THE EFFECT OF NICKEL ON THE FIBROBLAST-MEDIATED COLLAGEN MATRIX CONTRACTION

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Abstract.We report the effect of nickel chloride on fibroblast-mediated collagen gel contraction, fibroblast migration and pattern of matrix metalloproteinase (MMP) activities expressed by human embrzonary lung fibroblasts (HELFs). After the treatment with nickel chloride, gelatin zymography, casein zymography and also western blot assay revealed a significant decrease of MMP-2 and MMP-1 active forms. MMP-inhibition was associated with a reduction of HELF-mediated collagen matrix contraction. A dose of 10 mM $NiCl_2$ completely suppressed matrix contraction and cell migration.

Key words: lung fibroblasts, nickel, collagen matrix contraction, matrix metalloproteinases.

INTRODUCTION

Nickel is a metallic element that is naturally present in the earth's crust. Due to unique physical and chemical properties, metallic nickel and its compounds are widely used in modern industry. The high consumption of nickel-containing products inevitably leads to environmental pollution by nickel and its by-products at all stages of production, recycling and disposal. Human exposure to nickel occurs primarily via inhalation and ingestion. Significant amounts of nickel in different forms may be deposited in the human body through occupational exposure and diet over a lifetime. Since nickel has not been recognized as an essential element in humans it is not clear how nickel compounds are metabolized. It is known, however, that exposure to nickel compounds can have adverse effects on human health. Inhalation of metal dusts and fumes can cause a variety of pathophysiological responses including inflammation, airway hypersensitivity reactions, parenchymal diseases and cancer. The cellular and molecular mechanisms of action of metals in the lung are unresolved and involve complex pleiothropic effects. These effects are mediated by direct reaction of the metals with cellular macromolecules and indirect effects of reactive oxygen species generated when cells are exposed to metals. This article focuses on nickel which

remains on the list of the top 33 hazardous air pollutants of greatest concern, associated with high incidence of noncancerous respiratory diseases such as asthma, fibrosis and chronic obstructive pulmonary disease [11].

The movement of cells through extracellular matrix (ECM) and contraction of collagen containing tissues are fundamental to the biology of morphogenesis [21], development [19], wound healing, and scarring through the body [7, 13, 14]. Fibroblasts are key players in tissue repair. After injury, quiescent fibroblasts in the surrounding matrix are activated to proliferate and migrate into the wound site to deposit and remodel the new matrix.

The matrix metalloproteinases (MMP) are a family of enzymes capable of cleaving components of the ECM. The requirement of MMP activity for the penetration and movement of several cell types through the ECM has been identified [17, 18]. These reports suggest that movement of cells through ECM and subsequent matrix contraction may involve MMPs.

Fibroblast culture systems are routinely used to investigate tissue contraction under a wide range of experimental conditions. Fibroblast-collagen matrices have been extensively used to study fibroblast function. Fibroblast-mediated collagen matrix contraction has been used as *in vitro* model for investigating the biological mechanisms of tissue contraction [12, 16] and also the effects of various compounds on the rate of contraction [20].

In this study we examined the effects of nickel chloride on the fibroblast mediated collagen matrix contraction and cell migration. We also performed studies of the expression of MMPs by human embryonary lung fibroblasts (HELF) interacting with collagen gels.

MATERIALS AND METHODS

Cell Culture. HELFs were obtained in our laboratory by the explant culture method from lung biopsies. HELFs were seeded at a density of 2×10^5 cells per well in a six-well plate. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were studied between passages 5 and 7. After the culture reached 60 % confluence, the monolayers were rinsed with PBS and incubated at 37°C for 24 h in DMEM + 1% BSA (bovine serum albumin) without FBS plus various concentrations of $NiCl_2$ (100 µM-20 mM). Controls for these experiments consisted of similar cells incubated in DMEM + 1% BSA alone.

