A Newly Developed *In Vitro* Model of the Human Epithelial Airway Barrier to Study the Toxic Potential of Nanoparticles

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Summary

The potential health effects of inhaled engineered nanoparticles are almost unknown. To avoid and replace toxicity studies with animals, a triple cell co-culture system composed of epithelial cells, macrophages and dendritic cells was established, which simulates the most important barrier functions of the epithelial airway.

Using this model, the toxic potential of titanium dioxide was assessed by measuring the production of reactive oxygen species and the release of tumour necrosis factor alpha. The intracellular localisation of titanium dioxide nanoparticles was analyzed by energy filtering transmission electron microscopy.

Titanium dioxide nanoparticles were detected as single particles without membranes and in membrane-bound agglomerates. Cells incubated with titanium dioxide particles showed an elevated production of reactive oxygen species but no increase of the release of tumour necrosis factor alpha.

Our in vitro model of the epithelial airway barrier offers a valuable tool to study the interaction of particles with lung cells at a nanostructural level and to investigate the toxic potential of nanoparticles.

Zusammenfassung: Ein neu entwickeltes in vitro Modell für die menschliche epitheliale Lungenbarriere zur Prüfung des toxischen Potentials von Nanopartikeln

Die möglichen gesundheitlichen Auswirkungen von eingeatmeten Nanopartikeln sind bisher weitgehend unbekannt. Wir haben ein Zellkulturmodell der epithelialen Lungenbarriere, bestehend aus Epithelzellen, Makrophagen und dendritischen Zellen, aufgebaut, um die Interaktion von Partikeln mit Zellen und die Partikel-Toxizität zu studieren. Dieses Modell kann Toxizitätsversuche mit Tieren reduzieren und allenfalls ersetzen.

Die allfällige Auslösung von oxidativem Stress und Entzündungsreaktionen durch Titandioxid Nanopartikel wurde mit diesem Kulturmodel untersucht. Die intrazelluläre Lokalisation dieser Partikel konnte mittels Elektronen-Energie-Verlust-Spektroskopie nachgewiesen werden.

In den verschiedenen Zelltypen des Modells konnten Titandioxid Nanopartikel membranumhüllt wie auch frei im Zytoplasma detektiert werden. Die Inkubation mit Titandioxid Nanopartikeln löste in den Zellen oxidativen Stress aber keine Entzündungsreaktion aus.

Mit unserem in vitro Modell der epithelialen Lungenbarriere steht uns ein Testssystem zur Verfügung, um Partikel-Zell-Interaktionen und das toxische Potential von Nanopartikeln zu studieren, sowie die Zell-Zell-Interaktion nach Partikelex-position.

Keywords: epithelial airway barrier, nanoparticles, toxicity, oxidative stress, pro-inflammatory reactions

1 Introduction

During the last years there has been a substantial increase in the debate on the potential harmful effects of nanomaterials (Donaldson et al., 2005; Oberdörster et al., 2005a, Maynard and Michelson, 2006; Mühlfeld et al., 2008; Nel et al., 2006; Poland et al., 2008; Takagi et al., 2008). Among these nanomaterials, consisting of particles ≤100 nm, concern is expressed about their possible toxicity (Borm et al., 2006a). An important basis for the current concerns about the possible adverse health effects of NP has been provided by research in the field of inhalation toxicology (Oberdörster et al., 2005b). It has been observed that in rodent inhalation studies a number of different nanoparticulate materials show considerably stronger pulmonary toxicity when compared at equal mass dose with larger particles (Oberdörster et al., 2005a; Stoeger et al., 2006). Recent studies indicate the importance of exposed surface area as the most appropriate dose metric for NP since it has been found that particle surface area shows the closest correlation with the inflammatory response (Stoeger et al., 2006).

