Nickel Nanoparticles Enhance Platelet-Derived Growth Factor-Induced Chemokine Expression by Mesothelial Cells via Prolonged Mitogen-Activated Protein Kinase Activation

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Pleural diseases (fibrosis and mesothelioma) are a major concern for individuals exposed by inhalation to certain types of particles, metals, and fibers. Increasing attention has focused on the possibility that certain types of engineered nanoparticles (NPs), especially those containing nickel, might also pose a risk for pleural diseases. Platelet-derived growth factor (PDGF) is an important mediator of fibrosis and cancer that has been implicated in the pathogenesis of pleural diseases. In this study, we discovered that PDGF synergistically enhanced nickel NP (NiNP)-induced increases in mRNA and protein levels of the profibrogenic chemokine monocyte chemoattractant protein-1 (MCP-1 or CCL2), and the antifibrogenic IFN-inducible CXC chemokine (CXCL10) in normal rat pleural mesothelial 2 (NRM2) cells *in vitro*. Carbon black NPs (CBNPs), used as a negative control NP, did not cause a significant increase in CCL2 or CXCL10 in the absence or presence of PDGF. NiNPs prolonged PDGF-induced phosphorylation of the mitogen-activated protein kinase family termed extracellular signal-regulated kinases (ERK)-1 and -2 for up to 24 hours, and NiNPs also synergistically increased PDGF-induced hypoxia-inducible factor (HIF)-1 α protein levels in NRM2 cells. Inhibition of ERK-1,2 phosphorylation with the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, blocked the synergistic increase in CCL2, CXCL10, and HIF-1 α levels induced by PDGF and NiNPs. Moreover, the antioxidant, N-acetyl-L-cysteine (NAC), significantly reduced HIF-1 α , ERK-1,2 phosphorylation, and CCL2 protein levels that were synergistically increased by the combination of PDGF and NiNPs. These data indicate that NiNPs enhance the activity of PDGF in regulating chemokine production in NRM2 cells through a mechanism involving reactive oxygen species generation and prolonged activation of ERK-1,2.

Keywords: nanoparticles; metals; lung; pleura; chemokines

Pleural disease, such as fibrosis and mesothelioma, is a major concern for individuals exposed by inhalation to certain types of fibers, especially asbestos. Increasing attention has focused on the possibility that engineered carbon nanotubes (CNTs), a product of emerging nanotechnology, pose a similar risk due to their high aspect ratio, durability, and metal catalysts (1). Nickel nanoparticles (NiNPs) are a common catalyst used in

CLINICAL RELEVANCE

This research elucidates a fundamental signaling mechanism through which nickel nanoparticles (NiNPs) promote chemokine production by pleural mesothelial cells. This work has important implications for understanding the pathogenesis of pleural disease in individuals exposed occupationally or environmentally to NiNPs. Furthermore, we identify important molecular targets and signaling pathways in mesothelial cells that could be useful in determining the potential health risks of novel engineered nanomaterials that contain nickel.

the manufacture of multiwalled CNTs (MWCNTs) (2). Nickel (Ni) is known to cause a variety of pulmonary diseases, including fibrosis and cancer, and exposure to Ni particulates can also result in pleural and peritoneal mesothelioma in rodents (3–5). Furthermore, NiNPs are more potent inducers of pleural fibrosis compared with micron-sized Ni particles (6, 7). We recently reported that MWCNTs containing residual Ni catalyst reached the pleura of the lungs of mice after inhalation and caused pleural fibrosis and increased immune responses (8).

Platelet-derived growth factor (PDGF) is a key mediator of fibrogenesis, and is increased in the lungs of mice that are exposed to Ni-containing MWCNTs (8, 9). PDGF is a family of polypeptides (AA, BB, AB, CC, DD) that bind and dimerize cell surface receptors, termed PDGF receptor (PDGF-R) α and PDGF-R β , to form dimeric $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$ receptors with tyrosine kinase activity. Alveolar macrophages are a rich source of PDGF-BB, whereas fibroblasts are the central source of PDGF-AA. In addition, PDGF production by macrophages can be increased in a hypoxic environment stimulated by exposure to Ni (10, 11). PDGF acts as a stimulant of fibroblast growth and chemotaxis through the activation of the mitogen-activated protein kinases (MAPKs) termed extracellular signal-regulated kinase (ERK) -1 and -2 (collectively referred to as ERK), and plays a major role in fibroblast survival (10, 12). Fibroblasts are critical to fibrogenesis, as they differentiate into myofibroblasts in response to transforming growth factor (TGF)- β 1 to produce collagen, which defines fibrotic lesions (10). Moreover, PDGF is thought to play a role in pleural fibrosis and mesothelioma (13). PDGF-BB is produced by pleural mesothelial cells *in vitro* in an activating transcription factor-3 (ATF3)-dependent manner after exposure to asbestos fibers, and pleural mesothelial cells possess cell surface receptors for PDGF (14, 15).

