IN VITRO & MOLECULAR TOXICOLOGY
Volume 13, Number 1, 2000
Mary Ann Liebert, Inc.

Changes in F-Actin Organization Induced by Hard Metal Particle Exposure in Rat Pulmonary Epithelial Cells Using Laser Scanning Confocal Microscopy

JAMES M. ANTONINI, KENNETH STARKS, JENNY R. ROBERTS, LYNDELL MILLECCHIA, HUI-MIN YANG, and K. MURALI RAO

ABSTRACT

Chronic inhalation of hard metal (WC-Co) particles causes alveolitis and the eventual development of pulmonary fibrosis. The initial inflammatory response includes a change in the alveolar epithelial cell–capillary barrier, which has been shown to be regulated by the state of assembly and organization of the actin cytoskeletal network. The objective of this study was to evaluate the effect WC-Co particles have on F-actin organization of lung epithelial cells in an *in vitro* culture system. Rat lung epithelial (L2) cells were exposed to 5, 25, and 100 μg/mL of WC-Co particles, as well as the individual components (Co and WC) of the hard metal mixture particles for 24 h. The effect on F-actin organization was visualized by laser scanning confocal microscopy (LSCM) following Bodipy–Phallacidin staining. Minimal changes in the F-actin microfilaments of L2 cells were observed by LSCM after exposure to WC and WC-Co at 5 and 25 μg/mL, while at 100 μg/mL, there was a noticeable disruption in the uniform distribution of L2 cell F-actin microfilaments. After exposure to Co, a dose-dependent change in the F-actin organization of the L2 cells was observed. Little change in F-actin assembly was observed after treatment with 5 μg/mL of Co (the concentration equivalent to the 5% amount of Co commonly present in 100 μg/mL of the WC-Co sample mixture). However, at 100 μg/mL of Co, the microfilaments aggregated into homogeneous masses within the cells, and a significant loss in the organization of L2 F-actin was observed. These dramatic alterations in F-actin organization seen after exposure to the higher doses of Co were attributed to an increase in L2 cell injury as measured by lactate dehydrogenase and trypan blue exclusion. We conclude the pulmonary response evoked in the lung by inhalation of high levels of WC-Co particles is unlikely due to alterations in the F-actin microfilaments of lung-epithelial cells.

INTRODUCTION

HARD METAL (WC-Co) is made of a mixture of tungsten carbide (WC; 80–90% by weight) and cobalt metal powders (Co; 5–10% by weight). This alloy possesses extraordinary properties of hardness and resistance to wear, and thus has a wide range of industrial applications (Lison and Lauwerys, 1995). Occupational inhalation of WC-Co particles has been shown to induce bronchitis, alveolitis, pulmonary fibrosis, and asthma in workers (Chan-

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505.

Yeung and Lam, 1986; Kusaka et al., 1986; Lison, 1996; Ohori et al., 1989). Hard metal-induced lung inflammation is characterized by an increase in the number of neutrophils, macrophages, and eosinophils in bronchoalveolar lavage fluid (BALF) in exposed workers (Ohori et al., 1989). This initial inflammatory response has been shown to include a change in the alveolar epithelial cell–capillary permeability barrier, as evidenced by an increase in the presence of albumin and total protein in the BALF of exposed rats (Lasfargues et al., 1995). The presence of high levels of lactate dehydrogenase (LDH) in the BALF of rats treated with WC-Co particles has also been observed, indicating lung cell membrane damage (Adamis et al., 1977). The pathogenesis of hard metal-induced lung disease however still remains unclear.

Positioned at the interface between the external environment and the underlying tissues of the lung, airway epithelial cells serve as a barrier, limiting the movement of inhaled toxins into the lungs (Walters et al., 1997). It has been well-documented that different inhaled particles, such as silica, residual oil fly ash, and asbestos, damage the airway epithelium and alter the alveolar epithelial cell–capillary permeability barrier (Beck et al., 1982; Kodavanti et al., 1998; Lindenschmidt et al., 1990; Merchant et al., 1990; Peterson et al., 1993). Maintenance of the epithelial barrier is attributed to the assembly and organization of the cytoskeletal network, in particular the actin microfilaments (Ermert et al., 1996; Goldblum et al., 1993; Salva and Waters, 1998). The elaborate distribution of these microfilaments within the cytoplasm and the dynamic nature of their structural organization play an important role in determining cell shape, maintaining cell morphology, and anchoring intracellular organelles (Chou, 1989). The cytoskeleton of pulmonary cells has been shown to be a target of injury for inhaled toxins. Dramatic perturbations in the organization of actin microfilaments of cultured cells have been observed in a time- and dose-dependent manner to arsenic, cadmium, cobalt, chromium, and nickel (Chou, 1989). Ozone exposure has also produced significant changes in actin microfilaments of rat lung epithelial cells, implicating the cytoskeleton as a potential target for inhaled agents (Bhalla et al., 1990).

The objectives of the current study were: (1) to assess the effect WC-Co particles have on F-actin of a rat lung epithelial cell line and (2) to apply a novel system using laser scanning confocal microscopy (LSCM) by imaging the F-actin of lung epithelial cells and quantifying the hard metal-induced changes in F-actin organization. Mature cultures of rat lung epithelial (L2) cells were exposed to different concentrations of WC-Co particles for 24 h. Several experimental investigations have demonstrated that the toxicity of hard metal cannot be explained by its Co content, but results from an interaction between Co and WC (Huaux et al., 1995; Lasfargues et al., 1992; Lison and Lauwerys, 1992, 1994). The L2 cells were also exposed to varying concentrations of the individual components (Co or WC) of hard metal particles. The effect on F-actin organization was then visualized by LSCM following fluorescent labeling of the L2 cells.

