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The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis

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Abstract

Even though degradation products of biodegradable polymers are known to be largely non-cytotoxic, little detailed information is available regarding the degradation rate-dependent acidic byproduct effect of the scaffold. In vitro and in vivo scaffold degradation rate could be differentiated using a fast degrading polymer (e.g., poly D, L-lactic-glycolic acid co-polymer, PLGA, 50:50) and a slow degrading polymer (e.g., poly ε-caprolactone, PCL). We applied a new method to develop uniform 10 μm thickness of high porous scaffolds using a computer-controlled knife coater with a motion stage and exploiting phase transition properties of a combination of salts and water in salt-leaching method. We then verified in vitro the effect of fast degradation by assessing the viability of primary mouse aortic smooth muscle cell cultured in the three-dimensional scaffolds. We found that cell viability was inversely related to degradation rate and was dependent on the depth from the seeding (upper) surface toward the lower surface. The pH measurement of culture medium using fluorescence probes showed time-dependent decrease in pH in the PLGA scaffolds, corresponding to PLGA degradation, and closely related to cell viability. In vivo analysis of scaffolds implanted subcutaneously into the back of mice, showed significant differences in inflammation and cell invasion into PLGA vs. PCL. Importantly, these were correlated with the degree of the functional angiogenesis within the scaffolds. Again, PLGA scaffolds demonstrated less cell mobilization and less angiogenesis, further supporting the negative effect of the acidic environment created by the degradation of biocompatible polymers.
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1. Introduction

Synthetic biodegradable polymers have been used extensively for scaffold fabrication in tissue engineering applications because they can enhance the properties of constructs specifically the mechanical properties, while allowing control of degradation. Good biocompatibility and possibility to processed into desired configurations add to their popularity. A desirable feature would be synchronization of polymer degradation with the replacement by natural tissue produced from cells. Therefore, the degradation properties of a scaffold are of crucial importance for biomaterial selection and design but also the long-term success of a tissue-engineered construct.

We have used in our study poly D, L-lactic-glycolic acid co-polymer (PLGA) and poly ε-caprolactone (PCL), previously studied in respect to porosity, behavior in static and flow conditions, with temperature and cyclic loading [1–4]. Both polymers have been shown to degrade mainly by simple hydrolysis of the ester bond into acidic monomers, which can be removed from the body by normal metabolic pathways. Other factors that affect degradation include hydrophobicity [5] and molecular weight [6,7]. The degradation of a block co-polymer such as PLGA is affected by the ratio of hydrophilic poly glycolic acid (PGA) to hydrophobic poly lactic acid (PLA) [5]. PCL that has a higher

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molecular weight with higher hydrophobicity than PLGA, will degrade more slowly. The biocompatibility of these polymers also has been demonstrated in biological applications [8–11] and the previous reports implied that the rate of degradation might affect cellular interaction including cell proliferation, tissue synthesis and host response [12,13]. However, details of the potential effects of the acidic byproducts on the three-dimensional cell culture or upon in vivo host response remain understudied.

We are interested in developing small diameter tissue engineered vascular constructs and thus for the purpose of this study we developed 10 μm thick porous biodegradable scaffolds. We investigated polymer degradation and rate-dependent effects comparing a fast degrading polymer (PLGA, 50:50 ratio of PGA to PLA) and a slow degrading polymer (PCL). Mouse aortic smooth muscle cells (MASMC) were cultured in vitro on the scaffolds and we assessed cell viability and measured the degradation rate dependent pH change of culture medium. We also implanted the scaffolds in vivo and investigated in time their population by cells, especially by inflammatory cells. Interestingly, we found that the degree of inflammation was related to the level of vascularization of implants through angiogenesis.

2. Materials and methods

Scaffold fabrication: We used salt leaching to develop a porous structure as follows. Poly DL-lactide-*co*-glycolide (PLGA; Sigma Aldrich, St Louis, MO) with a 50:50 ratio of PGA and PLA and a molecular weight range of 40,000–75,000 Da and poly ε-caprolactone (PCL; Sigma; Molecular weight (Mw) = 114,000 Da) were dissolved by solvent casting from chloroform (Sigma). Salt crystals were generated by exploiting phase transition properties of a combination of salts and water. The salt size was controlled by ultrasonication time and filter size in the final step and calculated by image analysis of scanning electron microscope (SEM) at random 20 pores of each three samples of the both polymers. Computer-controlled knife coater with a motion stage [14] was used to generate an average 10-μm thick polymer matrix, at a constant velocity and angle.

