Embryonic Stem Cells for Tissue Biocompatibility, Angiogenesis, and Inflammation Testing

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Keywords

Angiogenesis · Macrophage differentiation · Cytokines · Biomaterials · Animal testing alternatives

Abstract

Aim: To introduce embryoid bodies derived from mouse embryonic stem (ES) cells, which differentiate blood vessel-like structures and leukocytes, as a novel in vitro model system for biocompatibility, inflammation, and angiogenesis studies. **Methodology/Results:** Punched spherical discs of bioabsorbable polymers (ϵ -caprolactone and L-lactide in different compositions) with a diameter of 2 mm and a thickness of 0.2 mm were inoculated with embryoid bodies for cocultivation. As reference material for biocompatible, nonbioabsorbable, and bioincompatible materials, polymer punched discs of petriPERM (PP) membrane (polytetrafluoroethylene) as well as polyvinylchloride (PVC) were used. Tissue outgrowth on the polymer discs decreased and cell toxicity increased upon confrontation on bioabsorbable biomaterials and PVC. Bioabsorbable polymers as well as PVC decreased the branching points and total tube length of CD31-positive vascular structures in embryoid bodies. With the exception

of PP, all applied materials increased the differentiation of CD68-positive macrophages and the generation of reactive oxygen species, which is indicative of proinflammatory processes upon contact of tissue with biomaterials. Consequently, cocultivation with polymers increased secretion of the cytokines interleukin-6, monocyte chemoattractant protein-1, and tumor necrosis factor- α . **Conclusion:** Three-dimensional tissues cultivated from ES cells are well-suited for testing the biocompatibility, the vascular response, and the inflammatory reaction towards bioabsorbable and nonbioabsorbable polymers.

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Introduction

Artificial implants are increasingly used in the clinical daily routine and they are applied diversely. In recent years, biocompatible and biodegradable polymers have been engineered for a variety of clinical applications. Moreover, the development of innovative biocompatible polymers is a basic requirement for the emerging field of tissue engineering and regenerative medicine which aims

Abbreviations used in this paper

CBA	cytometric bead assay
DCF	2',7'-dichlorofluorescein
EDTA	ethylenediaminetetra-acetic acid
ES	embryonic stem
H ₂ DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
IL	interleukin
iPS	induced pluripotent stem
max.	maximum
MCP	monocyte chemotactic protein
min.	minimum
ns	not significant
OL	oligolactide
PBS	phosphate-buffered saline
PBST	Triton X-100
PL	polylactide
PP	petriPERM
PVC	polyvinylchloride
ROS	reactive oxygen species
TNF	tumor necrosis factor

to generate complex 3-dimensional tissues for cell therapy approaches. The favored tissues should consist of various communicating organotypic cell types, including blood vessel structures which may interact differently with the scaffold on, or in, which the tissue grows. Very promising bioabsorbable polymer materials in terms of biodegradability and vascular biocompatibility are polymers consisting of ϵ -caprolactone and L-lactide [Araki et al., 2009; Matsumura et al., 2013; Larranaga et al., 2014]. Poly-L-lactic acid products were approved by the US Food and Drug Administration (FDA) for direct contact with biological fluids already in the 1970s [Arslan et al., 2016]. Four of its most attractive advantages are its renewability, bio-compatibility, processability, and energy saving properties [Rasal et al., 2010]. Copolymerization of L-lactide with ϵ -caprolactone allows specific adjustment of properties of the polymer with regard to firmness, melting point, and biodegradation. Such materials are currently used in the production of blood vessel grafts [Centola et al., 2010; Kim et al., 2010] and in cartilage and bone regeneration [Idris et al., 2010; Li et al., 2013], as well as for closure of gastric perforations in laparoscopic surgery [Bertleff et al., 2009].

Before their market launch, newly developed biomaterials have to pass extensive preclinical and clinical studies. The preclinical studies involve animal studies which – besides having ethical and financial constraints – bear the disadvantage of unpredictable side-effects of the materials and lack of transferability of the results to man. In

animal experiments as well as in patients, inflammatory reactions upon implantation of biomaterials consisting of ϵ -caprolactone and L-lactide, characterized by swelling and pain [Ekholm et al., 2003], increased accumulation of macrophages and neutrophils [van Tienen et al., 2002] and incapsulation of the implanted material [Pego et al., 2003], have been observed. Therefore, the development of new in vitro testing methods which include assay of inflammation markers is required to avoid rejection of the biomaterials in animals and humans.