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Various aspects of NP toxicity have been described in a number of recent reviews (Borm et al., 2006b; Donaldson et al., 2005; Oberdörster et al., 2005a; Unfried et al., 2007). Most important however, is that detailed mechanisms whereby NP may interact with their target cells and how this can impact on toxicity are still poorly understood. The principal interaction of NP with their biological environment occurs at the cellular level, i.e. by interaction with structural and functional cell compartments (organelles, nuclei). After NP translocation across the cell membrane, the generation of reactive oxygen species as a hallmark of the biological effects of NP and inflammatory responses are described in a number of publications (for a review see Donaldson et al., 2005).

Understanding the functional disorders and subsequent pathological alterations induced in the respiratory tract by NP requires the investigation of the direct effects of these particles on the state and activity of lung cells. So far, three approaches have been used: (1) animal experiments, (2) *ex vivo* studies of cells of bronchial lavage or biopsies and (3) *in vitro* systems of lung cells exposed to particles under controlled conditions (Aufderheide, 2005).

Risk assessment of nanomaterials is, however, mainly done using animals, like rats (Kapp et al., 2004; Geiser et al., 2005; Kuempel et al., 2006), mice (Vaughn et al., 2006), cats (Bice et al., 2000), as well as dogs (McIntire et al., 1998; Bice et al., 2000; Wellenius et al., 2003).

There is a strong need for *in vitro* test systems to assess the toxicity of particulate matter and especially NP (Ayres et al., 2008). Many culture models of lung cells exist, providing an alternative to animal exposure for analysing the effects of different types of particles (Rothen-Rutishauser et al., 2008).

In order to determine the translocation behaviour of NP and their potential to induce cellular responses, we have used a triple cell co-culture model of the airway wall composed of monocyte derived macrophages (MDM), epithelial cells and monocyte derived dendritic cells (MDDC) (Rothen-Rutishauser et al., 2005). Using this model the intracellular localization of titanium dioxide (TiO_2) NP was studied using energy filtering transmission electron microscopy (EFTEM). TiO_2 NP were among the earliest industrially produced and applied NP in everyday applications (Maynard and Michelson, 2006). The potential of these particles to induce a toxic reaction was investigated by measurement of reactive oxygen species (ROS) and tumour necrosis factor alpha (TNF-α).

2 Material and methods

Triple cell co-culture system of the epithelial airway barrier

Cultures were prepared as previously described (Rothen-Rutishauser, et al. 2005). Briefly, a monolayer of the epithelial cell line A549 was grown on a microporous membrane in a two chamber system. After isolation and differentiation of human blood derived monocytes into MDM and MDDC, the two cell types were added at the apical side and at the basal side of the epithelium, respectively (Fig. 1).

Particle incubation

Commercially available particles were used: Titanium (IV) oxide (TiO_2), anatase 99.9% (metal basis) with a mean diameter of 0.02-0.03 μm (Alfa Aesar, Johnson Matthey GmbH, Karlsruhe, Germany).

Cells were incubated with particles as described in detail in Rothen-Rutishauser et al. (2007). Briefly, the particle suspensions were sonicated for 2 min prior to incubation with cells. A stock solution of TiO_2 particles in millipore water (2.5 mg/ml) was diluted in RPMI 1640 to a final concentration of 5 μg/ml. One ml of this suspension was then added to cell cultures. Incubations were performed for 24 h before analysis of the cells.

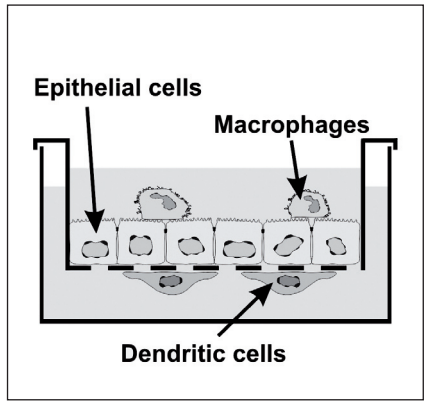

Fig. 1: The triple cell co-culture model of the human epithelial airway barrier. In order to obtain a triple co-culture, A549 cells were seeded and grown at the apical side of the filter insert. Differentiated MDDC were harvested and added to the basal side of the insert; then differentiated MDM were harvested and added on top of the epithelial cells.

