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Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells *in vitro*

Hanna K. Lindberg[a], Ghita C.-M. Falck[a], Satu Suhonen[a], Minnamari Vippola[b,c], Esa Vanhala[b], Julia Catalán[a,d], Kai Savolainen[a], Hannu Norppa[a,*]

[a] *New Technologies and Risks, Work Environment Development, Finnish Institute of Occupational Health, FI-00250 Helsinki, Finland*
[b] *Aerosols, Dusts and Metals, Work Environment Development, Finnish Institute of Occupational Health, FI-00250 Helsinki, Finland*
[c] *Department of Materials Science, Tampere University of Technology, FI-33101 Tampere, Finland*
[d] *Department of Anatomy, Embryology and Genetics, University of Zaragoza, Zaragoza, Spain*

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ABSTRACT

Despite the increasing industrial use of different nanomaterials, data on their genotoxicity are scant. In the present study, we examined the potential genotoxic effects of carbon nanotubes (CNTs; >50% single-walled, ~40% other CNTs; 1.1 nm × 0.5–100 μm; Sigma–Aldrich) and graphite nanofibres (GNFs; 95%; outer diameter 80–200 nm, inner diameter 30–50 nm, length 5–20 μm; Sigma–Aldrich) *in vitro*. Genotoxicity was assessed by the single cell gel electrophoresis (comet) assay and the micronucleus assay (cytokinesis-block method) in human bronchial epithelial BEAS 2B cells cultured for 24 h, 48 h, or 72 h with various doses (1–100 μg/cm^2, corresponding to 3.8–380 μg/ml) of the carbon nanomaterials. In the comet assay, CNTs induced a dose-dependent increase in DNA damage at all treatment times, with a statistically significant effect starting at the lowest dose tested. GNFs increased DNA damage at all doses in the 24-h treatment, at two doses (40 and 100 μg/cm^2) in the 48-h treatment (dose-dependent effect) and at four doses (lowest 10 μg/cm^2) in the 72-h treatment. In the micronucleus assay, no increase in micronucleated cells was observed with either of the nanomaterials after the 24-h treatment or with CNTs after the 72-h treatment. The 48-h treatment caused a significant increase in micronucleated cells at three doses (lowest 10 μg/cm^2) of CNTs and at two doses (5 and 10 μg/cm^2) of GNFs. The 72-h treatment with GNFs increased micronucleated cells at four doses (lowest 10 μg/cm^2). No dose-dependent effects were seen in the micronucleus assay. The presence of carbon nanomaterial on the microscopic slides disturbed the micronucleus analysis and made it impossible at levels higher than 20 μg/cm^2 of GNFs in the 24-h and 48-h treatments. In conclusion, our results suggest that both CNTs and GNFs are genotoxic in human bronchial epithelial BEAS 2B cells *in vitro*. This activity may be due to the fibrous nature of these carbon nanomaterials with a possible contribution by catalyst metals present in the materials—Co and Mo in CNTs (<5 wt.%) and Fe (<3 wt.%) in GNFs.

1. Introduction

Because of the growing industrial use of nanomaterials, there is an urgent need for information on their potential health effects. Due to the distinct physico-chemical properties of many nanomaterials, their possible toxicity may differ from that of the bulk material of similar chemical nature. As the lungs are the primary route of entry for inhaled nanoparticles, lung toxicity is of particular concern. It would appear especially important to identify nanomaterials that could act as lung carcinogens. Some fibrous nanomaterials with a high aspect ratio, such as carbon nanotubes (CNTs), might be able to induce lung cancer and mesothelioma in a similar manner as asbestos. CNTs seem to be very stable in biological systems and form aggregates in the micrometer size range (Lam et al., 2006), thus resembling asbestos. Because of their unique electrical, chemical, and thermal properties, CNTs and other carbon nanomaterials have wide applications in different industrial fields and are among the most important nanomaterials presently manufactured. Recently, low-iron (0.35%) multi-walled CNTs (MWCNTs) were observed to induce mesothelioma in *p53*+/− mice upon a single intraperitoneal injection, in a similar way as crocidolite asbestos (Takagi et al., 2008).

The carcinogenic effect of biopersistent fibres such as asbestos has been suggested to be linked with the local generation of reactive oxygen and nitrogen species and inflammatory reactions (Takagi et al., 2008); genotoxic effects associated with these phenomena or occurring independently probably also play a role. Studies conducted on rats and mice have indicated that MWCNTs and

* Corresponding author. Tel.: +358 30 4742336; fax: +358 30 4742110.
E-mail address: hannu.norppa@ttl.fi (H. Norppa).

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single-walled CNTs (SWCNTs) can induce oxidative stress, inflammation, fibrosis, and granulomas in the lungs (Lam et al., 2006, 2004; Li et al., 2007a,b; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004). Inflammatory responses have not, however, been seen in all *in vivo* studies with CNTs. In mice, the lung fibrosis induced by SWCNTs was associated with intercellular structures composed of nanotubes bridging lung macrophages (Mangum et al., 2006). Mice exposed to MWCNTs showed systemic immune function alterations (Mitchell et al., 2007).

Several *in vitro* studies have demonstrated the toxic potential of SWCNTs, as concerns oxidative stress in human epidermal HaCaT keratinocytes (Shvedova et al., 2003) and rat lung epithelial cells (Sharma et al., 2007), cytotoxicity in guinea pig alveolar macrophages (Jia et al., 2005), and G1 arrest and apoptosis in HEK293 human embryo kidney cells (Cui et al., 2005) at relatively low doses. Also MWCNTs were cytotoxic to guinea pig alveolar macrophages (Jia et al., 2005), induced apoptosis in T lymphocytes (Bottini et al., 2006), and altered protein expression in human epidermal keratinocytes (Witzmann and Monteiro-Riviere, 2006). However, studies indicating a low *in vitro* cytotoxicity of CNTs have also been published. SWCNTs showed only low toxicity against human monocyte-derived macrophages (Fiorito et al., 2006) and human A549 lung cells (Davoren et al., 2007) with no intracellular localization of SWCNTs seen in the latter cells. No acute toxic effects or inflammatory mediators were produced in rat NR8383 macrophages or A549 cells after treatment with commercial SWCNTs or MWCNTs, although the rat macrophages took up the materials (Pulskamp et al., 2007). In both NR8383 and A549 cells, there was a dose- and time-dependent production of intracellular oxygen species and decrease in mitochondrial membrane potential after treatment with the commercial SWCNTs and MWCNTs, which disappeared when the materials were acid-purified to remove metal catalysts (such as Co). Thus, the diverging findings might partly be explained by the catalyst metals in the CNTs used.

