Biomaterials 30 (2009) 141–148

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Mechanisms of cytotoxicity of nickel ions based on gene expression profiles

Xiaoying Lü*, Xiang Bao, Yan Huang, Yinghua Qu, Huiqin Lu, Zuhong Lu

State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Si Pailou 2#, Nanjing 210096, P.R. China

ARTICLE INFO

Article history:
Received 4 August 2008
Accepted 2 September 2008
Available online 14 October 2008

Keywords:
Gene expression microarray
Cytotoxicity
Biocompatibility
Nickel

ABSTRACT

This study investigated cytotoxic effects of Ni(II) to mouse fibroblast cells (L-929) on the level of gene expression profiles with cDNA microarray. The gene expression profiles of L-929 were detected after the cells were cultured in the medium with 200 μM Ni(II) for 24, 48 and 72 h, respectively, and the cytotoxicity of Ni(II) was evaluated with methylthiazoltetrazolium (MTT) assay. 20 up-regulated genes and 19 down-regulated genes were differentially expressed in all three-culture periods. Gene ontology analysis showed that the L-929 cells which responded to Ni(II) covered a broad range of functional gene groups including cellular biological process, molecular function, and cellular component. Ni(II) has extensive effects on cells by inhibiting cell proliferation and differentiation through inducing cell apoptosis, affecting cell development and influencing cholesterol metabolism.

1. Introduction

Biocompatibility evaluation of biomaterials has become an important scientific task in the research field of biomaterials. The key to understand biocompatibility is the determination of which, chemical, biochemical, physiological, physical or other mechanisms become operative (and why), under the highly specific conditions associated with contact between biomaterials and the tissues of the body, and what are the consequences of these interactions [1].

So far, pre-clinical evaluation of biocompatibility has been performed at three different levels: the animal level, the cellular level, and the molecular level. In vitro cytotoxicity test is always the first step in the assessment of biocompatibility of new biomaterials. However, with the results from a routine cytotoxicity test we can only confirm whether the cell died or not and how much the cell proliferation rate had been reduced. Nevertheless, we don't know how cells responded on a molecular level to the materials and what would have happened on the genomic level before the cells died.

Molecular biological methods, such as RT-PCR, in situ hybridization, Northern blotting, Western blotting and ELISA, have recently been used to evaluate molecular biocompatibility of biomaterials. Those methods have improved the evaluation method of biocompatibility from animal experiment level and cellular level to molecular level [2]. However, those earlier studies were limited by the technologies that only allowed analyzing a few genes at one time. The microarray techniques generate a quantitative analysis of over a thousand genes simultaneously [3–7], which have been used in many fields such as toxicity research [8,9], identification of genes relevant to diseases [10], filter of drugs [11,12] and in evaluation of biocompatibility [13–16]. Nowadays, the molecular biocompatibility study of biomaterials in genome scales has become possible with the introduction of the microarray technology [5–7,17,18].

The development of bioinformatics has provided some effective methods for analyzing gene expression data. Among them, cluster, gene ontology (GO) and pathway analysis are three important ones. GO is a controlled vocabulary used to describe molecular function, biological process, cellular component of genes and gene products in a generic cell. GO terms and their relationships are represented in the form of directed acyclic graphs. The more information two terms share, the more they are similar [19].

NiTi-based shaped memory alloys are useful in orthopedic and orthodontic applications because of their favorable mechanics and physical properties [20–23]. However, its biocompatibility remains controversial. Several studies have shown that the NiTi alloy can be regarded as a biologically safe implant material [24–26], while other studies reported the contrary results. For example, the Ni implants didn't show any bone contact during the whole experimental period. Histometric analysis revealed that NiTi implants showed a significantly ($P < 0.01$) lower percentage of bone contact and bone contact area than any other of the titanium or titanium alloy materials [27]. Biocompatibility results of NiTi screws showed a slower osteogenesis process characterized by no close contact between the implant and bone, disorganized migration of osteoblasts around the implant and a lower activity of osteonectin synthesis [28].

