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Review

Metallothionein: The multipurpose protein

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Abstract. Metallothioneins (MTs) are intracellular, low molecular, low molecular weight, cysteine-rich proteins. Ubiquitous in eukaryotes, MTs have unique structural characteristics to give potent metal-binding and redox capabilities. A primary role has not been identified, and remains elusive, as further functions continue to be discovered. The most widely expressed isoforms in mammals, MT-1 and MT-2, are rapidly induced in the liver by a wide range of metals, drugs and inflammatory mediators. In teh gut and pancreas, MT responds mainly to Zn status. A brain isoform, MT-3, has a specific neuronal growth inhibitory activity, while MT-1 and MT-2 have more diverse functions related to their thiolate cluster structure. These include involvement in Zn homeostasis, protection against heavy metal (especially Cd) and oxidant damage, and metabolic regulation via Zn donation, sequestration and/or redox control. Use of mice with altered gene expression has enhance our understanding of the multifaceted role of MT, emphasised in this review.

Key words. Metallothionein; gene regulation; zinc; pregnancy; inflammation; metabolism; cytokines; cadmium.

Introduction

Metallothioneins (MTs) belong to a superfamily of intracellular metal-binding proteins, present in virtually all living organisms, with features common to the archetypal MT first isolated from horse kidney and characterised over 40 years ago by Margoshes and Vallee [1]. These unique biomolecules have captured the attention of biologists and chemists alike due to their remarkable chemical structure that confers a degree of specificity, stability and dynamic behaviour almost impossible to predict from the properties of their organic and metallic ingredients. Typically, MTs have low molecular weight (<7000 Da), high metal content comprising predominantly Zn, Cu or Cd, highly conserved 18–23 cysteine residues and no aromatic amino acids or histidine.

It is frequently said that the primary biological role of MTs remains an enigma; however, it is increasingly clear that MT fulfils protean functions, the relative importance of which depends very much on specific evolved requirements of the particular organism. This should not be unexpected, as the unique structural characteristics of MT imbue it with potent metal binding and redox capabilities, which have bearing on almost all biochemical processes. Vital roles for this pleiotropic protein in more primitive life forms often result from sequestration of environmental toxic metals (e.g. Cd, Hg) or of physiologically important metals that are chemically disruptive in ionised form (Cu, Zn). Examples are the incorporation of cadmium into wMT-2 in earthworms [2] and the storage for recycling of large, potentially toxic, quantities of copper from hemocyanin (respiratory pigment) breakdown in molluscs and crabs [3]. Extensive research into vertebrate MT, chiefly using small mammals, has uncovered a diversity of important functions of MT. While these do not appear to be essential for life, as evidenced by apparently normal reproductive capacity and long-term survival of mice lacking functional MT genes, there is mounting ev-

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idence for a survival advantage of MT in situations of stress, including exposure to oxyradicals and toxic metals, inflammation, infection and low Zn nutrition.
Opposing views on the primacy of MT, either as ZnMT in the redox control of enzymes of intermediary metabolism and mitochondrial energy production, on the one hand, or passivation of metallic (e. g. Cd) and nonmetallic toxicants, on the other, have been forcefully put in reviews, by Vallee [4] and Klaassen [5], respectively. The focus of our review is on mammalian MT (m1 and m2 of family 1) with emphasis on findings from the increasing number of studies using MT-gene altered mice. For further discussion on MT the reader is directed to more comprehensive reviews [4–21] and to the proceedings of four symposia [22–25].

Structure and metal-binding properties, degradation

MT is currently classified into 15 families [26]. Other groups of metal binders that are more widespread in plants, including phytochelatins and nonprotein MT have not been included (plant MTs are reviewed by [20]).
Mammalian MTs are single-chain polypeptides of 61 to 68 amino acid residues. The number and position of the cysteine residues is highly conserved and forms cys-x-cys, cys-x-y-cys and cys-cys sequences, where x and y are noncysteine amino acids. There are no free thiol groups, and divalent metals are bound by sulphur atoms in thiolate clusters with a tetrahedral geometry (or trigonal for Cu^{+}) [10–12, 16]. The binding affinity varies between metals, with Cu having the greatest stability constant (10^{19}–10^{17}) followed by Cd (10^{17}–10^{15}) and then Zn (10^{14}–10^{11}). As many as 18 different metals may associate with MT, but only Cu^{+}, Cd^{2+}, Pb^{2+}, Ag^{+}, Hg^{2+} and Bi^{2+} can displace Zn [10, 21].
MT can incorporate up to 7 divalent metal or 12 monovalent Cu atoms per molecule (Cu^{++} is not bound by MT). Cu^{+} binds in multiple stoichiometries with a minimum of 7 Cu^{+}/mol [27]. MT has two subunits: the more stable α-domain (C-terminal), which incorporates four divalent metal atoms, and the more reactive β-domain (N-terminal), which contains only three [12, 21, 28–33]. The exchangeability depends upon the metal species, and in vivo MTs exist mainly in Zn form or as mixed-metal proteins [10, 34, 35]. The tertiary structure of MT is dynamic, and Zn and Cd exchange rapidly within the β-domain, more slowly in the α-domain, and may also exchange with other ions bound to intracellular ligands [21]. MT has also been found to donate metal ions to higher-affinity ligands on other proteins.
The rate of degradation of MT is determined by the identity of the metal atom bound to the protein, and half-lives for Cd-, Zn- and CuMT in liver have been estimated at 80, 20 and 17 h, respectively, with rates of degradation varying between animal species [16]. Differences in metal distribution between MT isoforms may also affect rate of degradation [36]. In hepatocytes, MT has been found to be degraded in both lysosomal and non-lysosomal compartments [15]. Studies in vitro at an acidic pH have shown cathepsin B to be the most important protease that degrades apoMT (metal-free thionein). Interestingly, under these conditions apoMT was degraded far more rapidly than ZnMT, which in turn was slightly more stable than CdMT [37]. Cu- and ZnMT appear to be degraded differently. Zn is rapidly released from the protein and is therefore able to participate in cellular function and to induce further MT synthesis, whereas due to the greater affinity of thionein for Cu, CuMT is oxidised to form insoluble polymers which accumulate in the lysosome, presumably in a nontoxic form and are eventually secreted in bile [16].

Isoforms

The majority of MT research has been performed on rodents, predominantly mice. The four known genes for murine MT are located in a 50-kb region (MT-1 and -2 within 6 kb) on chromosome 8, and encode MT-1 to -4 proteins [16]. In humans, at least 10 of 17 MT genes, clustered on chromosome 16, are functional, and these encode multiple isoforms of hMT-1 (designated by the letters a, b, e, f, g, h and x) and one isoform of hMT-2a. Single genes code for hMT-3 and -4 [10, 38–40] (see fig. 1). Heterogeneity of isoforms results from postranslational acetylation and/or variations in metal composition (metalloforms). Isoforms may be distributed in various ratios in individual tissues and have differing rates of degradation. Although the general physicochemical properties of MT isoforms are similar, there is some specialisation of biological function [10, 11, 41]. The most widely expressed isoforms in the body are MT-1 and -2. MT-2a appears to be expressed more in human tissues than MT-1. MT-3 is found mainly in the brain, but its message is expressed in tongue, stomach, heart, kidney and reproductive tissues [42–44]. There have been few studies on MT-4, and the gene has been detected only in certain squamous epithelia and the maternal deciduum [45, 46].

Tissue distribution, localisation and the cell cycle

The highest concentration of MT in the body is found in the liver, kidney, intestine and pancreas [7, 8, 10, 11]. There is considerable species variation in hepatic MT, with levels in human, dogs, cats, pigs and goats ranging from 400 to 700 µg/g of liver; monkey, cow and sheep around 200 µg/g of liver and rabbits, cavies and rodents 2–10 µg/g of liver [47].

