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

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Abstract
Carbon nanotubes (CNT) have been reported to induce lung inflammation and fibrosis in rodents. We investigated the direct and indirect cellular mechanisms mediating the fibrogenic activity of multi-wall (MW) CNT on fibroblasts. We showed that MWCNT indirectly stimulate lung fibroblast (MLg) differentiation, via epithelial cells and macrophages, whereas no direct effect of MWCNT on fibroblast differentiation or collagen production was detected. MWCNT directly stimulated the proliferation of fibroblasts primed with low concentrations of growth factors, such as PDGF, TGF-β or EGF. MWCNT prolonged ERK 1/2 phosphorylation induced by low concentrations of PDGF or TGF-β in fibroblasts. This phenomenon and the proliferative activity of MWCNT on fibroblasts was abrogated by the inhibitors of ERK 1/2, PDGF- and TGF-β-receptors. This activity was also reduced by amiloride, an endocytosis inhibitor. Finally, the lung fibrotic response to several MWCNT samples (different in length and diameter) correlated with their in vitro capacity to stimulate the proliferation of fibroblasts and to prolong ERK 1/2 signaling in these cells. Our findings point to a crosstalk between MWCNT, kinase receptors, ERK 1/2 signaling and endocytosis which stimulates the proliferation of fibroblasts. The mechanisms of action identified in this study contribute to predict the fibrogenic potential of MWCNT.

Introduction
Carbon nanotubes (CNT) are widely developed for various industrial and biomedical applications due to their unique mechanical, electrical and thermal properties (De Volder et al., 2013). However, the manipulation of these materials and their release into the environment may lead to human exposure, especially through inhalation. Many experimental studies showed that CNT can induce lung fibrosis in rodents (Lam et al., 2004; Muller et al., 2008; Shvedova et al., 2005), but the mechanisms involved are not completely elucidated. Several cell types may contribute to induce pulmonary fibrosis in response to inhaled particles. While alveolar macrophages and epithelial cells are in the short line to defend the lung by secreting pro-inflammatory and fibrogenic mediators, fibroblasts are the final performers in the establishment of pulmonary fibrosis. Excessive proliferation of fibroblasts, their differentiation in myofibroblasts and the exaggerated production and deposition of extracellular matrix (ECM) proteins, including collagen, are the key features of pulmonary fibrosis. Platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β play a critical role in this fibrotic process, since they stimulate fibroblast recruitment, proliferation and activation (Wynn, 2011). The fibrogenic activity of inhaled particles (e.g. crystalline silica, asbestos fibers) is usually ascribed to an indirect effect on fibroblasts mediated by macrophages and/or epithelial cells (Mossman & Churg, 1998). Recent in vitro studies showed that multi-wall (MW) CNT are also able to activate the secretion of TGF-β and PDGF by macrophages and epithelial cells (Li et al., 2013; Wang et al., 2011, 2012, 2013). He et al. (2011) observed that MWCNT stimulated the production of TGF-β and PDGF by macrophages that acted as paracrine signals to stimulate fibroblast differentiation. Increased levels of TGF-β and PDGF were also reported in bronchoalveolar lavage fluid of mice instilled with CNT (Vietti et al., 2013; Wang et al., 2011).
Because inhaled CNT can distribute in distal areas, such as the alveolar interstitial space (Mercer et al., 2010), it has been suggested that they can also directly interact with fibroblasts and new studies were carried out to understand the direct molecular interactions between CNT and fibroblasts. Several in vitro studies showed an increased fibroblast proliferation in response to multiwall (MW), double-wall (DW) and single-wall (SW) CNT exposure (Azad et al., 2013; Vietti et al., 2013; Wang et al., 2010a, 2010b), suggesting that a direct stimulation of fibroblasts by CNT could contribute to exacerbate or even initiate the development of lung fibrosis. Only few studies provided information on the mechanism of interaction between CNT and fibroblasts. Azad et al. (2013) reported that SWCNT induced reactive oxygen species-regulated phosphorylation of p38 mitogen-activated protein kinase (MAPK), leading to TGF-β and vascular endothelial growth factor (VEGF) secretion by fibroblasts that induced their proliferation and collagen production.
Recent studies have also suggested an interaction between CNT and membrane receptors (Hirano et al., 2008; Yan, 2014). However, it is still unclear which cellular mechanisms and receptors are involved in the proliferative activity of CNT on fibroblasts.

Furthermore, data indicate that the physicochemical properties of CNT determine the severity of their lung toxicity. Diameter, length, impurities and defects have been reported to influence the fibrogenic activity of CNT (Fenoglio et al., 2012; Liu et al., 2012; Muller et al., 2008; Palomaki et al., 2011). Our previous study suggested that the proliferative activity of MWCNT on fibroblasts was predictive of their lung fibrotic potential (Vietti et al., 2013), as demonstrated with several MWCNT samples.

The aim of this study was to identify the direct and indirect mechanisms mediating the fibrogenic activities of MWCNT on fibroblasts. We first confirmed that macrophages and epithelial cells exposed to MWCNT-stimulated fibroblast differentiation. Secondly, we observed that, in the presence of low concentrations of growth factors (PDGF, TGF-\(\beta\)), MWCNT directly in contact with fibroblasts stimulated their proliferation. Kinase receptors for these growth factors, ERK 1/2 signaling pathway and MWCNT endocytosis were essential to this proliferative activity. Finally, we strengthened the association between CNT capacity to induce lung fibrosis and their \textit{in vitro} fibroblast proliferative activity with MWCNT samples of varying length and diameter.

