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Metallothionein: An Overview on its Metal Homeostatic Regulation in Mammals

Metalotioneina: Una Visión General de su Regulación Homeostática de Metales en Mamíferos

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SUMMARY: Metallothionein (MT) is a ubiquitous protein with a low molecular weight of 6-7 kDa weight and it was first identified in the kidney cortex of equines as a cadmium (Cd)-binding protein responsible for the natural accumulation of Cd in the tissue. The mammalian MT contains 61 to 68 amino acid residues, in which 18 to 23 cysteine residues are present. The expression of MT starts by binding of metal transcription factor-1 (MTF-1) to the regulative region of MT gene called metal responsive elements (MREs). The induction of MT through the MREs region can be initiated by several metal ions such as zinc (Zn), copper (Cu) and Cd. However, Zn is the only heavy metal which can reversibly and directly activate the DNA-binding activity of MTF-1. In mammals four types of MT are expressed and they are termed metallothionein-1 (MT1), metallothionein-2 (MT2), metallothionein-3 (MT3), and metallothionein-4 (MT4). MT1 and MT2 are expressed in almost all tissues while MT3 and MT4 are tissue-specific. MT is a key compound involved in the intracellular handling of a variety of essential and nonessential post-transitional metal ions. In order to the heavy metal binding ability of MT, it is suggested to play roles both in the intracellular fixation of essential trace elements Zn and Cu, in controlling the concentrations, and in neutralizing the harmful influences of exposure to toxic elements.

KEY WORDS: Metallothionein; Cadmium; Zinc.

INTRODUCTION

Metal-binding proteins have been found in various mammalian and non-mammalian tissues. The first mammalian MT was discovered by Margoshes & Vallee (1957). It was isolated from the horse kidney cortex of equines and revealed to have a high affinity to Cd. This protein was later biochemically characterized and termed "metallothionein" (MT), due to its high content of metals and cysteine residues (Kagi & Vallee, 1960; 1961). Its three-dimensional protein structure has been reported by both X-ray crystallography (Robbins & Stout, 1991) and NMR spectroscopy in the 1990s (Otvos & Armitage, 1991). MT belongs to a superfamily of intracellular metal-binding proteins which is composed of 15 families, and has various consensuses sequences inferred from both amino acid and polynucleotide sequences. MT has been identified in all animal phyla examined to date and also in certain fungi, plants and cyanobacteria (Webb & Cain, 1982; Robinson *et al.*, 1993; Coyle *et al.*, 2002) as well as in humans (Miles *et al*., 2000).

In mammalian cells, MT has been known to be involved in the regulation of Zn- and Cu-level as well as several biological and chemical signals. Therefore, the potential role of MT in health and disease is evident. However, a huge number of factors stimulating the biosynthesis of MT make it difficult to identify its specific biological role. In fact, the expression of MT gene in higher species is stimulated by a variety of factors including heavy metals, glucocorticoids, hormones, oxidants, strenuous exercise and cold exposure. The induction of MT by heavy metals and its subsequent metal accumulation in the cell have been used as a biomarker in the field of environmental toxicology (Sakulsak *et al.*, 2009). The expression of MT in cells is also induced by superoxide and hydroxyl radicals generated by g-radiation. It is suggested that MT acts either as a scavenger of radicals or a Zn donor for enzymes participating in the repair processes (Ryvolova *et al*., 2011).

Hence, the precise functional significance of MT is still under debates. Some hypotheses have been advanced for the functional significance of mammalian MT, such as the homeostasis and transport of physiologically essential metals (Zn, Cu), the metal detoxification (Cd, Hg), the

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protection against oxidative stress, the maintenance of intracellular redox balances, the regulation of cell proliferation and apoptosis, the protection against neuronal injury and degeneration, and the regulation of neuronal outgrowth (Palmiter, 1998, Miles *et al.*, 2000). These historical reports about this unique molecule have captured the curiosity of biologists and chemists and inferred the mystery of this MT family for over five decades, as described in several reviews (Vasak & Hasler, 2000; Coyle *et al*., 2002; Cherian *et al.*, 2003; Cherian & Kang, 2006, Thirumoorthy *et al*., 2007; Carpene *et al.*, 2007).

