

Autoimmunity-Inducing Metals (Hg, Au and Ag) Modulate Mast Cell Signaling, Function and Survival

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Abstract: The three heavy metals, mercury, gold and silver commonly and specifically induce aberrant immunological responses leading to autoimmune disorders in genetically susceptible animals and humans. The disorders are characterized by autoantibody production, increases in serum IgG and IgE, polyclonal activation of B and T lymphocytes and renal immune complex deposition and glomerulonephritis. Mast cells play key roles in allergic and inflammatory reactions. A growing body of evidence suggests that mast cells are key players in innate and adaptive immunity and involved in autoimmune diseases. Mast cells are also direct targets for autoimmunity-inducing metals both *in vitro* and *in vivo* and play a role in the development of metal-induced autoimmune disorders. The three metals specifically modulate mast cell function, including degranulation and secretion of arachidonic acid metabolites and cytokines such as interleukin-4. Divergent signaling components, including mitogen-activated protein kinase activation, reactive oxygen and nitric oxide generation and Ca^{2+} influx are modulated by the metals. Furthermore, the metals have considerable impacts on mast cell survival, which also species seems to be involved in the development of metal-induced autoimmune disorders. In this review, we provide an overview of recent advances in our understanding of the impacts of the three metals on mast cell signaling, function and survival and their possible roles in the pathologies of metal-induced autoimmunity.

Keywords: ROS, nitric oxide, Ca^{2+} , mitochondria, apoptosis, necrosis.

INTRODUCTION

Xenobiotic heavy metals such as mercury, gold and silver are widely used as industrial products, medical drugs and items such as dental amalgam implants, metal stents and fashion accessories [1]. These metals are capable of inducing alterations in the immune system. Multiple rodent models exist for mercury-, gold- or silver-induced autoimmunity [2-8]. Among these metals, mercury is the best known to evoke autoimmunity in genetically susceptible animals. Druet *et al.* [2] were the first to show that subtoxic doses of HgCl_2 could induce systemic immune dysregulation in Brown Norway (BN) rats. This disorder is characterized by autoantibody production, increases in serum IgG and IgE, polyclonal activation of B and T lymphocytes and renal immune complex deposition, resulting in glomerulonephritis [9-11]. Metal-induced autoimmunity in mice shares many similarities with the corresponding disorder in BN rats, including the production of autoantibodies [7]. Fibrillarin, a 34-kDa ribonucleoprotein, is the main target of the autoantibodies, which appear in patients with the autoimmune disease systemic sclerosis (scleroderma). Dental amalgam fillings are the most important source of mercury exposure in the general population. Amalgam is a mixture of mercury with silver, tin, copper and zinc and all these metals can affect the immune system. However, among these constituents only mercury and silver induce autoimmune disorders in some populations [1]. Exposure to mercury vapors can also result in elevated IgE levels and increased numbers of T lymphocytes. In addition, there is a correlation between the presence of antinuclear antibodies in systemic sclerosis patients and urinary mercury levels. These observations suggest intriguing similarities between various animal models and the results of human exposure to heavy metals. Gold and silver compounds such as aurothiomalate [12-14] and AuCl [15] as well as AgNO_3 [4,5,16] are also capable of evoking responses that are nearly identical in nature to those caused by mercury, except for lower autoantibody productions [17-19]. The induction of the disorders by mercury is genetically restricted to

animals that express specified major histocompatibility complex (MHC) haplotypes. For instance, Rat strains express the RT-1ⁿ MHC haplotype, such as BN strain are highly susceptible, whereas inbred Lewis strain rats, which have the RT-1^l MHC haplotype are resistant [20]. In mice, the induction of anti-fibrillarin autoantibodies is most pronounced in strains that express I-A^s MHC alleles such as SJL or A.SW mice [21-23]. H-2^f (A.CA), H-2^g (DBA/1) and H-2^p (P/J) strains are also susceptible to mercury-induced autoimmune disorders, including the production of antinuclear antibodies, although their prevalence and titers are lower than in H-2^s strains [24, 25]. Similarly, susceptibility to silver is genetically restricted in mice. The genetic susceptibility is localized to the H-2A locus [26]. In addition, non-H-2 genetic background substantially influences both the response rate and the titer of anti-fibrillarin autoantibodies. Strains bearing H-2A^s on the SJL and A backgrounds (SJL, A.SW, A.TH) are significantly more responsible than H-2A^s mice on the B10 background (B10.S). A suppressive effect on the B10 background has been observed in mercury treatment, but the effect is stronger in silver-treated mice.

Mast cells play key roles in allergic and inflammatory reactions. Activation of the high-affinity IgE receptor (FcεRI) on their cell surface triggers a cascade of signal transduction pathways that lead to various outcomes, including degranulation and synthesis and secretion of arachidonic metabolite mediators, including leukotrienes (LTs), and proinflammatory cytokines and chemokines [27]. There is now strong evidence that mast cells are important for both adaptive and innate immunity [27, 28] as well as the development of autoimmune disorders [29]. Mast cells are also proposed to play roles in metal-induced autoimmunity, which is characterized by early tissue injury in the form of vasculitis [11, 30]. The early vasculitis is T cell-independent but accompanied by *in vivo* evidence for mast cell degranulation that can be reduced by treatment with mast cell stabilizers [1, 31], thereby indicating that mast cells play a central role in the vasculitis. It should be noted that mercury, gold and silver also commonly activate mast cells *in vitro*. HgCl_2 and several gold compounds, including aurothiomalate and AuCl and HAuCl_4 directly stimulate mast cell degranulation [32, 33]. Silver compounds such as Ag_2SO_4 and AgNO_3 also directly activate mast cells to release chemical mediators, including histamine and LTC_4 , by a unique mechanism that differs from the FcεRI-mediated

