The Physiological Roles of Extracellular Metallothionein

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Metallothionein (MT) is a low-molecular-weight protein with a number of roles to play in cellular homeostasis. MT is synthesized as a consequence of a variety of cellular stressors, and has been found in both intracellular compartments and in extracellular spaces. The intracellular pool of this cysteine-rich protein can act as a reservoir of essential heavy metals, as a scavenger of reactive oxygen and nitrogen species, as an antagonist of toxic metals and organic molecules, and as a regulator of transcription factor activity. The presence of MT outside of cells due to the influence of stressors suggests that this protein may make important contributions as a "danger signal" that influences the management of responses to cellular damage. While conventional wisdom has held that extracellular MT is the result of cell death or leakage from stressed cells, there are numerous examples of selective release of proteins by nontraditional mechanisms, including stress response proteins. This suggests that MT may similarly be selectively released, and that the pool of extracellular MT represents an important regulator of various cellular functions. For example, extracellular MT has effects both on the severity of autoimmune disease, and on the development of adaptive immune functions. Extracellular MT may operate as a chemotactic factor that governs the trafficking of inflammatory cells that move to resolve damaged tissues, as a counter to extracellular oxidant-mediated damage, and as a signal that influences the functional behavior of wounded cells. A thorough understanding of the mechanisms of MT release from cells, the conditions under which MT is released to the extracellular environment, and the ways in which MT interacts with sensitive cells may both illuminate our understanding of an important control mechanism that operates in stressful conditions, and should indicate new opportunities for therapeutic management via the manipulation of this pool of extracellular MT. Exp Biol Med 231:1548–1554, 2006

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Introduction

Metallothionein (MT) is a highly conserved family of closely related stress response proteins that have been linked to a number of different aspects of cellular homeostasis. MT is involved in the management of essential heavy metal divalent cations (e.g., Cu and Zn) (1–3), it serves to interfere with the toxic effects of other heavy metals (e.g., Hg and Cd) (4) and free radicals (5–8), and it has been reported to regulate specific transcription factor activity (9, 10). Induction of MT occurs as a consequence of a variety of initiators, including divalent heavy metals; endotoxin (11); interferon (12); glucocorticoids (13, 14); the acute-phase cytokines tumor necrosis factor-α (15), interleukin-1 (IL-1) (16), and IL-6 (17); reactive oxygen and nitrogen species; and toxic organic compounds (18) (reviewed in [19, 20]). While most studies focus on the role played by intracellular pools of MT, a number of reports suggest that an extracellular pool of MT also exists with important roles to play. This review explores the evidence that MT is found in significant amounts outside the cell, and considers potential roles for MT.

Metallothionein in Extracellular Environments

There have been a number of reports of MT in tissue extracellular spaces, in secretions, and in excretions. MT is found in serum in increasing concentrations following restraint stress (21, 22) and upon exposure to toxic metals (23). MT in the urine (metallothionuria) has also been used as an indication of cadmium toxicity (24, 25). MT has been found in milk (3), in bronchoalveolar (26) and prostatic (27) fluids, bile (28), liver sinusoids (29), pancreatic ducts (30), and renal tubules (29). This stress response protein is also found at sites of tissue wounding and inflammation (31–35). Indirect evidence for extracellular MT comes from the observation that metal stress can elicit anti-MT autoantibodies both in mice and in humans (36–38). Finally, MT has been reported to be selectively released by cells in culture (39–41).

It has been suggested that cadmium-exposed animals transfer cadmium from the liver to the kidneys by release of cadmium-MT from the liver (42, 43). The cadmium-MT

complex is filtered in the glomerulus, but is readsorbed in the proximal tubule. The major pathway for cadmium uptake by tubule cells *in vivo* is endocytosis of cadmium complexed with MT, and it has been suggested that this process is receptor-mediated (44). Renal tubular cells degrade the cadmium-MT complexes and the cadmium is released, subsequently inducing synthesis of new MT in the tubular cells.

The presence of MT as an extracellular protein is somewhat unexpected in light of the absence of signal peptide sequences or other protein trafficking signals in the protein that would target it to these environments. The vast majority of extracellular proteins are exported from cells by the classical secretory pathway, in which proteins pass through the endoplasmic reticulum (ER) and the Golgi apparatus, and which can be either constitutive or regulated. In the constitutive secretory pathway, both membrane-bound and soluble proteins are transported from the lumen of the ER to the Golgi apparatus and to the outer plasma membrane of the cell via transport vesicles. Soluble proteins subsequently leave the cell by an exocytic process, whereas membrane components become integrated in the outer membrane. Some specialized secretory cells have a second, regulated secretory pathway, in which soluble proteins and other molecules are initially concentrated and stored in secretory vesicles for later release upon specific intracellular cues, often in response to extracellular signals. Both of these conventional methods of protein secretion require a specific N-terminal signal peptide sequence that results in localization of the protein at the cytosolic membrane of the rough ER and subsequent movement into the lumen of the ER. From there, these proteins are transported in vesicles to the Golgi where they are sorted and packaged according to their final destination.

