metal responsive elements (MREs) which are present as multiple copies in the promoter region of all the MT genes.[84,85] These regions are conserved among many diverse organisms but are not all functionally equivalent.[66] Indeed, variations in the ability of different MREs to mediate metal-activated transcription of MT genes have been reported.[86] For example, of the five MREs in the mouse MT-1 promoter, MREd appears to be the strongest enhancer of MT gene transcription.[87] Similarly, glucocorticoid-stimulated MT induction is mediated via a glucocorticoid responsive element (GRE), although this is present in some but not all MT genes.[46,84,88-90] Antioxidant response elements (ARE) mediating induction by H_2O_2 and redox-active quinones are also present,[91] although MREs, such as MREa, MREb and MREd, also respond to oxidants.[72] Other cis-acting sequences as well as cyclic AMP responsive elements, TPA-responsive elements and interferon responsive elements have also been defined in a few MT promoters.[72,73,77,78]

During metal induction of MT, proteins which are thought to be positively acting transcription factors bind with MREs in a metal-dependent manner.[88] Following metal treatment, several different proteins from nuclear extracts of cells of both rodent and human origin have been identified as possible regulators of metal-mediated gene transcription.[72] These include among others (1) a 74-kDa protein, metal response element binding factor-I (MBF-I) from mouse fibroblasts,[92] (2) p108 (MEP-1), a 108-kDa protein also from mouse fibroblasts,[93] (3) zinc-activated protein (ZAP, approximately 108 kDa) from rat liver,[94] (4) zinc regulated factor (ZiRF1, which is distinct from ZAP) from mouse L cell fibroblasts and NIH3T3 cells[95] and (5) a 39-kDa protein from rat hepatoma Fao cells.[96] These proteins display different binding affinities for the various MREs. Thus, MBF-I and p108 are found to bind preferentially to MREe and MREa of the mouse MT-1 promoter, respectively.[92,93] Furthermore, ZAP has been identified in rat liver following exposure to zinc,[94] while the 39-kDa MRE binding protein has been detected following exposure to cadmium.[96] Binding of two metal-dependent proteins (approximately 28 kDa) to a synthetic consensus human MRE sequence has been observed in extracts from various cells of human origin (HepG2 cells, lung carcinoma A549 cells and leukaemia cell lines HL-60 and K562 cells) that have been exposed to cadmium, zinc, and copper.[97] However, although variability in binding activ-

ity with different metals has been reported, it is not clear if the binding of these proteins is metal specific.[97]

None of the metal-dependent factors described above have been shown to actually affect metal-induced transcription of MT genes. In contrast, two proteins identified from nuclear extracts of HeLa cells do affect metal-mediated MT gene transcription. These are MREBP (MRE binding protein) that specifically binds MREs of the human MT-2a gene and MTF-1 (MRE binding transcription factor), a constitutively active zinc-sensitive transcription factor. MREBP is thought to inhibit transcription,[98] whereas MTF-1 is thought to have an important role in the control of MT gene expression.[83,85,99]

Both the human MTF-1 (hMTF-1)[100] and the mouse MTF-1 (mMTF-1)[85] have been cloned. hMTF-1 is a 753 amino acid protein which shares a 93% amino acid sequence identity with mMTF-1, although it is 78 amino acids longer at the C terminus than mMTF-1.[100] Each contains six zinc fingers of the cys_2-his_2 type in the DNA binding domain and three different transactivation domains. The activity of MTF-1 is thought to be controlled via complex interdomain interactions and by the first zinc finger, which is saturated with zinc only at higher concentrations than the other zinc fingers.[101] Thus, unactivated MTF-1 with Zn-saturated fingers 2 to 6 can be activated by physiological increases in zinc.[102] Using mouse embryonic stem cells with targeted disruption of the MTF-1 gene, it has been found that this factor is essential for both constitutive and metal-induced MT expression, since neither MT-1 nor MT-2 were detectable in MTF-1 null cells even after metal exposure.[103]

Zinc is the only metal that activates MTF-1, but, surprisingly, there is evidence suggesting that oxidant stress also activates this transcription factor, resulting in increased binding to MREs.[83,104] It is possible that oxidation of cellular ligands binding zinc may release metal to activate MTF-1. There is also evidence that hypoxia activates MT gene expression through MREs and that this activation involves MTF-1.[105]

Palmiter has proposed that the constitutively active MTF-1 is under the control of a metal-sensitive metallothionein transcription inhibitor (MTTI) that prevents the MTF-1 from interacting with MREs.[106] Furthermore, he has also proposed that zinc is involved in the dissociation of MTTI from MTF-1 and that MTTI may be sensitive only to zinc. Because intracellular zinc is bound tightly to MT but less tightly to other proteins in the cell, other metals may cause zinc to dissociate from weak binding ligands and influence the association of MTTI with MTF-1. A subsequent increase in MT levels would bind labile zinc, thereby restoring equilibrium.[106] However, it is not clear if all metals stimulate the same transcription factor(s) and/or MREs and if there are different MTTIs.

