A new quantitative test method for cell proliferation based on detection of the Ki-67 protein

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A quantitative method to assess cell proliferation is one essential prerequisite for testing biomaterial cytocompatibility *in vitro*. Currently used methods, e.g. bromodeoxyuridine incorporation, show serious disadvantages concerning either sensitivity, specificity or handling. A new enzyme linked immunosorbent assay (ELISA) system for the quantification of cell proliferation based on detection of the Ki-67 protein is described. This protein has turned out to be strictly correlated with the active parts of the cell cycle but to be absent in G_0. The measurement of Ki-67 expression by different human cell types, e.g. endothelial cells and HeLa cells, was evaluated in order to answer the question of whether the data obtained using the Ki-67 ELISA method correlate with the proliferation measured with flow cytometrical DNA analysis and microscopical evaluation. Methods currently used for the evaluation of cell proliferation were compared to the new Ki-67 ELISA method. In addition, the functionality of adherent endothelial cells, and the viability and morphology of the cells were investigated. Cells were treated with standard culture medium with or without the transcription inhibitor, actinomycin D, or growth factors, e.g. endothelial cell growth factor (ECGF), and were exposed to metal ion standard solutions. These solutions were in a cytotoxic–non-cytotoxic range. Ki-67 ELISA was found to be a reliable quantitative method to assess proliferation of cultured human cells *in vitro*. It has advantages over methods that are currently being used. It is easy to perform and corresponds to the requirements for a test to be selected for biomaterial testing according to ISO standard 10 993.

1. Introduction

In previously published studies we investigated cell proliferation of primary isolated cells or permanent cell lines as one important parameter to improve cytocompatibility of biomaterials, especially in the context of cytotoxicity testing [1]. ISO 10 993 as well as diverse national standards, e.g. British standard BS5736 Part10, define test methods for the biological evaluation of medical and dental materials and devices. Cytotoxicity testing of the biological evaluation of medical devices is a central component of ISO 10 993-5 [2], which sensibly does not specify a single test, but rather attempts to present guidelines for the choice of suitable tests and define important principles of these tests. The latter include positive and negative control materials, extraction conditions, choice of cell lines and cell media, as well as important aspects of the test procedures, including tests on extracts, as well as tests by direct and indirect contact.

Existing concepts for determination of proliferation show serious disadvantages. Whereas total protein or DNA content do mirror the cell number analysis of tritiated thymidine incorporation requires extensive preparation, including the inherent problems of handling radioisotopes, time intensive film exposure or material intensive signal counting using a scintillation counter. Bromodeoxyuridine (BrdU) instead of thymidine is incorporated during DNA synthesis in the S-phase. Determination using an enzyme linked immunosorbent assay (ELISA) technique does show high background activity due to steric inhibition of the antigen and low labeling indices. This problem occurs especially for

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slowly proliferating cell types, e.g. endothelial cells (EC), muscle cells or osteoblasts. Analysis by flow cytometry requires quantitative detachment of cells from the surface by a technique avoiding cell damage. This procedure is time-consuming and carries a high risk of artifacts. Furthermore, it appears to be inappropriate for cells that are attached to material surfaces, e.g. fibroblasts, chondrocytes or EC.

In the present paper we describe a technique for investigating cell proliferation, based on nuclear detection of the Ki-67 antigen, a marker also currently used in diagnostic histopathology. The methods listed above were critically assessed in order to achieve a maximum standard of evaluation. An enzyme linked immunosorbent assay (ELISA) method for assessment of the cellular expression of the Ki-67 antigen has been developed in order to achieve a rapid, reliable and validated method to quantify cell proliferation in cell culture systems. In addition, the ease of use was one important aspect. The developed Ki-67 ELISA was compared to existing methods, which are considered to be current standards, such as cell counting, DNA measurement, BrdU incorporation or the MTT test. The developed method was validated by comparison to flow cytometric analysis. Furthermore, the cells were characterized by morphological means.

2. Materials and methods

2.1. Reagents and materials

Anti-Ki-67 antibody was bought from Dianova, Hamburg. Anti-mouse immunglobulins, POD-conjugated was obtained from DAKO Diagnostika GmbH, Hamburg. Blocking reagent (code No. 1112589) from Boehringer Mannheim. o-phenylenediamine dihydrochloride-tablets, MTT (3-[4,5-dimethylthiazol-2-yl]-2, S-biphenyl tetrazolium bromide), trypsin type III, triton X-100, tween 20, ethylenediaminetetre-acetic acid (EDTA), crystal violet were supplied by Sigma Chemie, Deisenhofen. RPMI 1640 including all supplements, PBS, fetal calf serum are from Life Technologies, Eggenstein. Ethanol, methanol, 2-propanol, acetic acid, hematoxylin, eosin yellow were obtained from Merck, Darmstadt, and RBS 35 from Roth, Karlsruhe. Tissue culture ware was supplied by Becton & Dickinson, Heidelberg. Microplate reader, Titertek II, and software (EIA3) were obtained from ICN-Flow, Meckenheim. The BrdU assays were supplied by Amersham, Braunschweig.

2.1.1. Culture of cell lines

HeLa cells (ATCC code: CCLZ) were obtained from ICN-Flow, Meckenheim. Cells were cultured in RPMI 1640 medium with additional L-glutamine, penicillin–streptomycin and 10% fetal calf serum in a humidified atmosphere containing 5% CO_2.

