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Cytotoxicity of amorphous silica particles against macrophage-like THP-1 cells depends on particle-size and surface properties

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Received December 17, 2009, accepted January 4, 2010

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Pharmazie 65: 596–599 (2010) *doi: 10.1691/ph.2010.9408*

Recent studies have indicated that amorphous silica particles (SPs) show cytotoxicity against various types of cells, including macrophages. However, the mechanism of cell death has not been determined, and systematic investigations of the relationship between particle characteristics and cytotoxicity are still quite limited. Here, we compared the cytotoxicity of SPs of various sizes (30–1000 nm) and surface properties against differentiated THP-1 human macrophage-like cells. We found that 300 and 1000 nm SPs showed cytotoxicity against THP-1 cells, whereas 30, 50, and 70 nm SPs did not induce cell death. We demonstrated that 1000 nm SP showed strong cytotoxicity that depended on reactive oxygen species but was independent of caspases. Furthermore, we showed that surface modification of 1000 nm SPs dramatically suppressed their cytotoxicity. Our results suggest that systematic evaluation of the association between particle characteristics and biological effects is necessary for the creation of safe SPs.

1. Introduction

Amorphous (noncrystalline) silica particles (SPs) possess useful properties, including straightforward synthesis, relatively low cost, easy separation, high hydrophilicity, and facile surface modification. In addition, SPs are usually considered to have low toxicity, in contrast to crystalline silica, which can cause silicosis and some forms of lung cancer (Mossman and Churg 1998; Huaux 2007). Therefore, SPs have been used for many applications, including cosmetics, foods, medical diagnosis, cancer therapy, and drug delivery (Hirsch et al. 2003; Bharali et al. 2005; Roy et al. 2005; Bottini et al. 2007; Verraedt et al. 2009).

However, the increasing use of SPs has raised public concern about their safety. In fact, recent studies have found that SPs induce substantial lung inflammation and are cytotoxic against various cells, including macrophages (Wiethoff et al. 2003; Cho et al. 2007; Napierska et al. 2009). Thus, the safety and overall biological effects of SPs have been questioned (Akerman et al. 2002; Kirchner et al. 2005; Dostert et al. 2008). In addition, it has recently become evident that particle characteristics, including particle size and surface properties, are important factors in pathologic alterations and cellular responses (Albrecht et al. 2004; He et al. 2008; Waters et al. 2009). Therefore, investigation of the mechanisms of SP-induced inflammation and cytotoxicity and of the relationship between particle characteristics and cytotoxicity is important for the development of safe SPs.

Here we demonstrate that SPs exhibit cytotoxicity against THP-1 human macrophage-like cells in a size-dependent man-

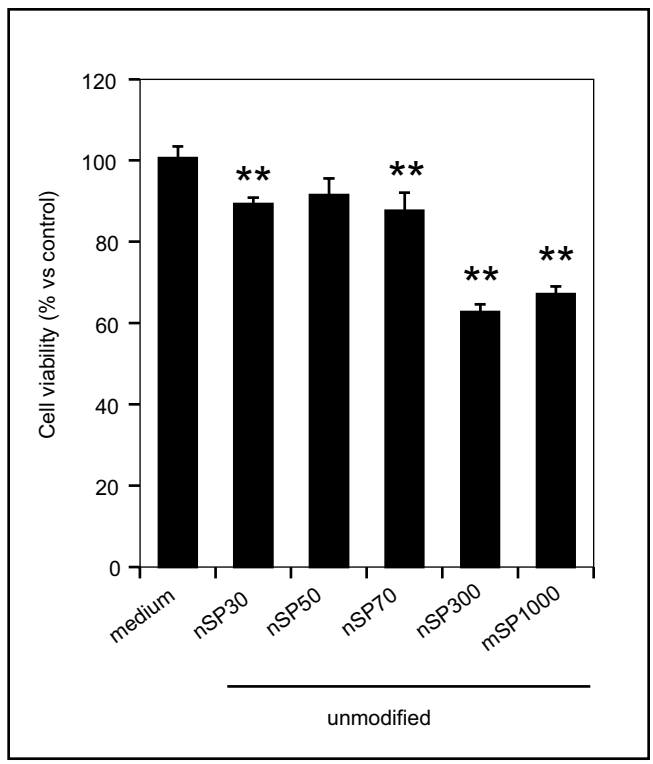

Fig. 1: Correlation between SP particle size and cytotoxicity against macrophage-like cells. PMA-primed THP-1 cells were treated with 100 μg/mL unmodified SPs for 24 h, and cell viability was evaluated by means of the standard methylene blue assay. The data represent the mean ± SD ($n = 5$; $^{**}P < 0.01$ versus value for medium control)