The discovery of so many factors with different sizes and properties that are able to bind MREs indicates that the regulation of MT genes is very complex and possibly involves interaction among these factors as well as negative regulators.[106,107] It has been suggested that MT genes might be regulated differentially by a family of transcription factors that are specific for different MRE sequences.[94] For instance, cadmium initiates MT transcription although activation of a transcription factor that is independent of MTF-1 but that binds to MREs.[108] How many different factors bind with MREs is unknown.

B. Posttranscriptional Control

It is well documented that expression of human MT genes is regulated at the tran-

scriptional level.[109] However, there is also evidence for posttranscriptional control of MT gene expression that is both metal specific[110] and isoform specific.[111] Thus, copper and zinc produce similar increases in the number of MT mRNA transcripts in the first 4 hours of treatment in HepG2 cells.[110] Thereafter, the levels of MT mRNA accumulation reach a plateau in copper-treated cells that are maintained for a further 15 hours in the presence of this metal, whereas levels begin to decline in zinc-treated cells. In contrast, pulse labeling of the treated cells has revealed that zinc induces a greater rate of transcription of MT mRNA than copper, with only some decline in rate observed over time, whereas a sharp decline in rate has been observed after exposure to copper for 4 hours.[110] Furthermore, inhibition of transcription and protein synthesis stabilises the number of zinc-induced MT mRNA transcripts, thus suggesting that zinc may activate a MT mRNA turnover pathway.

In addition, the increase in MT-1 mRNA in rat liver following treatment with copper or cadmium parallels both its recruitment into polyribosomes and the amount of MT-1 protein produced.[111] MT-2 mRNA, on the other hand, is more abundant than that of MT-1, but a lower proportion is associated with polyribosomes, indicating that MT-2 mRNA is not translated as efficiently as MT-1 mRNA.[111] Thus it has been proposed that there is translational control of the MT-2 mRNA in rat liver.[111]

C. Cell-Specific and Metal-Induced Differential Transcriptional Regulation of MT

Differences in constitutive levels of MT expression, in the isoforms expressed and their cellular levels, have been observed in response to various inducers.[47,67,71] Thus, both MT-1f and MT-1g have been induced in response to cadmium, zinc, and copper in HepG2 and Hep3B2 cells but not in lymphoblastoid-derived Wi L2 cells. Moreover, both constitutive and induced levels of MT-2a expression are considerably higher than those detected for MT-1f and MT-1g in all the cell types studied, while the induced levels of MT-1g mRNA are greater than those of MT-1f in embryonic kidney Hek 293 cells.[67]

Furthermore, there are cell-specific differences between metals in their ability to induce the various isoforms.[46,67] For example, cadmium induces the highest levels of MT-2a mRNA in Hek 293 and Wi L2 cells followed by zinc and then copper. In addition, only cadmium could induce MT-1g mRNA in Hek 293 cells,[67] while being the most effective inducer of MT-1a in TK cells, HeLa cells and human skin fibroblasts.[46] Following treatment of mouse Hepa 1A cells and L cells with cadmium, zinc, copper, or dexamethasone, the expression of MT is the same in response to each inducer, with about 1.5 times more MT-1 than MT-2 mRNA in each case due to the coordinated regulation of both isoforms.[70] The fact that optimal induction of MT has been found in response to very low concentrations of cadmium is clear evidence that this metal is a very efficient inducer of MT.

The regulation of the human MT genes is complex, involving both *cis*-acting and *trans*-acting components. DNA methylation is thought to regulate human MT gene expression.[112,113] For example, the differences observed in the cell-type-specific regulation and induction of the human MT genes is perhaps due to DNA methylation of the *cis*-acting elements and of the overall chromatin structure of the gene, thereby inhibiting the action of transcription factors.[67] The variable response of human MT genes to different metals are thought to be due to

varying levels of metal specific *trans*-acting factors present within the particular cell.[67] Nevertheless, the underlying mechanisms of MT regulation and the reasons for differential expression of the various genes are unclear, but the latter does suggest that there may be a specific physiological role for each of the isoforms.