MATERIALS AND METHODS

Cells

The rat lung epithelial cell line, L2 (CCL-149), was obtained from American Type Culture Collection (Rockville, MD). The cells were grown in F12 (HAM) culture medium supplemented with 15% fetal bovine serum (FBS), penicillin (110 U/mL), and streptomycin (100 mg/mL).

Hard Metal Particles

Co, WC, and WC-Co (~94% WC/~5% Co) were purchased from Alpha Chemicals (Wardhill, MA). The particle samples were washed with distilled water and sterilized by heating at 200°C overnight. Stock solutions of 1 mg/mL were made in the F12 (HAM) culture medium described above. The size of the different hard metal particles were measured using a TSI-aerodynamic particle sizer (TSI Inc., St. Paul, MN). Particles from all three samples were found to be of respirable size with a count mean diameter of <5 μm.

Hard Metal Particle Treatment

The L2 cells were grown on glass coverslips placed at the bottom of 24-well plates. The cells were exposed to 5, 25, and 100 μg/mL of the

particle samples for 24 hours at 37°C in 5% CO_2. Control cells were exposed to saline. After the incubation period, the cells were washed with phosphate-buffered salt solution (PBS; pH 7.4) and incubated in 100 μl of a cocktail consisting of 6 U/mL BODIPY-FL phallacidin (Molecular Probes, Eugene, OR), 2% paraformaldehyde, 0.1% Triton-X in PBS for 4 h. Fluorescent phallacidins have been shown to stain F-actin at nanomolar concentrations and are extremely water-soluble, thus providing convenient probes for labeling, identifying, and quantitating F-actin in tissue sections and cell cultures (Faulstich et al., 1988; Wang et al., 1982).

Confocal Microscopy

After the 4-hr incubation period, the treated L2 cells were washed with PBS, and the glass coverslips were mounted onto slides using Prolong Mounting Medium (Molecular Probes, Eugene, OR). The F-actin microfilaments of L2 cells were visualized using a Sarastro 2000 laser scanning confocal microscope fitted with an argon-ion laser (Molecular Dynamics, Inc., Sunnyvale, CA with a Optiphot-2, Nikon, Inc., Melville, PA). Micrographs were recorded through a 60× lens objective using a 488-nm laser line. Emission spectra >510 nm was diverted to a photodetector to image F-actin. Reflected light <510 nm was simultaneously passed to a separate optical path, and provided images of WC-Co particles. Images of L2 cell F-actin and WC-Co particles were combined to reveal the position of particles among cells. Changes in cell morphology were assessed by phase contrast microscopy.

Using Image Space software (Molecular Dynamics, Inc.), the changes in F-actin organization were quantified by measuring the number of pixels at a specific pixel intensity between 0–255 within a random image field of treated L2 cells. Forty image fields were measured for each of the particle samples at each dose. All scans

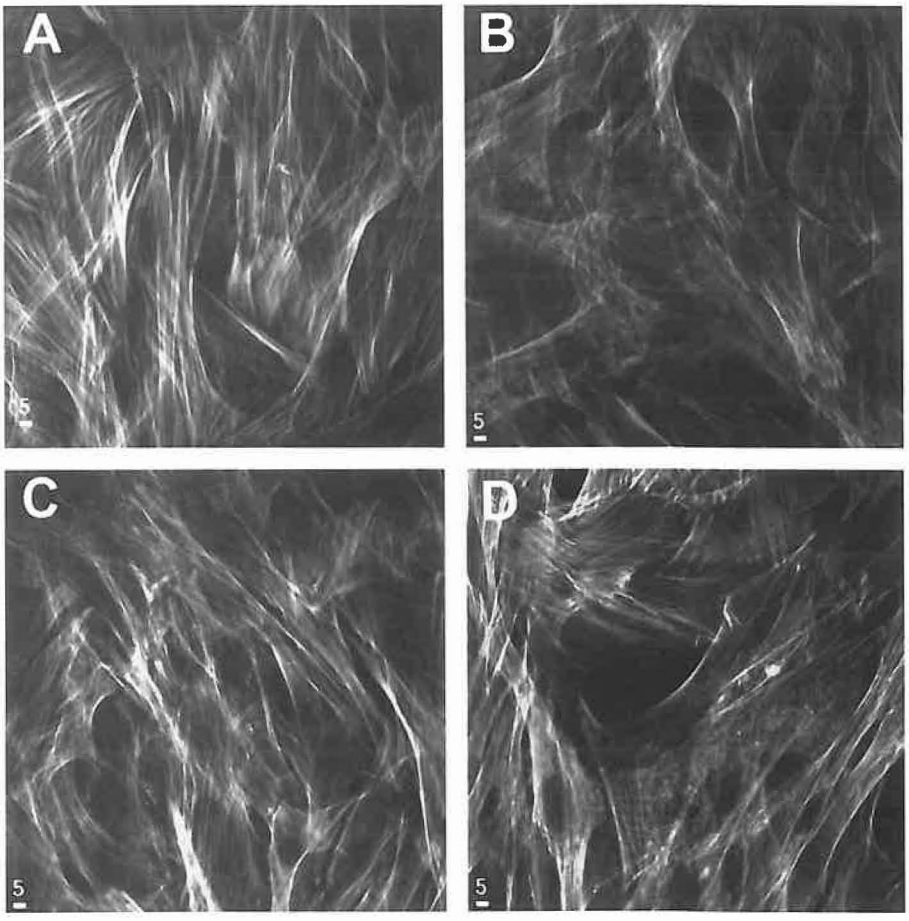

FIG. 1. Confocal micrographs of F-actin microfilaments from rat L2 cells exposed to different concentrations of WC particles: (A) Saline Control; (B) 5 μg/mL; (C) 25 μg/mL; (D) 100 μg/mL. Bars are 5 μm.

of L2 cells were recorded at the same photo multiplier tube settings of 400, a pinhole aperture setting of 50 μm, and a laser voltage setting of 20 mW. Random image fields were collected from four different experiments.