2.1.2. Culture of human endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from the lumen of umbilical veins as described by Jaffe *et al*. [3]. Immediately after receipt the lumen was washed thoroughly with HEPES-buffer (Gibco, UK) to remove coagulated blood, filled with 0.1% collagenase I (Worthington) and incubated for 15 min at 37 °C. The cell suspension was obtained by flushing the lumen with medium, which was a mixture of Ham's F12 (Gibco, UK) and Iscove's modified Dulbecco's media. The cell suspension was centrifuged at 1000 r.p.m. for 10 min. The supernatant was removed and the pelleted cells were resuspended in Ham's and Iscove's media. The cells were seeded at a density of between 12 000 and 24 000 cells cm^{-2} in T25 tissue culture dishes (Falcon, USA). Cells were cultured at 37 °C in a carbon-dioxide-enriched atmosphere with oxygen reduction to 10% by nitrogen addition. The culture medium was again a mixture of Ham's F12 (Gibco) and Iscove's modified Dulbecco's media with penicillin–streptomycin, L-glutamine (Gibco) and 20% human serum. Confluency was reached after one week in culture when the cells were measured at a density of about 50 000 cells cm^{-2}. Confluent monolayers were passaged at a splitting ratio of about 1 : 3 using 0.1% collagenase I (Worthington) with 0.01% EDTA and 0.25% bovine serum albumen (BSA). The suspension was centrifuged as described above, the cells were resuspended in fresh culture medium and cultured until the following passage. Cells of passage 1 have been used for all experiments.

2.1.3. Reference materials

Endothelial cell growth factor (ECGF) was obtained from Falcon, Heidelberg, zinc- and nickel-chloride from Sigma, Deisenhofen.

2.1.4. Preparation of cell cultures

In order to prepare test wells, both cell lines were detached from the culture flasks using a trypsin–EDTA solution (0.25–0.025%), and resuspended as a single cell suspension in culture medium. The cells were seeded into the wells of microtiter plates, for MTT test at the density of 100,000 HeLa cm^{-2} as well as 50 000 HUVEC cm^{-2}. For cell counting experiments, flow cytometrical analysis and Ki-67 ELISA 50,000 HeLa cm^{-2} or 10,000 HUVEC cm^{-2} were seeded in 96-well microtiter plates (Falcon 3072).

2.2. Microscopical evaluation

Cells have been characterized by using light microscopy, phase contrast microscopy as well as fluorescence microscopy and graded for cell loss, cell death or morphological changes. Photographs as well as digital images were taken using a Leica DMRB microscope. The number of cells in mitosis was determined by using computer-assisted image analysis (NIH image 1.57) and expressed in percentage of the untreated control group.

2.3. Cell cycle analysis using bi-parameter flow cytometric analysis of Ki-67 expression

In order to quantify Ki-67 expression in both resting and proliferating HUVEC a bi-parameter flow cytometric

analysis method was developed in order to analyze both specific cellular protein expression and the cell cycle by combination of a propidium iodide nuclear staining and a FITC-labeled second antibody method for the detection of cellular Ki-67. Cells were seeded on 6-well cell culture plates and were incubated with ECGF (30 ng ml^{-1}), actinomycin D (5 μg ml^{-1}) and solutions of $ZnCl_2$ at concentrations of 1000, 500, 250 and 100 μmol, respectively, for an 18 h period of time. Control cells were cultured in standard medium in parallel. After incubation the cells were suspended with the help of 0.2% collagenase in PBS–BSA solution and were washed twice. Fixation and permeabilization were performed with the help of a commercially available ''fix and perm'' kit (medac-diagnostica, Austria) following the supplied protocol. Primary antibody (Mib I, 5 μl) as well as the isotype control antibody (mouse IgG_1, 1 μl) were added and incubated for 15 min at room temperature. After incubation the cells were washed before 100 μl of the secondary antibody (rabbit anti-mouse, FITC conjugated, DAKO) were added. After further incubation at 4 °C for a 20 min period of time the cells were washed and 50 μl RNAse (Boehringer, Mannheim, 13.5 U ml^{-1}) was added followed by a further 15 min of incubation. A propidium–iodide solution (50 μg ml^{-1}) was added followed by a final incubation period of 15 min. The samples were measured flow-cytometrically (Coulter Epics XL MCL) by light scatter and bi-fluorescence analysis. Some 10^5 cells obtained from each sample were analyzed, samples were prepared in triplicate for each stimulus condition. The laser light emission was adjusted at wavelength $\lambda = 488$ nm, fluorescence intensities were measured at $\lambda = 525$ nm (FITC) in the first channel and $\lambda = 620$ nm (P1) in the third channel. Non-specific binding was excluded by photo-multiplier adjustment, which subtracted isotype control fluorescence. Coulter acquisition and listmode software systems as well as Microsoft Excel software were used for final evaluation of the results.

2.4. Enzyme linked immunosorbent assay for the Ki-67 antigen

HeLa cells, seeded for the proliferation assay, were washed twice with PBS, fixed with methanol–ethanol and permeabilized for 5 min with 0.1% triton X-100. After washing in PBS, non-specific binding was blocked for 30 min in blocking reagent. Another washing step, with PBS containing 0.05% tween 20 (used for all further washing steps), followed. Subsequently, four of the cells were incubated with 1 : 100 diluted primary (anti-Ki-67) antibody for 45 min at 37 °C (50 μl well^{-1}). For quantification of non-specific binding an anti-CD14 antibody has been used. After a third washing step, the 1 : 400 diluted anti-mouse POD-labeled antibody was incubated with the cells for 45 min at 37 °C (50 μl well^{-1}). After another washing step, the plates were developed using OPD as substrate. The reaction was stopped by addition of 1 M HCl. The values for the CD14 group were regarded as background and subtracted. The data obtained for the cells treated with standard medium were defined as being 100% values.