Visualisation and quantification of carboxy-dichlorodihydrofluorescein diacetate (H_2DCFD) oxidation in cells by laser scanning microscopy

Following particle incubation, the Image-iT™ LIVE detection kit for reactive oxygen species (Molecular Probes, Invitrogen AG, Basel, Switzerland) was used for the detection of ROS in live cells according to the user manual. Briefly, cells were incubated with 25 μM carboxy-H_2DCFDA and the cell nuclei were stained with Hoechst. After 30 min incubation in the dark, the cells were mounted in warm buffer and imaged immediately. The fluorescence in cells was visualised by conventional fluorescence microscopy.

Cell labelling and fixation

The cells were labelled as described in detail in Rothen-Rutishauser et al. (2005). Briefly, the cultures were fixed, permeabilised and incubated with the first and second antibodies. Preparations were mounted in Mowiol on microscopic slides. Antibodies were diluted in phosphate buffered saline (PBS) as follows: mouse anti-human CD14 1:20 (Clone UCHM-1, C 7673, Sigma), mouse anti-human CD86 1:20 (Clone HB15e, 36931A, PharMingen, BD Biosciences,), goat anti-mouse cyanine 5 1:50 (AP124S, Chemicon, VWR International AG, Life Sciences, Lucerne, Switzerland) and phalloidin rhodamine 1:100 (R-415, Molecular Probes, Invitrogen AG, Basel, Switzerland).

Laser scanning microscopy and image restoration

A Zeiss LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200M, Lasers: HeNe 633 nm, HeNe 543 nm and Ar 488 nm) was used. Image processing and visualisation was performed using IMA-

RIS, a 3D multi-channel image processing software for confocal microscopic images (Bitplane AG, Zurich, Switzerland) (Blank et al., 2007).

Energy filtering transmission electron microscopy

EFTEM analysis was performed as described (Rothen-Rutishauser et al., 2006, 2007; Mühlfeld et al., 2007a). Briefly, cells were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer and with 0.5% uranyl acetate in 0.05 M maleate buffer. Cells were then dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin (≤ 50 nm) sections were cut, mounted onto uncoated 600-mesh copper grids and stained with uranyl acetate and lead citrate. The presence and localisation of TiO_2 particles was investigated with a LEO 912 transmission electron microscope (Zeiss, Oberkochen, Germany) using electron energy loss spectroscopy (Kapp et al., 2004; Rothen-Rutishauser et al., 2007).

TNF-α detection

The determination of TNF-α concentration was performed as described in detail previously (Rothen-Rutishauser et al., 2007). Briefly, following particle incubation, supernatants from triple cell co-cultures in the upper and lower chamber were collected separately and stored at –70°C. After centrifugation, TNF-α was quantified by a commercially available DuoSet ELISA Development kit (R&D Systems, Catalogue Number: DY 210, Oxon, UK) according to the manufacturer's recommendations.

Abbreviations:

EFTEM	Energy filtering transmission electron microscopy
H_2DCFD	Dichlorodihydrofluorescein diacetate
LSM	Laser scanning microscopy
MDDC	Monocyte derived dendritic cells
MDM	Monocyte derived macrophages
NP	Nanoparticles
TiO_2	Titanium dioxide
TNF- α	Tumour necrosis factor alpha
ROS	Reactive oxygen species

3 Results

The human epithelial airway model

In order to identify clearly MDM and MDDC in the triple co-culture system, the expression of specific markers was investigated with laser scanning microscopy (LSM). For visualisation of the cells, their actin-containing cytoskeleton was stained with rhodamine-labelled phalloidin. After 24 hours in co-culture, the cells were fixed and stained for the specific surface markers CD14 and CD86 for the labelling of MDM and MDDC respectively. The MDM were localised on top of the epithelium, whereas MDDC could be observed at the bottom side (Fig. 2) (Rothen-Rutishauser et al., 2005). The model was evaluated carefully and compared with *in vivo* data and we found that the morphological (Rothen-Rutishauser et al., 2008) as well as the quantitative occurrence of macrophages and dendritic cells resembled very closely the *in vivo* situation (Blank et al., 2007).

