In vivo interaction of cells on poly L-(lactic acid) membranes containing plasticizer

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The development of biodegradable materials has lead to renewed interest in the study of their interactions with the host organism in order to make the resulting products appropriate for use as temporary materials in protheses. Poly L-(lactic acid)(PLLA)-based biodegradable devices have been used for several purposes. The physical properties of these materials can be modified by the addition of a plasticizer, such as the triethylcitrate, to provide flexibility and porosity to the implants and enhance control of the polymer degradation time. In this work we examined the biological properties of a PLLA porous membrane containing 7% triethylcitrate, by assessing the process of degradation and the interaction with dermal tissue. Samples of skin obtained from female Wistar rats 2–180 days after implantation with PLLA-based membrane were processed for light microscopy and scanning electron microscopy. The membranes became surrounded by a delicate network of connective tissue which gradually invaded the membrane structure. Polymer degradation began with the appearance of radial fractures in the globular units of the biodegradable membrane, especially by 90 and 180 days after implantation.

Introduction

Synthetic artificial materials have been used to correct disabilities in the human body. Important advances have been made in the development of products that meet the demands of implantable materials for protheses. The development of biodegradable materials has stimulated interest in the study of their interactions with host tissue in order to improve the likelihood of success in protheses.

Poly L-(lactic acid) (PLLA) is a bioabsorbable polymer that is degraded by hydrolysis. The resulting monomers enter the citric acid cycle and are excreted in the form of carbon dioxide and water or by direct renal excretion [1, 2].

PLLA-based implantable medical devices have been used in controled drug-release systems, fracture repair as a barrier for soft and hard tissues, as a support for cell culturing and for filling damaged tissues [2–6]. The kinetics of biomaterial degradation vary and are determined by the materials characteristics, including implant chemistry and surface texture, porosity, pore size, shape, and size of the implants, and the location and technique of implantation, as well as interfacial micromotion [7]. PLLA has potential use in implants with a spongy structure (Drylac™) [8], pins [9], and double layer [7], monolayer [10] and mixed material [7, 11] matrices.

Synthetic polymer matrices are highly reproducible, and their mechanical properties and degradation rates can be readily controled and manipulated [12]. Previous work [10] showed that adding a plasticizer triethylcitrate to PLLA alters the flexibility of the material, and allows the formation of pores in the polymer. Depending on the quantity of the plasticizer used, membranes of different porosities can be produced, and this allows control of the degradation of the polymer and of the invasion of the membrane pores by tissue elements. The accurate addition of plasticizer thus allows the production of material for different applications.

The aim of this work was to examine the biological and morphological properties of a porous membrane of PLLA containing 7% trietylcitrate for up to 180 days after implantation in the subcutaneous tissue of rats. Greater knowledge of the interaction between this polymer and tissue should allow the use of this material in the repair of damaged tissue.

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Materials and methods

Production of implants

PLLA (MW 300 000) was provided by Medsorb Technologies International L.P. (Cincinnati, OH, USA) as pellets. Ten grams of polymer were dissolved in 100 mL of methylene chloride (CH_2Cl_2, Merck) containing 7% triethylcitrate (Aldrich) in a closed recipient at room temperature [8, 13].

The mixture was then poured into glass plate (100 cm^2) each, which were air dried (air flow of 1 L/min) at room temperature. After 15 h, the membranes were removed from plates and vacuum dried for 24 h. Disks 5 mm diameter and 620 μm thick were cut and used in the studies described below.

Implantation

The membranes were immersed in 70% ethanol and then vacuum dried. Sixteen female Wistar rats 3 months old obtained from university's central animal house (CEMIB) were used. The rats were housed at $22 \pm 2\,^{\circ}C$ on a 12 h light/dark cycle with food and water *ad libitum*. Two membranes were implanted in the dorsal subcutaneous tissue of rats $(n = 16)$ anesthetized with ketamine and xylazine-HCl (16.6 and 3.33 mg/kg i.p., respectively) (Virbac, Brazil). The health and behavior of the rats were assessed daily until sample collection. The samples were obtained 2, 7, 14, 21, 28, 60, 90, and 180 days post-implantation.

