Induction of apoptosis in human microvascular endothelial cells by divalent cobalt ions. Evidence for integrin-mediated signaling via the cytoskeleton

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Wound healing following implantation is characterized by an acute inflammatory reaction and a subsequent reorganizing phase in which angiogenesis is involved. Endothelial cells (EC) participate in both inflammation and angiogenesis. Thus, the effects on functions of EC exerted by implanted materials could affect the progression of wound healing. The corrosion of metallic implants can cause high concentrations of heavy metal ions in the peri-implant tissues. The purpose of the present study was to test the effects of possible corrosion products on the function and viability of human EC *in vitro*. Long-term exposure of EC to $CoCl_2$ and $NiCl_2$ (3 days, 0.7 mM) leads to a decrease of cell number and changes in cellular morphology. However, the morphological changes between $CoCl_2$- and $NiCl_2$-treated cells differ significantly. The changed morphology of $CoCl_2$-treated EC and the fragmented DNA pattern indicates apoptosis. Nickel-treated cells demonstrated necrosis. The activity of integrins was tested by an assay of cellular adhesion on collagen-coated surfaces. It was shown that the number of adherent cells significantly decreased upon exposure to $CoCl_2$. Our studies suggest that induction of cell death in EC upon exposure to $CoCl_2$ could be attributed to impaired integrin signaling, which leads to a damaged cytoskeleton and culminates in apoptosis.
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1. Introduction

Wound healing following implantation is a complex process that involves cooperation of different cell types (e.g. monocytes/macrophages, mast cells, EC, smooth muscle cells/pericytes), cytokines and extracellular matrix components. Wound healing can be divided into an acute inflammatory reaction and a subsequent phase of reorganization, which is characterized by various stages resulting in the development of a granulation tissue. This reorganizational phase requires the establishment of new blood vessels, formed by the process of angiogenesis.

EC are major participants in inflammation. These cells exhibit cell surface adhesion molecules that mediate EC-leukocyte adherence and transmigration. Furthermore, EC are the crucial cell type in angiogenesis, as they invade the tissues to be vascularized [1]. Thus, effects on the function of EC exerted by an implanted material or its corrosion products could affect the progression of wound healing, both in the inflammatory and in the angiogenic phase.

The corrosion of metallic implants can cause high concentrations of heavy metal ions locally in the peri-implant tissues. Concentrations of cobalt ions detected in tissues of implant revision surgery have been shown to be at least 0.9 mM [2]. Studies on the regulation of the pro-inflammatory phenotype of EC *in vitro* have shown that brief treatment (4 h) with the divalent ions of cobalt and nickel induce a strong cell-surface expression of inflammation-related cell adhesion molecules E-selectin and VCAM-1 with no detectable cytotoxic effect [3]. However, longer exposure times of EC to divalent metal ions lead to cell death.

Recent studies from our laboratory revealed a correlation between heavy metal-induced cytotoxicity and restricted function of endothelial focal contacts (extracellular matrix–cytoskeleton linking regions) [4]. The main transmembrane linker molecules of the focal contacts are the integrins. These adhesion molecules connect proteins of the extracellular matrix to the cytoskeleton. In addition to their role in cell anchorage, integrins are involved in outside in– and inside out–

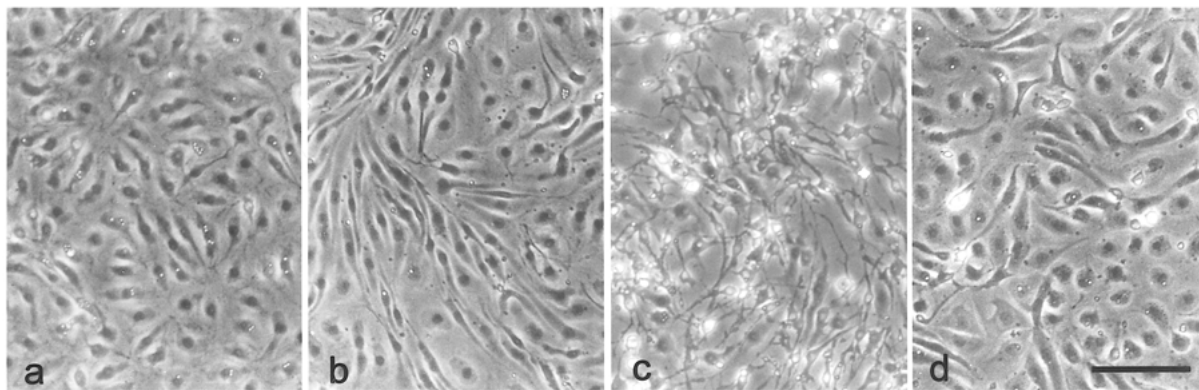* Author to whom correspondence should be addressed.