Currently, conventional biocompatibility testing is performed in vitro with monolayer cell cultures of mostly one cell type. The cells are plated on polymer materials and cell morphology, adhesion, and viability are assessed. The disadvantage of this conventional in vitro testing is the absence of an inflammatory response induced by the interaction of the tissue with the material, which normally occurs in vivo. To specifically address inflammation in vitro, the reaction of macrophages cultivated on different polymer materials was investigated and secretion of chemokines, phagocytotic activity, and protein absorption at the polymer surface were investigated [Pamula et al., 2008; Scislowska-Czarnecka et al., 2012]. However, these experiments ignored that cells are physiologically embedded into a 3-dimensional context and interact with each other to behave as a biological unit.

In the current project, we used embryonic stem (ES) cells which were differentiated to the 3-dimensional tissue of embryoid bodies. These tissues bear features of the cellular immune response, are vascularized, and contain organotypic cells of all 3 germ layers [Hannig et al., 2010; Bekhite et al., 2015; Sharifpanah et al., 2015]. Via the use of ES cell-derived embryoid bodies, vitality and inflammation parameters in response to confrontation with different bioabsorbable and non bioabsorbable biomaterials were assessed. Moreover, the in vitro model of embryoid bodies allowed the investigation of biomaterial effects on vascular structure formation in a complex 3-dimensional tissue.

Materials and Methods

Biomaterials

The biomaterials used in the bioassays were provided by IN-NOVENT e.V., Jena, Germany. The bioabsorbable polymer polylactide (PL) was synthesized by ring opening polymerization from 70% L-lactide and 30% ϵ -caprolactone under tin(II) 2-ethylhexanoate catalysis. The corresponding oligomer oligolactide (OL) was prepared in the same manner but followed by methacrylation of the end groups using methacryloyl chloride. Films with a thickness of 0.2 mm were prepared from both synthetic materials in

Fig. 1. Scheme of confrontation culture generation. Undifferentiated embryonic stem cells are cultivated in monolayer culture. After enzymatic dissociation they are transferred to spinner flasks and cultivated as embryoid bodies for 8 days. Subsequently one embryoid body is inoculated with one spherical biomaterial disc (2-mm diameter) in hanging drop and then transferred to bacteriological Petri dishes after 48 h of incubation. The transmission image shows an embryoid body on top of a punched spherical disc of biomaterial. Scale bar, 1 mm.

weight ratios of 80:20 and 50:50 after UV crosslinking using 2,2-dimethoxy-1,2-diphenylethan-1-one (Irgacure 651; BASF, Ludwigshafen, Germany). The tested biomaterials were used as punched spherical discs with a diameter of 2 mm. PetriPERM (PP) membranes (Heraeus, Hanau, Germany) made of fluoroethylene-propylene copolymer Teflon were used as a nonbioabsorbable polymer reference, whereas polyvinylchloride (PVC) stabilized with Pb (PVC-Pb) served as a non bioabsorbable material with limited biocompatibility. As controls, embryoid bodies grown on glass coverslips were used.

ES Cell Culture and EB Formation

The ES cell line CGR8 was cultured on gelatine-coated cell culture dishes in Glasgow Minimal Essential Medium (Sigma, Taufkirchen, Germany) to keep the cells in the undifferentiated stem cell stage for proliferation. Medium was supplemented with 9% heat-inactivated (56°C, 30 min) fetal bovine serum (Sigma), 2 mM L-glutamine (Biochrom, Berlin, Germany), 45 µM 2-mercaptoethanol (Sigma), and 10³ U/mL leukemia inhibitory factor (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO₂ at 37°C and passaged every 2–3 days. On day 0 of differentiation, adherent cells were enzymatically dissociated using 0.25% trypsin with 1 mM ethylenediaminetetra-acetic acid (EDTA) in Hank's balanced salt solution (Gibco Thermo Fisher Scientific, Darmstadt, Germany). Cells (1 × 10⁷) were seeded in 250-mL siliconized spinner flasks (Life Technologies, Darmstadt, Germany) containing 125 mL Iscove's medium (CGR8 medium) supplemented with 16% heat-inactivated fetal bovine serum (Sigma), 100 µM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Biochrom), and 2 mM nonessential amino acids (Biochrom). After 24 h, 125 mL medium was added to give a final volume of 250 mL. The spinner flask medium was stirred at 22.5 rpm using a stirrer system (Integra Biosciences), and 100 mL cell culture medium was exchanged every day.