While transcriptional response of MT genes is very rapid, with a high rate of transcription occurring after 1 to 2 hours, the rate and extent to which these transcripts accumulate, vary according to the inducing metal.[71,111] It has been demonstrated using HepG2 cells that the number of MT-1f mRNA transcripts induced by cadmium and zinc are twofold greater than those induced by copper, whereas there is a far lower number and rate of accumulation of MT-1g transcripts with copper compared to cadmium and zinc.[71] MT-2a appears to be strongly and efficiently induced by metals with zinc being an exceptionally good inducer.[46] These differences may indicate different physiological roles of MT-1 and MT-2.[46]

VI. DEGRADATION OF MT

In vitro studies show that MT degradation takes place mainly in the lysosomes[114-117] by proteolytic action of the enzymes cathepsins B and/or L,[118-122] although some degradation has been reported to occur in the cytosol by neutral soluble proteases.[115,11] Moreover, MT appears less susceptible to proteolysis in the metal bound state.[123-125] *Ex vivo*,[121,122] *in vivo*, and *in vitro* experiments have shown that metal-MTs have far longer half-lives than apo-MT and that the half-lives varies according to the nature of the bound metal[114,117,120,126,127] or the metal status of the organism.[125,127] In addition, in cultured cells (HeLa) zinc-induced MT is more stable than MT induced by dexamethasone.[128] Time differences in the degradation of rat MT-1 and MT-2 occur (MT-2 degrading more slowly than MT-1), thus influencing tissue concentrations of the isoforms.[129-132] Metal-dependent variations in the half life of MT have also been demonstrated in the rat liver *in vivo* where Cd-MT has a greater resistance to degradation than Zn-MT,[127,130,131,133,134] although there is some evidence to suggest the contrary.[119,120,125] Thus, metals bound to MT may protect the protein against intracellular degradation and the extent of protection may have an inverse relationship to the dissociation constants of the bound metals.[119]

Until recently, the mechanism by which bound metal is released from MT was thought to involve displacement by H^+ ions.[119] Lowering the pH *in vitro* increases the rate of metal release from MT and, for example, the zinc content of Zn-MT rapidly diminishes between pH 5 and 4.5, with zinc being fully released at pH 4. Lysosomes are acidic with a pH range of between 3.6 and 5 and so zinc should readily dissociate under these conditions. In addition, different metals are released more readily at different pH values; cadmium, for instance, is only fully released at pH 1.[119] *In vivo*, however, other factors may be involved in the degradation of MT.[119] The degradation products corresponding to the a-domain (carboxy terminal) of MT-1 and b-domain (amino terminal) of MT-2, but no other combinations, have been found in liver from zinc treated rats, indicating that they may occur independently.[135] This suggests that the metal clusters of Zn-MT-1 and Zn-MT-2 have different stability towards proteolytic degradation, which therefore may occur via two different pathways specific for the two isoforms.

From this evidence it is clear that the cause of the long-term retention of certain metals after chronic exposure is not the lack of metallothionein degradation,[126,136] as there

appears to be a concommitant rebinding of the MT-released metals to newly synthesized thionein polypeptide chains.[124,136-138]

VII. BIOLOGICAL ROLES OF MT

Since the discovery of MT, its primary biological role has proved difficult to identify and, indeed, remains elusive. MT has a relatively rapid rate of turnover and this has been taken as evidence for a regulatory function. Detailed analysis of the structure of MT has been helpful in delineating some of the possible biological roles for this protein. The metal-thiolate clusters within the MT molecules allow rapid exchanges of metal ions between clusters and with other MT molecules.[132,133] Hence, it is thought that this characteristic, which appears to be unique to MT, aids the transfer of metal ions and is fundamental to its biological role.[25] Proposed functions for MTs include metal absorption/excretion, metal homeostasis and metabolism, free radical scavenging, metal detoxification, apoptosis, and modulation of the intracellular redox balance. However, the search for the biological role of MT is further complicated by the cell specific expression of different isoforms and the complex pattern of induction of this protein. Thus, it is unclear whether each of the MT isoforms have their own specific functions and, if so, whether only the isoforms most suitable for the needs of a particular cell are those being expressed.

A. Metallothionein, Cell Proliferation and Apoptosis

The results of a number of investigations suggest that MT plays an important role in cell proliferation. For instance, in mammals, high levels of MT expression have been detected during the late stages of gestation and neonatal periods,[16,139-141] although significant changes in MT levels in several organs have been noted during rat foetal development.[142,143] MT levels are also transiently elevated during liver regeneration[78] as well as in liver and kidney of uninephrectomized rats.[144] Increased MT levels have also been reported *in vitro* during exponential cell growth.[145-147] MT is mainly a cytoplasmic protein but it is also present in the nucleus during cell proliferation and development.[146-150] For example, MT has been detected in both the nucleus and cytoplasm of human fetal hepatocytes, and the levels of MT increase with gestational age.[140,150] MT is also found in the nucleus of regenerating hepatocytes[146,148,149] as well as in rat foetal hepatocytes but in opposition to what occurs in human cells, the protein redistributed to the cytoplasm 2 to 3 weeks postpartum,[146] Although the mechanism behind the redistribution of MT is unclear, it is believed that nuclear retention is ATP dependent.[151] Cellular localization of MT appears to be cell cycle specific because it peaks in the nucleus during S, and G_2/M phases while maximal expression in the cytoplasm occurs during G_0 and G_1 phases.[145,146,152] MT is located mainly in the cytoplasm of both human fetal and adult renal proximal tubule cells[150,153] but no correlation between gestational age, levels of MT or cellular localization in the kidney has been found,[153] suggesting that the distribution and expression on MT during development may be organ specific.