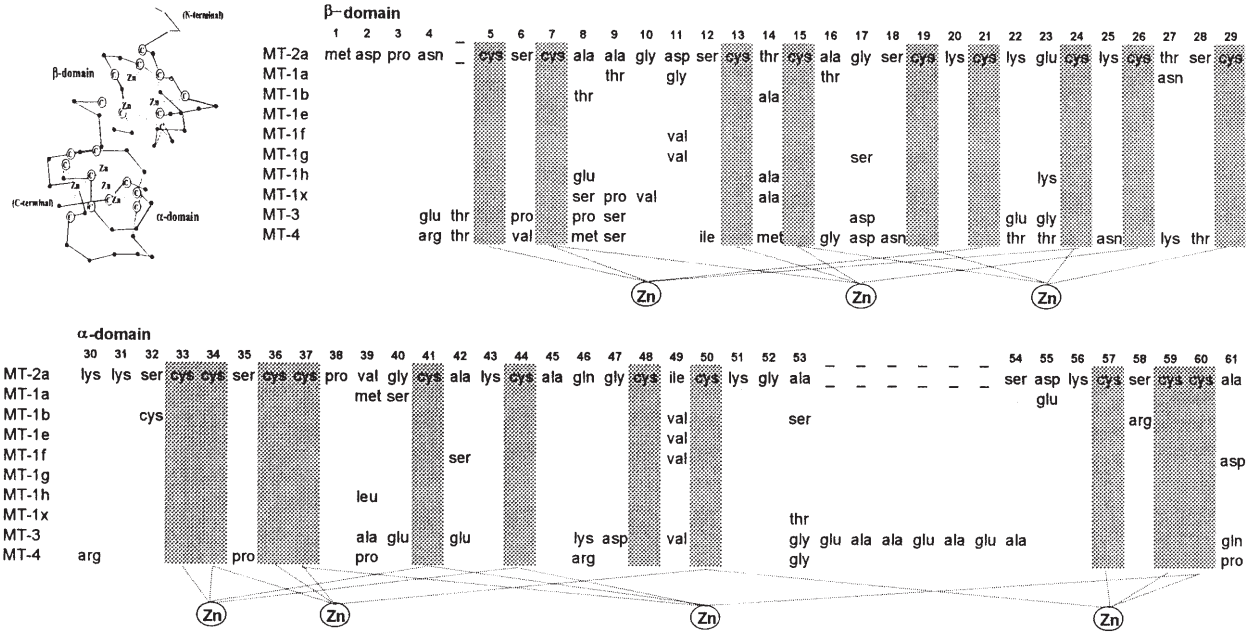

Figure 1. Schematic representation of the MT structure together with amino acid sequences for known functional human iso MTs. Shown are the α- and β-domains with the conserved positions of the cysteine residues highlighted in grey. Amino acid residues differing from MT-2a are given. Metal coordination positions are based upon those derived for rat MT-2 [32–33].

Immunohistochemical studies have demonstrated increased MT expression both in the cytoplasm and nucleus of rapidly proliferating cells (see reviews [17, 48]). The significance of the nuclear retention of MT is unknown, but it has been proposed that it might protect DNA from oxidative damage or regulate Zn supply to crucial enzymes and transcription factors involved in cell division [48–50]. In support of the first premise, preliminary evidence of Levadoux and co-workers indicated that DNA damage was more extensive when nuclear import of MT was prevented [51]. Then again, the translocation of MT from cytoplasm to nucleus in proliferating 3T3-L1 fibroblasts has been shown to coincide with an increase in intracellular Zn, which in turn was found to be essential for mitogenesis [52].

Increased nuclear MT expression has been found in fetal and newborn rat liver [53] and kidney [54], in hepatocytes during liver regeneration [55], in kidney during compensatory growth after uninephrectomy [56], in subconfluent cultures of human colonic cancer cells [57] and in adult rat hepatocytes stimulated into exponential growth with insulin and epidermal growth factor [58]. The mechanism(s) of nuclear translocation of MT is only beginning to be unravelled. In studies with human tumour cells, nuclear retention of MT was found to be both temperature and energy dependent [59]. In experiments where MT gene constructs were transfected into Chinese hamster ovary cells, nuclear uptake of MT-1 was targeted by a coding sequence within the 3′-untranslated region, which localised the MT messenger RNA (mRNA) to polysomes on the perinuclear cytoskeleton [60, 61]. This localisation was necessary for the newly synthesised MT protein to be shuttled into the nucleus and occurred at the start of S phase of the cell cycle [51]. In a recent study, nuclear MT translocation was found to require cytosolic factors other than known importins that target nuclear pores, as well as requiring the small GTPase, Ran [62].

MT regulation during cell cycle progression has been demonstrated in normally cycling cells. Maximal nuclear accretion of MT, two- to- three-fold basal, was found to coincide with the S and G_2 phases, whereas high cytoplasmic expression occurred during late G_1 and G_1/S transition and basal amounts were found in the G_0 phase [57, 58]. In another study, four-fold increases in MT-2 were found in proliferating Chang liver cells compared with those in growth arrest, and there was evidence for translational control as well as slower rate of MT-2 degradation in proliferating cells [63]. MT may also be involved in controlled cell death (apoptosis). In a study where fibroblast and embryonic cells from MT-1 and -2 gene knockout mice were treated with cisplatin, the levels of the tumour suppressor protein p53 and the death effector protein Bax were significantly higher than in normal cells, indicating that a lack of MT increases susceptibility to apoptosis [64]. Similarly, lung fibroblasts from MT knockout mice are more susceptible to copper-dependent apoptosis [65]. In other studies, overexpression of MT appears to protect against apoptosis, in mouse cardiomyocytes after both ischemia-reperfusion induced injury [66] and treatment with doxorubicin [67], in mouse keratinocytes after ultra-

violet B (UVB) irradiation [68] and in HL-60 cells challenged with cupric nitrilotriacetate [65]. MT expression directly correlated with a decrease in apoptotic cells in human liver tumours [69], laryngeal hyperplastic lesions [70] and in peripheral blood samples from children with acute leukemia [71]. In addition, in experimental autoimmune encephalomyelitis, ZnMT-II treatment reduced apoptotic cell death of neurons and oligodendrocytes [72]. On the other hand, in human kidney 293 cells treated with polymerised Cd-MT [73]; and in Cu-treated HL-60 cells after exposure to a nitric oxide (NO) donor [74], the protective role of MT as an antioxidant appears to be reversed, promoting apoptosis. For further discussion on the relationship of MT to apoptosis see [17].

Gene regulation and induction

Hepatic MT synthesis is induced by a number of metals, cytokines and stress hormones as well as by a wide range of chemicals, many of which act indirectly via a stress or inflammatory response [11, 15]. However in some cases, these MT responses may be considered as nonphysiological since pharmacological doses had been administered in vivo or added to cell culture systems. Although the metals, Zn, Cu, Cd, Hg, Au and Bi all induce MT, Zn is the primary physiological inducer since, Cu excepted, the other metals can be regarded as environmental toxicants. Interestingly, nontoxic Cu levels do not induce MT, although it is often bound to MT in vivo [7, 75, 76].

Metal regulation of MT genes has been covered in several recent reviews [17, 77]. Briefly, the binding of Zn to metal transcription factor (MTF-1) allows the protein to bind to metal response elements (MREs) in the promoter region which, in turn, initiates MT-gene transcription. It has been proposed that MTF-1 regulates the free zinc concentration by controlling the expression of MT as well as that of a Zn-transporter protein, ZnT-1 [78]. Basal expression of MTF-1 may be controlled by a Zn-sensitive inhibitor that prevents MTF-1 binding to MREs. Zn dissociates the inhibitor from MTF-1, thereby promoting transcription of MT [79]. Zn also prolongs the nuclear retention of MTF-1, but it is unclear whether this further promotes transcription [80]. MTF-1 is important in the regulation of a number of genes that play a role in cellular response to various stresses [81]. Indeed, MTF-1 is essential for normal development, and MTF-1 knockout mice die in utero from liver failure [82].

MREs are present in multiple copies in the MT promoter region, and they appear to be variable in their response to metal-induced transcription. In humans, only four out of seven MREs react with MTF-1 to mediate a Zn response [83]. Interestingly, Zn, Cd and Bi ions activate the promoter of the MT-gene via MREs [79], but only Zn is specific for binding and activating MTF-1 [77]. However, there are possibly other pathways of metal induction, because protein kinase C inhibitors have been found to inhibit Zn and Cd induction of MT in Chinese hamster cells [84]. Moreover, MREs can interact with a variety of nuclear proteins that either activate or inhibit transcription [17, 85, 86]. This may partly explain why Cd induction of MT-1 is inhibited by the administration of 17-β-estradiol and progesterone to ovariectomised mice [87]. However, the biological properties of individual MREs and the mechanisms that regulate cooperation between MREs and their interactions with putative regulatory proteins are only just being revealed. To add to the complexity, there is evidence that several MREs respond directly to hypoxia and oxidants, possibly via MTF-1, although these conditions may also dissociate Zn from protein ligands which could then activate MTF-1 [77, 88].

