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Metal ions potentiate microglia responsiveness to endotoxin

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ABSTRACT

Oral metal exposure has been associated with diverse adverse reactions, including neurotoxicity. We showed previously that dentally applied metals activate dendritic cells (MoDC) via TLR4 (Ni, Co, Pd) and TLR3 (Au). It is still unknown whether the low levels of dental metals reaching the brain can trigger local innate cells or prime them to become more responsive.

Here we tested whether dentally applied metals (Cr, Fe, Co, Ni, Cu, Zn, Au, Hg) activate primary human microglia in vitro and, as a model, monocytic THP-1-cells, in high non-toxic as well as near-physiological concentrations. In addition the effects of 'near-physiological' metal exposure on endotoxin (LPS) responsiveness of these cells were evaluated. IL-8 and IL-6 production after 24 h was used as read out.

In high, non-toxic concentrations all transition metals except Cr induced IL-8 and IL-6 production in microglia, with Ni and Co providing the strongest stimulation. When using near-physiological doses (up to 10× the normal plasma concentration), only Zn and Cu induced significant IL-8 production. Of note, the latter metals also markedly potentiated LPS responsiveness of microglia and THP-1 cells.

In conclusion, transition metals activate microglia similar to MoDCs. In near-physiological concentrations Zn and Cu are the most effective mediators of innate immune activation. A clear synergism between innate responses to Zn/Cu and LPS was observed, shedding new light on the possible relation between oral metal exposure and neurotoxicity.

1. Introduction

The use of metal alloys for dental reconstructions in the oral cavity is a contentious issue since local and systemic increased levels of metal ions (Milheiro et al., 2014; Muris et al., 2014) have been associated with neurodegenerative and other neurological diseases such as migraine (Shcherbatykh and Carpenter, 2007; Giacoppo et al., 2014; Dusek et al., 2014; Rosenberg et al., 2013). Little is known about how metal ions act locally and the precise mechanisms by which they contribute to central nervous system disorders have not yet been elucidated. Concentrations of various transition metals were reported to be increased in the cerebrospinal fluid (CSF) from patients with amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease (Roos et al., 2013; Hozumi et al., 2011). In particular Cu and Zn have been suggested to play important roles in the onset and progression of neurodegeneration (Giacoppo et al., 2014; Singla and Dhawan, 2014). Less information is available regarding the neurotoxic effects of Ni and Au (Pedersen et al., 2014; Kicinski et al., 2015). For Hg neurotoxic effects, in particular those manifested as neuropsychological complaints of patients exposed to amalgam, have been reported (Mutter, 2011; Carocci et al., 2014), although conclusive evidence of negative health effects of amalgam fillings, the most important source of Hg release, is lacking (Roberts and Charlton, 2009).

Pathogenic effects of transition metals could result from their ability to form complexes with peptides and proteins, thereby contributing to for example amyloid depositions, which may subsequently cause

Abbreviations: CD, cluster of differentiation; $CoCl_2$, cobalt (II) chloride; $CrCl_3$, chromium (III) chloride; $CuSO_4$, copper (II) sulphate; CSF, cerebrospinal fluid; CNS, central nervous system; DC, dendritic cell(s); DMEM, Dulbecco's modified Eagles' medium; DMSO, dimethylsulphoxide; EDTA, ethylene-diamine-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell scan; FCS, foetal calf serum; $FeCl_3$, iron (III) chloride; GM-CSF, granulocyte–macrophage colony stimulating factor; $HgCl_2$, mercuric (II) chloride; IL, interleukin; LPS, lipopolysaccharide; MS, multiple sclerosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MoDC, monocyte-derived dendritic cells; MW, molecular weight; $NiCl_2$, nickel (II) chloride; $Na_3Au(S_2O_3)_2.2H_2O$, sodium gold thiosulfate; OD, optical density; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; TLR, toll like receptor; $ZnCl_2$, zinc (II) chloride.