**Methods**

**MWCNT**

NM400 and NM402 MWCNT were obtained from the European Commission Joint Research Centre (Ispra, Italy) and originate from Nanocyl (Auvelais, Belgium). NM400c and MWCNTg 2400 were obtained as reported previously (Vietti et al., 2013). Briefly, a fraction of NM400 was crushed in an oscillatory agate ball mill (Fritsch, Idar-Oberstein, Germany), with a vertical vibration of a fraction of NM400 was crushed in an oscillatory agate ball mill from Nanocyl (Auvelais, Belgium). NM400c and MWCNTg 2400 were obtained from another sample of MWCNT modified by grinding and then heating at 2400 °C for the elimination of metal clusters and ablation of structural defects (Fenoglio et al., 2008; Muller et al., 2008). MWCNT called long (MWNT 20–30 nm od), short (short MWNT 20–30 nm od) and thick (MWNT >50 nm od) were obtained from Cheap Tubes Company (Cambridgeport, VT). MWCNT physicochemical characteristics and dispersion are presented in the Supplementary material.

**Cell culture and exposure**

Mouse peritoneal macrophage cells (J774) and mouse type-II alveolar epithelial cells (LA-4) were seeded at a concentration of 150.000 cells/cm\(^2\) (surface area of culture well) and mouse lung fibroblasts (MLg) at a concentration of 90.000 cells/cm\(^2\) (Supplementary material). After 24 h at 37 °C in complete culture medium (10% FBS), cells were washed once with their basal medium (no FBS, no AA) and then supplied with 200 μL (96-well culture plate) or 500 μL (48-well culture plate) medium with 1% AA containing 1/10 stock material dilutions. Cells were exposed during 24 h or other specified time points to 7.5–30 μg MWCNT/cm\(^2\). Depending on experiments, cells were also treated before MWCNT exposure with low concentrations of human platelet-derived growth factor (PDGF)-BB (3 ng/mL, R&D System, Minneapolis, MN) or transforming growth factor (TGF)-\(\beta\)-1 (1 ng/mL, R&D System) during 2 h. Before or after this priming, cells were treated with inhibitors during 2 or 4 h: SIS3, LY294002, PD98059, LY364974 were from Merck Millipore (Darmstadt, Germany); SB431542 and IRESSA from R&D systems; AG1296 from Santa Cruz (Dallas, TX); amiloride from Sigma Aldrich and Gleevec, a gift from Pr. JB Demoulin (de Duve Institute, Brussels, Belgium). For fibroblasts treated with macrophage or epithelial cell conditioned medium, J774 or LA-4 were first exposed 24 h to MWCNT. Cell supernatants were collected and MLg were then treated for 24 h with 100 μL of this conditioned medium supplemented with 100 μL medium with 1% AA. Control, TGF-\(\beta\) and PDGF conditions also derived from LA-4 and J774 culture supernatants.

**Proliferation/cytotoxicity assay**

WST-1 is a colorimetric assay that quantifies mitochondrial activity and reflects cell viability. In this study, cell proliferation/cytotoxicity activity was measured by a NM-adapted WST-1 assay described by (Vietti et al., 2013) (Supplementary material). Cell viability results are presented as relative cell activity, that is, as a ratio to nontreated cells (Ctl), calculated as follows:

\[
\text{Relative cell activity} = \frac{\text{Abs}_{\text{exposed}}}{\text{Abs}_{\text{non-exposed}}}
\]

where

\[
\text{Abs} = (\text{Abs}_{480} - \text{Abs}_{680})_{\text{no Triton}} - (\text{Abs}_{480} - \text{Abs}_{680})_{\text{Triton}}.
\]

**Immunoblotting**

About 80 μL/well (48-well plate) of Laemmli Sample Buffer (BioRad, Hercules, CA) was added to cells for extracting proteins. About 25 μL extracts was run on a 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, BioRad, Hercules, CA), transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore) and blocked with the Odyssey Blocking Buffer (Westburg, Leusden, the Netherlands). The membrane was blotted with the primary antibody at 4 °C overnight with shaking, followed by incubation with the secondary antibody for 1 h at room temperature protected from light. The primary antibodies used in this study were phospho-p44/42 MAPK (Thr 202/Tyr204) rabbit Ab (dilution 1:1000), p44/42 MAPK mouse mAb from Cell Signaling (dilution 1:1000, Danvers, MA) or β-actin from Sigma (dilution 1:2000) and the secondary antibodiesIRDye 680 RD anti-mouse IgG or IRDye 800 anti-rabbit IgG from LI-COR (dilution 1:15000, Lincoln, NE). Detection was performed using an Odyssey LI-COR platform.

