Chemico-Biological Interactions 210 (2014) 64–76

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Evaluation of cellular influences caused by calcium carbonate nanoparticles

Masanori Horie [a,b,*], Keiko Nishio [b], Haruhisa Kato [c], Shigehisa Endoh [d], Katsuhide Fujita [e], Ayako Nakamura [c], Shinichi Kinugasa [c], Yoshihisa Hagihara [b], Yasukazu Yoshida [b], Hitoshi Iwahashi [f]

[a] Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health Japan, 1-1 Iseigaoka, Kitakyushu, Fukuoka 807-8555, Japan
[b] Health Research Institute (HRI), National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31, Midorigaoka, Ikeda, Osaka 563-8577, Japan
[c] National Metrology Institute of Japan, AIST, 1-1-1, Higashi, Tsukuba, Ibaraki 305-8565, Japan
[d] Technology Research Association for Single Wall Carbon Nanotubes (TASC), 1-1-1, Higashi, Tsukuba, Ibaraki 305-8569, Japan
[e] Research Institute of Science for Safety and Sustainability (RISS), AIST, 16-1, Onogawa, Tsukuba, Ibaraki 305-8569, Japan
[f] Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 11 December 2013
Accepted 30 December 2013
Available online 8 January 2014

Keywords:
Calcium carbonate
Nanoparticle
Culture cell
Cellular influence

ABSTRACT

The cellular effects of calcium carbonate ($CaCO_3$) nanoparticles were evaluated. Three kinds of $CaCO_3$ nanoparticles were employed in our examinations. One of the types of $CaCO_3$ nanoparticles was highly soluble. And solubility of another type of $CaCO_3$ nanoparticle was lower. A stable $CaCO_3$ nanoparticle medium dispersion was prepared and applied to human lung carcinoma A549 cells and human keratinocyte HaCaT cells. Then, mitochondrial activity, cell membrane damage, colony formation ability, DNA injury, induction of oxidative stress, and apoptosis were evaluated. Although the influences of $CaCO_3$ nanoparticles on mitochondrial activity and cell membrane damage were small, "soluble" $CaCO_3$ nanoparticles exerted some cellular influences. Soluble $CaCO_3$ nanoparticles also induced a cell morphological change. Colony formation was inhibited by $CaCO_3$ nanoparticle exposure. In particular, soluble $CaCO_3$ nanoparticles completely inhibited colony formation. The influence on intracellular the reactive oxygen species (ROS) level was small. Soluble $CaCO_3$ nanoparticles caused an increase in C/EBP-homologous protein (CHOP) expression and the activation of caspase-3. Moreover, $CaCO_3$ exposure increased intracellular the Ca^{2+} level and activated calpain. These results suggest that cellular the influences of $CaCO_3$ nanoparticles are mainly caused by intracellular calcium release and subsequently disrupt the effect of calcium signaling. In conclusion, there is possibility that soluble $CaCO_3$ nanoparticles induce cellular influences such as a cell morphological change. Cellular influence of $CaCO_3$ nanoparticles is caused by intracellular calcium release. If inhaled $CaCO_3$ nanoparticles have the potential to influence cellular events. However, the effect might be not severe because calcium is omnipresent element in cell.

1. Introduction

Calcium carbonate ($CaCO_3$) is one of the important industrial materials in existence. In many cases, $CaCO_3$ is deeply intertwined with our lives. For example, $CaCO_3$ is used as an additive in plastics and gums, and it is conducive to more workability and combustion efficiency. According to manufacturer's information, $CaCO_3$ is also necessary for many industrial products such as paints, inks, paper, adhesives, and chalk. Moreover, $CaCO_3$ is used as a food additive in tooth pastes, tablets, and cosmetics. Therefore, we cannot live comfortably without $CaCO_3$. Nanoparticles whose sizes are 1–100 nm are used as a part of these products [1]. $CaCO_3$ nanoparticles are one type of nanoparticle that has a long history of utilization. $CaCO_3$ nanoparticles are mainly applied to paints, inks, sealants, and adhesive agents as a viscosity modifier. Although $CaCO_3$ nanoparticles have been used since the early 1990s, there is no report about their hazardous properties. Generally, manufactured nanoparticles have higher chemical and physical activities than micro-scale particles, and thus their biological influences are also higher. In fact, there are many reports about the cellular influences of manufactured nanoparticles including cytotoxic activity. The chemical and physical properties of nanoparticles manifested in its cellular influences. Solubility, adsorption ability, and cellular uptake are important factors of manufactured nanoparticles that exert cellular influences [2–5]. Some kinds of nanoparticles such as ZnO, CuO, NiO, and Cr_2O_3 release metal ions into culture media [6,7]. These nanoparticles are taken up into cells by endocytosis and release metal ions inside cells. As a result,

