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Review Article

METALLOTHIONEIN: CLASSIFICATION, BIOCHEMICAL FEATURES AND CLINICAL APPLICATIONS

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Abstract: Metallothionein (MT) is a ubiquitous low molecular weight protein with high cysteine content and has strong affinity for heavy metals. MT provides protection against heavy metal toxicity, oxidative stress, and participates in the regulation of physiological metals like zinc (Zn^{2+}) and copper (Cu). Abnormal MT expression and function presumably leads to various diseases like diabetes, cancer and neuro-degenerative diseases. MT gene expression is induced by a high variety of stimuli like metal exposure, oxidative stress, glucocorticoids, hydric stress etc. The level of the response to these inducers depends on the MT gene. These activities are regulated through intracellular metal ion modulation and free radical scavenging. MT participates in the uptake, transport, and regulation of zinc in biological system. It regulates zinc homeostasis by binding and releasing zinc ions which are a key element for the activation and binding of certain transcription factors through its participation in the zinc finger region of the protein. It also seems to be important for the regulation of tumor suppressor protein, p 53. Because MT plays an important role in transcription factor regulation, problems with MT function or expression may lead to malignant transformation of cells and ultimately cancer. There are various studies suggest that higher levels of MT expression may also lead to resistance to chemotherapeutic drugs.

Keywords: Metallothionein (MT); Molecular functions; Cancer; Neurodegenerative disease; Diabetes; Heavy metal toxicity

1. Introduction

Metallothioneins (MTs), discovered in 1957 by Margoshes and Vallee from horse (equine) cortex, are some of the most familiar proteins to researchers in the field of health and basic sciences. Due to their high metal content and fortuitous bio-organic structure, they are classified as metalloproteins (Thirumoorthy *et al.*, 2007). In mammals, MTs constitute a superfamily of non-enzymatic polypeptides having 61–68 amino acids, which correspond to low molecular weight (6–7 kDa), peculiar amino acid composition i.e. high cysteine content of approximately 30%, no aromatic amino acids, no or low histidine and a high amount of sulfur and metals in the form of metal-thiolate clusters (Pedersen *et al.*, 2009).

In mice, there are four *mt* genes that live in a 50-kb region on chromosome 8 (Quaife *et al.*, 1994) whereas in humans, mt inhabits various organs with numerous multiple molecular forms, including isoform of *mt1,mt2, mt3,* and *mt4* present on chromosome 16 (Cai *et al.*, 2006; Li *et al.*, 2007). This multi-gene family has around 14 closely related and pseudo genes which encode MT proteins. While a single mt-2A gene encodes MT-2 protein, MT-1 protein comprises many subtypes encoded by a set of mt-1 genes (*mt-1A, mt-1B, mt-1E, mt-1F, mt-1G, mt-1H* and *mt-1X*), accounting for the micro-heterogeneity of the MT-1 protein. The MT-3 and MT-4 are minor isoform which are normally found in specialized cells (Thirumoorthy *et al.*, 2007).

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The presumed functions of MT include intracellular metal metabolism and/or storage, metal donation to target apo-metalloproteins (especially zinc finger proteins and enzymes), and protection against electrophils (Davis and Cousins, 2000). They also provide protection against metal toxicity, regulation of physiological metals (Zn^{2+} and Cu), protection against DNA damage, oxidative stress, cell survival, angiogenesis, apoptosis, as well as increased proliferation (Thirumoorthy *et al.*, 2011) such as hydroxyl radical or nitric oxide; They have a role in apoptosis and in binding and exchange of heavy metals such as Zn, Cd, Hg or Cu (Thirumoorthy *et al.*, 2011) .

These remarkable findings discovered many significant contributions of MTs and its potential use for drug target. In this review, we have focused only on third era where enormous number of studies have been reported on their structure. Recently the role of MTs in cancer progression and its pathways has been extensively reviewed (Abdel-Mageed and Agrawal, 1998; Knipp *et al.*, 2005; Ostrakhovitch *et al.*, 2006). Here our goal is to review structural features of all MTs in association with its functions. Our review is mainly divided into two parts where first part deals with the biochemical composition, structure and types of all the MTs while second part with function and pathological role in concern to various diseases.