Fibre carcinogenesis is probably a complex process also involving a long-term genotoxic stress. In principle, fibrous carbon nanomaterial could be genotoxic via direct interaction with DNA or with the mitotic apparatus or indirectly via oxidative stress and inflammatory responses. Specific cationic functionalized CNTs are able to condense DNA. Nanotube surface area and charge density are considered to be critical in determining electrostatic complex formation with DNA (Singh et al., 2005). Positively charged polyelectrolyte coating of nanotubes functions as a counterpart for negatively charged DNA, so that CNTs can be wrapped with DNA to produce DNA sensors (He and Bayachou, 2005). SWCNTs complexes with double- and single-stranded DNA and peptide nucleic acid (Rajendra et al., 2004; Rajendra and Rodger, 2005; Zheng et al., 2003).

At present, only a few studies on the genotoxicity of fibrous carbon nanomaterials have been published. Mice exposed by intrapharyngeal instillation to SWCNTs (10 and 40 μg/mouse) developed aortic mtDNA damage accompanied by changes in aortic mitochondrial glutathione and protein carbonyl levels at 7, 28, and 60 days after the exposure. In ApoE(−/−) mice fed an atherogenic diet, accelerated plaque formation in the aortas was accompanied by increased mtDNA damage but not inflammation (Li et al., 2007a,b). A single intra-tracheal administration of MWCNTs increased the frequency of micronucleated type II pneumocytes in rat lungs *in vivo* in connection with a marked lung inflammatory response (Muller et al., 2008). Also *in vitro*, there was a significant increase of micronuclei in rat lung epithelial RLE cells and human MCF-7 epithelial cells treated with MWCNTs, with an induction of both centromere-positive and -negative micronuclei as judged by fluorescence *in situ* hybridization in MCF-7 cells (Muller et al., 2008). MWCNTs accumulated in cultured mouse embryonic stem (ES) cells and induced apoptosis, p53 activation, increased expression of DNA repair proteins, and a twofold increase in adenine phosphoribosyltransferase mutations (Zhu et al., 2007). SWCNTs produced no mutations in *Salmonella typhimurium* strains YG1024 or YG1029, but induced DNA damage (as measured by comet assay) in Chinese hamster V79 lung fibroblasts (Kisin et al., 2007); a non-significant increase in micronuclei was also seen. *Xenopus laevis* larvae grown in the presence of double-walled CNTs did not show an induction of micronuclei in blood erythrocytes, although gill toxicity and uptake of the test material to the organisms could be demonstrated (Mouchet et al., 2008).

The aim of the present study was to examine *in vitro* the potential genotoxicity of two commercially available fibrous carbon nanomaterials, CNTs (a mixture of SWCNTs and other CNTs) and graphite nanofibres (GNFs), in human bronchial epithelial cells (BEAS 2B). We applied the approach earlier proposed (Speit, 2002) for mutagenicity testing of fibres, utilizing the comet assay to assess DNA damage and the micronucleus assay to assess chromosomal damage.

2. Materials and methods

2.1. Nanomaterials and dispersions

The carbon nanomaterials examined in the present study were commercially available carbon nanotubes (product no. 636797; according to product sheets: >50% single-walled, ~40% other nanotubes; 1.1 nm × 0.5–100 μm) and graphite nanofibres (product no. 636398; according to product sheets: 95%; ~4% catalyst metals; outer diameter 80–200 nm, inner diameter 30–50 nm, length 5–20 μm) purchased from Sigma–Aldrich (Steinheim, Germany).

The size and morphology of the nanomaterials were characterized by using a transmission electron microscope (TEM; Jeol JEM 2010, Tokyo, Japan) and their composition and metal content by energy dispersive spectroscopy (EDS; ThermoNoran Vantage, Thermo Scientific, Breda, The Netherlands). Specific surface area was measured by adsorption, using the BET (Brunauer–Emmett–Teller) method (Coulter Omnisorp 100 CX, Florida, USA). The results of the nanomaterial characterization are summarized in Fig. 1. TEM analysis of dry CNTs and GNFs indicated that both materials were in the nano-scale, although agglomerated (Fig. 1A and B). As expected by product information, GNFs were clearly thicker than CNTs. The range of GNF outer diameter given by the manufacturer (80–200 nm) appeared to be an average range, as primary fibres with a diameter around and below 50 nm and above 200 nm were also seen in TEM (Fig. 1B). The composition of the materials was as expected, and EDS showed the presence of catalyst metals in both CNTs (<5 wt.% Co and Mo) and GNFs (<3 wt.% Fe). Specific surface was higher for CNTs (567 m^2/g) than for GNFs (32 m^2/g).

The materials were dispersed in BEGM cell culture medium (Clonetics, Walkerwille, MD) and subjected to ultrasonication (Elmasonic, Singen, Germany) for 20 min at 37 kHz prior to addition to the cell cultures. The nanomaterial dispersions were characterized by optical microscopy and TEM. 30 μl of each dispersion was placed on a microscopic glass slide and was covered with a cover slip. The samples were viewed using an optical microscope (Zeiss Axio Imager Z1, Jena, Germany) attached to a monochrome CCD camera (CVM-300, JAI Corporation, Yokohama, Japan) and Metasystems (Altlussheim, Germany) Isis Software. The images were quantified using a public domain image processing program (Image J Software, National Institute of Health, Maryland, USA), to determine mean particle sizes of the agglomerates. Image resolution (1 pixel) was 1.2 μm in the original sample. Thus, agglomerates smaller than 1 μm could not be distinguished in the optical images, and the average particle sizes obtained are valid only in the optical microscopic scale. As shown in Fig. 1(C and D), somewhat larger agglomerates in dispersion were seen with CNTs (average size 8.5 μm, S.D. 11.6) than with GNFs (3.8 μm, S.D. 4.8). Nanomaterial dispersions were also studied with TEM, and this analysis verified the presence of nano-scale agglomerates (<100 nm) in the BEGM dispersion in addition to previously determined μm-scale agglomerates (Fig. 1E and F).

2.2. Cell culture

BEAS 2B, a transformed human bronchial epithelial cell line exhibiting an epithelial phenotype (Reddel et al., 1988) was obtained from American Type Culture Collection through LGC Promochem AB (Borås, Sweden). The BEAS 2B cells were grown in serum-free BEGM medium (Clonetics, Walkerwille, MD, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Log-phase BEAS 2B cells were plated on 24-well plates (cell viability and comet assays), and 4-well chamber slides (micronucleus assay) (Nunc, Roskilde, Denmark) 2 days prior to exposure.

