J Mater Sci: Mater Med (2008) 19:645–649
DOI 10.1007/s10856-007-3192-5

In vitro biocompatibility of degradable biopolymers in cell line cultures from various ocular tissues: extraction studies

Anne Huhtala · Timo Pohjonen · Lotta Salminen · Antero Salminen · Kai Kaarniranta · Hannu Uusitalo

Received: 11 September 2006 / Accepted: 22 May 2007 / Published online: 10 July 2007

Abstract In vitro biocompatibility of 50:50 PDLGA, 85:15 PDLGA, and Inion GTR^{TM} membrane was evaluated in cell line cultures from various ocular tissues, in human corneal epithelial cells (HCE), rabbit stromal fibroblasts (SIRC), bovine corneal endothelial cells (BCE), human conjunctival epithelial cells (IOBA-NHC), and human retinal pigment epithelial cells (ARPE-19). To study the toxicity of degradation products, the biomaterials were extracted in phosphate buffered saline at 70 °C for 24 h. The cell cultures were exposed to biomaterial extract diluted in medium (1:1–1:8) and the biocompatibility was evaluated by the WST-1 cytotoxicity/cell proliferation test. In all experiments without pH neutralization, cell viability increased with decreasing biomaterial extract volume. The highest extraction ratio 1:1 of PDLGA 50:50 decreased viability from 5–20%, from the control level, depending on the cell type. The corresponding cell viability values for PDLGA 85:15 and Inion GTR membrane ranged from 47–87% and 66–92%, respectively. When the pH of biomaterial extract was neutralized, Inion GTR membrane and PDLGA 85:15 had no effect on viability. BCE, HCE, and IOBA-NHC appeared to the most sensitive cell types, while SIRC and ARPE-19 were more resistant. The results of our in vitro studies suggest that the polymers tested are satisfactorily biocompatible.

A. Huhtala (✉) · L. Salminen · H. Uusitalo
Medical School, University of Tampere, Tampere 33014, Finland
e-mail: anne.huhtala@uta.fi

A. Huhtala · L. Salminen · H. Uusitalo
Department of Ophthalmology, Tampere University Hospital, P.O. Box 2000, Tampere 33521, Finland

T. Pohjonen
Inion Ltd, Lääkärinkatu 2, Tampere 33520, Finland

A. Salminen · K. Kaarniranta
Department of Neuroscience and Neurology, University of Kuopio, P.O. Box 1627, Kuopio 70211, Finland

K. Kaarniranta · H. Uusitalo
Department of Ophthalmology, University of Kuopio, P.O. Box 1627, Kuopio 70211, Finland

Introduction

Synthetic biodegradable polymers have many potential therapeutic applications. In ophthalmology, biodegradable polymers have been used as viscoelastic agents and surgical implants. Potential other applications include controlled release of drugs [1] and growth factors [2], gene therapy [3] and tissue engineering [4, 5]. In the present study, in vitro biocompatibility of three biodegradable poly (lactic acid-glycolic acid) based biopolymers was evaluated in cell line cultures from various ocular tissues, in corneal epithelial, stromal fibroblasts and corneal endothelial cells, conjunctival epithelial cells and retinal pigment epithelial cells by investigating cell proliferation and potential acute toxicity. In vitro biocompatibility was tested as suggested by the ISO 10993 standards created by the International Standard Organization [6, 7], by preparing extracts from the polymers. Ocular cells lines were used as standard models, since they are easy to cultivate. The viability of the cells was quantified by a colorimetric assay for cellular growth, the WST-1 assay, based on the cleavage of slightly red tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to dark red formazan by various mitochondrial dehydrogenase enzymes.