Generation of Confrontation Cultures between Embryoid Bodies and Biomaterials

For the generation of confrontation cultures between embryoid bodies and biomaterials, the method of hanging drops and spinner flask 3-dimensional culture were used (Fig. 1). Embryoid bodies with a mean diameter of approximately 700 µm were selected for the experiments. Using the hanging drop technique, one 8-day-old embryoid body and one punched spherical disc (2-mm diameter, 0.2-mm thickness) of the bioabsorbable polymers PL, PL/OL (80:20), and PL/OL (50:50) were inoculated within a 40-µL drop of CGR8 medium placed onto the lid of a 10-cm Petri dish (40 drops per lid). As representative material for a conventional biocompatible, nonbioabsorbable polymer, punched discs of a PP membrane, which is made of a fluoroethylene-propylene copolymer Teflon membrane, were used. As representative material for a nonbiocompatible material, punched discs of PVC-Pb were applied. The lid was turned around and placed on the Petri dish, which was filled with 10 mL sterile PBS. Within 24 h, embryoid bodies closely attached to the biomaterials, and medium was changed within the hanging drop. After 48 h the confrontation cultures were transferred to 6-cm bacteriological Petri dishes containing 5 mL CGR8 medium and further incubated as indicated.

Immunohistochemistry

As primary antibodies, monoclonal anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31; Chemicon International Inc., USA) and monoclonal anti-mouse CD68 (AbD Serotec, Düsseldorf, Germany), both at 1:100 dilution, were used. The tissues grown on biomaterials were fixed in ice-cold methanol (7:3) for 60 min at -20°C and washed with phosphate-buffered saline (PBS) containing 0.01% Triton X-100 (PBST). Blocking against unspecific binding was performed for 60 min at room temperature with 10% fat-free milk powder (Heirler, Radolfzell, Ger-

many) dissolved in PBST. The tissues were subsequently incubated overnight at 4°C with primary antibodies dissolved in PBST supplemented with 10% milk powder. The tissues were thereafter washed 3 times with PBST and reincubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-rat IgG secondary antibody (Chemicon) diluted 1:100 in PBST containing 10% milk powder. After washing 3 times with PBST, the tissues were stored in PBST until inspection.

Quantification of Vasculogenesis in Embryoid Bodies

Fluorescence recordings were performed by means of a confocal laser scanning setup (LSM 510; Zeiss, Jena, Germany) connected to an inverted microscope (Axiovert 135; Zeiss). The confocal setup was equipped with a 5-mW helium/neon laser (single excitation 633 nm) and an argon ion laser (single excitation 488 nm). Emission was recorded using the band-pass filter BP 515–550 and long-pass LP 650 filter sets, respectively. A $\times 20$, NA 0.5 objective (Neofluar; Zeiss) was used. The pinhole settings of the confocal setup were adjusted to give a full-width half maximum of 5 μm . For quantification of capillary areas within embryoid bodies, an optical sectioning routine based on confocal laser scanning microscopy was used. Images (512 $\times$ 512 pixels) were acquired from PECAM-1-stained embryoid bodies using the extended depth of focus algorithm of the confocal setup. In brief, 10 full-frame images, separated by a distance of 10 μm in the z -direction, were recorded and they included the information of the capillary area and spatial organization in a 100- μm -thick tissue slice. The acquired images were processed to generate a single in-focus image projection of vascular structures in the scanned tissue slice. Using the image analysis software Metamorph (Molecular Devices, Sunnyvale, CA, USA), the branching points per square millimeter tissue and total tube length per square millimeter tissue within the 3-dimensional projection were identified and counted in relation to the size of the respective embryoid body.

Toxicity Assay

Cell toxicity was analyzed using the cell membrane permeant dye DRAQ5 (New England Biolabs, Frankfurt, Germany), which labels cell nuclei of living and dead cells, and Sytox green (Thermo Fisher Scientific), which is cell membrane impermeant and labels cell nuclei of dead cells. The viability of cells in confrontation cultures between embryoid bodies and biomaterials was determined 3 and 5 days after confrontation in hanging drops. As a control for 100% toxicity, embryoid bodies were fixed with ice-cold methanol for 30 min at -20°C . The confrontation cultures were stained with Sytox green (0.1 μM) and DRAQ5 (0.1 μM) for 30 min at 37°C in the dark and washed once with 1 mL PBS. The fluorescence of cell nuclei was determined by confocal microscopy using the argon ion laser with single excitation at 488 nm and emission at 515–525 nm for Sytox green excitation, and the helium/neon laser of the confocal setup for DRAQ5 excitation with single excitation at 633 nm and long pass filter 650 nm for emission recording. Fluorescence was recorded using a $\times 20$, NA 0.5 objective (Neofluar; Zeiss) from the top to a depth of 100 μm of tissue composed of 5 optical sections with an interval of 10 μm in the z -direction. The ratio of Sytox green/DRAQ5-labelled cells in the respective optical section was evaluated using Metamorph image analysis software, and it was presented as the ratio of dead cells/total cells (%).