**Real-time PCR**

Tripure was added to cell cultures and RNA extraction was performed according to manufacturer’s instructions (Roche). Total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen) with 350 pmol random hexamers (Eurogentec, Seraing, Belgium) in a final volume of 25 μL. Resulting cDNA was used as template in subsequent polymerase chain reaction (PCR). Sequences of interest were amplified using the following forward primers (Invitrogen): GGAGTAATGGGTTGAAATGGGC (α-SMA), CCAGCTCCTCTGCTCCTCTT (procollagen I), CCGCTCACATCCAGAAGAGAA (18 S RNA), CTGGTGTCACACCCTATTCC (PDGF R\(\beta\)), TCTATATCTTTTGTCGCCATCCCC (PDGF R\(\beta\)), GAAATGGTGACCATG (PDGF R\(\beta\)), GGRATAGTGG (TGF-RI), CACGACCCCAAGCTCACCTA (EGF R); and ACTGGTGGCA (PDGF R\(\beta\)), CAAAGAAATCCTTGACGAAGCC (TGF-RI), TGTGTA

**Relative cell activity**

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\]
mRNA expression of these genes was quantified by real-time PCR using SYBR Green technology on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the following program: 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) × 40. Five microliters of diluted cDNA or standards was amplified with 300 nM of the described primers using Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μL. PCR product specificity was verified by taking a dissociation curve. The results were expressed as a ratio of gene expression to the expression of the reference gene (18 S rRNA).

**Intracellular TEM analysis**

After 24-h incubation with NM400, mouse lung fibroblasts, type-II alveolar epithelial cells and peritoneal macrophages were harvested by trypsinisation or scraping, centrifuged and fixed for 150 min at 4 °C with 2.5% (v/v) glutaraldehyde (Agar Scientific) in 0.1 M cacodylate buffer (pH 7.4) in a pyramidal beam capsule (Agar Scientific). Cells were then washed 3 times with 0.2 M cacodylate buffer and postfixed in 1% (v/v) osmium tetroxide (Merck) in 0.1 M cacodylate buffer (pH 7.4) for 60 min at 4 °C. After fixation, cells were dehydrated in a graded series of ethanol solutions (30, 50, 70, 85 and 100%), infiltrated and embedded in epon resin LX 112 (LADD Research Industries). The samples were cut in 60–100 nm ultrathin sections with an ultramicrotome (Leica), placed on formvar coated copper/palladium grids (Agar) and contrasted with saturated lead citrate and uranyl acetate solutions. TEM images were taken using a transmission electronic microscope (Philips FEI Tecnai 10) at an acceleration voltage of 80 kV and a digital camera.

**Animals and treatments**

Eight-week-old C57BL/6 female mice were obtained from the local animal facility (Animalerie Centrale UCL, Brussels, Belgium), housed in positive pressure air-conditioned units (25 °C, 50% relative humidity) on a 12-h light/dark cycle with free access to water and laboratory animal food. MWCNT (NM400, long MWCNT, short MWCNT and thick MWCNT) were dispersed as previously described. MWCNT suspensions were diluted to 100 μg/50 μL H2O with 1.4 mg/mL BSA. After anesthesia with a mix of Ketalar, 1 mg/mouse (Warner-Lambert, Zaventem, Belgium), and Rompun, 0.2 mg/mouse (Bayer, Leverkusen, Germany) given intraperitoneally, 50 μL of particle suspensions were introduced into the lungs by pharyngeal aspiration. Six mice were included per group. Control animals were treated with an equivalent volume of sterile water with the dispersant (control group). Mice were sacrificed 2 months after instillation with an overdose of sodium pentobarbital (15 mg/mouse intraperitoneally). The left lobe was isolated by clamping the corresponding bronchi. This lobe was recovered in 3.65% paraformaldehyde (Sigma-Aldrich) in PBS for later histological analysis. The remaining lobes were perfused with NaCl 0.9%, excised and then placed in 3 mL ice-cold PBS for the determination of lung hydroxyproline (OH-proline) content. The protocols of this investigation were approved by the local committee for animal research at the Université catholique de Louvain, Comité d’Éthique pour l’Expérimentation Animale, Secteur des Sciences de la Santé.

**Lung homogenates and measurement of lung collagen content**

Lungs were homogenized on ice with an Ultra-Turrax T25 homogenizer (Janke & Kunkel, Brussels, Belgium) and stored at −80 °C. Lung total collagen was estimated by measuring OH-proline, a specific component of collagen. Part of lung homogenate was hydrolyzed in HCl 6 N at 108 °C during 24 h and OH-proline was quantified by high-performance liquid chromatography (Biondi et al., 1997).

**Histology**

Paraffin-embedded sections were stained with Sirius Red (type-I collagen staining) for light microscopy examination.

**Statistical analysis**

Data are presented as means ± standard error on the mean (SEM). Differences were evaluated by using t-test between control conditions and high concentrations of growth factors or one-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparison or Newman–Keuls multiple comparison tests. Statistical significance was considered at p ≤ 0.05. Data analysis was performed with GraphPad Prism 5.00 (GraphPad Software, San Diego, CA).

**Results**

**MWCNT characterization**

Physicochemical characteristics and morphology of the different MWCNT samples used in the present study are reported in Table 1. Long and short MWCNT are identical in diameter and impurity levels but differ by their length, while thick MWCNT have a similar length as long MWCNT but are thicker. NM400 are MWCNT selected as reference material by the JRC repository and present a similar diameter as long and short MWCNT with an intermediate length. NM400 contain high levels of Al impurity compared to the other MWCNT samples.