Since nickel release during the corrosion of NiTi is an important concern for its use as an implant material, several studies have been

* Corresponding author. Tel.: +86 25 83793430; fax: +86 25 83792882.
E-mail address: luxy@seu.edu.cn (X. Lü).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.09.011

Table 1
Cell proliferation percentage (P) and cytotoxicity grade for each Ni(II) treated group (72 h) in MTT assay

Group	*P*%	Toxicity grade[a]
Negative control	100	0
100 μmol/L	76.5	1
200 μmol/L	57.4	2
300 μmol/L	54.1	2
400 μmol/L	37.2	3
500 μmol/L	36.1	3
Positive control	5.5	4

[a] Toxicity grade 0 means $P \in$ [81%, 100%], grade 1 means $P \in$ [61%, 80%], grade 2 means $P \in$ [41%, 60%], grade 3 means $P \in$ [21%, 40%], and grade 4 means $P \in$ [0, 20%].

carried out. A comparative *in vitro* cell culture study by Ryhänen et al. measured Ni released from NiTi and 316L SS in a fibroblast and osteoblast cell culture media. The Ni level was higher in the NiTi group on the first day, and decreased rapidly as a function of time to achieve similar level of 316L SS after 8 days in both media. Although the Ni release was higher in the NiTi group, the cell proliferation or cell growth near the sample surface was not affected [29,30]. However, few studies have been reported on DNA microarray analysis of Ni(II) released from nickel–titanium alloy [16,31–33].

Therefore, Ni(II) was selected as a test agent in our study to investigate the interaction between Ni(II) and mouse fibroblast cells. The main purpose of our study was to determine the cytotoxic molecular mechanism of Ni(II) by using microarray technology and gene ontology (GO) analysis associated with cytotoxicity test to provide technical support and lay a foundation in establishing a quick, convenient, sensitive and reliable method for the biocompatibility evaluation.

2. Materials and methods

2.1. Cell culture and treatment

The mouse fibroblasts of cell line L-929 (presented by School of Basic Medical Science, Southeast University, China) were cultured using RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (Sigma) in a humidified 5% CO_2 atmosphere at 37 °C. The medium was changed every 2 days. When the cells reached a confluent monolayer, they were harvested using 0.25% trypsin (Sigma) for cytotoxicity assay.

To determine a suitable concentration, Ni(II) (prepared using $NiCl_2 \cdot 6H_2O$, Shanghai Chemical Reagent Corporation) was added to the medium at concentrations of 100, 200, 300, 400 and 500 μM, respectively, and was cultured with cells for 72 h. For MTT cytotoxicity test and gene expression microarray experiments, Ni(II) was added to the culture medium at a concentration of 200 μM and cultured with cells for 24, 48 and 72 h, respectively.

2.2. Cytotoxicity evaluation

The cytotoxicity of Ni(II) to the L-929 cells was evaluated using the methylthiazoltetrazolium (MTT) assay. L-929 cells were seeded in 96-well plates at a density of 6×10^3 in 100 μL/well. After seeding, the cells were exposed to Ni(II) for 24, 48 and 72 h. At the end of experimental incubation, MTT (Sigma) was added at a final concentration of 0.5 mg/mL PBS for 4 h. In the following 4 h incubation, 150 μL DMSO (Shanghai Chemical Reagent Corporation) was added to each well to dissolve the formazan crystals. The optical density (OD) values were recorded at 492 nm using an MRX (Dynatech) plate reader, and the cell proliferation percentage *P* was defined as:

$$P = \frac{\overline{\mathrm{OD}}_{\text{test-material}}}{\overline{\mathrm{OD}}_{\text{negative}}} \times 100\%$$

The value of *P* was used to evaluate the cytotoxicity grade of each group according to the 5-level (0–4) definition (see the notation under Table 1). Each experiment was repeated at least 3 times.