2. Biochemical composition of MT

MT has been traced in the entire animal kingdom, higher plants, eukaryotic microorganisms, and in some prokaryotes (Liang, 2013; Pan, 2013). The amino acid sequences of MT from many mammalian sources acknowledged by X-Ray Crystallography and ^{113}Cd-NMR (Boulanger et al., 1982; Kagi *et al.*, 1974; Otvos and Armitage, 1980; Winge and Miklossy, 1982) which suggests that mammalian MT contains approximately 61 amino acids of exceptionally similar composition which have affinity for both essential metals (Zn^{2+} and Cu) and toxic metals (Cd and Hg). More importantly, all contain 20 cysteine residues that remain unchanged along the amino acid sequence. They occur in the reduced form and are coordinated to the metal ions through mercaptide bonds, giving rise to spectroscopic features characteristic of metal-thiolate clusters (Otvos and Armitage, 1980). Aromatic amino acids are usually absent. The seven atoms of bound Cd are aligned in two separate poly-nuclear metal clusters, one containing three and the other four metal ions (Nielson and Winge, 1983).

Analogies of the primary structure of known mammalian MTs also disclose that most of the amino acid substitutions among species are conserved. The majority of non-conserved sequence is present in amino terminal signifies that there are lesser evolutionary constraints on this region of the polypeptide chain (Durliat *et al.*, 1999). Moreover, comparison of amino acid sequence tells us that MT-1 have evolved moderately at a rate intermediate to that of cytochrome *c* and hemoglobin (Pedersen *et al.*, 2009). Also, primary and secondary structure conservation of mammalian MTs suggests that the coding regions of MT genes are also homologous, whereas there is a difference in the non-coding sequences.

Altogether, these facts about MT structure probably can be cut into one simple principle: MT is functionally a very vital protein and its structural conservation is commanded by its functional need including the need for positional constancy of the cysteines. The significance of the overall structural conservation of MT can be more demonstrated by the finding that simply changing the length of the inter-domain hinge leads to a decline in its metal-binding ability. Hence, it cannot be overemphasized that the structure of mammalian MTs is the product of a functionally driven, evolutionarily selected process.

3. MT and its isoform

MT isoform are categorized on basis of various factors like molecular weight, metal to which it binds, genes encoded, chromosomes, amino acids sequence, etc. On a wide scale, MTs are categorized into two broad classes, major class and a minor class. Major class consists of MT-1 [MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H and MT-1X] and MT-2, both are ubiquitously expressed and are stress inducible whereas minor class includes MT-3 and MT-4 which are normally

found in specialized cells (Thirumoorthy *et al*., 2007; Thirumoorthy *et al*., 2011).

Four classes of MTs have been characterized in mammals.

- The MT-1 and 2 genes are expressed in many tissues including liver and kidney, where they regulate copper and Zn^{2+} which are involved in cell transcription, detoxification of heavy metals, play a role in immune and various G.I. tract functions.
- MT-3 expression is restricted to the brain and male reproductive organs. It plays a key role in development, organization and programmed death of brain cells.
- MT-4 is confined to stratified squamous epithelia i.e. skin and upper G.I. tract (Liang, 2013). It helps to regulate pH of stomach, taste and texture differentiation of the tongue and to protect against sunburn and other dermatological issues.

4. MT and its functions

4.1. MT as an antioxidant

MT is a vital constituent of cellular defenses against oxidative stress and negative switch of oxidative stress-mediated apoptosis as well as apoptosis independent of oxidative stress (Maret, 2009). An inspection of report have evinced that the antioxidant property of MT is augmented in presence of Zn^{2+} (Pedersen *et al*., 2009). Hence, the reduction of Zn^{2+} accessibility from MT advocates that the mutant MT is either less responsive towards nitric oxide or it is in an oxidized state and does not confine enough amount of Zn^{2+} (Cai and Cherian, 2003).

