JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 798–807

The role of metal corrosion in inflammatory processes: induction of adhesion molecules by heavy metal ions

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Prosthetic devices undergo corrosion processes after implantation including the release of certain amounts of metal ions into the adjacent tissues. On reaching the bloodstream, a systemic influence of those ions may be envisaged. Cell adhesion molecules (CAMs) are recognized as an essential component of the mechanisms of endothelial damage. To study the influence of selected heavy metals on human umbilical vein endothelial cells (HUVEC) EIA methods were used to evaluate cellular expression of E-selectin, ICAM-1, VCAM-1 and GMP-140 under the influence of high (cytotoxic) very low (non-cytotoxic) concentrations of Zn, Ni, Co and Cr. The *de novo* synthesis of CAMs was studied with the help of mRNA analysis. Intermediate voltage immuno electron-microscopical imaging was performed to detect the localization on the cell surface of the adhesion molecules E-selectin and ICAM-1 under the influence of cytokines, which represent important factors in inflammatory processes. Very low concentrations of metal ions, which gave no significant influence on cell morphology, elicited a significant expression of CAMs on endothelial cells *in vitro*. Thus, for example, zinc, nickel and cobalt ions in concentrations of 1×10^{-9} M increased the expression of endothelial E-selectin, compared to the control after a 5 h incubation. Similar findings were established for zinc, nickel and cobalt ions also with regard to ICAM-1, VCAM-1 and GMP-140. Northern blot analysis gave an increased ELAM-1 and ICAM-1 mRNA expression after incubation with high concentrations of zinc and nickel ions. The results should draw attention to possible effects of very low concentrations, which are released during processes of metal corrosion on prosthetic devices.

1. Introduction

It has been recognized that patients with artificial implants are predisposed to infection at the operative site [1]. Those infections are very resistant to non-invasive therapy and in most cases it is necessary to remove the device and debride the site of infection [2, 3]. Particles, metal corrosion complexes and metal ions are suspected of producing an inflammatory response in the surrounding tissues [4, 5]. Early removal of metal devices such as compression plates in cases of bone fractures is discussed in the literature to protect patients from prolonged exposure to metal ions [5]. On reaching the bloodstream, a systemic influence of those ions may be envisaged. Mechanisms of cell–cell interaction, with the recognition and the presentation of cellular adhesion molecules, can be strongly influenced by exogenic factors, e.g. heavy metal ions [6, 7]. Interactions may cause activation of leukocytes and initiate inflammatory processes [8]. Selectins like E-selectin (formerly: ELAM-1, endothelial leukocyte adhesion molecule-1) and GMP-140, as well as the intercellular adhesion molecule-1 (ICAM-1), are involved in the mechanisms of adhesion of platelets and leukocytes to endothelial cells [9–12]. Unstimulated endothelial cells (EC) do not present E-selectin, so that the molecule has to be produced by *de novo* synthesis at mRNA and protein level [13]. Endothelium-presented VCAM-1 is involved in the processes of allograft dysfunction [14, 15]. Various stimuli such as cytokines can induce CAM expression [16]. It has been suggested that cytokines like IL-1, TNFα and metal ions such as $NiCl_2$ may share a common pathway of activation of cellular adhesion molecules [17]. Heavy metal ions stimulate cytokine release by HUVEC [18].

We have been interested in the pathogenesis of inflammation caused by heavy metal ions. So far their effects have been studied in diverse *in vitro* experiments, animal trials and clinical studies, with special emphasis on morphological effects and specific and

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general alterations of cell metabolism of diverse cell types [19–21]. Metals such as cobalt, as well as chromium alloys containing zinc and nickel, are commonly utilized materials in prosthetic surgery. Metal corrosion is suspected of playing an important role in the mechanisms of metal ion release from prosthetic devices, which may contribute to the pathogenesis of inflammation around implants. Human umbilical vein endothelial cells (HUVEC) can be studied under standardized conditions and represent a good model to investigate components of the inflammatory response. Depending on the degree of stimulation, EC control the adhesion and extravasal migration of white blood cells by presenting cellular adhesion molecules [22–24]. The presentation of the CAMs, E-selectin, ICAM-1, VCAM-1 and GMP-140 by HUVEC under the influence of very high (cytotoxic) and very low (non-cytotoxic) molar concentrations of metal ions on the HUVEC cell surface, as well as their *de novo* synthesis at mRNA level were the subject of our studies to evaluate the effects of different doses of zinc, nickel, cobalt and chromium ions. To achieve this we used EIA (enzyme immuno assay) techniques and mRNA analysis. Intermediate voltage immunoelectron microscopy was performed to evaluate CAM expression on cell surfaces and to localize areas and structures on the HUVEC cell surface which show increased expression of CAMs under the influence of cytokines, which are regarded as especially relevant for the understanding of the pathogenesis of adhesional and inflammational processes and which are involved in the processes of metal ion toxicity [17, 18].

2. Materials and methods

2.1. Culturing of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated as described by Jaffe *et al.* [25] and cultured at 37 °C in a carbon dioxide enriched atmosphere with oxygen reduction to 10% by nitrogen addition. The medium was a mixture of Ham's F12 (Gibco, Paisley, UK) and Iscove's modified Dulbecco's media with penicillin-streptomycin and L-glutamine (Gibco). Confluent monolayers were passaged using 0.2% collagenase I (Worthington). In the experiments, EC from passage 1 were used.

2.2. Morphological analysis of HUVEC under exposure to heavy metal ions

HUVEC were seeded into 96-well microtiter pates at a density of 50 000 cells/cm^2. The cells were exposed to defined molar concentrations of zinc, nickel, cobalt and chromium ions for 5 h. After washing, the cells were fixed and stained with hematoxylin-eosin (HE) following standard protocols.