No single factor regulates MT synthesis in inflammation, rather a complex interrelationship exists between factors that in combination, and in different tissues, act synergistically on MT-gene transcription. A similar combination of inflammatory factors has been found to drive the MT and acute phase response in mice following restraint stress [89]. Nucleotide sequences other than MREs in the MT promoter have been found to respond to glucocorticoids [90, 91], interleukin (IL)-6 [92], phorbol esters [93] and hydrogen peroxide [94]. Many of the acute phase proteins appear to be regulated by combinations of the same factors, and these include catecholamines, glucocorticoids, glucagon and the cytokines IL-6 (in particular) as well as IL-1, tumor necrosis factor α (TNF-α) and γ-interferon [15, 95–98]. Unlike other acute phase proteins, MT induction by inflammatory mediators has been found to be conditional upon the presence of Zn. Reactive oxygen intermediates generated during the inflammatory response may induce MT through multiple pathways, including directly stimulating an antioxidant response element and specific MREs in the promoter region as well as by events associated with various second-messenger protein kinase pathways [99, 100]. Nitric oxide production inhibitors have been found to dampen the induction of MT by lipopolysaccharide (LPS) in rat primary cell cultures [101]; this effect was also demonstrated in vivo, where NO suppression was shown to blunt stress-related MT-1 upregulation in both brain and liver of mice [102], implicating a role of NO in MT induction. In addition, epidermal growth factor has been found to induce, and transforming growth factor-β to inhibit, MT-1 expression in the regenerating liver [103].

By analogy with the mechanism(s) of acute phase protein induction, one could speculate on MT gene regulation as illustrated in figure 2. Considering the extensive reorganisation of protein synthesis in the liver during the acute phase response, and the importance of Zn in protein synthesis, it is probably more than a coincidence that MT, a

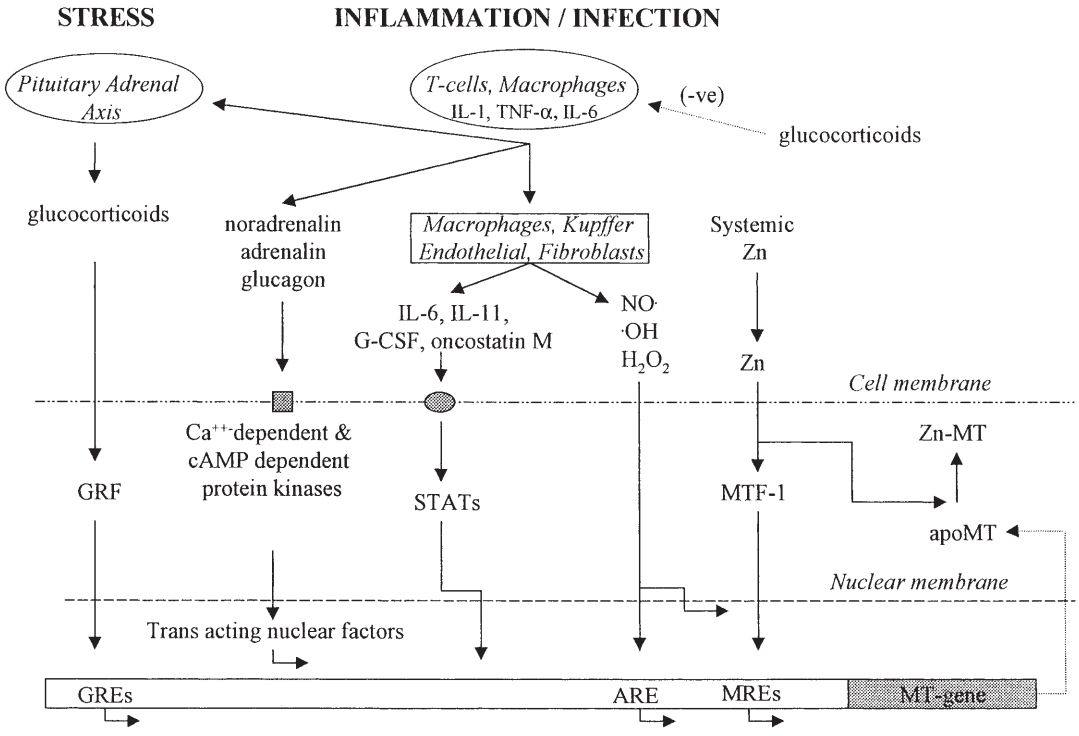

Figure 2. Overview of hepatic MT gene regulation in inflammation. In response to infection/inflammation, interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are released from activated T cells and macrophages. Glucocorticoids are increased by stress and by the abovementioned cytokines, which stimulate the pituitary/adrenal axis [98]. Glucocorticoids bind to the cytoplasmic glucocorticoid receptor complex (GRF), which in turn activates glucocorticoid responsive elements (GREs) on the MT gene, two of which have been located 1 and 7 kb upstream of MT genes [91]. A glucocorticoid-induced rise in MT initiates sequestration of Zn from the plasma, increasing the intracellular labile Zn pool, and thereby activating metal response elements (MREs) through a metal transcription factor (MTF-1) in a positive feedback loop. IL-6 secretion may be induced by IL-1 or TNF-α in a variety of tissues and by catecholamines [104–107]. IL-6 regulates the expression of MT and that of various acute phase proteins by inducing tyrosine phosphorylation of signal transducers and activator proteins (STATs) that interact with sites in the promotor region of the MT gene [92]. STAT3 synthesis is stimulated in response to IL-6, oncostatin M, leukemia inhibitory factor, IL-11, granulocyte colony stimulation factor and epidermal growth factor [108]. Glucocorticoids play a role in the IL-6-mediated induction of hepatic MT [109], and synergy between the two appears to require the physical interaction of their respective response elements [111]. Cytokines also cause variable expression of MT isoforms in different tissues, with IL-6 and TNF-α inducing more MT-2 than -1 in liver, whereas TNF-α is a stronger inducer than IL-6 of MT-1 in lung and heart [110]. Catecholamines and glucagon induced by inflammatory mediators attach to membrane-bound receptors and, via second-messenger systems activate trans-acting nuclear factors that interact with as yet unidentified control elements. Reactive oxygen species formed during the inflammatory response may interact with the antioxidant response element and/or several metal regulatory elements. Combinations of inducers may also effect posttranscriptional processes and further modulate MT levels in the cell [112]. It has been proposed that as the glucocorticoid concentration increases in inflammation, so does its ability to switch off the cytokine-driven response by inhibiting IL-1, TNF-α and IL-6 release from macrophages, thus terminating the acute phase response and restoring homeostasis to the liver [97, 104, 113].

source of exchangeable Zn, is one of the earliest proteins to be induced in the liver in response to inflammation.

Genetically modified mice

The generation of MT-1 and -2 gene knockout (referred to as MT–/–) mice [114, 115] gave new impetus to MT research. Two strains have been developed both using the same targeting vector, the pK-H clone containing MT-1 and -2 genes produced by Palmiter [116], although the final constructs differ. One strain is OLA129/C57 BL6J, backcrossed to C57/BL6J that reproduce as well as wild-type C57/BL6Jmice (referred to as MT+/+ mice). The other MT–/– strain, from an OLA129/SvCPJ genetic background, is commercially available with congenic normal controls from the Jackson Laboratories. Poorer reproductive rates have caused some investigators to backcross OLA129/SvCPJ mice against CD-1 mice for pregnancy-related experiments [117]. Some care may be required in interpreting more subtle differences between MT–/– and MT+/+ mice when there is doubt about the extent of backcrossing. At the MT IV meeting in Kansas in 1997 [25], some of these concerns were discussed, including the possibility of clonal selection when the gene pool of breeding colonies is limited. Nevertheless, there appears to be good agreement between the two strains of MT–/– mice in key areas, including susceptibility to

heavy metal toxicity, response to inflammation and zinc homeostasis, although some differences in phenotype and metabolic responses may exist.

Two other genetically modified mice have been generated. The first, transgenic mice with multiple MT-1 genes (MT-Tg), have 10–20-fold greater MT protein levels in the pancreas, liver and stomach, and 2–6-fold greater MT levels in a number of other organs including kidney, spleen and heart [118]. They have 50% more Zn in liver and 300% more in the pancreas. Female MT-Tg mice have 5-fold more MT in the liver than males. Pancreatic MT of these mice has been found to be a very sensitive indicator of Zn status [119]. The second, MT-3-gene knockout mice have decreased Zn in the hippocampus and other brain regions. No neuropathology or behavioural deficits have been detected, although they are more susceptible to seizures induced by kainic acid [120] (for discussion on MT-3, see section on MT and the brain.

Initial optimism that the availability of MT–/– mice would resolve, once and for all, the function of MT has been tempered by the realisation that MT is not essential for normal growth and development. This is perhaps not surprising given the experience with other knockout mice where the impact of gene deletion has often been more subtle than predicted. Nevertheless, use of MT-Tg and MT–/– mice has given further insight into the function of MT in a range of situations, including the ability of MT to limit heavy metal toxicity, protect against a range of noxious agents, modulate zinc homeostasis in particular settings and in brain research for differentiating the effects of MT-1 and -2 from MT-3.