In addition to stimulating the growth, chemotaxis, and survival of mesenchymal cells, PDGF stimulates the production of other cytokines, chemokines, and growth factors that play important roles in angiogenesis and fibrogenesis. For example, PDGF increases the production of CCL2 (monocyte chemoattractant

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protein [MCP]-1), a profibrogenic and proangiogenic chemokine, via a mechanism involving the activation of phosphoinositide 3-kinase and NF- κ B in NIH/3T3 fibroblasts (16, 17). Rat pleural mesothelial cells also produce CCL2 in response to asbestos fibers *in vitro*, which, in turn, serves as a potent chemoattractant for monocytes and macrophages (18, 19). CCL2 stimulates fibroblasts to produce TGF- β 1 and collagen (20). CCR2, the receptor for CCL2, is required for pulmonary fibrosis mice, as CCR2-deficient mice are protected from bleomycin or FITC-induced fibrosis (21, 22). In addition to its role in promoting fibrogenesis, CCL2 also stimulates angiogenesis via hypoxia-inducible factor (HIF)-1 α and consequent production of vascular endothelial growth factor (VEGF) (23).

Although CCL2 promotes fibrogenesis, the IFN-inducible chemokine, CXCL10, is antifibrogenic. For example, CXCL10 has been demonstrated to reduce bleomycin-induced lung fibrosis in mice, and is due, at least in part, to inhibition of angiogenesis (24). In addition to inhibition of angiogenesis, CXCL10 also mediates chemotaxis of activated T and NK cells, and regulates the expression of adhesion molecules (25, 26). CXCL10 mediates its biological effects through its receptor, termed CXCR3, and deletion of CXCR3 in mice has been shown to increase bleomycin-induced fibrogenesis (27). We have previously shown that the transition metal, vanadium pentoxide, increases the expression of CXCL10 in human lung fibroblasts through a mechanism involving activation of reduced nicotinamide adenine dinucleotide phosphate oxidase and autocrine production of IFN- β (28).

In this study, we hypothesized that PDGF would modify NiNP-induced expression of CCL2 or CXCL10 in rat pleural mesothelial cells. We discovered that NiNPs and PDGF-BB synergistically increased CCL2 and CXCL10 in rat pleural mesothelial cells through a mechanism involving the ability of NiNPs to prolong ERK phosphorylation that is initially induced by PDGF.

MATERIALS AND METHODS

Cell Culture

Normal rat pleural mesothelial (NRM2) cells were a kind gift from Edilberto Bermudez at the Hamner Institutes for Health Sciences (Research Triangle Park, NC), and were isolated from the parietal pleura (29). The details of cell culture and treatment with PDGF or NiNPs are described in the online supplement.

NPs

NiNPs (~20-nm diameter) were purchased from Sun Innovations (Fremont, CA). We determined NiNP size from digitized transmission electron microscopy (TEM) images. The diameter of NiNPs was measured using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA) by determining pixel length of >100 NiNPs and converting pixels to nanometers using a standard magnification bar contained within each TEM image. Carbon black NPs (CBNPs; ~8 nm) were in the form of Raven 5,000 Ultra II Powder (Columbian Chemicals, Marietta, GA). NPs were suspended in a sterile, 0.1% pluronic F-68 (Sigma, St. Louis, MO) in phosphate buffer solution and dispersed for 2 hours using a bath sonicator at room temperature. Further details on NP characterization are provided in the online supplement.

Taqman Real-Time RT-PCR

One-step, TaqMan quantitative real-time RT-PCR (qRT-PCR) was performed to quantify gene expression of our target genes. Total RNA was extracted and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentrations were determined by the Nano-drop1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA) and samples were normalized to a final concentration of 25 ng/ μ l. qRT-PCR was performed using reagents from the SuperScript

III Platinum One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) on the StepOne Plus instrument (Applied Biosystems, Foster City, CA). A comparative C_T method was used to quantify target gene expression for CXCL10 (Rn00594648_m1) and CCL2 (Rn00580555_m1) against the endogenous control, hypoxanthine phosphoribosyltransferase-1 (Rn01527840_m1). Each individual sample was analyzed in duplicate, and experiments were repeated at least three times. The StepOne Plus software calculated relative quantitation values and expressed results as fold change.

ELISA

Cell culture supernatants were assayed according to kit instructions for CCL2 (R&D Systems, Minneapolis, MN) or CXCL10 (Antigenix America, Inc., Huntington Station, NY) protein secretion. Absorbances were read at 450 nm by the Multiskan EX microplate spectrophotometer (ThermoFisher Scientific), with a correction wavelength set at 540 nm.

Western Blot

Cell lysates were collected at various time points and were separated by SDS-PAGE (Invitrogen), transferred to PVDF membranes, and blocked for 1 hour in 5% nonfat milk in TBST (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20). The blot was then incubated at 4°C overnight with primary antibody, followed by a 1-hour incubation of horseradish peroxidase-conjugated secondary antibody. The immunoblot signal was detected and visualized through enhanced chemiluminescence (ECL; Perkin Elmer Inc., Waltham, MA). Total and phosphorylated PDGF-R β primary antibodies, as well as all secondary antibodies, were purchased from Santa Cruz (Santa Cruz, CA). Total ERK, phosphorylated ERK (p-ERK), and β -actin primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). HIF-1 α antibody was purchased from Novus Biological (Littleton, CO).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 5.00 (San Diego, CA). Two-way ANOVA with a Bonferroni test was used to identify significant differences among multiple treatment groups. One-way ANOVA with a *post hoc* Tukey's test was used to test significance of treatment groups compared with control. The significance was set at $P < 0.05$ unless stated otherwise. Densitometric analysis was performed on Western blots using Image J analysis software (National Institutes of Health, Bethesda, MD).