2.3. Cell viability

Twenty thousand cells in 0.5 ml of BEGM medium were plated on 24-well plates (culture area 1.9 cm^2/well; culture medium volume 0.5 ml/well) and grown to semi-confluency after which the cells were exposed to CNTs or GNFs for 24, 48 and 72 h at

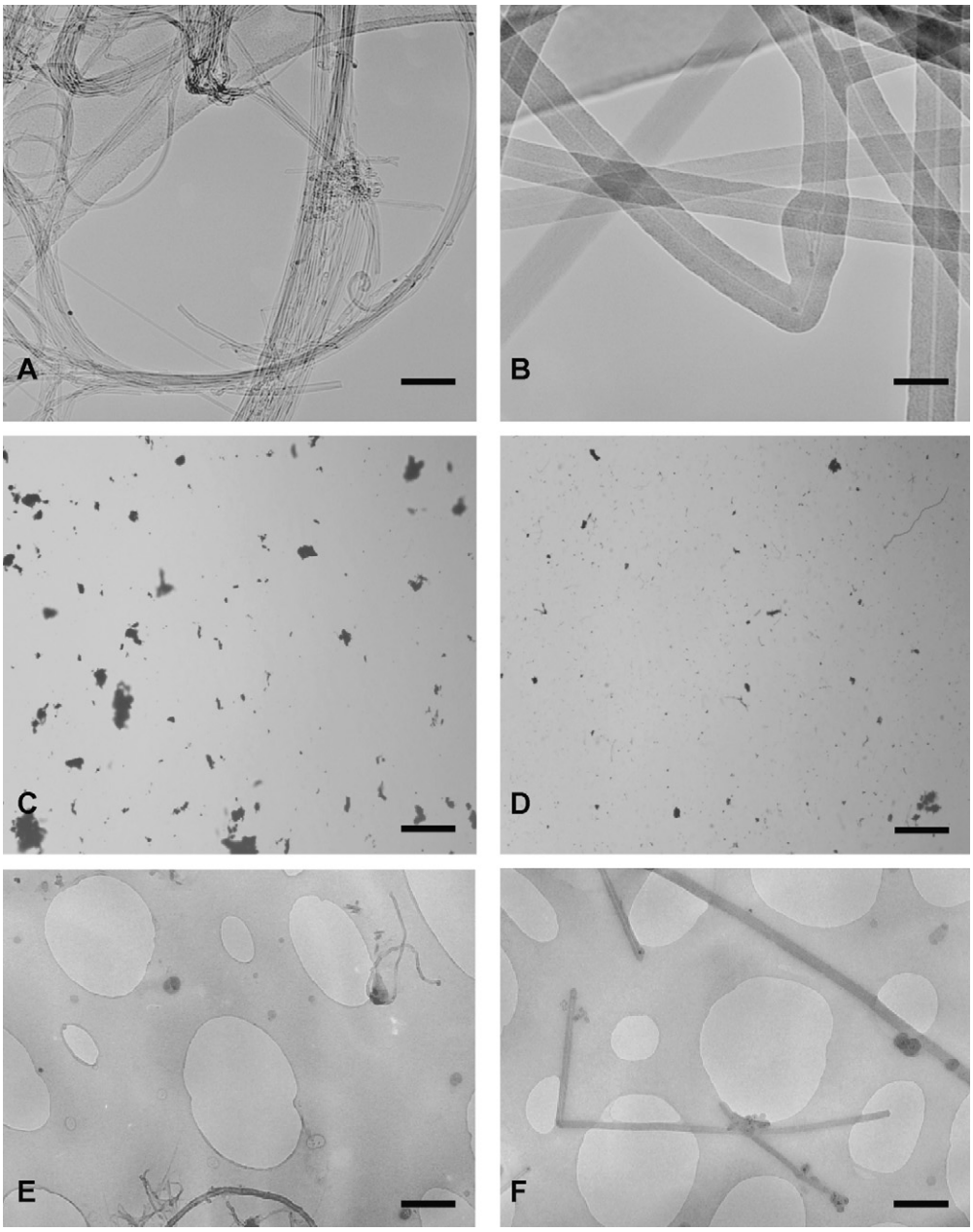

Fig. 1. Morphology of the carbon nanomaterials studied in the microscope. (A, C, and E) Carbon nanotubes (CNTs; >50% single-walled; ~40% other nanotubes; 1.1 nm × 0.5–100 μm; manufactured by chemical vapour deposition; Sigma–Aldrich product no. 636797) and (B, D, and F) graphite nanofibres (GNFs; 95%; ~4% catalyst metals; outer diameter 80–200 nm, inner diameter 30–50 nm, length 5–20 μm; production method not available; Sigma–Aldrich product no. 636) are shown by (A and B) transmission electron micrographs of dry nanopowders (measure bar is 50 nm), and (C and D) optical micrographs (measure bar is 100 μm) and (E and F) transmission electron micrographs (measure bar is 250 nm) of the carbon nanomaterials dispersed in BEGM medium and sonicated for 20 min. Measurements from light microscopic images indicated that the average size of the agglomerates in dispersion was 8.5 μm (S.D. 11.6) for CNTs and 3.8 μm (S.D. 4.8) for GNFs. Transmission electron microscopy verified that the BEGM dispersions also contained nano-scale (<100 nm) material in addition to the μm-scale agglomerates seen in the optical microscope. Specific surface area determined by the BET method was 567 m^2/g for CNTs (400 m^2/g by the vendor) and 32 m^2/g for GNFs (no information about specific surface area available from the vendor). According to energy dispersive spectroscopy, the catalyst metals were Co and Mo (<5 wt.%) in CNTs and Fe (<3 wt.%) in GNFs.

eight doses of each nanomaterial: 1, 5, 10, 20, 40, 60, 80, and 100 μg/cm^2 (cm^2 refers to chamber slide surface; in μg/ml culture medium, the corresponding doses were: 3.8, 19, 38, 76, 114, 228, 304, and 380 μg/ml). Untreated controls were included in all time points. After the nanomaterial treatment, the culture medium was replaced with fresh medium, and the cells were further cultured for 48 h to allow recovery. The cells were collected by trypsination (Trypsin-EDTA, Gibco, Paisley, UK) and thereafter resuspended in fresh BEGM medium. One scorer determined cell viability using the Trypan blue dye exclusion technique by counting the number of living (unstained) cells under phase-contrast microscopy.

2.4. Comet assay

The comet (single cell gel electrophoresis) assay was used to study DNA strand breaks and alkaline labile sites in BEAS 2B cells after the nanomaterial exposures. BEAS 2B cells in log-phase were plated in 24-well plates (culture area 1.9 cm^2/well, culture medium volume 0.5 ml/well; Nunc, Roskilde, Denmark) 2 days prior to exposure. Semiconfluent cultures were exposed for 24 h, 48 h or 72 h to eight doses of each nanomaterial: 1, 5, 10, 20, 40, 60, 80, and 100 μg/cm^2 (in μg/ml culture medium, the corresponding doses were: 3.8, 19, 38, 76, 114, 228, 304, and 380 μg/ml). The doses were chosen according to the viability assay. Untreated controls and positive controls treated with hydrogen peroxide (20 mM, Riedel-de Haen, Seelze, Germany) were included in all series.