**Indirect fibrogenic effects of MWCNT on lung fibroblasts**

Indirect effects of MWCNT (NM400) on fibroblasts were assessed in vitro using macrophages and lung epithelial cells. We first assessed the effect of NM400 on the viability of mouse peritoneal macrophages (J774) and type-II alveolar epithelial cells (LA-4) by a nanomaterial (NM)-adapted WST-1 assay (Vietti et al., 2013). Cells were exposed for 24 h to 7.5–30 μg MWCNT/cm² (surface area of the culture well). NM400 significantly stimulated epithelial cell proliferation, while they were cytotoxic to macrophages in the same dose range (Figure 1A and B). Cells were also treated with the pro-fibrotic mediators PDGF and TGF-β. Both growth factors stimulated epithelial cell proliferation but had no effect on macrophages, as expected. Transmission electron microscopy (TEM) studies showed the presence of intracellular CNT: dispersed CNT were found in macrophage and epithelial cell endosomes, while aggregated MWCNT were also observed in macrophage cytoplasm (Figure 1C and D).

Next, we exposed mouse lung fibroblasts (MLg) 24 h to supernatants from J774 and LA-4 collected after 24-h exposure to MWCNT. Procollagen I and α-SMA smooth muscle actin (SMA) were measured by quantitative RT-PCR as markers of collagen expression and fibroblast differentiation, respectively. Conditioned medium from both cell types increased fibroblast α-SMA expression (Figure 1E and F) significantly at the dose of 7.5 and 15 μg/cm² for LA-4 and J774 supernatant, respectively, while collagen expression was not altered (Figure 1G and H). These observations confirmed the indirect effects of CNT on fibroblasts, which might contribute to the establishment of lung fibrosis.

**Direct fibrogenic effects of MWCNT on lung fibroblasts**

In a previous study (Vietti et al., 2013), we demonstrated a direct proliferative activity of MWCNT on lung fibroblasts. This in vitro
response strongly reflected the lung fibrotic activity of several CNT samples in vivo. In the present experiments, we primed fibroblasts with low concentrations of pro-fibrotic growth factors (PDGF 3 ng/mL or TGF-β 1 ng/mL). These concentrations, contrary to the active doses used as positive controls (PDGF 30 ng/mL or TGF-β 10 ng/mL), were not observed to stimulate fibroblast proliferation. This priming represents a realistic scenario since PDGF and TGF-β are present in the CNT-exposed lung, potentially secreted by macrophages and/or epithelial cells (Cesta et al., 2010; Wang et al., 2013). When cells were additionally exposed to MWCNT (NM400), fibroblast proliferation was significantly and dose dependently increased (Figure 2A and B). The fact that MWCNT-stimulated fibroblast proliferation when primed with PDGF or TGF-β suggested the implication of their signaling pathways in this activity. These results were confirmed with other long CNT (NM402), whereas short MWCNT (NM400c and MWCNTg 2400) did not stimulate MLg proliferation (data not shown), in accordance with our previous results (Vietti et al., 2013). Like in macrophages and epithelial cells, we recorded loose MWCNT in fibroblast endosomes (Figure 2C).

Fibroblast collagen production and differentiation into myofibroblasts were also assessed after direct exposure to MWCNT. While TGF-β (10 ng/mL) increased both responses as expected (Wynn, 2011), procollagen I and α-SMA transcripts were not altered after direct exposure to MWCNT of cells primed with low concentrations of PDGF or TGF-β (Figure 2D–G). Interestingly, MWCNT even decreased α-SMA expression, similarly to PDGF 30 ng/mL (Figure 2D), as shown by other authors (Salabei et al., 2013). As our data confirmed that the tested MWCNT had a direct effect on fibroblast proliferation and suggested an involvement of PDGF and TGF-β as a mechanism of action, we next explored the impact of MWCNT at different levels of the PDGF/TGF-β stimulation axis.

PDGF or TGF-β secretion by fibroblasts and expression of their receptors in response to MWCNT

Several studies have reported an increased secretion of PDGF and TGF-β, and higher expression of TGF-β receptors after exposure to CNT, whereas other investigators did not record similar modifications (Azad et al., 2013; He et al., 2011; Lin et al., 2012; Wang et al., 2015). Thus, we investigated whether NM400 can alter PDGF and TGF-β secretion and the expression of their receptors. First, we assessed the basal level of PDGF and TGF-β receptor expression in the different cell lines used in this study. Figure S1A–D shows that PDGF (Rα and Rβ) and TGF-β (R1 and R2) receptors are significantly more expressed in MLg and LA-4 than in J774, which is consistent with the fact that both growth factors and MWCNT stimulated the proliferation of fibroblasts and epithelial cells but not of macrophages. Fibroblast exposure to MWCNT did not significantly modify PDGF and TGF-β secretion (Figure S1E–H) and receptor expression (data not shown).