MASMC culture and polymer seeding: Smooth muscle cells were harvested from explanted aortas of wild-type mice as previously described [15]. Cells were grown in DMEM (Cellgro, Herndon, VA) supplemented with 10% FBS (Sigma). SMC were seeded (1×10^5) onto polymer discs cut to fit into 24-well plates. Culture medium was changed twice weekly throughout the experiments. Discs were analyzed at 1, 7, 14, 21 and 28 days post-seeding.

Subdermal scaffold implantation in mice: The polymer discs were sandwiched between two nitrocellulose filters (Millipore, Boulder, CO) to block the non-specific tissue in-growth into the polymer. The discs were subcutaneously implanted in the back of wild-type 129/SvEv mice. The discs were harvested at 7, 14, 21 and 28 days post implanting for analysis.

Polymer degradation assay: The polymer molecular weight distribution for the sample polymer discs was determined by gel permeation chromatography (GPC) (Perkin-Elmer, Wellesley, MA) equipped with a refractive index detector (Perkin-Elmer). The dried samples were dissolved in tetrahydrofuran at a concentration of 8 mg/ml and eluted through the column at a flow rate of 1 ml/min at 37°C. Polystyrene standards (Polysciences, Warrington, PA) were used to obtain a primary calibration curve. All samples of the same polymer type were analyzed at a single run. Variability in the determination of the both polymer molecular weight by GPC for undegraded samples was about 1% relative standard deviation.

SMC viability test: For two-dimensional viability tests, MASMCs were dissociated from the scaffolds using an enzyme solution consisting of 100U collagenase 1A (Sigma) and 250 μg trypsin (Sigma) and incubated at 37°C for 1 h [16]. Cell viability was determined by staining with 3 μM calcein (Molecular Probes Inc., Eugene, OR) and non-viable cells were visualized by staining with 20 μM propidium iodide (PI) (Sigma) followed by flow cytometric analysis (Becton Dickinson, San Jose, CA) [17].

Three-dimensional sectional cell viability through the thickness of the polymer (from the upper to lower surface) was measured using optical sectioning of 3 μm intervals with a multi-photon LSM510 microscope (Zeiss, Oberkochen, Germany) fitted with a NLO 800 nm laser. Staining of SMC with calcein, PI and Hoechst 33342 (Sigma) for nuclei counterstaining was performed without dissociation from the scaffold. At each optical section, the total cell number was counted as total blue stained (Hoechst) nuclei. The viable and dead cell number was counted as green (calcein) and red (PI) cells, respectively. We defined cells that stained with both calcein and PI as dying cells. The viability was calculated as a percentage of green stained cells out of the total cell number. Average cell viability was calculated for three regions: top surface, inner layer, and bottom layer.

Scanning electron microscopy (SEM): The scaffolds were air- and vacuum-dried, and their surfaces were prepared for observation with the SEM at the beginning and end of the observation period. The polymer samples were gold coated using a sputter coater (Pelco sputter coater 91,000) set at 20 mA for a total time of 120 s (coating thickness, approximately 40 nm). The specimens then were imaged with JSM-5300 SEM (JEOL, Peabody, MA) operated at 20 kV.

Measurement of pH with fluorescence probe: To determine the effect of degradation on the pH around the scaffolds, the polymer discs were incubated in phenol-red free DMEM at 37°C for 1, 7, 14, 21 and 28 days. To determine pH, 10 μl of 10 μM fluorescein (Sigma) was incubated with 1 ml culture medium for 10 min at 37°C [18]. Fluorescence was measured using a CytoFluor multi well plate reader (Perseptive Biosystems, Framingham, MA) set at 485 nm excitation and 53 nm emission. A standard curve was generated by adding either HC1 or NaOH to the phenol-red free DMEM to give pH values in the range of 5–8, followed by the addition of the fluoroscein and measurement in the CytoFluor.

