Essential criteria that must be met by novel biomaterials prior to further in vitro and in vivo biological evaluation

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The development of biomaterials for use in humans has expanded at an ever increasing rate in the last 20 years. It is essential for the further evaluation of efficacy in *in vitro* model systems and for use in animal or eventual human patients that the materials used are sterile, free of cell-toxic compounds and free of endotoxin. Many of the biomaterials are being developed in university or small laboratories without access to large cell biology laboratories that will have the facilities or methodology to routinely screen samples to evaluate them for these factors. In this study three methods are described for rapidly testing samples for sterility, cell toxicity and the presence of endotoxin that only require equipment normally found in the most basic of cell biology laboratories. Not only are these methods useful for screening the end product, but they can also be used at any step of the manufacturing process to determine the origin and location of possible contaminations.

Keywords: biomaterial, nanoparticle, endotoxin, sterility, cell toxicity, biocompatibility testing, nanotoxicity

1. Introduction

The development of biomaterials in the last 20 years has increased at a tremendous rate. Biomaterials have been defined as "any substance (other than a drug) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body" by the National Institutes of Health Consensus Development Conference in 1982. Biomaterials can be made from natural products or synthesized in the laboratory. Examples of the most widely described natural products are collagen and gelatin, alginate, silk, fibrin, cellulose, chitosan, decellularized tissue, and extracellular matrix proteins. Synthetic biomaterials have been made from metals and their alloys, glass and ceramics and a wide range of polymers and composites as well as hybrids from any of the above (for review see [1]). Many different methodologies have been developed to prepare and modify natural and synthetic biomaterials. The manufacture of natural or synthetic biomaterials may involve steps including the incubation and washing with solvents, detergents, buffers or water that may include steps including chemical or biological modifications to generate a final biomaterial. Both the starting material and the modifying substances and solutions may not be sterile or contain compounds that are toxic for cells. For this reason, the final product is subjected to some form of sterilization.

Following sterilization the biomaterials are usually evaluated for "biocompatibility". The biocompatiblity of a biomaterial is a term to describe the response of the cells to which the biomaterial is targeted and the overall host response to the material. Biocompatibility "refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy" [2]. Biocompatibility testing involves the use of *in vitro* and *in vivo* studies to provide a safety evaluation of a specific biomaterial by examining biological effects that may include cytotoxicity, inflammatory potential, subcutaneous irritation, acute, subacute or chronic toxicity, genotoxicity, carcinogenicity, effects on reproduction or development and degradation effects.

The biocompatibility of a biomaterial is usually evaluated in a cell biology laboratory [3]. Cells are grown under sterile conditions and a material is added to the cells or cells are placed onto the material. The goals of these studies are to determine how the cells react to the material. If the material is to support the growth of the cells, studies are carried out to examine attachment, growth, morphology of cells, survival of cells and gene expression of cells in contact with or on the material. Further goals are to determine

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whether these cells show similar phenotypes to those observed *in vivo* and to ensure that negative characteristics are not observed. If the material is to interact with cells, such as a nanoparticle, studies are done to look at similar characteristics as above, to determine cell growth in the presence of the nanoparticles, the effects on their growth rate and morphology of the cells, gene regulation, the nanoparticles uptake by the cells, etc. The results of these studies will give the first indication to the developer which of the novel biomaterials or which of their modifications most closely reflect the *in vivo* situation or have the desired prerequsites in the biomaterial. Those meeting the desired prerequisites can then be tested using the ISO-10992 protocols and eventually be evaluated for their efficacy in *in vivo* studies.