Intracellular TiO_2 localisation

After its thorough evaluation, this model was exposed to TiO_2 NP (mean diameter of 0.032 μm) and the particles were visualised and analysed in cells using EFTEM (Rothen-Rutishauser et al., 2006). Bigger membrane-bound aggregates (>0.2 μm) of TiO_2 NP were identified in all cell types (Fig. 3). In addition we found single particles and smaller (<0.2 μm) aggregates that were not membrane bound (data not shown) (Rothen-Rutishauser et al., 2007).

Production of ROS upon particle exposure

After particle incubation, the production of ROS was visualised using carboxy-DCFH, a reliable fluorogenic marker for ROS in live cells. TiO_2 NP can induce a higher oxidative stress when compared with control cultures (Fig. 4).

TNF-a release into supernatants after particle incubation

The pro-inflammatory cytokine TNF-α released into the culture supernatants after incubation with particles for 24 h was determined. In the control cultures, minor TNF-α concentrations were measured. When LPS (lipopolysaccaride), a positive control, was added to the cell

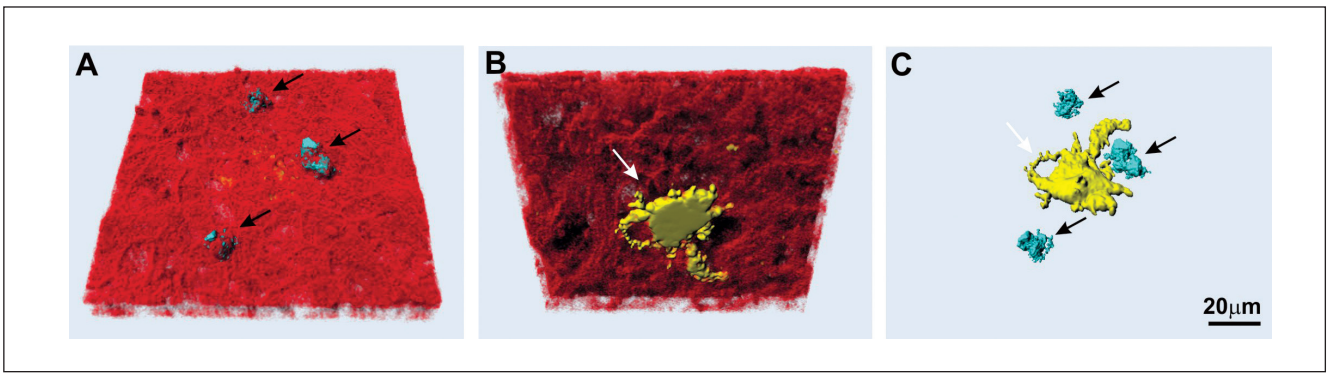

Fig. 2: LSM images of the triple cell co-culture model.
Epithelial cells (red, volume rendering), MDM (light blue, surface rendering; black arrows) and MDDC (yellow, surface rendering; white arrow) are shown. The same data set is shown from top (A), from bottom (B) and without epithelial cells from top (C).