2.5. Cell counting

Cells were detached from the material and serial dilutions were counted using a cell counter (Coulter ZM) or Schärfe (Casy).

2.6. BrdU-assay

The assay from Amersham (RPN210) was used as recommended.

2.7. MTT test

EC were seeded on 96-well microtiter plates and incubated for 24 h. HUVEC was exposed to culture medium, actinomycin D and different concentrations of $ZnCl_2$ for an 18 h period of time. MTT solution (50 μl well^{-1}) was added and incubated for 4 h at 37 °C in an atmosphere of air with 5% CO_2. Supernatants were removed from the wells and 2-propanol (100 ml) was added. Formazan in solution was quantified at 570 nm with the help of an ELISA reader.

2.8. Protein content

The cellular protein content of wells fixed with 2-propanol for 15 min has been measured using a commercial BCA protein assay as recommended by the supplier. Serial dilution of BSA served as standard. After, the quantitative reaction supernatants were transferred into an empty 96-well microtiter plate and extinction measured at 590 nm by means of a microplate reader (ICN, Meckenheim).

3. Results

3.1. Microscopical evaluation

EC were characterized by morphological means and showed typical cobblestone cell shape and cell behavior in the monolayer. After an 18 h exposure to 1 mmol $ZnCl_2$ EC showed cytoplasmatic retraction and partly detached from the surface. For ECGF, actinomycin D and all other concentrations of $ZnCl_2$, no morphological changes could be detected as demonstrated in Fig. 1. The number of cells in mitosis was increased for the ECGF-treated group to 122% in comparison to the medium control. Actinomycin D as well as $ZnCl_2$ in 1 mmol concentration both gave values of less than 8%. $ZnCl_2$ in 0.1 mmol concentration did not alter the number of cells in mitosis in comparison to the control group.

3.2. Flow cytometrical analysis of cell cycle and Ki-67 expression

Fig. 2 shows a differentiated image of the cell cycle distribution for HUVEC dependent on stimulus and metal ion concentration with the help of cell cycle analysis and combined bi-parameter cytometry. About 60% of the control cells were found to be in G_0/G_1 phases, while about 25% were detected to be S/G_2M cells as shown in Fig. 3. Apoptotic cells and detritus were subtracted in the present analysis.

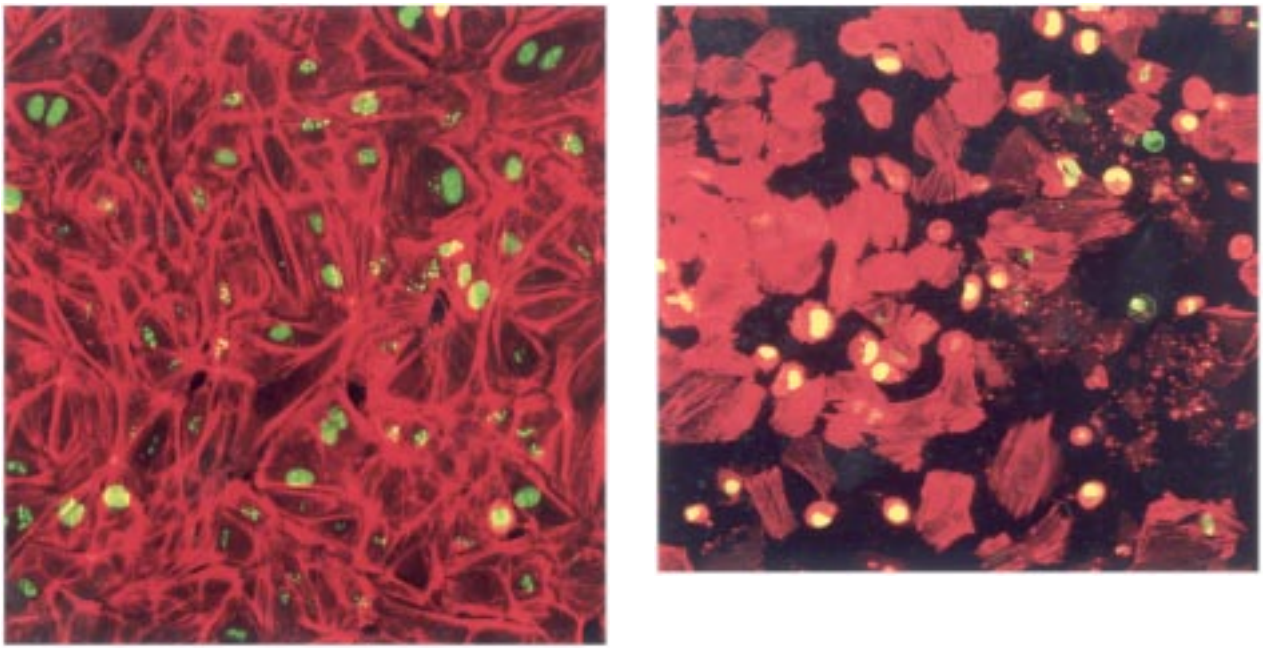

Figure 1 Morphology of primary isolated human endothelial cells after 18 h exposure to culture medium (a) or a 1 mmol concentration of $Zncl_2$ (b).