The majority of MT messenger RNA (mRNA) is translated on free and cytoskeleton-associated polysomes (45), and is responsible for the synthesis of MT that localizes in the cytoplasm and nucleus (46, 47). Shapiro and Cousins (48) compared the relative amounts of MT mRNA in free and membrane-bound rat liver polysomes. They reported that the amount of translatable MT mRNA in polysomes directly correlates to the rate of MT synthesis. More than 93% of rat liver MT mRNA was found to be located on free polysomes, and this percentage increased when MT synthesis was induced in zinc-treated rats (48). In the same study, membrane-bound polysomes were found to make a negligible contribution to MT synthesis. There is some evidence to suggest that MT release from cells is selective, implying that MT release is not the consequence of membrane permeability changes or cell lysis in all circumstances. For example, in studies of adipocytes in culture, MT was found to be released during *in vitro* differentiation, and this release occurred earlier than that of leptin, another secretory product of the differentiating cells (40).

Mechanisms of Nonclassical Protein Secretion

A growing assortment of molecules have been recognized to lack signal sequences necessary to target nascent polypeptide to the ER for secretion, yet which nevertheless can be selectively released from cells. Several nonclassical mechanisms by which proteins can be exported from mammalian cells have been suggested in the literature. Some cell types contain specialized lysosomes that are capable of fusing with the plasma membrane and releasing their contents extracellularly in a process called lysosomal secretion. Plasma membrane resident transporters, export through exosomes, and exovesicular membrane blebbing have also been suggested as nonclassical routes (49–51). Direct movement of proteins across membranes in the partially folded "molten globule state" has also been suggested (52). It is particularly intriguing to consider the possibility that MT in the molten globule form (53) may be released from the cell by this vesicle-independent mechanism.

Recently, tubular extensions of the plasma membrane have been discovered that link certain mammalian cells in culture, including natural killer cells, B cells, dendritic cells, and macrophages (54–57). These tunneling nanotubules (TNTs) are long, thin, tubular extensions between cells that result in membrane and cytoplasmic continuity and allow selective transport of membrane-proteins such as major histocompatibility complex class I molecules or GPI-GFP molecules, as well as organelles. Not only can cell-surface proteins be exchanged between cells, but cellular signals such as calcium fluxes can be propagated through TNTs, a discovery that is indicative of cytoplasmic continuity between the connected cells. These TNTs have been shown to be transient and highly sensitive to mechanical stress in culture, and their extremely thin diameter (50–200 nm for tubules up to 100 μm long) makes them prone to breakage by shear forces and oxidative damage that exist *in vivo* (58). Such breakage could result in the transient release of cytoplasmic proteins such as MT from immune cells that have been shown to form TNT networks *in vitro*.

Some of the types of proteins secreted by nontraditional mechanisms are, intriguingly, associated with cellular stress or the cellular response to stress. For example, IL-1α and IL-1β are examples of proteins secreted via a nonclassical pathway. These proinflammatory cytokines are part of the acute-phase response and are synthesized and released by activated macrophages and certain other cell types (59). Neither the human nor mouse isoforms of this cytokine display an identifiable N-terminal signal sequence, which has been interpreted to suggest that IL-1 reaches the extracellular environment by a nonclassical secretory route (52, 60, 61). IL-1β secretion is selective because its presence in the extracellular environment does not correlate with the presence of other cytosolic proteins such as lactate dehydrogenase, which remains inside the cell. The processing of IL-1α involves myristoylation and, following

insertion into the plasma membrane, a calpain-dependent cleavage that is believed to cause release of the mature form of IL-1α into the extracellular space (62) via a specialized subspecies of endolysosomes (63). The uptake of IL-1 into secretory lysosomes may be mediated by an ATP-binding cassette transporter (ABC-transporter). The secretion of another acute-phase protein, macrophage migration inhibitory factor, is also mediated by a nonclassical pathway involving an ABC-transporter (64). The secretion of IL-1β is increased by heat shock (65). IL-1 has known receptors, underscoring its role as an actor in extracellular events that occur during the acute phase of the immune response.