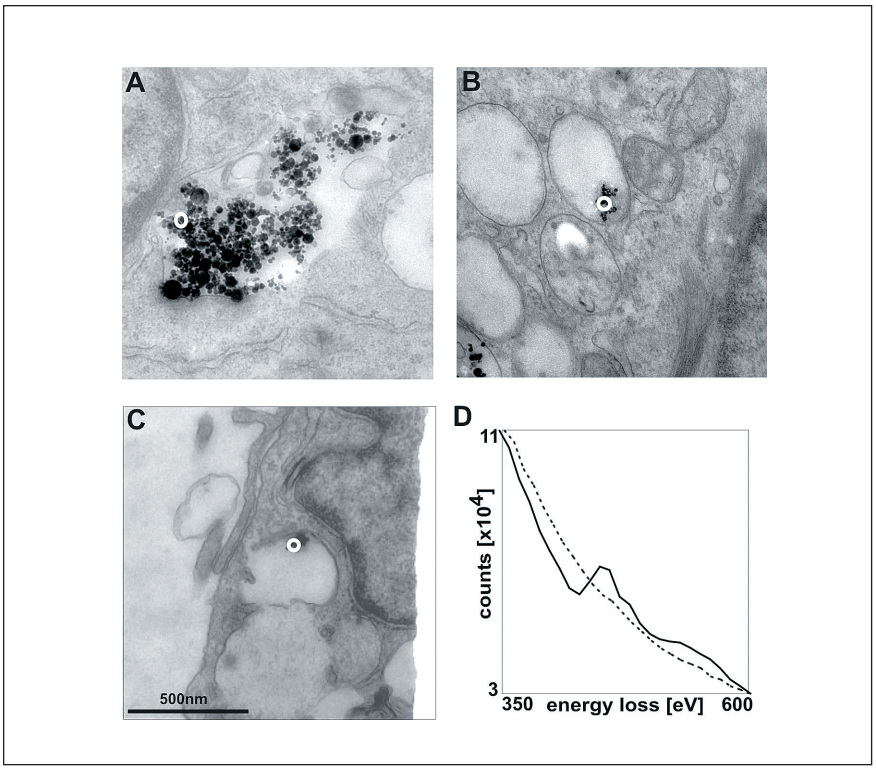

Fig. 3: Energy electron loss spectroscopy images of cells containing TiO_2 NP.
TiO_2 NP were found inside all cell types, i.e. MDM (A), epithelial cells (B) and MDDC (C). The circles mark the region in which the element analysis was performed. One representative energy loss spectrum (black lines) from picture (A) is shown (D). The dotted line shows the background.

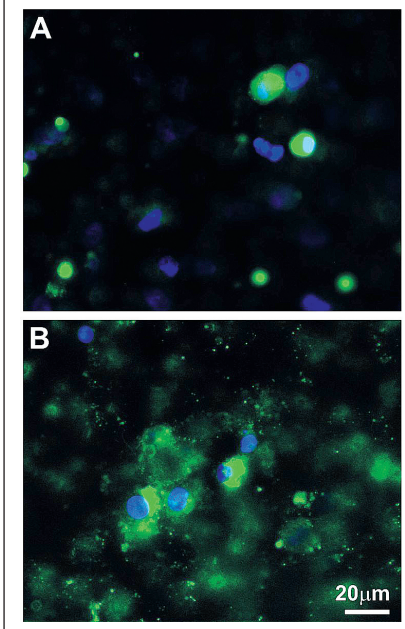

Fig. 4: Fluorescent micrographs of ROS in triple cell co-cultures.
Cell nuclei are shown in blue, ROS in green. In control cultures only few cells with ROS signals could be detected (A), whereas TiO_2 NP induce a higher oxidative response, as the green stain in the cytoplasm shows (B).

cultures, the TNF-α signal increased significantly. No increase in TNF-α concentration was seen when TiO_2 NP were added to the cultures (Fig. 5) (Rothen-Rutishauser et al., 2007).

4 Discussion

The understanding of the possible functional and pathological disorders induced in the respiratory tract by NP requires the investigation of the direct effects of these particles on the state and activity of lung cells. Therefore, we used our established *in vitro* model of the airway wall (Rothen-Rutishauser et al., 2005; 2008) to evaluate the translocation of TiO_2 NP into the cells and their potential to induce oxidative stress and TNF-α release.