RESULTS

NiNPs Form Agglomerates in Cell Culture Medium and Are Internalized by Pleural Mesothelial Cells *In Vitro*

TEM demonstrated that NiNPs formed agglomerates when suspended in tissue culture medium (Figure 1A). The size distribution frequency of individual NiNPs was calculated by measuring the diameter of particles from digitized TEM images, as described in MATERIALS AND METHODS. The average particle diameter was 25.43 ($\pm$ 11.62) nm, slightly larger than the 20-nm average diameter provided by the manufacturer (Figure 1B). Furthermore, greater than 90% of the NiNPs were between 10 and 50 nm in diameter. NRM2 cells were treated with NiNPs or CBNPs and observed by TEM after 24 hours of exposure. NiNP agglomerates were identified in contact with the cell membrane and within the cytoplasm of NRM2 cells (Figure 1C), as were CBNPs (Figure 1E). Higher magnification showed that both NiNP and CNBP agglomerates were contained within membrane-bound vesicles that resembled lysosomes (Figures 1D and 1F). NiNPs and CBNPs both retained their spherical shape and size within cells over a 24-hour time period under these experimental conditions.

Figure 1. Nickel nanoparticles (NiNPs) behave as NP agglomerates in cell culture medium, and are taken up by pleural mesothelial cells *in vitro*. NiNPs were dispersed in a bath sonicator for 2 hours in a 0.1% pluronic F-68 in phosphate buffer solution before dosing normal rat pleural mesothelial (NRM) 2 cells as indicated in MATERIALS AND METHODS. NiNP-exposed NRM2 cells were collected at 24 hours and characterized using transmission electron microscopy (TEM) imaging. (A) NiNP agglomerate in control medium. (B) Size distribution showing that the majority of NiNPs had a diameter between 20 and 50 nm. (C) NiNP agglomerates (black arrows) were observed in contact with the cell membrane and within the cytoplasm of a cultured NRM2 cell. (D) Higher magnification of the black box in (C) showing NiNP agglomerates enclosed within a membrane-bound vesicle in the NRM2 cell. (E) Carbon black NP (CBNP) agglomerates (black arrows) were also observed in contact with the cell membrane and within the cytoplasm. (F) Higher magnification of the black box in (E) showing CBNP agglomerates enclosed within a membrane-bound vesicle. Subcellular structures indicated are nucleus (Nu), microvilli (Mv), mitochondria (Mi), and lysosome (Ly).

NiNPs Synergistically Increase PDGF-Induced CCL2 and CXCL10 Expression in Rat Pleural Mesothelial Cells

CCL2 and CXCL10 mRNA and protein were measured using qRT-PCR and ELISA, respectively. The optimal concentrations of PDGF and NiNPs used in this study were determined by dose-response relationships. In addition, time course experiments were also performed to determine temporal expression patterns of CCL2 and CXCL10 (see the online supplement). The induction of CCL2 expression in NRM2 cells was due to the PDGF-BB isoform, referred to herein simple as “PDGF,” whereas PDGF-AA had no significant effect on the induction of CCL2 levels (data not shown). PDGF or NiNPs increased CCL2 mRNA levels by 6 hours and maximally increased CCL2 protein levels in cell supernatants at 24 hours after exposure (see Figures E1 and E2 in the online supplement). The combination of PDGF and NiNPs synergistically increased CCL2 mRNA and protein levels at 24 hours after treatment (Figures 2A and 2B). Similar to CCL2, CXCL10 mRNA and protein levels were synergistically increased in NRM2 cells by a combination of PDGF and NiNPs when compared with cells treated with either PDGF or NiNPs alone (Figures 2C and 2D). The time course of CXCL10 expression was different from CCL2 in that levels of CXCL10 mRNA and protein were maximally induced by NiNPs and PDGF at 48 hours after exposure (Figure E1). Treatment of NRM2 cells over all treatments and time points did not result in significant cytotoxicity,

as determined by lactate dehydrogenase (LDH) assay (data not shown). The synergistic response observed between PDGF and NiNPs on increasing chemokine production was observed in two separate isolates of NRM2 cells.

NiNPs Prolong PDGF-Induced Phosphorylation of MAPK, but Do Not Affect Phosphorylation of PDGF-R β

PDGF-induced ERK phosphorylation was observed in rat pleural mesothelial cells at 2 and 24 hours after exposure. ERK phosphorylation was measured by Western blot analysis using antibodies against total or p-ERK protein (Figure 3A). Densitometric analysis of the ratio of p-ERK to total ERK was performed for a quantitative evaluation of the different exposure groups (Figure 3B). At 2 hours, treatment of cells with either PDGF or a combination of PDGF and NiNPs caused a robust and significant increase in ERK phosphorylation over control and NiNPs. However, ERK phosphorylation induced by PDGF or PDGF plus NiNPs were not different from one another at 2 hours. In contrast, at 24 hours, ERK phosphorylation induced by PDGF plus NiNPs was significant over all dose groups, whereas ERK phosphorylation by PDGF alone at 24 hours was not significantly different from control (Figures 3A and 3B). To determine if NiNPs acted upstream at the PDGF-R to increase PDGF-induced phosphorylation of ERK at 24 hours, Western blot analysis was performed using antibodies specific