In vivo host reaction to implanted scaffolds: Implanted PLGA or PCL discs were harvested at 7, 14 and 28 post-implantation. The harvested disc was frozen-embedded with O.C.T compound (Tissue-Tek®, Sakura Finetek, Torrance, CA) in liquid nitrogen and sectioned using a cryostat (Leica, Nussloch, Germany). All cell nuclei were counterstained using hematoxylin-eosin. For detection of inflammatory cells we performed Geimsa staining (Richard–Allan Scientific, Kalamazoo, MI) at 45°C for 30 min and differentiated in 1% acetic acid solution. In Geimsa staining, the negatively charged phosphoric acid groups of DNA attract the purple polychromatic dyes. The blue basophilic granules are stained by the polychromatic cationic dyes. Cationic cellular components such as erythrocytes and eosinophilic granules are stained by red and pink anionic dyes.

In vivo angiogenesis: Immunohistochemical staining to identify vasculature was performed using the endothelial specific marker CD31 (PECAM-1) on sections of the frozen-embedded disc using rat anti-mouse PECAM-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by Rhodamine Red X (RRX)-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA). Nuclei counter staining was performed with Hoechst 33258 (Sigma) to identify the areas of cell invasion. As a measure of angiogenesis, red colored PECAM-1 positive areas were measured and normalized by the total cellular area determined by blue nuclear staining.

Statistical analysis: All data is shown as mean ± standard error. The paired student's *t*-test was used to determine significance and a *P* value less than 0.05 was considered significant.

3. Results

Scaffold fabrication: After preparing the polymer a disc, scanning electron microscopy (SEM) was used to verify the uniformity of the thickness and the pore size of each disc (Fig. 1). Changing the weight ratio of the salts to polymer controlled the scaffold porosity. The ratio of the salts to polymer was 4.4:1, thus the porosity was calculated to approximately 80% [2]. The maximum porosity was found to be 90%, as the 10 μm thickness thin polymer lost its mechanical strength at such high porosity (data not shown). The pore sized distributed to 8.2 ± 1.8 μm in PLGA and 9.8 ± 0.6 μm in PCL. They showed somewhat different size distribution because of viscosity, but overall pore size could be controlled less than 10 μm diameter.

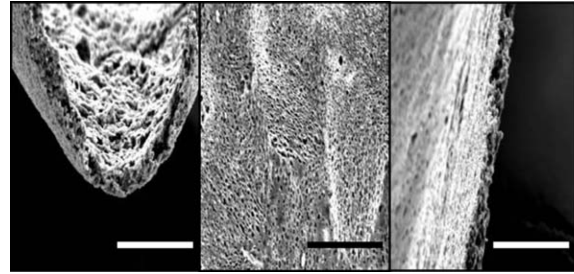

Fig. 1. PLGA can be used to create thin porous scaffolds of uniform thickness. Morphology of a PLGA scaffold as visualized by scanning electron microscopy (SEM). Left and right pictures demonstrate the possibility to control the scaffold thickness (left: 15 μm, right: 10 μm). Center image illustrate a surface view. Pore size: < 10 μm. Porosity: approximately 80%, Scale bars represent 50 μm.

Two- and three-dimensional cell viability test on the polymers: The two-dimensional (2D) viability of MASMC cultured on either type of polymers were compared against that of MASMC cultured in tissue culture plates (TCPS), for up to 28 days in culture. We found patterns of decreasing SMC viability on both polymers (Fig. 2). The 2D viability on PLGA was significantly lower than that on TCPS at 7, 21 and 28 days post-seeding. In comparison with PLGA, the viability on PCL was not significantly decreased compared to that on TCPS for the time investigated.

The three-dimensional (3D) viability test showed that MASMCs cultured on the surface of the scaffolds migrated into the scaffold through the inter-connected porous structure (Fig. 3(a)). Overall, at later time points cell viability decreased from the upper towards the lower surface of the scaffold. A significant decrease in cell viability within the scaffold compared to the upper surface of PLGA was detected starting at 14 post-seeding (Fig. 3(b)). In comparison, the significant decrease in cell viability was detected only on 28th days for PCL.