Reactive Oxygen Species Detection

Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$; Thermo Fisher Scientific), which is a non-polar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable, and it is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, embryoid bodies on biomaterials were incubated in E1 buffer (135 mM NaCl, 5.4 mM KCl, 1 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1 mM glucose $\times 1\text{H}_2\text{O}$, 10 mM HEPES, and 1.8 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, pH 7.5), and 20 μM $\text{H}_2\text{DCF-DA}$ dissolved in dimethyl sulfoxide was added. After 20 min the labeling solution was removed and intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3,600- μm^2 regions of interest using an overlay mask. For fluorescence excitation, the 488 nm band of the argon ion laser of the confocal laser scanning microscope was used. Emission was recorded at an emission band of 515–550 nm.

Cytokine Detection

For the detection of cytokine secretion from embryoid bodies, a mouse inflammation cytometric bead assay (CBA) (BD Biosciences, Heidelberg, Germany) was used. Briefly aliquots of cell culture supernatant from 20 confrontation cultures or embryoid bodies in suspension culture were taken on days 8+3 and 8+6 and frozen at -80°C for analysis of the cytokine content. The CBA allows quantitative measurement of interleukin (IL)-6, IL-10, monocyte chemotactic protein (MCP)-1, IFN- γ , tumor necrosis factor (TNF)- α , and IL-12p70 protein levels in a single sample. The test is based on micro-bead populations with distinct fluorescence intensities which are coated with capture antibodies specific for cytokines. A single set of diluted standards is used to generate a standard curve for each analyte. The assay was performed according to the instructions provided by the manufacturer (https://www.bdbiosciences.com/documents/CBA_MouseInflammation_Kit_Manual.pdf). A flow cytometer (BD FACSCalibur) equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm and BD CellQuest™ software were used for analysis.

Statistical Analyses

Data are given as means $\pm$ SEM, with n denoting the number of experiments performed with independent ES cell cultures. In each experiment at least 20 culture objects were analyzed unless otherwise indicated. The Student t test for unpaired data was applied for statistical analysis. $p < 0.05$ was considered statistically significant.

Results

Studies on Outgrowth and Cell Vitality of Embryoid Bodies on Biomaterials

The 3-dimensional tissue of embryoid bodies attached to the spherical discs of biomaterials started to grow out depending on the biocompatibility of the respective biomaterial (Fig. 2a). To assess cell migration from embryoid

Fig. 2. Effect of biomaterials on cell outgrowth from tissues. The minimum (min.) and maximum (max.) outgrowth cell distances from confrontation cultures on day 11 (day 8+3) of cell culture were determined by transmission light microscopy. **a** Representative image of an outgrowing embryoid body on a spherical disc demonstrating how min. and max. outgrowth distances were de-

termined. **b** Bar graphs of 3 independent experiments demonstrating the decrease in outgrow distance in the sequence PetriPERM (PP) > polylactide (PL)/oligolactide (OL) (80:20) = PL/OL (50:50) > PL > polyvinylchloride (PVC)-Pb. * $p < 0.05$, significantly different from PP.

bodies, the minimum and maximum outgrowth distances were determined after 3 days of confrontation culture (Fig. 2b). It was evident that the minimum as well as the maximum outgrowth distance decreased on biomaterials and PVC-Pb as compared to PP in the sequence PP > PL/OL (80:20) = PL/OL (50:50) > PL > PVC-Pb, which indicates that biomaterials, depending on the composition, differentially affect cell adhesion and migration from a 3-dimensional tissue.

To assess cell viability, embryoid bodies were stained on day 11 (day 8+3) and day 13 (day 8+5) of cell culture with DRAQ5 and Sytox green, and the number of Sytox green-positive cells was set in relation to the total (DRAQ5-positive) cell number and compared to a methanol-fixed tissue (100% toxicity). As shown in Figure 3, a significant increase in cell toxicity was observed on day 11 and 13 for the bioabsorbable materials PL, PL/OL (80:20) and PL/OL (50:50) as compared to the control (embryoid bodies plated on glass coverslips).