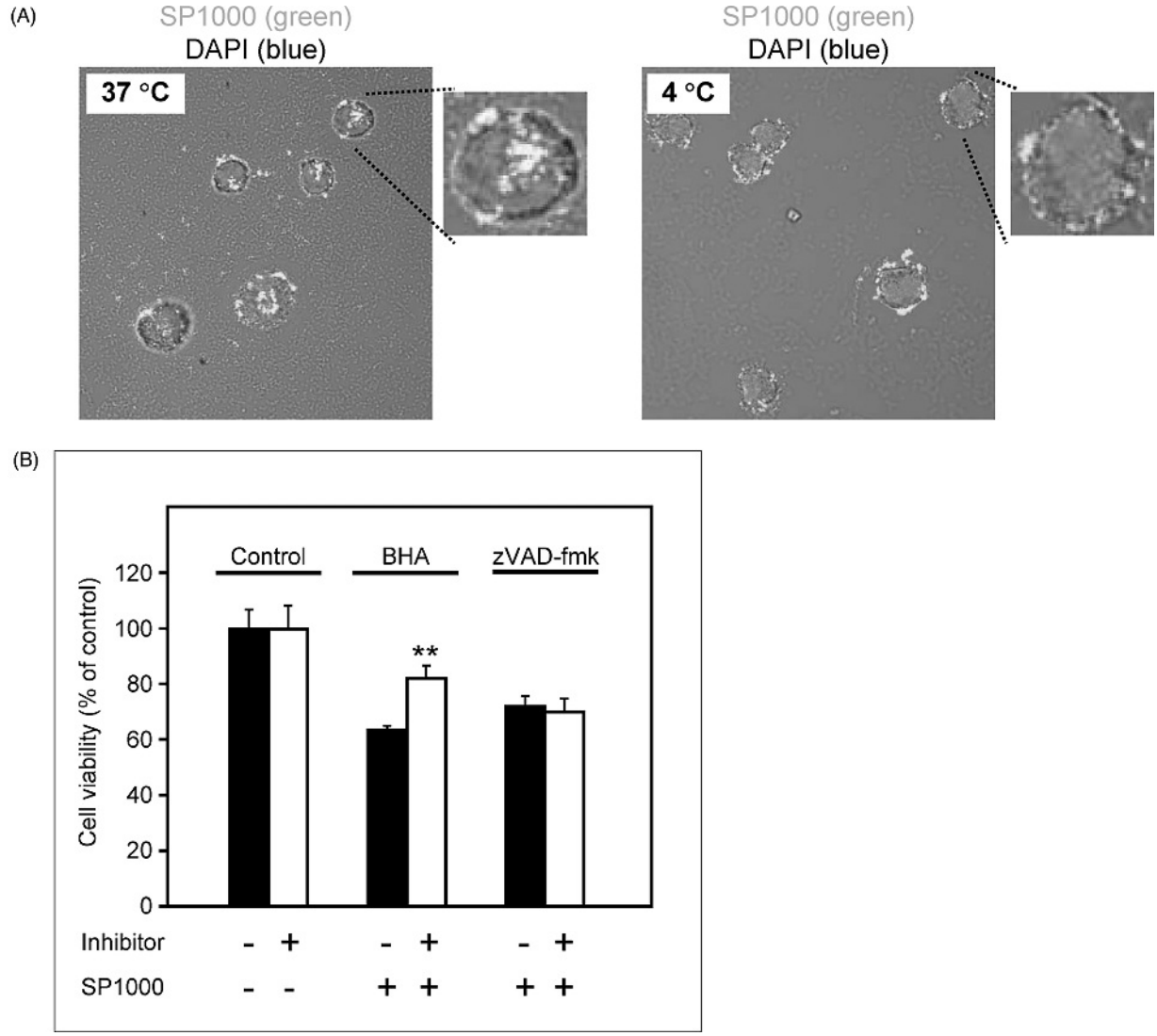

Fig. 2: ROS and caspase dependence of mSP1000-induced cell death. (A) Confocal microscopy images of the ingestion of mSP1000. FITC-conjugated mSP1000 (green) were added to the PMA-primed THP-1 cells at 100 μg/mL. Cells were incubated for 6 h at 37 °C (left) or 4 °C (right). The nucleus was stained with DAPI (blue). (B) Effect of a caspase inhibitor and an ROS scavenger on cytotoxicity of mSP1000. PMA-primed THP-1 cells were treated with 100 (g/mL SP1000s for 24 h in the presence or absence of BHA (150 μM) or zVAD-fmk (60 μM). Cell viability was measured by means of the methylene blue assay. The data represent the mean ± SD ($n = 4$; $^{**}P < 0.01$ versus value for inhibitor [-] control)

ner. Furthermore, we show that SPs with diameters of 1000 nm induce the production of reactive oxygen species (ROS), which triggers THP-1 cell death. We also demonstrate that surface modification of SPs with various functional groups significantly suppresses SP cytotoxicity.

2. Investigations, results and discussion

In this study, we examined whether the size and surface characteristics of SPs are correlated with their cytotoxicity. We also investigated the mechanism by which SPs induce the death of macrophage-like THP-1 cells, with the goal of providing information for the creation of novel safe SPs.

2.1. Amorphous silica particles induce cell death in a size-dependent manner