The comet assay was performed in alkaline conditions (pH > 13) as described previously (Nygren et al., 2004). Briefly, after the exposure the cells were trypsinised and centrifuged at 1100 rpm for 5 min. 1–3 × 10^4 cells were resuspended in 75 μl molten (37 °C) 0.5% low-melting-point agarose (LMPA; Bio-Rad Laboratories, Hercules, CA). The resuspended cells in agarose were put onto dry microscope slides (Assistant, Sondhiem/Röhn, Germany) pre-coated with 1% normal-melting agarose (Interna-

tional Biotechnologies, New Haven, CT, USA), and the agar was allowed to solidify for 10 min. The slides were thereafter immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100) for at least 1 h at 4 °C, after which they were transferred to an electrophoresis tank containing freshly made electrophoresis buffer (1 mM EDTA, 300 mM NaOH; pH > 13), where they were kept for 20 min at room temperature to allow DNA unwinding. Electrophoresis was performed in the same buffer at room temperature for 15 min at 24 V and 300 mA (0.8 V/cm). The slides were then neutralized three times with 0.4 M Tris buffer (pH 7.5), air-dried, and fixed in methanol. DNA was stained with ethidium bromide (2 μg/ml) in water for 5 min.

The slides were coded, and one scorer performed the comet analysis using a fluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) and an interactive automated comet counter (Komet 4.0, Kinetic Imaging Ltd., Liverpool, UK). The percentage of DNA in the comet tail from 100 cells per sample (two replicates, each with 50 cells/slide) was used as a measure of the amount of DNA damage.

2.5. Micronucleus assay

The micronucleus assay was applied to study chromosomal damage in BEAS 2B cells after exposure to the carbon nanomaterials. The cells were plated on 4-well chamber slides (culture area 1.8 cm^2/well, culture medium volume 0.5 ml/well; Nalge Nunc International, Rochester, USA) at a density of 20,000 cells per well and incubated for 48 h prior to the treatment.

Based on the viability assay, the cells were exposed to eight doses of each nanomaterial type: 1, 5, 10, 20, 40, 60, 80, and 100 μg/cm^2 (in μg/ml culture medium, the corresponding doses were: 3.6, 18, 36, 72, 144, 216, 288, and 360 μg/ml). Treatment time was 24 h, 48 h or 72 h. Cytochalasin B (9 μg/ml; Sigma–Aldrich Chemie, Steinheim, Germany) was added to the cell cultures simultaneously with the particles to induce binucleation of dividing cells. Untreated controls and positive controls treated with mitomycin C (Sigma–Aldrich, Steinheim, Germany; 500 ng/ml) were also included for the 24-h and 48-h time points.

After the end of the exposure, the medium and chambers were removed from the slides, and the cells were briefly rinsed in phosphate-buffered saline (PBS). The cells were stained with ethidium bromide (10 μg/ml) and acridine orange (3 μg/ml) for 5 min after which the slides were rinsed twice in PBS and fixed in absolute methanol for 5 min. Finally, the DNA of the cells was stained with 4′,6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml) for 5 min, after which the slides were rinsed in tap water and allowed to dry. The stained and fixed slides were kept at 4 °C and were protected from light until analysis.

The slides were coded and the frequency of micronucleated cells in 2000 binucleate cells (1000 cells/scorer) per culture were scored by two microscopists using an Axioplan 2E Universal microscope (Zeiss, Jena, Germany). Binucleate cells were identified using a 63× objective lens together with a DAPI filter and a green/red (FITC/TRITC) double filter. Micronuclei in the cells were verified by a 100× objective lens using DAPI, and FITC/TRITC double filters.

Cytokinesis-block proliferation index (CBPI) was calculated from 100 cells per sample (Surrallés et al., 1995) as follows: CBPI = [(no. mononucleate cells) + 2(no. binucleate cells) + 3(no. multinucleate cells)]/(total no. cells).

2.6. Statistics

The *t*-test (2-tailed) was applied to examine if the percentage of DNA in tail after the *in vitro* exposure was statistically significantly different from the result obtained for the control cultures. Fisher's exact test (2-tailed) was used to determine whether the treatment induced a statistically significant increase in the frequency of micronucleated binucleate cells as compared with the untreated control cultures. The difference in the level of DNA damage (comet assay) or micronucleated cells was interpreted to be significant if the *P*-value was <0.05. Linear regression analysis was applied to examine if a dose–response could be observed; the significance of the slope was assessed using the *t*-test (2-tailed). One-way analysis of variance was applied to examine if the CBPIs were statistically significantly different in treated cultures as compared with the control cultures.

3. Results

3.1. Cell viability

Treatment of the BEAS 2B cells with CNTs (Fig. 2A) and GNFs (Fig. 2B) decreased cell viability in a dose-dependent manner after all the exposure times used (24, 48, and 72 h) when the cells were allowed to recover for 48 h post treatment. In general, the number of viable cells decreased with the incubation time in all series, including treatments with CNTs and GNFs as well as the control cultures (Fig. 2A and B). Both carbon nanomaterials showed similar toxicity, with the number of viable cells dropping to 50% of the respective control value at about 10–40 μg/cm^2 in the 24-h treatment, and 40–60 μg/cm^2 in the 48-h and 72-h treatment.

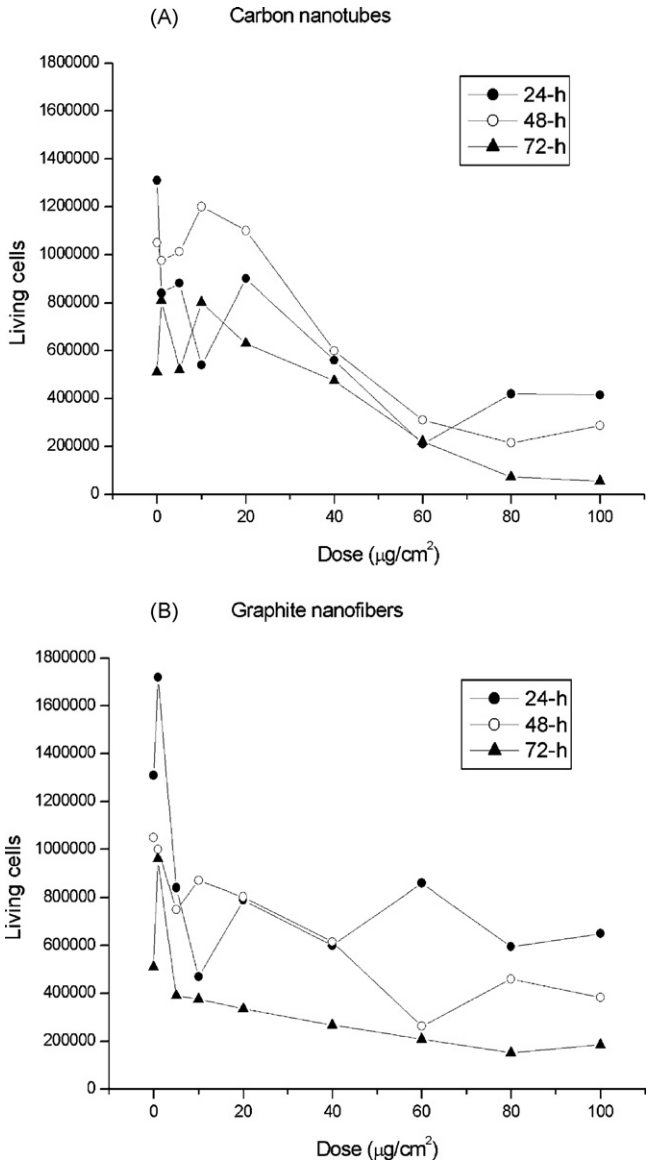

Fig. 2. Cell viability (number of living cells) in cultured human bronchial epithelial BEAS 2B cells after 24-h, 48-h, and 72-h exposure to carbon nanotubes (A) and graphite nanofibres (B). The cell viability was evaluated with the Trypan blue dye exclusion assay. The symbols show mean numbers of living cells in triplicate cultures.