Actinomycin D elicited no significant influence on the cell cycle of EC, while ECGF reduced G_0/G_1 cells to a level of about 40% and increased S/G_2M cells up to about 35%. S/G_2M cells were significantly reduced by zinc concentrations of 1 and 0.5 mmol, with levels of about 20% being found. About 20% of the G_0/G_1 cells gave a positive signal for Ki-67 in the control group. Ki-67 was highly expressed in proliferating S/G_2M control group cells, giving a control level of about 58% positive S/G_2M cells (data not shown). Actinomycin D treatment reduced Ki-67 expression significantly in both G_0/G_1 and S/G_2M cells, thus levels of 6% for G_0/G_1 cells and about 30% for S/G_2M cells were measured.

3.3. Ki-67 ELISA

The Ki-67 ELISA results are shown in Fig. 4 and were very similar to the data obtained by both flow cytometrical cell cycle analysis and morphological evaluation of cells. The expression of the Ki-67 antigen in EC is significantly increased to an extent of 120% after stimulation with ECGF in comparison to the medium

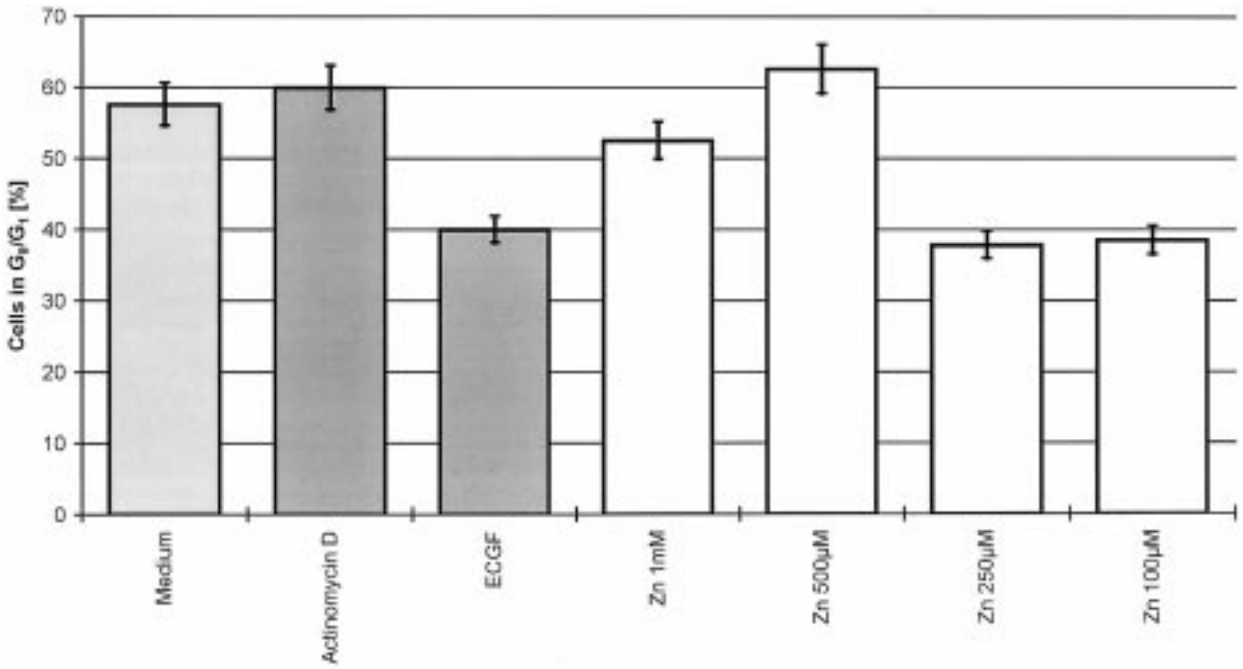

Figure 2 HUVEC in G_0/G_1 (per cent of total amount of analyzed endothelial cells) after an 18 h incubation with actinomycin D, ECGF and defined concentrations of metal ions. Data obtained from flow cytometrical cell cycle analysis of propidium iodide stained nuclei in permeabilized cells. Control represents the relative amount of cells in G_0/G_1 in standard culture (mean $\pm$ SD, $n = 5$).