Protection against metal toxicity

More than a third of the publications on MT–/– and MT-Tg mice involve heavy metal toxicity, mostly Cd. Whereas the characteristic phenotype of mice and cells lacking MT expression is sensitivity to Cd, the detoxification of this and other heavy metals is often seen as a fortuitous property of MT and not its evolutionary function [19]. Extensive evidence has emerged from Klaassen's laboratory ([121] for a overview) that the absence of MT-1 and -2 increases inorganic Cd-induced lethality and hepatotoxicity, whereas overexpression is associated with protection. Although MT protects against chronic $CdCl_2$ nephropathy, it does not protect against CdMT-induced renal injury, whereas Zn treatment does [122]. This highlights one of the strengths of the MT–/– model in that it acts as the definitive control. In the past, it was not possible to distinguish between Zn and MT effects due to the strong MT induction that follows Zn treatment.

Not all studies have shown significant protection by MT against the Cd hepatotoxicity. It is evident from the early [114, 115] and more recent [123] studies that there is considerable variability in the toxicity of Cd in MT–/– mice, including gender differences, indicating that factors other than MT are important in determining Cd toxicity. In one study with MT–/– mice [124], basal levels of MT were shown to be insufficient to protect against a single Cd injection. Once MT levels are increased however, either by Zn treatment or genetically (MT-Tg), protection against Cd toxicity is marked. Similarly, an initial low level exposure to a toxic metal, coinciding with MT induction, then results in resistance to more toxic doses of that metal.

Regarding the toxicity of physiologically important metals, Zn and Cu, only small increases in sensitivity to Zn have been observed in MT–/– mice, with large doses of Zn required to show any effect [125]. Surprisingly, Cu toxicity has not been investigated to any extent, but as with Zn, significant toxicity has not been reported [125, 126]. However, in the absence of Zn or Cu efflux tranporters, MT is necessary for protection against metal toxicity. Crossing of the mouse model of Menkes disease (absence of Cu-effluxing ATPase) with MT–/– proved to be lethal to embryos [127]. In a mouse model of Wilson's disease (toxic milk mutant), hepatic MT accumulates as a result of decreased protein degradation, and this appears to offer some protection from the high hepatic copper levels seen in this setting [128].

Protection against xenobiotics

Hepatic MT rises acutely following the administration of a range of noxious agents. It has long been assumed that this provides the liver with some protection from oxidant damage, and indeed, if MT is preinduced (e.g. by Zn administration) prior to administering a noxious agent, hepatotoxicity is often markedly reduced [129]. However, until the advent of the MT–/– mouse, it was difficult to prove conclusively that this was primarily related to MT induction.

MT–/– mice have now been widely used to investigate the role of MT in protecting against a number of toxic agents, including CCl_4, paracetamol, chemotherapy and UV and ionising radiation damage. The consensus from a number of these studies is that MT–/– mice are more sensitive to to toxic insults [CCl_4 being a typical example, 130], supporting an antioxidant role of MT. However, not all studies are in agreement in this regard showing that attention needs to be paid to proper controls to discriminate between MT, Zn and other contributing factors. For example, MT expression was associated with protection against CCl_4 hepatotoxicity, but MT overexpression, or Zn treatment (dietary) provided no further protection [131]. In another study, Zn treatment was shown to be protective against CCl_4 hepatotoxicity in MT–/– mice [129]. Other compounds (oleanoic acid, sakurasosaponin) also protect independent of MT [129, 132],

probably by limiting CCl_4 metabolism. Investigations with acetaminophen (paracetamol) toxicity indicate a greater susceptibility in MT–/– mice, which appears to be associated with an antioxidant function of MT [133, 134]. However, fasted mice are much more susceptible to paracetamol, and this masks the protective role of MT, indicating the importance of considering other factors, in this case glycogen stores, which affect the glucuronidation of paracetamol. These and other studies have not demonstrated significant differences in cytochrome P450 enzymes and glutathione levels between MT–/– and MT+/+ mice.

The amount of oxidative damage to liver DNA, lipids and proteins was found to be similar in MT–/– and wild-type mice following γ-irradiation or exposure to nitropropane [135]. The survival of MT-/- mice was the same as wild type following exposure to lethal dose of γirradiation. Zn treatment, however, increased survival in both genotypes [135]. This last observation is a recurring finding in a number of settings where Zn clearly has protective effects independent of MT. This is perhaps not surprising given that there are over 300 enzymes known to be Zn dependent, in addition to other actions, including membrane stabilisation.

In skin, MT–/– mice have been shown to have greater immunosuppression [136] and more damaged cells [137] following UVB irradiation, suggesting an antioxidant role for epidermal MT. On the other hand, MT–/– mice develop less epidermal hyperplasia after exposure to UVB and other proliferative agents, implicating MT (or Zn) in the proliferative process [138].

Zinc absorption and homeostasis

Induction of MT synthesis in mucosal cells is triggered by both fasting and high luminal Zn concentrations, but is not significantly induced at normal dietary Zn intakes [7, 139, 140]. MT has long been implicated in the regulation of absorption and excretion of Zn by the intestine [141, 142], and the action of MT in restricting Zn absorption at high Zn concentrations has gained acceptance [7]. It has been argued that MT limits Zn absorption by sequestering it in the intestinal wall, thereby transiently reducing its absorption and favouring Zn transfer back into the gut lumen [7, 143]. Davis and co-workers performed gastric intubation experiments on MT–/– and MT-Tg mice and found that Zn was absorbed more readily in the absence of MT [144]. Of particular interest in this study was the finding that the MT–/– mice retained more Zn in the intestinal wall than the MT-Tg mice, indicating that MT does not restrict Zn absorption solely by sequestering it in the mucosal cell.

Evidence that MT enhances Zn absorption/retention is less compelling. Many studies have demonstrated enhanced efficiency of Zn absorption in Zn-depleted animals [145–149]. This enhanced Zn uptake may result from increased absorption at low Zn intakes, decreased secretion of endogenous Zn or a balance between the two [139]. Basal levels of MT may play a role in Zn absorption by competing with or supplying Zn to a variety of transporter proteins, including ZnT-1 to ZnT-4 and DCT-1 [150–154]. Of most interest is ZnT-1, which has a wide pattern of tissue expression, including the intestine, and may function as a mucosal-to-serosal surface Zn effluxer [155]. Recent studies by Langmade and co-workers [78] found that in the visceral yolk sac during pregnancy, MTF-1 regulates the intracellular Zn concentration by coordinating the expression of MT synthesis with that of the basolateral Zn efflux protein, ZnT-1. In addition, dietary Zn deficiency during pregnancy caused the downregulation of ZnT-1 and MT in the visceral yolk sac. However, our understanding of the interactions between Zn transporters and cellular binding ligands that affect Zn uptake, absorption and secretion is still in its infancy.

We found evidence for a MT-mediated enhancement of Zn absorption when we gave ^{65}Zn to normal and MT–/– mice in solid food and as an oral gavage in aqueous solution [156, 157]. In those studies Zn-deficient wild-type mice absorbed/retained more ^{65}Zn from an intragastric solution than MT–/– mice [157]. Zn-replete normal mice also absorbed slightly more ^{65}Zn from a 50-mg Zn/kg egg-white-based test meal [156]. These differences were mainly due to an increased retention of ^{65}Zn in nongut tissues, in particular the liver. This may indicate that intestinal MT aids in sequestering Zn when it is present in the lumen, either attached to food ligands in a more complex form or when Zn supply is limited. However, this MT-mediated enhancement of Zn absorption appears to be of only minor significance relative to other mechanisms regulating Zn homeostasis. We were unable to demonstrate a conserving effect of MT on the intestinal processing of Zn in mice, either starved 48 h or fed a Zn-deficient diet for 37 days [158]. Nevertheless, MT–/– mice lost 10% of body weight over the first 25 days compared with no loss in MT+/+ mice, indicating a survival advantage of MT. A similar benefit of MT has been shown in MT-Tg mice [119].

MT–/– mice secrete more subcutaneously administered ^{65}Zn into the small intestine than do MT+/+ mice, and the absence of MT in the pancreas has been strongly implicated in causing this increase [159]. In vitro studies with isolated small intestinal segments have shown increased Zn absorption but not secretion in MT–/– mice, although the presence of a Zn-binding ligand (albumin) outweighed any MT effect [160]. ^{65}Zn-labelling experiments may thus be more sensitive than traditional Zn balance studies in demonstrating the influence of MT on certain aspects of intestinal Zn processing.

MT induction by dietary supplementation of Zn acetate is a recommended therapy for long-term management of

patients with Wilson's disease, an inherited disorder of Cu accumulation and toxicity [161, 162]. Zn induces intestinal MT, which sequesters Cu in the mucosal cell and prevents its transfer into the circulation. Intestinal cells turnover approximately every 6 days, thus removing the MT-bound Cu in the stool. Hepatic MT is also temporarily increased, presumably in the form of nontoxic CuMT. In the long-term (>18 months of Zn treatment), hepatic Cu concentrations remain the same or lower than pretherapy levels, and there is normal liver function.