Cell Viability

Lactate dehydrogenase (LDH) was measured spectrophotometrically in the supernatant of the L2 cells exposed for 24 hours at 37°C in 5% CO_2 to the different particle samples. The reduction of pyruvate coupled with the oxidation of NADH at 340 nm was measured according to the method of Wroblewski and LaDue (1955).

To determine the number of cells affected by the hard metal particle treatment, the L2 cells were incubated with the different samples for 24 h at 37°C in 5% CO_2. The control group was treated with saline. After the incubation period, a 0.1% solution of trypsin was added to the wells to remove the cells. Cell viability was measured on aliquots from each well by trypan blue exclusion.

Statistics

For quantification of F-actin changes and viability measurements, an analysis of variance (ANOVA) was performed using the Statview statistical program (Abacus Concepts, Inc., Berkeley, CA). If a significant interaction was present, the significance between each of the individual groups was analyzed using the Fisher's Least Significance Difference post-hoc test. The criterion of significance was $p < 0.05$.

RESULTS

Minimal changes were observed by LSCM in F-actin microfilaments of L2 cells after exposure

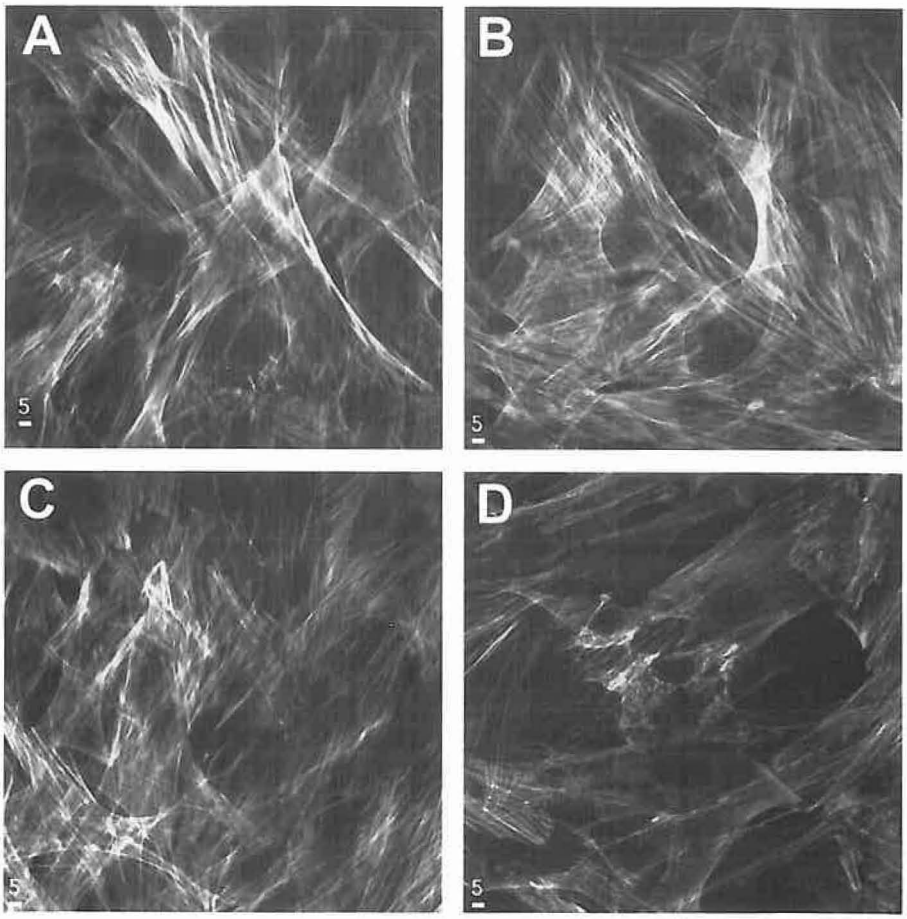

FIG. 2. Confocal micrographs of F-actin microfilaments from rat L2 cells exposed to different concentrations of WC-Co particles: (A) Saline Control; (B) 5 μg/mL; (C) 25 μg/mL; (D) 100 μg/mL. Bars are 5 μm.

to WC and WC-Co at 5 and 25 μg/mL (Fig. 1, and 2B, 2C). At 100 μg/mL, there was a noticeable disruption in the uniform distribution of L2 cell F-actin microfilaments (Figs. 1 and 2D). Areas of F-actin aggregation were observed in the L2 cells exposed to the 100 μg/mL concentration of the WC and WC-Co particles. After exposure to Co, there was a dose-dependent alteration in the F-actin organization of the L2 cells (Fig. 3). Little change in F-actin assembly was observed after treatment with 5 μg/mL of Co (the concentration equivalent to the 5% amount of Co present in 100 μg/mL of the WC-Co sample mixture). At the higher 100 μg/mL concentration of Co, there was a very apparent change in the distribution of L2 cell F-actin microfilaments. The F-actin microfilaments also aggregated into homogeneous masses within the cells (Fig. 3D).