We used five SPs with diameters between 30 and 1000 nm (nSP30, nSP50, nSP70, nSP300, and mSP1000); the mean secondary particle diameters of the SPs measured by means of a Zetasizer were 33, 44, 79, 326, and 945 nm, respectively (data not shown). To compare the cytotoxicities of the SPs with different diameters, we examined their cytotoxicity against macrophages, which are the first line of defense against infection or injury from various inhaled agents. We incubated phorbol 12-myristate 13-acetate (PMA)-primed human macrophage-like THP-1 cells with SPs and analyzed the levels of cell viability. Twenty-four hours after the incubation, we found that nSP300 and mSP1000 induced marked cytotoxicity, whereas nSP30, nSP50, and nSP70 showed no cytotoxicity (Fig. 1). These results indicate that the particle size of the SPs was intimately involved in their biological effects.

2.2. mSP1000-induced cytotoxicity depends on ROS but not on caspases

Macrophages remove inhaled agents including foreign particles by means of their phagocytic activity. To confirm that THP-1 cells took up mSP1000, we treated THP-1 cells with fluorescein-5-isothiocyanate (FITC)-conjugated mSP1000 at 37 °C or 4 °C. We visually confirmed that mSP1000 were ingested into THP-1 cells at 37 °C, whereas only adsorption of mSP1000 on the cellular surface was detected at 4 °C (Fig. 2A). These results