In vitro polymer degradation: GPC data confirmed that the two polymers degraded at different rates (Fig. 4(a)). Initially, the measured molecular weight was 40,000 ± 200 Da for PLGA, while it was 114,000 ± 350 Da for PCL. The molecular weight of PLGA had significantly decreased by 14 days (18%) and decreased to 56% ± 1% ($p < 0.01$) by 28 days. PCL degradation was slower, showing only a statistically

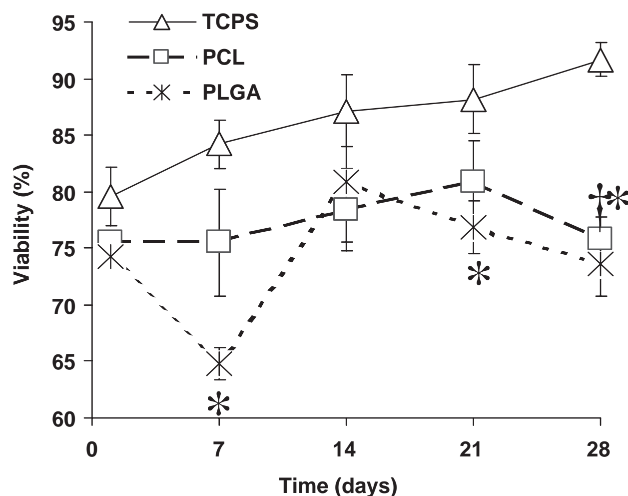

Fig. 2. Detection of cell viability using primary cultured mouse aortic smooth muscle cells (MASMC). Two-dimensional viability was measured for cells cultured on PCL or PLGA scaffolds compared to that of cells maintained under regular cell culture conditions, on a tissue cell culture plastic surface (TCPS) for up to 28 days. The means obtained from 4 independent experiments±SEM are shown. The viability was decreased at short time points on PLGA, then at later time points also on PCL. (*) $p < 0.05$ for PLGA vs. TCPS and (†) $p < 0.05$ for PCL vs. TCPS.

significantly decrease at 21 days (33%) and 28 days (39%±1%, $p<0.001$).

SEM micrographs illustrated the time-dependent morphological change of both polymer scaffolds (Fig. 4(b)). Significant morphological changes of PLGA could be seen at 14 days, with the disruption of internal structure changing the surface morphology. PCL showed relatively little change in morphology at 14 days. Huge disruption of inter-connected structure and changes in surface morphology was seen in PLGA at 28 days, while PCL scaffold structure appeared less affected.

Effects on pH: As polymer degradation leads to acidic byproducts, we investigated the potential effects on the pH surrounding the scaffolds. The pH measurements were performed in the absence of cells to eliminate potential changes caused by cell growth (Fig. 4(c)). In comparison with TCPS, the pH of medium with the PLGA discs was lower from 1 day through 28 days. PLGA degradation caused a significant decrease in pH by 7 days and continued decreasing to a final 0.5 pH units by 28 days. However, there was no significant decrease and difference of pH change between TCPS and PCL over the entire period investigated (28 days).

In vivo polymer degradation: In vivo degradation of PLGA or PCL discs implanted subdermally on mice was measured by GPC. The measured in vivo rates of degradation were found to be faster than those measured in vitro (Fig. 4(d)). The molecular weight of PLGA had decreased by 7 days and continued to decrease by 73.5%±6.5% ($p<0.05$) at 28 days. This degradation was significantly faster than in vitro degradation (56±1% at 28 days, $p<0.001$). PCL degraded more slowly than PLGA in vivo, degradation reaching significance by 21 days at 46.2%±2.3% ($p<0.05$). The degradation of PCL in vivo was also faster than that in vitro after the same length of time (39±1% at 28 days, $p<0.001$), supporting the notion that the degradation rate of polymers depends on the environment.

In vivo inflammatory cell invasion: We found that the cell density in the polymer regions remaining at 28 days was greater in the PCL compared to PLGA scaffolds (Fig. 5(a)). Taking into consideration the different degradation rates, the cell data was normalized to polymer area ($n = 3$ for each time point) to obtain a measure of cell migration into the scaffold. We found that a significantly greater number of total cells migrated into PCL than PLGA at all time points, the increased acidic environment likely inhibiting cell migration. We also determined that migration of inflammatory cells followed a similar trend at 7 and 14 days. At longer time points, we did not find a statistical difference in the migration of inflammatory cell into the polymers, however at this time point, quantification was less reliable due to significant polymer degradation.

