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TiO_2 Nanoparticles Induced Genotoxicity in Cultured Cells Using Atmospheric Scanning Electron Microscopy (ASEM)

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ABSTRACT

Nano-sized titanium oxide nanoparticles (TiO_2 NPs) are widely used as a dye in food and cosmetics. TiO_2 NPs are known to induce DNA damage when incorporated into cells. However, no bioassay is currently available to easily determine the cell incorporation of TiO_2 NPs or related DNA damage, and to date, few studies have examined the different degrees of incorporation into cells according to the size of the TiO_2 NPs particles and the presence or absence of cell specificity regarding DNA damage. This present study was therefore designed to examine COS7 cells that had incorporated TiO_2 NPs using atmospheric scanning electron microscopy (ASEM). The results indicated that absorption of TiO_2 NPs into cells and nuclear abnormalities had occurred. ASEM is a rapid and simple technique that enables the observation of samples immediately after fixation with glutaraldehyde and staining with phosphotungstic acid, and this method was suggested to be useful in screening for DNA damage.

Keywords: COS7 Cells; TiO_2; Atmospheric Scanning Electron Microscopy (ASEM); Nanoparticle

1. Introduction

Nano-sized titanium oxide (TiO_2) powder is widely used as a dye for food, cosmetic and plastic products. The size of the nanoparticles (NPs) is important in that NPs with sizes of up to 50, 25 and 200 nm are taken up by endocytosis into alveolar A549 epithelial cells [1], human keratinocytes [2], and red blood cells [3], respectively, but NPs with larger sizes are just adsorbed to the cell surface.

TiO_2 NPs incorporated into cells are known to cause oxidative stress [4,5], and TiO_2-induced inflammation [6]. In addition, it has also been reported that TiO_2 NPs induce DNA damage and micronuclei formation [6-10]. From these data, it has become clear that cell injuries including genetic damage are induced by TiO_2 NPs. Indeed, it has also been reported that lung cancer was caused by inhalation of TiO_2 NPs [11].

We are surrounded by a large amount of TiO_2 NPs. The complicated biochemical and morphological methods should be simply introduced and related literatures should be cited. Recently, atmospheric scanning electron microscopy (ASEM) has been developed with which samples in their natural state, either within a liquid or a body of gas, may be observed by allowing a gaseous environment in the specimen chamber. Moreover, the advantages and disadvantages between these complicated methods and ASEM method in this paper should be analyzed, which is important to the necessary and significance of the ASEM method in this paper. We report herein that nuclear changes in cultured cells could be rapidly and simply detected using ASEM.

2. Materials and Methods

COS7 cells were cultured on the SiN film of the polystyrene ASEM dish, in Dulbeco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μm/ml kanamycine, at 37˚C for one day in the 5% CO_2 atmosphere of a CO_2 incubator [12]. For experiments in cell culture, TiO_2 NPs powder form (with a diameter of 50 nm) dispersed in culture medium. After then, COS7 cells were cultured during 6 hr.

For ASEM (JASM-6200 Clair Scope™) observations, these cells were fixed with 1% glutaraldehyde in phosphate buffer solution for 10 minutes, washed with water, stained with 2% PTA (phosphor tungsten acid) in water for 20 minutes, and washed with water, Water was supplemented with 10 mg/ml glucose as a radical scavenger.

3. Results

When COS7 cells were observed with ASEM after culturing in a medium without TiO_2 NPs, the nuclei of all

cells were stained (**Figure 1**) and, in particular, nucleoli were strongly stained (**Figure 1**). In the cytoplasm, reticular or tubular endoplasmic reticulum was observed around the nuclei (**Figure 1**).

When COS7 cells were observed with ASEM after culturing in a medium with TiO_2 NPs, cells with stained nuclei and those with unstained nuclei were observed at a ratio of approximately 1:1 (**Figure 2(a)**). In cells with well-stained nuclei, abundant endoplasmic reticulum vesicles were observed around the nuclei (**Figure 2(b)**). In cells with phosphotungstic acid-unstained nuclei, nuclear membranes were not observed and the endoplasmic reticulum was also somewhat unclear around the nuclei. In addition, many granules (diameter, 600 - 1000 nm) were observed around the nuclei (**Figure 2(c)**). Such granules were not found in normal COS7 cells.

4. Discussion

When COS7 cells were cultured in a medium containing TiO_2 NPs with a diameter of 50 nm for 6 hours, granules stained with phosphotungstic acid were observed in the cells. These granules were not observed in normal COS7 cells and in TiO_2-treated cells with stained nuclei. The diameter of the granules ranged from 600 to approximately 1000 nm. It has been reported that TiO_2 NPs are incorporated into cells by endocytosis [1-3]. In addition, it was observed by transmission electron microscopy that many incorporated TiO_2 NPs aggregated in lysosomes [13]. This suggests that the intracellular granules observed in this study were lysosomes containing TiO_2 NPs.