Figure 2. NiNPs synergistically increase PDGF-induced CCL2 and CXCL10 mRNA and protein expression in NRM2 cells. NRM2 cells were grown to confluency, serum starved for 24 hours, and treated with PDGF-BB (50 ng/ml) alone or immediately prior to adding CBNPs or NiNPs (10 $\mu\text{g}/\text{cm}^2$). Either mRNA or cell culture supernatants were collected for analysis by Taqman quantitative real-time RT-PCR or ELISA, respectively. (A) CCL2 mRNA analyzed at 24 hours. *** $P < 0.001$ compared with control determined by one-way ANOVA with *post hoc* Tukey test; ## $P < 0.01$, between NiNPs and PDGF + NiNPs or *** $P < 0.001$ between PDGF and PDGF + NiNPs, as determined by two-way ANOVA with Bonferroni *post hoc* test. (B) Secreted CCL2 protein at 24 hours. *** $P < 0.001$ compared with control, determined by one-way ANOVA; ## $P < 0.01$, between NiNPs and PDGF + NiNPs or *** $P < 0.001$ between PDGF and PDGF + NiNPs, as determined by two-way ANOVA. (C) CXCL10 mRNA levels at 24 hours. * $P < 0.05$ compared with control determined by one-way ANOVA; # $P < 0.05$, between NiNPs and PDGF + NiNPs or + $P < 0.05$ between PDGF and PDGF + NiNPs as determined by two-way ANOVA. (D) Secreted CXCL10 protein at 48 hours. * $P < 0.01$ or *** $P < 0.001$ compared with control; ## $P < 0.01$, between NiNPs and PDGF + NiNPs or *** $P < 0.001$ between PDGF and PDGF + NiNPs, as determined by two-way ANOVA. All data represent mean values ($\pm$ SEM) of three experiments run in duplicate.

for phosphorylated PDGF-R α or PDGF-R β , or antibodies against total protein levels of these two receptors. Western blot analysis showed that treatment of NRM2 cells with NiNPs did not change PDGF-induced phosphorylation of PDGF-R β or total protein levels of PDGF-R β (Figure 3C). PDGF-R α levels were not detectable in NRM2 cells by Western blot analysis, but were present in positive control human lung fibroblasts (data not shown).

CXCL10 and CCL2 Induction by PDGF and NiNPs Is Mitogen-Activated Protein Kinase Kinase Dependent

We hypothesized that ERK is an upstream mediator of both CCL2 and CXCL10, because PDGF is known to induce ERK phosphorylation (10). To test this hypothesis, NRM2 cells were treated with 20 μM of PD98059 1 hour before PDGF and NiNP exposure. To make sure that the inhibitor worked properly, ERK phosphorylation was analyzed by Western Blot. As seen in Figure 4A, PD98059 significantly reduced ERK signaling in every treatment group, including control. In addition, the mitogen-activated protein kinase kinase (MEK) inhibitor significantly decreased CXCL10 mRNA and protein expression that was induced by the combination of PDGF and NiNPs (Figure 4B). Similarly, CCL2 mRNA and protein expression induced by the combination of PDGF and NiNPs were significantly decreased by PD98059 (Figure 4C).

PDGF Enhances NiNP-Induced HIF-1 α Levels Via a MAPK-Dependent Mechanism

NiNPs increased HIF-1 α levels in NRM2 cells 24 hours after exposure as measured by Western blot analysis. PDGF alone did not increase HIF-1 α levels, but PDGF synergistically enhanced NiNP-induced HIF-1 α expression (Figure 5A). Quantitative densitometry of HIF-1 α were normalized against β -actin in three independent experiments and revealed a statistically

significant effect of NiNPs on HIF-1 α . Furthermore, this effect was significantly enhanced by the addition of PDGF (Figure 5B). The role of ERK was further investigated as contributory to the additive effect of PDGF and NiNPs on HIF-1 α expression. Cells were treated with 20 μM PD98059 1 hour before PDGF and/or NiNP exposure. PD98059 blocked PDGF enhancement of NiNP-induced HIF-1 α expression, but did not block the induction of HIF-1 α by NiNPs alone. This indicated that ERK is responsible for only PDGF-driven induction of HIF-1 α , and that there is an additional mediator involved in the induction of HIF-1 α by NiNPs. HIF-1 α mRNA levels were not changed at 24 hours after treatment with PDGF, NiNPs, or PDGF combined with NiNPs (data not shown), suggesting that HIF-1 α regulation in response to PDGF and NiNPs is regulated at the post-translational level.