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oxidative stress, inflammation and neurotoxicity (Huang et al., 2004; Shcherbatykh and Carpenter, 2007). More recently, several metals, i.e. Zn, Mn and Co, were shown to directly induce the production of inflammatory mediators by microglia, the most important innate immune cells of the CNS (Dusek et al., 2014; Kauppinen et al., 2008; Mou et al., 2012). Moreover, excessive exposure to Mn and Co was shown to potentiate the release of inflammatory mediators induced by endotoxin (Dodd and Filipov, 2011; Mou et al., 2012). Indeed, inflammatory mediators generated by innate immune CNS cells such as microglia are considered the major culprits in neurodegenerative diseases (Lehnardt, 2010; Rosenberger et al., 2014; Amor et al., 2014) and new therapeutic strategies focus on the modulation of the inflammatory response by microglia (Fernandez et al., 2013; Hines et al., 2013).

Our previous research investigated the stimulatory capacity of dentally applied metals on innate immune cells (human monocyte derived dendritic cells, myelo-monocytic cell lines and TLR transfected cell lines) as assessed by the release of pro-inflammatory mediators including IL-8 (Rachmawati et al., 2013, 2015). In these studies Ni, Co and Pd were shown to induce IL-8 production via TLR4 binding. Au predominantly triggered TLR3, whereas Cu and Hg activated innate cells via thus far unidentified mechanisms. This activation of innate immune responses was, however, observed upon stimulation with relatively high, supra-physiologic, yet non-toxic concentrations of metal salts. Although the release of metal salts from dental restorations are increased due to corrosion for example (Chen et al., 2013; Matusiewicz, 2014), levels in plasma and cerebrospinal fluid (CSF) are generally lower than reported in our previous in vitro studies. Useful data on such physiologic concentrations has recently become available (Roos et al., 2013; Matusiewicz, 2014), but few studies evaluated the immunotoxicity of these concentrations using innate immune cells such as microglia (Mou et al., 2012; Wataha, 2000).

In the present study we focused on the direct response of primary human microglia following exposure to dentally relevant metals (Cr, Fe, Ni, Co, Cu, Zn, Pd, Au and Hg (Al-Hiyasat et al., 2002; Elshahawy et al., 2009; Matusiewicz, 2014)) in maximal non-toxic as well as 'physiological' concentrations. In addition to primary human microglia, the monocytoidr cell line THP-1 was explored as a model for microglia activation (Klegeris et al., 2007; Hendrickx et al., 2014).

Since the concept of microglial priming (i.e. alerting the cells to become more responsive to subsequent stimuli) is widely considered to be an important step in the development of neurodegenerative diseases (Perry and Holmes, 2014) we examined whether metal exposure of microglia and THP-1 cells potentiates their responsiveness to bacterial lipopolysaccharide (LPS), the most prominent microbial stimulatory ligand and relevant in the context of oral infections (Amor et al., 2014).

Here we show that such synergy between exposure to metal and LPS does exist, at least for Cu and Zn indicating that such combinations may contribute to or augment chronic inflammation and neurotoxicity in humans.

2. Materials and methods

2.1. THP-1 cells

THP-1 cells (passage 17; ATCC, Rockville, USA) were cultured in 100 ml flasks (Cellstar Greiner Bio-One) at a density of 1.10^6 cells/ml in RPMI 1640 medium (Biowhittaker, Verviers, Belgium) containing 2 mM L-glutamine (Merck, Darmstadt, Germany), 0.1 mg/ml streptomycin (Invitrogen), 100 IU/ml penicillin (Invitrogen) and 10% heated-inactivated foetal calf serum (FCS; Hyclone, Logan USA). The THP-1 cells were maintained in logarithmic growth by passaging every 3–4 days (1.10^6 cells/ml). For metal exposure the THP-1 cells were seeded in a 96 wells plate (flat bottom, Greiner Bio-One) in a concentration of 5.10^4 cells per well for 24 h with different concentrations of metal salts in a final volume of 200 μl.