The exocrine pancreas plays an important role in Zn homeostasis [163, 164]. Serum Zn increases with decreasing exocrine pancreatic function [165], pancreatectomy increases serum Zn [166] and Zn deficiency decreases the pancreatic secretory response [167]. There is a large amount of Zn in pancreatic-biliary secretions [164], and this is also dependent on Zn status [168]. In addition, the demonstration of high levels of MT in the pancreas [169] implicates a role for this protein in the regulation of Zn secretion. It has been shown that the pancreas and liver are the most responsive organs to Zn-induced MT synthesis [170]. Using MT-Tg mice, MT levels were shown to be dramatically raised in the pancreas [119], and to be a very sensitive indicator of Zn deficiency, declining almost absolutely in the face of a Zn-deficient diet (1.5 ppm), whereas MT in other organs decreased only moderately. This is a further indication that pancreatic MT is regulated by Zn [170–172]. MT has been shown to be secreted from the pancreas after Zn treatment [171]. MT in the pancreas appears to be less affected by inflammatory mediators, although cytokines and endotoxin are apparently effective inducers of MT in this organ [172].

MT–/– mice have been used to demonstrate that MT-1 and -2 not only protect against Zn deficiency but also prevent the toxic effects of Zn on the pancreas [119, 125]. MT–/– mice have lower Zn concentrations in the pancreas [159, 173], and less ^{65}Zn is sequestered in the pancreas of MT–/– mice under steady-state conditions, indicating higher rates of endogenous Zn secretion in MT–/– mice. MT has been shown to be present in the pancreatic secretions [171], and it has been suggested that the MT-2 isoform, which is more resistant to degradation, may commit some pancreatic Zn to excretion [168]. In the setting of adequate Zn supply, this difference in the handling of Zn between MT+/+ and MT–/– mice does not appear to be detrimental. However, with starvation or Zn restriction, the decreased ability to limit secretion of Zn could be deleterious and may be one of the reasons why MT–/– mice are less able to withstand Zn deficiency [125, 174]. It is interesting that transgenic mice that ectopically express MT-3, an isoform normally found in neurones, die at 2–3 months of age as a result of pancreatic acinar cell necrosis [175]. The reasons for this are not clear but indicate a distinct separation of biological properties between MT isoforms.

The ability of MT to protect against oxidant damage has been investigated in the pancreas; some of these studies have relevance to endocrine function. Pancreatitis induced by cerulein is more marked in MT–/– mice and diminished in MT-Tg mice [176]. MT does not protect endocrine cells against alloxan-induced damage, even when mice are given extra Zn to induce pancreatic MT [177]. However, in studies using streptozotocin (STZ) to induce diabetes, Zn was found to protect MT–/– but not wild-type mice. This effect was attributed to the greater availability of unbound Zn in the MT–/– mice, with Zn rather than MT acting as the main protective agent [178], prompting caution in interpreting the results of studies in which Zn is shown to be protective against streptozotocin in MT+/+ mice [179]. Nevertheless, MT-overexpressing transgenic mice with STZ-induced diabetes have been shown to have reduced hyperglycemia [180]. This hyperglycemia probably results from NAD depletion, DNA breakage and islet disruption. MT-2 may also contribute to endothelial cell protection against oxidative stress associated with high glucose concentrations, by a process involving glucose-induced cosynthesis with endothelin-1 [181]. This would be expected to ameliorate hyperglycemia-caused endothelial cell dysfunction of diabetes. MT, even at constitutive levels, has been shown to have a more general role in maintaining appropriate vascular myogenic tone under the relaxing influence of NO released from endothelial cells [182]. MT–/– and wild-type mice have similar insulin content in islet cells, but the glucose-stimulated insulin release is diminished in MT–/– islets [183]. In this study blood glucose levels were lower in MT–/– mice, consistent with other reports of low blood glucose in these mice in the fasted state [134, 158] and following an inflammatory insult [158, 184]. Although MT and Zn are implicated in the endocrine aspects of pancreatic function, the highest MT and Zn levels occur in exocrine tissue, with consequences for Zn homeostasis, as discussed above.

MT and inflammation

MT induction occurs most dramatically in response to tissue injury, infection, inflammation and neoplastic disease, and it is here that MT appears to have its greatest influence. In this context, MT has been considered to be a stress or acute phase protein, and one of the most striking experimental examples of its expression is seen in response to LPS injection, mediated through cytokine (primarily IL-6) and stress hormone release [17, 92, 109, 111, 185–190]. In this situation, cytosolic MT in liver parenchymal cells may increase up to 100-fold, sequestering Zn from the plasma compartment and thereby promoting a flux from Zn-donor tissues. Increases in MT protein can be demonstrated as early as 2–4 h after an in-

flammatory insult, preceding the appearance of other acute phase proteins in the plasma. Dependence of liver Zn accumulation on MT synthesis was first reported over 2 decades ago [191], and later confirmed in MT–/–mice following intraperitoneal injection of LPS [184, 189] and in MT–/– hepatocytes treated with dexamethasone and IL-6 [192]. Although there is a central effect on Zn, it must also be recognised that the ability of MT to act as an antioxidant may provide a survival advantage at a time of major infection and inflammation.

Advantages for MT-driven Zn redistribution are unclear, although various theoretical benefits have been proposed, including (i) lowering plasma Zn, which modulates leucocyte function, including cytokine production [193], (ii) increasing the pool of intracellular Zn, thereby facilitating metabolic processes during the acute phase response and (iii) sequestering Zn to allow maximal activity of enzymes which would be otherwise inhibited by this metal.

Attempts to rectify the hypozincemia of inflammation have not always been beneficial, with increased pyrexia and worsening of infectious disease outcome being recorded [194–197] lending support to theory i). In a study where Zn was administered by osmotic minipumps, prevention of the fall in plasma Zn and raising liver Zn levels led to improved survival from intravenous (iv) *Salmonella typhimurium*, whereas supranormal plasma and liver Zn levels, or low plasma Zn and elevated liver Zn, did not alter survival [197]. One report [198] of improved survival following intraperitoneal (ip) Zn was shown to be an indirect effect, where uptake of toxin from the peritoneum was limited by ip Zn treatment. This should be kept in mind when interpreting the results of other studies where ip Zn apparently limits the toxicity of ip LPS [199]. In a porcine model of endotoxemia [200], pretreatment with Zn prior to LPS infusion was shown to limit the production of IL-6, TNF-α and other inflammatory mediators. Other studies lend some support to (ii) and (iii) in that MT–/– mice are more metabolically compromised than MT+/+ mice after LPS injection and are less able to sustain blood glucose levels [158, 184]. MT is induced in concert with other acute phase proteins, which raises the possibility that it is linked with the synthesis of these proteins. However, the acute phase response in MT–/– mice appears largely intact [158, 184], although a recent report [201] showed increased sensitivity of MT–/– mice to LPS/galactosamine-induced liver injury, and this was associated with decreased mRNA for α-1 acid glycoprotein.

Conservation of body Zn during acute inflammation appears to rely mainly on increased ZnMT accumulation in the liver. MT in other tissues, including the small intestine [156, 157] and pancreas [159, 171] may restrict Zn loss from its major route of excretion, the gut, although findings regarding inflammation in this context are limited. The association in the rat between endotoxemia and a cytokine-driven increase in Zn absorption and retention by the gut was reported many years ago [202, 203]. The direct involvement of MT was confirmed only recently by a study in which MT+/+ mice fed a low Zn diet excreted 40% less Zn in the feces over 2 days following LPS administration than did their MT–/–counterparts [158]. In humans, LPS injection has been shown to limit Zn loss in the urine, presumably by cytokine-directed Zn redistribution [204]. Upregulation of MT-1 gene expression in response to IL-1α in Zn-deficient rats has also been reported [205]. Because chronic Zn undernutrition increases susceptibility to infection, and is often associated with a greater exposure to environmental pathogens, induction of MT confers a survival benefit.

Actions of MT with lesser direct dependence on Zn may also contribute to the stress response. The high cysteine content of MT has obvious implications for the reduction of reactive oxygen and nitrogen species (reviewed in [9, 17]). In certain oxidative and inflammatory environments MT has been shown to reduce apoptosis by inhibition of cytochrome c-mediated caspase-3 activation [67, 72]. MT-associated Cu is also important in the stress response, and this influence may extend to the extracellular environment, as demonstrated in vitro by the release of CuMT from peripheral blood monocytes during the oxidative burst [206]. Whereas CuMT has been shown to scavenge singlet oxygen and hydroxyl radicles and inhibit oxidative liver damage in galactosamine/endotoxin-induced hepatitis [207], Cu has been shown to be released from yeast CuMT by NO, making highly reactive Fenton Cu available during the oxidative burst [208]. Furthermore, MT has been shown in vitro to impair Cu-dependent lipid [209] and luminol [210] oxidation. This impairment was halved under mild oxidising conditions, leading the authors to speculate that Cu may be released from MT during oxidative stress. It is tempting to believe that MT is instrumental in first, providing redox cycling Cu to enhance the oxidative burst and second, removing Cu in the normal (more reducing) environment to prevent excessive tissue oxidation. Increased MT synthesis also renders more Cu inert. In the latter context, we have observed that the acute phase reaction in rats injected into the tailbase with Freund's adjuvant is characterised by an increase in hepatic Cu MT preceding that of Zn MT [unpublished].