Usually, phosphotungstic acid is often used for negative staining. It stains the nuclei, unit membranes and stromal collagen fibers in section staining. In addition, phosphotungstic acid is also known to stain basic proteins. In this study, phosphotungstic acid stained the cytoplasm, but not the nucleus, in some cultured cells, sug-

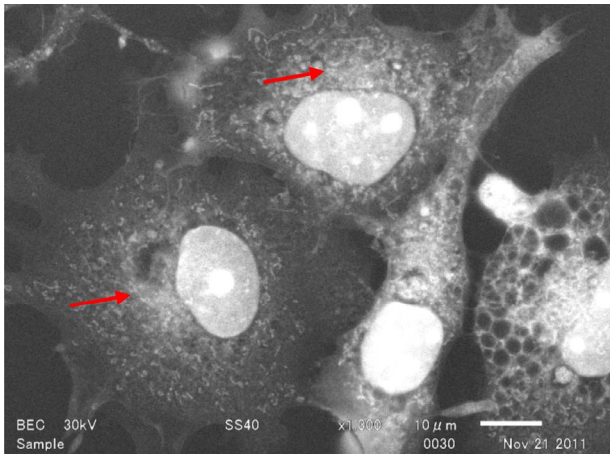
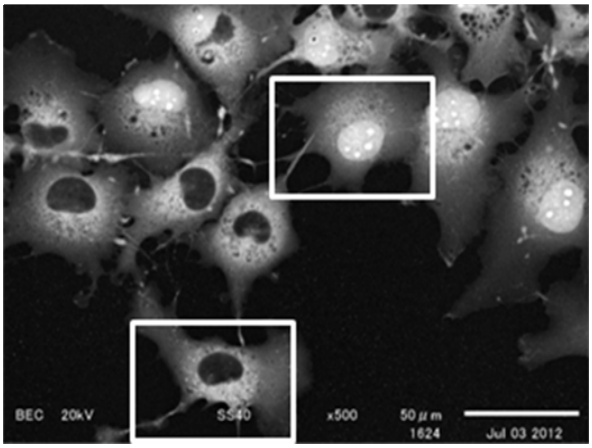
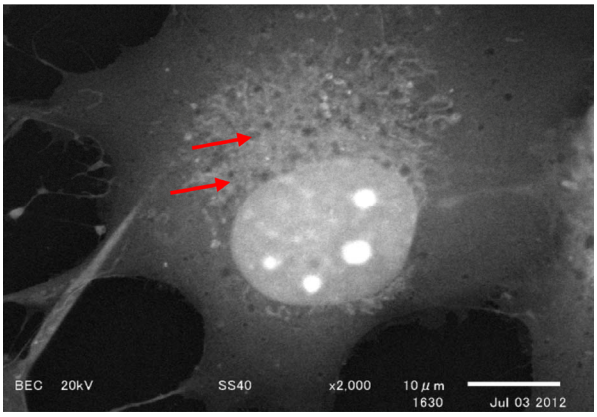


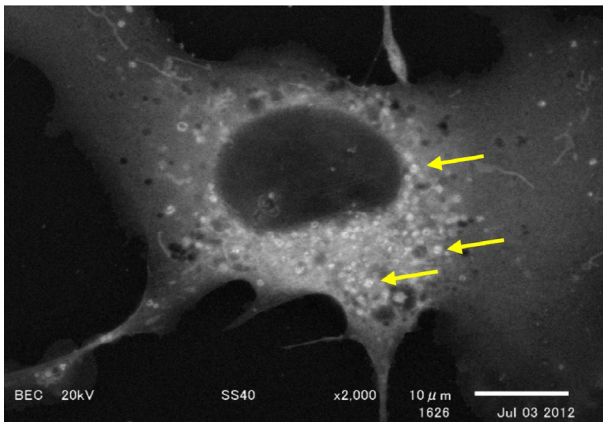
Figure 1. An ASEM photomicrograph of normal COS7 cells. (2% phosphotungstic acid staining) The nuclei and endoplasmic reticulum (Arrow) in the cytoplasm are well stained.



(a)



(b)



(c)

Figure 2. ASEM photomicrographs of cells cultured in medium containing TiO_2 NPs (2% phosphotungstic acid staining). (a) Cells with stained nuclei (upper insert) and those with unstained nuclei (lower insert) are observed; (b) The nucleus is stained and well-stained endoplasmic reticulum (Arrow) is observed in the cell; (c) The nucleus is not stained and there are many granules (Arrow) (600 - 1000 nm in diameter) in the cell.

gesting that basic proteins in the nuclei of cultured cells were denatured by TiO_2 NPs and became unstained. The genotoxicity of TiO_2 is a well-known fact [6-10,14]. However, it is necessary to clarify biochemically in the future what specific injuries the unstained nuclei have.

There were more granules in cells with unstained nuclei than in cells with stained nuclei. As described above, these granules were considered to be TiO_2 NPs. In other words, it can be said that only cells that incorporated a large amount of TiO_2 NPs had nuclear abnormalities. There was a variation in the amount of incorporated TiO_2 NPs within the same cultured cell population, and this is an issue to be addressed in the future.

There is an abundance of TiO_2 NPs in this world. Skocaj *et al.* described in their review [14] that "Until relevant toxicological and human exposure data that enable reliable risk assessment are obtained, TiO_2 nanoparticles should be used with great care." In addition, the toxicity of TiO_2 NPs varies depending on the size of the particles and the types of cells and tissues exposed to them [14]. This actually makes it difficult to understand various tissue and cell injuries induced by TiO_2 NPs.

Complicated techniques of biochemistry and transmission electron microscopy are needed to assess injuries induced by TiO_2 NPs, and a great deal of labor is required to assess many samples for analysis of the time course and dose dependence. However, the ASEM observation used in this study is a rapid and simple technique in that samples can be observed immediately after fixation with glutaraldehyde and staining with phosphotungstic acid. It is an excellent technique that can rapidly and simply detect nuclear abnormalities and intracellular incorporation of TiO_2 NPs. In the future, ASEM will be used frequently, not only to assess injuries induced by TiO_2 NPs, but also for application to drug screening, including screening of drug-induced toxicity.

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