Figure 1 Morphology of HDMEC upon exposure to CoCl$_2$ und NiCl$_2$ (phase contrast microscopy, $t = 48$ h; a: untreated HDMEC, b: HDMEC treated with 0.3 mM CoCl$_2$, c: HDMEC treated with 0.7 mM CoCl$_2$, d: HDMEC treated with 0.7 mM NiCl$_2$; scale bar = 100 µm).

signaling [5] and are thus involved in cell growth, differentiation and survival. Therefore, heavy metal-induced impaired function of integrins could influence the progression of wound healing by disturbance of the EC-triggered angiogenic response.

This study examines the cytotoxic effects of the divalent cations, cobalt and nickel, in human dermal microvascular EC (HDMEC) *in vitro* with respect to the type of cell death and pathway of induction.

2. Materials and methods
2.1. Cell culture
Human dermal microvascular endothelial cells (HDMEC) were isolated from juvenile foreskin and cultured in Endothelial Cell Basal Medium MV (PromoCell, Germany) supplemented with 15% fetal calf serum (FCS), basic fibroblast growth factor (bFGF, 2.5 ng/ml, Sigma), sodium heparin (10 µg/ml, Sigma), penicillin/ streptomycin (10.000 units penicillin/ml 10.000 µg streptomycin sulfate/ml, Gibco), cultivated in a humidi-fied atmosphere at 37 °C (5% CO$_2$) and used in passage 4.

2.2. Scanning electron microscopy (SEM)
Cells were seeded onto fibronectin-coated glass-cover-slips, treated with 0.7 mM CoCl$_2$- and NiCl$_2$-culture medium for 48 h and fixed in cacodylate-buffered glutaraldehyde (2.5%).

2.3. Cytotoxicity assay
Detection of cytotoxic effects was made indirectly by quantification of mitochondrial dehydrogenase activity via the enzymatic conversion of MTS tetrazolium (Promega Corporation) to a colored formazan product. MTS concentration was used according to the manu-facturer's instructions. The assay was performed in 96-well tissue culture plates and absorbance was quantified at 490 nm.

2.4. Nuclear staining
Hoechst dye 33342 (Sigma) was added to the culture medium (1.2 µg/ml final concentration) and cells were incubated for 10 min at 37 °C.

2.5. Isolation of fragmented DNA
Apoptotic DNA Ladder Kit (Roche) was used according to the manufacturer's instructions. Any remaining RNA was digested by the addition of 2 µg/ml RNase A (Roche). Isolated DNA was electrophoresed in an agarose gel (1% agarose in TBE-buffer) and visualized by UV-excitation of ethidium bromide-labeled DNA.

2.6. Adhesion assay
HDMEC in suspension were incubated with heavy metal ions (1 mM CoCl$_2$, NiCl$_2$ or CrCl$_3$) in serum-free culture medium supplemented with 0.1% bovine serum albumin and 12.5 ng/ml bFGF and seeded onto type I collagen-coated cell culture surfaces (12.000 cells/well, 96-well-plate). Forty-five minutes after seeding the surfaces were washed with pre-warmed phosphate-buffered saline solution and adherent cells were fixed with methanol. The amount of protein was determined by crystal violet staining. Optical density (OD, proportional to cell number) was quantified in a microplate photometer at 600 nm.

3. Results
Incubation of HDMEC with divalent ions of cobalt leads to dramatic morphological changes. In contrast to the typical endothelial cobblestone morphology (Fig. 1a, control, phase contrast microscopy) cobalt-treated cells became spindle-shaped (Fig. 1b, 0.3 mM CoCl$_2$, $t = 48$ h). Higher concentrations resulted in detachment of HDMEC (Fig. 1c, 0.7 mM CoCl$_2$). Treatment of HDMEC with NiCl$_2$ leads to an elongated morphology only at higher concentrations (0.7 mM) but no detach-ment was observable (Fig. 1d).

The dramatic phenotypic changes upon cobalt-treat-ment were confirmed by SEM images: The untreated HDMEC showed an even distribution with closed intercellular contacts (Fig. 2a), whereas CoCl$_2$-treated HDMEC were characterized by long cellular extensions and disintegrated intercellular contacts (Fig. 2b, 0.7 mM CoCl$_2$, $t = 48$ h). In comparable treatment of HDMEC with NiCl$_2$, a similar dramatic phenotypic alteration as observed with CoCl$_2$, did not occur (Fig. 2c, 0.7 mM NiCl$_2$, $t = 48$ h).