It has been demonstrated, using MT-null fibroblasts coupled to a regulation expression system allowing differential expression of nuclear and cytoplasmic MT, that the roles of MT in each subcellular compartment are different. Thus, while cytoplasmic MT seems to protect the cells against toxic insult caused by metals, reac-

tive oxygen species and oxidizing agents, nuclear expression of the protein may have an antimutagenic role.[154] Additional support has been obtained from experiments demonstrating that cytoplasmic expression of MT protected the cells against tert-butyl hydroperoxide toxicity but did not prevent this compound from damaging the DNA.[155]

Thus, it would appear that nuclear localisation of MT is either cell type specific[151] or characteristic of a particular proliferative state. MT may play a role as a zinc donor where required, such as in activating zinc-finger transcription factors.[156] The elevated concentrations of MT during proliferation could be related to the increased demand for certain metals such as copper and zinc,[143,157-159] although protection from oxidative stress is a possibility that has also been suggested.[155]

A number of MT inducers, such as glucocorticoids, lipopolysaccharides, steroid hormones, cytokines and tumour necrosis factors, among others, can influence apoptosis in certain cells.[160] This and other experimental data would seem to suggest that MT plays a role during the apoptotic process. Cells from MT-null mice have been found to be more susceptible to apoptosis after exposure to tert-butyl hydroperoxide and anticancer agents than normal MT-expressing cells.[161] Transfection of MCF-7 cells with a MT-antisense oligomer, inhibits cell growth, decreases bcl-2 protein levels and induces morphological changes characteristic of cell death, increases levels of c-fos and p53 transcripts and decreases c-myc transcripts. MCF-7 cells transfected with a MT-2 cDNA-containing plasmid, which increased MT levels, results in increased rates in cell proliferation, while decreasing c-fos and p53 and increasing c-myc transcripts.[162] Hepatic apoptotic lesions in cisplatin-treated MT-null mice are also more pronounced than in normal controls.[163] Overexpression of MT also inhibits ultraviolet B-induced apoptosis in mouse keratinocytes.[164] In man, MT expression and reduction in the number of apoptotic cells has been demonstrated in liver tumours,[165] laryngeal hyperplastic lesions[166] and squamous cell carcinoma of the tongue.[167] In summary, overexpression of MT appears to protect against apoptotic cell death, while lack of MT increases cell susceptibility to apoptosis.

However, there is also evidence to the contrary. Polymerized Cd-MT has been shown to promote apoptosis in human kidney 293 cells,[168] while in animals that accumulate copper, such as Bedlington terriers and the toxic milk mutant mouse, accumulation of the metal, and presumably, induction of MT, results in increased levels of apoptosis.[169,170]

With the involvement of MT in cell proliferation and apoptosis, it is not surprising that the possible role and significance of MT in cancer has also received considerable attention. The levels of MT expression have been investigated in a wide variety tumors.[78,165,167,171-183] The MT content in tumours has been related to the proliferative status of the cells,[184] although it is also a function of the degree of maturation and differentiation of the tumor.[185-187] Thus, expression of MT-2a has been reported to be lower in human colorectal tumours and derived cell lines due to a decrease in the basal activity of the MT-2a promoter that occurs concomitantly with inmortalization.[188]

MT overexpression in human tumours has also received considerable attention and generally it has been associated with a high degree of malignancy and poor chemotherapeutic outcome.[171,172,174-178,180,182,186,189-191] However, in some tumor types such as colon, bladder, and fibroblastic skin tumours, a correlation between MT overexpression and better prognosis has been found.[171,192] It has been suggested that MT prognosis may be related to the differential regulation of

human MT isoforms, particularly MT-1f and MT-1e,[171] on the basis of the specific differential regulation of MT genes according to the embryonic origin of the tissue.[171,189]

MT expression contributes to the resistance of mammalian cells to a wide variety of anticancer drugs, an area that has been studied extensively and reviewed elsewhere.[6,78,106,107,193-207]

B. Homeostasis of Essential Metals

MT is thought to be involved in homeostasis of the essential metals, copper, and zinc,[5,31] as it is the major zinc and copper binding protein in many tissues,[32] and there is a close relationship between tissue MT and zinc content.[208] The importance of zinc is evident from its role as a major component of many enzymes and cell processes and from the severe pathology observed in response to zinc deficiency in skin, neurological, immune, and reproductive tissues.[209] Indeed, second to iron, zinc is the most abundant trace element in the body.[208] The role of MT in the homeostasis of zinc and other essential metals has been studied extensively.[6-8,31,206,208,210]

