Supplementary Material

Towards predicting the lung fibrogenic activity of MWCNT: key role of endocytosis, kinase receptors and ERK 1/2 signaling

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Methods

MWCNT physico-chemical characteristics

Transmission electron microscopy (TEM) was used to study the diameter and morphology of CNT samples. Long, short and thick MWCNT have been dispersed in ethanol 99.9 % and then sonicated for 15 min in a water bath to ensure a maximum dispersion. TEM images were taken using a transmission electronic microscope (Philips FEI Tecnai 10) at an acceleration voltage of 80 kV and a digital camera. For NM400 characterization, methods are described in Vietti et al. (2013). The concentration of metals in the samples was quantified after mineralization in acid (HNO_3 14 N and HCl 12 N; Merck, Darmstadt, Germany) in a high pressure microwave (Multiwave, Anton Paar GmbH, Graz, Austria). The amount of Fe was measured by atomic absorption spectrometry on a Spectra AA 300 (Varian Zeeman Inc., Palo Alto, USA) equipped with a graphite furnace atomizer and a Zeeman system for correction of nonspecific absorbance. The amount of Al, Co and Ni was measured by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500G, Santa Clara, USA) with an octapole reaction system.

MWCNT dispersion

MWCNT were dispersed as previously described in Vietti et al. (2013). Briefly, MWCNT were weighed in a sterile glass vial and suspended to a concentration of 2.55 mg/ml. Dispersion media was prepared with autoclaved nanopure H_2O supplemented with 1.4 mg/ml bovine serum albumin (BSA, Sigma Aldrich, St Louis, USA) and filter-sterilized (0.22 μm). The suspension was vortexed and then sonicated (Virsonic 300 ultrasonic cell disrupter, Virtis, Gardiner, USA) for 16 min without interruption (power 15 %) on ice. In order to keep the final concentration of

the dispersant equal at all tested doses, the stock solutions (2.55 mg/ml) were diluted in the same dispersion medium and then sonicated 15 min in a waterbath.

Cell culture

Mouse peritoneal macrophage cells (J774) obtained from Pr Van Snick (Ludwig Institute, Brussels, Belgium) were cultured in DMEM GlutaMAX (Invitrogen) supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotic-antimycotic (AA) and maintained at 37 °C under 5 % CO₂. Cells were harvested in 5 ml of DMEM GlutaMAX, 10 % FBS and 1 % AA with a scraper. Viable cell number was determined with Trypan Blue (Sigma Aldrich). Cells were seeded in 96-well culture plates in complete medium at a concentration of 150.000 cells/cm² (surface area of culture well) and incubated at 37 °C under 5 % CO₂ for 24 h before exposure. J774 were sub-cultured for a maximum of 15 passages.

Mouse type II alveolar epithelial cells (LA-4) obtained from ATCC (catalog n°CCL-196) were cultured in F-12K (Invitrogen) supplemented with 15 % FBS and 1 % AA and maintained at 37 °C under 5 % CO₂. For harvesting, cells were incubated with 0.25 % trypsin-EDTA (Invitrogen) at 37 °C. Trypsin was neutralized by adjunction of complete medium (F-12K, 15 % FBS, 1 % AA). Cell suspensions were centrifuged for 10 min at 400 *g* and resuspended in fresh complete medium. Viable cell number was determined as described above. Cells were seeded in 96-well culture plates in complete medium at a concentration of 150.000 cells/cm² and incubated at 37 °C for 24 h before exposure. LA-4 were sub-cultured for a maximum of 15 passages.

Mouse lung fibroblasts (MLg) obtained from ATCC (catalog n°CCL-206) were cultured in MEM (Invitrogen) supplemented with 10 % FBS and 1 % AA and maintained at 37 °C under 5 % CO₂. Cells were harvested and counted as previously described for epithelial cells. Cells were seeded into 96-well or 48-well culture plates

in complete medium (10 % FBS) at a concentration of 90.000 cells/cm² and incubated at 37 °C under 5 % CO₂ for 24 h before exposure. MLg were sub-cultured for a maximum of 15 passages.