Preparation of cell-populated three-dimensional collagen matrices. Collagen matrices were prepared with rat tail type I collagen at 6 mg/ml in 0.5 M acetic acid solution. The collagen solution was then mixed on ice with DMEM containing 10% FBS and 0.1 N NaOH. Fibroblasts were immediately mixed with

the neutralized collagen solution (final concentration of 3 mg/ml). Gel contraction studies were performed in 24-well plates in which each well received 0.5 ml of this final mixture containing 5×10^5 cells plus.

Preparation of nested collagen matrices. Cells in neutralized solutions of collagen obtained as mentioned above were placed in 24-well culture plates. After 30 minute polymerization at 37°C in a CO_2-humidified incubator, matrices were gently released from the underlying culture dishes with a spatula. Matrices were cultured in growth medium for 24 h to allow remodelling and contraction. Collagen solution without cells was prepared as above and placed over the contracted collagen gel. After 30 minutes at 37°C, DMEM/FBS or DMEM/FBS plus 10 mM $NiCl_2$ were added. Nested collagen matrices were cultured for 72 h.

Gel contraction experiments. To assess the effect of nickel chloride on the fibroblast mediated matrix contraction, the overlaying media of the gels received varying concentrations of nickel chloride (100 μM-20 mM) for 24 h. Cell morphology within the collagen matrices was monitored by phase contrast microscopy (Nikon Eclipse TS 100). The gels were photographed with a digital camera (Nikon CoolPix 4500). The ability of the fibroblasts to contract the gels was determined by quantifying the area of the gels using an image analyzer software (SigmaScan).

Gelatin and casein zymography. Cell culture media were used for gelatin and casein zymography according to the method of Kleiner and Stetler-Stevenson [10] with minor modifications. To detect MMP activity, culture-conditioned, serum-free media were collected and analyzed by zymography. In order to normalize the MMP activity in the different samples, the volume of samples used was adjusted to the same quantity of total protein in each lane. Samples were electrophoresed on sodium dodecyl sulphate (SDS)-7.5% polyacrylamide gels containing 0.1 % gelatin and 0,1% casein, under non-reducing conditions, for MMP-2 and MMP-9 detection, respectively MMP-1 detection. Following electrophoresis, gels were washed in 2.5% Triton X-100 in order to eliminate SDS and then incubated in 0.05 M Tris buffer (pH 7.6) containing 0.01 M $CaCl_2$, 0.05 M NaCl and 0.05 % Brij 35 for 24 h at 37°C. Gels were stained with 0,1 % Coomassie Brilliant Blue R250 in 50 % methanol and 10 % acetic acid to reveal zones of lysis within the gelatin and casein gel and destained in a mixture of methanol:acetic acid:water (1:1:8). Molecular sizes of the bands displaying enzymatic activity were identified by comparison to prestained standard proteins (Sigma Chemical Co.).

Western Blot Analysis. To identify **MMP-2 and -9** proteins present in each fibroblast-conditioned medium, **western blot** analysis was performed using their specific antibodies. Equal loading of protein was assured by prior quantitation using the Lowry assay. Protein aliquots were separated by gel electrophoresis in 7.5% SDS-polyacrylamide minigels and transferred onto a polyvinylidene difluoride membrane (Sigma-Aldrich). Rabbit anti-human **MMP**-2 or anti-human **MMP**-9 antibodies (Sigma) were used at a dilution of 1:1000. A goat anti-rabbit

antibody conjugated to alkaline phosphatase (Sigma) was used as a secondary antibody (1:10000). Bands were visualised using BCIP/NBT as chromogenic substrate.