Examined in contrast with other antioxidants that can foster specifically against certain damage—SOD against superoxide radical, catalase against hydrogen peroxide and glutathione peroxidase against hydrogen peroxide and lipid peroxides—MT is an effective antioxidant against an advanced range of free radicals constituting the most effective radicals: hydroxyl radical and peroxynitrite. MT scavenges superoxide radical in a dose-dependent manner with increasing concentrations as evidenced by the inhibition of nitrite formation (Hussain et al., 1996). Therefore, MT as a potent antioxidant may prevent diabetic complications through suppression of diabetic oxidative damage (Abdel-Mageed and Agrawal, 1998).

4.2. MT as an anti-apoptotic factor

Latest studies have briefed that the enhanced expression of MT in cells generate the anti-apoptotic effects and a deficiency of MT in MT null cells boost the susceptibility to apoptotic cell death after exposure to certain anticancer drugs (Thirumoorthy *et al*., 2007). A study has brought in notice that down-regulation of MT in MCF-7 cells with an 18-mer antisense phosphorothioate not only restricts growth but also introduced apoptosis (Schwarz *et al*., 1995).

4.3. MT as anti-inflammatory factor

MT also plays a role of an anti-inflammatory agent including protection of liver injury, allergic response, and oxidative lung injury (Schwarz *et al*., 1995). A study has disclosed that MT defends against coagulatory and fibrinolytic disturbance and multiple organ damage including lung injury induced by lipopolysaccharide, at least partly, via inhibition of the local expression of pro-inflammatory cytokines including interleukin IL-1, IL-6, and interferon-γ (Schwarz *et al*., 1995). Conversely, mammalian cells that express excess MTs appear to be resistant to the toxic effects of nitric oxide and many electrophilic anti-neoplastic agents (Palmiter, 1998). Another study in China (2008) investigated how MT induced by dexamethasone (DEX) led to protection of ischemia/reperfusion (I/R) injury of myocardium in isolated rat heart (Zhuang *et al*., 2008).

As a well known fact, liver has high levels of Zn^{2+}- and Cu-bound MT and has a high capacity for regeneration and MT is reportedly involved in hepatocyte regeneration after partial hepatectomy and chemical injury (Fausto, 2000; Koniaris *et al*., 2003; Tohyama *et al*., 1993).

4.4. MT as scavenger for heavy metal detoxification

Amongst the hazardous environmental contaminants, Cd is listed at eighth in the top 20 hazardous polluting substances (Thirumoorthy *et*

al., 2007). Cd is noxious to number of tissues in body getting exposed to Cd results in hepatic, pulmonary, and testicular injury, whereas renal, bone injury and cancer, as well as toxicity to other organs are caused by the chronic exposure (Wasylenki and Goering, 1995). Nephrotoxicity, osteotoxicity and immunotoxicity are the consequences of prolonged exposure. This is also categorized as a human carcinogen causing genitourinary disorders like tumors of the lung, prostate, injection site, and other tissues. Most of Cd in the body is bound to a small, cysteine-rich, metal binding protein MT (Klaassen *et al.*, 2009).

The role of MT in Cd disposition had been examined in MT-transgenic mice. Using that model, it was observed that MT did not restrict intestinal Cd absorption nor did it alter initial Cd distribution to various tissues (Liu and Klaassen, 1996; Tohyama *et al.*, 1996). On the other hand, MT decreased Cd elimination through the bile (Klaassen, 1978) and was a chief agent for tissue retention of Cd (Liu *et al.*, 1996; Tohyama *et al.*, 1996).

5. MT in relevance to diseases

5.1. MT and ageing

Discovered with anti-apoptotic properties, MT is a low molecular weight protein that has been demonstrated to scavenge free radicals *in vitro* (Swindell, 2010). The 2010 study notify that abundance of MT is responsive to caloric restriction (CR) and inhibition of the insulin/ insulin-like signaling (IIS) pathway, and elevated MT gene expression has been observed in tissues from fasted and CR-fed mice, long-lived dwarf mice, worms maintained under CR conditions, and long-lived *daf-2* mutant worms. The deregulation of MT in these systems is probable to have tissue-specific effects on aging outcomes. Also, analysis was done to understand how MT contributes to the reaction of invertebrates and mice to CR and the endocrine mutations studied by aging research workers (Swindell, 2010).