2.3. Quantification of CAM's on the cell surface of HUVEC by enzyme immuno assay techniques

For EIA analysis 50 000 cells/cm^2 were seeded on cell-culture microtiter plates (Becton Dickinson, Lincoln Park, NJ). Cells were exposed to defined concentrations of heavy metal ions (Ni, Co, Zn, Cr) ranging from 1×10^{-9} M up to 1×10^{-2} M and the reference substances lipopolysaccharide (LPS) (1 µg/ml), tumor necrosis factor (TNFα) (30 U/ml, 300 U/ml) as well as IL-1β (10, 100 U/ml), and IFN (interferon-γ, 100 U/ml) for 5 h. Cells were washed with phosphate buffer (37 °C, pH 7.4) in a 96-well microplate washer (ICN, Meckenheim, Germany). The samples were fixed in ethanol/methanol for 45 min and were washed again as described above. Plates were stored overnight at 4 °C until analysis the next day. The microtiter plates were washed again and incubated with a blocking buffer (Boehringer Mannheim, Germany) containing 1% H_2O_2 at 37 °C for 60 min to inactivate unspecific binding-sites. Monoclonal anti-E-selectin in a dilution of 1 : 100 was added. As a second antibody a goat anti-mouse (Amersham, Braunschweig, Germany) antibody was added in a dilution 1 : 1000. The substrate development was achieved by using OPD (o-phenylendamine, Sigma, USA). To stop the reaction 4 M HCl was added. Photometrical evaluation took place with the help of a computer-controlled ELISA-reader (ICN, Meckenheim, Germany) at $\lambda = 492$ nm. Similar to the E-selectin assay procedure, samples were blocked and anti-ICAM-1, anti-VCAM and anti-GMP-140 (all monoclonal antibodies were obtained from British Biotechnology, UK) added at a 1 : 100 dilution. Increased sensitivity was achieved as described above and the same substrate and stopping reagent were used to develop the assay. The photometrical evaluation was performed by using the same methods presented above.

2.4. Determination of E-selectin, ICAM-1 and GMP-140 mRNA: Isolation of RNA and Northern blot analysis

Total RNA was isolated from confluent HUVEC-layers with the help of the guanidine isothiocyanate-phenol-chloroform extraction method described by Chomczynsky and Sacchi [26]. 15 µg RNA/lane were fractionated in a 1.2% agarose-formaldehyde gel, blotted onto nylon membrane filters (Hybond N, Amersham) by capillary blotting overnight and fixed by UV-crosslinking. cDNA-probes were α[^{32}P-d CTP]-labelled by the random prime method. Filters were pre-hybridized for 4 h at 42 °C and then hybridized for 15–18 h with 10^6 cpm/ml hybridization buffer (50% formamide deionized, 5 × SSC, 5% dextrane sulphate, 5 × Denhard's, 1% SDS, 25 mM phosphate buffer pH 6.8 and 0.25 mg/ml herring sperm DNA). Blots were washed twice for 30 min in 2 × SSC, 0.1% at 50 °C and twice in 1 × SSC, 0.1% SDS at 60 °C and then exposed to Kodak XAR-films at − 75 °C with the help of an intensifying screen. Filters were reprobed after stripping with 50% formamide, 1 × SSC at 70 °C.

2.5. Detection of cellular adhesion molecules on HUVEC surface by intermediate voltage electron microscopy

After a 5 h stimulation of HUVEC with IL-1β (100 U/ml) or TNFa (300 U/ml) samples were prepared in a way similar to preparation methods for

immunogold transmission electron microscopy as previously described in the literature [27, 28]. Immunocytochemistry was carried out on samples fixed in 1% glutaraldehyde in phosphate buffer (PBS, pH 7.4). Samples were washed in PBS and Gey's buffer containing 0.1% glycine, 2% bovine serum albumin, 2% gelatin and 5% normal goat serum as blocking agents for non-specific binding. Immunocytochemical processing took place by primary marking with mouse MoAb (monoclonal antibody) anti-E-selectin or anti-ICAM-1 (British Biotechnology) at a dilution of 1 : 100. Following primary antibody, the samples were again washed in Gey's buffer and incubated with a secondary, gold–labelled antibody. The mixture consisted of equal parts of Auroprob G-10 (internal control for non-specific binding) and G-30 (specific marker for the presence of CAM-antibody) (both substances were obtained from Amersham). Following washing, the samples were postfixed in 1% osmium tetroxide and stained with ethanolic uranyl acetate, dehydrated through ethanol and embedded in epoxy resin. Sections were viewed at 300 keV using a Philips CM-30 intermediate voltage electron microscope (IVEM). This latter procedure allowed determination of the three-dimensional distribution of immunogold probe within the endothelial cell monolayer [29].

2.6. Evaluation and statistics

Data were evaluated using student's t-test/f-test: p-values of less than 0.01 were considered to indicate statistically significant differences and calculated with the assistance of Microsoft Excel.

3. Results

3.1. Light microscopical analysis of HUVEC monolayers under exposure to heavy metal ions

Under the influence of the highest molar concentrations of Co (1×10^{-2} M) EC morphology was markedly altered (Fig. 1). The EC appeared spindle-like and degenerated. Cytoplasmatic retractions and naked nuclei could be observed. The exposure to concentrations of 1×10^{-3} M gave reduced but nevertheless distinct effects on the morphology (Fig. 2). Retractions of the cytoplasm as well as single naked nuclei could be found. The confluence of the monolayer was disturbed. Concentrations of 1×10^{-4} M Co gave only minor alterations (Fig. 3) and concentrations below 1×10^{-4} M down to 1×10^{-9} M elicited no significant effects on individual EC or the entire monolayer structure compared to the control (Fig. 4). Chromium ions gave even more severely damaged cells when observing concentrations between 1×10^{-2} M and 1×10^{-4} M. However, concentrations below 1×10^{-4} M elicited no significant effect on the light microscopic morphology of the cultured EC. Zinc and nickel gave only marked effects after incubation with the highest concentrations measured. Thus, a disturbance of the monolayer structure, as well as damage of the individual cells could be observed after exposure to concentrations between 1×10^{-2} M and 1×10^{-3} M. Those alterations were minor in comparison to cobalt and chromium.

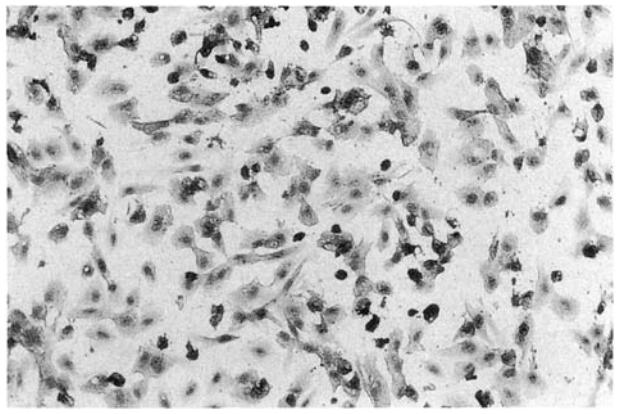

Figure 1 HUVEC monolayer after a 5 h incubation with cobalt at a concentration of 1×10^{-2} M. Cytoplasmic retractions, naked nuclei and spindle cells are shown. HE staining, × 150.

