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Aluminum induces inflammatory and proteolytic alterations in human monocytic cell line

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ABSTRACT

The increasing exposure to aluminum has been linked with the development of different human pathologies (e.g., breast cancer, myofasciitis, neurodegenerative diseases), probably due to the consistent presence of aluminum salts in widely diffused cosmetic products and vaccines. However, the mechanisms underlying immunologic and proliferative alterations still remain unknown.

In the present study we investigated the ability of different aluminum compounds (i.e., aluminum chloride vs Imject® Alum, a mixture of aluminum and magnesium hydroxide) to trigger both inflammatory and proteolytic responses in U-937 human monocytic cell line. We demonstrated, by multiplex immunoassay analyses, that monocytic cells treated with both Imject Alum and aluminum chloride showed different and peculiar expression profiles of 27 inflammatory mediators and 5 matrix metalloproteinases, with respect to untreated control cells. In particular, we found dose-dependent significantly increased levels of pro-inflammatory cytokines, growth factors, and chemoattractant chemokines; whereas among metalloproteinases, only collagenolytic protease showed a significant dose-dependent increase in Imject-treated cells with respect to controls and Al-chloride treated cells. Noteworthy, we found only in Imject Alum-treated cells the significant positive correlations among collagenolytic metalloproteinase and increased expression of pro-inflammatory chemokines, suggesting a possible involvement of aluminum in regulating the acute inflammatory responses.

In agreement to emerging evidences, for the first time we demonstrated that the treatment of monocyte cells with aluminum-based adjuvant is able to induce an inflammatory status and a proteolytic cascade activation. In fact, the cell treatment with Imject Alum induced increased levels of several cytokines and proteinases, suggesting these monocyte mediators as possible biomarkers for aluminum-linked diseases. The identification of the biochemical pathways involved in Al-induced cell injury pave the way for improving the knowledge on the potential impact of aluminum in human physio-pathology.

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1. Introduction

Aluminum represents a potential toxic element which has been linked to a plethora of human diseases, mainly through wide and continuous exposure by water, food, cosmetics and drugs containing significant amount of this metal. However, no physiological function has been associated with its intake and exposure. On the other hand, evidence supports the association between aluminum accumulation in human body and the development of a variety of pathological conditions, such as dialysis dementia, Alzheimer and Parkinson diseases, breast cancer, myofasciitis, osteomalacia and microcytic anemia [1].

Aluminum compounds and aluminum-based adjuvants have been widely investigated in vivo and in vitro to identify their possible involvement (role and mechanisms of action) in several human pathologies.

Aluminum compounds (such as Al chloride, Al nitrate, Al sulfate, Al hydroxide) are usually found in cosmetic formulations (e.g., deodorants, antiperspirants), antacid drugs, vaccine adjuvants, water and food treated or cooked with aluminum utensils [2,3]. The exposure to aluminum compounds has been related to some biochemical and metabolic alterations. In fact, several studies described pro-oxidant effects [4], modifications of the essential metal homeostasis [5], DNA double strand breaks [6], and altered release of some cytokines related to the main inflammatory pathways [7,8].

Aluminum-based adjuvants, commonly named "Alum", are the most common immune-stimulants found in widespread formulations of human vaccinations [9]. In particular, Alum has been used in human

Abbreviations: BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; IFN-γ, interferon gamma; IP-10, interferon gamma-induced protein 10; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PDGF, platelet derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factors alpha; VEGF, vascular endothelial growth factor.

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vaccines against diphtheria, hepatitis A and B and papilloma virus [10, 11], in order to potentiate both the efficacy of weak antigens and shape the immune response [10].

Alum is aluminum compounds dispersed in water to form heterogeneous suspensions or gels of hydrated colloid particles that consist of micron-sized aggregates [12–14].

The term "Alum" includes several formulations of aluminum-based adjuvants [15]. One of them, Imject® Alum, is commonly used in animals as a new alternative adjuvant [16–18]. Although it has been tested for immunogenicity [19–21], and adjuvanticity [15,22–25], it is not licensed for human immunization.

Even if Imject Alum, a combination of aluminum and magnesium hydroxide, is not used in commercially available vaccines, this study aimed to compare the specific cellular and biochemical effects of aluminum chloride vs Imject Alum, on the basis of some preclinical immunological studies, highlighting the strong capacity of aluminum salts to induce inflammatory responses [3,9,25].

Although there is a paucity of data about the mechanism promoting humoral responses [9], the uptake and cellular internalization of aluminum-based adjuvants by THP-1 monocytic cells have been recently demonstrated [26,27].