Phase contrast microscopy revealed little change in cell morphology after exposure to all three concentrations of the WC and WC-Co particles and 5 and 25 μg/mL of Co (Fig. 4B and 4C). However, at 100 μg/mL of Co, there was a significant disruption in the L2 cells. Many of the cell-to-cell connections were lost (Fig. 4F). Aggregates of WC-Co particles were easily observed among F-actin microfilaments using reflected light LSCM (Fig. 5).

The changes in F-actin organization were quantified by measuring the number of pixels at different pixel intensities between 0–255 (low intensity sites appear as blue, and increasingly high intensity sites appear as green, yellow, red, or white) within one random image field of L2 cells treated with saline (Fig. 6A) or 100 μ/mL of Co (Fig. 6B). A shift in the pixel number versus pixel intensity curve for control cells would

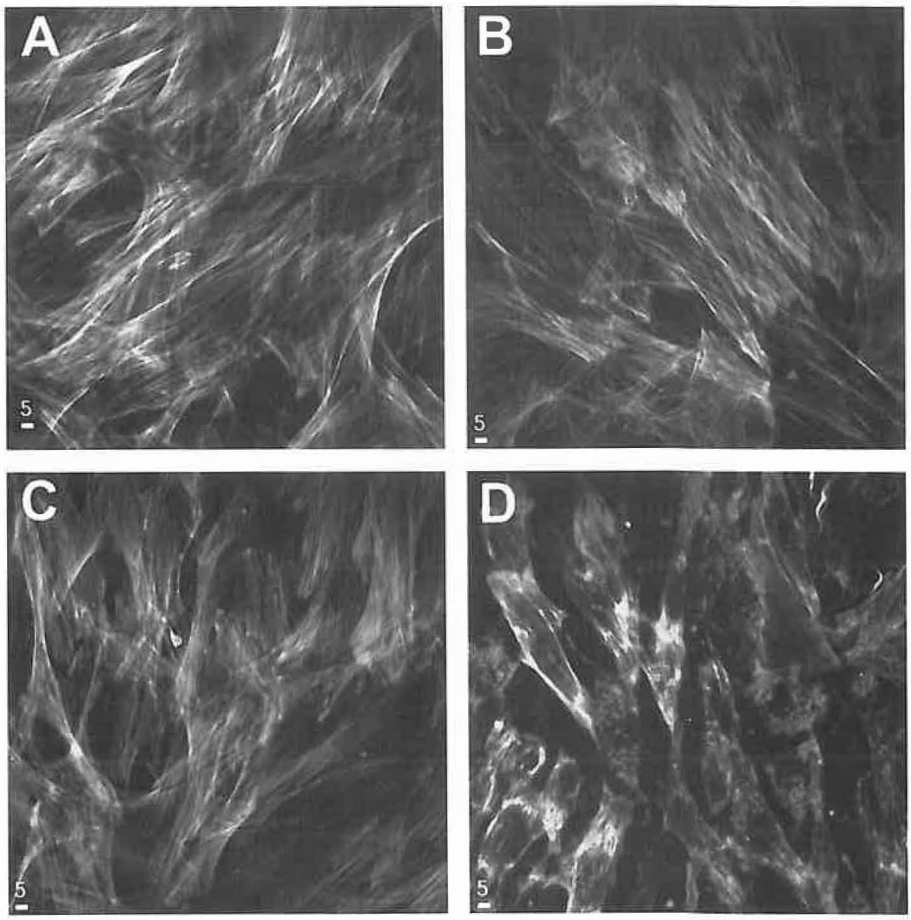

FIG. 3. Confocal micrographs of F-actin microfilaments from rat L2 cells exposed to different concentrations of Co particles: (A) Saline Control; (B) 5 μg/mL; (C) 25 μg/mL; (D) 100 μg/mL. Bars are 5 μm.