TiO_2 was found inside all three cell types of the triple cell co-culture model, i.e. MDM, epithelial cells and MDDC, as membrane-bound larger aggregates and as free smaller aggregates or individual particles in the cytoplasm (Rothen-Rutishauser et al., 2007). Further studies will include the intracellular quantification of NP with an unbiased stereological method (Mühlfeld et al., 2007b). NP are known to provoke oxidative stress causing inflammation (for a review see Donaldson et al., 2003 and Donaldson et al., 2005). We have therefore investigated whether TiO_2 NP can induce the production of ROS and the release of TNF-α into the supernatants. Interestingly, we found that TiO_2 increased the ROS production, but did not result in an inflammatory reaction. It is known that TiO_2 NP can induce oxidative stress in many different cell types, e.g. in cultured human epithelial cells (Gurr et al., 2005) or in cultured brain cells (Long et al., 2006) at low TiO_2 concentrations (2.5-10 µg/ml). The release of TNF-α has been shown to be concentration dependent in cultured macrophages exposed to TiO_2NP (Kang et al., 2008). In the latter study the concentration of TiO_2 NP had to be higher than 25 µg/ml to induce the secretion of pro-inflammatory mediators. In the present study a lower concentration was used, i.e. 5 µg/ml and this might be the reason why we did not observe a pro-inflammatory response. Further experiments need to be performed with higher TiO_2 NP concentrations.

In our triple cell co-culture model, monolayers of two different epithelial cell lines, A549 (Rothen-Rutishauser et al., 2005) and 16HBE14o epithelia (Blank et al., 2007), were grown on a microporous membrane in a two chamber system combined with MDM and MDDC. The transepithelial electrical resistance was monitored, because this is an important parameter of the epithelial tightness

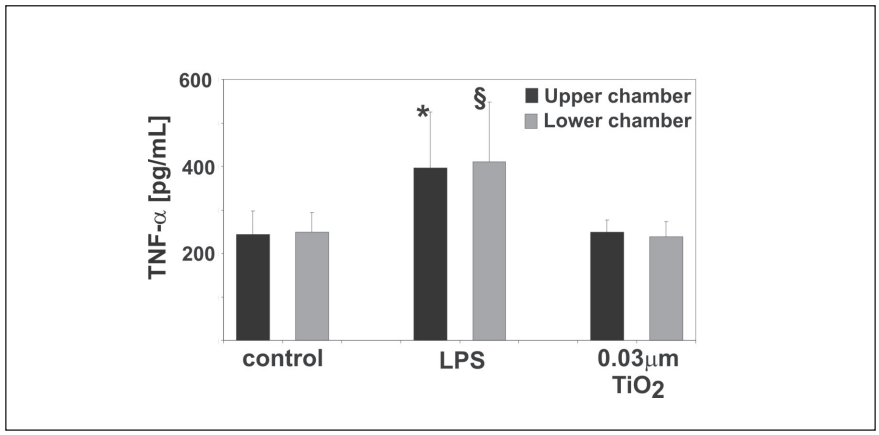

Fig. 5: TNF-α release in triple cell co-cultures upon particle incubation.
TNF-α levels in the supernatants (upper chamber, lower chamber) were measured by ELISA. TNF-α release in cells exposed to LPS and TiO_2 NP. Values are means ± SD of 3 experiments. * indicates a statistical difference to the levels in the supernatants in the control of the upper chamber, § indicates a statistical difference to the levels in the supernatants in the control of the lower chamber. (Modified from Rothen-Rutishauser et al., 2007.)

(Kwang-Jin, 2002). In the cultures with the 16HBE14o cells, a value of about 500 Ωcm^2 was measured (Blank et al., 2007), which is in agreement with other studies (Forbes, 2000). For the A549 cultures, values between 140 and 180 Ωcm^2 were measured (Rothen-Rutishauser et al., 2005), however, much higher values have been found in primary alveolar epithelial cultures, i.e. > 1000 Ωcm^2 (Elbert et al., 1999). Many cell culture models of the respiratory tract have been developed for pulmonary drug delivery studies (for a review see Steimer et al., 2005) and our model might also be used for safety and efficacy aspects in pharmaceutical research, since immune cells have been included. However, when a high epithelial tightness is required, the A549 or 16HBE14o cells might need to be replaced by other epithelial cells, i.e. primary cultures.