RESULTS

Cellular morphology in collagen matrices. During collagen matrix contraction, the cells were initially round within the matrix and most cells progressed from the initial development of small processes to a stellate and spindle-shaped appearance. Exposure of HELFs to different doses of nickel chloride (100 µM, 1 mM, 10 mM and 20 mM) resulted in dose-dependent morphological changes of cells (Fig.1). HELFs, grown for 24 h in serum-free DMEM medium, have an acicular shape with slender lamellar expansions. The treatment with doses higher than 1 mM NiCl2 for 24 h showed cytotoxic effects, characterized by the death of cells which display a round shape.
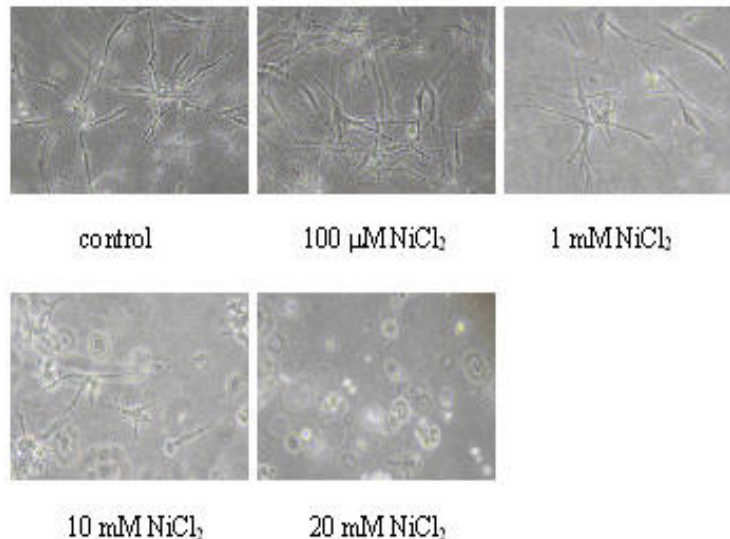

Fig. 1. Morphological appearance in phase-contrast light microscopy of human embryonary lung fibroblast cultures in three dimensional collagen matrices after 24 h of treatment with $NiCl_2$.

The production of MMPs during HELF-dependent collagen matrix contraction. By gelatin zymography of the HELF culture media we have identified the presence of lytic bands at approximately 59, 62, 72, 82 and 92 kDa. These bands were not observed in the presence of 1,10-phenantroline (10mM), confirming that they represent MMP activities. These bands correspond to MMP-2 and MMP-9 in their active and latent form (Fig. 2).The most abundant form of gelatinolytic activity in the conditioned medium of HELFs was detected at an apparent molecular weight of 62 kDa. Following the treatment with nickel, HELFs

displayed a MMP-2 (62 kDa and 59 kDa) gelatinolytic activity lower than that detected in the control cell conditioned medium.
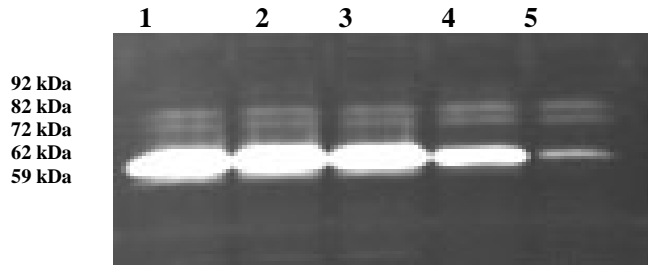

Fig. 2. MMP activity expressed during collagen contraction by HELFs seeded within collagen matrix, in conditioned medium treated with different concentrations of nickel chloride: lane 1: control; lane 2: 100 μM $NiCl_2$; lane 3: 1 mM $NiCl_2$; lane 4: 10 mM $NiCl_2$; lane 5: 20 mM $NiCl_2$.

Casein zymography revealed the presence of the active forms of MMP-1 (48 and 42 kDa) (Fig. 3). Nickel treated HELFs exhibited a significant decrease of the MMP-1 active forms.
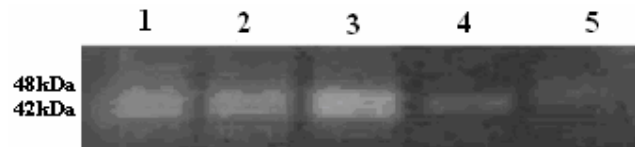

Fig. 3. Casein zymography of the conditioned medium of HELFs seeded within collagen matrix treated with $NiCl_2$: lane 1: control; lane 2: 100μM $NiCl_2$; lane 3: 1 mM $NiCl_2$; lane 4: 10 mM $NiCl_2$; lane 5: 20 mM $NiCl_2$.