* Corresponding author at: Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health Japan, 1-1 Iseigaoka, Kitakyushu, Fukuoka 807-8555, Japan. Tel.: +81 93 691 7466; fax: +81 93 691 4284.
E-mail address: masa-horie@med.uoeh-u.ac.jp (M. Horie).

0009-2797/$ - see front matter © 2014 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cbi.2013.12.013

noteworthy cellular influences such as the induction of oxidative stress, apoptosis, the inhibition of cell growth, and cell death are induced. However, although increased solubility is an important feature of nanoparticles, not all types of nanoparticles dissolve. For example, TiO_2 and CeO_2 nanoparticles do not dissolve in culture medium and their cellular influences are smaller than "soluble" nanoparticles [6]. On the other hand, TiO_2 and CeO_2 nanoparticles show large protein and calcium adsorption abilities [6,8]. We previously reported that one type of CeO_2 nanoparticle has a strong calcium adsorption ability [9]. Calcium adsorbed CeO_2 nanoparticles were taken up into cells by endocytosis, and then the intracellular calcium level and calpain activity were increased. Calcium is an essential element of cells. Calcium has many physiological activities; for example, Ca^{2+} is involved in cell signaling via calcium signaling. In mammalian cells, Ca^{2+} activates CaM-kinase via calcium binding proteins such as calmodulin [10]. Activated CaM-kinase enhances or suppresses the transcription of a specific gene. The Ca^{2+} concentration in the intracellular fluid of a quiescent cell is very low.

The intracellular calcium concentration is controlled by the influx of extracellular calcium via calcium channels and the release of intracellular calcium from the endoplasmic reticulum (ER), which is an intracellular calcium storage organelle. The ER plays an important role in regulating the intracellular calcium level. The intracellular calcium concentration is strictly controlled and imbalances cause cellular responses such as the induction of apoptosis [11–13]. Additionally, Ca^{2+} release from the ER leads to mitochondrial-related oxidative stress [14]. CeO_2 nanoparticles adsorb calcium in culture media, including that which contains approximately 2 mM $CaCl_2$. Following this, calcium-adsorbed CeO_2 increases the intracellular calcium level after cellular uptake. Similarly, $CaCO_3$ nanoparticles may cause increases in the intracellular calcium level. Moreover, $CaCO_3$ nanoparticles behave as an intracellular source of calcium. Therefore, understanding the cellular influences of $CaCO_3$ nanoparticles is important for their effective utilization. However, there is no report about the cellular influences of $CaCO_3$ nanoparticles. As well as some types of cytotoxic metal oxide nanoparticles, whether or not $CaCO_3$ nanoparticles show some cellular influences are unknown. Specifically, the cellular influence-related properties of $CaCO_3$ of solubility, adsorption ability, and cellular uptake, have not been explored. In the present study, we examined the potential effect of $CaCO_3$ nanoparticles on cultured cells. In particular, we focused on the release of Ca^{2+} from $CaCO_3$ nanoparticles. Two kinds of cell lines, human lung carcinoma A549 cells and human keratinocyte HaCaT cells, were employed as inhalation and skin exposure models.