NiNP-Induced HIF-1 α Levels and NiNP Enhancement of PDGF-Induced Chemokine Expression Is Reduced by the Antioxidant, N-Acetyl-L-Cysteine

NRM2 cells were pretreated with 5 mM of N-acetyl-L-cysteine (NAC), an antioxidant, for 1 hour before treatment with NiNPs, PDGF, or the combination of NiNPs and PDGF. Treatment with NAC decreased HIF-1 α protein levels that were induced after exposure to NiNPs or the combination of NiNPs and PDGF (Figure 6A). Quantitative densitometry of HIF-1 α Western blots were normalized against β -actin in three independent experiments, and revealed a statistically significant effect of NAC on reducing HIF-1 α levels induced by NiNPs or the combination of NiNPs and PDGF (Figure 6B). In addition, NAC significantly reduced p-ERK-1,2 and CCL2 protein levels in NRM2 cell supernatants that were induced by the combination of NiNPs and PDGF (Figures 6C and 6D). The effect of NAC on CXCL10, which was induced after 48 hours of treatment with NiNPs with or without PDGF, was not evaluated,

Figure 3. NiNPs enhance and prolong PDGF-induced extracellular signal-regulated kinase (ERK) phosphorylation, but not PDGF receptor (PDGF-R) β in rat pleural mesothelial cells. NRM2 cells were grown to confluency, serum starved, and treated with PDGF-BB (50 ng/ml) or PDGF-BB plus NiNPs (10 $\mu\text{g}/\text{cm}^2$), and cell lysates were collected at 2 and 24 hours after exposure. (A) Western blot analysis showing strong PDGF-induced phosphorylated ERK (p-ERK) at 2 hours that is almost completely diminished by 24 hours. NiNP coexposure did not alter PDGF-induced ERK phosphorylation at 2 hours, but enhanced PDGF-induced activation at 24 hours. Total ERK protein levels were not changed by any of the treatments. (B) Densitometry of the blots in (A) showed the relative ratio of p-ERK to total ERK protein for each time point. Densitometric analysis was performed using Image J analysis software, as described in MATERIALS AND METHODS, and represent mean values ($\pm$ SEM) of three experiments. (C) Western blot analysis showed PDGF-BB-induced phosphorylation of PDGF-R β at 15 minutes that is not affected by NiNPs. PDGF-BB-induced PDGF-R β phosphorylation was diminished by 24 hours and not affected by NiNPs. ** $P < 0.01$ compared with untreated (control) and NiNP-exposed cells; *** $P < 0.001$ compared with cells treated with control or NiNPs alone, as determined by one-way ANOVA with *post hoc* Tukey's test and $^{\wedge}P < 0.05$ compared with cells treated with PDGF alone, as determined by two-way ANOVA with Bonferroni's *post test*. Both Western blots shown are representative of at least three independent experiments that produced similar results.

because the combination of antioxidant and NiNPs or NiNPs plus PDGF caused significant cytotoxicity at this time point (data not shown).

DISCUSSION

Nanomaterials have increasingly gained popularity due to the novel physical and chemical properties they possess compared with their bulk counterparts. Their nano-size dimensions, shape, and surface area make them attractive for use in numerous applications, yet the potential risks they pose to human health remain largely unknown (1). In this study, we investigated the effects of NiNPs on pleural mesothelial cell signaling and chemokine production. When particle size is decreased to nanoscale proportions, this alters physiochemical properties (e.g., increased surface area and reactivity), and has the potential to change particle interaction with the cellular microenvironment and subcellular structures (30). Nano-sized Ni particles, when compared with larger Ni particles, have greater surface area, higher magnetism, and lower melting point (31). In addition, NPs cause more lung injury compared with larger particles, due to their higher surface area per unit mass and increased potential to generate reactive oxygen species (ROS) (30). Previous studies have shown that NiNPs cause more inflammation and toxicity in the lungs of rodents after intratracheal instillation when compared with micron-sized Ni particles, demonstrating

that pulmonary toxicity increases as particle size decreases (1, 6, 7, 31).

NPs are more likely to migrate to the distal areas of the lung and reach the pleural mesothelium (1). Previous studies have shown that NiNPs and Ni-containing nanomaterials can reach the pleura, causing fibrosis and mesothelioma in rodents (5–8). It is therefore important to gain a better understanding of the cellular and molecular mechanisms behind the progression of NiNP-induced pleural diseases. We found that NiNPs are taken up by cultured rat pleural mesothelial (NRM2) cells and stimulate marginal increases in chemokines (CCL2 and CXCL10) and HIF-1 α . However, in the presence of PDGF-BB, a fibroblast mitogen and chemoattractant, NiNP-induced chemokine and HIF-1 α expression were synergistically increased. Our data support the hypothesis that synergy between NiNPs and PDGF involves initiation of ERK phosphorylation by PDGF-BB and prolonged activation of PDGF-induced ERK signaling by NiNP-generated ROS. This hypothetical mechanism is illustrated in Figure 7.