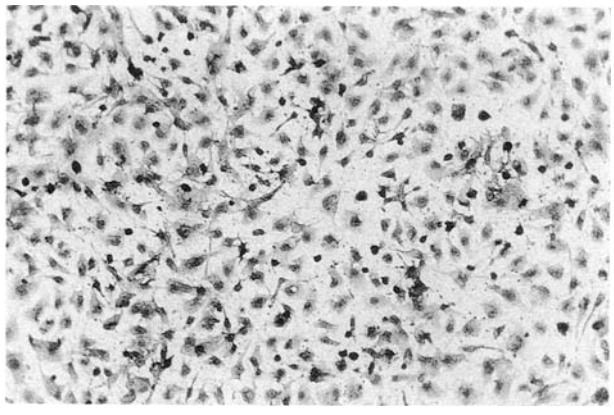

Figure 2 HUVEC monolayer after a 5 h incubation with cobalt at a concentration of 1×10^{-3} M. Cytoplasmic retractions, some naked nuclei and spindle cells are shown. HE staining, × 150.

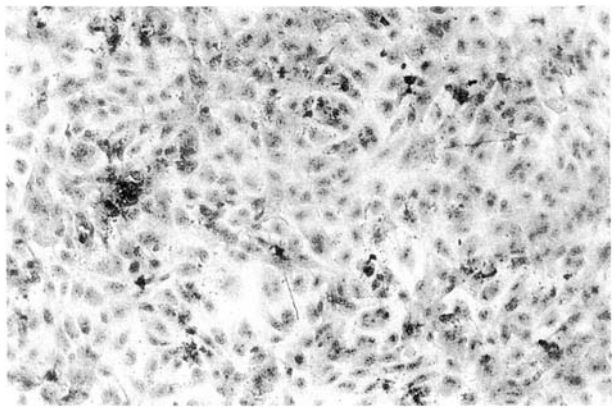

Figure 3 HUVEC monolayer after a 5 h exposure to cobalt at a concentration of 1×10^{-3} M. The monolayer appears generally intact. A few cytoplasmic retractions can be observed. HE staining, × 150.

3.2. CAM expression on endothelial cells after incubation with cytokines and lipopolysaccharide-data from EIA analysis

The expression of the constitutively presented adhesion molecule ICAM-1 was increased under the

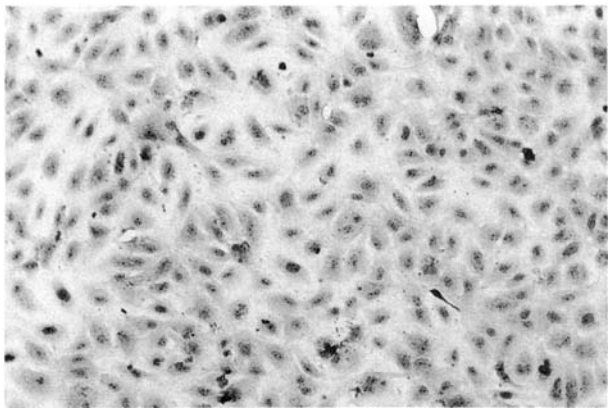

Figure 4 HUVEC monolayer after a 5 h incubation with cobalt at a concentration of 1×10^{-9} M. The monolayer appears intact and undisturbed. HE staining, × 150.

influence of LPS at a concentration of 1 μg/ml. TNFα increased ICAM-1 presentation at a concentration of 30 U/ml. Ten times higher doses of 300 U/ml did not give a further significant stimulation of ICAM-1 expression. IL-1β at concentrations of 10 U/ml and 100 U/ml elicited an increased expression which was however non-significant (Fig. 5). E-selectin expression on HUVEC could be clearly and significantly induced by the applied agents in the concentration range studied. Increased concentrations of TNFα and IL-1β gave no further significant stimulation of E-selectin presentation (Fig. 5). VCAM-1 presentation was significantly induced after a 5 h incubation with LPS, TNFα and IL-1β in the defined concentrations. Increased concentrations of TNFa from 30 U/ml to 300 U/ml gave a slight but significant further increase of VCAM-1 expression. A similar result was found in the IL-1β experiments when increasing the concentration from 10 U/ml to 100 U/ml, as is shown in Fig. 5. GMP-140 could not be stimulated by the agents LPS, TNFα and IL-1β, compared to controls (Fig. 5). The original assay data were settled on average close to the limits of sensitivity and specificity of the assay (GMP-140 was expressed on a low level).

3.3. CAM expression on endothelial cells after incubation with defined concentrations of metal ions-data from EIA analysis

As shown in Fig. 6, E-selectin was clearly presented on HUVEC and increased compared to the negative control (HUVEC under standard culture conditions) after a 5 h incubation to the reference substances LPS and IFNγ. In the zinc experiments, E-selectin expression on the EC was significantly increased after a 5 h incubation to Zn ions at a concentration of 1×10^{-3} M up to about 1.6-fold of the control. Severely cytotoxic concentrations of 1×10^{-2} M elicited no significant E-selectin stimulation, as well as moderate concentrations lower than 1×10^{-4} M and higher than 1×10^{-7} M. A second peak of E-selectin presentation was found after an incubation to very low molar concentrations between 1×10^{-8} M and 1×10^{-9} M. The nickel experiments elicited similar results and gave a similar biphasic profile of E-selectin presentation analogous to the zinc experiments (Fig. 6). The highest E-selectin stimulation was reached by an incubation with nickel concentrations of 1×10^{-3} M, corresponding to the zinc results. The highest evaluated metal ion concentration (1×10^{-2} M) gave a slight increase of E-selectin expression on HUVEC. The moderate concentrations of nickel ions (1×10^{-4} M–1×10^{-7} M) elicited no significant stimulating abilities