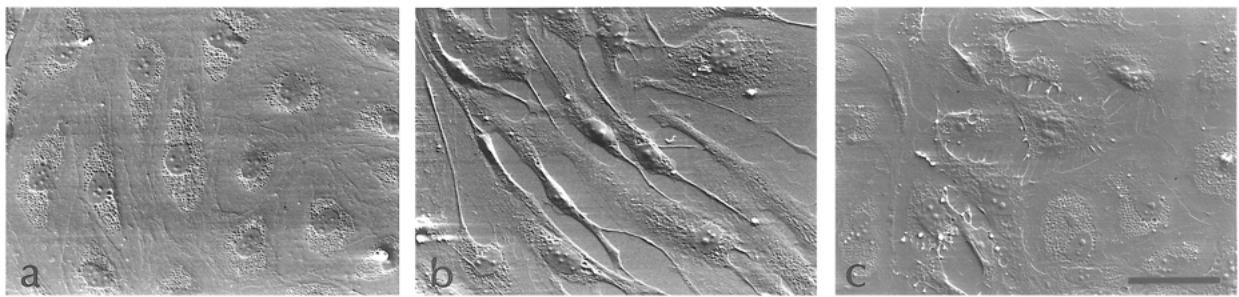Although only the divalent ions of cobalt exert effects

Figure 2 Morphology of HDMEC upon exposure to CoCl$_2$ und NiCl$_2$ (SEM images, $t = 48$ h; a: untreated HDMEC, b: HDMEC treated with 0.7 mM CoCl$_2$, c: HDMEC treated with 0.7 mM NiCl$_2$; scale bar $= 50$ μm).

on endothelial morphology both metal ions (Co^{2+} and Ni^{2+}) exhibited nearly similar, concentration-dependent cytotoxic effects (Fig. 3, $t = 72$ h), as demonstrated by mitochondrial enzyme activity.

Nuclear staining of CoCl$_2$-treated HDMEC reveals a number of condensed, partially fragmented nuclei indicating apoptosis as the process responsible for cell death (Fig. 4). Untreated control and NiCl$_2$-treated HDMEC exhibited no such phenomena.

For further examination of this type of cell death we isolated DNA from cell extracts. DNA from CoCl$_2$-treated HDMEC (0.7 mM, $t = 72$ h) exhibited an internucleosomal fragmentation, the so-called 'apoptotic ladder', in the subsequent electrophoresis (Fig. 5). The NiCl$_2$-treated cells failed to show this phenomenon.

The occurrence of morphological changes and the subsequent detachment upon CoCl$_2$-treatment suggests a disruption of endothelial adherence. Since the function of the adhesion mediators, the integrins, is dependent upon divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}), we tested the adhesion of HDMEC to type I collagen-coated surfaces (substrate for a number of β$_1$-integrins) in the presence of the divalent ions cobalt and nickel, and – as a control – the trivalent ions of chromium.

The short-term exposure of HDMEC to divalent cobalt and nickel ions leads to reduced adhesion, whereas the treatment with the trivalent chromium ion did not (Fig. 6). This supports the hypothesis that integrins are a target for the divalent cobalt and nickel ions in this *in vitro* assay.
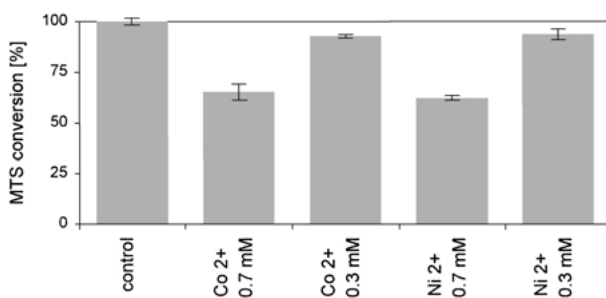

Figure 3 Cytotoxicity assay. MTS conversion of HDMEC exposed to CoCl$_2$ and NiCl$_2$ (respective 0.3 or 0.7 mM) for 70 h. Untreated control was set as 100%.

4. Discussion

In this study we demonstrated that exposure of microvascular EC to Co^{2+} results in changes of the typical cell morphology and long-term incubation leads to cell death by apoptosis. The early, initial events of exposure to Co^{2+} preceding apoptosis, resulting in dramatic morphological changes, indicate an involvement of the cytoskeleton. The connection between Co^{2+}-toxicity and cytoskeleton derangement is strengthened by the detection of reduced adhesion upon heavy metal ion-exposure, since the adhesion to the extracellular matrix by the cells is dependent on the cytoskeleton-linking transmembrane molecules, the integrins. The physiological function of integrins is dependent upon divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}) and their ion binding sites may be a target at which divalent heavy metal ions exert their effects. The link between heavy metal ion-induced cytotoxicity and the cytoskeleton has been previously suggested by our group [4], on the basis of experiments in which heavy metal ion exposure evoked the derangement of focal contacts. Since integrins are involved in cell growth, differentiation and survival [5], impaired integrin function could lead to drastic alterations of endothelial functions. This includes the signaling of apoptosis. The apoptotic reaction in response to Co^{2+}-treatment is unequivocal, since HDMEC exhibited the classical features of apoptosis, including condensed and fragmented nuclei [6] as well as the occurrence of the 'apoptotic ladder' [7, 8].