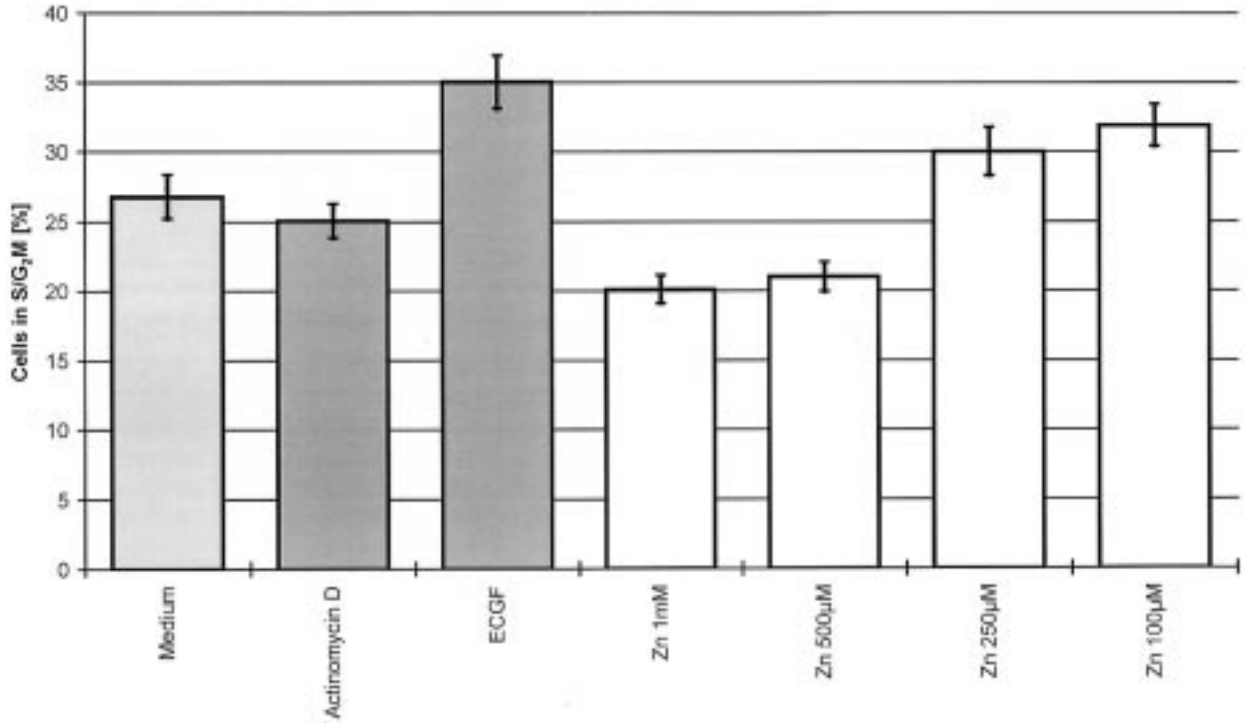

Figure 3 HUVEC in S/G_2M (per cent of total amount of analyzed endothelial cells) after an 18 h incubation with actinomycin D, ECGF and given molar concentrations of metal ions. Data obtained from flow cytometrical cell cycle analysis of propidium iodide stained nuclei in permeabilized cells. Control represents the relative amount of cells in S/G_2M in standard culture (mean $\pm$ SD, $n = 5$).

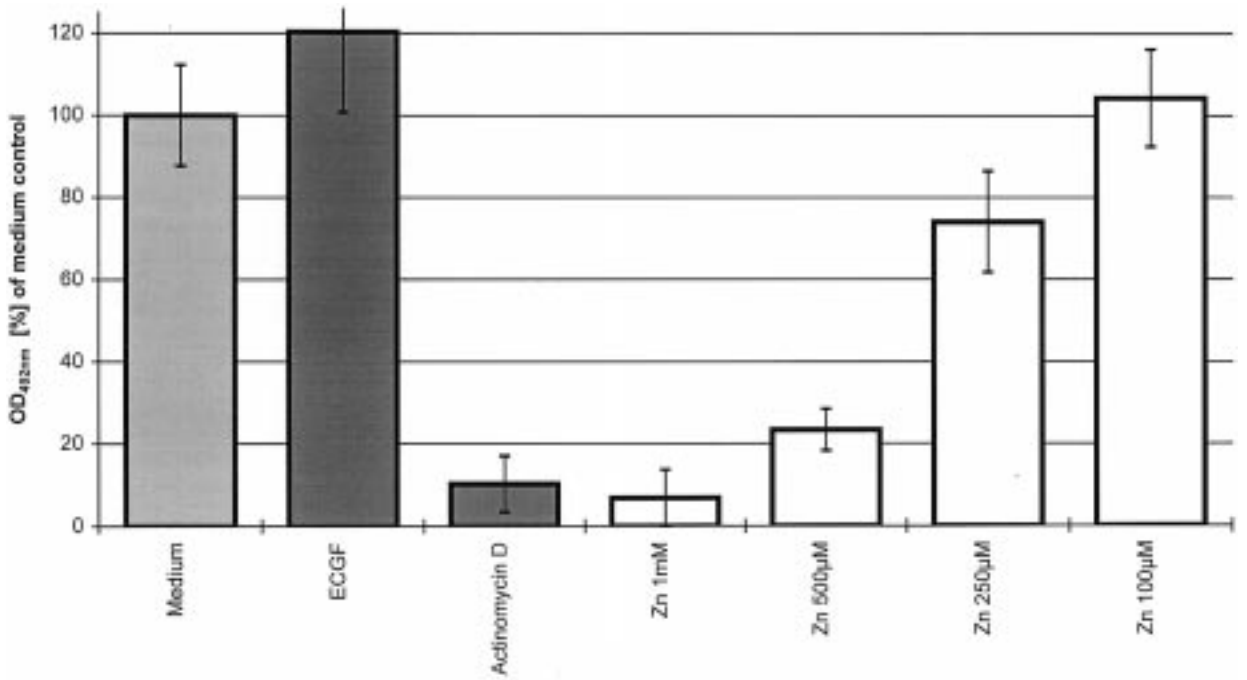

Figure 4 Nuclear expression of the Ki-67 antigen by primary isolated human endothelial cells after an 18 h exposure to culture medium, ECGF, actinomycin D or the indicated molar concentrations of $ZnCl_2$. OD_{492nm} (%) of the medium control, $n = 8$ mean $\pm$ SD.

control. Actinomycin D as well as an acute cytotoxic concentration of 1 mmol $ZnCl_2$ for 18 h reduce the expression to a basic level of 9 and 7%, respectively. Further dilution of the defined concentration of $ZnCl_2$ gives stepwise increasing levels of the signal reaching the control *niveau* in the case of a concentration of 0.1 mmol $ZnCl_2$. The same effect was observed using other substances reducing or inhibiting cell proliferation *in vitro*. The method shows a good specificity with a low non-specific signal, which has been subtracted from all values. Sensitivity was high, giving optical densities at 492 nm from 0.05 up to 1.2 with a conventional microtiter plate reader.