In vivo angiogenesis: To measure the extent of angiogenesis within the implanted scaffolds, we detected the endothelial cell specific marker CD31 in histological sections of scaffold harvested at various times after implantation (Fig. 5(b)). We hypothesized that more in vivo cell invasion relating with inflammation (Fig. 5(a)) would result in a greater angiogenic response. A higher density of CD31 positive cells was detected in the PCL than PLGA from 14 to 28 days. The CD31 positive area was normalized to total tissue area ($n = 3$ for each time point) to determine the extent of angiogenesis. Significantly more angiogenesis was observed in PCL than PLGA scaffolds from 14 to 28 days, supporting the hypothesis that angiogenesis was related to increased invasion of scaffolds, specifically by inflammatory cells.

4. Discussion

This study demonstrated the negative effect of fast degradation of scaffolds upon the in vitro viability of cultured primary smooth muscle cells. Analysis of scaffolds implanted into the back of mice, further supported this observation by showing less population with cells and angiogenesis within rapidly degrading scaffolds. We suggest that these effects were due at least partially to the increasingly acidic environment resulting from the degradation of PLGA vs. PCL. Use of dynamic flow would likely reduce the buildup of the acidic local pH of PLGA.

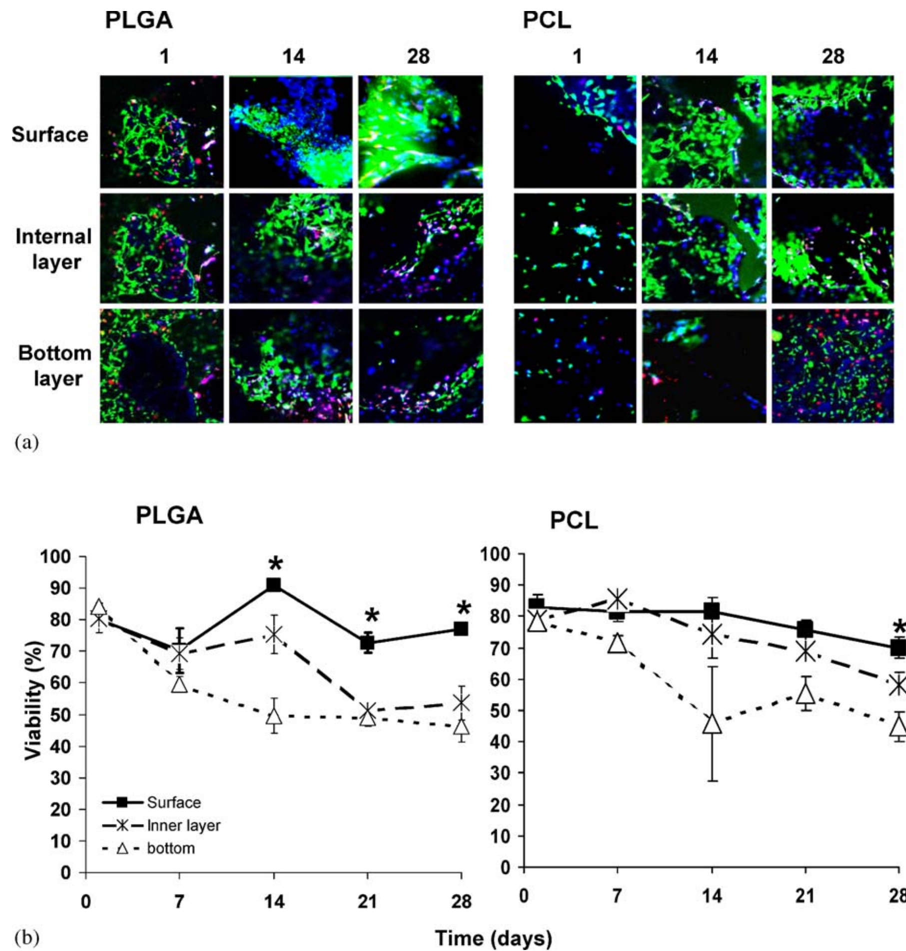

Fig. 3. Cell viability within scaffolds (3D) decreased with depth and as a function of time. Polymer discs cultured with MASMC for various length of time were analyzed by multi-photon microscopy using *z*-series at 3 μm interval optical sections. Top panels (a) illustrate cell viability in the three main regions (upper surface, middle region, bottom surface) after culture on PLGA (left) and PCL (right) for 1, 14, 28 days (viable cells are green, dead cells are red, all nuclei are blue) (b) Quantitative analysis of 3D viability in the three regions of PLGA (left) and PCL (right) scaffolds. The mean values obtained from 3 experiments ± SEM are shown. (*) $p < 0.05$ for surface vs. the other layers—internal and bottom layers.