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mechanism [34-36]. On the contrary, other metals, including zinc, copper, nickel, cadmium, palladium and platinum at non-toxic concentrations do not induce mast cell degranulation and/or LTC₄ secretion [34, 35]. Silver also modulates mast cell survival by a unique mechanism [37]. Based on the unique effects of mercury, gold and silver on mast cell functions and survival, we will focus the three metals in this review. A growing body of evidence suggests that the three metals exert their effects by at least partly overlapping mechanisms. These include generation of intracellular reactive oxygen species (ROS) and nitric oxide (NO) [36, 38], Ca²⁺ influx through thiol-regulated Ca²⁺ channels [35, 37] and mitochondrial deregulation [37]. In this article, we provide an overview of recent advances in our understanding of the effects of mercury, gold and silver on mast cell signaling, function and survival. We pay special attention to their possible roles in dysregulated mast cell activation and survival, which are relevant to T helper 2 (Th2)-independent and -dependent pathologies of metal-induced autoimmunity.

AUTOIMMUNITY-INDUCING METALS AFFECT MAST CELL FUNCTION

As mentioned above, mast cell degranulation plays a role in the early Th2-independent process during the development of metal-induced autoimmune disorders in rodents. Therefore, much attention has been paid to the impact of autoimmunity-inducing metals on mast cell degranulation. It has been shown that distinct metal ions, including Hg²⁺, Au³⁺ and Ag⁺, can be released from dental alloys or other biomaterials and may cause toxic effects on various cell types. These metal ions (0.33 mM each) induce histamine release by mast cells and basophils but this release is due to cytotoxicity, because ultrastructural signs of necrosis are associated [39]. Lower concentrations ($\leq 1 \mu\text{M}$) of HgCl₂ also induces release of granule-associated β -hexosaminidase and production of interleukin (IL)-4 and tumor necrosis factor (TNF)- α in bone marrow-derived mast cell (BMMC) degranulation with minimal effects on cell viability [40], indicating that these effects are due to cell activation. Mercury appears to exert its effects by a mechanism that differs from that activated by Fc ϵ RI stimulation, including protein tyrosine phosphorylation. At a wide range of non-cytotoxic concentrations (10^{-9} - 10^{-6} M), HgCl₂ enhances IgE-dependent histamine release from BN rat mast cells and human mast cells and basophils [41, 42]. However, the effects are additive, rather than synergistic, suggesting that the two stimuli activate at least partly overlapping mechanisms. HgCl₂ has similar effects on human LAD-2 mast cells and umbilical cord blood-derived cultured mast cells, and acts synergistically with substance P [42]. In rodents, the enhancement of (IL)-4 production from mast cells facilitates Th2 development and responses and IgE production. In humans, allergens stimulate the release of the Th2 cytokines IL-4 and IL-13 from granulocytes, primarily basophils, thereby causing Th2-dependent responses. Consequently, IL-4 and IL-13 secretion from mast cells/basophils may be important targets for metal-mediated modulation of the immune system. Non-toxic concentrations of HgCl₂ were shown to stimulate the transcription, translation and/or secretion of IL-4 and/or IL-13 in rodent mast cells and human basophils [40, 41, 43]. In addition, HgCl₂ stimulates the secretion of other cytokines, including IL-6 and vascular endothelial growth factor in human basophils, and acted synergistically with substance P in their release [42]. Additive IL-4 secretion was observed in the presence of HgCl₂ and Fc ϵ RI stimulation [41], suggesting that the underlying mechanisms are at least partly overlapping. In addition, IL-4 secretion occurs at HgCl₂ concentrations, which are comparable to those required to induce the upregulation of IgE production in experimental animals. Collectively, the IL-4 secretion induced by HgCl₂ may play a role in the Th2 pathologies observed in the autoimmune disorders.

Compared with mercury, there are few reports regarding the stimulatory effects of gold and silver on mast cell function. Re-

cently, non-cytotoxic concentrations ($\leq 10^{-5}$ M) of HAuCl₄ have been found to induce robust degranulation (β -hexosaminidase and histamine release) in RBL-2H3 cells and BMMCs [33]. Gold also induces moderate LTC₄ secretion (approximately 50% of the optimal Fc ϵ RI stimulation) and IL-13 secretion, indicating that it can directly activate mast cells to release mediators. Silver compounds such as AgNO₃ and Ag₂SO₄ [34-36] as well as silver nanoparticles [44] also strongly stimulate mast cell degranulation and/or secretion of LTC₄, but not cytokines. On the contrary, silver at concentrations that induce degranulation and LTC₄ secretion has minimal effects on the secretion of cytokines in RBL-2H3 cells and BMMCs [35]. However, cytokines such as IL-4, IL-6 and TNF- α as well as the costimulatory molecule CD28 have been shown to play important roles in the autoimmunity induced by mercury, gold and silver in mice [12]. Taken together with the fact that the onset of metal-induced autoimmunity is strictly dependent on the genetic background, there is an intriguing possibility that silver can activate cytokine secretion in mast cells derived from certain highly genetically sensitive strains of mice such as H-2^s strains. Alternatively, it may stimulate cytokine secretion by other cell types such as T cells. Collectively, as summarized in Table 1, mercury, gold and silver appear to commonly activate mast cells but their manners of action are different. The three metals are almost equally effective in the induction of degranulation but are distinct in terms of their abilities to evoke LTC₄ and cytokine secretion. Mercury and silver have reciprocal effects on LTC₄ and cytokine secretion. Specifically, mercury is a strong inducer of IL-4 secretion and weak inducer of LTC₄ secretion, whereas silver can strongly induce LTC₄ secretion and has minimal effects on cytokine secretion. On the other hand, gold can moderately activate both events. Fyn-deficient mast cells are unable to secrete LTC₄, IL-6 and TNF- α in response to antigen [45]. This failure is associated with defects in IgE-dependent arachidonic acid generation and the induction of cytokine mRNA expression. In the cells, c-Jun NH₂-terminal kinase (JNK), which is associated with the generation of arachidonic acid and p38 mitogen-activated protein kinase (MAPK) activation, is defective, while extracellular signal-regulated kinase (ERK) 1/2 and cytosolic phospholipase A₂ phosphorylation at Ser505 are normal. In addition, the NF- κ B DNA-binding activity, which drives IL-6 and TNF production, is markedly decreased through defects in I κ B kinase β and I κ B α degradation [45]. These facts strongly suggest that Fyn plays a crucial role in intracellular signaling pathways that lead to cytokine production. It is plausible that activation of both Lyn and Fyn kinases results in more pronounced effects than single kinase activation on tyrosine phosphorylation of cellular substrates. In this regard, it should be noted that compared with mercury and gold, silver induces tyrosine phosphorylation of much smaller number of proteins and the effect is less sustained; the increase in response to silver often disappears within 10 min [35], whereas those in response to mercury and gold are observed at least 30 min (Inoue and Suzuki, unpublished data). Taken together, there is an intriguing possibility that different abilities of the three metals to induce cytokine production are at least partly attributed to their different effects on Lyn and Fyn kinases. The species-specific effects of the three metals on mast cell functions might be also attributed to some extent to their different dependences on L-type Ca²⁺ channels (LTCCs), as an emerging machinery for mast cell Ca²⁺ entry and mediator release [46, 47]. We will address this issue in the latter part of the review.