Evaluating the efficacy of a biomaterial generally begins with examining the biocompatibility of the compound in more complex quantitative toxicity assays. This can be followed by examining the effects on gene regulation or the uptake of nanoparticles in *in vitro* cell culture model systems with standardized cell lines and, optimally, with primary human cells isolated from the target tissue [4–10]. For example, a bone biomaterial may be cultured with osteoblasts and endothelial cells to determine whether and how osteoblasts attach to, grow and spread on the material and whether these cells exhibit bone characteristics on the materials [11]. Studies with endothelial cells can provide information about the effects on growth as described above for osteoblasts but also on essential endothelial gene expression such as cell structure, inflammatory response or influence on angiogenesis potential [12–14]. Complex *in vitro* coculture systems consisting of two (for example, osteoblast and endothelial cells) or more cell types that mimic the *in vivo* environment have also been developed. These can be used to evaluate and compare different biomaterials destined for bone regeneration by simultaneously looking at osteoblast differentiation to bone and endothelial cell capillary formation [15, 16]. The best and most promising of these can then be selected for further *in vivo* studies by direct implantation into animal models [17].

Each lot or new synthesis of a biomaterial must undergo a screening for biocompatibility. International standards have been developed with standard protocols for testing the biocompatibility of medical devices. These are known as ISO 10993 and specific relevant studies depending on the material must be carried out prior to clinical studies [18]. However, for developers of novel products, be it large companies or researchers at universities, following these protocols for each new biomaterial synthesized would be incredibly time consuming and expensive.

Certain initial criteria must be met by biomaterials developers so that cell biology studies are possible. To be able to analyze the direct effects of the novel biomaterial on the cells, the novel material must be sterile, the sterile biomaterial must be free of endotoxin and the biomaterial must be free of substances that are toxic and affect the normal functioning of the cells (unless specifically designed for this purpose). Obviously, the presence of bacteria represents an infection in the body and this would result in a similarly undesirable effect in cell culture. Endotoxin, or bacterial lipopolysaccharide (LPS) (the terms are synonymous), from the outer membrane of gram-negative bacteria is a powerful stimulator and activator of a number of cell types in the human body [19]. When even small amounts of endotoxin enter the bloodstream this can result in fever and shivering, to systemic inflammation, cell and tissue damage, adult respiratory distress syndrome and in the worst case irreversible septic shock and death [20, 21]. So even if a novel biomaterial is sterile, then the biomaterials may have a substantial quantity of endotoxin present if bacteria are present prior to sterilization. This contamination will have a substantial effect in cell biological experiments, especially those with endothelial cells. Finally, remnants of chemical solutions, solvents, polymerizing compounds, etc. used in the synthesis process of biomaterials, can result in the detachment and death of cells *in vitro* even in very small amounts.

Instead of extensive biocompatibility and efficacy testing studies *in vitro* in cell biology laboratories, biomaterial developers can carry out a few simple and rapid testing procedures to determine whether the biomaterial is sterile, free of endotoxin and of residual toxic compounds from the synthesis process. These methods can also be used to follow the manufacturing process and to identify sources of potential contamination or toxic compounds. The respective studies can easily be carried out in small cell biology laboratories without the need for exclusive or expensive equipment.

2. Materials and methods

2.1. Cells

Human umbilical vein endothelial cells (HUVEC) were isolated according to a previously published method [22]. Cells were cultured in medium M199 (Sigma-Aldrich, Steinbach, Germany) + 20% fetal calf serum (FCS) (Life Technologies, Karlsruhe, Germany) + 2 mM Glutamax I (Life Technologies) + 100 U/100 µg/ml Penicillin/Streptomycin + 25 µg/ml sodium heparin (Sigma-Aldrich) + 25 µg/ml endothelial growth factor supplement (ECGS, Becton Dickinson, Bedford, MA). Cells were used in the 3rd or 4th passage.

The human alveolar type-II (ATII)-like cell line A549 (ATCC number CCL-185) was purchased from LGC Promochem (Wesel, Germany). Cells were cultured in complete cell culture medium composed of RPMI 1640 with L-Glutamine (Invitrogen Corporation, Germany) supplemented with 10% (v/v) fetal bovine serum (Sigma Aldrich, Germany) and 1% (v/v) 10000 U/ml Penicillin and 10000 U/ml Streptomycin (Invitrogen Corporation, Germany).