Light microscopy

Fragments of skin were fixed in Bouin solution and embedded in paraffin. Sections 5 μm thick were stained with Masson's trichromic and sirius red. Membrane fragments that had adhered to adjacent tissue were fixed in 4% paraformaldehyde and embedded in glycol methacrylate. Sections 2 μm thick were stained with toluidine blue. These samples were observed and photographed with a Nikon Eclipse E800 photomicroscope. The samples stained with sirius red were observed and photographed under polarized light to assess invasion by collagen fibers.

Scanning electron microscopy (SEM)

Samples from the different periods of implantation were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde containing 0.5% tannic acid in 0.1 M phosphate buffer, pH 7.4, followed by post-fixation in 1% osmium tetroxide in the same buffer. After dehydration in a graded ethanol series, the samples were freeze-fractured in liquid nitrogen then critical point dried (CPD 030, Balzers) and coated with sputtered gold (SCD 050, Balzers). The samples were examined in a Jeol JMS 5800 LV SEM (Japan).

Results

Analysis of the membranes by SEM before implantation showed that the membrane pores were delimited by juxtaposed globular units with a rugged surface. Examination of the polymer fracture surface showed that there were also structural differences between the faces of the membrane: one half had globular units intermixed with pores, while the other was composed of a dense layer with no pore but had juxtaposed units of different diameters (Fig. 1). The pores were of various sizes (Fig. 2) and had a heterogenous distribution in the membrane.

Light microscopy

In samples obtained early after implantation (2, 7, and 14 days), there was a separation between the membrane and its adjacent tissue. However, after longer periods of implantation (90 and 180 days), the polymer was surrounded by adjacent tissue in the subcutaneous compartment. Increasing vascularization of the implant area was seen during removal of the samples, but there were no macroscopic signs of an inflammatory response.

Two days after implantation, there was a massive infiltrate of polymorphonuclear cells embedded in a fibrin net and edema was observed (Fig. 3). By seven days after implantation, a fibrous capsule had formed around the surface of the implant and there were numerous fibroblasts and macrophages inside the pores of the membrane (Fig. 4). Polarized light microscopy revealed that the fibrous capsule consisted of a delicate meshwork of collagen fibers. On the 14th day post-implantation, a massive cellular infiltrate was observed inside the membrane including the presence of giant multinucleated cells (Fig. 5). Twenty-one days after implantation, the blood vessels present in the capsule were observed penetrating the pores of the implant (Fig. 6). Analysis of the samples obtained 28 days after implantation revealed that a highly vascularized connective tissue infiltrate within the polymer particles, whose organization can be seen by conventional (Fig. 7) or picrosirius-polarization microscopy (Fig. 8).

Sixty days after the implantation, intense tissue invasion was noticed. A marked process of degradation isolated the globular units from the membrane (Fig. 9). Samples collected 90 days after the implantation showed that the implant was surrounded by a capsule with a great number of collagen fibers. In this case, the polymer was intensely fragmented and invaded by an extensive amount of connective tissue (Fig. 10).

By 180 days after implantation, the connective tissue inside the membrane was observed to be subdividing the polymer in small fragments surrounded by a delicate network of connective tissue (Fig. 11). Polarized light revealed that groups of collagen fibers surrounded the units of the membrane and thereby separated them from each other (Fig. 12). A more detailed examination of these samples revealed the presence of giant cells containing many nuclei (Fig. 13) and irregular polymeric units surrounded by connective tissue with conspicuous blood vessels. PLLA membrane fragments were observed within cells in the connective tissue (Fig. 14).

Scanning electron microscopy (SEM)

Samples collected two days after the implantation showed intact polymer units surrounded by cells (Fig. 15) and a fibrin network.