MWCNT stimulate fibroblast proliferation via ERK 1/2 signaling pathway

Because PDGF and TGF-β signaling pathways appeared to play a role in the proliferative activity of MWCNT, we tested the possible implication of several intracellular signaling pathways known to be activated by these growth factors. Smad2/3 (TGF-β), PI3K (TGF-β and PDGF) and ERK 1/2 (TGF-β and PDGF) signaling were inhibited with SIS3, LY294002 and PD98059, respectively. Fibroblasts were first treated with inhibitors for 2 h, then primed with low concentrations of PDGF (3 ng/mL) or TGF-β (1 ng/mL) for two additional hours and finally exposed to MWCNT for 24 h. Smad3 and PI3K inhibitors had no effect on MWCNT proliferative activity (Figure 3A–D). On the contrary, the proliferative activity of MWCNT was inhibited by PD98059 when fibroblasts were primed with PDGF or TGF-β (Figure 3E and F). The effect of MWCNT on the ERK 1/2 signaling pathway was confirmed in a kinetic study where we showed that MWCNT prolonged ERK 1/2 phosphorylation induced by low concentrations of PDGF or TGF-β (Figure 4A and B). Two hours after the addition of PDGF 3 ng/mL (time 0), ERK 1/2 phosphorylation was increased. This phosphorylation decreased time-dependently in the absence of CNT, while after the addition of MWCNT, ERK 1/2 phosphorylation was prolonged compared to fibroblasts only treated with PDGF 3 ng/mL. Moreover, PD98059 (administered before or after priming, Figures 3 and 4, respectively) inhibited MWCNT-stimulated ERK 1/2 phosphorylation 2 h after exposure to MWCNT and also reduced the phosphorylation of ERK 1/2 induced by high concentration of PDGF and TGF-β. The inhibition of the proliferative activity of MWCNT by PD98059 was associated with a reduction of ERK 1/2 phosphorylation (Figure 3G and H). Our data suggest that MWCNT interfere with fibroblast proliferation at a level upstream of ERK 1/2 phosphorylation.

Table 1. Physicochemical characteristics and morphology of MWCNT.

| Characteristics | NM400 | Long | Short | Thick |
|-----------------|-------|------|-------|-------|
| Diameter (nm)   | 5–35  | 11–59| 10–47 | 15–74 |
| Length (μm)     | 0.7–3 | 10–30| 0.5–2 | 10–30 |
| Impurities      |       |      |       |       |
| % Al            | 5.38  | 0.01 | 0.01  | 0.01  |
| % Co            | 0.24  |      | 0.04  | 0.03  |
| % Fe            | 0.58  | 0.05 | 0.04  | 0.03  |
| % Ni            |      | 1.35 | 0.81  | 2     |
| Morphology      |       |      |       |       |

*Refer to Vietti et al., 2013.

*Refer to Cheap Tubes Inc (http://www.cheaptubes.com).

*SEM (bar 1 μm) for NM400, TEM (bar 500 nm) for long, short and thick MWCNT.
Implication of kinase receptors in the proliferative activity of MWCNT

To understand whether PDGF and TGF-β receptors (R) are involved in the fibrogenic activity of MWCNT, fibroblasts were treated with PDGF or TGF-β receptor kinase inhibitors after priming with low concentrations of PDGF or TGF-β before NM400 exposure. The stimulation of fibroblast proliferation by PDGF (30 ng/mL) and TGF-β (10 ng/mL) was inhibited by Gleevec and AG1296 (PDGF R inhibitors), and LY364947 and SB431542 (TGF-β R inhibitors), respectively (Figure 5A, C, E, and G). MWCNT-induced stimulation of fibroblast proliferation was reduced by these inhibitors except LY364947. Interestingly, PDGF R inhibitors did not reduce the proliferation of TGF-β-primed fibroblasts exposed to MWCNT, and SB431542 (TGF-β R inhibitor) of PDGF-primed fibroblasts exposed to MWCNT (data not shown). The absence of inhibitory activity of LY364947 on fibroblast proliferation in the presence of MWCNT could be explained by the fact that SB431542 reduced more efficiently ERK 1/2 phosphorylation compared to LY364947 when fibroblasts were treated with TGF-β 10 ng/mL (Figure 5H). None of TGF-β R inhibitors affected ERK 1/2 phosphorylation after fibroblast treatment with PDGF (30 ng/mL) (Figure 5).
PDGF R inhibitors or SB431542 (TGF-β R inhibitor) also reduced the prolonged ERK 1/2 phosphorylation induced by MWCNT after priming with low concentrations of PDGF (A) or TGF-β (B) (3 and 1 ng/mL, respectively). Cell viability was assessed by the adapted WST-1 assay. PDGF (30 ng/mL) and TGF-β (10 ng/mL) were used as positive controls. Results are presented as relative cell activity, that is, as a ratio to nontreated cells (Ctl). *p < 0.05, ***p < 0.001 versus nontreated cells (n = 4, t-test or Dunnett’s multiple comparison test as appropriate). (C) Intracellular localization of CNT was assessed by TEM 24 h after fibroblast exposure to MWCNT (30 μg/cm²). Effect of MWCNT on fibroblast differentiation and collagen production was measured by quantifying α-SMA (D, E) and pro-collagen I (F, G) mRNA, respectively, by quantitative RT-PCR. Fibroblasts were primed with low concentrations of PDGF (D, F) or TGF-β (E, G) (3 and 1 ng/mL, respectively) and then exposed to MWCNT (7.5–15 μg/cm²) for 24 h. TGF-β (10 ng/mL) was used as a positive control. RT-PCR results are presented as a ratio of gene expression normalized on the expression of the reference gene, 18S rRNA gene. *p < 0.05, ***p < 0.001 versus nontreated cells (n = 3, t-test or Dunnett’s multiple comparison test as appropriate).
participate to the fibrogenic activity of MWCNT. Of note, IRESSA completely inhibited EGF-stimulated (10 ng/mL), but not PDGF-stimulated proliferation of fibroblasts (data not shown).