NiNPs are used as a catalyst in the fabrication of MWCNTs, and there are concerns that they could be a contributing factor to pulmonary fibrosis or mesothelioma (5, 6, 12). We have previously reported a significant fibrotic response in the lungs and pleura of mice after inhalation exposure to MWCNTs containing residual Ni (8, 32). It is unknown whether the NiNPs used in this study closely represents the residual Ni catalyst

Figure 4. CXCL10 and CCL2 expression in rat pleural mesothelial cells is regulated by the MEK-ERK signaling pathway. NRM2 cells were grown to confluency, serum starved, and then pretreated with 20 μ M PD98059 (black bars) for 1 hour before adding PDGF (50 ng/ml) or NiNPs (10 μ g/cm²), alone or in combination, for 24 or 48 hours. (A) Western blot analysis showing p-ERK and total ERK protein levels in cell lysates from NRM2 cells. (B) CXCL10 mRNA levels at 24 hours and secreted protein levels at 48 hours from NRM2 cells were measured using TaqMan quantitative real-time RT-PCR or ELISA, respectively. (C) CCL2 mRNA and secreted protein levels were measured at 24 hours after exposure. Data represent mean values ($\pm$ SEM) of three experiments run in duplicate. ** P < 0.01 or *** P < 0.001 compared with cells in the same exposure group treated with mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, and determined by two-way ANOVA with Bonferroni's post test.

found in MWCNTs, although TEM photomicrographs coupled with energy-dispersive X-ray (EDX) spectroscopy indicate NiNPs within MWCNTs (8). Moreover, the dose of NiNPs used in the *in vitro* studies presented here are likely much higher than doses of Ni that would occur at the pleura of mice after lung exposure to MWCNTs. However, aside from exposures to MWCNTs that contain residual Ni, it is important to consider that much higher exposures to NiNPs alone could occur occupationally where Ni is used for manufacturing or catalytic processes.

Our study also shows that the cellular effects of NiNPs are not merely related to the physical size of the NPs, as CBNPs also were avidly taken up by NRM2 cells and did not cause significant ERK phosphorylation, induction of HIF-1 α , or chemokine expression. These findings suggest that the reactivity of NiNPs, in addition to nanometer size, is important for the biological effects observed in NRM2 cells. We observed that NiNPs retained their spherical shape and size within NRM2 cells over a 24-hour period, with no indication of degradation (Figure 1). This observation suggests that no dissolution of Ni is necessary to produce the biological responses reported in this study, and that surface reactivity of NiNPs plays an important role in generating ROS to initiate cell signaling and HIF-1 α or chemokine induction. Nevertheless, we cannot rule out the possibility that some Ni ions were generated that could have caused significant effects on biological responses. Finally, it is unclear whether the observed effects reported here might be seen with other types of metal NPs. Therefore, other transition metals used to manufacture engineered NPs should be tested to determine whether or not they act synergistically with PDGF to promote HIF-1 α and chemokine expression in mesothelial cells.

In our previous work, rats or mice exposed to MWCNTs containing residual Ni exhibited increased levels of PDGF (9, 32).

PDGF is an important mediator of fibrotic diseases (10), and has been implicated in the pathogenesis of pleural disease (13). During the pathogenesis of fibrosis, PDGF contributes to the production and deposition of collagen by driving myofibroblast proliferation and chemotaxis (33, 34). After exposure to MWCNTs, mice have increased levels of PDGF throughout the lung that could be available to mesothelial cells *in vivo* (8, 9). Alveolar macrophages are a major source of PDGF-BB in the lungs, whereas PDGF-AA is mainly produced by fibroblasts and epithelial cells (10, 35). Pleural mesothelial cells are another source for increased levels of PDGF-BB after asbestos exposure and injury. This suggests that PDGF-BB could act in an autocrine fashion to increase CCL2 expression in mesothelial cells (18, 19). We have previously shown that macrophages engulf inhaled MWCNTs and then migrate to the pleura, where they accumulate beneath the mesothelium (8). In our experiments, we found that PDGF-BB, but not PDGF-AA, enhanced NiNP-induced chemokine production by rat pleural mesothelial cells. Moreover, we have observed that the majority of NiNPs delivered to the lungs of mice by oropharyngeal aspiration are taken up by alveolar macrophages, and many of these macrophages are found in the subpleural region of the lungs adjacent to the pleural mesothelium (E.E. G.-B. and J.C.B., unpublished observation). As illustrated in Figure 7, paracrine signaling between macrophages and mesothelial cells via PDGF-BB could be important to the amplification of NiNP-induced signaling in pleural mesothelial cells *in vivo*. Nevertheless, it is unclear how PDGF-BB produced by macrophages would translocate across the epithelial barrier that separates the subpleural region of the lung from the mesothelial lining. Moreover, cell types other than macrophages cannot be ruled out as a source of PDGF-BB, and this issue requires further study.

Figure 5. PDGF enhancement of NiNP-induced hypoxia-inducible factor (HIF)-1 α is mediated by MAPK signaling. NRM2 cells were grown to confluency, serum starved, and then pretreated with 20 μ M PD98059 (black bars) for 1 hour before adding PDGF (50 ng/ml) or NiNPs (10 μ g/cm²), alone or in combination, for 24 or 48 hours. (A) Western blot analysis of HIF-1 α protein levels in cell lysates from NRM2 cells showing that PDGF enhancement of NiNP-induced HIF-1 α is reduced by PD98059. (B) Densitometry of Western blots in (A) showing the relative ratio of HIF-1 α to β -actin protein for each treatment. Densitometric analysis was performed using Image J analysis software, as described in MATERIALS AND METHODS. All data represent mean values ($\pm$ SEM) of three independent experiments. *** P < 0.001 compared with control or PDGF treatment with or without PD98059, as determined by one-way ANOVA with *post hoc* Tukey's test; ### P < 0.001 comparing NiNP to PDGF + NiNP treatment without PD98059; or ^ P < 0.05 compared with cells in the same exposure group treated without MEK inhibitor, PD98059, as determined by two-way ANOVA with Bonferroni's post test. Western blot shown is a representative of at least three independent experiments that produced similar results.