EFFECTS OF AUTOIMMUNITY-INDUCING METALS ON MAST CELL SIGNALING

Intracellular Signaling Pathways Regulating IL-4 Gene Expression

MAPKs

Table 2 shows the effects of the xenobiotic metals on mast cell signaling and their possible targeted functions. Mercury and gold

Table 1. Effects of Mercury, Gold and Silver on Mast Cell/Basophil Function

Metals	Effects	Doses	Cells	Remarks	Refs.
Mercury*	Histamine release	$0.33 \times 10^{-3} \text{M}$	Human mast cells Basophils KU-812 HMC-1	Toxic histamine release	[39]
Mercury*	β -Hex release, IL-4, TNF- α secretion	10^{-6} - 10^{-5}M	BMMCs	Non-toxic	[40]
Mercury*	β -Hex release, VEGF, IL-6 secretion	10^{-7} - 10^{-5}M	LAD-2 hCBMCs	Synergistic with Substance-P	[42]
Mercury*	No histamine release Enhancement of IgE-dependent histamine release, LTC ₄ , IL-4, IL-13 secretion	10^{-9} - 10^{-6}M	Human basophils	Promotion of a Th2-cytokine profile	[41]
Mercury*	Enhancement of IgE-dependent serotonin release,	10^{-9} - 10^{-6}M	BN rat mast cells	Inhibition by antioxidants	[32]
Mercury*	Up-regulation of IL-4 mRNA, its promoter activity, IL-4 secretion	10^{-5}M	RBL-2H3	Inhibition by antioxidants similar effects of H ₂ O ₂	[43]
Gold**	Histamine, β -Hex release, LTC ₄ , IL-13 secretion	10^{-5}M	RBL-2H3 BMMCs	Inhibition by antioxidants	[33]
Silver***	Histamine, β -Hex release, LTC ₄ secretion	3×10^{-6} - 10^{-4}M	RBL-2H3 BMMCs	MAPK activation without proximal signals	[34] [35]
Silver***	β -Hex release, LTC ₄ secretion	10^{-5}M	RBL-2H3 BMMCs	Inhibition by antioxidants	[36]
Silver****	Histamine release	50 $\mu\text{g/ml}$	RBL-2H3	Optical quantitative phase microscopy	[44]

* HgCl₂** HAuCl₄*** AgNO₃, Ag₂SO₄

**** Silver nanoparticles

trigger intracellular signaling pathways that appear to be partially similar to those activated by Fc ϵ RI stimulation. Among these pathways, the molecular basis of IL-4 secretion induced by mercury has been exclusively studied. Several signaling pathways, including MAPKs and ROS, have been shown to play roles in mercury-induced IL-4 secretion. HgCl₂ upregulates the phosphorylation of JNK at concentrations that induce IL-4 secretion in C1.MC/C57.1 mouse mast cells. This upregulation is associated with increased JNK activity, and the JNK-specific inhibitor SP600125 abolishes both the mercury-induced c-Jun phosphorylation and IL-4 secretion in mast cells [48]. Thus, JNK appears to be one of the signaling proteins that mediate the effects of mercury on IL-4 expression in mast cells.

ROS

Several lines of evidence suggest that ROS are also critical for mediating the effects of mercury on IL-4 secretion in mast cells. Divergent agents such as HgCl₂ and gold salts that induce Th2-dominant autoimmune disorders commonly induce a robust increase in intracellular ROS in mast cells [32]. The enhancement of IL-4 gene and protein expression by HgCl₂ is significantly reduced by both thiol- and non-thiol-containing antioxidants, thereby imply-

ing a role for ROS in these events [32]. In fact, exogenous application of H₂O₂ causes upregulated transcription of IL-4 mRNA and IL-4 promoter activity [43]. Moreover, ROS have been shown to be critical regulators of the IL-4 promoter. In T cells, the P1 and P4 elements of the IL-4 promoter interact with the typical ROS-regulated transcription factors, NF- κ B and AP-1, and the potent antioxidant vitamin E can block the binding of transcription factors to these binding sites and downregulate IL-4 protein production [49-51]. Apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) is an enzyme that displays both APE endonuclease DNA repair activity and nuclear redox activity, thereby regulating multiple transcription factors. In mast cells, H₂O₂ induces the nuclear translocation of APE/Ref-1, while the inactivation of APE/Ref-1 expression abolishes H₂O₂-induced IL-4 expression [52, 53]. Moreover, impaired APE/Ref-1 expression inhibits oxidative stress-induced IL-4 secretion, but not IgE-dependent IL-4 secretion, despite the fact that H₂O₂ and Fc ϵ RI stimulation additively induce the secretion [53]. H₂O₂ has also been shown to induce the formation of AP-1 and NFAT complexes that recognize the IL-4 promoter. Strikingly, the effects of H₂O₂ treatment on BMMCs are similar but distinct from those of Fc ϵ RI stimulation. H₂O₂ causes