3.2. DNA damage

CNTs induced a dose-dependent increase in DNA damage ($r = 0.73$, $P = 0.03$) after the 24-h treatment, and a significant effect was seen at 1, 60, 80, and 100 μg/cm^2 ($P = 0.003$, $P = 0.004$, $P < 0.001$, $P < 0.001$, respectively; *t*-test, 2-tailed). After the 48-h and 72-h treatments with CNTs, the effect was also very clear; a statistically significant increase in DNA damage was observed at all tested doses, with a significant dependence on dose ($r = 0.87$, $P = 0.003$; $r = 0.80$, $P = 0.01$, respectively) (Fig. 3).

A significant increase in DNA damage ($P < 0.05$; *t*-test, 2-tailed) was seen at all tested doses after the 24-h treatment with GNFs, but there was no clear dependence on dose. After the 48-h treatment with GNFs, the level of DNA damage significantly increased at 40 and 100 μg/cm^2 ($P = 0.01$ and $P = 0.001$, respectively; *t*-test, 2-tailed), and the effect was dose-dependent ($r = 0.88$, $P = 0.002$). The 72-h treatment with GNFs induced a significant increase in DNA damage at 10, 20, 60, and 100 μg/cm^2 ($P = 0.003$, $P = 0.004$, $P < 0.001$,

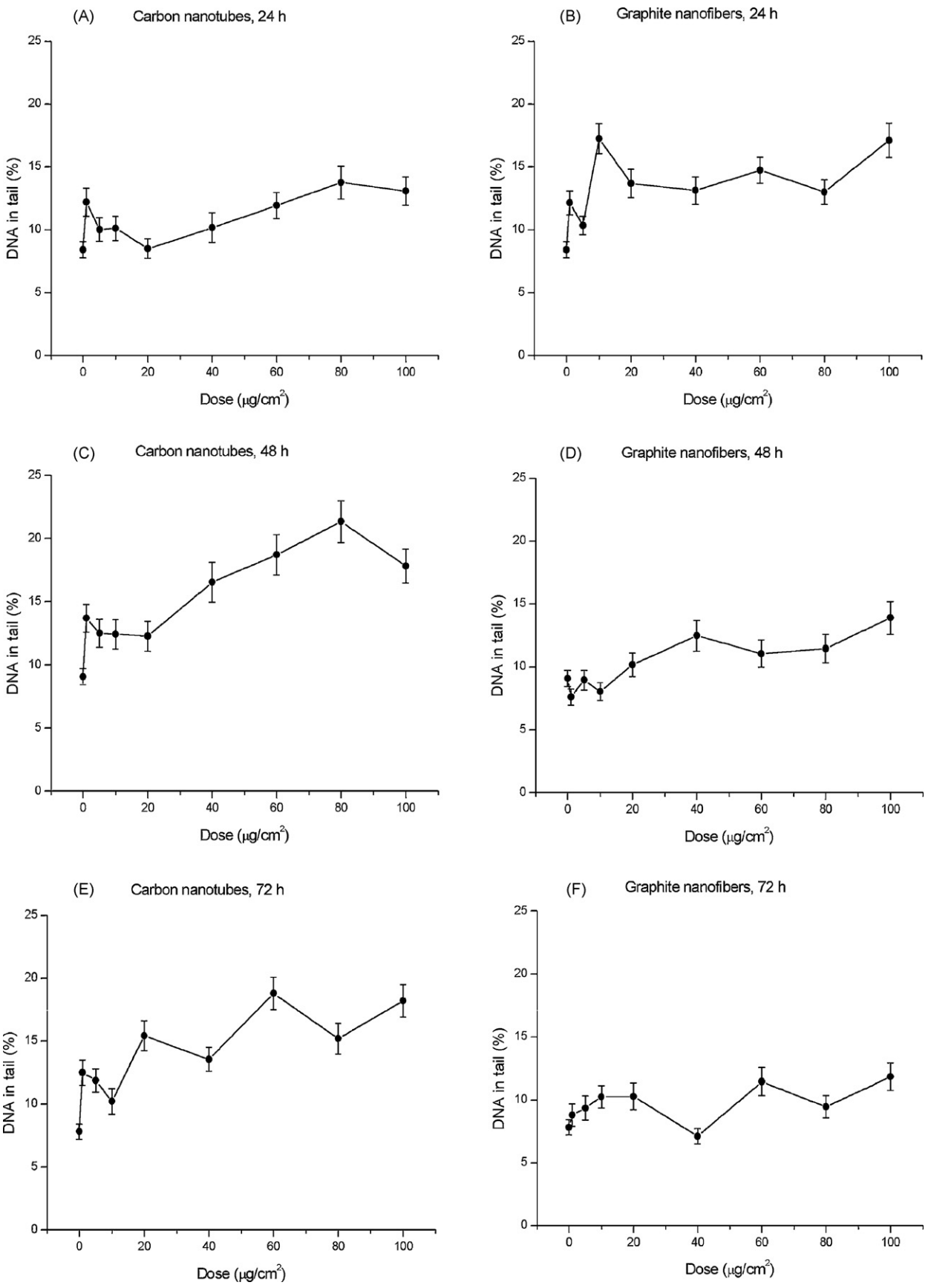

Fig. 3. DNA damage as measured by the comet assay (percentage of DNA in tail) in cultured human bronchial epithelial BEAS 2B cells after exposure to carbon nanotubes (A, C, and E) and graphite nanofibres (B, D, and F) for 24-h (A and B), 48-h (C and D), and 72-h (E and F). The symbols show means (±S.E.) of duplicate cultures. Hydrogen peroxide (20 mM), which was used as a positive control, showed a clear increase in tail percentage (24-h exposure 40%; 48-h exposure 35.8%; 72-h exposure 30.8%).