Lung cell culture models may help to elucidate the mechanisms of how particles that are inhaled and deposited on the lung surface can interact with the cells and induce cellular responses. Even though *in vitro* models exhibit a number of limitations, they can be used for high-throughput screening and the screening of large numbers of newly developed particles, in particular NP, within a short time. An essential disadvantage is that cell culture models often do not exhibit all the differentiated and functional characteristics of the corresponding native cells, tissue or the entire organ. However, the 3D model of the epithelial airway barrier presented here is a step forward since, by including several cell types, it offers the possibility not only to study the reaction of individual cell types but also the interaction of the different cell types with each other.

References

Aufderheide, M. (2005). Direct exposure methods for testing native atmospheres. *Exp. Toxicol. Pathol. 57*, 213-226.

Ayres, J. G., Borm, P., Cassee, F. R. et al. (2008). Evaluating the toxicity of airborne particulate matter and nanoparticles by measuring oxidative stress potential – a workshop report and consensus statement. *Inhal. Toxicol. 20*, 75-99.

Bice, D. E., Seagrave, J. and Green, F. H. (2000). Animal models of asthma: potential usefulness for studying health effects of inhaled particles. *Inhal. Toxicol. 12*, 829-862.

Blank, F., Rothen-Rutishauser, B. and Gehr P. (2007). Dendritic cells and macrophages form a transepithelial network against foreign particulate antigens. *Am. J. Respir. Cell Mol. Biol. 36*, 669-677.

Borm, P. J. and Müller-Schulte, D. (2006a). Nanoparticles in drug delivery and environmental exposure: same size, same risks? *Nanomed. 1*, 235-249.

Borm, P. J., Robbins, D., Haubold, S. et al. (2006b). The potential risks of nanomaterials: a review carried out for ECETOC. *Part. Fibre. Toxicol. 3*, 11.

Donaldson, K., Tran, L., Jimenez L. A. et al. (2005). Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part. Fibre. Toxicol. 2*, 10.

Donaldson, K., Stone, V., Borm, P. J. et al. (2003). Oxidative stress and calcium signaling in the adverse effects of environmental particles (PM10). *Free Radic. Biol. Med. 34*, 1369-1382.

Elbert, K. J., Schafer, U. F., Schafers, H. J., et al. (1999). Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. *Pharm. Res. 16,* 601-608.

Forbes, B. (2000). Human airway epithelial cell lines for in vitro drug transport and metabolism studies. *Pharm. Sci. Technolo. Today 3*, 18-27.

Geiser, M., Rothen-Rutishauser, B., Kapp, N. et al. (2005). Ultrafine particles cross cellular membranes by non-phagocytic mechanisms in lungs and in cultured cells. *Environ. Health Perspect. 113,* 1555-1560.

Gurr, J.R., Wang, A.S., Chen, C.H. and Jan, K.Y (2005). Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative DNA damage to human bronchial epithelial cells. *Toxicol. 15*, 66-73.

Kapp, N., Kreyling, W., Schulz, H. et al. (2004). Electron energy loss spectroscopy for analysis of inhaled ultrafine particles in rat lungs. *Microsc. Res. Tech. 63,* 298-305.

Kang, J. L., Moon, C., Lee, H. S. et al. (2008). Comparison of the biological activity between ultrafine and fine titanium dioxide particles in RAW 264.7 cells associated with oxidative stress. *J. Toxicol. Environ. Health A. 71*, 478-85.

Kuempel, E. D., Tran, C. L., Castranova, V., Bailer, A. J. (2006). Lung dosimetry and risk assessment of nanoparticles: evaluating and extending current models in rats and humans. *Inhal. Toxicol. 18*, 717-724.

Kwang-Jin, K. (2002). Bioelectrical characterization of epithelial cell (mono) layers and tissues. In C.-M. Lehr (ed.), *Cell culture models of biological barriers. In-vitro test systems for drug absorption and delivery*. (3:41-51). London, New York: Taylor and Francis.