Heat shock proteins (Hsps) such as Hsp-70 are also generally considered to be intracellular proteins with a variety of roles to play inside the cell. Nevertheless, Hsp-70 has been reported to be selectively released from cells under stress, and has been found to accumulate in extracellular compartments (66). Hightower and Guidon (67) showed that cultured rat embryo cells can be stimulated to rapidly release a select subset of proteins that include Hsp110, Hsp70, Hscp73, and actin. This release was not blocked by either monensin or colchicines, inhibitors of the common secretory pathway. The extracellular accumulation of these proteins was inhibited if the proteins were synthesized in the presence of the lysine analogue aminoethyl cysteine, suggesting that a selective release mechanism is involved (67). Hsp secretion was further supported by the observation that the sorting of proteins into exosome membranes is correlated with the presence of cholesterol/sphingomylein lipid rafts (68). Hsp70 and Hsp90 associate with such membrane microdomains (69). Furthermore, the release of Hsp70 from a human colonic adenocarcinoma cell line is dependent on the presence of intact lipid rafts (70). Like IL-1, the Hsp family of proteins has been suggested to bind to certain surface receptors (71), further underscoring a potential role for that pool of protein. Hsp70 has been suggested to exit the cell by membrane shedding or blebbing (72), which involves the formation of so-called exovesicles (for a review see [49]). Hsps have been found to bind to surface receptors (73) and can act as immune modifiers when in extracellular environments (74).

The majority of the members of the fibroblast growth factor (FGF) family are exported by ER/Golgi-dependent secretory transport. However, FGF-1 and FGF-2 have been shown to be secreted by an alternative pathway (75). These proteins regulate a variety of activities such as angiogenesis, tumor growth, and neurogenesis via extracellular interactions (76). As with MT and the Hsps, FGF can be secreted as a consequence of stress. In the case of FGF, it is secreted as an inactive homodimer (77) that is sensitive to reduction and activation with dithiothreitol, suggesting that the dimer forms via disulfide bonds between monomers. Once active, FGF interacts with several FGF receptors to influence cell division and differentiation. It was originally assumed that angiogenic growth factors would be released from mechanically injured tissues in order to promote wound healing. But subsequent evidence has shown that FGF-1 and FGF-2 are exported from cultured cells in the absence of an appreciable amount of cell death (78–81). Unlike IL-1β, which has been reported to be secreted by a vesicular nonclassical export pathway (63, 65), FGF-1 and FGF-2 may be directly translocated from the cytoplasm into the extracellular space because intracellular FGF-1 and FGF-2 have been localized to the cytoplasm in many FGF-secreting cell types with no apparent localization in vesicular structures (80, 82). In other studies, it has been suggested that some FGF-2 may be released by shedding of membrane vesicles (83).

In some circumstances, MT may be released from cells that have suffered membrane permeability changes or during necrotic cell death. There is as yet no conclusive data to suggest a specific mechanism by which MT might be secreted from cells, yet the protein does share some features with other proteins known to be secreted by nonclassical pathways. Many of these proteins are secreted at elevated levels under various forms of cellular stress (e.g., inflammation, increased temperature, tissue wounding, and toxicant exposure). Although the route of MT exit from cells remains to be established (and the route may differ under individual circumstances of stress, development, and other conditions), it is inescapable that MT does exit cells, and has intriguing effects on cellular activities.

Extracellular MT Activities

Once outside the cell, "intracellular" proteins may have functions that are either related or distinct from the activities in which they participate within the cell. MT is an example of a protein that may have unique activities linked to its extracellular role in addition to those similar to its role inside the cell. The redistribution of heavy metals between tissues may be one result of extracellular MT (84). MT plays an important role in zinc homeostasis, particularly in the pancreas, where it not only protects against zinc deficiency, but it also prevents any toxic effects of zinc on the pancreas (30). MT also might act as an antioxidant in extracellular environments. Kainic acid–induced seizures cause up-regulation of MT-I and MT-II genes in the hippocampus (33). MT-I/II–deficient mice display increased oxidative stress in the hippocampus and neuronal cell death following kainic acid treatment compared with wild-type controls. (85). Recently, it has been reported that transgenic mice overexpressing MT display decreased hippocampal inflammation induced by kainic acid when compared with wild-type control animals (33). Significant amounts of extracellular MT were found around hypertrophic reactive astroglia in the transgenic mice. This evidence suggests that MT may be protecting cells via its anti-inflammatory actions. A similar antioxidant role for MT has also been invoked in various forms of ischemic damage (e.g., focal cortical ischemia in the brain [86], renal ischemia [87], and ischemia-reperfusion–induced myocardial injury [88]).