We also report a method to control the thickness of biodegradable scaffolds using a knife-edge coater with a computer-controlled motion stage at a constant velocity and angle. Pore size within the scaffold was controlled by the use of phase-transitional salts. For our particular application we created 10 μm scaffolds with pore of 8.2 ± 1.8 μm for PLGA and 9.8 ± 0.6 μm for PCL. Such scaffold will likely be useful to generate a highly porous three-dimensional structure to optimize growth of vascular smooth muscle cells from aortic media [19,20].

We found that degradation of scaffolds made of either PLGA or PCL was faster in vivo than in vitro. Hydrolysis of PLA-PGA and PCL materials is influenced by the concentration of carboxylic acid end-groups, thus the degradation products of those materials may serve as catalysts for the reaction in static conditions, which are likely to accelerate degradation [21,22]. We also confirmed that more hydrophilic side chains and lower molecular weight of PLGA accelerated its degradation compared to PCL [23]. On the other hand, our study suggest that it is possible to accelerate the degradation of PCL, which although popular as a soft- and hard-tissue compatible material had somewhat limited application because of its slow degradation kinetics. Various approaches have been previously employed to enhance its degradability, for example, PCL diol with acryloyl chloride [10]. However, degradation is also affected by the scaffold thickness and porosity. In our application, the faster than expected PCL degradation was likely to be related to the fact that the PCL thin films had a greater surface area to volume ratio and thus a greater water uptake [6].

We found that the population with cells of scaffolds implanted in vivo was also dependent on the nature of the scaffold. Under similar conditions, the population of scaffolds with host cells, specifically with inflammatory and endothelial cells, were found to be significantly lower in the PLGA compared to the PCL scaffolds,

