Evidence That Ultrafine Titanium Dioxide Induces Micronuclei and Apoptosis in Syrian Hamster Embryo Fibroblasts

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Inhaled ultrafine titanium dioxide (UF-TiO2) particles cause pronounced pulmonary inﬂammation, in contrast to ﬁne TiO2. Previous studies provide evidence for the production of reactive oxygen species by alveolar macrophages, after loading with UF-TiO2 particles and cytotoxicity of UF-TiO2 in rat lung alveolar macrophages. UF-TiO2 also causes pulmonary ﬁbrosis and lung tumors in rats. UF-TiO2 particles are photogenotoxic, but in general, information on the genotoxicity of UF-TiO2 is still limited. We studied the potential of UF-TiO2 (particle size ≤ 20 nm) and ﬁne TiO2 (particle size > 200 nm) to induce chromosomal changes, which can be monitored by the formation of micronuclei (MN) in Syrian hamster embryo (SHE) cells. We also analyzed UF-TiO2-treated cells for apoptosis induction. The MN assay revealed a signiﬁcant increase in MN induction (p ≤ 0.05) in SHE cells after treatment with UF-TiO2 (1.0 µg/cm2) for 12 hr (mean, 24.5 MN/1,000 cells), 24 hr (mean, 31.13 MN/1,000 cells), 48 hr (mean, 30.8 MN/1,000 cells), 66 hr (mean, 31.2 MN/1,000 cells), and 72 hr (mean, 31.3 MN/1,000 cells). Bisbenzimide staining of the ﬁxed cells revealed typical apoptotic structures (apoptotic bodies), and the apoptosis-speciﬁc “DNA ladder pattern” resulting from internucleosomal cleavage was identiﬁed by gel electrophoresis. Furthermore, transmission electron microscopy of the exposed cells revealed the typical chromatin compaction of apoptosis. Key words: apoptosis, genotoxicity, kinetochore, micronuclei, ultrafine titanium dioxide. Environ Health Perspect 110:797–800 (2002). [Online 21 June 2002. http://ehpnet1.niehs.nih.gov/docs/2002/110p797-800 rahman/abstract.html

Titanium dioxide (TiO2) is a naturally occurring mineral found primarily as rutile, anatase, brookite, and ilmenite (1). It is widely used in the cosmetics, pharmaceutical, paint, and paper industries.

TiO2 was previously classiﬁed as biologically inert, both in animals and in humans (2,3). However, recent investigations on the basis of both experimental studies on rats and epidemiologic surveys revealed the development of inﬂammation, pulmonary damage, ﬁbrosis, and lung tumors after exposure to ultraﬁne TiO2 (UF-TiO2) (4–9). Dimensions of the TiO2 particles are pivotal, as demonstrated by the fact that UF-TiO2 causes more pronounced toxicity compared with ﬁne TiO2 particles (10–12).

UF-TiO2 particles (≤ 20 nm) induce impairment of macrophage function, persistently high inﬂammatory reactions, and increased pulmonary retention compared to ﬁne TiO2 (particle size > 200 nm) (6). They also enter the epithelium faster and are translocated in greater proportion to the subepithelium space compared with ﬁne particles (13). In situations such as “particle overload” after exposure to UF-TiO2 particles, the activated alveolar macrophages produce excessive amounts of such mediators as oxygen radicals, proteases, and growth-regulating proteins (14,15). Furthermore, an increase in the level of mRNA speciﬁc for interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α in human alveolar macrophages has been found after exposure to UF-TiO2 (16). Rahman et al. (17) also showed the production of reactive oxygen species (ROS) in human and rat alveolar macrophages after exposure to UF-TiO2 using a luminol-dependent chemiluminescence assay. Nakagawa et al. (18) reported the photogenotoxic potential of TiO2 using different in vitro genotoxicity assays and found that without ultraviolet (UV)/visible light it is weakly mutagenic, but combined with irradiation it is strongly mutagenic. Furthermore Lu et al. (19) reported the potential of TiO2 to induce micronuclei (MN) sister chromatid exchanges in Chinese hamster ovary cells.

