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Proteomic profile of mouse fibroblasts exposed to pure magnesium extract

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ABSTRACT

Magnesium and its alloys gain wide attention as degradable biomaterials. In order to reveal the molecular mechanism of the influence of biodegradable magnesium on cells, proteomics analysis was performed in this work. After mouse fibroblasts (L929) were cultured with or without Mg degradation products (Mg-extract) for 8, 24, and 48 h, changes in protein expression profiles were obtained using isobaric tags for relative and absolute quantitation (iTRAQ) coupled two dimensional liquid chromatography-tandem mass spectrometry (2D LC MS/MS). A total of 867 proteins were identified (relying on at least two peptides). Compared to the control group, 205, 282, and 217 regulated proteins were identified at 8, 24, and 48 h, respectively. 65 common proteins were up or down- regulated within all the three time points, which were involved in various physiological and metabolic activities. Consistent with viability, proliferation, and cell cycle analysis, stimulated energy metabolism as well as protein synthesis pathways were discussed, indicating a possible effect of Mg-extract on L929 proliferation. Furthermore, endocytosis and focal adhesion processes were also discussed. This proteomics study uncovers early cellular mechanisms triggered by Mg degradation products and highlights the cytocompatibility of biodegradable metallic materials for biomedical applications such as stents or orthopaedic implants.

1. Introduction

Magnesium (Mg) and its alloys are attracting more and more attention due to their good biocompatibility and suitable mechanical properties [1], and are developed in the fields of hard tissue implantation and cardiovascular stents to provide temporary scaffolding during tissue remodeling. They are fabricated into screws [2], intramedullary nailing [3] and other orthopaedics not only because of their mechanical properties closer to the ones of bone (less or no stress shielding effect or osteolysis to wear) [4], but also because they are believed to be osteoinductive according to animal experiment [5]. In addition, magnesium stents are developed as the latest generation (degradable stent) under clinical evaluation [6]. Two clinical applications of bare magnesium stents and drug-eluting magnesium stents [7] have been reported in Lancet, indicating a prospective future of biodegradable magnesium stents.

During their biodegradation Mg-based materials release Mg^{2+}, hydrogen, and other degradation products which in turn lead to a local increase of pH and osmolality. These events, arising directly after implantation, are changing the environment of the surrounding tissue. In response, tissue and cells have to maintain their homeostasis and to adjust their biochemistry to survive, including different bioprocesses such as changes in enzyme activity, in cell proliferation rate, and in membrane transport [8]. As these processes are mediated by proteins, the study of protein expression profiles provide one of the most effective and direct way to understand the effect of biomaterial degradation on cells.

Pure Mg was here chosen to avoid the potential influence of alloying element such as aluminum (e.g., present in AZ91 alloy) which can affect DNA synthesis and cell cycle [9]. In order to mimic in vivo environment, cells were exposed to extract of pure Mg prepared according to ISO 10993-16:2009 [10] and ISO 10993-12:2012 [11]. As routinely chosen for cytotoxicity studies in biomaterial applications, mouse fibroblasts L929, which are very sensitive to their environment [12], have been selected to study the early influence of initial degradation.

A rather new technique, isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two dimensional liquid chromatography-tandem mass spectrometry (2D LC MS/MS) [13], was applied to determine the proteome profiles of L929 after 8 h, 24 h, and 48 h exposition to Mg-extract or to pure media. At each time point, the expressions of the detected proteins in presence of Mg-extract were normalized to

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their respective control ones (i.e., without Mg-extract). Further in silico analyses were performed to find in which pathways and associated functions these differentially expressed proteins are implicated, revealing possible molecular mechanisms and biological processes influenced by Mg degradation.

2. Experimental

2.1. Material and extract preparation

As-cast pure Mg (99.95% purity) was purchased from Rare-Earth Metal Research Institute (Hunan, China). Surface of the samples were ground by different silicon carbide water-proof sandpapers with granulations from 600 to 2000 grit (Panda, Dongxinyanmogongju, Beijing, China). Afterwards, samples were cleaned ultrasonically in absolute ethanol for 15 min. After being dried at room temperature, samples were sterilized by ultraviolet radiation for at least 2 h. The Mg-extract was prepared according to ISO 10993-12 [11] with a surface area/extraction medium ratio of 1.25 cm^2/mL. Samples were immersed in complete medium (minimum essential medium (MEM) supplemented with 10% horse serum, 100 IU/L penicillin, and 100 μg/L streptomycin (all chemicals were purchased from Gibco, New York, USA)) under cell culture condition (5% CO_2, 95% humidity, 37 °C) for 24 h.

The concentrations of magnesium and calcium contained in the Mg-extract were measured by inductively coupled plasma atomic emission spectrometry (ICP; Profile ICP-AES, Leeman, USA), after microwave digested with 5 mL HNO_3 (Sigma-Aldrich Chemie GmbH, Munich, Germany).

2.2. Cell culture

The mouse fibroblasts cell line L929, provided by National Institutes for Food and Drug Control (Beijing, China), were cultured in complete medium. Cells were seeded at a density of 3×10^4 cells/mL and cultured for 24 h before replacing the medium by Mg-extract or fresh complete medium (Control). The cells were further cultured for 8 h, 24 h, and 48 h. At each time point, cell viability, cell cycle, and proteomics analyses were performed.