Materials and methods

Test materials

The biodegradable copolymers used in this study were a 50:50 molar ratio of poly(DL-lactide-*co*-glycolide) (PDLGA, Resomer® RG 50:50), a 85:15 molar ratio of PDLGA (Purasorb® PDGL 85:15), and Inion GTR™ membrane, a blend of 85:15 poly(L-lactide-*co*-glycolide) (PLGA) and 70:30 poly(L-lactide-*co*-1,3-trimethylene carbonate) (PLTMC) copolymers in a molar ratio of 70:30. PDLGA 50:50 copolymer was obtained from Boehringer Ingelheim (Ingelheim, Germany), PDLGA 85:15 from PURAC Biochem (Goerinchem, The Netherlands) and Inion GTR™ membrane from Inion Ltd (Tampere, Finland). All materials were manufactured into thin films by compression moulding at 160–170 °C in a zirconium nitride coated stainless steel mould and distributed by Inion Ltd. The test specimens were prepared as discs of diameter 5 mm, thickness 0.20–0.25 mm, surface area 42–43 mm^2, and average mass about 5.5 mg. The polymers were sterilized by γ-radiation (25 KGy) and subsequently handled in sterile manner.

Cell culture

Biocompatibility was assessed in five cell line cultures from various ocular tissues, in human corneal epithelial cells, HCE cells [8], rabbit corneal stromal fibroblasts, SIRC cells [9], bovine corneal endothelial cells, BCE cells [10], human conjunctival epithelial cells, IOBA-NHC (Normal Human Conjunctiva) cells [11], and human retinal pigment epithelial cells, ARPE-19 cells [12]. SIRC, BCE and ARPE-19 cells were obtained from the American Type Cell Collection (Manassas, Virginia, USA). HCE cells were kindly provided by Dr. Araki-Sasaki (Kinki Central Hospital, Hyogo, Japan) and IOBA-NHC cells by Dr. Calonge (IOBA-University of Valladolid, Valladolid, Spain). HCE cells were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere in a culture medium containing 1 vol of Dulbecco's Modified Eagle's Medium (DMEM) and 1 vol of Ham's nutrient mixture F-12 (from Gibco, Paisley, UK) supplemented with 15% (v/v) fetal bovine serum (FBS, from Gibco), 1% (v/v) antibiotic, antimycotic solution (penicillin 10,000 U/ml, streptomycin 10,000 µg/ml and amphotericin B 25 µg/ml, from Gibco), 2 mmol/l L-glutamine (Gibco), 5 µg/ml insulin (Sigma, St. Louis, MO), and 10 ng/ml human epithelial growth factor (EGF; Sigma). SIRC cells were grown in Eagle's Minimum Essential Medium (MEM, from Gibco) supplemented with 10% (v/v) FBS, 2 mmol/l L-glutamine, 1% (v/v) antibiotic, antimycotic solution, and 1 mmol/l sodium puryvate (Gibco). BCE cells were maintained in DMEM (Gibco) supplemented with 10% FBS, 2 mmol/l L-glutamine, 1% (v/v) antibiotic, antimycotic solution, and 1 mmol/l sodium puryvate. IOBA-NHC cells were grown in DMEM/F12 (1:1) medium supplemented with 10% (v/v) FBS, 2 mmol/l L-glutamine, 1% (v/v) antibiotic, antimycotic solution, 5 µg/ml hydrocortisone (Sigma), 5 µg/ml insulin and 2 ng/ml EGF. ARPE-19 cells were maintained in DMEM/F12 (1:1) medium (Gibco) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic, antimycotic solution. The cells were harvested with trypsin-EDTA (Gibco) twice a week at the split ratios 1:4–1:5.

Biocompatibility assessment

Cells were exposed as recommended by ISO-10993-12 and 10993-5 standards [6, 7]. The extraction ratio was 6 cm^2/ml in phosphate-buffered saline (PBS) and the extraction of the materials was carried out at 70 °C for 24 h without shaking. In a set of experiments the pH of biomaterial extract was not adjusted, while in another set of experiments the pH of biomaterial extract was adjusted with sodium hydroxide to 7.2. All cell culture studies were performed in 96-well tissue culture polystyrene (TCPS) plates. Cell cultures were inoculated at the density of 9×10^4 cells/cm^2 (3×10^4 cells/well) and grown for 24 h. The biomaterial extracts (without pH neutralization or with pH adjusted to 7.2) were diluted with growth medium to ratios 1:1, 1:2, 1:4 and 1:8, were directly given to the cell cultures, and incubated for 24 h at 37 °C. Medium produced in the same way without the addition of polymer extract provided as a control. Benzalkonium chloride (BAC), a known eye irritant, was used as a positive control. Cells were exposed to 0.001% BAC for 24 h.