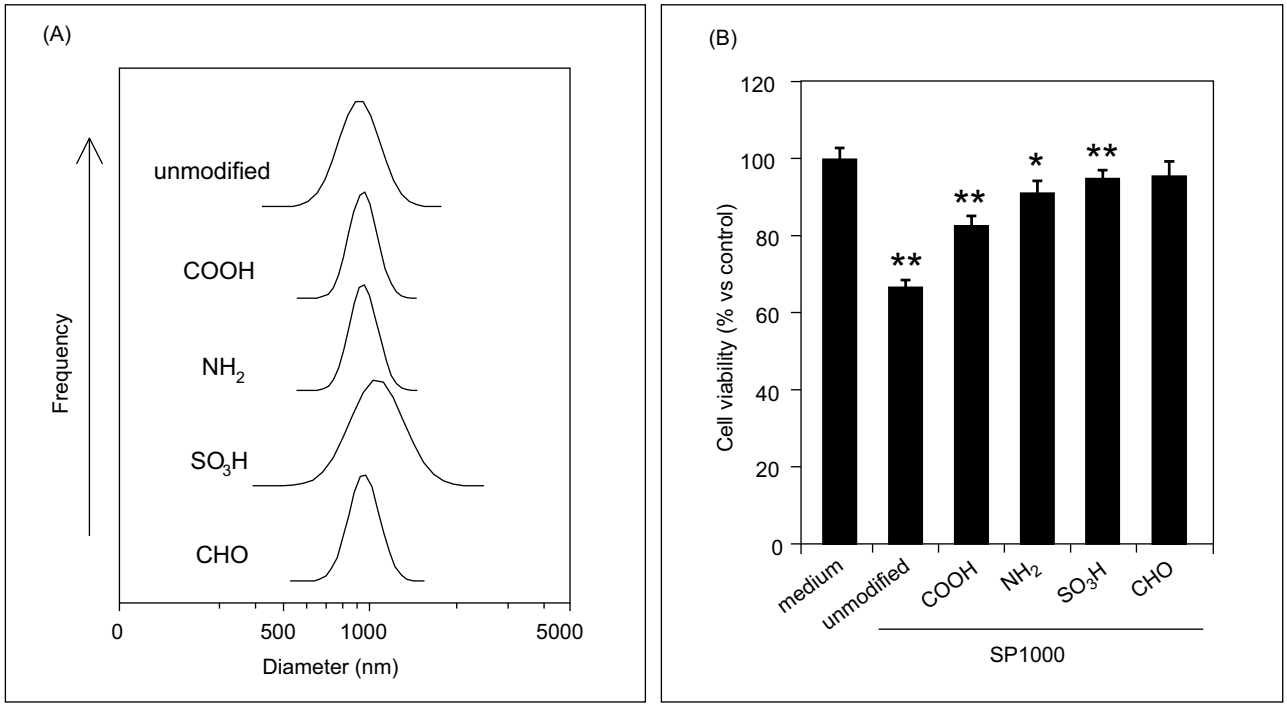

Fig. 3: Correlation between surface modification of mSP1000s and cytotoxicity against macrophage-like THP-1 cells. (A) Particle size distributions of unmodified and surface-modified mSP1000s. Particle size distributions were measured with a Zetasizer 3000HS after sonication at a particle concentration of 300 (μg/mL in H_2O. (B) Cytotoxicity of surface-modified mSP1000s. PMA-primed THP-1 cells were treated with the surface-modified mSP1000s at 100 μg/mL for 24 h. After the stimulation, cell viability was measured by means of the standard methylene blue assay. The data represent the mean ± SD ($n = 5$; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for medium control)

indicate that mSP1000 were recognized and taken up into THP-1 cells by energy-dependent phagocytosis.

We next examined the mechanism of mSP1000-induced cytotoxicity. To determine whether there was an association between caspases and mSP1000-induced cell death, we treated cells with mSP1000 in the presence or absence of zVAD-fmk, a broad caspase inhibitor (it inhibits caspase-1, -3, -4, and -7). We found that zVAD-fmk did not affect the mSP1000-induced cytotoxicity, which indicates that the cytotoxicity was independent of caspases (Fig. 2B). Recently, four kinds of cell death pathways were reported: apoptosis, necrosis, pyroptosis, and pyronecrosis (Ting et al. 2008). Apoptosis and pyroptosis are dependent on the activity of caspases, whereas necrosis and pyronecrosis are independent of caspases. Therefore, our results suggest that mSP1000-induced cell death might have been necrosis or pyronecrosis. Both pathways elicit substantial inflammation, whereas apoptosis is a non-inflammatory cell death that does not affect the area around the dying cells (Ting et al. 2008). Therefore, we suspected that SP-induced cell death might be associated with inflammatory responses induced by mSP1000.

However, the stimulation of macrophages with materials such as silica is known to induce ROS production (Msiska et al. 2009). Excessive production of ROS itself causes irreversible cellular injuries and contributes to the pathogenesis of several inflammatory diseases (Cross et al. 1994; Terman et al. 2006). To determine whether ROS were involved in mSP1000-induced cell death, we stimulated THP-1 cells with mSP1000 in the presence of a broad ROS scavenger, butylated hydroxyanisole (BHA), and found that the scavenger significantly inhibited the cytotoxicity of mSP1000 (Fig. 2B). These results indicate that ROS played an important role in the mSP1000-induced cell death and that the cytotoxicity induced by mSP1000 depends on ROS production but is independent of caspases, which suggests that mSP1000-induced cell death is inflammatory necrosis or pyronecrosis.