Table 2. Effects of Mercury, Gold and Silver on Mast Cell Signaling

Metals	Effects	Doses	Cells	Function of Target	Refs.
Mercury*	Up-regulation of JNK phosphorylation, activity	10^{-6} - 10^{-5} M	C1.MC/C57.1 mouse mast cells	IL-4 secretion	[48]
Mercury*	Production of intracellular ROS	10^{-9} - 10^{-6} M	BN rat mast cells	IL-4 secretion	[32]
Mercury*	STAT6 tyrosine phosphorylation	10^{-5} M	RBL-2H3	IL-4 gene expression, promoter activity	[43]
Gold**	Production and release of ROS, Ca^{2+} influx, LTCC activation	10^{-5} M	RBL-2H3 BMMCs	β -Hex release, LTC ₄ secretion	[33]
Silver***	Tyrosine phosphorylation of ERK1/2, Ca^{2+} influx	3×10^{-6} - 10^{-4} M	RBL-2H3 BMMCs	β -Hex release, LTC ₄ secretion	[35]
Silver***	Production of intracellular ROS, thiol-sensitive Ca^{2+} influx	10^{-5} M	RBL-2H3 BMMCs	β -Hex release, LTC ₄ secretion	[36]
Silver***	Production and release of NO (NOS-independent)	10^{-5} M	RBL-2H3 BMMCs	β -Hex release, LTC ₄ secretion	[38]

* HgCl_2 ** HAuCl_4 *** AgNO_3 , Ag_2SO_4

robust increases in tyrosine phosphorylation of Lyn, Fyn, phosphatidylinositol-3-kinase (PI3K), Akt and p38 MAPK, but not the adaptor molecule linker for activation of T cells (LAT). In addition, analyses using knockout mice or pharmacological specific inhibitors revealed that Fyn, PI3K and p38 MAPK, but not Lyn, are required for H_2O_2 -mediated induction of IL-4 gene and protein expression [53]. These findings are similar to those obtained for IgE-dependent cytokine production in mast cells, which seems to be primarily driven by Fyn but not Lyn [54]. Interestingly, antigen stimulation, but not H_2O_2 induces changes in the association of Fc ϵ RI with detergent-resistant membranes (the lipid rafts) [55] and Lyn, but not Fyn is considered to transduce signals in the lipid rafts. Taken together, Fc ϵ RI and H_2O_2 appear to induce cytokine production by different mechanisms in which the lipid rafts and Lyn and Fyn play different roles.

Transcription Regulatory Elements

cis- and *trans*-acting regulatory elements for mercury-induced IL-4 gene expression were characterized in rat mast cells. HgCl_2 has been shown to induce increased tyrosine phosphorylation of signal transducer and activator of transcription (STAT) 6, and STAT6 and TATA box binding sites are important for HgCl_2 -induced IL-4 gene expression and promoter activity [56].

Protein Tyrosine Phosphorylation

Similar to the observations in T cells [57], treatment of mast cells with mercury and gold results in increased tyrosine phosphorylation of multiple proteins, which is similar to the phosphorylation induced by Fc ϵ RI stimulation. On the contrary, silver results in no or very little tyrosine phosphorylation of a few proteins. Subsequently, it was revealed that silver triggers mast cell activation by bypassing early and prerequisite events in Fc ϵ RI signaling, including increased tyrosine phosphorylation of Fc ϵ RI β , Lyn, Syk and LAT [35]. Consistent with these observations, the biological effects of silver are minimally affected by the general tyrosine kinase inhibitor genistein [35] and Fc ϵ RI β gene knockout (Inoue and Suzuki, unpublished data). Collectively, silver appears to evoke intracellular signaling pathways that differ from those stimulated by Fc ϵ RI stimulation or mercury.

Intracellular Signaling Pathways Regulating Degranulation and LTC₄ Secretion

ROS

An increasing body of evidence suggests that mast cells are ROS producers and that endogenous ROS play roles in IgE-dependent degranulation and LTC₄ secretion [58-60]. Besides endogenous ROS, ROS from external sources (e.g. macrophages) also enter the mast cells and cause their activation and degranulation [59, 60]. ROS are not only critical for the induction of IL-4 secretion but also crucial for mast cell degranulation and LTC₄ secretion induced by metals. Similar to mercury and gold, silver evokes the production of intracellular ROS, including H_2O_2 , and antioxidants such as ebselen and dithiothreitol (DTT) block mast cell degranulation and LTC₄ secretion at concentrations that abolish the production of ROS [36]. Similar effects are observed for gold-induced secretion, but not IgE-dependent secretion [33]. Thus, metal-induced mast cell activation is characterized by its high sensitivity to thiol-containing antioxidants. Given that DTT may predominantly exist outside of cells owing to its cell impermeability, it is possible that external ROS play a selective role in metal-induced mast cell activation. In support with this view, these metal ions are more effective than Fc ϵ RI stimulation for inducing the release of ROS, including superoxide ($\text{O}_2^{\cdot -}$) into the extracellular space [35, 36]. Although silver appears to have minimal effects on the tyrosine phosphorylation of early signaling molecules, including Fc ϵ RI β , Lyn, Syk and LAT, it increases the tyrosine phosphorylation of ERK1/2 and the ERK-specific inhibitor PD98059 dose-dependently inhibits silver-induced LTC₄ secretion, but not degranulation [35]. These observations suggest that silver can evoke comparatively downstream signaling events, such as ERK activation, thereby inducing LTC₄ secretion.

