From cytotoxicity to biocompatibility testing *in vitro:* cell adhesion molecule expression defines a new set of parameters

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Determination of potential cytotoxicity is a central issue in current biocompatibility testing standards such as ISO and ASTM. Most of these tests do not assess biocompatibility of a biomaterial with regard to cell function. This study was aimed at screening a number of potential parameters that could be included in assessment of cell functional aspects of biocompatibility. Human umbilical vein endothelial cells (HUVEC) were seeded directly on titanium, NiCr alloy, CoCr alloy, PMMA, PE, PU, PVC, and silicone, or were exposed to the material extracts. Cytotoxicity was assessed for these materials through MTT conversion, crystal violet protein determination and Ki67 expression. In addition, expression of the cell adhesion molecules E-selectin, cadherin-5 and PECAM, as well as of the adhesion-associated proteins fibronectin and vinculin (focal adhesions), was determined by immunocytochemistry and western blotting. Cytotoxicity was not detected with the material extracts. Cells were able to adhere to bare metals, but not polymers. Fibronectin preadsorption resulted in adhesion and spreading also on the polymers. Cells were able to establish cell–cell contacts and focal adhesions. Western blotting, in combination with differential detergent extraction, indicated that linkage of cell–cell adhesion markers to the cytoskeleton may be used as an additional parameter relevant to cell function.

1. Introduction

Determination of potential cytotoxicity is an important aspect of biomaterial testing standards such as ISO and ASTM. The absence of cytotoxicity, however, does not confer knowledge about the biocompatibility of a biomaterial. Enclosed in this general term are many aspects of cell behaviour and cell function that deal with the appropriate cell response to the presence of a biomaterial. For bone prostheses, for example, this implies that bone-forming cells are able to deposit a proper bone matrix with all the natural constituents balanced [1]. Each application needs to be tested in the proper settings reflecting the natural environment and the demands posed on the application. Although this will result in separate sets of test methods for each application, there are common parameters that can be studied. These involve cell–cell adhesion molecules or their receptors, extracellular matrix proteins and proliferation markers.

In this study we determined the cytotoxicity of a number of materials currently used in the clinic. We additionally started to analyse the potential of tests incorporating cell adhesion molecules and proliferation markers in order to allow better statements about true biocompatibility.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated as described elsewhere [2]. Cells for experiments were pooled from one or more passage 1 cultures after about 10 days in culture after isolation. The cells were maintained in tissue culture flasks coated with 0.2% gelatine (Sigma G1393) in the following culture medium: Ham's F12 (Gibco 31765-027) and Iscove's (Gibco 31980-022) 1:1, both with glutamax I, containing penicillin, streptomycin, and 20% human serum pool. Cells were passaged with 0.25% collagenase (CLS I, Worthington). Depending on the test, cells were seeded in 6-well plates (100 000 cells/well), 8-well LabTeks (15 000–20 000 cells/well),

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24-well plates (with biomaterial discs, 20 000 cells/well), or in 96-well plates (8000–16 000 cells/well).

2.2. Materials

The following materials were used: titanium (Ti), NiCr alloy, CoCr alloy, PMMA, PE, PU, PVC, and silicone, all obtained through the University of Liverpool. Serially diluted ion solutions of $ZnCl_2$ (Riedel de Häen 31650) and $NiCI_2x6H_2O_2$ (Merck 6717.0250), prepared in full medium, were used as model systems for cytotoxicity. Materials were pre-adsorbed with $5\,\mu g\,ml^{-1}$ fibronectin overnight at 4 °C for selected experiments. The materials were screened using two principal methods: exposure of cells to 24 h extracts or direct cell seeding. Extracts were prepared from the material samples based upon a $1\,ml\,cm^{-2}$ ratio. Extracts were incubated for 24 h at 37 °C while shaking at 83 r.p.m.