**Role of endocytosis in the proliferative activity of MWCNT**

In a previous section, we showed that NM400 were endocytosed by fibroblasts (Figure 2C). To understand the role of MWCNT uptake in the stimulation of fibroblast proliferation, we used amiloride, a nonspecific inhibitor of endocytosis. Fibroblasts were treated during 2 h with amiloride, then primed with low concentrations of PDGF or TGF-β for two additional hours and finally exposed to MWCNT. Figure 6 shows that amiloride reduced the proliferative activity of MWCNT and the associated phosphorylation of ERK 1/2, while it did not alter PDGF- (30 ng/mL) or TGF-β- (10 ng/mL) induced proliferation or ERK 1/2 phosphorylation. These data indicate that the fibrogenic activity of MWCNT is also associated with the process of endocytosis in fibroblasts.

**ERK 1/2 signaling as a mechanism-based predictor of the lung fibrogenic activity of MWCNT**

We recently reported that the in vitro proliferative activity of a range of MWCNT on fibroblasts reflected their fibrotic activity in vivo (Vietti et al., 2013). Since the present study indicated that...
the pro-fibrotic activity of CNT was correlated with the prolongation of ERK 1/2 phosphorylation promoted by growth factors, we verified this hypothesis with three other MWCNT samples. Thus, MWCNT with different physicochemical properties (such as length and diameter, described in Table 1) were selected to assess the potential predictive value of ERK 1/2 signaling phosphorylation in terms of lung fibrosis. We observed that long MWCNT, but not short and thick MWCNT, stimulated fibroblast proliferation and prolonged ERK 1/2 phosphorylation when primed with a low concentration of PDGF (Figure 7A and B). Fibroblast proliferation stimulated by long MWCNT was reduced by the ERK 1/2 inhibitor PD98059. Two months after administration of MWCNT by pharyngeal aspiration in mice, we observed a significant increase of lung collagen fibers and content with NM400 and long MWCNT, but not with short and thick MWCNT (Figure 7C and D). These data indicate that the MWCNT-induced prolongation of ERK 1/2 signaling in vitro correlates with their activity on fibroblast proliferation, and, most interestingly, may predict the lung fibrotic response to MWCNT.

Discussion

A number of studies have revealed that CNT can cause pulmonary fibrosis in rodents (Lam et al., 2004; Muller et al., 2008; Shvedova et al., 2005). As already mentioned, both indirect and direct mechanisms appear essential for the fibrogenic activity of CNT. Here, we show that CNT-exposed epithelial cells and macrophages are important mediators for fibroblast differentiation and that the additional direct proliferative activity of MWCNT on fibroblasts results from a prolonged activation of ERK 1/2 signaling mediated by kinase receptors and endocytosis. Moreover, the effect of different MWCNT samples on ERK 1/2 phosphorylation strongly reflected the proliferative activity of MWCNT on fibroblasts and the extent of lung fibrosis, suggesting that this in vitro mechanism of action is relevant for and predictive of the pulmonary fibrotic activity of MWCNT. Figure 8 depicts a schematic overview of the mechanisms identified in this study and participating in the indirect and direct fibrogenic activities of MWCNT on fibroblasts.

In the first part of our study, we confirmed the indirect effects of MWCNT on fibroblasts, via epithelial cells and macrophages. We found that, at equivalent doses, MWCNT induce a dose-dependent proliferative and cytotoxic effect on epithelial cells and macrophages, respectively. Furthermore, the intracellular localization analysis showed loose MWCNT in endosomes of both cell types and aggregated MWCNT in the macrophage cytoplasm. Macrophages are professional phagocytes highly specialized in the clearance of particles in the lung. The presence of aggregated MWCNT in their cytoplasm suggests a substantial endocytosis of CNT but an incapacity to clear them, leading to phagosome destabilization and cell death (Murphy et al., 2012). Conditioned media from epithelial cells and macrophages exposed to MWCNT were shown to upregulate α-SMA, suggesting a role of these cells in fibroblast differentiation via the secretion of (a) pro-fibrotic...
mediator(s). Though conditioned medium might still contain CNT, we ascribed this effect to (a) factor(s) released by macrophages and epithelial cells, since NM400 did not directly increase α-SMA expression. Indeed, several investigators have amply shown that macrophages and epithelial cells exposed to CNT secrete key pro-fibrotic mediators such as PDGF, TGF-β and IL-1β (Cesta et al., 2010; Li et al., 2013; Wang et al., 2011). However, other mediators, such as EGF and fibroblast growth factor (FGF), could also contribute to the indirect effects of CNT on fibroblasts and further studies should be carried out to clarify this important issue. Our observations confirm, therefore, the indirect action of MWCNT on fibroblasts mediated by macrophages or epithelial cells that could contribute to the development of CNT-induced lung fibrosis.