Ca^{2+} Signaling

Ca^{2+} is a highly versatile intracellular second messenger that regulates many complicated cellular processes, including cell functions, proliferation and death. It is widely accepted that mast cell secretion responses depend on an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and that entry of Ca^{2+} from the extracellular

fluid (i.e. Ca^{2+} influx) is the major cause of the increase required for full activation of Ca^{2+} -dependent events. Similar to the effects induced by Fc ϵ RI stimulation, silver-induced degranulation and LTC₄ secretion requires a Ca^{2+} influx [38] and comparable data are obtained for gold [33]. Silver stimulates a robust Ca^{2+} influx independently of Fc ϵ RI β , Lyn, Syk and LAT [35]. These molecules play critical roles in the production of the second messenger inositol-1,4,5-triphosphate and the induction of Ca^{2+} mobilization from intracellular stores [61]. The resulting depletion of the stores leads to the activation of store-operated Ca^{2+} (SOC) channels such as Ca^{2+} release-activated Ca^{2+} (CRAC) channels, the main pathway for Ca^{2+} influx in mast cells [62]. Silver evokes a minimal increase in $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} , suggesting that it induces a Ca^{2+} influx in a store-independent manner. Consistent with this view, silver, but not antigen, induces a robust Ca^{2+} influx in cells that have been depleted of thapsigargin (Tg)-sensitive stores and this effect is resistant to SOC channel antagonists such as 2-aminoethoxydiphenyl borate and SK&F96365. However, the Ca^{2+} influx induced by low concentrations ($\leq 1 \mu\text{M}$) of silver is completely abolished by the depletion of the intracellular stores [28]. Therefore, silver appears to utilize different modes of Ca^{2+} influx, depending on the concentration employed. Similar to the secretion, the silver-induced Ca^{2+} influx is completely blocked by thiol-containing antioxidants such as DTT, N-acetylcysteine (NAC) and glutathione monomethylester (GSH-MEE) [38]. On the contrary, exogenous superoxide dismutase and catalase have minimal effects on the influx. The Ca^{2+} influx induced by gold is also blocked by these thiol-containing antioxidants and resistant to SOC channel antagonists and depletion of the intracellular Ca^{2+} stores [33]. Therefore, similar thiol-regulated non-SOC channels may play a common role in metal-induced Ca^{2+} influxes. At present, the molecular entities of the metal-sensitive Ca^{2+} channels are unknown. Recently, it was shown that $\text{Ca}_v1.2$, a subtype of LTCCs, which possesses α_{1C} subunit, are activated independently of Ca^{2+} store emptying, comprise another route of Ca^{2+} influx into mast cells [46, 47]. Analyses using $\text{Ca}_v1.2$ -knockdown cells revealed that $\text{Ca}_v1.2$ LTCCs plays a role in gold-induced Ca^{2+} influx [33] and mercury-induced Ca^{2+} influx (Inoue and Suzuki, unpublished data), but only plays a minor role in silver-induced Ca^{2+} influx [38]. In addition, this channel activation is sensitive to inhibition by DTT and ebselen (Inoue and Suzuki, unpublished data). Collectively, $\text{Ca}_v1.2$ LTCCs may form one of the thiol-regulated store-independent Ca^{2+} channels activated by mercury and gold. In addition, not only the CRAC channels [62] but also $\text{Ca}_v1.2$ LTCCs play important roles in cytokine secretion [46, 47]. Consequently, the failure of silver to induce cytokine secretion may be attributed to the insufficient activation of the two Ca^{2+} channel functions that are required for cytokine secretion.

NO Signaling

NO is a potent radical with diverse roles in biological systems, including vasodilatation, platelet aggregation and neuronal transmission. It also regulates the functions, death and survival of various cell types, including cells involved in immunity and inflammation, depending on the cell type and concentration [63, 64]. NO also suppresses mast cell activation and the subsequent features of inflammation as well as IgE-dependent degranulation, LT secretion and cytokine expression and secretion in mast cells [65-67]. NO is biosynthesized from L-arginine by NO synthase (NOS). There are three different NOS isoforms, NOS-1 (neuronal, nNOS), NOS-2 (inducible, iNOS) and NOS-3 (endothelial, eNOS). Befus's groups have shown that mast cells express various types of rodent and human mast cells and cell lines express NOS-1 and NOS-3 [67-69]. Moreover, significant increase in NO production was demonstrated in mast cells using confocal microscopical analysis after stimulation with A23187 and IgE crosslinking [68]. We have shown that mast cells can produce robust NO upon Fc ϵ RI stimulation in a PI3K- and NOS-dependent manner and that this NO production is associated