Long, T. C., Saleh, N., Tilton, R. D. et al. (2006). Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. *Environ. Sci. Technol. 40*, 4346-4352.

Maynard, A. and Michelson, E. (2006). The Nanotechnology Consumer Products Inventory. http://www.nanotechproject.org/inventories/consumer/

McIntire, G. L., Bacon, E. R., Toner, J. L. et al. (1998). Pulmonary delivery of nanoparticles of insoluble, iodinated CT X-ray contrast agents to lung draining lymph nodes in dogs. *J. Pharm. Sci. 87*, 1466-1470.

Mühlfeld, C., Rothen-Rutishauser, B., Vanhecke, D. et al. (2008). Interactions of nanoparticles with pulmonary structures and cellular responses. *Am. J. Physiol. Lung Cell. Mol. Physiol. 294*, L817-29.

Mühlfeld, C., Rothen-Rutishauser, B., Vanhecke, D. et al. (2007a). Visualization and quantitative analysis of nanoparticles in the repsiratory system by transmission electron microscopy. *Part. Fibre Toxicol. 4*, 11.

Mühlfeld, C., Mayhew, T.M., Gehr, P. and Rothen-Rutishauser, B. (2007b). A novel quantitative method for analysing the distribution of nanoparticles between different tissue and intracellular compartments. *J. Aerosol. Med. 20*, 395-407.

Nel, A., Xia, T., Madler, L. and Li, N. (2006). Toxic potential of materials at the nanolevel. *Science 311*, 622-627.

Oberdörster, G., Oberdörster, E. and Oberdörster, J. (2005a). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. *Health Perspect. 113*, 823-839.

Oberdörster, G., Maynard, A., Donaldson, K. et al. (2005b). ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group, Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Part. Fibre. Toxicol. 2*, 8.

Poland, C. A., Duffin, R., Kinloch, I. et al. (2008). Carbon nanotubes introduced into abdominal cavity display asbestos-like pathogenic behaviour in a pilot study. *Nat. Nanotechnol.,* doi:10.1038/nnano.

Rothen-Rutishauser, B. M., Kiama, S. G. and Gehr P. (2005). A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *Am. J. Respir. Cell. Mol. Biol. 32*, 281-289.

Rothen-Rutishauser, B. M., Schürch, S., Haenni, B. et al. (2006). Interaction of fine and nano- particles with red blood cells visualized with advanced microscopic techniques. *Environ. Sci. Technol. 40*, 4353-4359.

Rothen-Rutishauser, B. M., Mühlfeld, C., Blank, F. et al. (2007). Translocation of particles and inflammatory responses after exposure to fine particles and nanoparticles in an epithelial airway model. *Part. Fibre. Toxicol. 4*, 9.

Rothen-Rutishauser, B., Blank, F., Mühlfeld, C. and Gehr, P. (2008). In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter. *Exp. Opin. Drug Metabol. Toxicol.* (in press).

Takagi, A., Hirose, A., Nishimura, T. et al. (2008) Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. *J. Toxicol. Sci. 33*, 105-16.

Unfried, K., Albrecht, C., Klotz, L. O. et al. (2007). Cellular responses to nanoparticles: Target structures and mechanisms. *Nanotoxicology 1*, 1-20.

Steimer, A., Haltner, E., Lehr, C. M. (2005). Cell culture models of the respiratory tract relevant to pulmonary drug delivery. *J. Aerosol. Med. 18*, 137-182.

Stoeger, T., Reinhard, C., Takenaka, S. et al. (2006). Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. *Environ. Health. Perspect. 114*, 328-333.

Vaughn, J. M., McConville, J. T., Burgess, D. et al. (2006). Single dose and multiple dose studies of itraconazole nanoparticles. *Eur. J. Pharm. Biopharm. 63*, 95-102.

Wellenius, G. A., Coull, B. A., Godleski, J. J. et al. (2003). Inhalation of concentrated ambient air particles exacerbates myocardial ischemia in conscious dogs. *Environ. Health Perspect. 111*, 402-408.

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