Mice overexpressing or ectopically expressing MT and also MT knock-out mice have been used as models to test the proposed functions of MT *in vivo*. For example, transgenic mice that overexpress MT-1 are more resistant to zinc deficiency. This has been attributed to a larger pool of zinc bound to MT, thereby supporting the view that MT is an important source of intracellular zinc.[211] In addition, evidence from MT-1 and MT-2 deficient null mice fed zinc-deficient diets suggests that zinc-MT is required for normal kidney development and maturation.[212] However, because MT null mice appear to live normally, this raises questions about the role of MT during normal physiological processes. An obese phenotype has been noted in some MT-null mouse colonies, which suggests perturbation of energy metabolism,[213] but it is not yet clear whether this is caused by lack of MT.

A recent hypothesis has proposed that MT acts as a chaperone during synthesis and modulation of metalloproteins and MT appears to be stabilized at high cellular GSH concentrations. Metal-requiring apoenzymes[78,214] can abstract metals from MT as demonstrated *in vitro*.[215-219] GSH can form a complex with MT;[220] release of zinc from MT mediated by interactions with GSH and GSSG through *S*-thiolation has recently been reported.[218,221,222] Recent evidence suggests that zinc release from MT is facilitated by direct coupled interaction of GSH and GSSG with MT[223] via the thiolate ligands that confer redox activity on zinc clusters resulting in an oxidoreductive MT-Zn complex. MT therefore may be acting as a sensor of the localised intracellular redox balance and may itself influence redox balance through GSH and the known antioxidant properties of zinc. It has been suggested that "the control of cellular zinc distribution as a function of the energy state of the cell is the long sought role of MT".[223] In cells in culture MT can donate Cu to Cu-Zn superoxide dismutase[224,225] and at least *in vitro* the Cu–MT interaction is also under redox control.[226] These facts support the hypothesis that MTs act as physiological transporters of essential metals (such as Cu and Zn) and that their distribution is regulated in a redox-sensitive manner. This, however, has not yet been demonstrated *in vivo*[227] and the validity of such a role for MT on the basis of the "apparent normality" of MT-1 and/or MT-2 null mice has been raised by others, while arguing that MT can "provide a reservoir of zinc that can be mobilized under zinc-limiting conditions".[214] It is conceivable though that (although tissue specific),

yet undiscovered or unidentified mammalian MTs, or indeed other proteins are also performing this role in the absence of MT-1 and MT-2.

C. MT as Free Radical Scavenger

MTs may provide mammalian cells with a primitive antioxidant defence mechanism.[21,200] Metallothionein is induced by treatments (hyperoxia,[228] ionising radiation,[229-231] exercise,[232,233] or cold exposure [232,234]) and substances (ethanol,[235,236] paraquat,[229,237] or tert-butyl hydroperoxide[155,238]) that cause oxidative stress,[19,155,198,239,240] as well as agents involved in inflammatory processes (interleukins,[241-245] interferon,[53,81,246] and tumor necrosis factor alpha[246,247]). This suggests that MT may protect against reactive oxygen and nitrogen species. Moreover, animals or cells in culture that are deficient in metallothionein isoforms exhibit greater susceptibility to oxidative stress caused by electrophilic mutagens, antineoplastic drugs, nitric oxide as well as cadmium.[161,199,201,206,248,249] Overexpression of metallothionein reduces the sensitivity of cells and tissues to free radical damage [201,247] and metallothionein genes are transcriptionally activated in cells and tissues in response to oxidative stress.[72,73,83,247,250] Differences in the contribution of the different MT isoforms to the scavenging properties of the protein have also been reported.[20]

The mechanism by which MT protects against oxidative stress is unknown. It has been suggested that the thiolate clusters of MT are primary targets for the reaction of hydroxyl radicals.[251] Dithiothreitol[252] and GSH[253] only cause a limited reduction in peroxidation compared with that of MT. At least *in vitro,* hydrogen peroxide, superoxide anions, and peroxyl radicals interact with MT and result in oxidative modification of the protein, thus suggesting that *in vivo* this may affect the capacity of MT to bind metals. Some authors therefore favour MT-released zinc as the primary element of protection via interaction and stabilisation of membranes.[252] It has been postulated that the free metals (cadmium and zinc) interact with the cell membrane and interfere with iron redox reactions (by either competing for binding sites or causing structural changes which reduced binding), thereby reducing the conversion of H_2O_2 to $OH\Sigma$ radicals. A similar effect has been proposed in relation to Zn- and Cd,Zn-MT, where free radicals were thought to interact with the MT to release the metals and influence redox events.[252] However, Cu-MT enhances lipid peroxidation initiated by organic hydroperoxides,[254] suggesting that MT may act as an antioxidant or a pro-oxidant depending on its association with metals.[170] Embryonic cells from MT null mice are more sensitive to the free radical-mediated damage caused by the compounds *tert*-butylhydroperoxide and paraquat, thereby indicating that basal levels of MT may be involved in the regulation of intracellular redox status in mammalian cells.[248] MT is also induced in tissues subjected to a rapid increase in metabolic activity, such as brown adipose tissue during thermogenesis,[234] and may indicate a physiological role for this protein in scavenging free radicals, the levels of which are elevated under these conditions.