Cytotoxicity tests

In vitro toxicity was assessed by using the WST-1 proliferation and cytotoxicity test. WST-1 test is based on the cleavage of the tetrazolium salt WST-1 (slightly red) to formazan (dark red) by various mitochondrial dehydrogenase enzymes. WST-1 test was purchased from Roche, Basel, Switzerland. To perform a WST-1 test, 10 µl of WST-1 reagent was added to the cells in medium in each well. The cells were incubated with the WST-1 reagent in a humidified atmosphere at 37 °C in 5% CO_2/95% air for 1 h with the ARPE-19 cells and for 2 h with the other cell cultures, then the multititer plate was thoroughly shaken for 1 min, after which the absorbances were measured with a Victor 1420 Multilabel Counter (Wallac Oy, Turku, Finland) using a wavelength of 450 nm. The use of the 1 h incubation period with the ARPE-19 cells and the 2 h incubation period with the other cells was based on a series of preliminary experiments. Cytotoxicity data were

obtained from at least three independent experiments testing four wells per material. The results were expressed as percentages of the mean absorbance (optical density) of treated *vs.* negative controls (normal growth medium). The mean optical density of the negative control was set to represent 100% viability.

Results

After an exposure time of 24 h, in all experiments when the pH of biomaterial extract was not neutralized, cell viability increased with the decreasing biomaterial extract volume. In those experiments, from the biodegradable materials studied, the extract of PDLGA 50:50 material affected cell viability the most (Fig. 1a), following PDLGA 85:15 material (Fig. 2a). Inion GTR^{TM} membrane material affected the viability the least (Fig. 3a). When the pH was not neutralized, the highest extraction ratio 1:1 of PDLGA 50:50 decreased viability from 5 to 20%, depending on the cell type. The corresponding cell viability values for PDLGA 85:15 and Inion GTR membrane ranged from 47–87% and 66–92%, respectively. The rank order for the different cells types was ARPE-19 > SIRC > BCE > IOBA-NHC > HCE. The positive control, 0.001% BAC for 24 h, decreased cell viability close to zero with all cell types.

When the pH of biomaterial extract was neutralized, Inion GTR membrane had no effect on viability (Fig. 3b). PDLGA 85:15 had a minor effect on BCE cell viability (Fig. 2b) and PDLGA 50:50 had an effect on cell viability, especially on BCE and HCE cells, even when the pH was neutralized (Fig. 1b). In these set of experiments in general, BCE cells appeared to be the most sensitive and ARPE-19 cells the most resistant.

Discussion

Polylactic acid and polyglycolic acid and their copolymers degrade in vivo by hydrolysis into lactic acid and glycolic acid, which are then incorporated into tricarboxylic acid cycle and excreted [13]. Degradation time for PDLGA 50:50 has been estimated to be about 2–4 months, 6–12 months for PDLGA 85:15, and 1–2 years for the Inion GTR membrane. PDLGA 50:50 degrades faster than PDLGA 85:15 due to a higher content of hydrophilic glycolic units [14]. On the other hand, the addition of carbonate units to the biomaterial (PLTMC) reduces degradation rates [15, 16]. The disadvantage of the materials tested is that they produce acidic byproducts during degradation which can lead to pH decrease in the vicinity of the scaffolds and inflammatory responses [17].