Effect of Biomaterials on Vascular Structures in Embryoid Bodies

When ES cells are grown in the 3-dimensional tissue of embryoid bodies, they efficiently differentiate vascular structures [Wartenberg et al., 1998; Bekhite et al., 2011; Sharifpanah et al., 2016]. Since the future clinical applica-

bility of biomaterials is critically dependent on their effects on vascular integrity and function, the impact of biomaterials on vascular structure formation in confrontation cultures of embryoid bodies with biomaterials was assessed (Fig. 4a–c). To achieve this aim, vascular structures in confrontation cultures on day 13 of cell culture (day 8+5) were labeled with the vascular marker CD31, and the branching points per square millimeter tissue (Fig. 4b) as well as the total tube length per square millimeter (Fig. 4c) were assessed. All biodegradable materials decreased the branching points of vascular CD31-positive vascular structures as well as the total tube length per square millimeter as compared with embryoid bodies grown on glass coverslips (control), suggesting that even biomaterials with a low cytotoxicity like PL significantly impaired vascular structure formation. Vascular structure formation was not impaired on nondegradable PP membranes but it was severely inhibited on PVC-Pb.

Generation of ROS upon Confrontation of Embryoid Bodies with Biomaterials

One major impediment for the applicability of biomaterials in patient treatment is the potential proinflammatory reaction of the tissue towards the transplanted material. The hallmark of inflammation is generation of ROS by, for example, NADPH oxidase or the mitochondrial

Fig. 3. Toxicity of biomaterials on embryoid body tissues. Tissues on biomaterials were analyzed for cell toxicity using the nuclear lethal marker Sytox green and the life/dead cell marker DRAQ5 on day 11 (day 8+3) and day 13 (day 8+5) of cell culture. As a positive control (Fix) (100% toxicity), cells were fixed in methanol before staining. Cell toxicity was determined as the number of dead (Sytox green-positive) cells/total (DRAQ5-positive) cells (%). Shown is a bar graph of 3 independent experiments demonstrating that biodegradable biomaterials exerted significant toxicity towards the tissue. * $p < 0.05$, significantly different to embryoid bodies on glass coverslips on day 11. # $p < 0.05$, significantly different to embryoid bodies on glass coverslips on day 13. PP, PetriPERM; PL, polylactide; OL, oligolactide; PVC, polyvinylchloride; ns, not significant.

respiratory chain. To assess ROS formation in embryoid bodies upon confrontation culture with biomaterials, the tissue was labeled with the redox sensitive fluorescence dye $H_2DCF\text{-}DA$ and DCF fluorescence was determined on day 1 (day 8+1), day 2 (day 8+2), and day 3 (day 8+3) of confrontation culture. Apparently all used materials increased ROS generation as compared to PP, with maximum effects observed on PVC-Pb (Fig. 5a). Interestingly, ROS generation decreased during prolonged confrontation culture times, indicating either improvement of the tolerance towards the respective materials or a reaction with nitric oxide for generation of peroxynitrite. The source of ROS generation was investigated on day 2 (day 8+2) in embryoid bodies grown on glass coverslips (control), on PP membranes and PL/OL (50:50), respectively, by applying the NADPH oxidase inhibitor DPI (1 μM), the respiratory chain complex I inhibitor rotenone (1 μM), or the free radical scavenger trolox (100 μM). It was observed that DPI as well as rotenone significantly decreased ROS generation as compared to the untreated control, indicating that both NADPH oxidase and the mi-

tochondrial respiratory chain were involved in ROS generation upon confrontation with biomaterials (Fig. 5b). Likewise, ROS generation was significantly decreased by the free radical scavenger trolox.

Secretion of Cytokines upon Confrontation of Embryoid Bodies with Biomaterials

The interaction of tissues with biomaterials causes a foreign body response which is accompanied by secretion of a variety of proinflammatory and anti-inflammatory cytokines [Morris and Kyriakides, 2014]. To assess whether embryoid bodies grown on biomaterials would provoke an inflammatory response, the release of the cytokines IL-6, IL-10, MCP-1, IFN- γ , TNF- α , and IL-12p70 to the cell culture medium was determined after either 3 days (day 8+3) or 6 days (day 8+6) of confrontation culture. It was shown that confrontation with biomaterials elicited IL-6, MCP-1, and TNF- α release to the medium, indicating that the tissue was able to react towards biomaterials with an inflammatory response (Fig. 6a–c). The highest cytokine concentration in the cell culture medium was observed for MCP-1, which attracts monocytes to the sites of inflammation, where they differentiate into macrophages. Notably the cytokines IL-10, IL-12p70, and IFN- γ remained below the detection limit (data not shown).