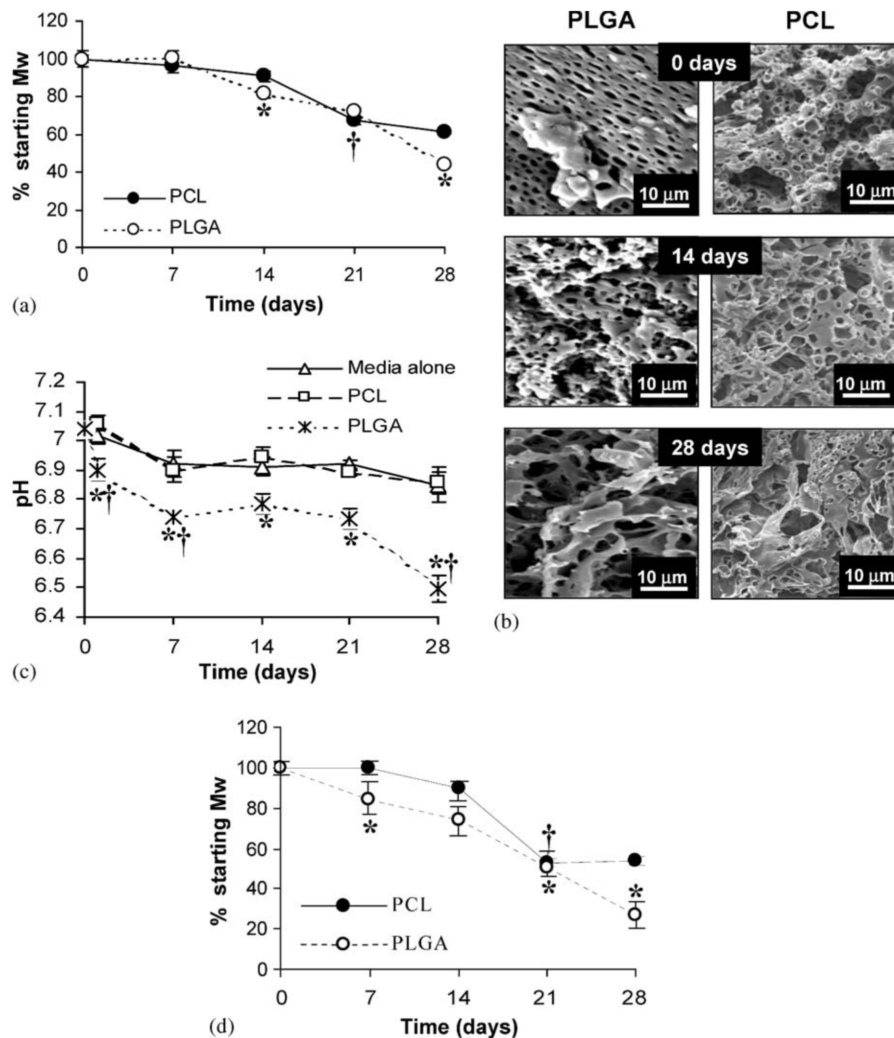

Fig. 4. Characterization of PLGA and PCL scaffolds degradation in vitro and in vivo. (a) Measurement of in vitro degradation by GPC. *Y*-axis represents the % of starting Mw. Error bars represent SEM for $n=3$. (*) $p < 0.05$ for percent decrease from previous value for PLGA degradation and (†) $p < 0.05$ for percent decrease from previous value for PCL degradation. (b) Investigation of scaffold morphology by SEM reveals effect of degradation at 14 and 28 days compared to day 0. (c) The environment pH changed as function of polymer and time, as measured using fluorescent probes. (Δ) Media minus scaffold (negative control), (□) PCL and (×) PLGA. The mean values obtained from 4 experiments ± SEM are shown. (*) $p < 0.05$ for each polymer vs. control (culture medium alone) and (†) $p < 0.05$ for the value change compared to the previous value. (d) Degradation of polymer scaffolds was faster in vivo, measurement by GPC—compare to graph illustrated in (a). *Y*-axis represents the % of starting Mw. The mean values obtained from 3 experiments ± SEM are shown. (*) $p < 0.05$ represents PLGA value compared to value at previous time point and (†) $p < 0.05$ represents PCL value compared to previous time point for PCL.

suggesting that the acidic pH and/or the rapid degradation of the scaffold prevented integration. Thus, we suggest that PCL may be more desirable for the survival of thin scaffolds implanted in vivo. Interestingly, the degree of inflammation seemed to be related to the degree of angiogenesis, further demonstrating the biocompatibility of degradation products [24]. Importantly, our results support the necessity of eliciting an appropriate host inflammatory responses in order to enhance the local recruitment of vasculature to tissue engineered constructs, essential for their integration as well as the long-tern survival and function.

5. Conclusion

We developed three-dimensional biodegradable scaffolds using a new method to control the thickness using a knife-edge coater with a computer-controlled motion stage and the pore size using phase-transitional salts. The comparative study of fast vs. slow degrading three-dimensional scaffolds indicated that fast degradation negatively affects cell viability and migration into the scaffold in vitro and in vivo. This effect is likely due to the significant acidification of the local environment due to the polymer degradation. We also report that the