D. Metal Detoxification

MT plays a major role in metal detoxification, a fact supported by extensive evi-

dence from both *in vivo* and *in vitro* studies.[6,11-14,16,206,255-259] After exposure to various metals there is a significant increase of MT in tissues such as kidney, liver and intestine.[13,31] Likewise, various cell types have been shown to accumulate MT after metal exposure.[260] However, although some metals such as nickel and cobalt are able to induce MT, there is conflicting evidence as to whether they bind *in vivo*.[258,261] Some metals (such as lead) are also known to induce and bind to other intracellular proteins, which may also play a role in their detoxification.[262]

Stress proteins may also be involved in metal detoxification. Organ-specific increases in the levels of various stress proteins in rats following exposure to inorganic mercury[263] and overexpression of stress proteins in embryonic stem cells results in greater resistance to the toxic effects of cadmium, mercury, and cisplatin.[264]

GSH has also been implicated in metal detoxification. Indeed, there are several studies both *in vivo* and *in vitro* that have reported increased sensitivities towards the toxic effects of mercury[265,266] and cadmium[267,268] following depletion of GSH levels. GSH, however, appears to be the first line of defence against cadmium toxicity preceding MT induction.[267]

There are many examples in the literature demonstrating both *in vivo* and *in vitro*, that exposure to a non-toxic, low dose of metal allows a higher, toxic dose to be tolerated. This has been attributed to the priming of MT induction by low doses of metal which facilitates subsequent MT expression and sequestration of higher metal doses.[269] Indeed, much of the information regarding metal toxicity has been gleaned from studying the toxic effects of cadmium that accumulates in the body under normal conditions. For example, tolerance to cadmium following low dose pretreatment with cadmium or zinc is directly related to the concentration of hepatic MT in rats.[269,270] In addition, cadmium-resistant cell lines express very high levels of MT and these cells show increased resistance to the toxic effects of several other metals.[261] Similarly, protection against the toxic effects of several heavy metals via increased MT expression has been detected after pretreatment of rat hepatocytes with zinc.[258] Pretreatment with non-metal inducers of MT such as vitamin A have also been shown to reduce the hepatotoxic effects of cadmium.[271]

In addition to metal sequestration, MT may reduce the toxic effects of metals by reducing the uptake of metal into cells.[16,272] Indeed, metal-induced metal resistance also occurs without any increase in intracellular MT. This has been demonstrated in bovine endothelial cell cultures after simultaneous administration of toxic doses of cadmium with zinc[273] or copper.[274] These protective effects appear to be due to a decrease in the uptake of cadmium into the cells since less intracellular accumulation of cadmium and more of zinc or copper were found on exposure to both metals. Likewise, pretreatment of these endothelial cells with zinc for 24 hours protects against cadmium cytotoxicity, without the induction of MT by decreasing the accumulation of cadmium within the zinc-pretreated cells, indicating that uptake of cadmium is inhibited by the increase in intracellular zinc.[275,276]

Simultaneous administration of bismuth and cadmium fails to protect against the toxic effects of cadmium in bovine endothelial cells, despite inducing MT.[277] Bismuth induces maximal MT expression after 48 hours exposure in cultured human proximal tubular cell cultures,[278] but it fails to reduce the toxic effects of cadmium, mercury, and zinc[278,279] or cisplatin.[279] In contrast, *in vivo* studies with mice,[280] rats,[281] and humans[282] have demonstrated that prior administration with bismuth induces MT and reduces the nephrotoxic effects of the

anticancer drug cisplatin. Similarly, pretreatment with bismuth and subsequent induction of MT reduces the cardiotoxic effects of adriamycin in mice.[283]

Taken together, the mechanism(s) whereby metal-induced MT reduces the toxicity of metals may be dependent on the metal and in addition they may be cell type specific. Additionally, the differences observed between treatments performed *in vivo* and *in vitro* could be due to the absence of *in vivo* homeostatic mechanisms that can be difficult to mimic *in vitro*.