3.4. Cell counting

ECs of the control group were counted twice, i.e. at the time when exposure started (0 h) and at the end of exposure after 18 h. The number of cells increased from 60 to 100% in this course of time. The actinomycin D as well as the 1 mmol and 500 µmol $ZnCl_2$ treated cells

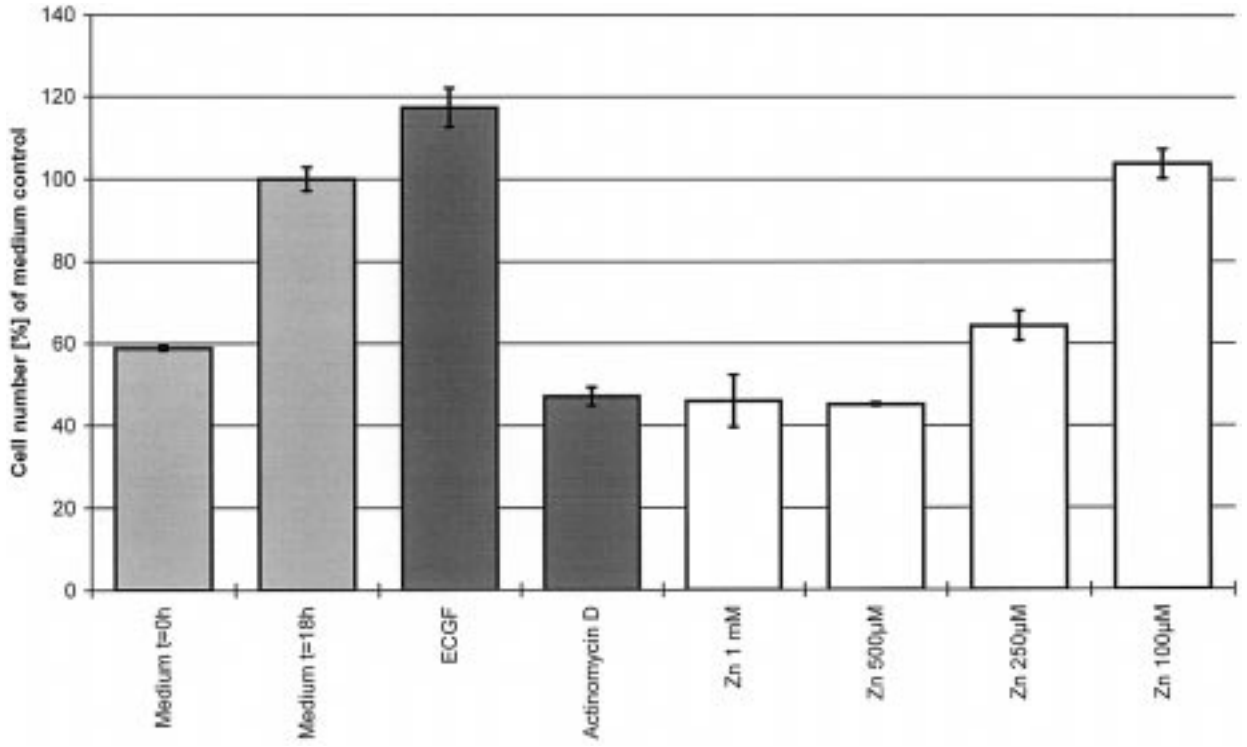

Figure 5 Cell number of primary isolated human endothelial cells after an 18 h exposure to culture medium, ECGF, actinomycin D (transcription inhibitor) or the indicated molar concentrations of $ZnCl_2$. OD_{570nm} (%) of the medium control, $n = 6$ mean $\pm$ SD.