with increased tyrosine phosphorylation of Akt Ser473 and NOS-3 Ser1177 [70], which are known to contribute to increased activity of Akt and NOS-3, respectively. In accordance with these observations, the mast cells express NOS-3, but not NOS-1 and downregulated expression of NOS-3 abolishes IgE-dependent NO production [71]. Despite the substantial evidence of NOS expression and NO production by mast cells, whether mast cells can produce NO was a matter of debate. Swindle *et al.* have reported that rodent and human mast cells produce intracellular ROS but do not generate NO or express NOS-2 (NOS-1 and NOS-3 were not studied) [72]. Recently, Befus's group has shown that the conflicting results reflect at least partly different systems used [67]. Swindle *et al.* [72] used rat peritoneal mast cells from BN rats while many of the studies by others used mast cells from Sprague-Dawley (SD) rats. It was shown that mast cells from BN rats are poor producers of NO and IFN- γ stimulates NO production by mast cells from SD rats but not BN rats. In addition, NOS-2 mRNA expression is detected after IFN- γ stimulation in mast cells from SD rats but not BN rats [67]. It should be noted that BN rats are a good model for allergy and autoimmune disorders. Given that endogenous NO is an inhibitor of mast cell functions, the high IgE levels and strong allergic and autoimmune responses observed in these rats may be related to the low NO production by mast cells. Thus, the emerging view is that mast cells themselves are NO producers in response to physiological stimuli. Silver was also found to evoke production of intracellular NO in mast cells. Interestingly, however, silver is much effective than Fc ϵ RI stimulation for evoking NO release into the extracellular space [38]. Moreover, the effects of the two stimuli are distinct in terms of their sensitivities to PI3K and NOS inhibitors including arginine and tetrahydrobiopterin competitors. Consistent with the essential role for PI3K [70], IgE-dependent NO production is completely blocked by wortmannin while silver-induced NO production is minimally affected by wortmannin. NOS inhibitors such as L-N^G-nitroarginine methyl ester hydrochloride and L-erythro-1',2'-dihydroxypropyl-5,6,7,8-tetrahydropteridine, which inhibit NOS activity by competing with the substrate arginine and essential cofactor tetrahydrobiopterin, respectively [73], block IgE-dependent NO production, but not silver-induced NO production [38]. On the other hand, silver-induced NO production is specifically highly sensitive to inhibition by DTT and hemoglobin (Hb), a NO scavenger [74]. These observations show that silver-induced NO production specifically involves thiol-mediated process but do not require NOS activity. Moreover, the NOS-independent NO production appears to positively regulate silver-induced degranulation and LTC₄ secretion, since DTT and Hb blocked the secretion at concentrations that inhibit the NO production. This is in sharp contrast to the negative regulation of IgE-dependent mast cell activation by NOS-dependent NO production [65-67]. Moreover, it was revealed that NOS-independent NO production is necessary for non-store-operated Ca^{2+} entry but not store-operated Ca^{2+} entry [38]. The molecular basis of the different actions of NO produced by the NOS-dependent and -independent pathways is currently unknown. However, there are several possible explanations for such different effects. One explanation is that different reactive nitrogen species are produced by the two pathways and play distinct roles, since diamino fluorescein, which is widely used for the detection of NO, was shown to also react with peroxynitrite [75]. Alternatively, NO produced by the two different mechanisms may be localized at different intracellular sites or subjected to different metabolism and export processes. Interestingly, mitochondria from several organs such as the brain, heart and liver were recently shown to be capable of significant reactive nitrogen species production *via* the respiratory chain rather than through arginine-dependent NOS activity [75]. Ca^{2+} is necessary for activation of the respiratory chain and silver-induced NO production is Ca^{2+} -dependent [38]. In addition, silver increases the Ca^{2+} concentration in the mitochondrial matrix [37, 38]. Collectively, it is possible to speculate that the respiratory

chain plays a role in the NOS-independent NO production by silver, although further studies are necessary to confirm this role.

GPI-Anchored Proteins

Since these metal ions each possess a positive charge, they are more likely to interact with putative surface receptors than with intracellular receptors unless they are transported inside the cell by a transport system. In addition, mercury, gold and silver possess a very strong affinity for thiol groups. Therefore, it is possible to speculate that these metals target thiol groups existing in the extracellular milieu. The metals may bind directly to the vicinal thiol groups of Ca^{2+} channels or some modulators *via* a sulfur-metal co-ordinate bond, thereby modulating the functions of these molecules, because many signaling molecules and Ca^{2+} channels are known to possess cysteine residues that are necessary for their functions. Alternatively, ROS induced by the metals may indirectly modulate the functions of Ca^{2+} channels or their regulators by affecting the thiol redox status. As mentioned above, current evidence suggests that the latter mode of modulation occurs for silver through the release of NO. Intriguing possible metal receptors on the cell surface are glycosphosphatidylinositol-anchored receptors (GPI-ARs). GPI-ARs are relatively susceptible to cluster formation, and their clustering occurs in response to not only relevant specific ligands but also non-specific ligands. For instance, colloidal gold particles can induce clustering of GPI-ARs such as CD59, which results in the activation of Lyn, phospholipase $\text{C}\gamma$ (PLC γ) and Ca^{2+} mobilization [76]. Mast cells were shown to express multiple GPI-ARs, including CD14, CD48, CD59 and CD160 on their cell surface [77-79]. Ligand-mediated clustering of GPI-ARs such as Thy-1 (CD90) on the mast cell surface activates Src family kinases and intracellular signaling pathways that lead to Ca^{2+} influx and mediator release. Clustering of Thy-1 (CD90) induces increased tyrosine phosphorylation of multiple proteins, including Syk, LAT, NTAL and PLC γ , independently of Fc ϵ RI γ and expression of Fc ϵ RI on the cell surface [80-82]. In addition, it was preliminarily showed that lipid rafts are differently involved in the biological effects of the metals (see Table 3). Considering the fact that GPI-ARs exist in lipid rafts, it is possible that metal-induced clustering of GPI-ARs leads to activation of the metal signaling Fig. (1).