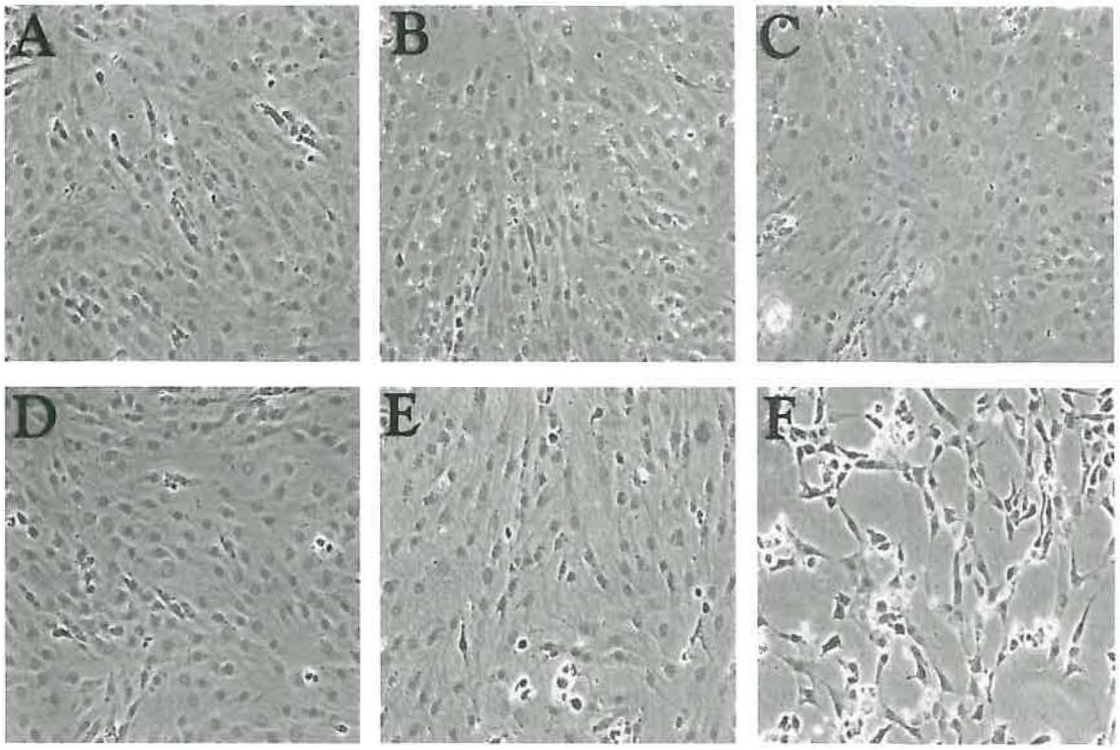

FIG. 4. Phase contrast micrographs of rat L2 cells exposed to hard metal particles: (A) Saline Control; (B) 100 μg/mL, WC; (C) 100 μg/mL, WC-Co; (D) 5 μg/mL, Co; (E) 25 μg/mL, Co; (F) 100 μg/ml, Co. (Magnification; 20×.)

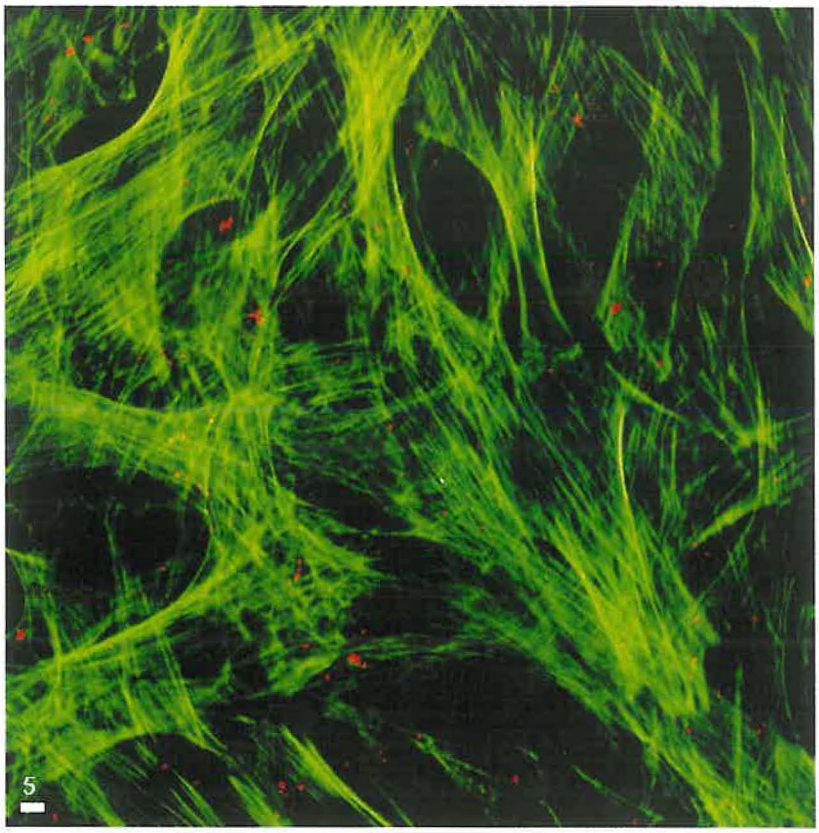

FIG. 5. Confocal micrograph of rat L2 cells exposed to 5 μg/mL WC-Co particles. Accumulations of WC-Co particles (red) are easily identified among F-actin microfilaments. Bar is 5 μm.