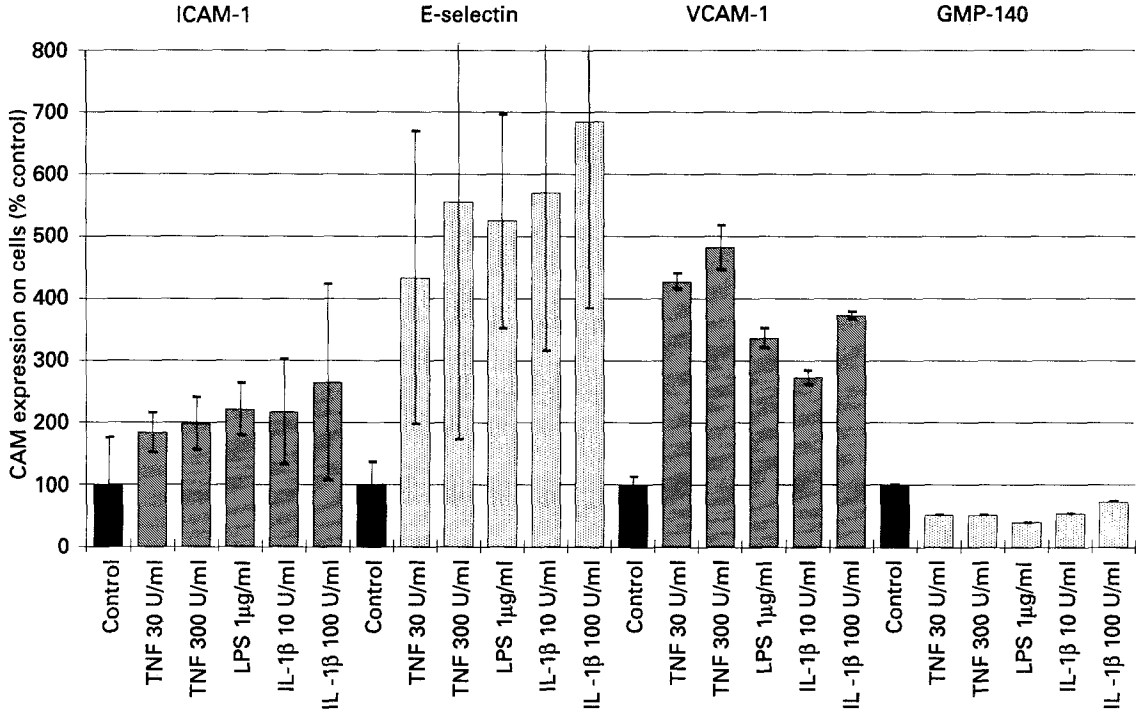

Figure 5 CAM-expression on HUVEC after a 5 h incubation to defined concentrations of LPS (lipopolysaccharide), TNFα (tumour necrosis factor) and IL-1β (interleukin-1β), ICAM-1, E-selectin, VCAM-1 and GMP-140 presentation on cell surfaces, EIA data, mean/SD, $n = 4$.

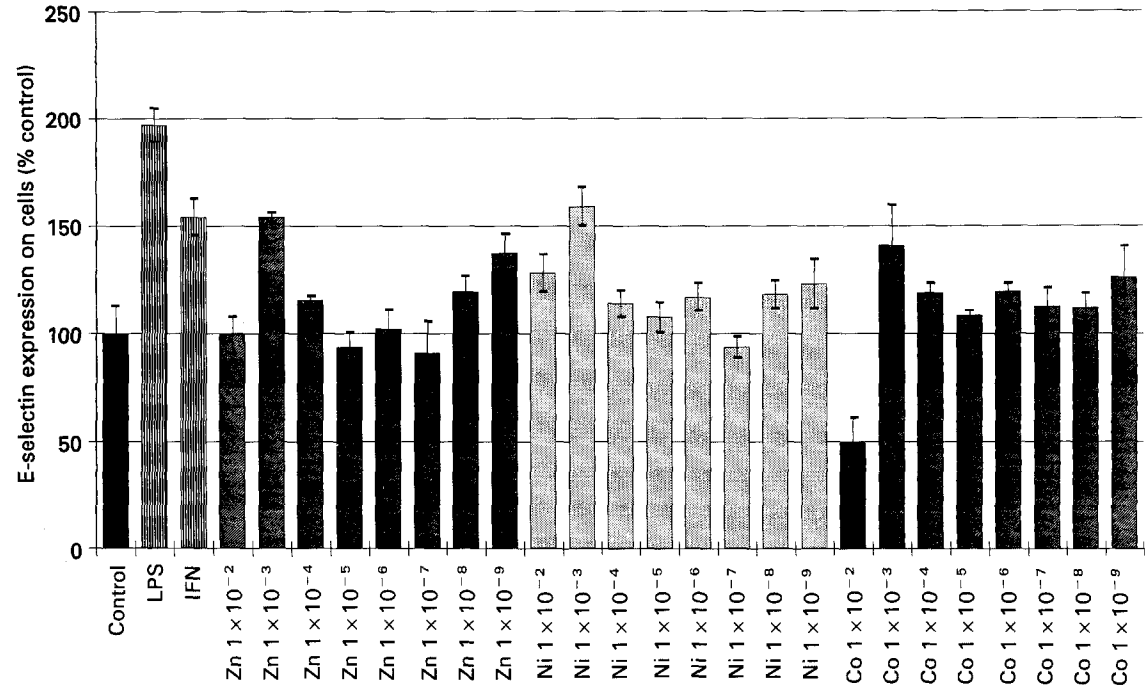

Figure 6 Expression of E-selectin on HUVEC after a 5 h incubation with defined concentrations of Zn, Ni and Co ions (M) and to the reference substances LPS (lipopolysaccharride) and IFNγ (interferon γ). Control is E-selectin presentation on unstimulated HUVEC. EIA-data, percentage of control, mean/SD, $n = 8$.