The osteoblast-like human cell line MG-63 (ATCC number CRL-1427) was purchased from LGC Promochem (Wesel, Germany). The cells were cultured in Eagle's minimum essential medium (EMEM) (Gibco) + 10% fetal bovine serum (Invitrogen) + 2 mM Glutamax I (Life Technologies) + 100 U/100 µg/ml Penicillin/Streptomycin.

For sterility testing, the above media were used without the inclusion of Penicillin and Streptomycin.

3. Sterility testing

The test for determining the sterility of biomaterials was carried out under aseptic conditions. Sterilized dry biomaterials were transferred to a disposable culture tube (15 or 50 ml cell culture flask or well-plate depending on the size of the biomaterial) containing cell culture medium described above without antibiotics. A few drops of liquid from biomaterials in solution were transferred to vessels above using sterile micropipetting techniques and sterile solids were placed directly into containers described above. Sufficient medium was added to cover the materials. In the case of materials in solvents, high dilutions (1:5000) into medium were used. The various vessels with medium and test biomaterial as well as vessels without the test biomaterial as control were then placed into a 37°C incubator and incubated for 24 to 96 h. At various time points the vessels were visually examined for turbidity.

4. Identification of toxic compounds in formulations of biomaterials

To determine the presence of cell-toxic substances in biomaterials, biomaterials were placed onto confluent layers of cells. HUVEC, A549 or MG-63 were grown in 12, 24, or larger well-plates and then the biomaterials were added. For nanoparticles, microparticles or small biomaterials, 24-well plates were generally sufficient. For larger biomaterials, larger wells were used. In all cases, a size was selected so that 60–70 % of the monolayer was visible after addition of the test biomaterial. A biomaterial exhibiting a toxic effect was identified by revealing a rapid change to the confluent monolayer of cells. After addition of the material, cells were examined for changes to morphology, disappearance of the cell-cell contact between confluent cells and for rounding-up and detachment after 4 h and at regular intervals up to 24 h.

5. Conditions for evaluating the presence of endotoxin on solids and in liquids

The presence of endotoxin/LPS in solutions or on biomaterials was tested as previously described. This test is based on the finding that HUVEC cells exposed to endotoxin rapidly express E-selectin and that this can be observed by immunofluorescent (IF) staining [23]. To determine the expression of E-selectin on endothelial cells exposed to different types of biomaterials or other compounds or solutions, various methods were used. Solutions (such as water, stock solutions or buffers, etc., nanoparticles or other granulate containing solutions), were added to cells grown in Lab-Tek™ II Chamber Slides™ coated with fibronectin (5 µg/ml), cultured until nearly confluent. After 4 h, the solutions were removed and cells were washed 3x with phosphate buffered saline (PBS). For studies of 3D materials three different approaches were used. In the first, materials were incubated overnight in cell culture media after which the media was removed and added to cells growing in chamber slides (as described above, also known as extraction or leaching experiment). In the other two methods, cells were either added onto and grown directly on the material, or the material was placed directly on a monolayer of HUVEC growing in a chamber slide and removed after 4 h. If the chamber slides were too small to hold the test material, cell numbers were scaled up for growth in 35 mm Petri plates. As in chamber slide studies, cells were incubated for 4 h in the presence of the test material and then washed 3x with PBS before proceeding with the IF staining protocol for detection of E-selectin expression (see below). To evaluate the expression and staining pattern of E-selectin on endothelial cells either exposed to solutions or growing on different materials, controls with cells incubated with and without 1 µg/ml LPS for 4 h were carried out.