MT is a standard stress response protein and a lot of studies have concluded the participation of MT in response to various types of stress like heavy metal toxicity, drug toxicity, bacterial endotoxin, viral infection, alkylating agents, endoplasmic reticulum (ER) stress and oxidative stress (Bakka *et al.*, 1982; Satoh *et al.*, 1997; Viarengo *et al.*, 2000). The anti-oxidant potential of MT is of special interest in the context to ageing biology, which is talked about a lot of times, with disagreeing results and uncertainty about whether *in vitro* findings are in relevance to its activity *in vivo* (Yang *et al.*, 2006).

5.2. MT and neuro-degenerative diseases

The physiological role of MT remains a matter of debate even after 50 years of its discovery. It is assumed as an ambiguous protein. This is due to its function in the central nervous system (CNS), where it is difficult to link its known biochemical properties such as metal binding and free radical scavenging to intricate the working of brain. There are a large number of neuro-degenerative diseases linked to up-regulation and down-regulation of MT. Some of them as cited below:

Parkinson's Disease- Brain MT-I and II can be driven by Cd, Zn^{2+}, endotoxin, kainic acid and 6-hydroxydopamine (Choudhuri *et al.*, 1995; Ebadi *et al.*, 1996; Wasylenki and Goering, 1995). A study in 2011 approved MT expression by reactive astrocytes in Parkinson's disease and supports a neuro-protective role for these cells. The classical theory that says that nigral astrocytes are non-reactive in Parkinson's disease is clearly incorrect. However, it is possible that astrocytes are themselves affected by the disease process which may explain their comparatively modest and previously overlooked response (Michael, 2011).

Autism- Heavy metal toxicity in children with autism spectrum disorder, especially mercury has been a matter of debate from a long time. Getting exposed to mercury can worsen immune, sensory, neurological, motor and behavioural functions in a child. These traits are similar with autism. The ethyl-mercury containing preservative, thiomersol, has been implicated as a source of heavy metal contamination in Autism Spectrum Disorder children (Dufault, 2012). Ejection of heavy metals requires the expression of the *mt* gene, which amalgamate the Zn^{2+} dependent metal binding protein MT. With dietary Zn^{2+} loss and Cu gain from the consumption of high fructose corn syrup (HFCS) (Ivaturi and Kies, 1992), metabolic processes occur to eliminate

heavy metals which are impaired in children with autism (Dufault et al., 2009).

Alzheimer's disease- MT plays a role in Zn^{2+} homeostasis in brain and neurodegenerative diseases (Amoureux et al., 1997; Erickson *et al.*, 1994; West *et al.*, 2008). Brain MT-III has been discovered as an agent that curb growth and neuronal sprouting factor in culture (Zheng *et al.*, 1995). MT-III was originally thought to be down-regulated in Alzheimer patients (Dalton *et al.*, 1995; Zheng *et al.*, 1995). However, further studies could not validate the association of MT-III with Alzheimer's disease (Dalton *et al.*, 1995; Rojas *et al.*, 1996).

It has also been validated by immunofluorescence staining, that astrocytes and microglia/ macrophages surrounding the plaques express MT-I&II. In common places, MT-I regulation follows a similar but less potent response than glial fibrillary acidic protein (GFAP) expression. MT-III mRNA expression is not significantly modified in any of the models evaluated in contrast to MT-I suggesting that the various MT isoform may play different role in these experimental systems and perhaps also in human AD (Carrasco *et al.*, 2006).

5.3. MT and Cancer

MT has a known role in regulation of programmed cell death (Dutsch-Wicherek *et al.*, 2008). An increased level of MT has been found to prevent apoptosis in cell cultures. Hence, MT regulates intracellular Zn^{2+} concentration and interacts with some proteins involved in apoptosis. Zn^{2+} is an intracellular mediator of apoptosis, which also interferes with action of calcium. Zn^{2+} is able to inhibit many proteins linked with apoptosis, e.g. caspase-3 and some proteases. Zn^{2+} also prevents DNA fragmentation and restrains calcium-magnesium dependent proteases. MT can also regulate the biological activity of p53 via Zn^{2+} exchange. MT-1 and MT-2 keep a check on the level, activity and cellular location of the transcription factor NF-κB (Butcher *et al.*, 2004; Wang *et al.*, 1999). NF-κB is required to protect cells from the apoptotic cascade induced by TNF and other stimuli through activation of anti-apoptotic genes and proto-oncogenes such as Bcl-2, c-myc and TRAF-1. In addition, apo-MT-1 (metal-free form of MT-1) but not MT-1 (MT-1 with metal ion) forms a complex with p53 (Abdel-Mageed and Agrawal, 1998; Knipp *et al.*, 2005; Ostrakhovitch *et al.*, 2006). p53 plays an important role by increasing metal-dependent expression of metal-responsive-elements (Ostrakhovitch *et al.*, 2007).