In the present study, an accelerated in vitro degradation model of polymers, combined with an extraction technique as suggested by the ISO 10993-12 standard [7] was used to evaluate the adverse toxic effects of these three materials in five cell types from various ocular tissues. Experiments with the polymer extracts, especially when the pH of the extract was not neutralized, showed increasing toxicity with faster degradation rates. However, it should be reminded that in vivo, due to small quantities produced, the acidic degradation products would likely not result in substantial decrease in local pH and thus would be expected to have minimal effects on the surrounding tissues [4]. From the cell types studied, corneal endothelial cells, corneal epithelial and conjunctival epithelial cells appeared to the most sensitive cell types, while stromal fibroblasts and retinal pigment epithelial cells were more resistant. When pH was neutralized, no toxic effects was observed on Inion GTR and PDLGA 85:15, apart from corneal endothelial cells. In

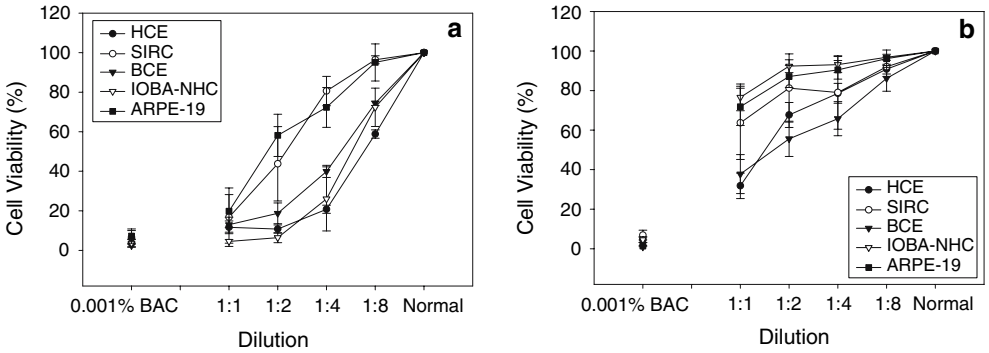

Fig. 1 (**a**) Results from the WST-1 assays with 50:50 PDLGA polymer (a 50:50 molar ratio of poly(DL-lactide-co-glycolide)) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was not neutralized. Extract dilutions in medium from 1:1 and to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (± SEM). (**b**) Results from the WST-1 assays with 50:50 PDLGA polymer (a 50:50 molar ratio of poly(DL-lactide-co-glycolide)) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was adjusted to 7.2. Extract dilutions in medium from 1:1 and to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (±SEM)

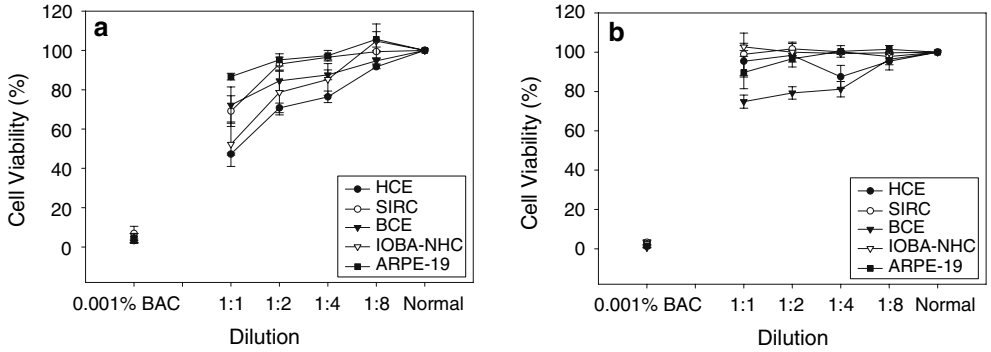

Fig. 2 (**a**) Results from the WST-1 assays with 85:15 PDLGA polymer (a 85:15 molar ratio of poly(DL-lactide-co-glycolide)) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was not neutralized. Extract dilutions in medium from 1:1 to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (±SEM). (**b**) Results from the WST-1 assays with 85:15 PDLGA polymer (a 85:15 molar ratio of poly(DL-lactide-co-glycolide)) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was adjusted to 7.2. Extract dilutions in medium from 1:1 to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (±SEM)