6. Immunofluorescent staining method for detection of E-selectin

The methods used for the detection of E-selectin expression on endothelial cells have been previously described. Briefly, E-selectin expression on endothelial cells was visualized by immunofluorescent (IF) staining. After exposure to the LPS control solution or biomaterials of interest for 4 h, the cells were rinsed twice with PBS, fixed in 3.7% paraformaldehyde for 15 min, permeabilized in 0.2% (v/v) buffered Triton X-100 for 10 min, and then incubated in 1% bovine serum albumin (BSA)/PBS) for 1 h. Cells were subsequently incubated with mouse anti-human E-selectin (1:2000 Bender MedSystems, Cat. BMS110 in 1% BSA/PBS) for 1 h, washed 3x with PBS and then incubated with anti-mouse Alexa Fluor 488-conjugates (Molecular Probes) diluted 1:1000 in 1% BSA/PBS for 60 min. Cell nuclei were counterstained with Hoechst 33342 (Molecular Probes) diluted in PBS (0.5 µg/mL) for 10 min or with DAPI [24]. The samples were mounted with Gel/Mount (Biomeda) and observed using a fluorescent microscope or by confocal laser scanning microscopy. Negative controls were performed by omitting the primary antibodies. Endothelial cells without LPS and with 1 µg/ml LPS were used as negative (basal level E-selectin expression) and positive controls, respectively.

7. Results and discussion

An essential prerequisite for the evaluation of novel biomaterials in *in vitro* and *in vivo* studies requires that the materials are sterile, exhibit no toxic effect on the cells and are free of endotoxin. Novel biomaterials are generally manufactured in university laboratories where sterile and aseptic conditions are not possible. Many compounds are synthesized and handled under conditions where contaminating bacteria are killed; however, contamination can still be introduced by improper handling at any point in the production steps, for example, when re-suspending a precipitated or dried compound with water or buffer containing bacteria or endotoxin. These novel materials are then analyzed by many different methods to obtain information on structure, size, shape, porosity and surface charges in addition to being further modified by the addition of peptides, proteins, chemotherapeutics, biological active agents, etc. Since most developers do not have access to cell biological laboratories for extensive evaluation of the biomaterials at each manufacturing step, a contamination, endotoxin or a residual toxic compound may be introduced at any time point, remain in the system, be passed on at each manufacturing step and end up in the final product.

We have been involved in the *in vitro* analysis of biomaterials for over 15 years and have developed numerous model systems of different tissues and organs using primary human cells for the evaluation of toxicity, cell compatibility, cell functioning and gene expression on or exposed to biomaterials [8, 9, 13, 14, 25–27]. Early on many biomaterials were either not sterile or contained endotoxin or a residual toxic compound that led to the death of cells after exposure to the biomaterials or to unexplained results in the studies examining the effects on cell gene regulation or functions. By applying the three relatively simple tests described below, we have been able to assist manufacturers in identifying the sources of the three problematic factors outlined above early on in the manufacturing process, and, in nearly completely eliminating these factors rapidly and inexpensively from the final product.

A sterile material is essential for use in humans as well as animals, and obviously it is necessary if further *in vitro* studies with cells are to be conducted. A contamination can easily be determined by placing the biomaterial into sterile cell culture media (Fig. 1).

Bacterial or yeast cells replicate in the medium, resulting in a visible turbidity throughout the medium. Containers with medium can be stored in the biomaterial manufacturer labs and used whenever needed to screen and identify potential sources of contamination. Surprisingly, in many cases the source of contamination was “sterile” water or buffers in the laboratory used to store the compounds or to re-suspend the compounds at the final step of synthesis. These were often utilized by all personnel and not handled employing aseptic techniques.

Fig. 1. Images of cell culture flasks with and without contamination. Cell culture medium without antibiotics was placed into the culture flasks: a control flask (a), a flask inoculated with 1 µl of a gelatin nanoparticle suspension in solution (b). The two samples were incubated 24 h at 37° C. Note that no turbidity is observed in (a) while (b) is highly turbid (medium is cloudy due to bacterial growth—ruler marks behind flask not visible), indicating a bacterial contamination of the gelatin nanoparticle suspension in solution.

The absence of toxicity is essential for a successful tissue regeneration biomaterial. This may not necessarily be the case for nanoparticles designed for the delivery of drugs such as chemotherapeutic compounds. An early toxic evaluation is targeted at identifying residual substances such as solvents or polymerizing chemicals that may not have been completely washed from materials in the final manufacturing steps. The presence of such a compound(s) can easily be determined by adding the biomaterial directly to cells in monolayer culture and following changes to the morphology of the cells at various time points after addition. This method can identify compounds that are directly toxic to cells (Fig. 2).