There is also evidence that extracellular MT has effects not traditionally associated with its intracellular properties. There is one report of an MT-specific receptor on astrocytes (89), and MT can be detected on the surface of leukocytes harvested from congenitally autoimmune animals (90), from animals immunized in the presence of adjuvant (unpublished data), and on cells cultured in the presence of mitogen (e.g., lipopolysaccharide) or exogenous MT (91–93). Extracellular MT can influence cell proliferation in different ways depending on the conditions of exposure and the type of cell exposed. For example, MT-III (also known as growth inhibitory factor, or GIF) can act to suppress neuronal growth survival and elongation in culture (39, 94). In contrast, MT-II increases neurite elongation in culture (95). MT-I and MT-II have been shown to stimulate lymphocyte proliferation, both alone and in concert with other polyclonal activators of proliferation (e.g., Concanavalin A) (91, 96, 97). The consequences of these interactions can be observed in the effects that exogenous MT has on various immune functions. MT does not alter macrophage phagocytosis, but it does enhance the superoxide anion production by activated macrophages, and the candidacidal activity of these cells. Moreover, MT also suppressed the ability of macrophages to stimulate T cell proliferation (93). MT-mediated immunosuppression is also observed when cytotoxic T lymphocytes are cocultured with MT during the initiation and effector phases (98). Supporting these observations are *in vivo* studies of exogenous MT influences on immune activities. MT has been shown to suppress the development of a T-dependent humoral response, while having no effect on T-independent responses (99). This *in vivo* suppression can be blocked with a monoclonal anti-MT antibody, and in the absence of exogenous MT, this same antibody enhances the humoral response to T-dependent antigen challenge (100). This latter observation suggests that extracellular MT is released during a normal immune challenge to moderate the vigor of the humoral response, and that the monoclonal antibody can release the immune response from that endogenous extracellular MT. This conclusion is supported by the observation that MT-null mice develop a more robust humoral response to T-dependent antigen challenge than their wild-type counterparts (101), as well as by the observation of detectable levels of surface MT on leukocytes harvested from mice immunized in the presence of adjuvant (unpublished data). Exogenous MT can also suppress the development of both collagen-induced arthritis (102) and experimental autoimmune encephalomyelitis (103, 104).

Extracellular MT can promote some forms of wound healing. There are reports of MT produced at tissue wound sites (105, 106), and suggestions that MT present at those sites enhances tissue repair (35, 107–109).

Recent work from our laboratory has been based on the sequence similarities between MT and the chemokines (chemotactic cytokines). These similarities, combined with the observation that the MT gene cluster maps closely to some of the chemokine genes, suggested that MT might evince chemotactic behavior. MT can stimulate a variety of primary leukocytes and leukocyte cell lines to migrate in a directional fashion (110). Additionally, this chemotactic response can be blocked by antagonists that are known to interfere with G-coupled protein receptors. This suggests that the chemotactic response is a specific one, and may represent a means by which tissue damage initiates an inflammatory response that can progress to wound healing. This suggests that leukocytes may express an MT receptor that is part of the G-coupled protein receptor family.

Danger Signals

Stress response proteins have been suggested to serve as "danger signals": agents that augment the immune response as a consequence of tissue damage (111). According to this hypothesis, the molecular "nonself" motifs that the immune system recognizes are insufficient to initiate an effective immune response. In order to stimulate a fully functional immune response, molecular signals that indicate tissue damage or stress must also be present. In light of their synthesis during instances of tissue damage, and their capacity to influence various aspects of the immune system, Hsps have often been suggested as one form of these danger signals (112). In light of the influences that MT has on wound healing, inflammation, and the immune response, combined with the variety of immunomodulatory agents that induce MT, it is possible that MT may represent another form of danger signal.

Conclusions

MT is synthesized as a consequence of agonists that each can influence the progression of tissue damage and wound healing. A significant number of experiments have demonstrated that endogenous synthesis of MT, or exogenously added MT, can influence the progression of tissue damage, the inflammatory process, and the resolution of tissue damage. In light of the observations that exogenous MT can be used to manipulate the immune response and tissue repair, and observations that MT gene dose can influence these same activities, it seems reasonable to hypothesize that manipulation of MT may represent a novel therapeutic intervention. Moreover, in light of the suggestions in the literature that levels of MT synthesis produced by individuals under stress may vary significantly (113), there may be an opportunity to use MT synthesis and secretion as a diagnostic indicator of wound healing capacity.

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