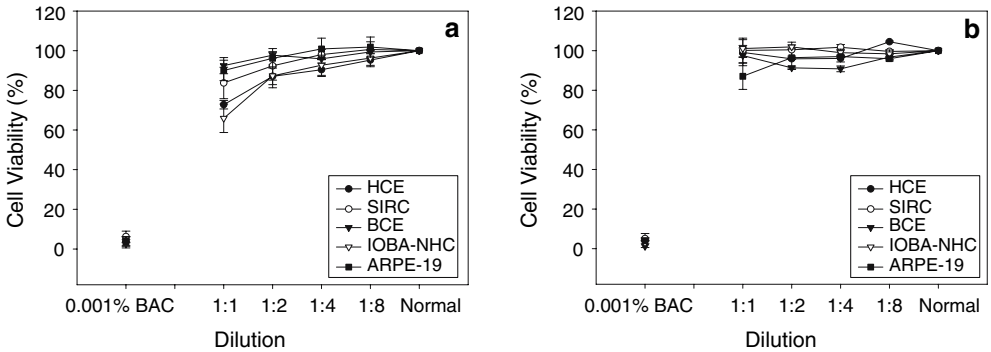

Fig. 3 (**a**) Results from the WST-1 assays with the Inion GTR™ copolymer (a blend of 85:15 poly(L-lactide-co-glycolide) (PLGA) and 70:30 poly(L-lactide-co-1,3-trimethylene carbonate) (PLTMC) co-polymers in a molar ratio of 70:30) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was not neutralized. Extract dilutions in medium from 1:1 to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (±SEM). (**b**) Results from the WST-1 assays with the Inion GTR™ copolymer (a blend of 85:15 poly(L-lactide-co-glycolide) (PLGA) and 70:30 poly(L-lactide-co-1,3-trimethylene carbonate) (PLTMC) co-polymers in a molar ratio of 70:30) following 24 h extraction in phosphate-buffered saline at 70 °C. The pH of biomaterial extract was adjusted to 7.2. Extract dilutions in medium from 1:1 to 1:8 were exposed to the cells for 24 h. 0.001% benzalkonium chloride (BAC) is a positive control. Data represents the mean of 12 replicates (±SEM)

the case of PDLGA 50:50, there appeared to be a non-pH-dependent toxic factor.

In vitro biocompatibility tests seem to be often more sensitive than studies with experimental animals [18]. Therefore, in vitro biocompatibility tests can be used to screen a series of biomaterials to reduce to use of experimental animals. It is unlikely that the toxicity studies in experimental animals can be completely replaced by in vitro studies, since biocompatibility of medical devices and drug delivery systems is a complex phenomenon including immune reactions and interactions with different inflammatory cells [19]. Biomedical implants often exhibit the formation of an avascular fibrous capsule or new blood vessels appearing at the polymer surface [20, 21]. Altered circulation around implants may thus affect on tissue homeostasis in vivo depending on the biomaterial implant type.

The results of our in vitro studies suggest PDLGA 50:50, PDLGA 85:15, and the Inion GTR are satisfactorily biocompatible in cells from various ocular tissues. The toxicity of these materials is mainly dependent on pH changes and is related to the cells used in this study. Thus, these biopolymers have potential to be used as scaffolds for tissue engineering or surgical implants, which may provide promising new therapies for ocular diseases.

Acknowledgements This study was financially supported by the National Technology Agency of Finland and the Medical Research

Fund of Tampere University Hospital. The authors thank Dr. Araki-Sasaki and Dr. Calonge for the generous supply of cells. Mrs. Maija Koskela is gratefully acknowledged for technical assistance.