2.3. Cytotoxicity testing

The following parameters were measured on cells exposed to material extracts: MTT conversion, crystal violet staining and Ki67 expression. Endothelial cells were seeded on 96-well plates, preadsorbed with human fibronectin, at a density of 16 000 cells/well for MTT and crystal violet and 8000 cells/well for Ki67. The cells were incubated for 24 h before extracts and solutions of nickel and zinc ions were given. Subsequently, cells were incubated in 100 µl extract for 24 h. After this incubation, 20 µl MTT-solution (3-[4,5-Dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue; Sigma M5655; 1% in PBS) was given to each well, except for the blanks. Cells were incubated for a further 4 h. Medium was decanted and cells washed with 100 µl PBS. After decanting the PBS wash, the blue formazan crystals were solved with 2-propanol (100 µl/ well) by shaking rigorously for at least 15 min. Supernatants were transferred in a new 96-well plate and the optical density measured at 550 nm. Crystal violet staining was performed on the MTT test plate containing the cells. The wells were washed three times with PBS with 0.05% Tween 20 (Sigma). Crystal violet solution was added to the wells (Sigma, 50 µl/well, 0.1%), except for the blanks. After an incubation time of 20 min with continuous shaking, wells were washed with water four times. The cell-bound stain was extracted with 33% acetic acid (Merck, 100 µl/well), while shaking for 10–15 min. Supernatants were transferred in another 96-well plate and the optical density measured at 600 nm.

For Ki67 cells were washed twice with warm PBS and fixed with 100 µl methanol/ethanol (2 : 1 vol/vol) for 15 min at RT, and washed again with PBS + 0.05% Tween 20 (Sigma). After blocking with blocking solution + 1% H_2O_2 + 1% rabbit serum (10x blocking solution, Boehringer, no. 1112589, diluted with a.dest) at RT for 30 min, cells were washed in PBS + 0.05% Tween 20 once more. Then they were incubated with mouse-anti-human Ki-67 Antigen (MIB-1, 1 : 100 diluted in blocking solution, Dianova no. DIA 505) at 37 °C for 45 min, while gently shaking. After washing three times in PBS + 0.05% Tween 20, cells were further incubated with POX-conjugated rabbit-anti-mouse immunoglobulin (1 : 400, Dako no. P0260) at 37 °C for 30–45 min with gentle agitation, 50 µl per well, while shaking at moderate speed (e.g. 47 r.p.m.). Wells were washed six times in PBS + 0.05% Tween 20. Then 170 µl/well of freshly prepared substrate solution (1 tablet o-PD (20 mg, Sigma P7288) + 20 µl 30% H_2O_2 in citrate buffer, pH 5.0) was added. Cells were incubated at 37 °C for at least 15 min until a well-developed colour reaction could be observed. Colour development was stopped with 50 µl/well of 3M HCl (Merck) pipetted in a new 96-well plate to which the supernatants were transferred. The optical density was measured at 492 nm.

2.4. Additional testing

Tests were performed on cells directly seeded on the materials or exposed to extracts, including defined nickel ion suspensions. The cell response was studied using immunocytochemical methods and also western blotting. Antibodies against human vinculin (clone h-vin-l, Sigma), human fibronectin (Sigma, polyclonal), E-selectin (clone RR1/1, Bender MedSystems, no. BMS 110), Ki67 (MIB-1, Dianova), PECAM (cloneJC/70A, DAKO) and cadherin-5 (clone 75, Transduction Laboratories) were used.

3. Results and discussion

3.1. Cytotoxicity testing

MTT conversion was not affected by the material extracts, as shown in Fig. 1a and b. In Fig. 2a and b, the MTT data are normalized relative to the protein content as measured with crystal violet. Protein content measured through crystal violet is generally considered to be a good representation of cell number [3–5]. It can be seen from Fig. 2 that MTT converting activity over 4 h incubation depends on the extract given to the cells and that in this respect MTT cannot be used to make a statement about the proliferative status of cell populations, as suggested by some authors [6,7] and companies (Boehringer Mannheim, cell proliferation kit I-MTT). In the proliferation assay with Ki67, some small dilution effects were seen, as shown in Fig. 3a and b. It is highly unlikely that these dilution effects are caused by edge effects in 96-well plates, because the outer wells were avoided.