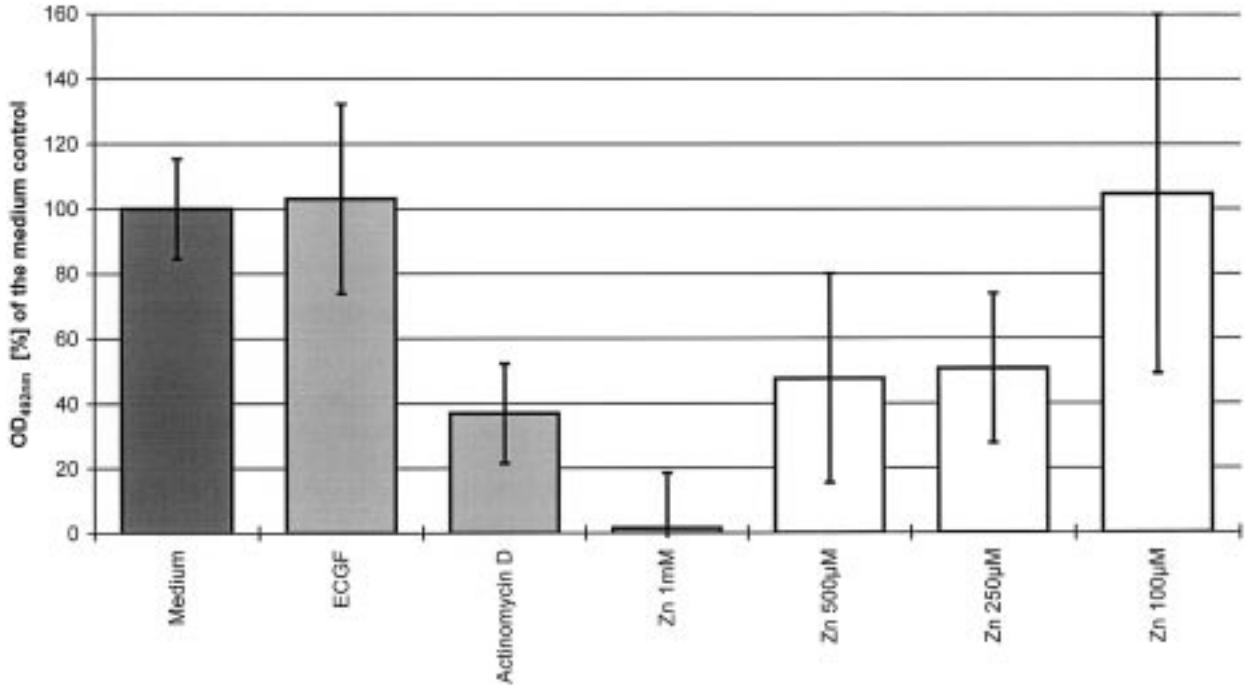

Figure 6 Incorporation of BrdU by primary isolated human endothelial cells after a 18 h exposure to culture medium, ECGF, actinomycin D (transcription inhibitor) or the indicated molar concentrations of $ZnCl_2$. OD_{492nm} (%) of the medium control, $n = 8$ mean $\pm$ SD.

show a decreased cell number of around 48%. Concentrations of 100 µmol $ZnCl_2$ and lower demonstrated no changes in comparison to the medium control group, as shown in Fig. 5.

3.5. BrdU incorporation

The results for incorporation of BrdU over a 18 h period of time elicit a high background, which is typical for the assay, as shown in Fig. 6. The data do not necessarily correspond to the data obtained by flow cytometrical cell cycle analysis or cell counting. This could be clearly demonstrated for the ECGF-treated group as well as for a concentration of 0.5 versus 250 µmol $ZnCl_2$.

3.6. MTT reduction test

MTT conversion under the influence of $ZnCl_2$ decreased in a concentration dependent manner, compared to the control medium. The results reflect the metabolic activity of HUVEC exposed to substances influencing both

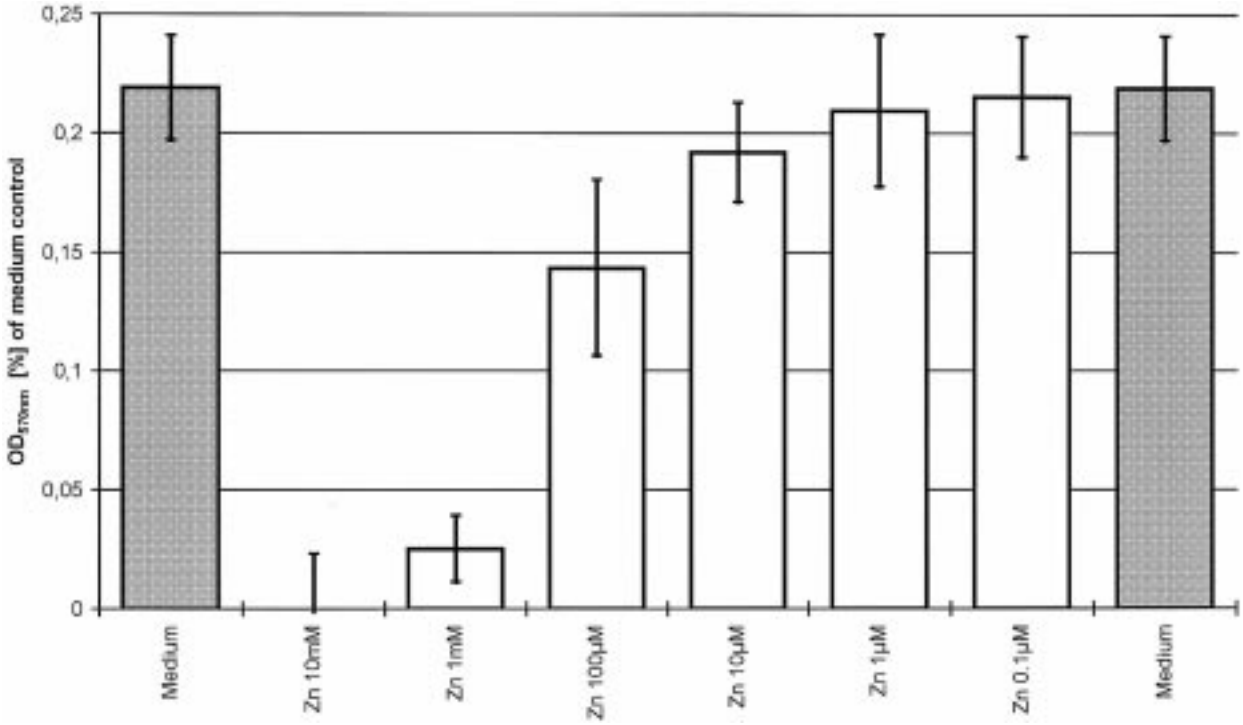

Figure 7 Mitochondrial activity quantified by studying MTT reduction to formazan salt by primary isolated human endothelial cells after an 18 h exposure to culture medium or the indicated molar concentrations of $ZnCl_2$. OD_{570nm} (%) of the medium control, $n = 8$ mean $\pm$ SD.

metabolic activity and cell proliferation. The lower metabolic activity of EC, for example under the influence of 100 µmol $ZnCl_2$, should *not* be misinterpreted as a reduction of proliferation rate.