References

1. T. YASUKAWA, Y. OGURA, H. KIMURA, E. SAKURAI and Y. TABATA, *Expert Opin. Drug Deliv.* **3** (2006) 261
2. C. ANDRIEU-SOLER, A. AUBERT-POUESSEL, M. DOAT, S. PICAUD, M. HALHAL, M. SIMONUTTI, M. C. VENIER-JULIENNE, J. P. BENOIT and F. BEHAR-COHEN, *Mol. Vis.* **11** (2005) 1002
3. R. A. BEJJANI, D. BENEZRA, H. COHEN, J. RIEGER, C. ANDRIEU, J. C. JEANNY, G. GOLLOMB and F. F. BEHAR-COHEN, *Mol. Vis.* **11** (2005) 124
4. L. C. LU, M. J. YASZEMSKI and A. G. MIKOS, *Biomaterials* **22** (2001) 3345
5. L. P. ANG, Z. Y. CHENG, R. W. BEUERMAN, S. H. TEOH, X. ZHU and D. T. TAN, *Invest. Ophthalmol. Vis. Sci.* **47** (2006) 105
6. INTERNATIONAL ORGANIZATION FOR STANDARDIZATION, in "Biological Evaluation Of Medical Devices – Part 5: Tests for In Vitro Cytotoxicity (ISO 10993-5:1999)" (International Organization for Standardization, Geneva, 1999). p. 3
7. INTERNATIONAL ORGANIZATION FOR STANDARDIZATION, in "Biological Evaluation Of Medical Devices – Part 12: Sample Preparation And Reference Materials (ISO 10993-12:1996)" (International Organization for Standardization, Geneva, 1996). p. 5
8. K. ARAKI-SASAKI, Y. OHASHI, T. SASABE, K. HAYASHI, H. WATANABE, Y. TANO and H. HANDA, *Invest. Ophthalmol. Vis. Sci.* **36** (1995) 614
9. J. LEERHOY, *Science* **149** (1965) 633
10. D. GOSPODAROWICZ, A. L. MESCHER and C. R. BIRDWELL, *Exp.Eye Res*. **25** (1977) 75
11. Y. DIEBOLD, M. CALONGE, A. E. DE SALAMANCA, S. CALLEJO, R. M. CORRALES, V. SAEZ, K. F. SIEMASKO and M. E. STERN, *Invest. Ophthalmol. Vis. Sci.* **44** (2003) 4263
12. K. C. DUNN, A. E. AOTAKIKEEN, F. R. PUTKEY and L. M. HJELMELAND, *Exp. Eye Res.* **62** (1996) 155
13. K. A. ATHANASIOU, G. G. NIEDERAUER and C. M. AGRAWAL, *Biomaterials* **17** (1996) 93
14. T. YASUKAWA, H. KIMURA, Y. TABATA and Y. OGURA, *Adv. Drug Deliv. Rev*. **52** (2001) 25
15. J. CAI, K. J. ZHU and S. L. YANG, *Polymer* **39** (1998) 4409
16. K. J. ZHU, J. X. ZHANG, C. WANG, H. YASUDA, A. ICHIMARU and K. YAMAMOTO, *J. Microencapsul.* **20** (2003) 731
17. M. S. TAYLOR, A. U. DANIELS, K. P. ANDRIANO and J. HELLER, *J. Appl. Biomater*. **5** (1994) 151
18. J. B. ULREICH and M. CHAVAPILIN, in "In Vitro Toxicity Testing: A Quantitative Microassay", Edited by: S. A. Brown (ASTM, Philadelphia, 1983), p. 102
19. R. ZANGE, Y. LI and T. KISSEL, *J. Control. Release* **56** (1998) 249
20. K. R. KIDD, D. Dal PONTE, A. L. STONE, J. B. HOYING, R. B. NAGLE and S. K. WILLIAMS, *Tissue Eng.* **11** (2005) 1379
21. E. BINZEN, A. LENDLEIN, S. KELCH, D. RICKERT and R. P. FRANKE, *Clin. Hemorheol. Microcirc.* **30** (2004) 283