Apoptosis is a regulated form of cell death which is controlled by a genetic program in the affected cell itself [9]. Apoptosis is energy-dependent since it is an active form of cell death that requires synthesis of mRNA and the activity of specialized enzymes (e.g. caspases) [10]. Necrosis, in contrast to apoptosis, is an alternative form of cell death, that is uncontrolled, mostly concerning cell groups, and characterized by cell lysis [11].

Inhibition of cellular attachment induces apoptosis via an integrin-mediated pathway [12, 13]. Therefore, the reduced adhesion upon Co^{2+}-treatment indicates integrin-impairment, which potentially leads to the initiation of endothelial apoptosis.

Surprisingly, Ni^{2+}-induced cell death of HDMEC appears to be due to necrosis. A possible explanation is that direct cytotoxicity of Ni^{2+} overrides the energy-dependent induction of apoptosis. Since ATP depletion during the early phase of apoptosis does not involve caspase activation, the presence of Ni^{2+} may induce a
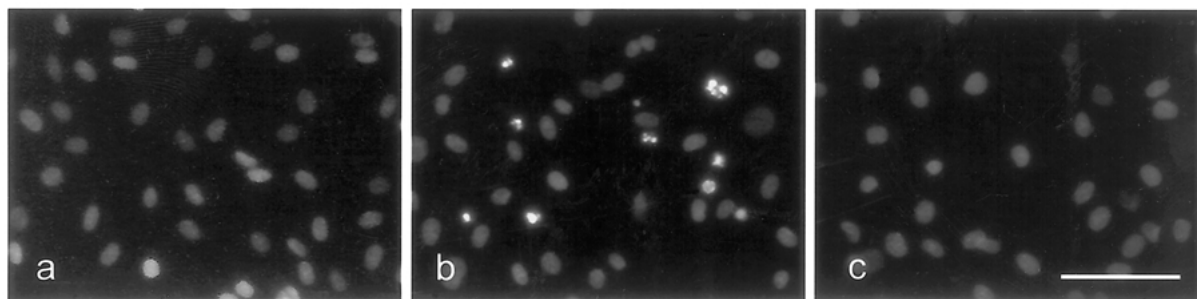
Figure 4 Nuclear staining of HDMEC ($t = 48$ h; a: untreated control, b: HDMEC treated with 0.7 mM CoCl$_2$, c: HDMEC treated with 0.7 mM NiCl$_2$; scale bar = 100 µm).
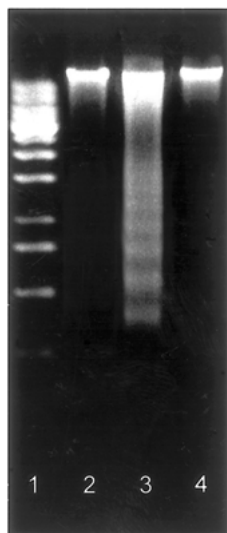

Figure 5 DNA cleavage ('apoptotic ladder') in CoCl$_2$-treated HDMEC (lane 1: DNA size marker, lane 2: control, lane 3: 0.7 mM CoCl$_2$-treated HDMEC, lane 4: 0.7 mM NiCl$_2$-treated HDMEC).
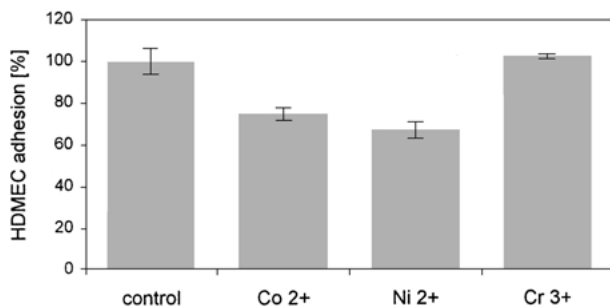

Figure 6 HDMEC adhesion to type I collagen coated surfaces. Amount of protein (corresponding to cell number) was determined by crystal violet staining and the subsequent quantification of optical density. Untreated control was set as 100%.

switch from apoptosis to necrosis [14]. Alternate forms of cell death (apoptosis versus necrosis) in response to different heavy metal ions are currently being examined in further studies.

To summarize, the corrosion product Co^{2+} appears to affect the progression of wound healing upon implantation by disturbance of EC viability and function. The concentrations of cobalt ions detected in the peri-implant tissues *in situ* (0.9 mM) [2] lead to EC activation and apoptosis *in vitro*.

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