Direct contact data indicated that cells were not able to adhere to and spread on the bare polymeric materials. Clearly these materials were too hydrophobic to support adhesion. Prior adsorption with fibronectin resulted in adhesion and spreading on all materials used in this study, supporting similar data obtained by others [6, 8]. The three metals also supported adhesion without prior adsorption. It has been observed that non-adherence can induce apoptosis in endothelial cells [8–11], and that consequently these cells will die. The question is whether or not cell death due to non-adherence can be called cytotoxicity [8]. To extend this point a little bit further, it can be

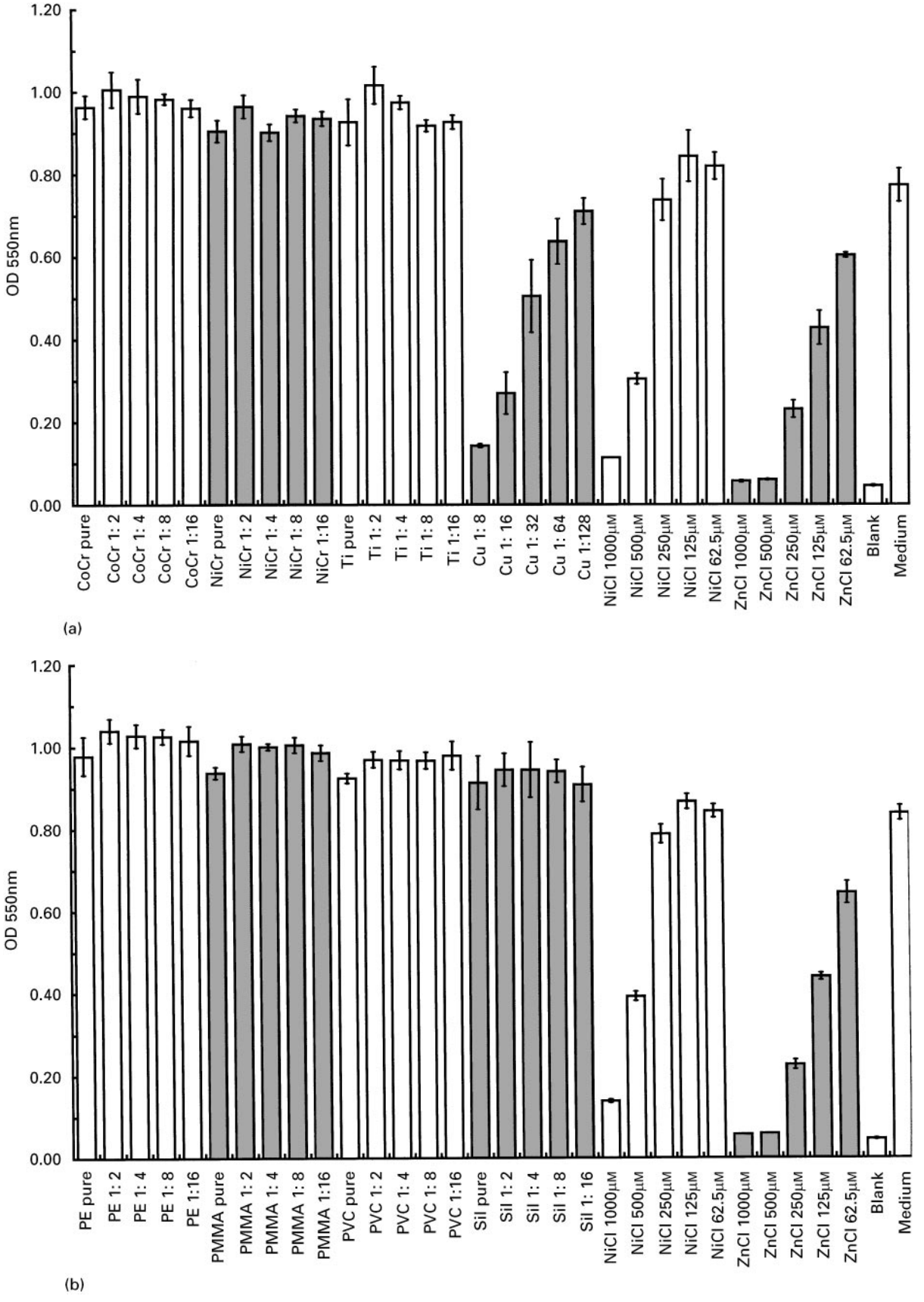

Figure 1 MTT conversion by HUVEC exposed to (diluted) extracts from (a) three metal alloys and controls (copper extracts, nickel and zinc ion solutions), and (b) four polymers and controls (nickel and zinc ion solutions); mean values ± S.D. ($n = 3$).