Proliferation/cytotoxicity assay

WST-1 is a colorimetric assay that quantifies mitochondrial activity and reflects cell viability. In this study, cell proliferation/cytotoxicity was measured by a NM-adapted WST-1 assay (Vietti et al., 2013). Briefly, cells exposed for 24 h to test materials in 96-well plates were washed twice with their basal medium. Half of the cultures were supplied with 100 µl of medium and the other half with 100 µl medium containing 0.2 % Triton X-100 to lyse cells. Triton-treated wells were used as blanks since they contain the same amount of test material as test samples but have no mitochondrial activity. This procedure was introduced to eliminate the possible interferences of test materials with the assay either by interacting with the absorbance wavelength and/or with the WST-1 substrate. After 15 min at 37 °C, 100 µl WST-1 (diluted 10 times in medium, Roche Diagnostics GmbH, Mannheim, Germany) were added to the cells and absorbance was measured after 60-90 min at 480 nm and at 680 nm (Spectrophotometer Infinite F200, Tecan, Mannedorf, Switzerland). Data are presented as relative cell activity ($\text{Abs}_{\text{exposed}}/\text{Abs}_{\text{non-exposed}}$) where $\text{Abs} = (\text{Abs}_{480} - \text{Abs}_{680})_{\text{no Triton}} - (\text{Abs}_{480} - \text{Abs}_{680})_{\text{Triton}}$. Exposure to PDGF-BB 30 ng/ml, TGF-β1 10 ng/ml or EGF (10 ng/ml) was used as a positive control to stimulate fibroblast and epithelial cell proliferation.

References

Vietti G, Ibouaadata S, Palmai-Pallag M, Yakoub Y, Bailly C, Fenoglio I, Marbaix E, Lison D, Van Den Brule S. 2013. Towards predicting the lung fibrogenic activity of nanomaterials: experimental validation of an in vitro fibroblast proliferation assay. Part Fibre Toxicol 10, 52.

Figures

Figure S1

Figure S1. Effect of MWCNT on PDGF and TGF- β secretion by fibroblasts and expression of their receptors. Expression of PDGF R α (A), PDGFR β (B), TGF- β RI (C) and TGF- β RII (D) mRNA was assessed by quantitative RT-PCR on mouse lung fibroblasts (MLg), type II alveolar epithelial cells (LA-4) and peritoneal macrophages (J774). Results are presented as a ratio of gene expression normalized on the expression of the reference gene, 18S rRNA. Secretion of PDGF-BB (E,F) and TGF- β 1 (G,H) was measured in MLg culture supernatants by ELISA. MLg were primed with low concentrations of PDGF (E,G) or TGF- β (F,H) (3 and 1 ng/ml, respectively) and then exposed to MWCNT (7.5-15 $\mu\text{g}/\text{cm}^2$) for 24 h. * $p < 0.05$, *** $p < 0.001$ vs non-treated cells ($n = 3$, t-test or Dunnett's Multiple Comparison test as appropriate).

Figure S2

Figure S2. Implication of EGF R in the fibrogenic activity of MWCNT in fibroblasts.

(A) Expression of EGF R mRNA was measured by quantitative RT-PCR in mouse lung fibroblasts (MLg), type II alveolar epithelial cells (LA-4) and peritoneal macrophages (J774). Results are presented as a ratio of gene expression normalized on the expression of the reference gene, 18S rRNA. (B) MWCNT activity on fibroblast proliferation in presence of the EGF R inhibitor IRESSA was assessed by the adapted WST-1 assay. Fibroblasts were primed with a low concentration of EGF (1 ng/ml), treated with IRESSA and then exposed to MWCNT (7.5-15 μg/cm²) for 24 h. EGF (10 ng/ml) was used as a positive control. WST-1 results are presented as **relative cell activity, i.e. as** a ratio to non-treated cells (Ctl). * p < 0.05, *** p < 0.001 vs non-treated cells (n = 4, Newman-Keuls Multiple Comparison test).