In this paper we report on the occurrence of mitotic distortions, genetic damage, and oxidant-mediated DNA damage resulting in apoptosis (20) and induction of MN by UF-TiO2 using Syrian hamster embryo (SHE) ﬁbroblasts. Previous studies, by several groups as well as our own, have shown that this cell system is an efﬁcient and reliable in vitro model to study the genetic damage induced by ﬁbers or particles (21). Clastogenic and aneugenic effects have been monitored by the assessment of MN in combination with kinetochore analysis. We studied the induction of apoptosis using the DNA ladder assay and transmission electron microscopy (TEM).

Materials and Methods

Cell culture and treatment conditions. We isolated SHE ﬁbroblasts from 13-day-old embryos and grew the cells in a humidiﬁed atmosphere with 12% CO2 at 37°C. The culture medium was modiﬁed Dulbecco’s Eagle’s reinforced medium (Gibco, Karlsruhe, Germany), supplemented with 15% fetal calf serum (Flow Laboratories, Meckenheim, Germany), NaHCO3 (7.5%), 1% glucose, 10,000 IU penicillin, and 10 mg/mL streptomycin according to the standard procedure described by Pienta (22).

UF-TiO2 and ﬁne TiO2 were a gift from G. Oberdorster, University of Rochester (Rochester, New York, USA). The particle size was ≤ 20 nm for UF-TiO2 and > 200 nm for TiO2. Particles were sterilized by heating to 120°C for 2 hr and suspended in phosphate-buffered saline (PBS; 1 µg/µl). We treated cells with different concentrations of ﬁbers (0.5, 1.0, 5, and 10 µg/cm2) for different periods (12, 24, 48, 66, and 72 hr). We also treated cells separately with ﬁne TiO2 using similar concentrations and time periods.

MN assay. We grew SHE cells on coverslips after treatment with different doses of UF-TiO2 and ﬁxed them in cold ﬁxative after different incubation periods. We then stored them at –20°C for at least 30 min. We carried out DNA staining using bisbenzimide (1 µg/mL; Hoechst 33258; Sigma, St. Louis, MO, USA) for 4 min; after washing in PBS, the slides were mounted for microscopy. We scored only MN smaller than one-third the diameter of the nucleus under a ﬂuorescence microscope at 630 × magniﬁcation.

Kinetochore staining. For further MN analysis, we stained kinetochores by incubating the ﬁxed cell preparations with CREST serum (Chemicon, Temecula, CA, USA) for 1 hr in a humidified chamber at 37°C. After

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rinsing with PBS, we incubated the cells with fluorescein isothiocyanate (FITC)-conjugated goat anti-human antibodies (Sigma, Heidelberg, Germany) before applying bisbenzimide 33258 (Hoechst). We examined at least 100 MN for the presence of kinetochores in each case.

**TEM.** We exposed SHE cells to 5 µg/cm² and 10 µg/cm² UF-TiO₂ or 100 µM cisplatin (positive control) for 48 and 66 hr and fixed them with 4% glutaraldehyde in 0.1 M phosphate buffer. The fixed samples were dehydrated, embedded, polymerized, and sectioned in the usual manner. After preparing ultrathin sections with an LKB-ultratom III (LKB Instrument, Rockville, MD, USA) and treating them with uranyl acetate and lead citrate, we examined samples using a TEM (EM 902A; Zeiss, Oberkochen, Germany).

**Analysis of DNA fragmentation.** We used agarose gel electrophoresis to analyze the fragmentation of DNA during apoptosis. SHE cells were treated with 5 µg/cm² or 10 µg/cm² UF-TiO₂ or 100 µM cisplatin for 48 hr and 66 hr, respectively, and then digested at 50°C overnight and incubated with RNAse (10 µg/mL). DNA was extracted with phenol/chloroform and precipitated in ethanol; the extracted DNA was then separated by electrophoresis in an agarose gel (1% agarose gel containing 0.4 µg/mL ethidium bromide) and visualized under UV light. We repeated the experiment twice with consistent results.