A key step in the progression of fibrosis is the deposition of collagen, which is stimulated primarily by TGF- β 1 (36). CCL2 is considered to be a profibrotic chemokine, as it plays a role in

stimulating fibroblasts to produce collagen by induction and activation of TGF- β 1 (20). In addition, CCL2 is a potent chemoattractant produced by pleural mesothelial cells that induces the migration of macrophages to an area of inflammation and injury (19). Time course experiments demonstrated that CCL2 mRNA and protein were induced transiently by PDGF-BB, whereas NiNPs caused a sustained increase in CCL2 expression (Figures E1 and E2). The combination of PDGF and NiNPs caused a synergistic increase in CCL2 mRNA and protein at 24 hours (Figures 2A and 2B). These data demonstrate a potentially important interaction between NP exposure and an endogenous growth factor that could be important in progression of subpleural fibrosis or pleural cancer. We also demonstrated that PDGF and NiNPs synergistically increased CXCL10 mRNA and protein levels in mesothelial cells (Figures 2C and 2D). The chemokine, CXCL10, promotes the recruitment of many inflammatory cells, such as T cells, monocytes, fibroblasts, and endothelial cells (24). In addition, mesothelial cells are a significant source of CXCL10 during inflammation (37). Elevated levels of CXCL10 have been found to be indicative of injury and inflammation associated with pulmonary fibrosis (38). Unlike CCL2, which is profibrogenic, CXCL10 is antifibrogenic, and transgenic mice that lack CXCL10 or its receptor, CXCR3, have exaggerated lung fibrosis (24, 27). Furthermore, CXCL10 is involved in the early stages of peritoneal wound healing by preventing the formation of fibrotic adhesions (39). Therefore, CXCL10 is likely a protective response to injury. Although CCL2 and CXCL10 have opposing roles in fibrosis, it is equally important to understand the regulation of both of these chemokines, because they likely have important functions in pleural fibrosis and the resolution of pleural fibrosis, respectively.

We identified ERK-1,2 as a key signaling intermediate in the molecular mechanism by which PDGF-BB and NiNPs synergistically increased CXCL10 and CCL2. PDGF-BB binds to the extracellular domain of PDGF-R α and/or PDGF-R β transmembrane subunits to form dimeric PDGF-R $\alpha\beta$ or PDGF-R $\beta\beta$ on the cell surface (10). The autophosphorylation on tyrosine residues within the intracellular domain of these dimeric receptors initiates activation of the intracellular ERK-1,2 signaling pathway. ERK-1,2 signaling can regulate multiple processes, including cellular proliferation and cell cycle progression, depending on the duration of the signal and cell type (40). Furthermore, prolonged ERK-1,2 phosphorylation leads to differences in

Figure 6. HIF-1 α and CCL2 expression induced by NiNPs and PDGF is reduced by the antioxidant, N-acetyl-L-cysteine (NAC). NRM2 cells were grown to confluency, serum starved, and then pretreated with 5 mM NAC for 1 hour before adding PDGF (50 ng/ml) or NiNPs (10 μ g/cm²), alone or in combination, for 24 hours. (A) Representative Western blot of HIF-1 α protein levels in cell lysates from NRM2 cells showing that induction of HIF-1 α levels after treatment with NiNPs or NiNPs and PDGF-BB were both reduced by NAC. (B) Densitometry of three independent Western blots, including the representative blot displayed in (A) showing the relative ratio of HIF-1 α to β -actin protein for each treatment. Densitometric analysis was performed using Image J analysis software, as described in MATERIALS AND METHODS. (C) Western blot showing increased levels of p-ERK-1,2 after 24-hour treatment with the combination of PDGF and NiNPs, which were reduced by 1-hour pretreatment with NAC. Note that ERK-1,2 phosphorylation is transiently induced by PDGF alone at 2 hours, and returns to basal levels by 24 hours (see Figure 3). (D) ELISA results showing that CCL2 protein

induced by the combination of NiNPs and PDGF is significantly reduced by pretreatment with NAC. The ELISA data are from a single experiment with samples run in triplicate. * P < 0.05 or ** P < 0.01 compared with cells in the same exposure group treated without NAC, as determined by two-way ANOVA with Bonferroni's post test.

