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Dental metal-induced innate reactivity in keratinocytes☆

Dessy Rachmawati [a,b,*], Jeroen K. Buskermolen [d], Rik J. Scheper [a], Susan Gibbs [c,d], B. Mary E. von Blomberg [a], Ingrid M.W. van Hoogstraten [a]

[a] Department of Pathology, VU University Medical Center, Amsterdam, Amsterdam, The Netherlands
[b] Department of Biomedical Sciences and Prostodontics, Faculty of Dentistry, Jember University, Indonesia
[c] Department of Dermatology, VU University Medical Center, Amsterdam, Amsterdam, The Netherlands
[d] Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, The Netherlands

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ABSTRACT

Gold, nickel, copper and mercury, i.e. four metals frequently used in dental applications, were explored for their capacity to induce innate immune activation in keratinocytes (KC). Due to their anatomical location the latter epithelial cells are key in primary local irritative responses of skin and mucosa. Fresh foreskin-derived keratinocytes and skin and gingiva KC cell lines were studied for IL-8 release as a most sensitive parameter for NF-kB activation. First, we verified that viral-defense mediating TLR3 is a key innate immune receptor in both skin- and mucosa derived keratinocytes. Second, we found that, in line with our earlier finding that ionized gold can mimic viral dsRNA in triggering TLR3, gold is very effective in KC activation. It would appear that epithelial TLR3 can play a key role in both skin- and mucosa localized irritation reactivities to gold. Subsequently we found that not only gold, but also nickel, copper and mercury salts can activate innate immune reactivity in keratinocytes, although the pathways involved remain unclear. Although current alloys have been optimized for minimal leakage of metal ions, secondary factors such as mechanical friction and acidity may still facilitate such leakage. Subsequently, these metal ions may create local irritation, itching and swelling by triggering innate immune reactions, potentially also facilitating the development of metal specific adaptive immunity.

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1. Introduction

Metal alloys in dental appliances are located intra-orally for years to decades. During this time period metal components begin to dissolve or corrode with various bio-pathological consequences. The most frequent manifestation of metal-induced complaints is local irritation, potentially facilitating systemic contact hypersensitivity (Martin, 2015; Muris et al., 2014; Rachmawati et al., 2015). To study potential direct effects on the outermost cells in skin and mucosa, keratinocytes, we selected four most widely employed dental metals, i.e. gold, mercury, copper and nickel.

Gold alloys are widely used for dental restorations in developed countries because of their corrosion resistance and biocompatibility. Next to their use in oral applications, gold-based alloys are also widely used in skin appliances, e.g. by jewelers for earrings and piercing studs. These frequent uses of metallic gold are complemented by the medical use of gold salts for the local treatment of chronic inflammations such as in rheumatoid arthritis. Still, despite its biocompatibility or even immunosuppressive capacities, gold has also obtained some disrepute as an irritant and contact allergen (Ahlgren et al., 2002; Moller, 2002). The use of mercury-based amalgam for material fillings in dentistry has declined over recent years due its potential negative health effects. These may vary from autoimmunity (Nielsen and Hultman, 2002; Pigatto and Guzzi, 2010; Rowley and Monestier, 2005) to neurological problems (Kern et al., 2012; Mutter, 2011). Yet, negative reports are still scarce or disputed, and certainly in developing countries amalgam fillings are still widely being used nowadays. Of note, amalgam also contains nickel and copper. Copper has been used for a long time in dentistry as a strengthener and color enhancer in Au–Ag–Cu crowns and bridge alloys (Muris, 2015). Copper is an abundant element, poisonous to higher organisms but at lower concentrations an essential trace nutrient to all animal life. Allergic reactivity to copper is usually associated with other metal allergies (notably nickel) potentially resulting from concomitant sensitization or cross-reactivity (Pistoor et al., 1995). Nickel is a major component of stainless steel alloys and is widely used in orthodontics e.g. for brackets and orthodontic retention wires (Milheiro et al., 2012). Actually, these appliances can release distinct amounts of nickel and could also be responsible for extra-oral eczema even in the absence of local reactions. Allergies to nickel are very common and usually associated with exposure to jewelry, piercing, consumer products, and/or medical devices (Thyssen et al., 2009).