a point of discussion whether or not this phenomenon is a lack of biocompatibility. The answers to these questions will depend on the ultimate application of the biomaterial.

3.2. Additional testing

Cell proliferation on the fibronectin preadsorbed materials was observed, as demonstrated in Fig. 4. Confluence of the cell layer was not reached on bare

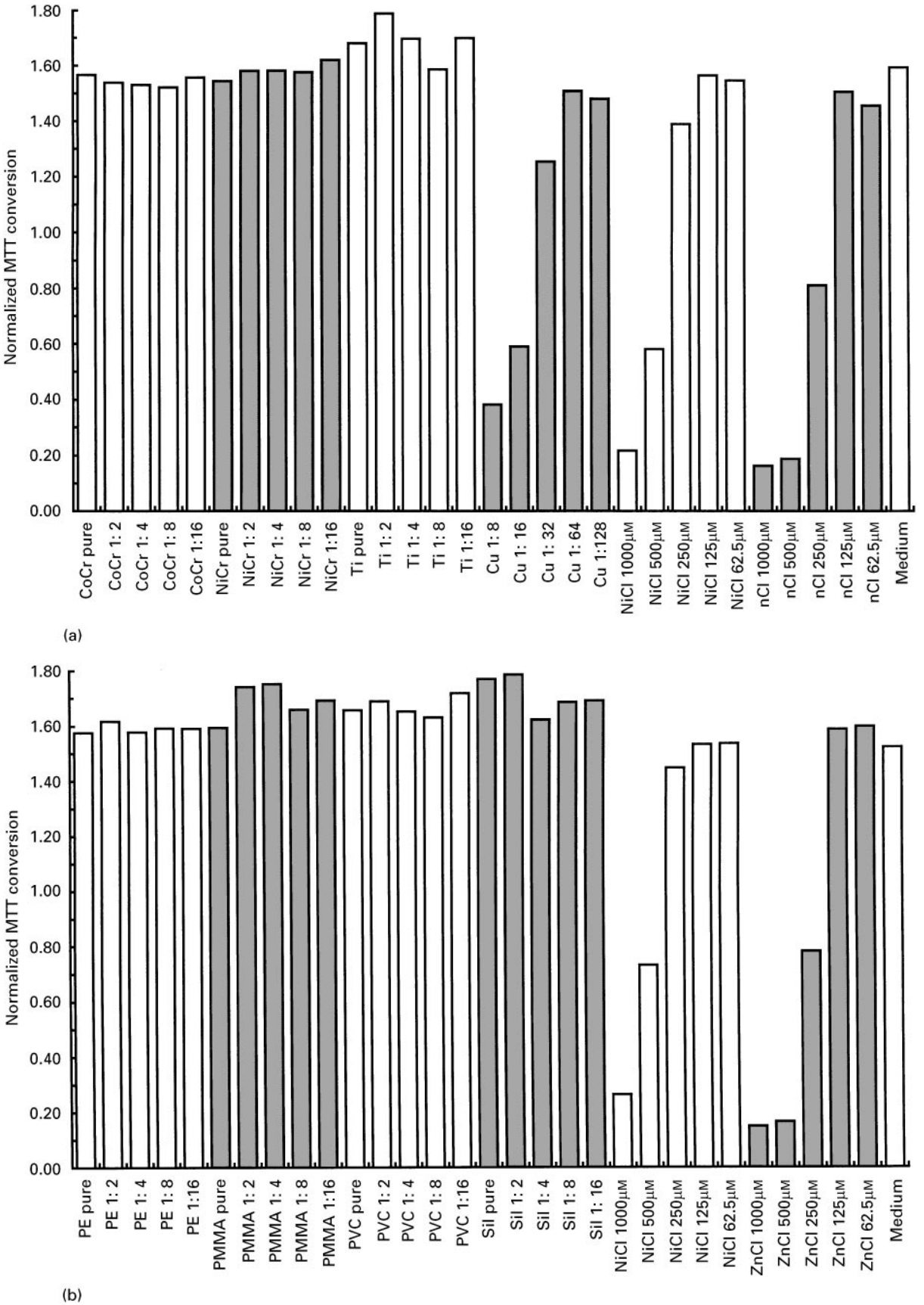

Figure 2 MTT conversion data of (a) Fig. 1a and (b) Fig. 1b, normalized on the basis of protein content as determined by crystal violet staining of HUVEC exposed to (diluted) extracts from (a) three metal alloys and controls (copper extracts, nickel and zinc ion solutions), and (b) four polymers and controls (nickel and zinc ion solutions).

metals, as shown in Fig. 5, in which Ki67 labelling was restricted to a few cells despite the fact that a monolayer was far away from being completed. Expression of the marker E-selectin, which is specific for activated endothelium, was determined in the case of direct contact and extract testing. Whereas extracts did not induce activation, cells seeded on the materials directly all expressed E-selectin at the cell surface 4 h after