Figure 7. Schematic illustration showing the hypothetical cell signaling mechanism involved in the synergistic induction of HIF-1 α and chemokines by NiNPs and PDGF in rat pleural mesothelial cells. NiNPs are taken up by pleural mesothelial cells to generate reactive oxygen species (ROS), increase HIF-1 α , and stimulate chemokine production. PDGF-BB, a growth factor produced by activated alveolar macrophages or other cellular sources, binds to PDGF-R β on mesothelial cells to stimulate ERK phosphorylation and marginally increase chemokine production. NiNPs are postulated to increase the duration of PDGF-induced ERK phosphorylation, and thereby synergistically increase the production of CCL2 and CXCL10, which play important roles in the progression and resolution of pleural fibrogenesis, respectively.

the expression of immediate early genes (IEGs), which can allow for additional cell growth, differentiation, and survival associated with the development of cancer and disease (41). We observed that NiNPs caused sustained PDGF-induced ERK-1,2 phosphorylation (Figures 3A and 3B), suggesting that this activity could contribute to the increased expression and stabilization of IEGs, which include CCL2 and CXCL10 (17, 42). This was verified upon treatment of NRM2 with the MEK-1/2 inhibitor, PD98059, which blocked ERK-1,2 activity and significantly decreased IEG expression that was induced by the combination of PDGF plus NiNPs (Figure 4A). However, in the absence of NiNPs, ERK-1,2 phosphorylation induced by PDGF was not sustained. Moreover, NiNPs did not alter phosphorylation at the PDGF-R (Figure 3C), suggesting that cross-talk between NiNPs and growth factor signaling occurs downstream of the PDGF-R.

Ni is known to cause oxidative stress by generating a variety of intracellular ROS, such as hydrogen peroxide and oxygen radicals, while decreasing antioxidants, such as glutathione (43). This creates a hypoxic environment in the cell and induces hypoxia-inducible genes, specifically the stabilization and accumulation of the HIF-1 α protein, a primary biomarker of oxidative stress (44). The HIF-1 complex consists of two subunits, HIF-1 α and HIF-1 β , which bind to the hypoxia response element in the promoter region. Whereas HIF-1 β is constitutively expressed, the HIF-1 α protein is stabilized by low oxygen levels and becomes resistant to proteasomal degradation (45). In addition, the transcription of HIF-1 can also be regulated by intracellular signaling intermediates, including MAPKs, such as ERK (44, 46). Our results show that PDGF-BB synergistically increased NiNP-induced HIF-1 α protein levels in NRM2 cells,

but the combination of PDGF and NiNPs had no effect on HIF-1 α mRNA levels (data not shown). This suggests that the increase in HIF-1 α caused by the combination of PDGF and NiNPs was due to HIF-1 α protein stabilization. The induction of HIF-1 α by the combination of PDGF and NiNPs was also significantly reduced by the MEK inhibitor (Figure 5). However, NiNP-induced HIF-1 α levels were not affected by the MEK inhibitor, PD98059. These data indicate that PDGF enhancement of NiNP-induced HIF-1 α was ERK dependent, whereas NiNP-induced induction of HIF-1 α was ERK independent.

As stated previously here, others have shown that ROS, such as hydrogen peroxide, contribute to the stabilization of HIF-1 α protein (43, 44). Recent studies have also shown that nano-sized Ni particles can sustain HIF-1 α activity in human cell lines (47, 48). Therefore, we hypothesized that ROS mediated the induction of HIF-1 α and chemokines that were induced by NiNPs or the combination of NiNPs and PDGF in NRM2 cells. Our results show that NAC significantly decreased total HIF-1 α protein levels after exposure to NiNPs alone and also after the combination of PDGF plus NiNPs (Figures 6A and 6B). In addition, we observed that NAC treatment also significantly reduced p-ERK-1,2 levels and CCL2 protein levels that were induced by the combination of NiNPs and PDGF (Figures 6C and 6D). These data suggest that ROS mediate induction of HIF-1 α by NiNPs and play a role in the synergistic increase in chemokine production stimulated by the combination of NiNPs and PDGF. However, NAC had no effect on CCL2 production induced by PDGF alone (Figure 6D), suggesting that ROS are not required for the PDGF signaling component of this mechanism. Moreover, although HIF-1 α induction and ERK-1,2 phosphorylation by NiNPs and PDGF were almost completely inhibited by NAC at 24 hours, the induction of CCL2 by NiNPs and PDGF was only partially reduced by NAC, suggested that both ROS-dependent and ROS-independent signaling is involved in the induction of CCL2 by the combination of NiNPs and PDGF.

Ni compounds are classified as carcinogenic in humans, causing lung and nasal cancer as well as pulmonary inflammation and fibrosis after chronic exposure (49). Our data demonstrate that PDGF-BB synergistically increased NiNP-induced production of chemokines in rat pleural mesothelial cells via an ERK-1,2-dependent mechanism that appears to be due to NiNPs prolonging ERK phosphorylation after initiation by PDGF-BB. In the context of carcinogenesis, others have shown that an ERK-2 survival pathway mediates resistance of human mesothelioma cells to asbestos injury (50). Therefore, it would be important to know whether mesothelioma cells are resistant to NiNP or MWCNT exposure as a consequence of ERK-2 signaling. In addition, we also found that ERK activation is important for PDGF-BB to increase HIF-1 α , typically associated with hypoxia and oxidative stress, whereas ROS mediated NiNP-induced HIF-1 α . These findings are highly relevant to *in vivo* exposures, as macrophages have previously been shown to accumulate at the subpleural region after exposure to other nanomaterials that cause pleural fibrosis. Macrophages could influence the biological responses of mesothelial cells by triggering increased expression of profibrotic growth factors, such as PDGF-BB, when exposed to NiNPs (Figure 7). In summary, our findings suggest that PDGF-BB is a critical coactivator of NiNP-induced chemokine production at the pleural mesothelium that relies on ERK phosphorylation and ROS generation as a central part of the signaling mechanism.

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