MT has an antioxidant property that along with glutathione (GSH), it is able to protect easily oxidizable molecules from free radicals by decreasing reactive oxygen species (ROS) level. The role of MTs as a tumor disease marker or as a cause of resistance in cancer treatment is reviewed and discussed.

Prostate Cancer- Prostate glands contain heavy metals such as Zn^{2+} and Cd, which are said to be linked with prostate cancer development. In order to understand the heavy metal metabolism in prostate glands, A study was conducted to investigate the MT regulation by metal-responsive promoter element-binding transcription factor (MTF) and zinc transporter in human prostate cells and tissues (Hasumi et al., 2003). The disturbance of Zn^{2+} homeostasis emphasized a significant decrease of cellular Zn^{2+} level was well documented to associate with the development and progression of human prostate malignancy (Wei *et al.*, 2008). In normal prostate tissue, the MT-1A, 1E, 1X and MT-2A isoform existed but there was a down-regulation of the MT-IX isoform in advanced prostate cancer. It has been imparted that MT-1 and MT-2 isoform may be related to the proliferative activity of breast, colon and prostate human cancers (Anania, 2011).

Papillary Thyroid Cancer- The thyroid follicular epithelium results in Papillary thyroid carcinoma (PTC) and symbolizes the most recurrent thyroid malignancy. PTC is correlated with gene rearrangements generating RET/PTC and TRK oncogenes, and to the BRAFV600E activating point mutation (Anania, 2011). MT 1G performs as an onco-suppressor in papillary thyroid carcinoma. MT isoform have not been much examined in papillary thyroid cancer. The role of MT-1 and MT-2 isoform in papillary thyroid cancer cells (KAT5) authenticated that KAT5 cells expressed eight functional MT-1 and MT-2 isoform induced by Cd. Elevated calcium and activated ERK1/2 predated MT expression. The

variation in cell cycle disappeared when the expression of MT isoform was obstructed by calcium inhibitor or ERK1/2 inhibitor. Bodily, KAT5 cells express eight functional MT1 and MT2 isoform in a pathway regulated by calcium and ERK1/2. The elevation of the MT isoform contribute to the decreased G_0/G_1 phase but increased G2-M phase revealed a novel pathway for the expression of the functional MT in papillary thyroid cancer. Thyroid cancers are assorted as papillary, medullary, follicular and undifferentiated or anaplastic (Liu *et al.*, 2009).

Breast Cancer- Globally many women are haunted by the affliction of Breast cancer (Lai, 2011). MTs have been associated in breast cancer boosting as oncogenic proteins, assisting cell invasion in numerous types of cancers (Yap et al., 2009). These isoform are manifested in a tissue specific arrangement and may play definite isoform in various human tumors. The mRNA of MT-1 series termed as A, E, F, G, H, X and MT-3 isoform but not MT-1B and MT-4 isoform have been detected in breast cancer tissues (Thirumoorthy *et al.*, 2011). The MT-2A mRNA transcripts are reported to be the highest among all distinct roles in the different cell type. There are several reports on the expression of certain functional isoform detected in breast tissues and is positively correlated with cell proliferation and histological grade (Kim, 2010). Expression of MT-1F isoform has also been found to clout histological differentiation in invasive breast cancer since estrogen is known to act importantly in breast cancer tumorgenesis, the MT-1E isoform has been advanced to participate in alternative manners that replace the function of estrogen. It has also been documented that MT-3 isoform over expression is associated with a poor prognosis for patients with breast cancer (Cherian *et al.*, 2003).