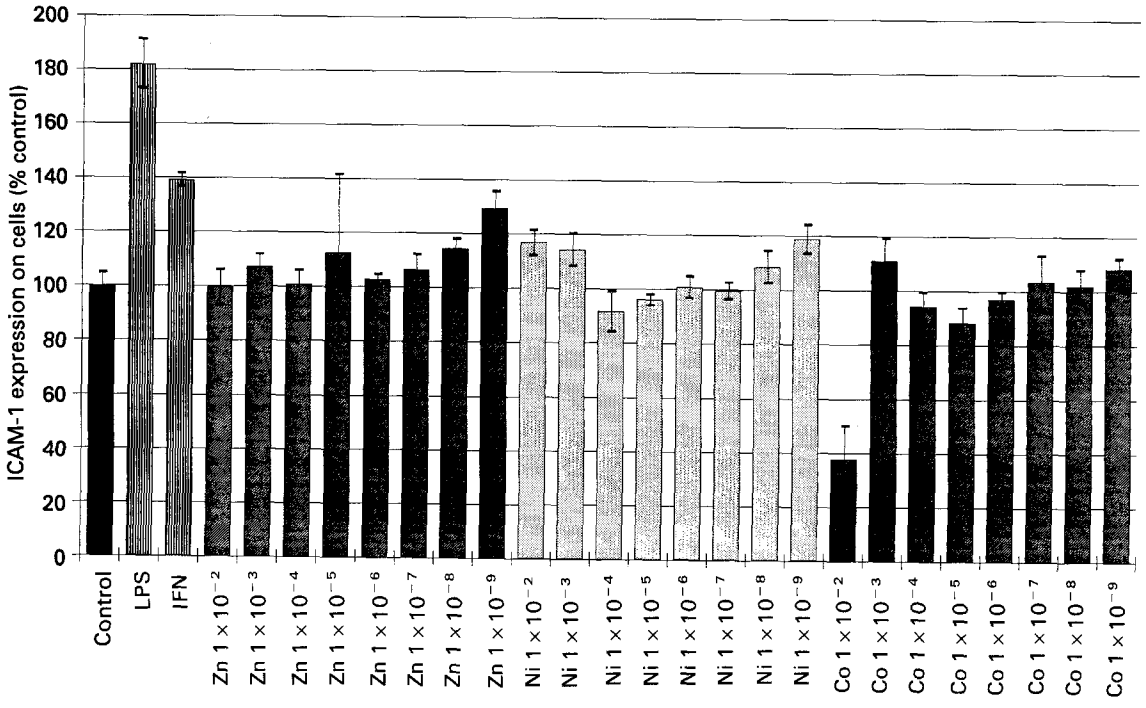

Figure 7 Expression of ICAM-1 on HUVEC after a 5 h incubation with defined concentrations of Zn, Ni, and Co ions (M) and to the reference substances LPS (lipopolysaccharride) and IFNγ (interferon γ). Control is ICAM-1 presentation on unstimulated HUVEC. EIA-data, percentage of control, mean/SD, $n = 8$.

on E-selectin expression, while, corresponding to the results of the zinc experiments, the lowest evaluated Ni concentrations between 1×10^{-8} M and 1×10^{-9} M, gave a second maximum of the presentation of this adhesion molecule of nearly 1.3-fold of the control. Fig. 6 additionally illustrates the results of the cobalt experiments with regard to E-selectin. Cobalt at highest concentrations (1×10^{-2} M) gave a decreased presentation of E-selectin on the endothelial cells. The maximum stimulation of E-selectin presentation on HUVEC after incubation to cobalt ions was reached by concentrations of 1×10^{-3} M of about 1.4-fold of the control value. The stimulation profile with regard to the concentrations corresponds to the findings of the zinc and nickel experiments. Thus an increase of E-selectin presentation of about 1.3-fold was found in the 1×10^{-9} M experiments. An additional significant stimulation was found in the 1×10^{-6} M experiments. The other concentrations gave no significant effect on E-selectin presentation on the endothelial cells. Fig. 7

illustrates, that the presentation of ICAM-1 was increased by the positive controls LPS and IFNγ up to about 1.8-fold and 1.4-fold of the control values, respectively.

In the zinc experiments an increased presentation of ICAM-1 was found after a 5 h incubation to metal ion concentrations of 1×10^{-8} M and 1×10^{-9} M. The simulation settled between about 1.18-fold and 1.25-fold in comparison to the control value, which represented the ICAM-1 expression of HUVEC under standard culture conditions. The other concentrations of zinc, between 1×10^{-2} M and 1×10^{-7} M, did not affect the ICAM-1 presentation in any significant way. Nickel ions stimulated ICAM-1 expression at concentrations of 1×10^{-2} M and 1×10^{-3} M, as well as after incubation with concentrations of 1×10^{-9} M. In general the biphasic profile was obvious also in the nickel experiments. Cobalt at concentrations of 1×10^{-2} M, which had cytotoxic effects, decreased the detectable ICAM-1 in the experiments. These results correspond to the cobalt experiments with regard to E-selectin, as has been described above. A stimulation was given after incubation to concentrations of 1×10^{-3} M, as well as there was a significant increase of ICAM-1 presentation on HUVEC after a 5 h incubation to nickel in concentrations of 1×10^{-9} M. All other concentrations studied elicited no significant effects. The biphasic profile of ICAM-1 stimulation is illustrated in Fig. 7. Nickel experiments were performed to evaluate the effects of metal ions on the presentation of VCAM-1 on HUVEC. As is illustrated in Fig. 8, the positive reference LPS gave maximum stimulation of VCAM-1 expression on endothelial cells compared to the control in the EIA data. VCAM-1 expression increased with decreasing ion concentration. A maximum metal ion stimulation was given after a 5 h incubation with nickel at concentrations of 1×10^{-8} M. The mean value was of the order of about 1.6-fold of the control (presentation of VCAM-1 on unstimulated HUVEC). The stimulation of VCAM-1 expression was also significant after incubation of HUVEC to Ni at concentrations of 1×10^{-6} M (Fig. 8). GMP-140 presentation on HUVEC was evaluated in chromium experiments by EIA analysis. LPS gave no stimulation compared to the control, which corresponds to the results described above for the reference cytokines and LPS (Fig. 5). A maximum stimulation was given after a 5 h incubation of HUVEC to Cr ions at a concentration of 1×10^{-9} M, giving mean values of 6.5-fold in comparison to the control. Significant stimulation of GMP-140-presentation on HUVEC was also given after exposure to Cr ions at a concentration of 1×10^{-8} M, which was about 5-fold compared to the control. Higher metal ion concentrations (1×10^{-3} M–1×10^{-6} M) elicited no significant effects. It has to be pointed out that the values of the effects of LPS and Cr 1×10^{-3} M–1×10^{-6} M) were near the range for non-specific binding.