MT and pregnancy

Throughout gestation there is highly regulated and coordinated expression of the mouse MT genes in both maternal and fetal tissues (fig. 3). In the preimplantation mouse embryo, gestational day (GD) <4.5, MT-1 mRNA is expressed from the time of fertilisation (one cell), and is responsive to metal induction by the blastocyst stage

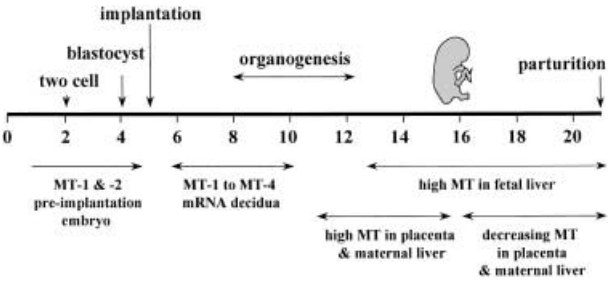

Figure 3. Fetal and maternal expression of MT during the gestational period of a mouse. MT-1 and -2 proteins are expressed in the preimplantation embryo. After implantation, the mRNA for all four MT isoforms is expressed in the deciduum, peaking by GD 10. Placenta and maternal liver MT are maximum by GD 14, decreasing thereafter. Fetal liver MT increases exponentially from GD 12, peaks before parturition and declines thereafter.

[211]. MT-1 and -2 proteins have also been demonstrated in the preimplantation embryo [212]. Post-implantation, all four isoforms of MT mRNA have been detected in the decidua, with increases in expression to maximum levels attained by GD10 [46]. At this point, levels (MT-1, -2) in the placenta begin to rise, peaking at GD16, as decidual levels decrease concomitantly [213, 214]. At the same time (GD12-16), there are high levels of MT-1 and -2 mRNA in both maternal and fetal liver, and the visceral yolk sac [46, 214]. Although MT mRNA is expressed in fetal liver shortly after formation (around GD 11), it is maximal during latter gestation GD16–17 [214–216], after which there is a gradual decline, such that by 12 days postpartum basal adult levels are attained [215].

During the gestational and postnatal periods, MT protein concentrations in maternal and fetal tissues exhibit rises and falls temporally related to mRNA levels, although the increases are more sustained. Our unpublished data with mice indicate that the expression of MT protein in the maternal liver changes dramatically during gestation: MT levels in the maternal liver begin to rise shortly after implantation, reaching 4-fold basal by GD 9, and peaking at concentrations 20-fold basal near GD 14. Maternal liver mass also doubles over gestation. Over the last stage of gestation, maternal hepatic MT begins a gradual decline, which continues after parturition. Evidence from studies in the rat indicate that there may be species differences with regard to the magnitude and timing of changes in maternal liver MT, with those in the rat possibly being delayed and not as marked compared with the mouse [217, 218]. It seems highly likely that this induction of maternal MT is initiated at least in part by circulating glucocorticoids and uterus-released IL-6, the concentrations of which rise and fall over a similar time course during gestation [216, 219]. The probable reason behind the staged hepatic MT induction (and resulting Zn accretion) is at first to provide for the extreme metabolic and growth demands of the dam and after, in late gestation when the fetus gains competence in Zn homeostasis, to release stored Zn for placental transfer. This decline in maternal hepatic MT begins at a time when the fetal liver appears to be anatomically fully developed and becoming functional. MT concentrations in fetal liver are extremely high at GD18, four to five times higher than the already elevated maternal levels [220] but after birth fall to adult levels by 4 weeks post-partum [53, 221]. MT concentrations in the placenta appear to have similar gestation-coordinated changes to those of the maternal liver, rising gradually to a peak between GD12 and GD15, and declining (to 60% below peak value at GD 18) towards parturition [220].

Although there is clear evidence that expression of MT in the maternal liver, placenta, fetal liver and other tissues such as the yolk sac at various stages during pregnancy facilitates movement of Zn into the fetus, where it is required for the processes underlying growth and development, it has also been demonstrated that, in response to maternal cadmium exposure, MT in the placenta prevents potentially toxic cadmium from entering the fetus [222]. Thus, as well as facilitating the flow of vital nutrient metals to the embryos, MT may also provide a barrier to entry of toxic metals.

In unstressed conditions, MT–/– fetuses grow and develop normally during gestation [114, 115]. However, their liver Zn levels are some 50% lower than wild-type levels at GD18 [174, 220] and at parturition [125]. Zn concentrations in bone are also lower at birth [125]. These fetal Zn levels appear to be at the threshold of Zn deficiency, whereas in the MT+/+ environment the Zn sequestering/storage ability of MT provides a surplus of Zn. This difference is particularly important in maternal Zn deficiency where MT–/– fetuses are at a significant developmental disadvantage. Compared to wild-type counterparts, MT–/– dams maintained on Zn-deficient diets have fewer fetuses (indicative of resorptions) and an increased incidence of grossly abnormal fetuses [117, 174]. Further underlying the importance of MT in this setting is the observation that mice which overexpress MT in turn have a reproductive advantage over wild-type mice [119]. In the postnatal environment, continued feeding of Zn-deficient diets to MT–/– pups leads to impaired renal development [125].

Despite fetoprotection from Zn deficiency by MT when physiologically regulated during pregnancy, the inappropriate induction of MT in the maternal liver at the critical time of organogenesis has been shown to be deleterious to the fetus (fig. 4). It is well established that maternal hepatic MT induction results in an accumulation of Zn within the liver to the detriment of plasma Zn concentration (see [220]). This decrease in maternal plasma Zn restricts Zn supply to the fetus, compromising the range of processes for which Zn is essential. A wide variety of teratogens including ethanol, TNF-α, urethane, α-hederin, 2-ethylhexanoic acid, 2-ethyl hexanol, valproate, melphalan

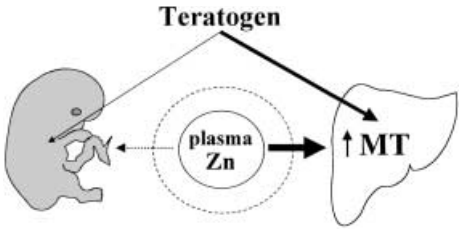

Figure 4. Proposed mechanisms of teratogenicity associated with maternal MT induction. A diverse group of teratogens, including inflammatory mediators, drugs and chemicals when administered to the mother during the critical stage of organogenesis, can impair Zn transfer from mother to fetus. The inappropriate induction of maternal liver MT results in liver Zn accumulation as MT sequesters Zn from the plasma. The fall in maternal plasma Zn concentration restricts the fetal Zn supply, which in turn disrupts the processes underlying organogenesis, and this may leads to birth defects. A direct action of the teratogen with the fetus may coincide with the impairment of Zn supply.

and arsenic are known inducers of MT, and have been shown to impair the transfer of Zn from mother to fetus when administered during the organogenic period [223–228]. Furthermore, the types of fetal abnormalities resulting from these teratogenic insults are very similar to those caused by Zn deficiency itself, thus implying, at least to some extent, a common underlying etiology [220]. The ultimate control in these types of experiments is the MT–/– mouse, as demonstrated by the observation that when MT–/– dams are exposed to ethanol at GD 8–9, the MT-driven changes in Zn homeostasis do not occur and a disruption to fetal Zn supply is not apparent. As a result, fewer birth defects are found in MT–/– than MT+/+ mouse pups, supporting the concept that induction of MT at a critical gestational stage causes a detrimental change in materno-fetal Zn homeostasis, increasing the incidence of teratogenicity [220]. It should be reiterated that the situation with ethanol treatment and the inappropriate rise in maternal MT and short-term limitation in Zn supply to the fetus is quite different from that seen with prolonged Zn deficiency, as discussed earlier, where MT expression is associated with a survival advantage.

MT and metabolism

MT is strongly implicated in metabolic regulation by its intracellular liganding of Zn, a structural and/or catalytic element in over 300 enzymes from all six classes. The large number of cysteine residues in MT raises the important possibility of redox control of Zn. Furthermore, Zn participates in apo-enzyme synthesis by influencing DNA stability as well as being an integral part of DNA-binding protein (Zn-finger) motifs. A direct relationship between cellular Zn concentrations and altered carbohydrate metabolism in rat hepatocytes has been described [229], supporting previous studies showing Zn stimulation of muscle glycolysis [230], inhibition of glycogen synthesis [231] and alterations of cellular energy metabolism [232].