Differentiation of Macrophages upon Confrontation of Embryoid Bodies with Biomaterials

The major issue during inflammatory processes is the differentiation and recruitment of inflammatory leukocytes to the site of inflammation. Hence, studies on the biocompatibility of biomaterials should be performed with tissues which are able to show a cellular immune response. Since ES cells efficiently differentiate various types of leukocytes including monocytes/macrophages [Hannig et al., 2010; Finkensieper et al., 2013; Huang et al., 2016], we investigated the differentiation of CD68-positive macrophages upon confrontation culture with biomaterials. For this purpose, embryoid bodies on glass coverslips (control) or punched discs of biomaterials were incubated in bacteriological Petri dishes until day 14 (day 8+6) and the percentage of CD68-positive macrophages in relation to DRAQ5-positive total cells was assessed by immunohistochemistry (Fig. 7a, b). It was apparent that confrontation with biomaterials significantly increased the percentage of CD68-positive cells per total cell number in the area of inspection, supporting the notion that embryoid bodies reacted towards confrontation with biomaterials with an inflammatory response involv-

Fig. 4. Antiangiogenic effect of biomaterials. Embryoid body tissues grown on biomaterials were analyzed for CD31-positive vascular structures on day 13 of cell culture (day 8+5). **a** Representative images of CD31-positive vascular structures (red) in embryoid body tissues. The tissue was counterstained with DRAQ5 (green) to show cell nuclei. Scale bars, 100 μ m. **b, c** Confrontation culture

with biomaterials resulted in a decrease in branching points per square millimeter (3 independent experiments) (**b**) and a reduction in total tube length per square millimeter (**c**). * $p < 0.05$, significantly different from the control (embryoid bodies on glass coverslips). ns, not significant; PP, PetriPERM; PL, polylactide; OL, oligolactide; PVC, polyvinylchloride.

Fig. 5. Reactive oxygen species generation in embryoid body tissues upon confrontation culture with biomaterials. **a** Embryoid body tissues on biomaterials were labelled with the fluorescent reactive oxygen species indicator H₂DCF-DA on day 9 (day 8+1), day 10 (day 8+2), and day 11 (day 8+3) of cell culture and analyzed for DCF fluorescence. Reactive oxygen species increased in biomaterials with maximum effects on polyvinylchloride (PVC)-Pb (3 independent experiments). Notably, reactive oxygen species decreased with prolonged confrontation culture. * $p < 0.05$, significantly different from PetriPERM (PP). **b** Effect of the NADPH oxidase inhibitor DPI, the mitochondrial respiratory chain inhibitor rotenone, or the free radical scavenger trolox on reactive oxygen species generation in embryoid bodies plated on either glass coverslips (control), PP or polylactide (PL)/oligolactide (OL) (50:50) and investigated on day 8+2 (3 independent experiments). * $p < 0.05$, significantly different from the untreated control. # $p < 0.05$, significantly different from untreated PP. § $p < 0.05$, significantly different from untreated PL/OL (50:50). DCF, 2',7'-dichlorofluorescein; ns, not significant.

Fig. 6. Cytokine secretion upon confrontation culture of embryoid body tissues with biomaterials. Embryoid bodies on biomaterials or in suspension culture (control) were cultivated until day 11 (day 8+3) and day 14 (day 8+6) and the cytokines interleukin (IL)-6 (**a**), monocyte chemotactic protein (MCP)-1 (**b**), and tumor necrosis factor (TNF)- α (**c**) were determined in the cell supernatant. The highest cytokine secretion was observed for MCP-1, which attracts monocytes to the site of inflammation; note that on day 8+6 no MCP-1 secretion was observed in the control sample ($n = 3$). * $p < 0.05$, significantly different from control day 8+3. # $p < 0.05$, significantly different from control day 8+6. PP, PetriPERM; PL, polylactide; OL, oligolactide; PVC, polyvinylchloride; ns, not significant.

Fig. 7. Differentiation of macrophages upon confrontation culture of embryoid body tissues with biomaterials. Embryoid bodies on biomaterials were cultivated until day 14 (day 8+6) and immunostaining for CD68-positive macrophages was performed. The number of CD68 positive cells (%) was evaluated by labelling the total cells with DRAQ5. **a** Representative stainings of tissues with an antibody directed against CD68. **b** Bar graph of 3 independent experiments showing the increase in the percentage of CD68-positive macrophages upon confrontation of embryoid body tissues with biomaterials. * $p < 0.05$, significant as compared to PetriPERM (PP). PL, polylactide; OL, oligolactide; PVC, polyvinylchloride; ns, not significant.

ing a cellular immune response. These data underscore that the in vitro model of embryoid bodies represents a superior test system for investigation of the complex interaction of biological tissues with biomaterials.