AUTOIMMUNITY-INDUCING METALS AFFECT MAST CELL SURVIVAL

It is widely accepted that transition metals act as potent pro-apoptotic agents. Carcinogenic heavy metals such as cadmium,

chromium and nickel promote apoptosis along with DNA base modifications, strand breaks and rearrangements. The molecular basis of the pro-apoptotic activity is not fully understood, although the generation of ROS such as $\text{O}_2^{\cdot-}$, accumulation of Ca^{2+} , upregulation of caspase-3, downregulation of anti-apoptotic Bcl-2 family molecules were shown to play roles [83]. Such disorders of apoptosis may play critical roles in some of the most serious metal-induced effects, including hepatotoxicity, renal toxicity, neurotoxicity, autoimmunity and carcinogenesis. Compared with these carcinogenic heavy metals, little is known about the impacts of mercury, gold and silver on cell survival except that gold is far less cytotoxic than mercury and silver. Therefore, we began to clarify the impacts of the three metals on mast cell survival with special attention to their abilities to evoke necrotic cell death, since this type of cell death may cause inflammation, which often develops into autoimmune reactions [22]. Among these, silver is the most potent inducer of apoptotic and necrotic mast cell death. Silver induces the two modes of cell death depending on the concentration used, cellular sensitivity and cellular redox status [37]. Substantial apoptotic cell death occurs within 20 h in low-responding cells treated with silver and is accompanied by mitochondrial membrane depolarization, cytochrome c release and caspase-3/7 activation. Strikingly, however, the cell death is not prevented by pan-caspase and caspase-3/7-specific inhibitors or by the mitochondrial permeability transition pore complex antagonists such as cyclosporine A and bongkreikic acid. Collectively, silver appears to exert its pro-apoptotic effects through a pathway that differs from those mediated by permeability transition pore complex or permeabilization of the outer mitochondrial membrane by the pro-apoptotic bcl-2 family proteins Bak and Bax, since apoptosis mediated by permeability transition pore complex and Bak/Bax pores can be usually blocked by caspase-3/7 inhibitors. The cell death is preceded by elevations in the cytoplasmic and mitochondrial Ca^{2+} levels, and both the Ca^{2+} responses and cell death are prevented by thiol-containing antioxidants, including DTT, NAC and GSH-MEE. Silver treatment of high-responding cells results in necrotic cell death within 1 h without inducing caspase activation, and this cell death is significantly reduced by depleting the extracellular, but not intracellular, Ca^{2+} . Silver also strongly induces Ca^{2+} -dependent oxidation of the mitochondria-specific phospholipid, cardiolipin and depletion of intracellular ATP, both of which are blocked by thiol-containing antioxidants. Taken together with the aforementioned activation of thiol-regulated Ca^{2+} channels by silver, these observations suggest that Ca^{2+} influx through such Ca^{2+} channels results in Ca^{2+} -

Table 3. Comparison of Biochemical and Biological Properties of Mercury, Gold and Silver

	Mercury	Gold	Silver	Antigen
Degranulation	strong	moderate	strong	strong
LTC ₄ secretion	weak	moderate	strong	strong
IL-4 production	strong	no effect	no effect	weak
IL-13 production	weak	moderate	no effect	strong
TNF- α production	weak	moderate	no effect	strong
Ca^{2+} mobilization / SOCE	no	no	no	yes
Non-SOCE	yes	yes	yes	yes
$\text{Ca}_v1.2$ LTCC	yes	yes	no	yes
β chain / Lyn	not determined	yes	no	yes
Raft	yes	no	yes	yes
GPI-anchored proteins	yes	no	yes	yes (Thy-1)
Cell death	strong (A)	weak	strong (A/N)	weak (A)

A; apoptosis; N: necrosis

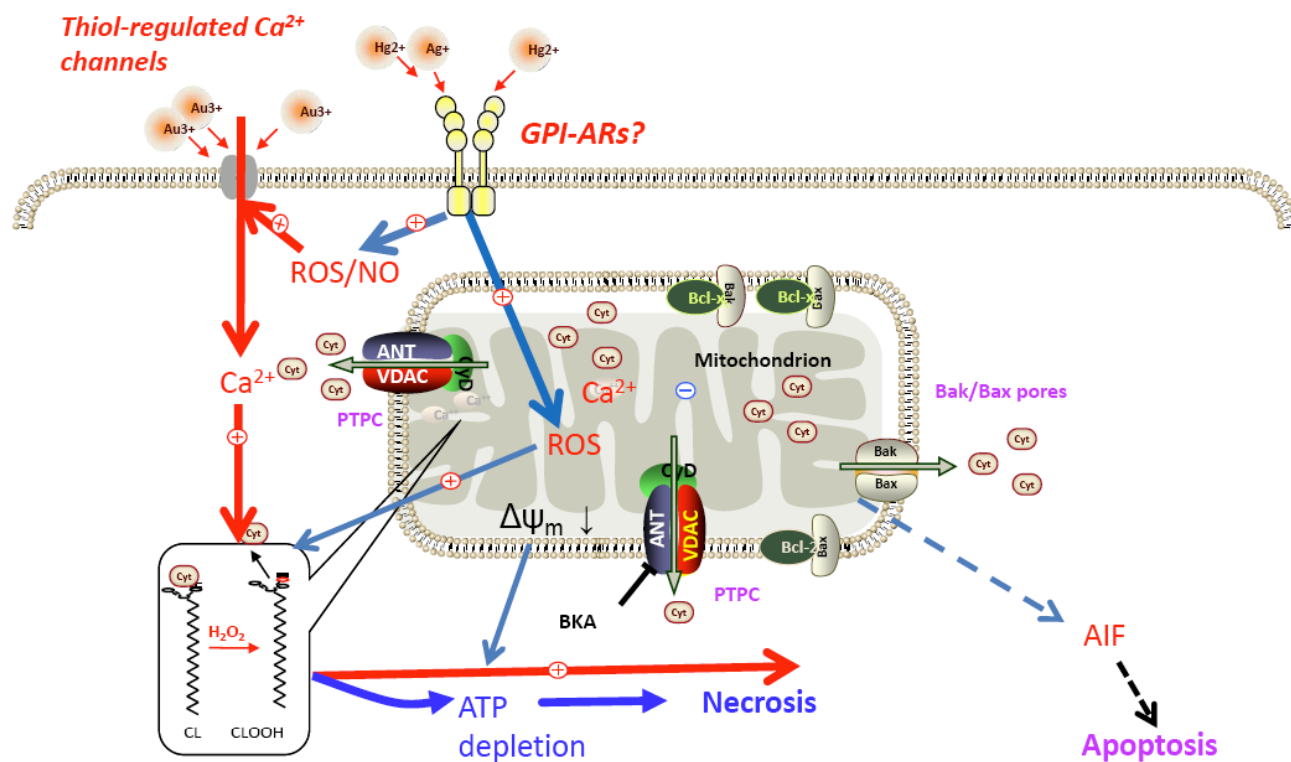


Fig. (1). Current model for metal-induced mast cell death.