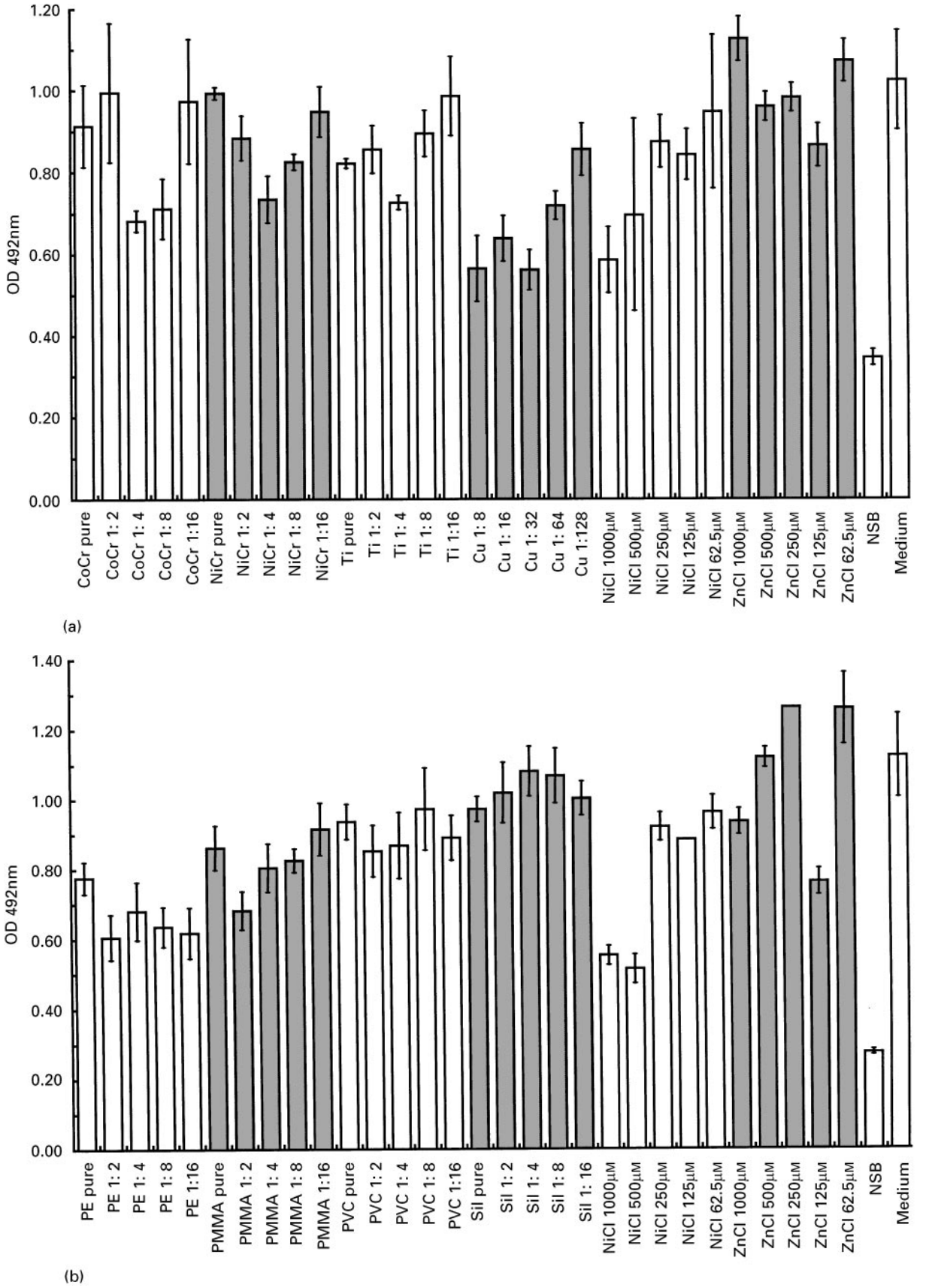

Figure 3 Ki67 expression on HUVEC exposed to (diluted) extracts from (a) three metal alloys and controls (copper extracts, nickel and zinc ion solutions), and (b) four polymers and controls (nickel and zinc ion solutions). The results in this figure are obtained from the same cell batch and with the same extracts as those depicted in Figs 1a and 2a, and Figs 1b and 2b, respectively; mean values $\pm$ S.D. ($n = 3$).

seeding, irrespective of the material (data not shown). E-selectin disappeared after 24 h incubation. This activation pattern resembles stimulation effects induced by cytokines or other inflammation mediators. It is not clear if this stimulation is induced by the harvest-seeding procedure or by the materials or even by the fibronectin layer.

Cell–cell adhesion molecules PECAM and cadherin-5 were also studied as a model for biocompatibility testing. Cells were able to establish cell–cell