Metal-binding properties of MT. Mammalian MT is a single chain polypeptide containing 61-68 amino acid residues. It is a non-enzymatic intracellular protein with a low molecular weight of 6 to 7 kDa (< 7,000 Da) and it is ubiquitous in eukaryotes. The cysteine-containing residues (18-23 cysteine-residues) comprise about 30% of the molecule and they juxtapose to basic amino acids such as lysine and arginine without aromatic amino acids or histidine residuals. MT binds metals through the thiol (-SH) group of their cysteine residues. Typically, MT has a high affinity to both divalent essential metals such as Zn and Cu, and nonessential (or toxic) metals such as Cd and Hg which give rise to metal-thiolate clusters (Kagi & Vallee, 1960; Robbins *et al.*, 1991). The MT binding affinity to metal ions is different between various metals or metal-dependent as reported in several in vitro studies using MT common to rat liver, and it showed a slightly different relative order of the affinity to metals as shown in Table I.

Table I. The binding affinity of MT for metal ions.

The MT binding affinity for metal ions	References
Cd > Pb > Cu > Hg > Zn > Ag > Ni > Co	Waalkes *et al*., 1984
Hg > Cu > Cd > Zn > Ni = Co	Nelson *et al*., 1985
Hg > Ag >> Cu > Cd > Zn	Hamer *et al*., 1986

Moreover, MT have the greatest stability constant estimated for Cu (1019-1017) followed by that for Cd (1017-1015) and then Zn (1014-1011), indicating that CdMT has a 100-fold higher stability than ZnMT. According to both binding affinity and stability of MT indicate that the other metal ions such as Cd, Hg or Cu are more capable to displace Zn to further forming the more stable CdMT, HgMT or CuMT complexes than other low affinity and stability metal ions (Sabolic *et al.*, 2010).

MT is composed of two distinct clusters: a more stable a-domain closer to C-terminal which incorporates four divalent metal atoms bound to 11 cysteines with five bridging sulfur atoms, and a more reactive b-domain closer to N-terminal which contains only three metal atoms bound to nine cysteines with three bridging sulfur atoms (Coyle *et al*., 2002; Cherian *et al.*, 2003). The MT binding to metals starts with the a-domain and follows by the b-domain. These arrangements (28 intramolecular Zn-sulfur bonds) account for the very tight Zn- binding in both clusters and for the Zn-donating properties of MT. Although the exchangeability depends on metal species, in vivo mammalian MT appears as the predominant form of ZnMT complexes. (Orlowski & Piotrowski, 1998). The tertiary structure of MT is dynamic, especially for Zn and Cd. The exchange of Zn and Cd occurs rapidly within the b-domain, more slowly in the a-domain and perhaps also exchange with other ions bound to intracellular ligands. Furthermore, MT also donates metal ions to ligands with a higher affinity on the other proteins (Coyle *et al.*, 2002).

However, it has recently been reported in mammals that under the metal-excess condition, the N-terminal domain is responsible for the formation of non-oxidative metal-bridge dimers. In contrast, under the aerobic condition, a specific intermolecular disulfide is formed between two C-terminal domains. Both forms of the dimers show radical differences in the reactive properties of their respective clusters bound to the metal ions (Carpene *et al*., 2007).

The rate of MT degradation varies among animal species. The rate of MT degradation is determined by identity of metal-atoms bound to the protein, and the half-lives for Cd-, Zn- and Cu-MT in the liver have been reported to be approximately 80, 20 and 17 hrs, respectively (Richard, 1989). In addition, the differences in the metal distribution between MT isoforms may also influence the rate of MT degradation. According to its metal binding properties, MT has been suggested to be involved in the cellular homeostatic control and the regulation of trace elements (Tapiero & Tew, 2003).

GENE REGULATION OF MT. The induction of MT is specifically metal-dependent. The presence of metal response elements (MREs) in the upstream sequences of MT gene represents the evidence for this specific metal-induced transcription of MT (Stuart *et al*., 1985). The candidate MRE-binding protein termed metal transcription factor-1 (MTF-1), acts as the positive mediator to initiate the expression of MT gene. This factor, MTF-1 required increased Zn concentrations for optimal DNA binding (Westin & Schaffner, 1988). Based on studies of mice (Heuchel *et al*., 1994; Gunes *et al.*, 1998), MTF-1 is a ubiquitous expressed Zn finger protein that is necessary for basal and heavy metal-induced expression of MT. Subsequently, a constitutively active MTF-1 is initially inhibited by a Zn-sensitive inhibitor termed metal transcription inhibitor (MTI). In the presence of Zn, Zn-ions are bound to Zn-finger binding sites, resulting