Metal-induced clustering of putative receptors, possibly glycosphosphatidylinositol- anchored receptors (GPI-ARs), or metal binding to the thiol groups of cysteine residues within GPI-ARs triggers intracellular signaling pathways, including the production of reactive oxygen species (ROS), nitric oxide (NO) and a Ca^{2+} influx. Alternatively, direct metal modulation of thiol-regulated Ca^{2+} channels or their regulators activate a Ca^{2+} influx. The increase in the mitochondrial Ca^{2+} concentration may facilitate the production of ROS within mitochondria. Mitochondrial ROS, most probably hydrogen peroxide (H_2O_2), cause oxidation of cardiolipin (CL) to CL hydroperoxide (CLOOH), which decreases its contact with cytochrome (Cyt), thereby facilitating its release from the inner mitochondrial membrane, and leading to dysfunction of the respiratory ATP production. In turn, this dysfunction may accelerate ROS generation, thereby facilitating mitochondrial membrane potential ($\Delta\psi_m$) collapse and integrity disruption, and resulting in apoptotic and necrotic cell death. Although apoptotic cell death is associated with the release of the apoptogenic protein Cyt and robust caspase-3 activation, it is minimally affected by caspase inhibitors and mitochondrial permeability transition pore complex (PTPC) antagonists such as bongkreikic acid (BKA), thereby suggesting that the metal-induced pathway may differ from the usual apoptotic pathway triggered by Cyt released through the PTPC and Bak/Bax pores. Since apoptosis-inducing factor (AIF) is released from mitochondria for export to the nucleus to mediate caspase-independent apoptosis, metal-induced disruption of the mitochondrial membrane integrity may activate the AIF-mediated death pathway.

dependent cardiolipin oxidation, cytochrome c release and ATP depletion, thereby causing necrotic cell death [37]. Collectively, Silver appears to stimulate a Ca^{2+} influx through thiol-regulated Ca^{2+} channels, thereby evoking Ca^{2+} -dependent, but caspase-independent apoptosis and necrosis, depending on the conditions. The currently proposed mechanism is depicted in Fig. (1). Since mercury and gold also activate similar thiol-regulated Ca^{2+} channels, and their cytotoxic effects are caspase-independent, similar mechanisms may be involved in their cytotoxic effects. Although the molecular basis of caspase-independent apoptosis is largely unclear, apoptosis-inducing factor (AIF) was shown to mediate this type of cell death. AIF can be exported from mitochondria to the nucleus to participate in large-scale DNA fragmentation and chromatin condensation [84]. Therefore, there is a possibility that metal-induced disruption of the mitochondrial membrane integrity causes activation of the caspase-independent death pathway via AIF export.

CONCLUSIONS

The heavy metal, mercury, gold and silver commonly induce aberrant immunological responses such as hypersensitivity and autoimmunity in genetically susceptible animals and humans. Accumulating evidence suggests that mast cells are direct targets for these metals both *in vitro* and *in vivo* and play a role in the devel-

opment of these disorders. The metals considerably modulate mast cell signaling, function and survival, thereby affecting innate and adaptive immunity. Recent studies have shown that silver induced apoptotic cell death in low-responding cells, but necrotic cell death in high-responding cells, although it stimulates similar Ca^{2+} -dependent death pathways that are independent of caspase activation in both cell types. Given that necrosis, but not apoptosis, causes inflammation that often develops into autoimmune reactions, these observations raise an intriguing possibility that the mode of cell death is a critical determinant of the susceptibility to autoimmunity, which may be critically defined by the genetic background of the host. Thus, it is worth evaluating the effects of these metals on mast cell biology in more detail. Better understanding of the molecular basis of the impacts of the genetic background may become increasingly attractive for developing innovative therapeutic approaches for treating or preventing metal-induced autoimmunity.

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ence and Technology, Japan and Grants-in-Aid from Nihon University.

ABBREVIATIONS

AIF	=	Apoptosis-inducing factor
APE/Ref-1	=	Apurinic/aprimidinic endonuclease/redox factor-1
BMMC	=	Bone marrow-derived mast cell
BN	=	Brown Norway
$[Ca^{2+}]_i$	=	Intracellular Ca^{2+} concentration
CRAC	=	Ca^{2+} release-activated Ca^{2+}
DTT	=	Dithiothreitol
ERK	=	Extracellular signal-regulated kinase
FcεRI	=	High-affinity IgE receptor
GPI-AR	=	Glycophosphatidylinositol-anchored receptor
GSH-MEE	=	Glutathione monoethylester
Hb	=	Hemoglobin
IL	=	Interleukin
JNK	=	c-Jun NH ₂ -terminal kinase
LAT	=	Linker for activation of T cells
LT	=	Leukotriene
LTCC	=	L-type Ca^{2+} channel
MAPK	=	Mitogen-activated protein kinase
NAC	=	N-acetylcysteine
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
PI3K	=	Phosphatidylinositol-3-kinase
ROS	=	Reactive oxygen species
SD	=	Sprague-Dowley
SOC	=	store-operated Ca^{2+}
STAT	=	Signal transducer and activator of transcription
$O_2^{\cdot -}$	=	Superoxide anion
Th2	=	T helper 2
TNF	=	Tumor necrosis factor

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