Discussion

The objective of the present study was to develop a complex in vitro system for biosafety screening of biomaterials. Currently biomaterials are tested in laboratory animals, which is time consuming, costly, and ethically questionable. In vitro cell culture models are available.

However, they commonly use only a single cell type, thereby neglecting that in organs and organisms cells interact with each other in a complex manner and tissues are 3-dimensional vascularized entities with distinct oxygen and nutrient gradients [Gentile, 2016] which may affect the response of the tissue towards the biomaterial.

To overcome the detriments of 2-dimensional cell culture models, we used the embryoid body model of ES cells. Within the 3-dimensional tissue of embryoid bodies blood vessel-like structures are formed and – most importantly – leukocytes are differentiated [Finkensieper et al., 2010; Sharifpanah et al., 2015]. These immune-competent cells may be critically involved in the inflamma-

tory and cellular immune response towards the biomaterial. The ES cell model has an emergent property as compared to animal models and in vitro cell culture models: differentiated human cells can be reprogrammed to induced pluripotent stem (iPS) cells. iPS cell technology allows the development of human tissues which can be compared in terms of their response towards biomaterials with animal tissues. Moreover, iPS cells can be generated from human patients suffering from diseases. This allows investigation of whether a patient with, for example, a compromised immune system or a situation of chronic inflammation, would differently respond towards a specific biomaterial as compared to a healthy person.

The major focus of the present study was to collate the reaction of ES cell-derived tissue towards biodegradable biomaterials of different compositions and to relate data on biodegradable materials to nonbiodegradable materials, either the well-tolerated PP membrane or PVC which – though used in various medical applications – displays a low biocompatibility presumably due to the release of toxic stabilizers like lead and tin and the presence of plasticizers [Blass, 1992; Smith et al., 1995]. Using embryoid body tissues, different parameters of biocompatibility (e.g., outgrowth, vitality, ROS generation, vasculogenesis, leukopoiesis) as well as secretion of cytokines was investigated, thereby proving that the ES cell-derived embryoid body model is superior to conventional cell culture models which are mostly used just for cell vitality studies.

Our data demonstrated that outgrowth of the embryoid body tissue on the biomaterials did not inevitably correlate to the toxicity of the material used. For instance, PL strongly inhibited the outgrowth of cells from the tissue even though the toxicity of this material was moderate and comparable to that of the PL/OL preparations. Consistent with the data on outgrowth on biomaterials, all materials used in the present study decreased the CD31-positive branching points and tube lengths in the embryoid body tissue as compared to embryoid bodies grown on glass coverslips or PP, indicating that vascular sprout formation was correlated to the outgrowth capacity of the tissue on biomaterials. In embryoid bodies, hypoxia in the center of the tissue may play a role in ROS-driven vascular network formation [Gentile, 2016]. In the absence of vascular structures, hypoxia becomes severe and may cause apoptotic or necrotic cell death and contribute to the toxicity of biomaterials observed in the present study. Presumably outgrowth on the biomaterial causes mechanical tension within the tissue, which is an important signal for vascular growth and differentiation [Schmelter et al.,

2006; Potter et al., 2014; Zhu et al., 2015; Sharifpanah et al., 2016]. It should be mentioned that confrontation culture was performed with 8-day-old embryoid bodies, where vasculogenesis is already initiated [Wartenberg et al., 1998], which suggests that the materials affected already existing vascular structures and angiogenic sprouting processes rather than impairing vasculogenesis, i.e., de novo blood vessel differentiation from stem cells. Biocompatibility of biomaterials is largely dependent on their effects on blood vessel integrity since vascular dysfunction is correlated to tissue necrosis and biomaterial rejection. Moreover, cardiovascular diseases (e.g., coronary heart disease) are treated by the use of intravascular stents, which are inserted by a minimally invasive method and protect the vessel wall against elastic recoil, thereby safeguarding blood flow in diseased vessels [Shedden et al., 2009]. Currently biodegradable stents made of poly-L-lactic acid (XINSORB) are commercially available and are currently used in experimental animal studies and human patient treatments [Wu et al., 2012, 2016]. Whether biodegradable drug-eluting stents will be superior to metal stents will primarily depend on the tolerance of the blood vessel intima towards the bio-material and oxidative stress generated upon biomaterial/blood vessel interaction.