in dissociation of MTF-1/MTI complex. This condition allows MTF-1 to interact with MREs in the MT promoter to further activate the MT transcription. The binding of newly synthesized MT to Zn results in the reform of MTF-1/MTI complex (Pamiter, 1994). Other metals such as Cd, Cu and Hg, which can induce MT, do not activate the MREs directly (Seguin, 1991) and it is suggested to follow a pathway that results in increased levels of the concentration of intracellular free Zn (Pamiter, 1994). For example, Cd, Cu and Hg have greater affinities for their ligands than Zn, and would be expected to displace Zn from other Zn-binding sites through a metal-metal exchange reaction. The displaced Zn also provides for binding of the inhibitor, releasing the transcription factor from inhibition, and further initiating the expression of MT (Roesijadi, 1996). Moreover, MREs is able to interact with many proteins, which can regulate MT expression (Miles *et al.,* 2000). The chemicals producing free radicals as well as various organic solvents, such as ethanol (Waalkes *et al.,* 1984), chloroform and alkylation agents can also induce the expression of MT (Ryvolova *et al.,* 2011). It has been reported that the highest levels of MT expression is shown in the late G1 phase and during entering the S phase. Hence, the role of MT is currently focused on the cancerogenesis and on its relation to the cancer cell cycle (Coyle *et al.*, 2002; Thirumoorthy *et al.*, 2007).

CLASSIFICATION OF MT. The classification of MT into families, subfamilies, subgroups and isoforms are based on the sequence similarities and phylogenetic relationships (Binz & Kagi, 1999). All vertebrates examined contain four distinct MT isoforms designated MT1 through MT4 (Moffatt & Denizeau, 1997). In rodent, MT is located on chromosome 8, whereas human MT is controlled by a family of genes which are located on chromosome 16q13 and encoded by a multigene family of at least 12 tightly linked genes, with numerous non-functional and processed pseudogenes (West *et al.,* 1990).

In mammals, four MT isoforms (MT1-MT4) and 13 MT-like human proteins have been identified (Simpkins, 2000). For MT1 isoform, eleven genes (MT1-A, B, E, F, G, H, I, J, K, L and X) have been discovered, whereas one gene for each isoform (Ghoshal & Jacob, 2001). MT1 and MT2 are major isoforms, in which the most prevalent thionein isoforms expressed in mammalian tissues. The cystein thiol (SH-) group of these metal-free proteins (apothionein) are able to complex 7 divalent metals like Zn and Cd or 12 monovalent metal ions (Nielson *et al*., 2006). In addition, mammalian MT1 and MT2 have the ability to form stable aggregates either with physiological or xenobiotic metals, and they exhibit the ubiquitous metal-induced expression of their genes, enhance the process of homeostasis, transport, and detoxification of metals as their main biological roles (Tio *et al*., 2004). Regarding to Zn metabolism using transgenic mice studies, MT1 and MT2 offer as a driving force for Zn-uptake by the transient production of apo-metalloprotein (apo-MT). Hence, they are suggested that mammalian MT1 and MT2 may function as chaperons for the synthesis of metalloproteins (Suhy *et al.,* 1999). These protein inductions by Zn are regulated by the (MTF1). In the embryos, MTF-1 absence abolished MT1 and MT2 gene transcriptions and reduced the transcription of g-glutamylcysteine synthetase, a key enzyme in glutathione synthesis. Moreover, MTF-1 null embryos resulted in increased susceptibility to Cd and hydrogen peroxide including liver degeneration (Gunes *et al.,* 1998). These proteins could serve as a reservoir for Zn while preventing the metal toxicity, and involved in Zn transfer to newly synthesized apo-MT. Furthermore, these two isoforms are inducible by a number of stress conditions and compounds, including glucocorticoids, cytokines, reactive oxygen species, and metal ions (Vasak & Hasler, 2000). Several studies on MT protein have been performed without discriminating effects on MT1 and MT2, and have been represented only as MT. Especially in most immunohistochemical studies, the anti-MT antibody did not differentiate these two isoforms. Hence, the term MT often is used as a term common to both MT1 and MT2.

MT3 and MT4 are minor isoforms which are generally found in specialized cells. MT3 is expressed mainly in the CNS which has a unique inhibitory effect on neurite outgrowth of cultured neurons that has not been observed for MT1 and MT2. Conversely, the MT4 is most abundant in certain stratified tissues. The expression of MT3 was first detected in specific structures of the mouse and human brain, such as Zn-rich neurons and astrocytes in the cortex, hippocampus, and amygdala (Ucida *et al*., 1991; Palmiter, *et al.,* 1992). MT3 is identified as a growth inhibiting factor (GIF) or neuronal growth-inhibitory factor, and it is synthesized in neural tissues and down-regulated in patients of Alzheimer's disease (Tsuji *et al.,* 1992; Aschner *et al.,* 1997). In contrast to isolated mammalian MT1 and MT2 from liver usually composes of 7 divalent metals of Zn, MT3 isolated from human and bovine brains contains 4 of Cu divalent metal ions, and 3 or 4 divalent metals of Zn. Hence, MT3 demonstrated the presence of 2 homometallic clusters, a Cu-thiolate and Zn-thiolate clusters (Bogumil *et al*., 1998). It is immediately brought about a high commitment to the biomedical research and later characterized as the brain specific isomer as well as the potential therapeutic targets for some neurodegenerative diseases therapy (Hozumi *et al*., 2004). In addition, it has been reported that Cd is involved in the reduction of MT3 mRNA in male and female urogenital tracts and a few other peripheral organs of rats, mice and humans (Hozumi *et al*., 2008). In 1994, MT4 was