The role that MT plays in metal detoxification, the mechanism(s) by which it acts and to what extent it is involved in the long term handling of metals is unclear. Certainly there is compelling evidence for the importance of MT in the short-term detoxification of metals, although its involvement in the long-term metabolism of metals is less clear. Indeed, chronic exposure to cadmium results in the transport of cadmium from the liver to the kidneys in the form of the Cd-MT complex, which was thought to be fundamental to the long-term nephrotoxicity of cadmium.[11] Recently, however, Klaassen's group, in a series of elegant experiments, has questioned the role of CdMT in the induction of nephrotoxicity.[284,285]

Studies using transgenic mice that either overexpress or are deficient in MT have provided further insight into the role that MT plays in the protection against metal toxicity. Liu and co-workers have found that MT-1-overexpressing mice (MT-TG) (which carry 56 copies of the MT-1 gene) are more tolerant to hepatotoxicity and lethality caused by cadmium than control mice;[286] others have reported no protection against the toxic effects of cadmium on the reproductive system.[287] Similarly, blastocysts from MT-TG mice are also able to tolerate very high concentrations of cadmium *in vitro*.[288] In addition, mice with disrupted MT-1 and MT-2 genes (MT null mice) are more sensitive to cadmium-induced toxicity than control mice.[284,289,290]

Maximal induction of MT-1 by metals[46] occurs at the same metal doses which cause toxicity,[261] suggesting a role for this isoform in metal management. Bylander and co-workers have shown that after exposure of human proximal tubular cells to a non-toxic dose of cadmium, various MT isoforms, but not MT-1a, were expressed.[291] However, cytotoxic doses of cadmium induce MT-1a expression, which correlates with cell toxicity. MT-1a therefore is thought to prevent cell toxicity by heavy metals and it could possibly be used as a biomarker for metal toxicity.[44,281]

MT may also have a "rescue" function within the cell in addition to its binding of excess metals.[292] The proposed model is an extension to that described by Palmiter[106] and is based on the evidence that MT is able to activate zinc proteins such as alcohol dehydrogenase and alkaline phosphatase[216] and that zinc-MT restores the functions of actin and carboxypeptidase after these have been rendered dysfunctional by exposure to cadmium.[292] The displacement of zinc from its weakly bound state to intracellular proteins by the displacing metal are perhaps toxic interactions that precede the induction of MT. The free zinc is then able to bind to MT-1, thereby allowing MT gene transcription to take place (as described above). Hence, according to this model the newly synthesized MT, which has a higher affinity for the metal, serves to bind the foreign metal so as to "rescue" the proteins.[293]

VIII. MT-3 AND OTHER BRAIN METALLOTHIEINS

Mammalian brain contains three different isoforms of MT (MT-1, MT-2, and

MT-3). MT-3 was initially called "growth inhibitory factor" or GIF because it was discovered as a consequence of its growth inhibitory property,[294] which is not shared with the other MTs.[295] MT-1 and MT-2 have been localized in the astroglia of cerebral cortex and basal ganglia, whereas MT-3 is predominantly expressed in the dentate gyrus and hyppocampal structures as well as the neocortex and olfactory bulb.[8,239,296] Remarkably, the neuroanatomical distribution of MTs and particularly that of MT-3 rich areas is well correlated with zinc-rich areas of the brain as demonstrated by the Timm's stain.[10,297] Experiments carried out in mice lacking or overexpressing the MT-3 gene have demonstrated that this isoform does not alter the presence of synaptic zinc.[298,299] Similarly, it has also been demonstrated that MT-3 is not required for protection against exogenous zinc or cadmium[299] and in mice, its level of expression does not affect learning and memory processes.[8,299]

Recently, there has been much interest in the role of the MT isoforms and particularly MT-3, in the etiology of neurodegenerative disorders, since they have been shown to correlate with zinc alterations, and brain MTs[8,10,300] and confer protection against oxidative stress in brain[301] a potential causative factor in Alzheimer (AD) and Parkinson disease . Thus, down-regulation of MT-3 and disturbances in zinc homeostasis have been implicated in excessive neurite sprouting, increased susceptibility to oxidative stress and metal induced neurotoxicity.[8] Zinc metabolism is altered in AD as demonstrated by increased extracellular Zn-metalloproteinase activity in AD hippocampus,[302] decreased levels of MT-3[294] as well as increased hepatic zinc and reduced amounts of Zn-MT.[303] In addition, the distribution of MT isoforms correlates with the distribution of amyloid plaques.[297] However, the role of brain MTs in the distribution of neural zinc and the causative relationship between MT and AD remain to be determined.