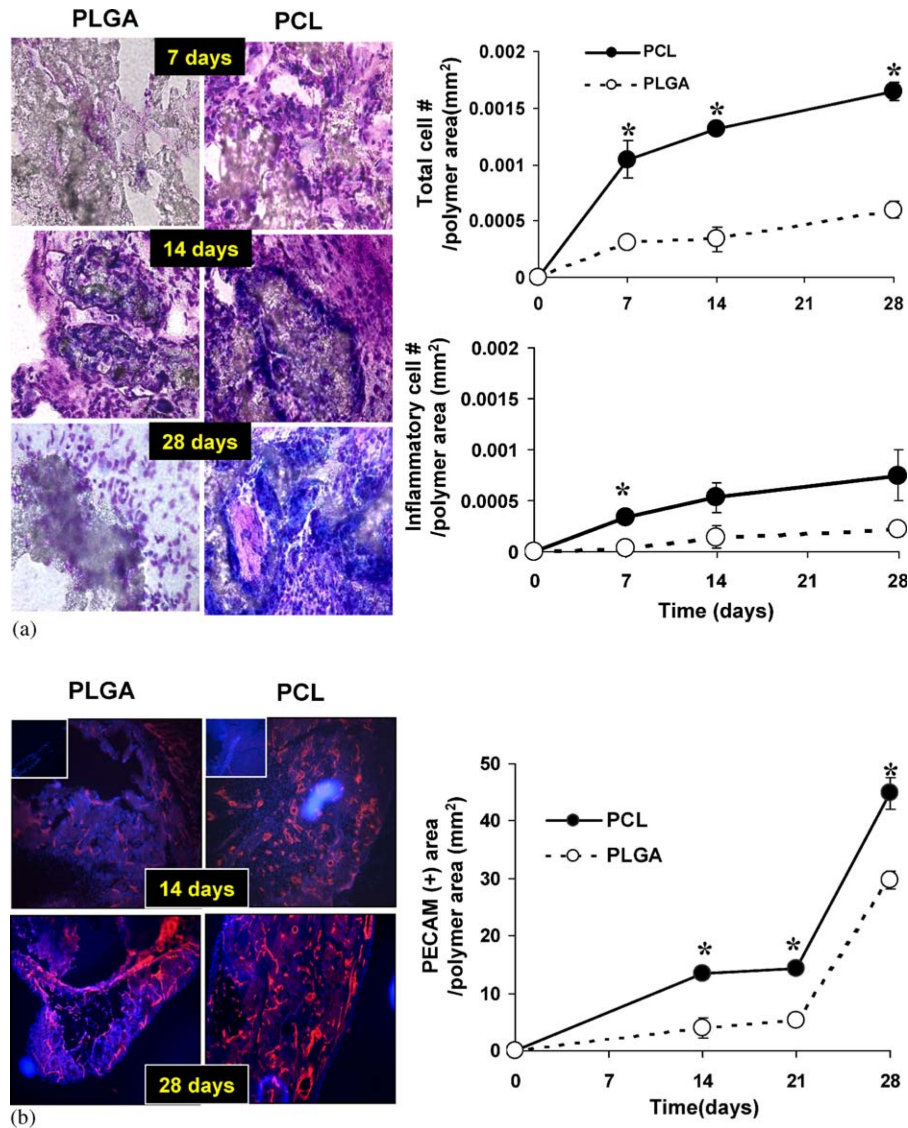

Fig. 5. Analysis of cell population of biodegradable scaffolds PLGA (right panel) and PCL (left panel) implanted in vivo was determined initially (day 0) and at 14 and 28 days (frozen sections). (a) Presence of cells is indicated by the purple nuclear staining. Inflammatory cells are detected by Geimsa staining (blue and pink staining). Quantification of total cell density (right upper graph) and inflammatory cell density (right lower graph) indicated a significantly increased cell invasion into PCL vs. PLGA scaffolds implanted in vivo. (b) To determine the extent of angiogenesis, frozen sections were stained with an antibody against the endothelial marker CD31 (PECAM-1). Positive profiles were detected using red fluorescence. All nuclei were counterstained with Hoechst (blue). The data obtained for endothelial positive staining, considered to represent angiogenesis, normalized to polymer area for both PLGA and PCL, indicated a significantly increased vascularization of PCL scaffolds implanted in vivo. Error bars represent SEM for three separate implanted scaffolds ($n=3$). (*) $p < 0.05$ for PLGA vs. PCL.

angiogenic response developed within the scaffolds implanted in vivo was related to the presence of inflammatory response. Our results should contribute to a better understanding of the cell-biodegradable scaffold interactions necessary to optimize the manufacturing and survival of tissue constructs.

Acknowledgements

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