3.4. CAM-mRNA synthesis by HUVEC after exposure to metal ions – data from Northern blot analysis

It is illustrated in Fig. 10a that LPS (4 μg/ml) and TNFα (300 U/ml) markedly stimulated ICAM-1 mRNA expression. It is also shown that Co concentrations of 1×10^{-3} M elicited a maximum increase of ICAM-1 mRNA in the metal ion experiments after a 5 h incubation time compared to the control (ICAM-1 mRNA in HUVEC cultured in standard medium, lane 9). All further analysed concentrations of Co ions (1×10^{-3} M down to E-10 M) gave a minor, however,

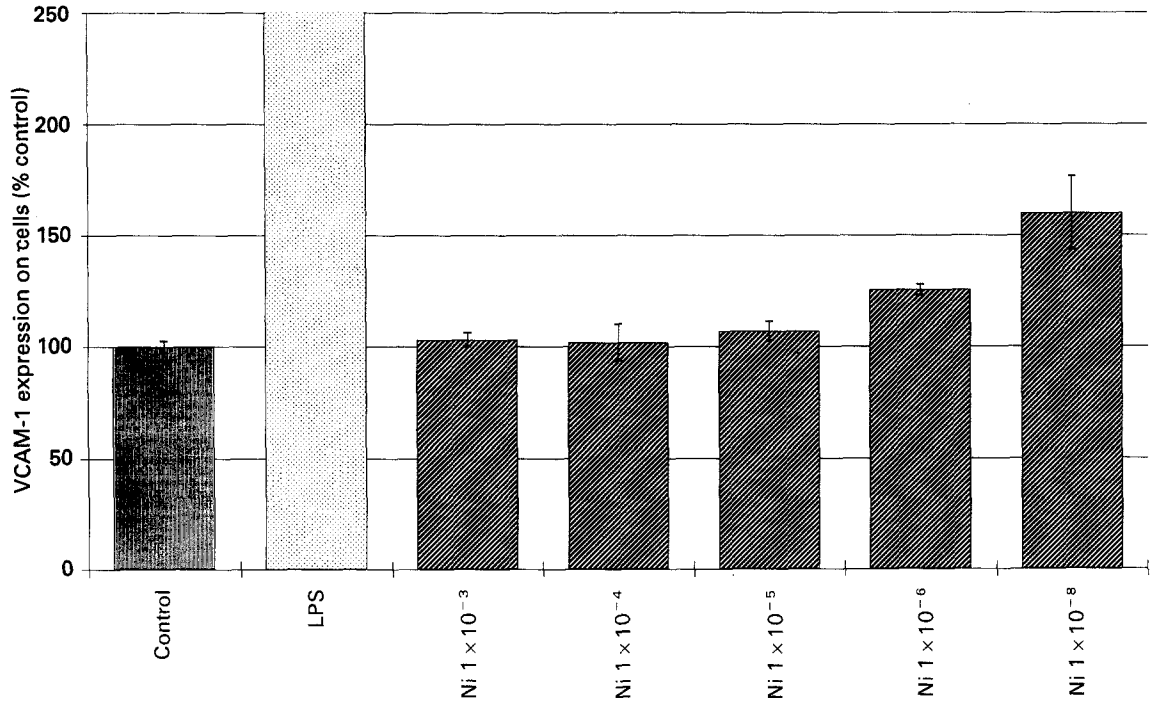

Figure 8 Expression of VCAM-1 on HUVEC after a 5 h incubation with defined concentrations of Ni ions (M) and to the reference substances LPS (lipopolysaccharide) and IL-1β (interleukin 1β). Control is VCAM-1 presentation on unstimulated HUVEC. EIA-data, percentage of control, mean/SD, $n = 7$.

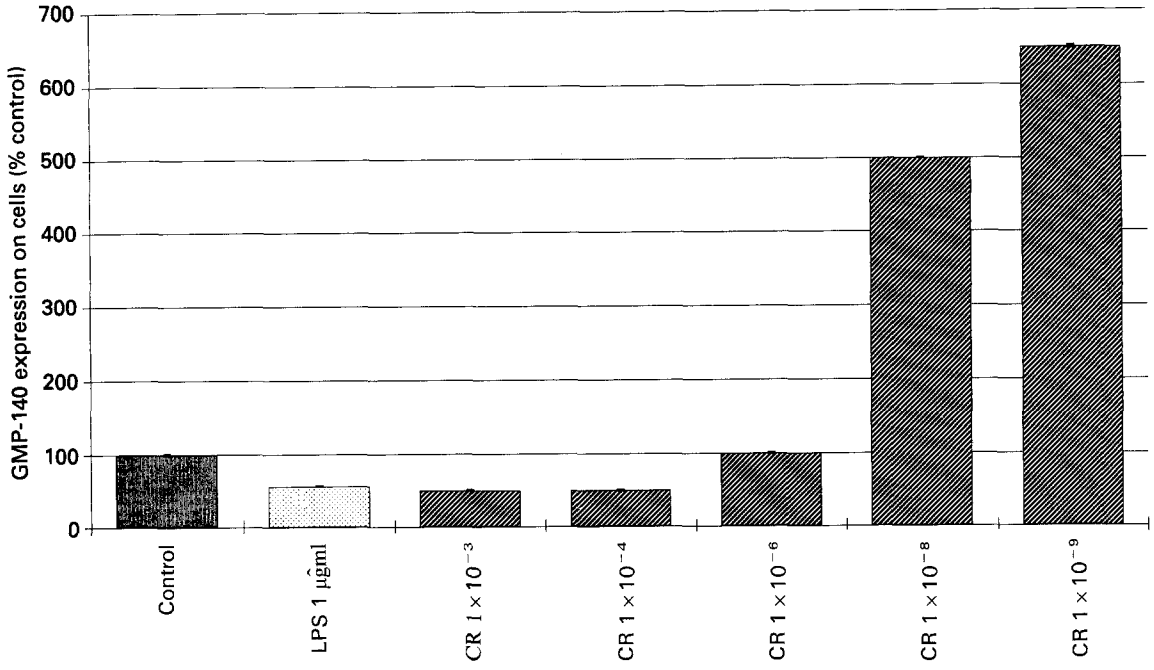

Figure 9 Expression of GMP-140 on HUVEC after a 5 h incubation with defined concentrations of Cr ions (M) and to the reference substance LPS (lipopolysaccharride). Control is GMP-140 presentation on unstimulated HUVEC. EIA-data, percentage of control, mean/SD, $n = 4$.