To assess oxidative stress during confrontation with biomaterials, ROS generation was determined during different days of contact with the biomaterials. ROS generation was inversely correlated to vascular sprouting and outgrowth of the tissue on the respective material. Low levels of ROS are physiologically generated in differentiating ES cells and determine the cardiovascular cell fate [Bekhite et al., 2013; Mascheck et al., 2015]. However, high levels of ROS cause oxidative stress, which may cause vascular dysfunction and result in a low biocompatibility. The strong ROS generation upon confrontation with PCV-Pb correlated well with the observed prominent macrophage differentiation but not with PL/OL (80:20) since under these conditions ROS generation was moderate despite efficient macrophage differentiation. ROS generation was not significantly different between PL, PL/OL (80:20), and PL/OL (50:50), which may explain the comparable effects of these polymers on toxicity, outgrowth, and vascular branching observed in the present study. The oxidative stress that arose during confrontation culture was obviously derived from NADPH oxidase and the mitochondrial respiratory chain since the NADPH inhibitor DPI and the complex I inhibitor rotenone largely abolished the observed effect. Since previous studies by us excluded the participation of ROS from the respiratory chain in physiological ROS generation of dif-

ferentiating ES cells [Sharifpanah et al., 2008; Bekhite et al., 2015], the involvement of mitochondria in ROS generation upon confrontation with biomaterials appears to be a distinct stress response.

ROS may be derived from leukocytes during the oxidative burst reaction. Moreover, the overall proinflammatory microenvironment contributes to the oxidative stress condition and may induce differentiation as well as attraction of leukocytes to the site of inflammation. Indeed, oxidative stress in the tissue upon confrontation with biomaterials increased the number of CD68-positive macrophages, which are major players in the inflammatory stress response and react with the secretion of various cytokines and chemokines to orchestrate the inflammatory condition [Castoldi et al., 2015]. Since the recruitment of macrophages towards the site of inflammation, which is observed in vivo, cannot occur under the conditions of embryoid body culture, we assumed that upon confrontation culture with biomaterials, resident monocytes may be activated. In line with this notion, it has been recently demonstrated that resident macrophages densely populate the normal arterial wall of blood vessels. These cells arise embryonically from CX3CR1(+) precursors and postnatally from bone marrow-derived monocytes that colonize the tissue immediately after birth [Ensan et al., 2016]. Recently the macrophage reaction towards different poly-L-lactic acid containing biomaterials was investigated, and it was shown that conditioned medium from macrophages stimulated endothelial cell migration in a scratch wound-healing assay [Boccafroschi et al., 2013]. Since differentiation of macrophages is a rather late event during the differentiation of ES cells [Hannig et al., 2010], it can be concluded that in the present study the biomaterials enhanced the differentiation of macrophages presumably due to ROS generation in the tissue.

To underscore the notion that confrontation culture of embryoid bodies with biomaterials elicited an inflammatory response, the release of cytokines to the cell supernatant was investigated. It was observed that the biomaterials differentially caused a release of cytokines to the cell culture medium. The highest level of cytokine secretion was observed for MCP-1 (CCL2), which is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages [Deshmane et al., 2009].

In summary, the data of the present study demonstrate the suitability of 3-dimensional vascularized tissues derived from ES cells to investigate the biocompatibility of biomaterials. This in vitro model is superior to conventional cellular models since it contains not only vascular structures, which support the supply of oxygen and nu-

trients to the tissue, but also connective tissue, cardiac cells, and leukocytes, which are critical players in the cellular immune response as well as in processes of wound healing and regeneration. Moreover, ES cells can be stably kept in culture for many years and passages, therefore enabling reproducible cell culture conditions on a defined genetic background. If extended to iPS cell cultures, this in vitro model will allow investigation of the biocompatibility of biomaterials with tissues from diseased patients (e.g., diabetic patients) who suffer from a chronic inflammatory condition and may differently react towards biomaterials as compared to healthy individuals. In the spinner flask 3-dimensional culture used in the present study, approximately 1,000 embryoid bodies can be simultaneously grown. With the availability of robotic cell culture systems, the production of confrontation cultures with embryoid bodies and biomaterials could be automatized. This will enable high throughput screenings for the biocompatibility of biomaterials, thereby reducing costs and avoiding ethically questionable animal experiments.

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Disclosure Statement

The authors have nothing to declare.

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