**Statistical analysis.** Each data point represents the mean of 2,000 nuclei counted from each of three treated separate cultures from one experiment. We carried out the experiments three times with consistent results. The chi-square test was used to compare the results of the MN assay and to compare kinetochore analysis of each treatment group with the control.

**Results**
We observed formation of MN and appearance of characteristic changes typical for apoptosis in SHE cells after treatment with different concentrations of UF-TiO₂ (0.5, 1.0, 5.0, and 10.0 µg/cm²) for different exposure times (12, 24, 48, 66, and 72 hr). Figure 1 shows MN in SHE cells treated with 10.0 µg/cm² UF-TiO₂. UF-TiO₂ induced MN, which significantly (p ≤ 0.05) increased at concentrations between 0.5 and 5.0 µg/cm² (Figure 2), whereas fine TiO₂ did not induce significant alterations in the MN induction rate (data not shown). Cytotoxicity increased after exposure of cells to higher concentrations (> 10.0 µg/cm²).

Kinetochore analysis of MN from all groups revealed only an insignificant increase in the kinetochore-positive MN compared with unexposed control cells (data not shown).

We observed induction of apoptosis after bisbenzimide DNA staining of the fixed cells showing apoptotic bodies after 24, 48, and 72 hr of UF-TiO₂ exposure (Figure 3).

Figure 4 shows the TEM results. The typical compaction and marginalization of chromatin toward the nuclear periphery is clearly visible in the exposed SHE cells. Both early and late stages can be recognized, whereas unexposed cells do not show these specific features.

Figure 5 shows the typical internucleosomal fragmentation of UF-TiO₂-treated DNA (concentration, 10.0 µg/cm²; exposure times, 24 and 48 hr) and cisplatin-treated DNA (concentration, 100 µM; exposure time, 24 hr) after DNA agarose gel electrophoresis. In contrast, the DNA of untreated cells did not show this typical DNA ladder pattern in electrophoresis.

**Discussion**
We found UF-TiO₂ to induce MN; the number of MN was significantly increased (p ≤ 0.05) at all concentrations between 0.5 and 5.0 µg/cm² (Figure 2). Cytotoxicity was clearly detectable after exposure of cells to higher concentrations (> 10.0 µg/cm²). The time course of MN induction may reveal a saturation effect because the MN equency is identical at 24, 48, and 72 hr, whereas at 12 hr it was lower. In contrast, fine titanium dioxide did not induce MN to a significant extent (data not shown). MN induction results from very early genetic damage; therefore, linking these events directly to tumorigenesis would be too speculative. Nevertheless, this assay is one of the tests that are able to indicate the carcinogenic potential of chemicals and particles. Because the same effects would be expected in lung macrophages (no metabolism involved), these cells are also at risk.

Kinetochore analysis revealed no significant increase in the kinetochore-positive MN compared with unexposed control cells (data not shown). This indicates that the MN mainly arise from clastogenic events.

The frequency of the induced MN reflects the extent of chromosomal changes induced (23). Furthermore, staining of kinetochores with anti-kinetochore serum (CREST) allows discrimination of clastogenic effects from aneuploidy. The observed formation of MN may be due to ROS and/or the physical presence of these particles around the mitotic apparatus, as reported earlier by Hesterberg et al. (24) for mineral fibers. Further investigations are required to identify the causative mechanisms.

Apoptosis is a unique type of programmed cell death, and oxidant-mediated

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**Figure 1.** SHE cells exposed to UF-TiO₂ (10 µg/cm², 24 hr), showing induction of MN.

**Figure 2.** Formation of MN in SHE fibroblasts after treating cells with different concentrations of UF-TiO₂. (A) After 12, 24, and 48 hr. (B) After 66 and 72 hr. *p < 0.001.