2.3. Total RNA isolation

After the treatment with 200 μM Ni(II) for 24, 48 and 72 h, the total RNA from each sample was extracted from L-929 cells using a Unizol total RNA isolation Kit (Biostar Inc., China) according to the instructions provided by the manufacturer. The quality and integrity of the RNA samples were determined by appearance of distinct 28S and 18S bands of ribosomal RNA on agarose gel electrophoresis. Total RNA concentration and purity were measured spectrophotometrically by the absorbance ratio 260:280 nm.

2.4. Gene expression study

Briefly, a cDNA probe was synthesized from total RNA isolated as described above. Fluorescent labeling and hybridization were carried out using BioStar LAB/HYB Kit and BioStar M-140s gene expression microarray containing 14,112 genes (United Gene Holdings, Ltd., Shanghai, China) according to the manufacturer's recommendation. Cy3 and Cy5 (Amersham Pharmacia Biotech, USA) were used for cDNA labeling. The RNA isolated from untreated cells was labeled with Cy3 and used as control against the Cy5-labeled treated (Ni(II)) cDNA in the experiment. After hybridizing the samples for 16 h, the gene chips were washed, fluorescently stained and scanned with ScanArray4000 Scanner (General Scanning, USA) to collect the images of post-hybridization chips. The ratios of intensity (Cy5/Cy3) were calculated and normalized with Genepix Pro 3.0 software (BioDiscovery Inc.). Filtering of the results was done as follows: genes were considered as up-regulated when the Cy5/Cy3 ratio was higher than 2 and as down-regulated when the Cy5/Cy3 ratio was lower than 0.5 in two parallel experiments. Genes were considered as unregulated when the Cy5/Cy3 ratio was between 0.5 and 2. Genes that were consistently up-regulated or down-regulated at all three time points (24 h vs. control, 48 h vs. control and 72 h vs. control) were tabled for further analysis.

2.5. Gene ontology analysis of gene expression data

To determine biologically relevant gene ontology (GO, provided by NCBI) terms of differentially expressed genes in L-929 cells, a free software GoSurfer (Version 1.1, http://bioinformatics.bioen.uiuc.edu/gosurfer/) was used, which creates a global gene expression profile from microarray data by integrating the annotations of the GO Project (http://www.geneontology.org). The analysis included three categories GO such as molecular function, biological processes, and cellular component. Unigene ID was used as the identifier.

3. Results

3.1. Cytotoxicity

The cytotoxicity results of the five concentrations of Ni(II) (100, 200, 300, 400 and 500 μM) for 72 h cell treatment are presented in Table 1. Fig. 1 shows the cell growth states after Ni(II) treatment under the concentration of 200 μM for 24, 48 and 72 h, respectively. As indicated in Table 2, treatment of L-929 cells with 200 μM Ni(II)

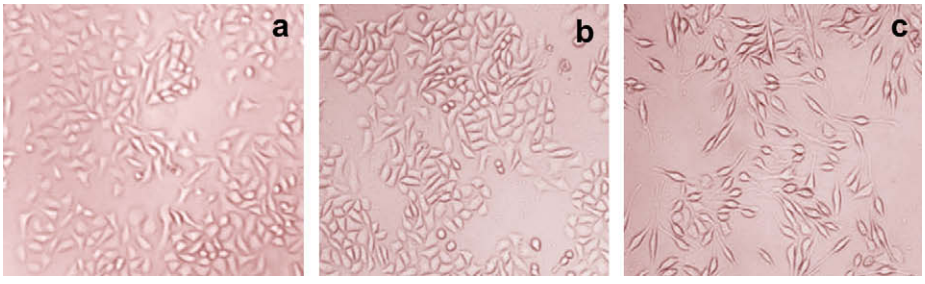

Fig. 1. Cell morphology of L-929 treated with 200 μM Ni(II) for 24 h (a), 48 h (b) and 72 h (c) visualized by light microscopy.