4. Discussion

Biocompatibility has undergone a change of emphasis over the past few years and is now generally understood and defined by the ISO [4, 5] as involving two principal areas. The first is the principle of biosafety, including the exclusion of cytotoxicity. The principle of biofunctionality deals with the ability of a material and a device to perform in the specific application with an appropriate host response [6]. The pressure in industrialized societies to reduce experiments in animals, coupled with considerable advances in *in vitro* methodology as well as cell and tissue culture techniques have paved the way for a variety of tests that are suitable for testing regimens, and in particular, for the ISO standards. The assessment of cell structural changes by a trained morphologist must be regarded as a reliable method. However, we recommend, if possible, an objective evaluation by *quantitative* methods. Theoretical and practical elements of performing such *in vitro* tests have been published elsewhere [1, 6–10]. In ISO 10 993, categories of evaluation are presented including cell attachment, cell growth and cell proliferation. A variety of tests is used in this context and assumed to measure cell proliferation, the process by which cells pass through the cell cycle, including mitosis. Many related phenomena can be perceived during the course of proliferation but do also occur under different conditions. In some cases test methods tend to concentrate on related phenomena that are potentially easier to detect. The quantifiable MTT test is a measure of cell metabolic function, dependent on the intact activity of a mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to, for example, toxic surroundings. As shown in the present study, this test is unsuitable to measure either cell attachment, growth or even cell proliferation [11]. In brief, the test involves the conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to an insoluble formazan product, which can be quantitated by spectrophotometry [1, 12]. The number of cells on a surface or in an indirect contact test is an important parameter of *in vitro* evaluation of biomaterials [1, 6, 10]. Cells can be counted by using video microscopy and computer-assisted image analysis [13]. Total protein or DNA content is a measure for cell number, if non-specific activity can be excluded for the system used [1, 10]. Dye uptake or enclusion is quantified and has to do with the integrity of the cytoplasmatic membrane. FDA, calcein derivatives, toluidine blue and other substances have been described and reflect the cell number, their viability, metabolic activity and membrane integrity [1, 7, 8, 10]. Cells *in vitro* in a non-confluent state in monolayer undergo mitosis, prior to which DNA synthesis occurs (in the S-phase of the cell cycle). Cultures that are entering the log growth phase can be pulse labeled with a nucleotide building block of DNA, which has been tagged, for example, with a radioactive atom to enable detection, as is the case of tritiated thymidine. One alternative is the use of non-radioactive bromodeoxyuridine (BrdU), which can be detected by a fluorescent dye-conjugated monoclonal antibody against BrdU [1, 7] or in a cell ELISA using an enzyme coupled antibody [10]. Using parallel cultures it is possible to compare control cultures with test cultures that contain either the biomaterial itself or its extract. By this means one can determine if material exposure causes reduced DNA synthesis, which is expressed as the labeling index, i.e. the proportion of cells with positively tagged nuclei. The methods for quantitation vary from direct counting

in the microscope, to flow cytometric analysis, to a cell ELISA. The presented results demonstrate the difficulties of this experimental approach. Incubation time has to be prolonged when a cell population is chosen that normally proliferates slowly. The signal intensity in the assay is strongly dependent on the amount of BrdU available to compete with thymidine [14]. It has to be added to the culture medium and may change the biophysiological environment of the cells [15]. The non-specific signal intensity, which is found for cells without BrdU addition, is remarkably high, showing values of 30% for HeLa cells up to 80% for EC in comparison to a 100% value defined for cells treated with culture medium containing BrdU. The determination using flow cytometry [1] has been strongly regarded as inappropriate to investigate cells attached to a surface. In all likelihood artifacts may occur by detaching cells from different biomaterial surfaces, if we consider the different adhesive properties of biomaterials [16]. Furthermore, a pulse chase experiment seems to be unnecessary. Once the cells have been detached from the surface, a DNA cell cycle analysis can be performed by using a DNA specific dye for flow cytometric evaluation, as shown in the present study.

The monoclonal antibody, MIB-1, detects a nuclear proliferation-associated antigen, which corresponds to a non-histone protein [17]. Cell cycle analysis shows that the Ki-67 nuclear antigen is expressed in G_1S_1, G_2 and mitosis, but not in G_0. A highly significant correlation between the mean values of the proliferating fraction of given human cell populations as determined using the Ki-67 antibody, and the biological behavior of certain malignant tumors has been reported [18–23]. Different retrospective studies demonstrated that assessment of the proliferating fraction using the Ki-67 antibody is a prognostic marker and can be of help outlining individual therapy protocols [24, 25]. The Ki-67 equivalent murine monoclonal antibody was introduced by Key *et al.* in 1993 [26]. It could be demonstrated that assessment of cell proliferation in the *in vitro* cell culture systems could be performed using the developed ELISA based on detection of the Ki-67 protein. In agreement with the findings of several other authors the detection of Ki-67 correlates strongly with the proliferation activity quantified by microscopic methods and flow cytometry.

5. Conclusions

In conclusion, the cell ELISA detecting the Ki-67 protein is a rapid, reliable and non-radioactive method for assessment of human cell proliferation and thus provides a better alternative to tritiated thymidine or BrdU assays. It can be used as one suitable method, according to ISO 10 993, part 5, together with other test methods, in order to exclude cytotoxicity. Already presented as an important parameter within risk assessment and evaluation of biofunctionality, cell proliferation determination is a central component of studying materials designed to be integrated into host tissues, as in the case of osteointegration of a joint prosthesis or dental implant. The requirement to use the relevant cell type in *in vitro* assays in order to explore biofunctionality in a relevant cell culture model has to be stressed.

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