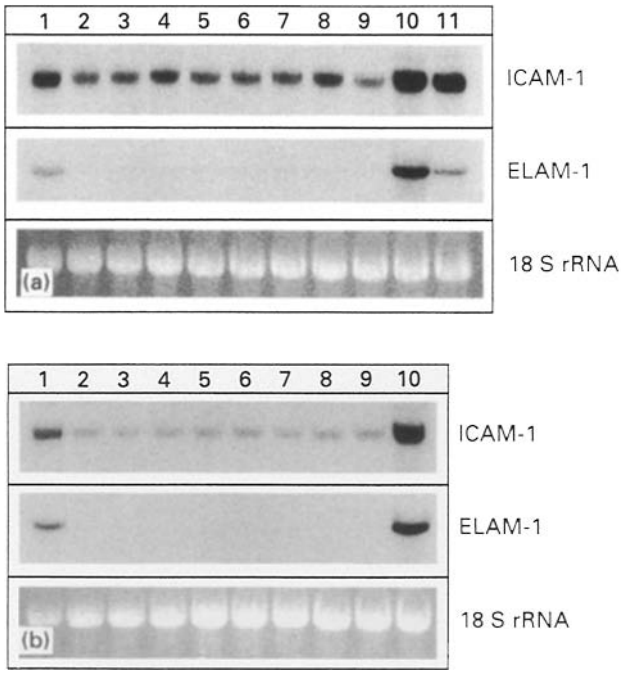

Figure 10 Northern blot analysis of ICAM-1 and E-selectin expression after a 5 h incubation with defined molar concentrations of Co and Ni ions. HUVEC monolayers were treated with medium containing different concentrations of $CoCl_2$ (a) and $NiCl_2$ (b) (lane 1–8 metal ions 1×10^{-3} M– 1×10^{-8} M), with standard medium (lane 9) and medium containing LPS (4 µg/ml) (lane 10) or TNFα (300 U/ml) (lane 11) for 5 h. Total RNA was isolated and Northern blots were prepared as described. Blots were hybridized with an ICAM-1 specific probe and after stripping reprobed for E-selectin transcripts.

definite increase of ICAM-1 mRNA expression in HUVEC after exposure to those concentrations. Further maxima could be observed after a 5 h incubation with Co ions at concentrations of 1×10^{-6} M and 1×10^{-10}M. E-selectin mRNA was not expressed by unstimulated HUVEC in standard culture medium. E-selectin mRNA-synthesis was stimulated by the LPS positive control even more markedly than after incubation with TNFα. A Co ion concentration of 1×10^{-3} M elicited a marked stimulation of E-selectin mRNA. The other evaluated concentrations gave no detectable increase compared to the negative control. The results of the Northern blot analysis of the nickel ion experiments are presented in Fig. 10b. ICAM-1 mRNA could be detected in unstimulated HUVEC. The expression could be markedly increased by LPS. A stimulation was also given after exposure to a Ni concentration of 1×10^{-3} M. Lower concentrations (1×10^{-4} M–E-10 M) elicited no increase of ICAM-1 RNA expression compared to the control. Corresponding to the results of the cobalt ions experiments, E-selectin mRNA was detected after a 5 h incubation with LPS and ion concentrations of 1×10^{-3} M in the nickel experiments. Lower concentrations did not give a stimulation of E-selectin mRNA synthesis compared to the negative control (Fig. 10b). GMP-140 mRNA was not detected with the help of the Northern blot analysis method in the 5 h experiments. Experiments were performed to examine GMP-140 mRNA expression after a 5 h incubation with LPS, TNFa, and defined concentrations of zinc, nickel, cobalt and chromium as described above. However, an 18 h incubation with LPS, TNFα and high molar concentrations (1×10^{-3} M) of metal ions elicited a weak, but definite signal in the Northern blot analysis.

3.5. Presentation of CAM's on HUVEC cell surface after incubation with cytokines – results of the intermediate voltage immunoelectron microscopy

E-selectin was clearly detectable by intermediate voltage immuno electron microscopy after treatment with