2.2. Primary human microglia

Postmortem human brain tissue was obtained according to the protocol of The Netherlands Brain Bank (Amsterdam, The Netherlands), in agreement with the Medical Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands) (Peferoen et al., 2015). All patients had provided written informed consent for autopsy, the use of their brain tissue and clinical details for research purposes. At autopsy, 10 to 15 g of brain white matter was collected in 30 ml of Dulbecco modified Eagle medium, Ham's nutrient mixture F10 (DMEM; Invitrogen), and 1% (vol/vol) gentamicin (Invitrogen) and stored at 4 °C until further isolation procedures. Microglia were isolated from 10 patients with various neuro-degenerative diseases (Table 1). Isolation procedures were performed as previously described and validated by assessment of the purity of microglia by FACS analysis, showing more than 95% CD68 positive cells, expressing $CD11b^{high}$ and $CD45^{low}$ to distinguish them from macrophages ($CD11^{low}$ and $CD45^{high}$) (Peferoen et al., 2014). After isolation, microglial cells were maintained in 25 ml flasks in DMEM, supplemented with 10% FCS, 1% L-glutamine, 1% β-mercaptoethanol and 1% streptomycin-penicillin. After culturing for 5 days at indicated doses, cells were detached using 0.01% ethylene-diamine-tetraacetic acid (EDTA; Biowhittaker, Verviers, Belgium) in phosphate buffered saline (PBS; Braun, Melsungen, Germany), and a cell scraper (Greiner Bio-One). Cells were counted (CASY®Cell-Counter + analyser system TT; Schärfe System) and seeded in a 96 wells plate (flat bottom, Greiner Bio-One; 5.0×10^4 cells/well). Upon one day of culturing, cells were exposed for 24 h to different concentrations of metal salts in a final volume of 200 μl.

2.3. Metal salts and LPS exposure

Microglia and THP-1 cells were exposed to maximum non-toxic concentrations of metal salts (Rachmawati et al., 2013, 2015), or exposed to physiological concentrations of metal salts. For stock solutions, metal salts obtained as analytical grade metal salts, purchased from Fluka/Riedel de Haen, Seelze, Germany except for $(Na_3Au(S_2O_3)_2.2H_2O$ were purchased from Chemotechnique Diagnostics, Vellinge, Sweden, were dissolved in distilled water and further dilutions were made in culture medium prior to cell culture. In the first experiment supra-physiologic concentrations were examined, i.e. for $CrCl_3$, $NiCl_2$, $CoCl_2$, $CuSO_4$, $ZnCl_2$, $Na_2(PdCl_4)$, $Na_3Au(S_2O_3)_2$: 750, 500 and 250 μM and for $HgCl_2$ 750, 500 and 250 nM. $FeCl_3$ was not tested in these high concentrations because of low solubility. In the second study physiological doses were tested of $CrCl_3$, $FeCl_3$, $NiCl_2$, $CoCl_2$, $CuSO_4$, $ZnCl_2$, $Na_3Au(S_2O_3)$ and $HgCl_2$. These concentrations were based on the maximum plasma concentrations of metals as determined by Roos and colleagues (Roos et al., 2013) (Table 2). In addition, 3-fold ('3× phys') and 10-fold ('10× phys') of these concentrations were used to mimic possible levels obtained locally i.e. orally. To mimic exposure to multiple metals as may be expected in the oral cavity or in plasma a 'metal mix' of 6–8 metal salts were made as 10×, 3× and 1× physiological plasma concentration (Table 2).

Supernatants were collected at 24 h after metal exposure. To evaluate the impact of metal exposure on LPS responsiveness, microglia and THP-1 cells were exposed to 'physiological' concentrations of metal salts or metal mixes in the absence or presence of LPS (50 ng/ml E.coli 055:B5; Sigma Aldrich, St. Louis, MO, USA).

2.4. MTT assay

To determine the viability of the microglia and THP-1 cells after 24 h of metal salt exposure, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma, St. Louis, MO, USA) assays were performed. MTT was freshly prepared by dissolving 7.5 mg/ml in water and filtering it through a 0.22 μm filter prior to addition of 50 μl MTT solution to each well. The 96-wells plate with THP-1 cells and