The reactivation of Zn requiring apo-forms of aldolase, alkaline phosphatase and carbonic anhydrase by ZnMT was first demonstrated over 20 years ago [233, 234]. More recently, further insight into fundamental Zn-biochemical interactions of MT was provided by Maret and co-workers, who have used purified systems to demonstrate that the GSH-GSSG redox couple via thionein/MT can control Zn removal and addition to inhibitory sites on enzymes involved in glycolysis and signal transduction (see [235] for a review).

In 1994, Maret demonstrated that GSSG at pharmacological concentrations could release Zn from ^{65}Zn-labelled rabbit MT-2, suggesting redox control of Zn bioavailability (Zn by itself, having only one valence state, is redox neutral [236]). It was later demonstrated that the presence of GSH enabled a higher transfer rate, and caused the release of more of the available Zn from MT by GSSG for reactivation of Zn-depleted sorbitol dehydrogenase [237]. Certain selenium compounds were shown to facilitate Zn release from MT by disulphides in the presence of excess GSH [238], giving greater credence to a redox-sensitive regulatory function of MT in the cellular environment, where GSH:GSSG typically exceeds 50:1.

Later attention has focused on the activation/inhibition by thionein/MT at Zn inhibitory sites on enzymes, some of which are not regarded as Zn-metalloproteins. As thionein cannot remove Zn from Zn-metalloenzyme catalytic sites, which have stability constants in the nanomolar range, there is greater potential for two-way control at Zn inhibitory sites, with stability constants three orders of magnitude less. A range of enzymes have been examined and shown to be inhibited by Zn at nanomolar concentrations and reactivated by addition of thionein [239]. For example, 150 nM Zn inhibited glyceraldehyde-3-phosphate dehydrogenase by 50%, and 200 nM thionein reactivated this enzyme by 90% in the presence of 1 μm Zn [240].

An influence on energy production by ATP binding to lysine residues on MT [237], as well as the modulation of mitochondrial respiration [240–244], suggests a more central area of MT bioactivity. It was earlier reported that Zn can inhibit mitochondrial electron transport [243] and also inhibit the oxidation of microsomally generated NADPH [244]. (Zn,Cd)MT has been shown to increase mitochondrial succinate-initiated oxygen uptake, inhibit ADP-stimulated oxygen uptake, and to facilitate the reduction of cytochrome c [240, 241]. ZnMT has also been shown to increase the permeability of the inner mitochondrial membrane [245].

Most recently, it has been reported that incubation of liver mitochondria with ZnMT leads to localisation of MT in the intermembrane space with a consequent inhibition of respiration [242]. Reduction of mitochondrial oxygen

consumption was similar between MT-2, MT β-domain and Zn sulphate on a molar basis, indicating that the MT inhibitory activity derives from the release of one Zn atom from the β-domain. The addition of 2 µM thionein stoichiometrically reversed inhibition of respiration by 10 µM Zn, and this was shown to be due to Zn binding, not reducing, capacity of thionein. Fine control of inhibitory Zn may derive from its displacement at one site on MT. Based on molecular mechanics calculations, Brouwer [246] proposed that GSH could be accommodated within a cleft in the β-domain of MT at Cys-26 (Zn-2), the only GSH docking site he found, to produce a MT-GSH complex thermodynamically more stable than MT.

Although redox controlled regulation of Zn by thionein, and hence MT influence, on specific metabolic reactions has been demonstrated in the test tube, evidence from experiments in the more complex milieu of the living cell has been of a more general nature. The glycolytic response to Zn differs between hepatocytes from MT+/+ and MT–/– mice [247]. Exposure to physiological Zn concentrations in hepatocytes from MT–/– mice, although associated with increased intracellular Zn, caused little effect on glycolysis, whereas a direct correlation between increased intracellular Zn, hepatic MT and glycolysis was found in MT+/+ hepatocytes. The 3-hydroxybutyrate/acetoacetate ratio increased in parallel with ZnMT, consistent with a more reduced mitochondrial redox state in MT+/+ mice. Other studies [184, 229] have raised the possibility that mitochondrial oxidative metabolism is diminished by Zn sequestration, with greater reliance on glycolysis for maintenance of energy levels. The metabolic and energy deficits seen in vitro align with growing evidence of a metabolic disorder in intact MT–/– mice. Beattie and co-workers [248, 249] reported adiposity in OLA129/BL6J MT–/– mice, with 20% of males weighing 46–59 g at 22–39 weeks. It should be noted that obesity is not apparent in OLA129/SvCPJ MT–/– mice [19]. However, C57BL6J mice have a greater tendency to obesity, which can be unmasked by dietary and other challenges, even if MT+/+. They may provide a more sensitive model than SvCPJ mice for the clinical effects of metabolic dysregulation. Beattie found that the degree of obesity correlated with greater ob gene mRNA and plasma leptin concentration. Leptin resistance associated with increased food consumption and higher plasma insulin concentration was present, in common with leptin receptor deficient (db/db) mice. Unlike these mice however, MT–/– mice have lower than normal blood glucose and delayed onset of obesity. Given the association between obesity and defective carbohydrate metabolism, a requirement for MT in the activation of enzymes of intermediary metabolism [239, 250] may be sufficient to cause adiposity in MT–/– mice. This does not preclude a defect in mitochondrial energy production. These mice also have subnormal hepatic glycogen levels, but lack evidence of increased utilisation [134, 184]. In addition to the differences in glycogen storage, MT–/– mice have now been found to have significantly lower hepatic ATP levels at all times throughout the feeding cycle [A. M. Rofe, unpublished data], indicating an altered energy state. This may be the chief cause of other observed deficiencies, including reduced glycogen storage and inability to sustain hepatic gluconeogenesis after an inflammatory or noxious insult [134, 184]. Overall, MT–/– mice appear to have a reduced ability to synthesise, store and utilise carbohydrate fuels. Following 1–2 days of starvation, weight loss from MT–/– mice is not significantly different from that of MT+/+ mice; however, the added stress of LPS injection causes MT–/– mice to lose one-third less weight, with evidence of an MT-associated mismatch between lipid mobilisation from peripheral tissues and hepatic oxidation. That is, livers from MT–/– mice contained grossly more fat than those from MT+/+ mice subjected to the same LPS treatment [158]. This, and greater torpor in the MT–/– mice, suggests a lower metabolic rate. It is tempting to believe that the MT–/– mice do well in a nonchallenging environment, despite perturbations in intermediary metabolism, because of the redundancy inherent in evolved mammalian systems. Severe metabolic challenge, however, unmasks defective feedback control of the respiratory chain, which ultimately results in failure of oxidative phosphorylation. Calorimetry (BMR) and respiratory quotient determinations are required to determine the degree of hypometabolism and/or altered fuels ratio in MT–/– mice.

MT and the brain

Interest in the actions of MT in nervous tissue has followed the discovery of a brain-specific isoform, MT-3. First purified and characterised by Uchida a decade ago [251], it is the only MT isoform with a proven specific (but not sole) function, resulting in its original name of neuronal growth inhibitory factor (GIF).

Caution is necessary when interpreting reported brain MT concentrations. MT-3, as well as -1 and -2, is generally measured in nervous tissues by in situ hybridisation (MT mRNA) and/or immunohistochemistry (MT protein), methods which have been shown to give poor agreement with each other, especially for MT-3. For example, in two reports comparing MT–/– to normal mice by the same authors in the same year, immunoreactive MT-3 protein was found to be dramatically increased in MT–/– mice [252], but MT-3 mRNA expression to be similar [253]. Lack of sensitivity of the immunocytochemical method may explain some of the poor agreement, but anomalies still exist, such as MT-3 message being higher in neurones, but MT-3 protein higher in astrocytes [review 6]. The discrepancies are largely unresolved.

MT-1, -2 and -3 isoforms are expressed in the central nervous system (CNS) and in general have similar regional relative abundance but differences in their cellular distribution. In brain, constitutive expression follows the order MT-1 > MT-3 > MT-2 with MT-3 and MT-2 being 70 and 50% of the MT-1 levels, respectively [254]; also see reviews [6, 17, 255]. Constitutive mRNA expression of all three isoforms is greatest in the olfactory bulb of mice [254]. The cerebellum contains high constitutive levels of MT-1 and -2 and is low in MT-3 [254], but these differences are reversed in the hippocampus, piriform cortex and the amygdala. In these regions, the high constitutive expression of MT-3 correlates with increased concentrations of Zn in synaptic vesicles [42]. MT-3 is associated predominantly with neurones and the choroid plexus epithelium, whereas MT-1 and -2 are found mainly in astrocytes [255]. MT-1 and -2 genes are coordinately expressed and together respond to a variety of inducers, including metals, oxidants, hormones and cytokines [254, 256]. The nature of these inducers and the relative abundance of MT-1 and -2 in astrocytes, which are intricately linked with neurones, suggest that these isoforms function chiefly to protect the brain from oxidative intermediates which arise during stress, infection or inflammation. In this regard, after freeze injury to the cortex, MT–/– mice have depressed CNS wound healing with delayed astrocytosis [257].