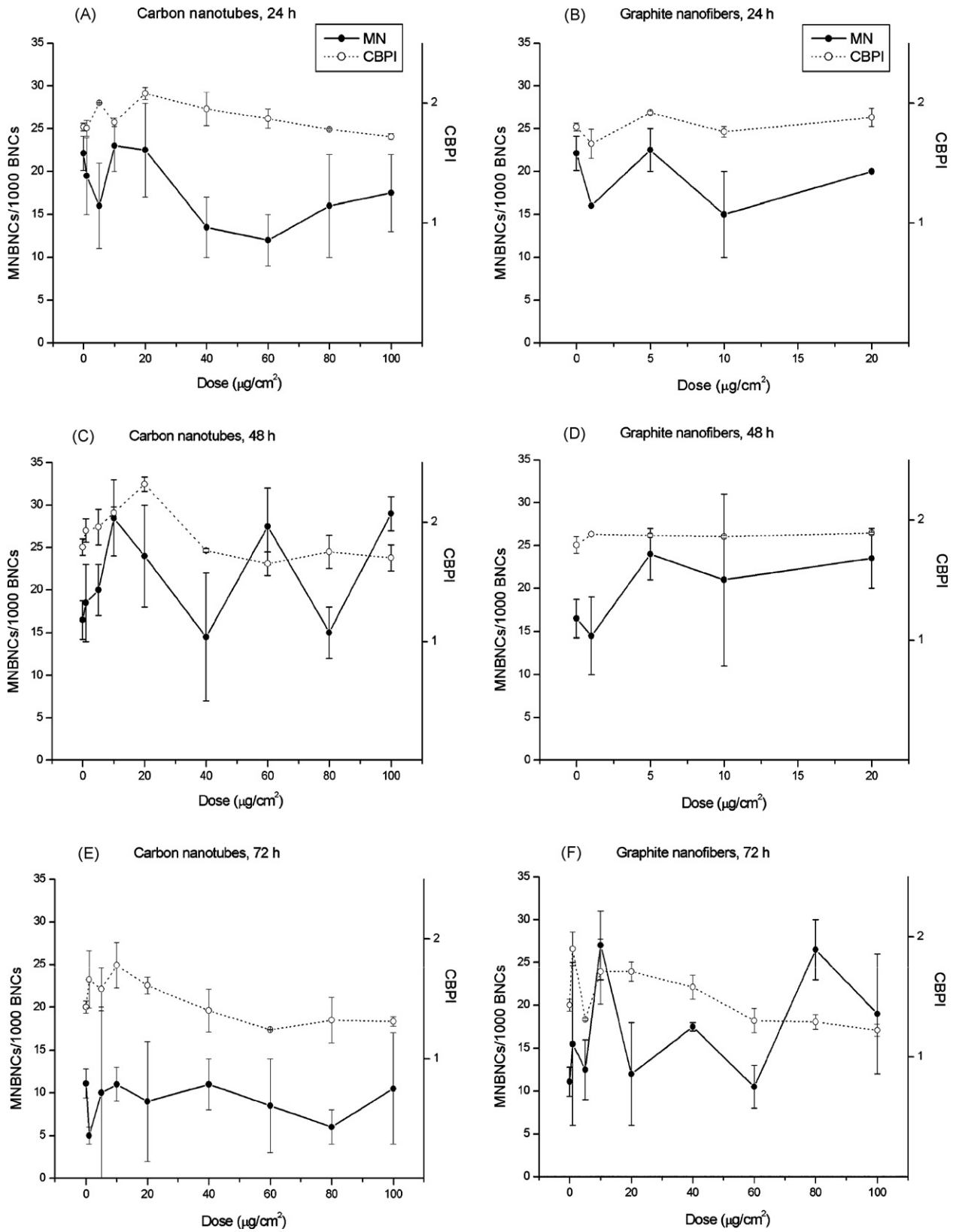

Fig. 4. Frequency (mean (±S.E.) of duplicate cultures) of micronucleated binucleate cells (MNBNCs) per 1000 binucleate cells (BNCs) and cytokinesis-block proliferation index (CBPI) in cultured human bronchial epithelial BEAS 2B cells after exposure to carbon nanotubes (A, C, and E) and graphite nanofibres (B, D, and F) for 24-h (A and B), 48-h (C and D), and 72-h (E and F). Mitomycin C (500 ng/ml), used as a positive control, clearly increased the frequency of MNBNCs (24-h exposure 79.5 MNBNCs/1000 BNCs; 48-h exposure 70.2 MNBNCs/1000 BNCs).

$P < 0.001$, respectively; *t*-test, 2-tailed), but no dose-dependency was seen (Fig. 3).

3.3. Micronuclei

CNTs did not increase the frequency of micronucleated cells at any of the tested doses after the 24-h or 72-h treatments. In fact, the frequency of micronucleated cells was significantly lower as compared with the controls at two doses after the 24-h treatment (40 and 60 μg/cm^2) and the 72-h treatment (1 and 80 μg/cm^2). The variation in micronucleus frequencies appeared to be due to the presence of CNTs on the microscopic slides, which disturbed the analysis. However, the 48-h treatment induced a significant increase in micronucleated cells at three different doses, 10, 60, and 100 μg/cm^2 ($P < 0.001$, $P = 0.002$, $P < 0.001$, respectively; Fisher's exact test, 2-tailed), but a clear dose-dependency was not seen. CBPI differed significantly as compared with the controls at two doses after the 24-h (5 and 20 μg/cm^2, $P < 0.05$; one-way ANOVA) and 48-h (10 and 20 μg/cm^2, $P < 0.05$) CNT treatments, and at one dose (10 μg/cm^2, $P < 0.05$) after the 72-h treatment (Fig. 4).

Micronucleus analysis turned out to be still more difficult to perform after the GNF treatment, since the test material covered a large part of the slides. For this reason, 20 μg/cm^2 was the highest dose that could be evaluated after the 24-h and 48-h treatments. The 24-h treatment did not induce micronuclei, but the 48-h treatment caused a significant increase in micronucleated cells at two of the tested doses, 5 and 20 μg/cm^2 ($P = 0.03$ and $P = 0.04$, respectively; Fisher's exact test, 2-tailed). The clearest effect was seen in the 72-h treatment, where a significant increase in the number of micronucleated cells was observed at four different doses, 10, 40, 80, and 100 μg/cm^2 ($P < 0.001$, $P = 0.03$, $P < 0.001$ and $P = 0.007$, respectively; Fisher's exact test, 2-tailed). Yet, no significant dose–response was observed. The 24-h and 48-h GNF treatments did not affect CBPIs as compared with the controls, but after the 72-h treatment CBPI differed significantly from the control at 1 μg/cm^2 ($P < 0.05$; one-way ANOVA) (Fig. 4).

As there were several statistically significant data points for both CNTs and GNFs, the results of the micronucleus assay were considered positive for both materials, despite the lacking dose–responses.