The western blot analysis confirmed the gelatin zymography results (Fig. 4). A progressive reduction of MMP-2 expression with the increase of nickel chloride concentration was observed.
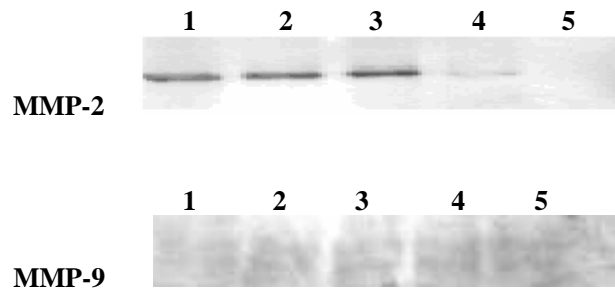

Fig. 4. Western blotting for MMP-2 and MMP-9 of the conditioned medium of HELFs seeded within collagen matrix treated with $NiCl_2$: lane 1:control; lane 2: 100 μM $NiCl_2$; lane 3: 1 mM $NiCl_2$; lane 4: 10 mM $NiCl_2$; lane 5: 20 mM $NiCl_2$.

Migration of fibroblasts in nested collagen matrices. Human fibroblasts were cultured in floating collagen matrices 24 h. Fig. 5 shows the cells within contracted matrices, visualized by phase-contrast microscopy. Cells tended to be bipolar with dendritic extensions. No particular cell orientation in the matrices was evident. Contracted gels were embedded in cell-free matrices. Subsequently, cell migration into the surrounding cell-free matrix was analyzed. Elongated fibroblasts were seen oriented towards and stretching across the contracted gel border (Fig. 6) Migrating cells typically were bipolar with leading dendritic processes. No cell migrating from contracted gels was seen in the presence of 10 mM $NiCl_2$. Moreover, in this case cells became round as a result of their death. This finding was also supported by lactate dehydrogenase assay (data not shown).

Fig. 5. Appearance of fibroblasts in contracted collagen gel prior to embedding in cell-free collagen matrices.
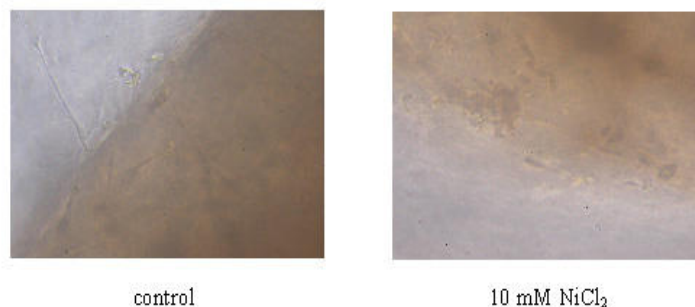

control   10 mM $NiCl_2$

Fig. 6. Cell migration in the nested collagen matrix model. Nested collagen matrices were cultured 72 h in DMEM/FBS or DMEM/FBS plus 10 mM $NiCl_2$.

Effect of nickel on collagen gel contraction. Exposure of fibroblast populated collagen matrices to varying concentrations of nickel for 24 hours influenced gel contraction. A concentration of 10 mM completely suppressed matrix contraction (Fig. 7). Imagistic analysis indicated that collagen matrix area decreased in a nickel dose dependent manner.
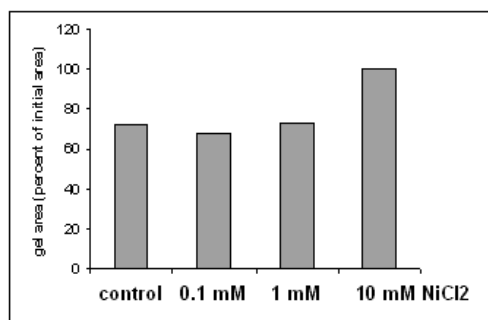
Fig. 7. Effect of nickel chloride on fibroblast mediated collagen matrix contraction.
Each column represents the mean of 3 assays.