2.3. Cell viability

Cell Counting Kit-8 (CCK-8; Neuronbc, Beijing, China) was employed to assess cell viability. CCK-8 is a sensitive colorimetric assay based on a water-soluble tetrazolium salt (WST-8). The amount of orange-colored formazan generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. CCK-8 assay was carried out following the ISO 10993-5 standard [14] with complete medium as negative control and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Shanghai, China) in complete medium as positive control under the same conditions as described in Zhen et al. [15].

2.4. Cell cycle assay

Cell cycle assay was conducted following cell cycle assay kit (Biolegend, Beijing Korad Biotechnology, Beijing, China). Briefly, cells were dissociated using trypsin (Gibco, Life Technologies, Canada), washed and suspended in cold phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL. Cells were further fixed in 70% ice-cold alcohol (Beijing Chemical Works, Beijing, China), centrifuged and resuspended in 0.5 mL PBS with a final concentration of 100 μg/mL RNase and 50 μg/mL propidium iodide (PI, Biolegend, Beijing Korad Biotechnology, Beijing, China) for 30 min in the dark to stain DNA. The samples were then analyzed using a flow cytometer (BD FACSCalibur, Becton-Dickson, USA).

At least 20,000 cells of each stained suspension cells were collected by BD FACSCalibur. Cell groups were circled in the scatter plots of FSC (forward scatter) and SSC (side scatter) to remove the cell debris, and single cell gate was circled in the scatter plots of FL-2-W (fluorescence width) and FL-2-A (fluorescence area) to remove the clustered cells. DNA content histogram was showed in FL-2-A histogram. The obtained FCS files were fitting analyzed by ModFit LT software to get the data of each cell cycle phase.

2.5. Proteomics profile analysis

2.5.1. Cell lysis, protein digestion and iTRAQ reagent labeling

Treated L929 cells were collected by trypsin and washed twice with PBS before whole cell protein extraction. Total proteins in cells were extracted with freshly prepared lysis buffer [8 M urea (GibcoBRL Carlsbad, CA, USA), 30 mM 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (MHEPES, Sigma-Aldrich, Shanghai, China, pH 8.0–8.3), 10 mM dithiothreitol (DTT, Promega, Beijing, China), 2 mM ethylenediaminetetraacetic acid (EDTA Amresco, Solon, OH, USA), and 1 mM phenylmethanesulfonyl fluoride (PMSF Amresco, Solon, OH, USA)]. The solution was dispersed by sonication for 5 min (power 180 W, pulse 2 s on and 3 s off), then centrifuged at 20,000*g* for 30 min. The supernatant was collected and reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide (IAM, Promega, Beijing, China). They were then precipitated by cold acetone (Beijing Chemical Works, Beijing, China), stored at −20 °C for 3 h, and concentrated by centrifuging at 20,000*g* for 30 min. The precipitates were resuspended in solution buffer (50% tetraethylammonium bromide (TEAB, Sigma-Aldrich, Shanghai, China), 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich, Shanghai, China)), and then treated by sonication and centrifuge again to get the supernatant. The supernatant was collected and proteins were quantified by the Bradford method. Each protein sample (100 mg) supplemented with 3.3 g trypsin was incubated in 37 °C for 24 h and then again 1 μg trypsin was added before another 12 h incubation. Lyophilized precipitates were resuspended in 30 μL solution buffer (50% TEAB, 0.1% SDS), and then labeled by isobaric tag according to the iTRAQ kit protocol (Applied Biosystems, Foster City, CA) as follows: control group 8 h "114", control group 24 h "115", control group 48 h "116", Mg extract 8 h "118", Mg extract 24 h "119", and Mg extract 48 h "121".

2.5.2. Online 2D Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

After labeling and quenching, the samples were sent to online 2D Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. For the first dimensional separation, protein digests were diluted by 10 times in buffer A pH 3 (25% acetonitrile (ACN) and 10 mM KH_2PO_4 all chemicals from Fisher Scientific, Pittsburgh, PA, USA), and centrifuged (15,000 rpm, 10 min). Supernatant were further fractionated by strong cation exchange chromatography (SCX, Phenomenex Luna SCX 100 A, Torrance, CA) using high performance liquid chromatography strong (HPLC, Shimadzu, Kyoto, Japan). SCX fractionation was performed at a flow rate of 1 mL/min using a gradient of 0–50% buffer B (25% ACN, 2 M KCl Fisher Scientific, Pittsburgh, PA, USA, 10 mM KH_2PO_4) for 56 min, up to 100% B at 61 min, then held for an additional 10 min at 100% B, reduced to 0%B at 76 min and stopped. To remove high salt concentrations from the ion source of SCX elution, reverse-phase liquid chromatography was performed.

For the second dimensional separation, the fractionated samples were sent to an UltiMate 3000 nano HPLC system (Dionex, Idstein, Germany) equipped with a Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). The desalted fractions were loaded onto a homemade analytical column (Venusil XBP, C18 (L), 75 mm * 100 mm, 5 μm, 300 A, Agela Technologies, Delaware, USA) and separated using a mobile phase containing buffer C (0.1% formic acid in water) and buffer D (0.1% formic acid in acetonitrile) with 400 nL/min flow rate (the gradient separation was 5% D for 10 min, 5% to 30% D for 30 min, 30% to 60% D for 5 min, 60% to 80% D for 3 min, 80% D for 7 min, 80% to 5% D for 3 min, and 5% D for 7 min). A