Figure 5. Implication of PDGF and TGF-β receptors in the fibrogenic activity of MWCNT in fibroblasts. MWCNT activity on fibroblast proliferation and ERK 1/2 phosphorylation in the presence of PDGF and TGF-β inhibitors were assessed by the adapted WST-1 assay (A, C, E, G) and immunoblotting (B, D, F), respectively. Fibroblasts (MLg) were primed with a low concentration of PDGF (A–D) or TGF-β (E–G) (3 or 1 ng/mL, respectively), treated with PDGF R inhibitors Gleevec (A, B) or AG1296 (C, D), TGF-β R inhibitors SB431542 (E, F) or LY364947 (G) and then exposed to MWCNT (7.5–15 µg/cm²) for 24 h (WST-1 assay) or 2 h (immunoblotting). PDGF and TGF-β (30 and 10 ng/mL, respectively) were used as positive controls. WST-1 results are presented as relative cell activity, that is, as a ratio to nontreated cells (Ctl). *p<0.05, **p<0.01, ***p<0.001 versus nontreated cells (n=4, Newman–Keuls multiple comparison test). (H) Inhibitory activity of TGF-β receptor inhibitors on ERK 1/2 phosphorylation in fibroblasts was assessed by immunoblotting. MLg were treated with TGF-β R inhibitors LY364947 or SB431542 and then with active concentrations of PDGF or TGF-β (30 or 10 ng/mL, respectively).
Figure 6. Implication of endocytosis in the fibrogenic activity of MWCNT in fibroblasts. MWCNT activity on fibroblast proliferation and ERK 1/2 phosphorylation in presence of the endocytosis inhibitor amiloride were assessed by the adapted WST-1 assay (A–B) and immunoblotting (C–D), respectively. Fibroblasts (MLg) were treated with the endocytosis inhibitor amiloride, primed with low concentrations of PDGF (A, C) or TGF-β (B, D) (3 and 1 ng/mL, respectively) and then exposed to MWCNT (7.5–15 µg/cm²) for 24 h (WST-1 assay) or 2 h (immunoblotting). PDGF and TGF-β (30 and 10 ng/mL, respectively) were used as positive controls. WST-1 results are presented as relative cell activity, that is, as a ratio to nontreated cells (Ctl). *p < 0.05, **p < 0.01, ***p < 0.001 versus nontreated cells (n = 4, Newman–Keuls multiple comparison test).

Figure 7. Activity of MWCNT on fibroblast proliferation and ERK 1/2 phosphorylation in vitro correlates with the induction of lung fibrosis. Fibroblasts (MLg) were exposed to different MWCNT samples (NM400, long, short or thick) in presence or absence of ERK 1/2 inhibitor, PD98059. Cell viability and ERK 1/2 phosphorylation were assessed by the adapted WST-1 assay (A) and immunoblotting (B), respectively. Fibroblasts were treated with ERK 1/2 inhibitor PD98059, primed with low concentrations of PDGF (3 ng/mL) and then exposed to MWCNT (15 µg/cm²) for 24 h (WST-1 assay) or 2 h (immunoblotting). PDGF and TGF-β (30 and 10 ng/mL, respectively) were used as positive controls. WST-1 results are presented as relative cell activity, that is, as a ratio to nontreated cells (Ctl). *p < 0.05, **p < 0.01, ***p < 0.001 versus nontreated cells (n = 4, Newman–Keuls multiple comparison test). (C) Sirius red lung sections (12x) and (D) OH-proline lung content were analyzed 2 m after pharyngeal aspiration of 100 µg MWCNT/mouse (NM400, long, short, thick). **p < 0.01 versus nontreated cells (n = 6, Dunnett’s multiple comparison).
The ability of CNT to reach the alveolar interstitium and thus to directly interact with fibroblasts and stimulate their proliferation may also participate in the development of pulmonary fibrosis (Azad et al., 2013; Mercer et al., 2010; Vietti et al., 2013; Wang et al., 2010a; Wang et al., 2010b). In the present study, we showed that MWCNT stimulate fibroblast proliferation when cells are primed with low concentrations of PDGF or TGF-β. Interestingly, Wang et al. (2013) observed that MWCNT increased lung fibroblast number in vivo after intratracheal instillation by using the fibroblast-specific protein-1 (FSP-1) marker. Although it was not shown that increased fibroblasts number resulted solely from proliferation, these observations support the importance of this process in the development of fibrosis.

To explore the mechanisms responsible for fibroblast proliferation after MWCNT exposure, different hypotheses have been investigated. Some authors have reported increased TGF-βRII protein levels and the secretion of TGF-β by fibroblasts exposed to SWCNT (Azad et al., 2013; Lin et al., 2012; Manke et al., 2014). We did not observe increased PDGF or TGF-β secretion or expression of their receptors by fibroblast exposed to MWCNT. However, we observed a stronger expression of PDGF and TGF-β receptors in fibroblasts and epithelial cells compared to macrophages, suggesting the implication of their signaling pathway in the proliferative activity of MWCNT.