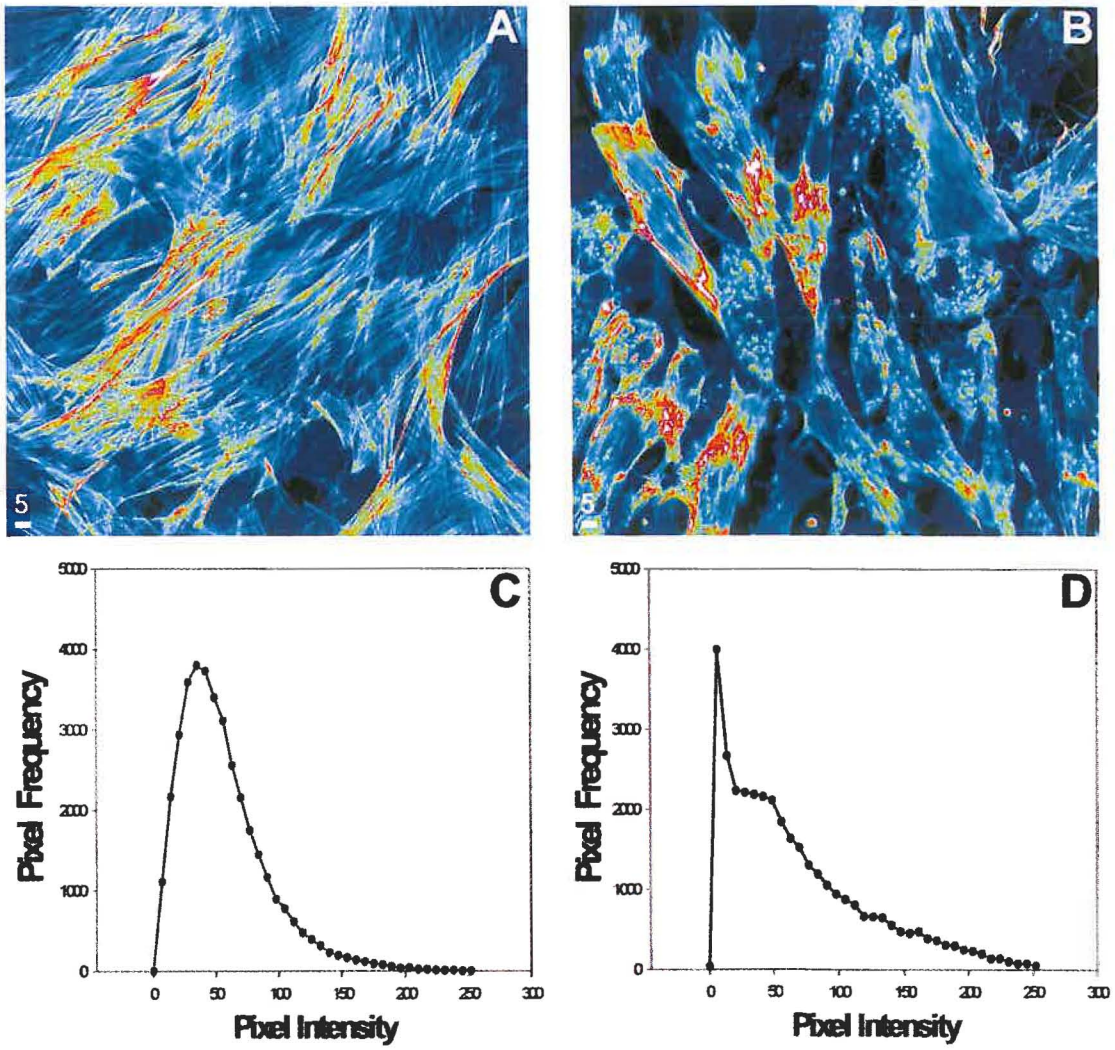

FIG. 6. Pseudo-color confocal micrographs of F-actin microfilaments and quantification curves of F-actin changes from one random field of imaged rat L2 cells exposed to: (A, C) Saline Control; (B, D) 100 μg/mL, Co. Low-intensity sites appear as blue, and increasingly high-intensity sites appear as green, yellow, red, or white in (A) and (B). Bars are 5 μm in (A) and (B).

be representative of changes in fluorescent labeling (i.e., cytoskeletal organization) of F-actin. A shift to the left in the quantification graph of Co was observed, indicating an increase in areas of little or no fluorescence and a loss in the distribution of the F-actin microfilaments as compared to the saline control (Fig. 6C and 6D). In randomly analyzing forty different fields after particle exposure, little or no differences in F-actin changes were observed for the WC and WC-Co groups at all three doses when compared with the saline control (Fig. 7A and 7B). At 100 μg/mL, there was a significant shift to the left in the quantification curve for Co, indicating an increase in regions of minimal fluorescence and a loss in the distribution of the F-actin microfilaments as compared to the saline control (Fig. 7C).

To assess L2 cell viability after exposure to the particle samples, LDH was measured in the supernatant of the treated cells (Fig. 8). Treatment of L2 cells with WC and WC-Co at all three concentrations studied caused no change in LDH release as compared to the saline control. At 25 and 100 μg/mL of Co, a significantly greater release of LDH by the L2 cells was observed as compared to the other groups. However, after treatment with 5 μg/mL of Co (the concentration equivalent to the 5% amount of Co present in 100 μg/mL of WC-Co sample mixture), no differences in LDH release were seen when compared with the WC, WC-Co, and