4. Discussion

Both of the carbon nanomaterials examined were found to be genotoxic in human bronchial epithelial BEAS 2B cells, as measured by the alkaline comet assay and the micronucleus assay. Dose-dependent increases in DNA damage were seen in the comet assay with both materials, and the effect was more marked at the longer treatment times. Also in the micronucleus assay, the longer treatment times resulted in a clearer effect than the 24-h treatment, but no obvious dose-dependent effects were seen at any treatment time. In both tests, positive results were obtained already at doses that were not toxic to the cells. The lowest doses giving a positive effect were 1 μg/cm^2 (3.8 μg/ml; the lowest dose studied) for both carbon nanomaterials in the comet assay and 10 μg/cm^2 (36 μg/ml) for CNTs and 5 μg/cm^2 (18 μg/ml) for GNFs in the micronucleus assay.

At increasing doses of both carbon nanomaterials, and especially GNFs, the microscopic analysis of micronuclei became difficult due to the agglomerated nanomaterial that partly covered the cells. In the case of GNFs, doses higher than 20 μg/cm^2 were unreadable at the two shorter treatment times. This is a limitation of the present micronucleus assay based on the use of chamber slides where insoluble test materials tend to stay.

In general, test substance insolubility and agglomeration are important technical issues in the *in vitro* testing of this type of nanomaterials. In the dispersions examined, the CNTs and GNFs were mostly as rather large agglomerates, and the dispersions were not uniform. Naturally, these materials form agglomerates and remain insoluble also in inhalation exposure of the lungs. Thus, much of the exposure of the tissue always occurs to larger agglomerates also *in vivo*.

Our results agree with the two studies published previously on the effect of CNTs in the comet and micronucleus assays *in vitro*, although the CNTs material tested and the cell lines used were different. Kisin et al. (2007) observed that SWCNTs induce DNA damage starting at 48 μg/cm^2 in a 24-h treatment (a dose–response) and at 96 μg/cm^2 in a 3-h treatment of Chinese hamster V79 lung fibroblasts; also their micronucleus results appeared similar to ours, with no significant effect in the 24-h treatment (longest treatment time applied), although we could see an effect with longer treatment times. Muller et al. (2008) demonstrated micronucleus induction in rat lung RLE epithelial cells after 24-h exposure to MWCNT at 10–50 μg/ml and in human epithelial MCF-7 cells after 48-h exposure at 10 μg/ml, while our micronucleus analysis was positive starting from 36 μg/ml (10 μg/cm^2) for CNTs and from 19 μg/ml (5 μg/cm^2) for GNFs. The genotoxicity of the CNTs was not restricted to cultured cells, since an increase was seen in DNA damage in aortic mitochondria after a single intrapharyngeal instillation of SWCNTs to mice (Li et al., 2007a,b) and in micronucleated type II pneumocytes after a single intratracheal administration of MWCNTs in rats (Muller et al., 2008). Therefore, CNT genotoxicity does not appear to be an *in vitro* artefact.

The lack of a dose-dependent effect in our micronucleus assays with CNTs and GNFs might be explained by the increasing size of agglomerates at higher dose levels. Larger agglomerates may be taken up less efficiently by the cells than smaller ones, and few larger agglomerates may have less effect on the cells than numerous smaller agglomerates. Furthermore, uptake of the particulate material into the epithelial cells may not depend on the dose of the material in the medium, if the medium contains enough of the material. The fact that a dose–response was seen with the comet assay but not with the micronucleus assay may reflect differences between the endpoints, better sensitivity of the comet assay, and the technical limitations of the micronucleus assay.

In a biological system, the absorption, distribution, metabolism, and toxicity of CNTs depend on the inherent physical and chemical characteristics such as functionalization, coating, length, and agglomeration state which is influenced by external environmental conditions (Helland et al., 2007). Functionalized water-soluble SWCNTs conjugated with a peptide responsible for G protein function were reported to enter the nucleus of human 3T6 fibroblasts and keratinocytes and murine 3T3 cells (Pantarotto et al., 2004); fluorescein isothiocyanate (FITC)-conjugated functionalized water-soluble SWCNTs were able to enter the cells in less than an hour. The mechanisms for the uptake were unknown.

For the insoluble carbon nanomaterials examined in the present study, it is not known, how well they are taken up by the BEAS 2B cells and if they are able to pass the nuclear envelope. The particle size of these materials is highly diverse due to aggregation and agglomeration. It may be assumed that the smallest particles can enter the cells. Larger particles may be taken up only partly, which may induce oxidative stress. The nanomaterials may also affect the cells mechanically. To be capable of direct interaction with DNA, the nanomaterials should be able to reach the nucleus of interphase cells. Nano-sized particles free in the cytoplasm may get access to the nucleus in cell division, when the nuclear envelope disappears. When the nuclear envelope is reformed after mitosis, some particles may stay inside the nucleus where they have the possibility to interact with DNA. The chemical and physical characteristics of the nanomaterial determine, if it can have genotoxic effects, directly or indirectly. Nanomaterials, especially long fibrous materials such as

the CNTs and GNFs examined in the present study may also interfere with cellular functions, e.g., the mitotic apparatus and chromosome segregation physically. Previous studies in human epithelial MCF-7 cells suggested that MWCNTs can induce micronuclei by both clastogenic and aneugenic mechanisms, but it is not known if this occurs via direct or indirect action (Muller et al., 2008).

Transition metals, used as catalysts in the manufacture of carbon nanomaterials, may contribute to the observed genotoxicity. In the present study, the EDS analysis of metal impurity indicated that the CNTs contained Co and Mo (<5 wt.%) while the GNFs contained Fe (<3 wt.%). Muller et al. (2008) recently suggested that metals may not play an important role in the genotoxicity of MWCNTs, since the MWCNTs (~0.5% Fe, 1% Co) did not generate reactive oxygen radicals in an acellular system, but showed, on the contrary, radical scavenging capacity (Fenoglio et al., 2006). On the other hand, Co was reported to induce chromosome loss at doses that were compatible with the Co content of the MWCNT tested (Muller et al., 2008). At present the possible influence of metal catalysts on the genotoxicity of carbon nanomaterials is unclear.

In conclusion, our results show that both CNTs and GNFs are genotoxic to human bronchial epithelial BEAS 2B cells *in vitro* at doses that are not overly cytotoxic. This activity may be due to the fibrous nature of these materials with a possibly contribution by catalyst metals (Co, Mo, and Fe) present in the carbon nanomaterials.

Conflict of interest

None.

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References

Bottini, M., Bruckner, S., Nika, K., Bottini, N., Bellucci, S., Magrini, A., Bergamaschi, A., Mustelin, T., 2006. Multi-walled carbon nanotubes induce T lymphocyte apoptosis. Toxicol. Lett. 160 (2), 121–126.

Cui, D., Tian, F., Ozkan, C.S., Wang, M., Gao, H., 2005. Effect of single wall carbon nanotubes on human HEK293 cells. Toxicol. Lett. 155 (1), 73–85.

Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H.J., Lyng, F.M., 2007. In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. Toxicol. In Vitro 21 (3), 438–448.

Fenoglio, I., Tomatis, M., Lison, D., Muller, J., Fonseca, A., Nagy, J.B., Fubini, B., 2006. Reactivity of carbon nanotubes: free radical generation or scavenging activity? Free Radic. Biol. Med. 40 (7), 1227–1233.

Fiorito, S., Serafino, A., Andreola, F., Bernier, P., 2006. Effects of fullerenes and single-wall carbon nanotubes on murine and human macrophages. Carbon 44, 1100–1105.

He, P., Bayachou, M., 2005. Layer-by-layer fabrication and characterization of DNA-wrapped single-walled carbon nanotube particles. Langmuir 21 (13), 6086–6092.

Helland, A., Wick, P., Koehler, A., Schmid, K., Som, C., 2007. Reviewing the environmental and human health knowledge base of carbon nanotubes. Environ. Health Perspect. 115 (8), 1125–1131.

Jia, G., Wang, H., Yan, L., Wang, X., Pei, R., Yan, T., Zhao, Y., Guo, X., 2005. Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. Environ. Sci. Technol. 39 (5), 1378–1383.

Kisin, E.R., Murray, A.R., Keane, M.J., Shi, X.C., Schwegler-Berry, D., Gorelik, O., Arepalli, S., Castranova, V., Wallace, W.E., Kagan, V.E., Shvedova, A.A., 2007. Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells. J. Toxicol. Environ. Health A 70 (24), 2071–2079.

Lam, C.W., James, J.T., McCluskey, R., Arepalli, S., Hunter, R.L., 2006. A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Crit. Rev. Toxicol. 36 (3), 189–217.

Lam, C.W., James, J.T., McCluskey, R., Hunter, R.L., 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci. 77 (1), 126–134.

Li, J.G., Li, W.X., Xu, J.Y., Cai, X.Q., Liu, R.L., Li, Y.J., Zhao, Q.F., Li, Q.N., 2007a. Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. Environ. Toxicol. 22 (4), 415–421.

Li, Z., Hulderman, T., Salmen, R., Chapman, R., Leonard, S.S., Young, S.H., Shvedova, A., Luster, M.I., Simeonova, P.P., 2007b. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. Environ. Health Perspect. 115 (3), 377–382.

Mangum, J.B., Turpin, E.A., Antao-Menezes, A., Cesta, M.F., Bermudez, E., Bonner, J.C., 2006. Single-walled carbon nanotube (SWCNT)-induced interstitial fibrosis in the lungs of rats is associated with increased levels of PDGF mRNA and the formation of unique intercellular carbon structures that bridge alveolar macrophages in situ. Part. Fibre Toxicol. 3, 15.

Mitchell, L.A., Gao, J., Wal, R.V., Gigliotti, A., Burchiel, S.W., McDonald, J.D., 2007. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. Toxicol. Sci. 100 (1), 203–214.

Mouchet, F., Landois, P., Sarremejean, E., Bernard, G., Puech, P., Pinelli, E., Flahaut, E., Gauthier, L., 2008. Characterisation and in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. Aquat. Toxicol. 87 (2), 127–137.

Muller, J., Decordier, I., Hoet, P., Lombaert, N., Thomassen, L., Huaux, F., Lison, D., Kirsch-Volders, M., 2008. Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29 (2), 427–433.

Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J.F., Delos, M., Arras, M., Fonseca, A., Nagy, J.B., Lison, D., 2005. Respiratory toxicity of multi-wall carbon nanotubes. Toxicol. Appl. Pharmacol. 207 (3), 221–231.

Nygren, J., Suhonen, S., Norppa, H., Linnainmaa, K., 2004. DNA damage in bronchial epithelial and mesothelial cells with and without associated crocidolite asbestos fibers. Environ. Mol. Mutagen. 44 (5), 477–482.

Pantarotto, D., Briand, J.P., Prato, M., Bianco, A., 2004. Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem. Commun. (Camb) 1, 16–17.

Pulskamp, K., Diabate, S., Krug, H.F., 2007. Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol. Lett. 168 (1), 58–74.

Rajendra, J., Baxendale, M., Dit Rap, L.G., Rodger, A., 2004. Flow linear dichroism to probe binding of aromatic molecules and DNA to single-walled carbon nanotubes. J. Am. Chem. Soc. 126 (36), 11182–11188.

Rajendra, J., Rodger, A., 2005. The binding of single-stranded DNA and PNA to single-walled carbon nanotubes probed by flow linear dichroism. Chemistry 11 (16), 4841–4847.

Reddel, R.R., Ke, Y., Gerwin, B.I., McMenamin, M.G., Lechner, J.F., Su, R.T., Brash, D.E., Park, J.B., Rhim, J.S., Harris, C.C., 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. 48 (7), 1904–1909.

Sharma, C.S., Sarkar, S., Periyakaruppan, A., Barr, J., Wise, K., Thomas, R., Wilson, B.L., Ramesh, G.T., 2007. Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. J. Nanosci. Nanotechnol. 7 (7), 2466–2472.

Shvedova, A.A., Castranova, V., Kisin, E.R., Schwegler-Berry, D., Murray, A.R., Gandelsman, V.Z., Maynard, A., Baron, P., 2003. Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. J. Toxicol. Environ. Health A 66 (20), 1909–1926.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V., Baron, P., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 289 (5), L698–L708.

Singh, R., Pantarotto, D., McCarthy, D., Chaloin, O., Hoebeke, J., Partidos, C.D., Briand, J.P., Prato, M., Bianco, A., Kostarelos, K., 2005. Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors. J. Am. Chem. Soc. 127 (12), 4388–4396.

Speit, G., 2002. Appropriate in vitro test conditions for genotoxicity testing of fibers. Inhal. Toxicol. 14 (1), 79–90.

Surrallés, J., Xamena, N., Creus, A., Catalán, J., Norppa, H., Marcos, R., 1995. Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. Mutat. Res. 341, 169–184.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S., Kanno, J., 2008. Induction of mesothelioma in p53+/− mouse by intraperitoneal application of multi-wall carbon nanotube. J. Toxicol. Sci. 33 (1), 105–116.

Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A., Webb, T.R., 2004. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol. Sci. 77 (1), 117–125.

Witzmann, F.A., Monteiro-Riviere, N.A., 2006. Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. Nanomedicine 2 (3), 158–168.

Zheng, M., Jagota, A., Semke, E.D., Diner, B.A., McLean, R.S., Lustig, S.R., Richardson, R.E., Tassi, N.G., 2003. DNA-assisted dispersion and separation of carbon nanotubes. Nat. Mater. 2 (5), 338–342.

Zhu, L., Chang, D.W., Dai, L., Hong, Y., 2007. DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. Nano Lett. 7 (12), 3592–3597.