MT–/– mice are also more sensitive to 6-aminonicotinamide, which is toxic to bone marrow cells and grey matter astrocytes [252]. Recent work indicates that both Zn and antioxidant functions are involved in the neuropathology seen in MT–/– mice [258]. Further evidence for MT neuroprotection is provided by the finding that MT-1-overexpressing TG mice are more resistant to cerebral ischemia-reperfusion damage [259]. The MT-1 TG mice had treble the MT-1 mRNA response in the ischemic cortex 24 h after reperfusion, associated with increased MT immunoreactivity in astrocytes, neurones and vascular endothelial cells, as well as significantly improved motor performance over normal control mice.

It has been proposed that MT-3 has a different function in the brain to that of MT-1 and -2. Supporting this viewpoint are the observations that (i) MT-3 is poorly induced and appears to be regulated differently [254, 256], (ii) its expression pattern is different, being predominantly found in the hippocampal neurones which sequester Zn in synaptic vesicles and (iii) it is the only MT isoform that has neuroinhibitory activity, first demonstrated by its inhibition of survival and neuritic sprouting of rat cortical neurones in culture [251]. At physiological pH, MT-3, which contains a six-amino-acid (three glutamates) insertion at position 55, is more acidic than other MT isoforms. These insertion and sequence differences, particularly the Thr insertion at position 5, and two Pro residues at positions 7 and 9, are thought to contribute to a putative receptor recognition site which gives the protein its biological activity [255, 260]. Interestingly, the neuroinhibitory activity is solely associated with the N-terminal, β-domain, is independent of the metal ions but does require the two proline residues at position 7 and 9 [260, 261]. This property together with the early finding that MT-3 was deficient in extracts from Alzheimer's-diseased (AD) brains pointed to a role of MT-3 in AD pathology. However, it was later shown that MT-3 downregulation was not necessarily associated with AD, and thus its involvement in the disease remains controversial [262]. Further interest in the association of MT-3 with AD may follow the recent report that MT-3 ameliorates the deleterious affect of amyloid β_{1-40} peptides [263] on cerebral cortical neurones in vitro. Amyloid β aggregates formed in the absence of MT-3 were predominantly of the SDS-resistant fibrillar neurotoxic form; in the presence of MT-3 they were mainly SDS soluble. Neither MT-1 nor MT-2 gave this effect.

There is considerable evidence that Zn metabolism is altered in AD and a variety of other neurodegenerative diseases (see review [255]). Zn is essential not only for normal function of a variety of enzymes, structural proteins and transcription factors but it is also known to modulate the activity of certain neurotransmitters via their ionotropic receptors [264, 265]. MT-3 knockout mice have decreased Zn in the hippocampus and other brain regions but in a normal laboratory environment show no neuropathology or behavioural deficits [120]. Thus it is unclear whether MT influences synaptic Zn concentrations, although the finding that MT-3 knockout, and MT–/– mice are more sensitive to seizures from kainic acid, a glutamate receptor agonist, suggests that it may [120, 258, 266]. In this situation, MT may both protect neurones from oxidative stress as well as modulate neurotransmission. A clear indication that MT-3 ameliorates glutamate neurotoxicity by reducing oxidative stress has been provided using cultured cerebellar neurones, in which MT-3 reduced NO-induced formation of cyclic GMP, but did not prevent a rise in intracellular calcium [267].

The relative contributions to brain chemistry of individual MT isoforms with regard to specific neuronal functions, or general redox and metal-regulating actions remains to be unravelled, although it would appear that MT-3 has both. Furthermore, MT-3 mRNA has now been detected in nonneuronal tissue, including testis, prostate, epididymus, tongue, ovary, stomach and heart, albeit at lower concentrations than in brain [44].

Conclusions

MT is a protein with redox and metal-binding properties that endow it with wide-ranging functional capabilities in

biosystems. To prove a single 'primary' role may not be possible, in view of MT's ubiquity, present in most if not all eukaryotes, and active within most tissues and organ systems of higher animals. Functions depend on individual species and tissue/organ requirements. Constitutive MT may play a background role in certain homeostatic mechanisms, whereas highly induced MT concentrations are adaptive to various environmental stresses.

Advocates for toxic metal (Cd) sequestration as the principal function for MT have used an evolutionary argument that because Cd renal impairment already afflicts 7% of the general population, increased Cd toxicity in the absence of MT would cause greater damage to a higher percentage of the population, thereby bestowing a selective advantage to having MT [5]. For support, the proven increased susceptibility of MT–/– mice to Cd toxicity is cited. It can be argued, however, that this case arises only from recent activities of one species, and therefore lacks evolutionary relevance, although in past geological ages volcanic activity and disruptions of the Earth's crust may have released large quantities of Cd into the environment. The widespread ability of more primitive organisms to adapt to high environmental Cd is evidence for natural releases of Cd. Supporters of detoxification claim that Zn homeostasis/metabolic regulation is secondary because MT–/– mice survive and breed well, with no apparent selective advantage of MT. This claim can be countered by clear evidence from studies in MT–/– mice that MT bestows evolutionary fitness via control of Zn homeostasis under stressful conditions, e.g. infection, especially with lowered food and/or Zn intake, where influence on breeding success can be extreme. Evolution is driven by environmental change, not stability, and survival in a protected laboratory environment does not predict success in the wild.

Redox control of the metabolic influence of Zn has been proposed as the core function of MT [4, 235]. However, despite the demonstration of relevant mechanisms at physiologically appropriate concentrations of biomolecules in vitro, a necessarily high degree of reductionism has rendered confirmation in the more complex milieu of living cells problematic. Nevertheless, phenotypical characteristics of MT–/– mice are consistent with some degree of metabolic decompensation, and it may only be a matter of time before confirmation in cell systems is achieved. Cells from MT–/– mice provide perhaps the best means to prove the biological relevance of the in vitro findings. Whether or not the regulation of Zn in controlling metabolism is the primary role of MT is another question, however. One must first examine the probable evolutionary history of MT-related adaptations of a broader range of life forms.

MTs, or at least polypeptides with remarkable similarity to the β-domain of MT, are to be found from the lowest limbs to the tallest branches of the phylogenetic tree; virtual proof of an evolutionary history exceeding 600 million years. In the shallow PreCambrian seas it seems reasonable to surmise that proto-MT structure evolved under selective pressure from toxic metals and free radicals. Molecules with metal thiolate clusters in common with MT have enabled even prokaryotes, such as waterborn cyanobacteria, to survive and proliferate under high concentrations of toxic heavy metals. Harnessing toxic Fenton metals such as Cu for biologically useful roles (e.g. in respiration) must also have influenced selection. In blue crabs, a complex coordinated system for control of Cu by GSH and two iso MT-2s exists [3]. An example of divergence of MT function within the one species has been described in the land snail, *Helix pomatia* [268]. One MT isoform, found in the mid-gut gland, binds Cd and the other, in the mantle, binds Cu, presumably to regulate supply to hemocyanin. As life forms diversified to fill every possible environmental niche, their constituent MTs have responded to the resulting chemical challenges.

Research into MT in invertebrates has been largely directed at Cu and Cd metalloforms, with the emphasis shifting to ZnMT in vertebrates, making it tempting to believe that the primary role of MT has become more Zn oriented in higher animals. However, the extraordinary degree of conservation of the functional structure of MT across phyla suggests that most of its evolutionary shaping was complete hundreds of millions of years ago, with relatively minor further structural changes, fine-tuning the chemical adaptation to specific (external) environmental and metabolic (internal environment) requirements. With the exception of the investigation of inherited disorders of Cu metabolism (Wilson's Disease, Menke's syndrome) and the generation and study of relevant rodent models, CuMT in mammals has been somewhat neglected, possibly because, unlike Zn, there is higher intracellular binding of Cu by GSH and non-MT proteins. Nevertheless, there is no convincing evidence that CuMT is less important in higher animals. Indeed, the oxidation state of Cu can change after release from MT, with potentially greater implications for intracellular redox than Zn. CuMT can also donate Cu to Cu Zn superoxide dismutase [269], has influence on heme transport [270] and participates in other important processes.

It cannot be denied that the quest for the primary function of metallothionein has given direction and impetus to research, especially when opposing views are held by different groups. It may be, however, that the primary role for MT is more philosophical than physiological.

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