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* Corresponding author at: Lab Medical Immunology, Dept. of Pathology, VUmc Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands; Depts. of Biomedical Science and Prosthodontics, Faculty of Dentistry, Jember University, JL Kalimantan 37, 68121 Jember, East Java, Indonesia.
E-mail addresses: d.rachmawati@unej.ac.id (D. Rachmawati), i.vanhoogstraten@vumc.nl (I.M.W. van Hoogstraten).

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The innate immune response is the first line of host defense against exogenous toxic threats, including metals and microorganisms. Recognition of microbial factors involves signaling through specific receptors, of which the TLRs represent a major group. TLRs recognize various so-called pathogen-associated molecular patterns (PAMPs) conserved in microorganisms, including triacylated lipoproteins (TLR1/2 agonist), diacylated lipoproteins (TLR2/6 agonist), double-stranded RNA (TLR3 agonist), LPS (TLR4 agonist), flagellin (TLR5 agonist), single-stranded RNA (TLR7, TLR8 agonist) and CpG motifs in DNA (TLR9 agonist) (Cloonan and Choi, 2012). Following pathogen binding, TLRs mediate activation of innate immune responses through modulation of inflammatory gene expression by immune cells. This may subsequently facilitate adaptive immune responses involving pathogen-specific T and/or B cells. Whereas TLR receptors are abundantly present on dendritic cells, primary contacts with exogenous microorganisms are with epithelial keratinocytes. Also keratinocytes express TLRs, thus providing a first layer of defense in skin and mucosa (Baker et al., 2003; Mempel et al., 2003). Indeed, exposure of KC to microbial constituents known as TLR ligands led to activation of the NFkB pathway as revealed by release of a.o. IL-8 (Lebre et al., 2007). Still, no consensus exists on which TLR members show functional expression on KC. Some studies report functional expression of at least 4 members, i.e. TLR3, 4, 5 and 9 (Flacher et al., 2006; Lebre et al., 2007; Olaru and Jensen, 2010) whereas other data suggest only substantial expression of TLR3 (Kollisch et al., 2005; Oosterhoff et al., 2013). After the discovery that nickel and cobalt metal ions could associate with TLR4 thus activating downstream signaling (Schmidt et al., 2010), we recently reported that palladium ions show the same capacity (Rachmawati et al., 2013). Subsequently we found that gold-ions could trigger TLR3, whereas mercury and copper also activated IL-8 release, the latter metal potentially via TLR5 (Rachmawati et al., 2015). In these studies next to TLR-transfected cells, various immune cells were used, including PBMC, MoDC and THP-1 cells.

In the present study, we decided to explore the potential of distinct metals, frequently used in dental applications, to activate the innate immune pathway in keratinocytes. Due to their anatomical location and critical role in skin and mucosal inflammatory and immunological reactions, these epithelial cells are key in primary local responsiveness. Next to fresh foreskin-derived keratinocytes, skin and gingiva KC cell lines were used. IL-8 was selected as a read-out while this chemokine is the most abundantly produced inflammatory cytokine (Coquette et al., 2003; Toebak et al., 2006). Most importantly, NF-kB signaling as revealed by IL-8 release highlights not only signaling via TLR but also other major innate immune receptor pathways, such as NLR, CLR, RLR (Caruso et al., 2014; Kawamura et al., 2014) (. and EGFR (Frankart et al., 2012).

We first set out to identify most prominent TLR receptors on KC. Then, with TLR3 surfacing as the major receptor we verified that Au, as frequently used in dental and skin contexts, would not only directly stimulate DC but also keratinocytes. Secondly, we hypothesized that other dental relevant transition metals, in particular Ni, Hg and Cu might also directly activate the TLR-NF-kB-IL8 pathway in keratinocytes: via TLR4 (Ni) or unknown (Cu, Hg).