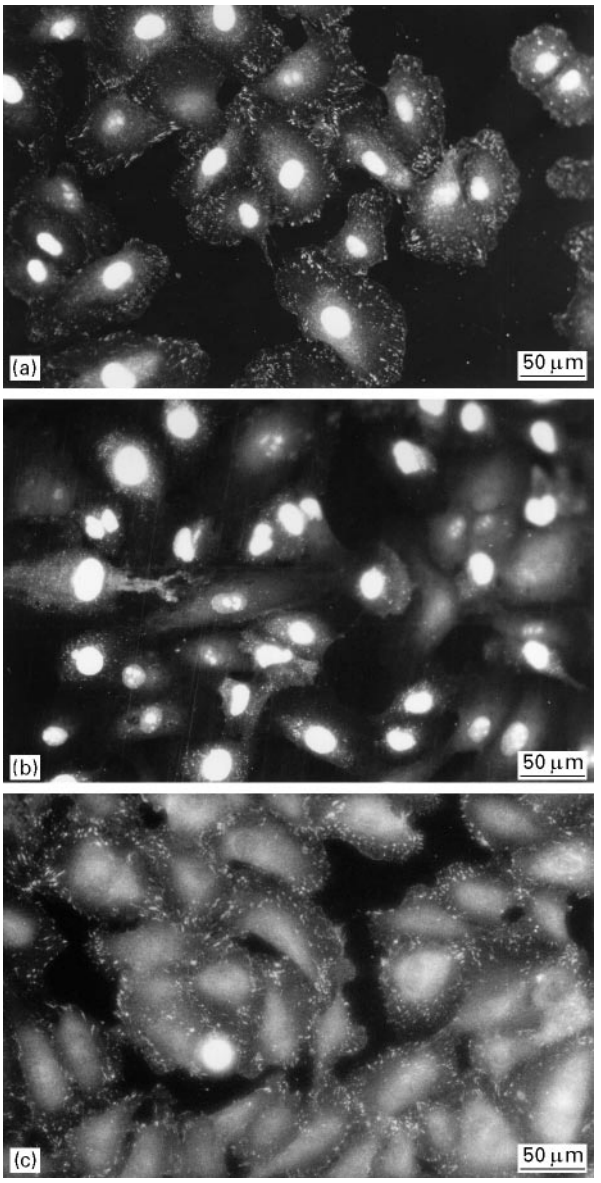

Figure 4 (a,b) Ki67 and (a,c) vinculin expression of HUVEC seeded on fibronectin preadsorbed titanium and incubated for (a) 24 h and (b,c) 48 h. Ki67 labelling is limited to the cell nuclei and vinculin-labelled focal adhesions are mainly seen in the periphery. Note that after 48 h most cells are still positive for Ki67.

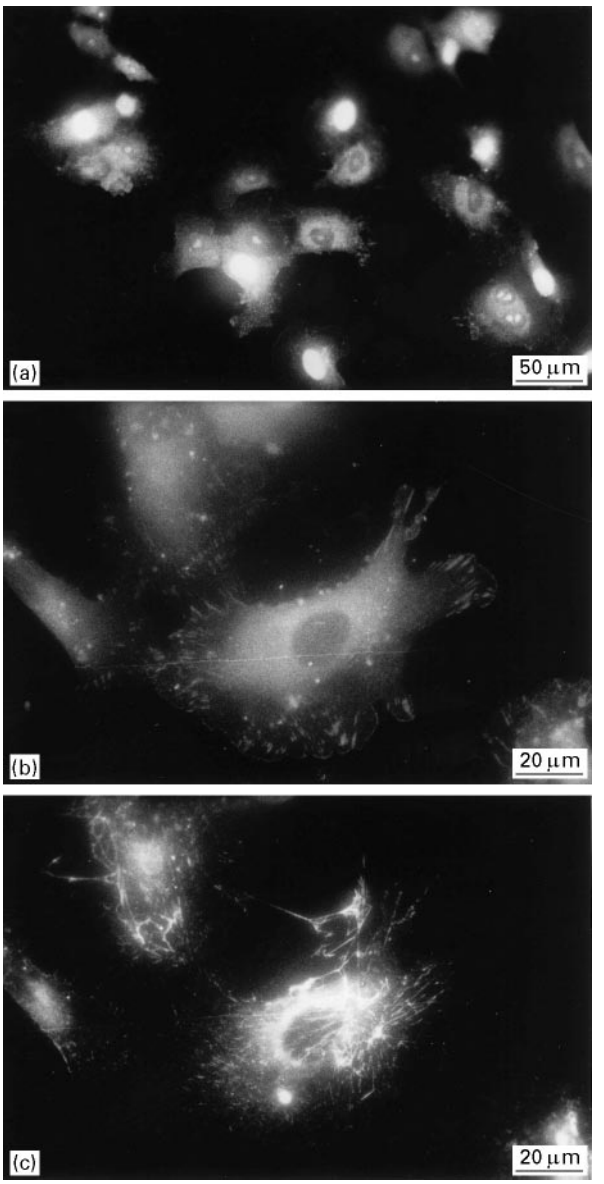

Figure 5 (a) Ki67, (b) vinculin and (c) fibronectin expression of HUVEC seeded on bare titanium and incubated for (a) 48 h and (b,c) 96 h. After 48 h, many cells are negative for Ki67 despite the low cell density. Cells are well-spread and have developed focal adhesions (b). Fibronectin assembly is minimal, even after 4 d adhesion.

contacts (Fig. 6) as well as focal adhesions (see Fig. 4) on all materials pre-adsorbed with fibronectin. Cadherin-5 expression in the model system with nickel ions demonstrated a clear dependence of the staining on nickel ion concentration (Fig. 6). Western blotting in combination with differential detergent extraction indicated that linkage of cell–cell adhesion markers to the cytoskeleton was also dependent on the nickel concentration, as shown in Fig. 6. Data derived from these techniques are qualitative in nature but may be rendered quantitative with additional analysing techniques. Experiments using immunofluorescence techniques and western blotting are not as practical as, for example, ELISAs. Often they are time-consuming and results are not always easy to interpret. The latter, however, is also true for many tests described in current ISO standards. Therefore, we need to intensify our efforts in the direction of understanding cell function in relation to cell–biomaterial interactions. Although practicability and potential predictive power are important aspects to be considered in developing new test systems, the complex biological environment created by the interplay of a number of cell types, extracellular matrix and circulating mediators and growth factors might force us to accept complex, tedious test systems as part of a standard for biocompatibility testing.