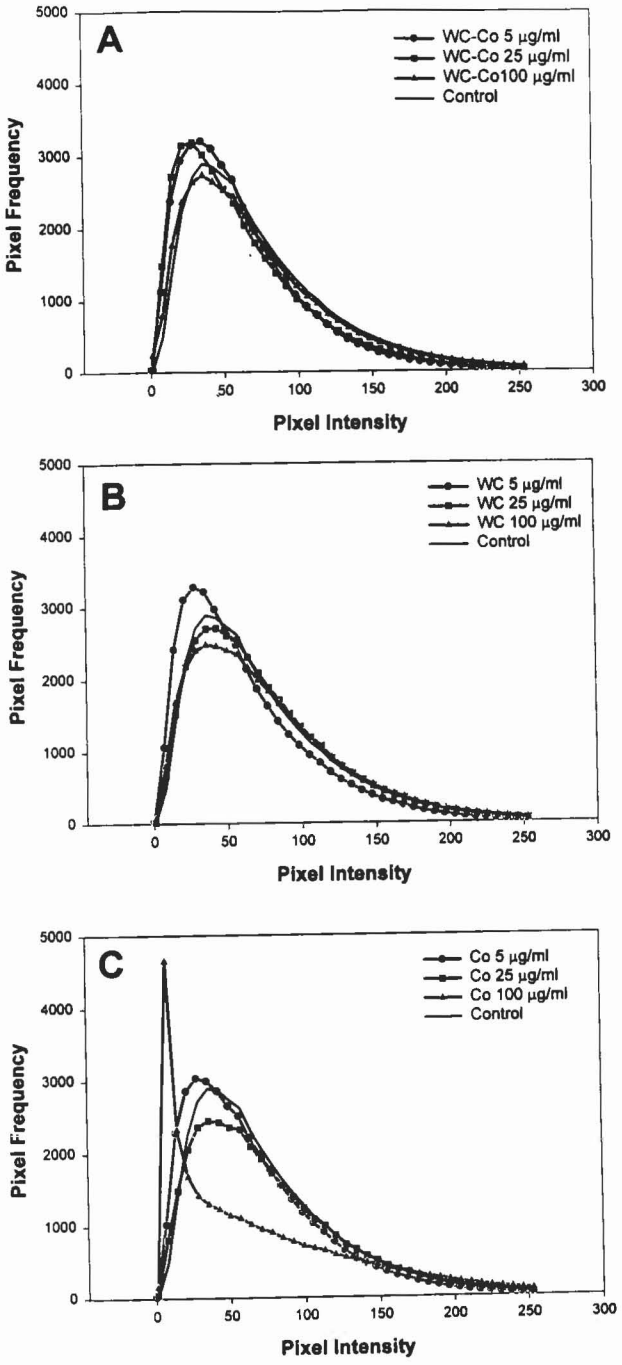

FIG. 7. Quantification curves of F-actin changes in rat L2 cells exposed to: (A) WC-Co; (B) WC; (C) Co. Values are means; $n = 40$ random fields of imaged rat L2 cells after treatment with the different particle samples.

saline control groups. To determine the number of L2 cells affected by the hard metal particle treatment, the ability of the L2 cells to exclude trypan blue was determined (Table 1). As seen with the LDH assay at 25 and 100 µg/mL of Co, significantly more L2 cells were affected as compared with the other groups.

DISCUSSION

It is well established that occupational exposure to WC-Co particles has been associated with adverse respiratory health effects in humans (Kusaka et al., 1986; Lison 1996; Ohori et al., 1989). The pathogenesis of hard metal-induced lung diseases as well as the early pathological changes observed after exposure to WC-Co particles still remains undefined. Most animal *in vivo* studies examining hard metal disease have been purely descriptive and have failed to offer any definitive information on the mechanism of the human response to WC-Co particles (Lison et al., 1996). Numerous *in vitro* studies have contributed a better understanding to the basic biochemical and molecular mechanisms of how WC-Co particles interact with cells to elicit inflammation and injury (Lison and Lauwerys, 1992, 1994, 1995). These studies, however, have been limited to the effects of WC-Co particles on macrophages since this cell type constitutes one of the first steps in the initiation of the inflammatory process.

It was our goal to evaluate the effect WC-Co particles have on another cell type—the lung epithelial cell. Airway epithelial cells are in a particularly vulnerable location because they are

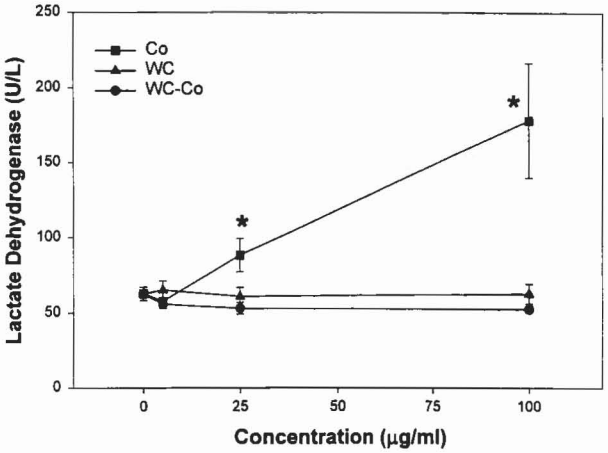

FIG. 8. Lactate dehydrogenase release from rat L2 cells exposed to WC, WC-Co, and Co particles for 24 h. Values are means ± S.E. *Significantly greater than control value ($p < 0.05$).

TABLE 1. L2 CELL VIABILITY

Treatment Groups	*Concentration (μg/mL)*	*% Dead L2 Cells*
Saline control	0	6.75 ± 1.60
Co	5	9.75 ± 0.85
	25	31.8 ± 2.69*
	100	54.3 ± 2.87*
WC	5	6.50 ± 0.87
	25	8.75 ± 1.31
	100	10.5 ± 0.65
WC-Co	5	8.50 ± 0.65
	25	7.75 ± 1.11
	100	10.3 ± 1.31

Note: Values are means ± S.E.: $n = 4$.
*Significantly greater than control value ($p < 0.05$).

constantly exposed to inhaled particulates and form the first physiological barrier to these agents (Rochelle et al., 1996). Experimental studies have suggested that any inhaled particle that lands on an epithelial surface and is not phagocytized by a macrophage or removed via mucociliary transport will likely enter the underlying epithelial cell (Churg, 1996; Keeling et al., 1994). The cytoskeleton of lung epithelial cells has been shown to be a target of injury for inhaled metals and oxidants. Arsenic, cadmium, cobalt, chromium, and nickel (Chou, 1989) as well as ozone exposure (Bhalla et al., 1999) have been shown to induce significant alterations in the organization of actin microfilaments of cultured cells.

The mechanisms by which metals cause injury to cytoskeletal components are not completely understood. Cadmium-induced changes in cytoskeletal elements have been shown to occur by activation of calmodulin (Perrino and Chou, 1986). Disassembly of the cytoskeleton mediated by cadmium and calcium can be prevented by calmodulin inhibitors. Both cobalt and nickel have been observed to cause polymerization of cytoskeletal components (Lin and Chou, 1990; Wallin et al., 1977). Cellular glutathione has also been shown to play an important role in protecting the cytoskeleton after insult from cadium nickel, and arsenic exposure (Li and Chou, 1992; Li et al., 1993).