DISCUSSION

Remodelling of lung architecture is a hallmark of many lung diseases, for example, loss of alveolar walls in emphysema, subepithelial fibrosis in asthmatic airways, intra-alveolar fibrosis in idiopathic pulmonary fibrosis, cavity formation in tuberculosis, and bronchiectasis in cystic fibrosis. All of these pathologic changes involve extensive alterations of lung extracellular matrix. Fibroblasts are the major type of mesenchymal cells present in the connective tissue matrix. Besides being a structural cell, the fibroblast has the capacity to secrete a number of inflammatory mediators that can drive fibrotic tissue remodelling. For example, matrix components, matrix metalloproteinases (MMP) and pro-fibrotic mediators like TGF-β are produced and secreted by fibroblasts.

Fibroblasts cultured in three-dimensional collagen gels are capable of remodelling their surrounding matrix. Fibroblasts attach to the collagen gels by integrin mediated mechanisms, and by exerting mechanical tension can determine these gels to contract [1, 6]. This contractile process can be modulated by a variety of exogenous mediators and has been used to model the tissue contraction that characterizes both the formation of fibrous scar and fibrosis [15]. Matrix metalloproteinases have been proposed to be key enzymes in inducing these changes because of their capacity to cleave structural proteins such as collagens and elastin. Recent studies showed a relation between high exposure to nickel compounds and a decrease of pulmonary function. Tissue damage caused by inflammation and tissue remodelling contribute to the structural and functional alterations in the lungs. Therefore, *in vitro* models have been developed using HELFs seeded in collagen matrix.

Members of the MMP family are upregulated during a number of biological processes, including tissue morphogenesis, embryonic development, and wound healing [7, 13, 14]. Inhibition of MMP activity may impair the ability of fibroblasts

to migrate into the wound site.The fibroblast-populated collagen *in vitro* model is thought to represent matrix contraction exerted by tractional forces generated by cells as they migrate through the matrix *in vivo* [4, 5, 8]. The MMPs are a unique family of zinc-dependent endopeptidases capable of degrading all the components of the extracellular matrix. They are secreted as inactive proenzymes, activated extracellularly by limited proteolytic cleavage, and inhibited by specific tissue inhibitors of metalloproteinases. MMPs are a continually growing group of enzymes and are classified according to their structural and enzymatic properties: interstitial collagenases, gelatinases, stromalysins, membrane type MMP and others. In this study we explored the pattern of MMP activity expressed by HELFs treated with different concentrations (100 µM – 20 mM) of nickel chloride.

Over a 24 hours period, treatment of HELFs with nickel chloride resulted in inhibition of fibroblast-mediated collagen matrix contraction. There is an inverse relationship between the nickel dose and the degree of contraction. The matrix contraction could be related to the fibroblast migration capacity. Thus, studies by using collagen nested matrices showed migrating cells into the surrounding cell-free matrix.

Our study showed that nickel inhibited MMP activity expressed by HELFs and significantly reduced fibroblast-populated collagen matrix contraction at high doses. These results are according to Daniels et al. [3] who showed that MMP-inhibition significantly reduced fibroblast-mediated collagen lattice contraction. This effect appears to be due to its cytotoxicity because the cells did not remain viable within the collagen matrices at concentrations higher than 1 mM. Accumulating evidence suggests that nickel toxicity is mediated by oxidative stress-induced cell death. However, the molecular signaling underlying nickel-induced apoptosis remains unclear. Since nickel induces oxidative damage resulting in an increase of ROS production [2], it is possible that nickel-induced ROS are involved in apoptosis.

The movement of cells through extracellular matrix and the subsequent contraction of collagen-containing tissues are important components of the wound healing. Our study showed that nickel inhibits fibroblast mediated gel contraction as a result of cell death induction and inhibition of MMPs. This might explain nickel involvement in diseases like lung fibrosis characterized by an imbalance of extracellular matrix remodelling.

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