2.3. mSP1000-induced cell death is suppressed by surface modification with functional groups

To assess the correlation between surface modification and SP cytotoxicity, we used mSP1000 modified with various surface functional groups (-COOH, -NH_2, -SO_3H, and -CHO). The mean secondary particle diameters of unmodified mSP1000 was 945 nm, and the corresponding values for the modified particles were 1022, 958, 1023, and 969 nm, respectively (Fig. 3A). We compared the cytotoxicity of the modified and unmodified particles against THP-1 cells and found that mSP1000-induced cytotoxicity was suppressed by the surface modification (Fig. 3B). Interestingly, we confirmed that all the surface-modified mSP1000 were taken up equally into the cells (data not shown). We expect that surface modification can be used as a novel method to create safe SPs.

In summary, we confirmed that the cytotoxicity of SPs depended on particle size and surface properties. We confirmed that mSP1000-induced cell death was dependent on ROS production but independent of caspases. We believe that this information will be useful for the creation of novel safe SP-based materials.

3. Experimental

3.1. Materials and reagents

We used SPs with diameters between 30 and 1000 nm (nSP30, nSP50, nSP70, nSP300, and mSP1000), and mSP1000s with various surface functional groups (-COOH, -NH_2, -SO_3H, and -CHO). The SPs (Sicaster) were purchased from Micromod Partikeltechnologie (Rostock/Warnemünde, Germany). PMA and BHA were purchased from Sigma (St. Louis, MO). zVAD-fmk was purchased from Merck Calbiochem (Darmstadt, Germany).

3.2. Cell treatment

THP-1 cells (human acute monocytic leukemia cell line) were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented

with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics at 37 °C. Treatment of THP-1 cells with PMA reportedly induces differentiation to a macrophage phenotype (Hoff et al. 1992; Rutault et al. 2001).

3.3. Size distribution of silica particles

The size distributions of the SP were measured with a Zetasizer 3000HS (Malvern, Worcestershire, UK) after sonication at a particle concentration of 300 μg/mL in H_2O.

3.4. Cytotoxicity of various silica particles

THP-1 cells (1.5×10^4 cells/well) were seeded in 96-well plates, differentiated to macrophages by incubation with 0.5 (M PMA for 24 h, and then washed once with incubation medium. After the PMA priming, cells were treated with 100 (g/mL SPs for 24 h. The cytotoxicity of the SPs against THP-1 cells was assessed by means of the standard methylene blue assay. In brief, after the SP treatment, cells were fixed with 100 μL of 2.5% glutaraldehyde for 15 min and stained with 100 μL of 0.05% methylene blue for 15 min. Then, the cells were lysed with 200 μL of 0.33 N HCl. The $OD_{655–415}$ was measured using a multiwell spectrophotometer (Molecular Devices, Inc., Tokyo, Japan).
For the inhibitory assays, PMA-primed THP-1 cells were pre-incubated with BHA (150 μM) or zVAD-fmk (60 μM) for 30 min and then treated with 100 μg/mL SPs for 24 h in the presence or absence of each inhibitor.

3.5. Laser scanning confocal microscopy analysis

THP-1 cells (1.0×10^5 cells/well) were seeded on Lab-Tek II Chambered Coverglass (Nunc, Rochester, NY), differentiated to macrophages by incubation with 0.5 μM PMA for 24 h, and treated for 6 h with 100 μg/mL mSP1000s. Then the cells were washed and fixed with 4% paraformaldehyde and mounted with Prolong Gold with 2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine (DAPI, Invitrogen, Carlsbad, CA) for nuclear staining. Fluorescence was observed with a laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

3.6. Statistical analysis

All results are presented as means ± standard deviation (SD). Differences were compared using Student's *t*-test or Scheffé's method after analysis of variance (ANOVA).

Acknowledgements: The authors declare that they have no conflicts of interest. This study was supported in part by grants from the Ministry of Health, Labor, and Welfare in Japan.

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