identified by chance in human and mouse DNA as a fourth transcriptional active family member. MT4 is located about 20 kb upstream from the 5' of the MT3 gene in both mouse and human genome (Quaife *et al*., 1994). This protein consists of 62 amino acids including an insert glutamate in position 5 relative to the classical MT1 and MT2 proteins (Vasak & Meloni, 2011). Although the amino acid sequence of MT4 is more similar to that of MT1 and MT2 than that of MT3, a differential biological function was readily assigned to MT4 by its unique tissue and developmental precise expression pattern (Quaife *et al*., 1994). According to the structure of MT4, its function in handling divalent Zn ion or monovalent Cu metal ions has been inferred. In vitro study using Zn-, Cd-, or Cu-supplement media and their comparison with the well-characterized metalloforms of MT1, it has been reported that MT4 shows an increased preference for monovalent Cu ions over divalent Zn or Cd ions. These results suggested that MT4 differentiated to a MT with a "copper thionein" character rather than to a MT with a "zinc thionein" character (Tio *et al*., 2004). Taken together, the biological function of MT4 is probably both its Cu-thionein character and its developmentally controlled, tissue-specific expression pattern. By DNA microarray analyses of genes involved in the differentiation of mouse-epidermis, MT4 is identified as a target of the transcriptional activator Whn, and its expression is seen in the squamous epidermis and also in all epithelial developing tissues including the hair follicles and the back skin. Therefore, functional genomics not only corroborates but also extends the physiological role of MT4 as involving Zn transport or exchange in mammalian epithelia differentiation.

Furthermore, due to the increased metal binding capacity, MT has been suggested as a biomarker of both environmental and biological monitoring reflecting exposure to metal (Ryvolova *et al*., 2011). Since MT is present in most tissues and cell types, it is generally considered as a "house keeping" protein. Nevertheless, its concentration in cells can be altered by a variety of physiological condition such as the changes in the concentration of hormones as well as growth factors, and the accumulation of certain metals. The transcriptional control of MT induction and the changes in its nuclear and/or cytoplasmic localization during the cell proliferation and differentiation has been suggested that altered levels of MT could be expected in any situations where abnormal cell growths such as cancer occur (Coyle *et al.*, 2002; Cherian *et al.*, 2003; Thirumoorthy *et al.,* 2007).

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SAKULSAK, N. Metalotioneina: Una visión general de su regulación homeostática de metales en mamíferos. ***Int. J. Morphol., 30(3)***:1007-1012, 2012.

RESUMEN: Metalotioneina (MT) es una proteína, con bajo peso molecular de kDa 6-7 y que fue primero identificada en la corteza renal de equinos como cadmio (Cd)-proteína responsable por la acumulación natural de Cd en los tejidos. La MT en mamíferos contiene 61 a 68 residuos de aminoácidos, de los cuales están presentes 18 a 23 residuos de cisteína. La expresión de MT se inicia por la unión del factor-1 de transcripción de metal (MTF-1) a la región reguladora del gen de la MT llamado elementos metálicos responsable (MREs). La inducción de MT a través de la región MREs puede ser iniciada por varios iones metálicos tales como zinc (Zn), cobre (Cu) y Cd. Sin embargo, el Zn es el único metal pesado que puede revertir y activar directamente la unión ADN de MTF-1. En los mamíferos se expresan cuatro tipos de MT y ellos se denominan metalotioneína-1 (MT1), metalotioneína-2 (MT2), metalotioneína-3 (MT3), y metalotioneína-4 (MT4). MT1 y MT2 se expresan en casi todos los tejidos mientras que MT3 y MT4 son tejido-específico. La MT es un compuesto clave implicado en la manipulación intracelular de una variedad de iones metálicos esenciales y no esenciales post-transicionales. Con el fin de evaluar la capacidad de unión de metales pesados de MT, se sugiere que éste desempeña ambos roles tanto en la fijación intracelular de trazas de elementos de Zn y Cu como en el control de las concentraciones, y neutralizando las influencias perjudiciales a la exposición de elementos tóxicos.

PALABRAS CLAVE: Metalotioneina; Cadmio; Zinc.

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