As with the other MTs, many functions have been proposed for the brain MTs such as sequestration and distribution of metal ions, regulation of biosynthesis and activity of zinc metalloproteins, removal of metals from the extracellular space, protection from free radical damage, cellular adaptation to stress and neuromodulation, particularly in zinc and MT-3 containing neurones, of chatecolaminergic, glutamatergic and GABAergic transmission.[8,9,74,75,304,305] In addition, the preferential localization and distribution of MT-1 and MT-2 in the pia arachnoid, ependymal cells, glial cells of Purkinje cell layer of the cerebellum and astrocytes may be related to the cytoprotective properties of these proteins or to the supply of essential metals to neurones.[9,296,304-306]

IX. ARE THERE SEPARATE FUNCTIONS FOR THE DIFFERENT MT ISOFORMS

Whether there are distinct functions for each of the human MT isoforms or whether they merely represent a duplication of function has not yet been established. Recent evidence, however, supports the former possibility. Transgenic mice expressing MT-3 ectopically (i.e., in the same expression domain as MT-1) died of pancreatic acinar cell necrosis 2 to 3 months after birth.[307] Although the mechanism by which MT-3 disrupts pancreatic function is unclear, these findings together with the specific distribution and function of MT-3 in the brain would indicate that MT-1 and MT-3 have distinct properties and biological roles.[307] MT-4 is only expressed in stratified epithelia and it has been suggested that it may play a spe-

cial role during differentiation of this tissue.[52] Both human and mammalian MT isoforms are differentially expressed in various tissues and in cultured cells,[40,46,47,49,55,66,67,71,74,241,308-313] as well as during development.[141,149,153,247,314-317] Furthermore, investigations concerning expression of MT isoforms in human breast cancer cell lines have revealed that all four cell lines expressed MT-2a mRNA with modest variation and MT-1x mRNA consistently.[318] However, estrogen receptor positive cells had no MT-1e mRNA expression whereas estrogen receptor negative cells expressed high levels of MT-1e mRNA and contained significantly higher levels of MT protein. Thus, there may be a relationship between estrogen receptor status and MT-1e gene expression in human breast cancer.[318]

In addition and despite the coordinated regulation of mouse MT-1 and MT-2 isoforms,[68,70] their level of expression at least in newborn mice is unequal[319] as is their response to different stimuli.[320,321] In several mammalian species, including man, the MT-1 and MT-2 isoforms show different levels of constitutive expression, are independently regulated,[72,73,129,322] and show differential response to inducers.[71,73,128,309,323-328]

It is likely that the basic function of the different isoforms may partly overlap. It is also likely, in view of the differential regulation and induction of some isoforms, that, perhaps as a result of evolution, some of them have acquired specific functions, which they perform in those defined tissues or cell types where they are expressed.

X. CONCLUSION

The conservation of the basic structure of MT throughout evolution and its ubiquity underline the biological significance of this small metal-binding protein. However, the discovery of a number of functional isoforms in several mammalian species, including man, raises the question of whether these isoforms have different biological roles or if they simply reflect a duplication of function.

The use of transgenic mice has been particularly useful in furthering our understanding of this molecule and in the elucidation of the different roles the isoforms may play. The fact that different MT isoforms are concentrated in particular tissues or cell-types in the body suggests that they may have distinct tissue or cell-dependent roles. Furthermore, the cell specific expression of MT isoforms indicates that they may not only have different biological functions but that the isoforms may be expressed according to need in different cell types.

The transcriptional regulation of MT appears to be a complex event that varies between species and cell type. Transcription is very rapid (although this varies depending on which inducer is present) and may be activated by a number of positively and negatively acting transcription factors. In addition, posttranscriptional regulation may occur in some cases. Less is known about the degradation of MT but it is clearly a lysosomal event. There is also some evidence indicating that the different isoforms have separate degradation pathways.

Although a primary biological role for MT has yet to be identified, the ability of a wide range of stimuli to induce MT would suggest that MT is a stress response protein, which acts either directly or indirectly as part of a cellular defence mechanism. Under normal conditions however, it seems likely that the primary role of MT is in the homeostasis of essential metals and the maintenance of the intracellular redox status, donating and accepting copper and zinc where and when required. The close association between zinc and MT, of which the

brain is the prime example, seems to testify to its role in metal homeostasis. Thus, the involvement of MT in cell proliferation and modulation of apoptosis as well as its role as free radical scavenger and in metal detoxification are fortuitous and perhaps accidental secondary roles that relate to the unique chemico-biological structure of this protein.

However, the importance of the role MT plays in metal detoxification, especially in the long-term handling of metals, is not evident. The relationship between type of metal and the extent to which MT is directly involved in its detoxification is unclear, as are the mechanisms by which metal-induced metal resistance occurs, which also appear to be both metal and cell type specific. There is evidence to suggest that the MT-1 isoforms and particularly the MT-1a isoform may play an important part in metal detoxification.

Nevertheless, whatever the reason for the existence of MTs, there is no doubt that its structure and unique chemistry affects cellular metal metabolism. It may be less important to assign a specific function to MT than it is to understand the mechanisms by which it is able to ameliorate the toxicity of metals and confer resistance to therapeutic agents or to elucidate its role in cell proliferation and apoptosis.

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