Renal Tumor- MT expression has been detected in different types of human tumors (Cherian *et al.*, 2003; Theocharis *et al.*, 2002) including neoplasias of the urogenital tract (Bahnson et al., 1991; Tuzel *et al.*, 2001). The foetal and the adult kidneys consistently express MT-1 and MT-2 mRNA (Mididoddi *et al.*, 1996) and the corresponding protein, while the MT-3 isoform is also expressed in developing renal tissue, adult proximal tubule and renal cell carcinoma cell lines (Hoey *et al.*, 1997; Mididoddi *et al.*, 1996). The MT-0 isoform are not present in adult kidneys but it can be located in non-neoplastic tissue from renal and transitional cell carcinoma (Hellemans *et al.*, 1999). The renal cell cancer tissue exhibits three contrasting type of expression as up-regulation of MT-2A, down-regulation of MT-1A and MT-1G transcripts. Expression of the MT-3 isoform has been reported in the tubules of normal kidney and also in renal cell carcinoma along with other isoform of MT. The expression of the MT-3 isoform in cancerous bladder tissues which were not present in normal bladder tissues, suggested its application as a potential biomarker for bladder cancer. They have also shown high levels of MT-1X mRNA expression in bladder cancer. The MT-3 isoform which was formerly reported as specific to brain has been evinced in normal human kidney, renal carcinoma, bladder cancer and prostatic adenocarcinoma (Cherian *et al.*, 2003).

5.4. MT and diabetes

Diabetes is an endemic disease which is caused due to increased oxidative stress. Genetic or pharmacological increase in MT expression in heart, kidney and other organs protects organ dysfunctions like cardiomyopathy and nephropathy due to diabetes (Garrett *et al.*, 1999). Oxidative stress is the one of root cause of diabetic onset and its complications, and antioxidants can prevent both of these (Li *et al.*, 2007). T-cell–mediated inflammatory autoimmune reaction and STZ- or alloxan-induced diabetes are all regarded because of ROS and reactive nitrogen species (RNS) formation that causes β-cell destruction. Also, due to very low concentrations of antioxidants in animal pancreatic islets and heart, these organs are susceptible to STZ or alloxan-induced diabetes through ROS and RNS generation (Rosen *et al.*, 2001). A preclinical research in 2008 concluded the result that the acute angiotensin II administration to wild type mice or neonatal cardiomyocytes increased cardiac apoptosis, nitrosative damage, and membrane translocation of the nicotinamide adenine dinucleotide phosphate oxidase (NOX) isoform p47phox (Thirumoorthy *et al.*, 2011).

Over expression of MT in various metabolic organs has also been exhibited to reduce

hyperglycemia-induced oxidative stress, organ specific diabetic complexities and DNA injury in diabetic experimental animals, which have been further substantiated by the results from MT-knockout mice. Furthermore, supplementation with Zn^{2+} has been shown to actuate *in vivo* MT synthesis in experimental animals and to decrease diabetes relevant complications in both humans and animal models (Wang *et al.*, 1999).

6. Conclusions

MT is ubiquitous protein that regulates metal level in animal and human body. It attains fingers like arrangement when metal level is elevated in the body, these fingers are triggered to bind them which reverse when metal level falls down in the body. Any change in MT expression causes anamolies due to change in metal concentration including neuro-degenerative disease like autism etc. Here we concluded that different isoform of MT plays a key role in patho-physiology of organism i.e. anti-oxidant, anti-apoptotic, anti-inflammatory properties and heavy metal scavenging. Furthermore, it regulates the level of glucocorticoids, interferon, interleukin-1, progesterone, vitamin D3 endotoxins, serum factors, heavy metals, storage of metal ions in the body.

Abbreviations

MT, Metallothionein; Zn^{2+}, zinc; Cu, copper; Cd, cadmium; Hg, Mercury; GI, Gastro Intestinal; CR, Caloric Restriction; IIS, Inhibition of the Insulin/insulin-like Signaling; CNS, Central Nervous System, MPTP, 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydro Pyridine, HFSC, High Fructose Corn Syrup; GSH, glutathione; PTC, Papillary Thyroid carcinoma; RTK, Receptor Tyrosine Kinase; ERK, Extracellular signal-Regulated Kinases; ER, Endoplasmic Reticulum

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