2. Methods

2.1. Cell culturing

Human keratinocyte HaCaT cells were purchased from the German Cancer Research Center (DKFZ, Heidelberg, Germany). Human lung carcinoma A549 cells were purchased from the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). These cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen Corporation, Gland Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; CELLect GOLD; MP Biomedicals Inc., Solon, OH), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Nacalai Tesque Inc., Kyoto, Japan). In this study, this DMEM cocktail is called "DMEM–FBS." The DMEM cultures were placed in 75-cm^2 flasks (Corning Incorporated, Corning, NY) and incubated at 37 °C in a 5% CO_2 atmosphere. For cell viability assays, the cells were seeded in 96-well or 6-well plates (Corning Incorporated) at 1×10^5 cells/ml and incubated for 24 h. Subsequently, they were removed from the culture medium, subjected to metal oxide dispersion, and incubated for another 24 h.

2.2. $CaCO_3$ particles and preparation of $CaCO_3$ dispersions in DMEM–FBS

$CaCO_3$ nanoparticles were provided by Shiraishi Kogyo Kaisha Ltd. (Amagasaki, Hyogo, Japan). The product names of the $CaCO_3$ nanoparticles are Hakuenka O, Hakuenka T-DD, and Hakuenka PZ. We examined 3 kinds of $CaCO_3$ particles. Details of the particles are described in Table 1. The crystal structures and particle shapes of all of the particles were calcite and cubic, respectively. Two kinds of particles were surface treated with rosin acid. These $CaCO_3$ particles were mainly used for ink and paint applications. There are two major crystalline phase in $CaCO_3$ for industrial use; calcite and aragonite. And majority of manufactured $CaCO_3$ "nanoparticles" are calcite. Because the aim of this study is evaluation of cellular influences of manufactured nanoparticles, we employed calcite in this study.

For the cytotoxicity assays described in Figs. 1–3, $CaCO_3$ powder was dispersed directly in DMEM–FBS at concentrations ranging from 0.1–50 mg/ml without a dispersant. For some examinations described in Figs. 4–9, stable and uniform dispersions were prepared by using a previously described pre-adsorption and centrifugation method [15]. Nanoparticles induce artificial cellular influences by medium depletion because of their protein adsorption ability [8]. To prevent cell starvation caused by the adsorption of medium components onto the particle surfaces, the $CaCO_3$ particles were initially dispersed in FBS at 80 mg/ml. Subsequently, the dispersion was centrifuged at 16,000×*g* for 20 min. The precipitated $CaCO_3$ particles were washed once with FBS-free DMEM and re-dispersed in an equivalent volume of fresh DMEM–FBS. This dispersion of ultrafine particles in DMEM–FBS was centrifuged at 8000×*g* for 20 min. After discarding the supernatant, the precipitate was re-suspended in an equal volume of fresh DMEM–FBS and the resulting $CaCO_3$ dispersion was centrifuged at 4000×*g* for 20 min. The supernatant was collected as a stable $CaCO_3$ dispersion.

2.3. Characterization of $CaCO_3$-DMEM–FBS dispersions

In this study, we defined "secondary particles" as being complex aggregates of primary particles, proteins from FBS, and other

Table 1
Properties of $CaCO_3$ particles and characterization of the $CaCO_3$-DMEM–FBS dispersion used in this study.

Material	Product name	Code in this study	Primary particle size[a] (nm)	Specific surface area[a] (m^2/g)	Surface coating reagent[a]	Concentration of $CaCO_3$ (μg/ml)	Concentration of Ca^{2+} (mM)	Inorganic salt concentration in the dispersion (mM)			Zeta potential (mV)
								Na	P	K	
$CaCO_3$	Hakuenka PZ	A	80	16.3	None	39	2.25 ± 0.30	19.23 ± 3.84	0.57 ± 0.17	6.23 ± 0.85	−12.3 ± 1.2
	Hakuenka T-DD	B	80	18.0	Rosin acid	19	2.07 ± 0.15	17.83 ± 3.39	0.57 ± 0.06	6.33 ± 0.95	−14.2 ± 2.0
	Hakuenka O	C	30	52.6	Rosin acid	89	2.72 ± 0.35	16.55 ± 2.90	0.25 ± 0.05	4.99 ± 0.24	−18.7 ± 1.3
DMEM							1.94 ± 0.22	19.83 ± 5.40	0.51 ± 0.22	6.44 ± 0.73	

[a] The primary particle was detected by TEM and the specific surface area was measured by BET. These data were obtained by the manufacture's data sheet.