Furthermore, the presence of aluminum has been confirmed in the skin [28], in subcutaneous nodules of children after injection of vaccines containing aluminum hydroxide [29]. Intramuscular administration in cynomolgus monkeys of a Diphtheria–tetanus vaccine containing aluminum oxyhydroxide or aluminum hydroxyphosphate-based adjuvants provoked an increased aluminum concentration in the injection site [30]. Finally, it has also been demonstrated the presence of Alum crystalline inclusions in macrophages [31], suggesting their intracellular uptake via phagocytic mechanisms [27,32,33].

The altered metabolic pathways induced by aluminum have not been discovered and/or completely understood; although this metal is recognized as biochemically highly reactive, it has been suggested that aluminum is critical for activating and driving the inflammosome, as well as apoptosis and modifications of nucleic acids in different cellular models [15,34,35].

On the basis of these evidences, the aim of this study was to investigate if Imject Alum and $AlCl_3$ could modulate both the inflammatory and proteolytic profiles of U-937 human monocytic cell line, evaluating the release in culture media of 32 biomolecules, including cytokines, chemokines, growth factors and matrix metalloproteinases (MMP).

2. Methods

2.1. Cell culture and treatments

U-937 cells (ATCC® CRL 1593.2™) were cultured in a complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% antibiotics). The monocyte cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. To detect the biomarkers released from monocytes treated with aluminum salts, we performed experiments in serum free media. Monocytic cells were cultured until confluence, and subsequently exposed to increasing concentrations of $AlCl_3$ (25, 50, 100, 200 μM) or Imject® Alum (25, 50, 100, 200 μg/ml) for 24 h. In particular, we selected the μM range for aluminum chloride and μg/ml range for the Imject Alum, in full agreement to previous studies demonstrating that at these concentrations aluminum salts are able to induce cellular and biochemical alterations [6,15].

Untreated U-937 monocytic cells, maintained for 24 h in serum free culture media, were used as control. Each of the cell treatments was performed in duplicates in 96 well plates, with a final volume of 200 μl per well. Each experiment was carried out twice, and intra- and inter-experiment variabilities were evaluated for the statistical analyses. The number of intact cells after co-culture with and without aluminum compounds was counted through the trypan blue exclusion test (commonly used method to enumerate the amount of viable cells).

To correctly evaluate the proteolytic and inflammatory pathways in U-937 monocytic cell line after the treatment with aluminum chloride and Imject Alum adjuvant, we treated 0.8×10^6 cells with 1 μg/ml of lipopolysaccharide (LPS) in a serum-free medium at 37 °C during 24 h in culture dishes [36].

2.2. Biochemical determinations

Total protein concentrations in serum-free media were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Milan, Italy).

Cytokine and MMP concentrations have been analyzed by BioPlex multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with a monoclonal antibody specific for the target proteins, according to the manufacturer's instructions (Bio-Rad Lab, Hercules, USA) [8].

Serum free culture media from U-937 cells have been analyzed for the cytokine content using the 27-plex panel of Pro™ Human Cytokines, including IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α and VEGF.

The evaluation of the MMP in serum free culture media has been performed by using the 5-plex panel of Human MMP Panel 2 (including MMP-1, MMP-2, MMP-7, MMP-9, MMP-10).

Levels of cytokines and MMPs were determined using a Bio-Plex 200 array reader, based on Luminex X-Map Technology (BioRad, Hercules, USA) that detects and quantifies multiple targets in a 96-well plate within a single small fluid volume (~50 μl).

Analysis of each sample was performed in duplicate. Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v.6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve; the lower detection limit was 0.6 pg/ml for the cytokines and 2.0 pg/ml for the MMPs, according to the manufacturer's indications (Bio-Rad, Hercules, USA) [8].

2.3. Statistical analysis

Each variable was expressed as the mean ± SD, unless otherwise specified. The differences were analyzed and compared by the chi-squared test or 2way-ANOVA, Student t-test or Mann–Whitney test, according to variable parametric or non-parametric characteristics, respectively. Pearson's or Spearman's correlations explored the relationships of variables. All statistical tests were two-tailed, and the significance was set at $p < 0.05$. Data were analyzed with Prism software for Windows-7, version 3.1 (Graph-Pad, San Diego, USA).

3. Results and discussions

3.1. Assay controls

According to the manufacturer's protocols and indications, the intra- and inter-assay CVs of serum-free medium samples containing aluminum compounds were always below 10%. The curve generated using spiked samples paralleled the standard curve (data not shown). All these data suggest that the "matrix" of serum-free media containing aluminum compounds did not affect the metalloproteinases and cytokines immunoassay performance, even if originally developed for plasma/serum specimens.

Aluminum compounds induce different cell viabilities.

The aim of the resent study was to investigate the possibility of using the human monocytic cell line U-937 as a relevant in vitro model to evaluate the effects of $AlCl_3$ and Imject Alum on the release in serum-

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