2. Materials and methods

2.1. Metal chemicals

As metal allergens the following chemicals were used: nickel (II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$), sodium gold thiosulfate ($Na_3Au(S_2O_3)_2 \cdot 2H_2O$; Chemotechnique Diagnostics, Vellinge, Sweden), Copper sulfate ($CuSO_4$), mercuric chloride ($HgCl_2$; Riedel-de Haën, Seelze, Germany). LPS was obtained from Escherichia coli 055:B5 (Sigma, St Louis, MO, USA). $Na_3Au(S_2O_3)_2 \cdot 2H_2O$, $HgCl_2$, $CuSO_4$ and $NiCl_2$ were dissolved in H_2O as stock solutions and further diluted with culture medium just before use.

2.2. Primary keratinocytes

Human foreskin was obtained from healthy donors. The VU University medical center approved all experiments. The study was conducted according to the Declaration of Helsinki Principles. Epithelial keratinocytes (KC) were isolated from healthy skin essentially as described earlier (Kroeze et al., 2012). In brief, KC were cultured in KC medium (Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland)/Ham's F-12 (Gibco, Grand Island, USA) (3:1) containing 1% UltroserG (BioSepra S.A. Cergy-Saint-Christophe, France), 1% penicillin–streptomycin (Gibco), 1 μmol/l hydrocortisone, 1 μmol/l isoproterenol, 0.1 μmol/l insulin containing 2 ng/ml keratinocyte growth factor (KGF) at 37 °C, 7.5% CO_2. Cultures were passaged when 90% confluent, using 0.5 mM EDTA/0.05% trypsin (Gibco) and used for experiments at passage 2.

2.3. NCTC 2544 cells

Skin-derived NCTC 2544 keratinocytes (Institute Zooprofilattico di Brescia, Brescia, Italy) were used at passage 37. The cells were grown in 75 cm^2 culture flasks and maintained in RPMI containing 2 mM L-glutamine, 2% pen/strep, supplemented with 10% heated-inactivated fetal calf serum. 1.25×10^6 cells were cultured per flask (10 ml culture medium) for 3 days at 37 °C in 5% CO_2. NCTCC 2544 cells were reported earlier to show different responses to irritants and contact allergens (Corsini et al., 2013).

2.4. Gingival keratinocyte cell line

The gingiva keratinocyte cell line OKG4 cells (Lindberg and Rheinwald, 1990) was provided by J.G. Rheinwald, Harvard Skin Disease Research Centre, Boston, MA, USA, and cultured in KC medium exactly as described above for primary keratinocytes. Cells were used for experiments at passage 39–41.

2.5. TLR ligand exposure

Primary KC, NCTC 2544 and OKG4 cells (4×10^5 cells/well) were cultured in 6 well plates and exposed to TLR ligands: TLR3 (poly Ic, working concentration: 1 & 10 μg/ml); TLR4 (LPS: 50 & 100 ng/ml); TLR5 (Flagellin: 10 & 100 ng/ml); TLR7/8 (Loxoribine: 1 & 5 mM); TLR9 (CpG: 1 & 10 μg/ml). Total volume in each well was 2 ml. Cells (4×10^5 cells/well), supernatants were collected after 24 h and kept at −20 °C until IL-8 assessment.

2.6. Metal toxicity experiments

In order to design appropriate concentration ranges of metals, the maximal non-toxic concentration was determined by the MTT reduction test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). 2 ml of cells (4×10^5/well) were plated in 6 well culture plates and exposed to increasing concentrations of the metals. After 24 h incubation supernatants were removed and 1 ml of MTT solution (7.5 mg/ml) was added per well. MTT solution was prepared freshly and dissolved with PBS. The plates were incubated in the dark at 37 °C. After 2–3 h of incubation, 1 ml DMSO (Merck, Darmstad, Germany) was added to each well and after shaking, the solution was measured using an ELISA reader at optical density (OD) 490 nm. The viability of the cells in the absence of metal was considered as 100%. Viabilities of exposed cells were determined by the formula: OD experimental sample/OD of control cells × 100%. The MTT analysis was performed in 96-well plates, essentially as described in detail previously (Gibbs et al., 2013).

2.7. Metal exposure

Primary KC and NCTC 2544 (4×10^5 cells/well) were exposed to metals ($Na_3Au(S_2O_3)_2 \cdot 2H_2O$, $CuSO_4$, $HgCl_2$ and $NiCl_2$) at concentrations

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