**Figure 3.** SHE cells exposed to UF-TiO₂ (10 µg/cm², 24 hr) showing formation of apoptotic bodies after staining with bisbenzimide.
DNA damage is one of the key factors for its induction. According to Jimenez et al. (25), it may be an important factor in both the induction and/or promotion of carcinogenesis and certain proliferative diseases.

Apoptotic cells are characterized by a number of morphologic, molecular, and biochemical features, including shrinkage of cells, blebbing of cells and nuclear membranes, compaction and condensation of chromatin toward the nuclear periphery, and fragmentation of DNA into oligonucleosomes (26,27). Detection of more than one of these parameters is essential for a clear characterization of apoptotic cells. The present results confirm UF-TiO\textsubscript{2}–mediated induction of apoptosis in SHE cells through different endpoints such as DNA ladder formation (internucleosomal cleavage) and chromatin compaction as analyzed by TEM.

Dopp et al. (28) demonstrated that chrysotile asbestos induces apoptosis after 48 hr of exposure in SHE cells. Eastman (29) showed that other inducers of apoptosis such as cisplatin induce apoptosis after 18–24 hr, whereas camptothecin or teniposide induce apoptosis in rat thymocytes even after 6 hr of exposure. The induction of apoptosis appears to depend on the inducing agent, although apoptosis itself is a basic cellular inherent process (29). Because the manifestation of the genetic damage is one of the main prerequisites for apoptosis, the concentrations for the induction of this effect by UF-TiO\textsubscript{2} are considerably higher than those for MN induction.

TEM revealed the typical compaction and marginalization of chromatin toward the nuclear periphery of exposed SHE cells, which is an important characteristic of the initial stages of apoptosis (Figure 4). We encountered both early and late stages, whereas unexposed cells did not show such specific features.

For the quantification of apoptosis, we also carried out dual staining of the nuclei of UF-TiO\textsubscript{2}–exposed SHE cells with acridine orange and propidium iodide in a modified standard assay (30) (data not shown). According to Broaddus et al. (31), acridine orange enters the cells during the early stages of apoptosis and stains the nucleus bright green, but in later stages of apoptosis, due to loss of membrane integrity, both dyes enter the cell and stain the nucleus orange-red. We did not find this method appropriate because the cells showed staining in a number of shades between bright green and bright orange-red, making differential counting literally impossible.

Degradation of internucleosomal (linker) DNA segments as a consequence of activation of endogenous endonucleases is considered a characteristic endpoint of apoptosis and is analyzed using gel electrophoresis. DNA agarose gel electrophoresis depicted a characteristic end point of apoptosis and is analyzed using gel electrophoresis. DNA agarose gel electrophoresis depicted a typical internucleosomal fragmentation in DNA of UF-TiO\textsubscript{2}–treated (concentrations, 5.0 and 10.0 µg/cm\textsuperscript{2}; exposure times, 24 and 48 hr) and cisplatin-treated (concentration, 100 µM; exposure time, 24 hr) cells. In contrast, the DNA of untreated cells did not show this typical DNA ladder pattern in electrophoresis. DNA fragmentation by UF-TiO\textsubscript{2} and cisplatin showed similar cleavage patterns. Because cisplatin is a very potent apoptosis inducer, these results indicate that UF-TiO\textsubscript{2} also has similar effects (Figure 5).

On the basis of the present findings and previous studies, we hypothesize that the size of UF-TiO\textsubscript{2} particles may stimulate phagocytosis. This evidence is supported by the results of the TEM analyses. The reaction of particles with cell membranes results in the generation of ROS, and the generated oxidative stress may cause a breakdown of membrane lipids, imbalance of intracellular calcium homeostasis, and alterations in metabolic pathways.
chelators and OH radical scavengers has also been demonstrated (44).

To our knowledge, this is the first study showing that UF-TiO$_2$ induces apoptosis. However, the precise mechanism of MN formation and induction of apoptosis by ultrafine particles and fibers is still unknown. Further insight is required into intracellular signaling and the role of oxygen radicals.

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