4. Conclusion

Cytotoxicity tests will generally confirm that a material will not induce an adverse tissue response due to a measurable cytotoxicity. Other factors, however, are more important in mediating such a tissue response. Therefore, research efforts should focus on other aspects of application-related biocompatibility, such as cell function. Cell function can be studied with

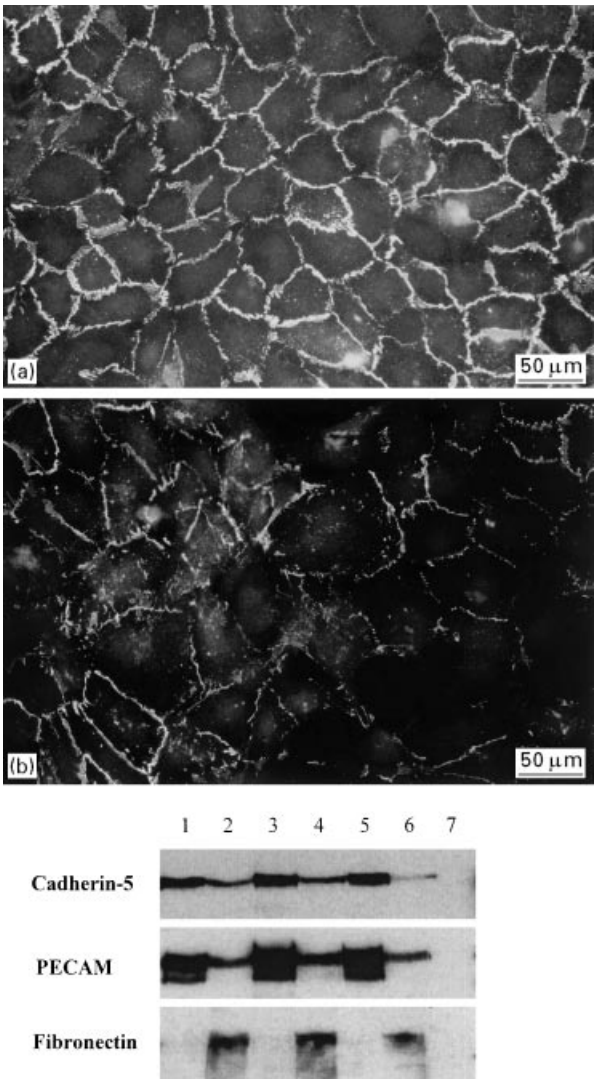

Figure 6 Cadherin-5 expression of HUVEC seeded in LabTeks and exposed to (a) 0.1 mM nickel and (b) 1.0 mM nickel, for 48 h. The total adhesion time of these cells was 72 h. The western blot shows the expression of cadherin-5, PECAM and fibronectin as a function of the following treatments: lane 1, medium Tx; lane 2, medium CS; lane 3, Ni 0.1 mM Tx; lane 4, Ni 0.1 mM CS; lane 5, Ni 1.0 mM Tx; lane 6, Ni 1.0 mM CS; lane 7, isotype controls. Stimulations were performed over 24 h, the total incubation time was 72 h. All blots are from the same experimental run. Tx, triton X-100 soluble protein fraction; CS, cytoskeleton-associated, triton X-100 insoluble protein fraction. Clearly the lesser extent of maturation of cell–cell contacts in (b) is associated with less cadherin-5 being associated with the cytoskeleton.

current cell and molecular biological methods. These methods are not easily translated into tests suitable for ISO standards, but a committed and concerted effort towards this goal seems to be worthwhile. Experimental conditions such as cell sources, relevant markers and cell culture conditions (e.g. three-dimensional) must be adapted to the ultimate application.

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