Figures 2a and 2b show monolayers of HUVEC and A549 cells, Figs. 2c and 2d show the same monolayer after a 3 h exposure to poly(ethylene oxide) PEO and poly(propylene oxide) block copolymer (PEO-PPO-PEO) nanoparticles. As can be seen in Fig. 2c, the HUVEC monolayer was disrupted and a substantial number of cells were rounded up. After further incubation all of the HUVEC became detached (data not shown). Little or no effect was observed on the A549 cells (Fig. 2d). When the PEO-PPO-PEO nanoparticles were centrifuged and the supernatant was removed and placed on a HUVEC monolayer, the same results were obtained (Fig. 2f) as those observed in Fig. 2c. The supernatant was toxic. When the nanoparticle pellet was washed 2x with PBS and the washed nanoparticles were

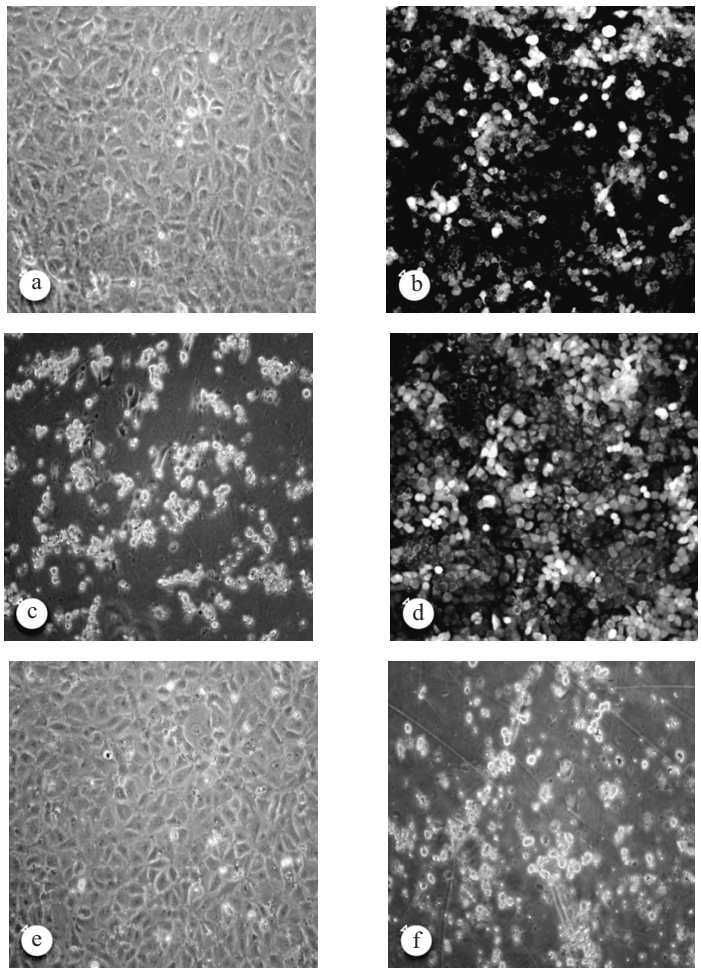

Fig. 2. Images of HUVEC and A549 cells exposed to PEO-PPO-PEO nanoparticles in a 24 well cell culture plate: (a) and (b) are images of HUVEC and A549 monolayer control samples, (c) is an image of HUVEC cells 4 h after 1µl of the nanoparticle solution was added to the culture medium, (d) is an image of A549 cells exposed as in (c), (e) are HUVEC cells after exposure to 1µl of the PEO-PPO-PEO nanoparticles that were washed 2x with PBS and then re-suspended in sterile PBS, (f) are HUVEC cells exposed to the supernatant recovered from the centrifuged nanoparticles prior to washing.

placed on the cells, no changes were observed in the HUVEC monolayer (Fig. 2e). Thus, the toxicity was due to a leachable component in the original solution. No differences were observed on the A549 cells. Also, MG63 cells exhibited results identical to the A549 (data not shown). Interestingly, it appears that the primary HUVEC were more sensitive to residual toxic compounds in materials compared to A549 or numerous other cell lines that have been used in studies in our laboratory although this has not been confirmed (data not shown).