In the current study, we assessed the effect WC-Co particles have on F-actin of a rat lung epithelial cell line using LSCM. LSCM offers many advantages over conventional microscopic techniques. The shallow depth of field of confocal microscopes allows information to be collected from a well-defined optical section rather than from most of the specimen as in the conventional light microscope (Weight et al., 1993). LSCM optics remove flare emitted in the plane of focus and reject out-of-focus light from underlying and overlying structures of a sample, resulting in an increase in contrast, clarity, and detection sensitivity (Inoue, 1989; Shotton, 1989). Using basic image analysis and morphometric procedures, LSCM is, therefore, a potentially powerful tool for quantitative studies.

Confocal images of fluorescently labeled F-actin of rat L2 cells were generated using LSCM after exposure to different concentrations of WC-Co particles and its individual components, Co and WC. Only at the highest concentration (100 μg/mL) examined were noticeable changes observed in F-actin organization after treatment with WC-Co particles. The F-actin response to WC treatment was similar to what was observed after exposure to the WC-Co particles. This similarity in response between the two particle samples was not surprising because WC-Co used in this study is comprised of ~94% WC. While the concentration of 100 μg/mL of WC-Co and WC needed to induce slight changes in F-actin was high, it still did not cause L2 cell injury. LDH release by L2 cells after treatment with WC-Co and WC was no different from saline controls.

A small fraction of the WC-Co particles is Co (~5%). No change in F-actin assembly was observed after treatment with 5 μg/mL of Co (the concentration equivalent to the 5% amount of Co present in 100 μg/mL of the WC-Co sample mixture which caused a noticeable response). This small effect in F-actin organization induced by 5 μg/mL of Co as compared with WC-Co particles is supported by other studies. The WC-Co particles produced lung parenchymal lesions in the rat that were much more severe than those induced by Co particles alone (Lasfargues et al., 1992). This difference in response between the WC-Co and Co particles has also been reproduced ***in vitro*** in macrophages (Lison and Lauwerys, 1990, 1995). However, in the current investigation, when concentrations of Co were increased above the 5% normally found in WC-Co particles, a dramatic alteration in F-actin organization of L2 cells was observed. These significant changes in cytoskeletal assembly found at the higher concentrations of Co were likely due to L2 cell damage. At 25 and 100 μg/mL, a significant increase in the release of LDH by the cells was observed.

To quantitate the changes in F-actin organization after exposure to WC-Co particles, the number of pixels at different pixel intensities of randomly scanned areas of F-actin of L2 cells was measured. A shift in the pixel number/pixel intensity curve would be representative of changes in fluorescent labeling (i.e., cytoskeletal organization) of F-actin. No changes in F-actin assembly were measured by this method for Co at 5 μg/mL and the WC and WC-Co groups at all three concentrations when compared with the saline control. Thus, the slight but noticeable changes in the F-actin microfilaments after exposure to WC and WC-Co as observed by microscopy did not cause significant differences in the pixel number versus pixel intensity curve. The effects then of WC and WC-Co particles on F-actin of L2 cells may be minimal. However, in the quantification of the significant alterations in F-actin induced by the high 100 μg/mL concentration of Co, a dramatic shift to the left in the pixel number versus pixel intensity curve was observed as predicted when the cytoskeleton was perturbed. This shift in the quantification curve is indicative of an increase in the number of pixels with less pixel intensity (i.e., an increase in areas of little or no fluorescence).

In summary, *in vitro* exposure of L2 cells to WC-Co particles was performed in an attempt to determine a potential mechanism by which WC-Co particles may injure the lungs. Slight but noticeable changes in F-actin organization of L2 cells were observed after exposure to high concentrations of WC-Co and WC particles. Co (at 5% of the WC-Co concentration shown to alter F-actin) had no effect. We then conclude the pulmonary response evoked in the lung by inhaled WC-Co particles is unlikely due to alterations in the F-actin of lung epithelial cells. Additional studies are underway examining the effects of hard metals on the cytoskeleton of other lung cells (i.e., macrophages) and also on other cytoskeletal components (i.e., microtubules).

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Address reprint requests to:
James M. Antonini, Ph.D.
Health Effects Laboratory Division
National Institute for Occupational Safety and Health
1095 Willowdale Road (M/S 2015)
Morgantown, WV 26505

E-mail: jga6@cdc.gov

Volume 13, Number 1, Spring 2000
ISSN: 1097-9336

IN VITRO & MOLECULAR TOXICOLOGY

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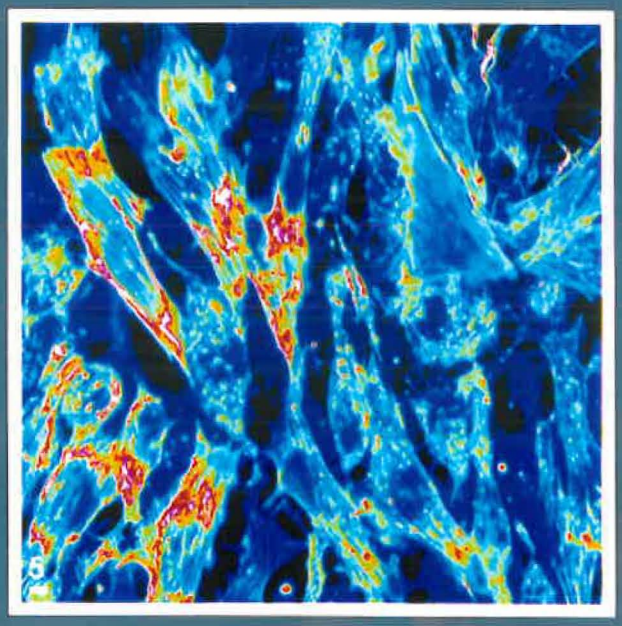

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