To assess this, several intracellular pathways of PDGF and TGF-β receptors (Smad3, PI3K and ERK 1/2) were probed by inhibitors. Results showed the involvement of ERK 1/2 in the proliferative activity of MWCNT. We also observed that MWCNT prolonged PDGF and TGF-β-induced ERK 1/2 phosphorylation in fibroblasts and that this effect was prevented by an ERK 1/2 inhibitor administered before or after priming, suggesting that MWCNT interfere with the signaling pathway upstream of the phosphorylation of ERK 1/2. The association between CNT exposure and activation of ERK 1/2 signaling was already shown in some studies but never in relation with the stimulation of fibroblast proliferation.
proliferation. For instance, phosphorylation of ERK 1/2 was detected in malignant human mesothelial cells after SWCNT exposure and during the induction of neoplastic-like transformation (Lohcharoenkal et al., 2014; Pucariri et al., 2008). Also, MWCNT were shown to activate ERK 1/2 in human skin fibroblasts, neuronal neurites and macrophages (Ding et al., 2005; Lee et al., 2012; Matsumoto et al., 2010).

Since we showed that MWCNT act upstream of ERK 1/2 in fibroblasts, we investigated whether PDGF and TGF-β receptors were involved in the proliferative activity of MWCNT. Inhibiting PDGF or TGF-β receptor after priming with their respective ligands drastically reduced MWCNT-induced fibroblast proliferation and ERK 1/2 phosphorylation. We observed that MWCNT also stimulated the proliferation of EGF-primed fibroblasts and the inhibitor of EGF R prevented this effect, suggesting that the proliferative activity of MWCNT on fibroblasts can be mediated by several kinase receptors. The EGF kinase R also regulates the activation of specific signaling pathways, including ERK 1/2 (Jorissen et al., 2003), and interacts with ultrafine carbon black particles and asbestos fibres to activate cells (Sydlik et al., 2006; Tamaoki et al., 2004; Taylor et al., 2013; Unfried et al., 2008). Currently, we ignore whether there is a direct interaction between the CNT and kinase receptors and further studies need to be performed. We can, however, conclude that the proliferative effect of MWCNT is mediated by kinase receptors.

As mentioned above, we observed the internalization of MWCNT in the endosomes of lung fibroblasts and, thus, decided to investigate whether the cellular uptake of CNT might play a role in their proliferative activity. We showed that an unspecific endocytosis inhibitor, amiloride, abolished the proliferative activity of MWCNT on primed fibroblasts as well as ERK 1/2 phosphorylation. Recent studies suggest that internalization of kinase receptors might act as a positive signal for their activation (Pahara et al., 2010; Wang et al., 2004). However, our data show that the inhibitor of endocytosis did not decrease fibroblast proliferation induced by high concentrations of PDGF and TGF-β, suggesting that receptor internalization alone is not sufficient to explain the pro-fibrotic activity of MWCNT.

Finally, we tested the proliferative activity of several MWCNT and found that their capacity to stimulate fibroblast proliferation was related to their lung fibrotic activity, as we already reported with other CNT samples (Vietti et al., 2013). Additionally, the proliferative activity of MWCNT was also associated with the prolongation of ERK 1/2 phosphorylation in fibroblasts in vitro, suggesting that this in vitro mechanism is predictive of a fibrogenic potential. We also confirmed the importance of MWCNT length and diameter in their fibrotic activity. Other studies also showed that MWCNT induce cytotoxic or fibrotic effects in a length-dependent manner and that thick MWCNT are less toxic than thinner ones both in vivo and in vitro (Fenoglio et al., 2012; Liu et al., 2012; Murphy et al., 2012; Nagai et al., 2011; Palomaki et al., 2011; Vietti et al., 2013). Thus, our data support the hypothesis that thin and long fibers can induce more chronic inflammation and lung fibrosis, as proposed in the fiber paradigm (Donaldson et al., 2010).

## Conclusion

The present findings, summarized in Figure 8, demonstrate that MWCNT act both indirectly, via macrophages and epithelial cells, and directly on fibroblasts to stimulate their pro-fibrotic activities: (i) the interaction of MWCNT with macrophages and epithelial cells can promote the differentiation of lung fibroblasts, (ii) MWCNT interaction with fibroblasts can stimulate their proliferation. We showed that the proliferative activity of MWCNT is kinase receptor-, ERK 1/2 pathway- and endocytosis-dependent. As suggested in our previous study (Vietti et al., 2013), we confirmed with other MWCNT samples that the proliferative activity of MWCNT on fibroblasts strongly reflects their capacity to induce lung fibrosis. This was also associated with the prolongation of ERK 1/2 phosphorylation in fibroblasts, indicating that this in vitro mechanism of action is relevant for and predictive of the pulmonary fibrogenic activity of CNT. Other mechanisms of action have been shown to modulate the pro-fibrotic activity of CNT, and a better understanding of all pathways involved in the fibrotic potential of CNT is thus needed to better establish the value of our in vitro fibroblast proliferation assay to predict CNT lung fibrotic activity.

## Declaration of interest

The authors declare that they have no competing interests.

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Supplementary material available online

Supplementary material