Another major problem for further *in vitro* or *in vivo* studies is the presence of endotoxin in materials. Endotoxin, also known as bacterial lipopolysaccharide (LPS), is practically ubiquitous. LPS is part of the outer membrane of gram-negative bacteria and is secreted in small amounts during replication. A substantial amount of LPS may be released during the destruction of the cells by antibiotics or host immune cells and by killing and rupturing bacteria through heating solutions or adding solvents [19, 28]. Endotoxin is very stable and highly resistant to destruction by high temperatures or non-neutral pH levels [29]. In addition, endotoxin can adhere strongly to many materials and once present is difficult if not impossible to remove completely [30]. In humans and animals, endotoxin stimulates and activates different cell types, primarily those in the blood and especially with endothelial cells, making up the blood vessels. Endotoxin in the body can result in fever and shivering, as well as in larger amounts, to systemic inflammation, cell and tissue damage, adult respiratory distress syndrome and even irreversible septic shock and death [20, 21, 31]. Endotoxin or LPS from gram-negative bacteria is increasingly reported in biomaterials such as medical devices, albumin, collagen or gelatin, scaffolds for tissue engineering or nanoparticles for drug delivery. Unfortunately,

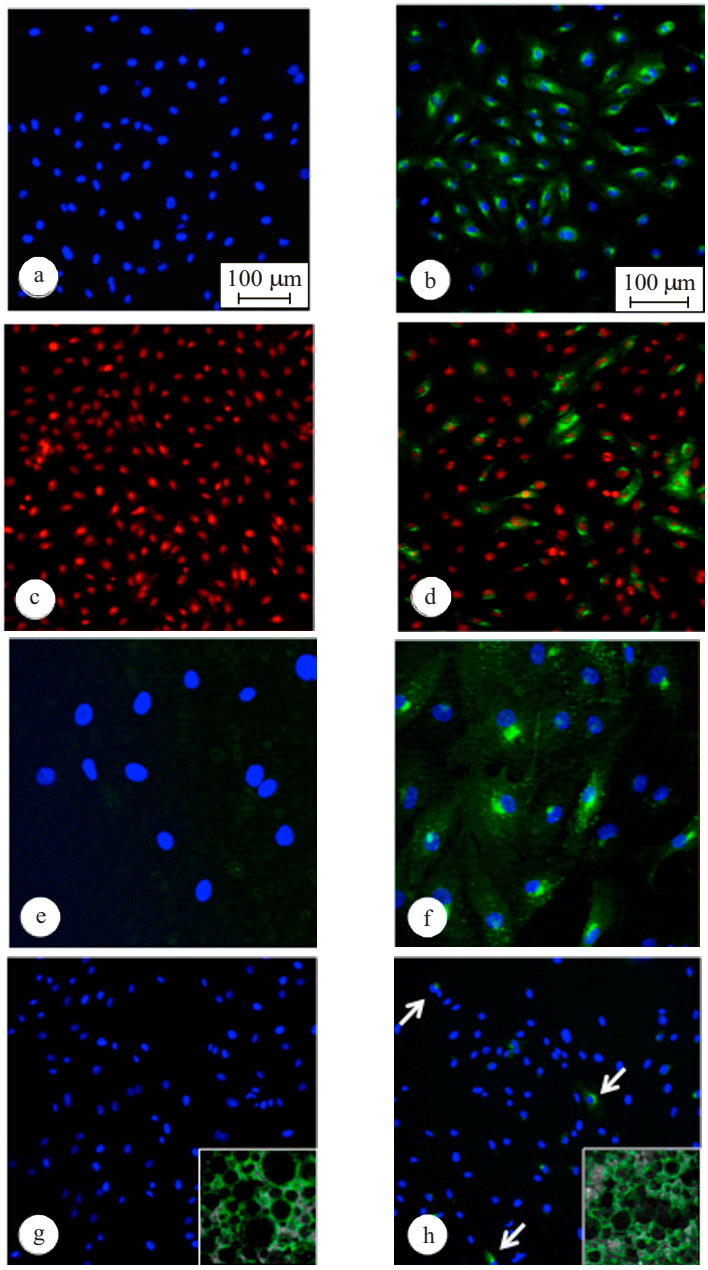

Fig. 3. Images of HUVEC cells stained for E-selectin after exposure to different biomaterials. Green is the positive staining for E-selectin, blue or red are nuclei stained with DAPI or Propidium iodide: (a) HUVEC control, (b) HUVEC 4 h after exposure to 1µg/ml LPS, (c) and (d) HUVEC 4 h after exposure to two different PET membranes, respectively, (e) and (f) HUVEC 4 h after exposure to two different samples of gelatin nanoparticles, respectively, and (g) and (h) HUVEC exposed to two different lots of hydroxyapatite granules. Inserts show calcium-AM stained cells on the two biomaterials, respectively. Arrows in (h) show the positive green staining for E-selectin.

in most cases, no studies have been done to determine the source of the contamination. Endotoxin in biomaterials has been described as "the uninvited guest" or "the invisible companion" [32, 33]. Currently, the limulus amebocyte lysate (LAL) gel clot assay and its derivatives are the most widely used assay systems for detecting endotoxin [28, 34].

We have previously described an assay system using human endothelial cells that offers an alternative method of similar sensitivity to the commonly used LAL assay system to detect endotoxin [23]. The E-selectin assay uses methods and equipment similar to those found in normal cell biology laboratories using the standard LAL assay. With this method it is possible to rapidly detect E-selectin induction of endothelial cells in contact with the materials as well as growing on the biomaterial (Fig. 3). As can be seen in Figs. 3a and 3b, HUVEC in the absence of LPS show no E-selectin