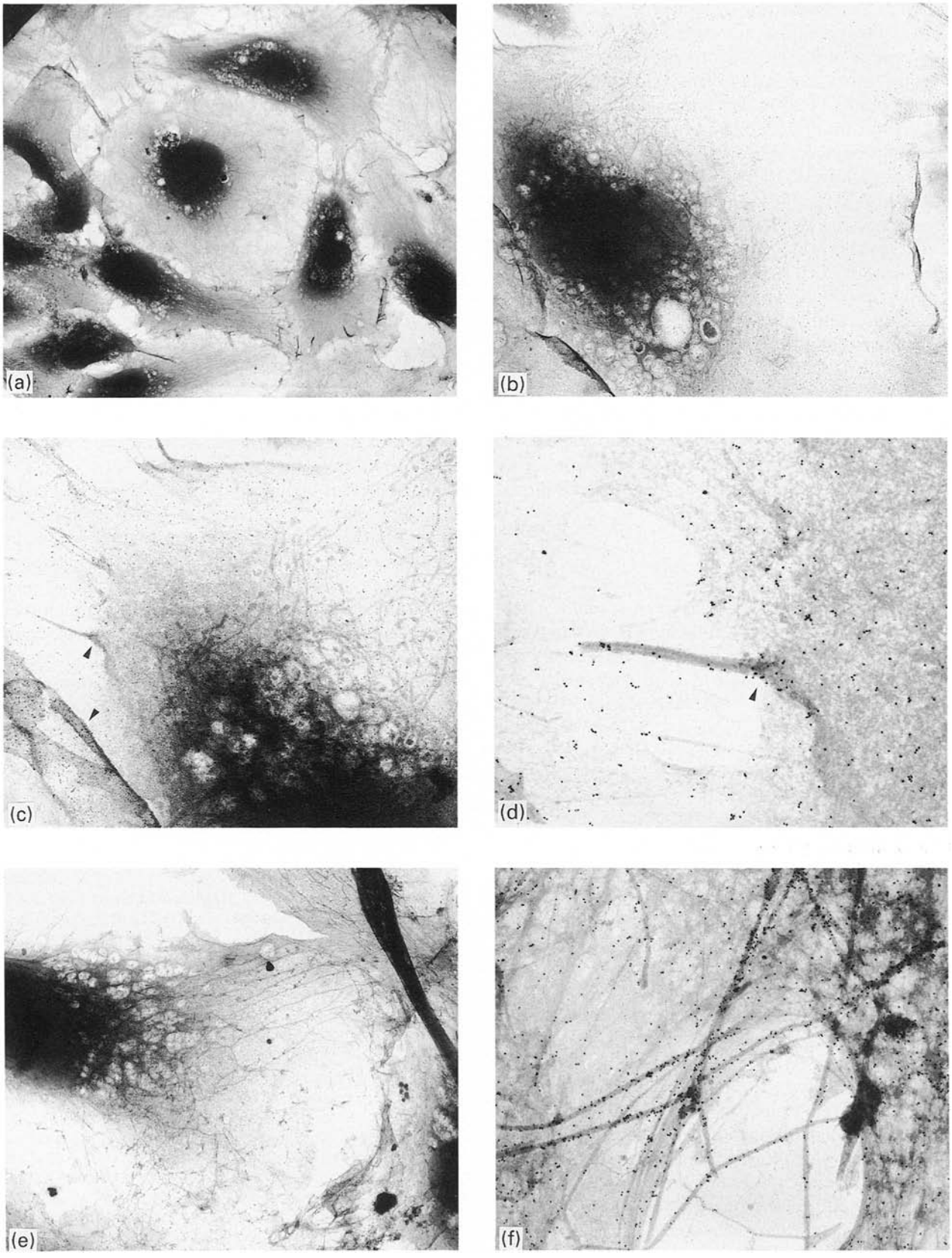

Figure 11 Intermediate voltage immunoelectron microscopy of HUVEC after a 5 h incubation with medium containing IL-1β (100 U/ml) or TNFa (300 U/ml). Gold labelling against ICAM-1 and E-selectin antibody. Intact monolayer after incubation with IL-1β (a) (magnification × 570). E-selectin detected on cell surface (b) (magnification × 1700). Distribution of E-selectin, concentrations of signals on microvilli and cellular fold structures (arrows) (c) (magnification × 3900). Single microvilli with a concentration of E-selectin at the base (arrow) (d) (magnification × 13500). ICAM-1 distribution on HUVEC surface after incubation with TNFα (e) (magnification × 1700). Concentration of ICAM-1 antibody on cytoplasmic extensions (f) (magnification × 10500).

IL-1β at a concentration of 100 U/ml. The ultrastructure of the individual endothelial cells, as well as the appearance of the HUVEC monolayer appeared unaffected after incubation with this cytokine, as is shown in Fig. 11a. Fig. 11b illustrates that gold-labelled second antibody, which marks anti-E-selectin, can be found on the surface of an endothelial cell after incubation with IL-1β. Higher magnifications, as illustrated in Fig. 11c, indicate a typical distribution of the location of E-selectin. Thus signals concentrate especially on areas of cytoplasmic extensions or folds, while the remaining cell surface gave a more or less homogenous distribution of gold-labelled antibodies. A single observation of an EC microvillous structure is shown in Fig. 11 d. Signals were concentrated on the base of this structure. ICAM-1 was found on the HUVEC surface after a 5 h exposure to TNFα (300 U/ml) (Fig. 11e). High magnification gave a corresponding image of distribution, analogous to the results of the E-selectin experiments. The signals were concentrated on cytoplasmatic extensions and microvillous structures (Fig. 11f).

4. Discussion

Adhesion processes, e.g. adhesion of granulocytes to the stimulated vascular endothelium is mediated predominantly by β_2-integrin molecules on granulocytes and by E-selectin and ICAM-1 on endothelial cells [30–33]. Lymphocytes bind to E-selectin, ICAM-1 and VCAM-1 [34–36]. GMP-140 is involved in the processes of monocyte, platelet and granulocyte interaction with the vascular endothelium. These abilities suggest an important role for the adhesion molecules during interactions between leukocytes and EC in the case of an inflammatory response.

It has been shown in our experiments that heavy metal ions have the ability to upregulate the adhesion molecules E-selectin, ICAM-1, VCAM-1 and GMP-140 after exposure to high, as well as after an incubation with very low concentrations of heavy metal ions. Increased expression of CAMs on vascular endothelial cells was therefore observed after exposure to non-cytotoxic metal ion concentrations, which gave no significant effect on the morphology of the HUVEC. The highest evaluated concentrations of cobalt ions (1×10^{-2} M) gave a significant decrease of ICAM-1 and E-selectin presentation on the monolayer compared to the controls, which might be an effect of the loss of cytoplasm caused by the cytotoxic concentration of this metal ion. High concentrations (1×10^{-3} M) of metal ions significantly stimulated ICAM-1 and E-selectin presentation after incubation with Zn, Ni and Co ions. Parts of our results correspond to the observations of Goebeler *et al.* [17], who found a dose-dependent stimulation of CAM presentation after exposure to heavy metal ions. The authors only studied high metal ion concentrations between 0.5 and 5 mM. The low doses, which gave significant effects in our experiments, appear more relevant to us with regard to metal ion toxicity in human systems and inflammatory processes around, e.g. prosthetic devices. Goebeler *et al.* [17] suggested that induction of adhesion molecules by $NiCl_2$ requires mRNA *de novo* and protein synthesis. Experiments with actinomycin D and cycloheximide were performed by this group to study possible decreasing effects of these substances and an inhibitory effect was observed after stimulation by high concentrations of metal ions. The results of our mRNA analysis partly underline this theory. E-selectin mRNA, however, was only detected after stimulation with the highest evaluated Ni and Co concentrations. Low concentrations, which stimulated CAM presentation on cell surfaces, did not stimulate E-selectin mRNA expression. Our experiments on actinomycin D-effects on HUVEC, which were stimulated by high *and* low metal ion concentrations, gave an inhibitory effect on CAM presentation after incubation with high concentrations, but a lack of effect or only minor inhibition after exposure to low concentrations [authors' unpublished results]. The processes of CAM regulation after exposure to low concentrations have to be investigated in further experiments.

CAM expression on endothelial cells follows a characteristic scheme of distribution. This was illustrated by the intermediate voltage electron microscopy in our experiments on cytokine-mediated CAM stimulation. It has been suggested that cytokines and metal ions have similar effects on CAM presentation on endothelial cells. Moreover, different concentrations of metal ions can modify cytokine release by EC [18]. Metal ions at lowest concentrations, released from commonly utilized prosthetic alloys in the case of metal corrosion, might, on the basis of their broad spectrum of effects, be an important factor in the pathogenesis of inflammation around implants.

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