expression while after 4 h of exposure to LPS nearly 100 % of the cells show a strong expression of E-selectin. In cells added to two different polyethylene terephthalate (PET) membranes and stained for E-selectin, one of the membranes showed no staining for E-selectin, whereas the other did (Figs. 3c and 3d). Two gelatin nanoparticle preparations were examined by placing a solution of each onto HUVEC cells. As can be seen in Figs. 3e and 3f, one sample did not show staining for E-selectin, while the other cells did, respectively. Finally, HUVEC were cultured on two different lots of hydroxyapatite biomaterials. Cells attached and grew well on both biomaterials (inserts, Figs. 3g and 3h). However, materials stained for E-selectin showed that biomaterial in Fig. 3h resulted in a slight expression of E-selectin in a few cells (arrows), whereas the cells on the biomaterial in Fig. 3g did not show any E-selectin expression. A LAL assay of all samples confirmed that E-selectin positive samples were also LAL positive and negative samples were also negative in LAL (data not shown). Thus, in a little over 6 h a result can be obtained demonstrating whether biomaterials contain substances that produce the induction of E-selectin on endothelial cells. (HUVEC cells exposed to biomaterials for 4 h, staining with antibody to E-selectin followed by a second staining with a fluorescent-labeled antibody (~2 h). The E-selectin staining method on endothelial cells has been used for many years to evaluate the effects of LPS on endothelial cells and biomaterials [13, 14, 16, 27].

Three methods have been described that can be applied in biomaterial manufacturing laboratories to rapidly determine whether the biomaterials are sterile, free of cell-toxic compounds and whether the materials contain endotoxin. These methods can be used at all steps of the manufacturing process to identify steps where an introduction of bacterial contamination occurs, where insufficient processing is done to remove cell-toxic agents and can identify sources where endotoxin is introduced into the processing. These methods can help biomaterial manufacturers generate biomaterials that are free of contaminants and that can be used in studies for further *in vitro* or *in vivo* evaluation.

Acknowledgements

Parts of this research were generously supported by the the German-Israeli-Foundation (Grant No. 1092-28.2/2010), the BMBF (German Federal Ministry of Education and Research, Grant No. 0313405C) and the European Commission (Network of Excellence EXPERTISSUES, Contract No. 500283-2).

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