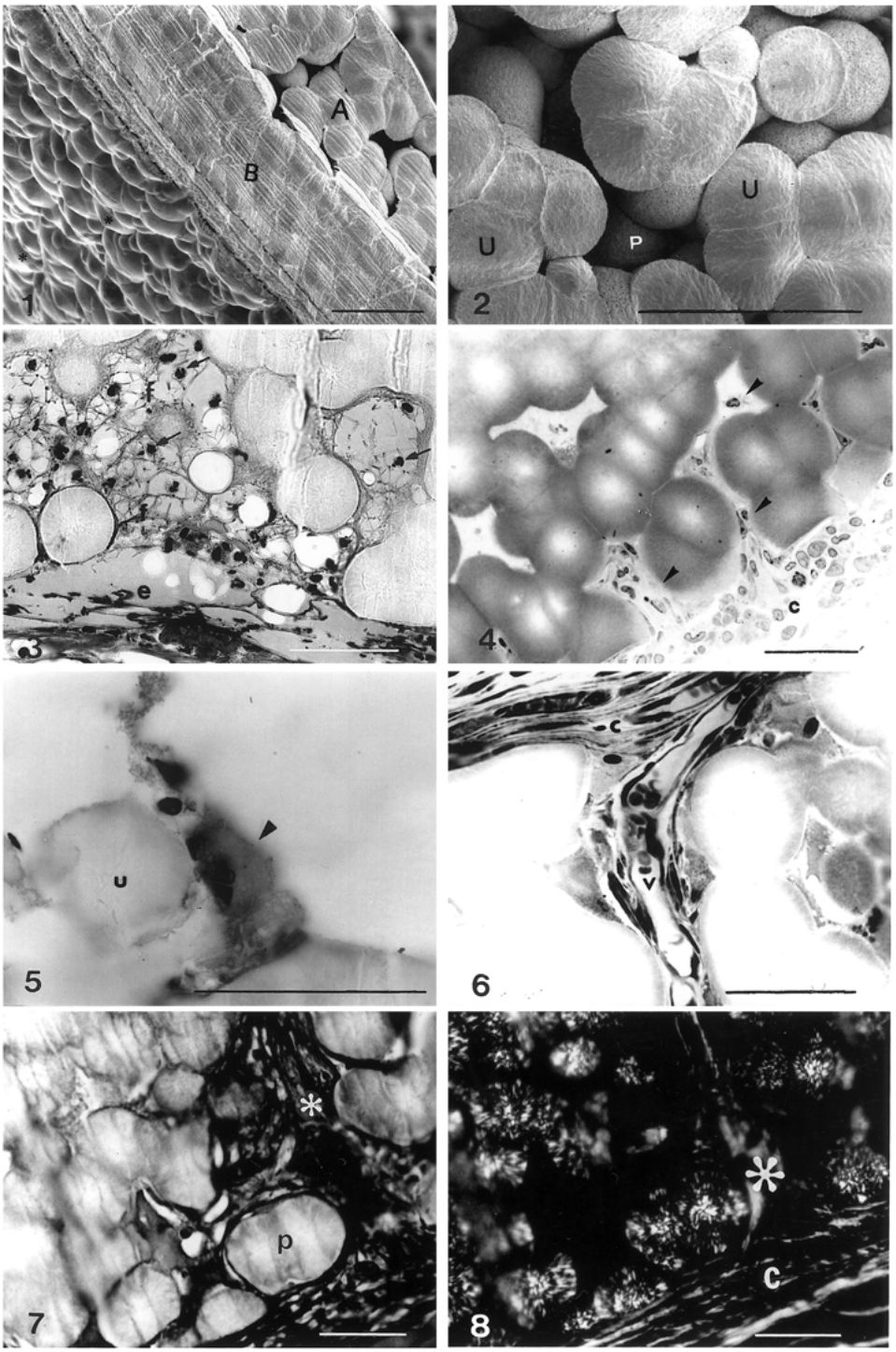

Figure 1 PLLA membrane before implantation. Note the presence of porous (A) and dense (B) layers containing globular units (*). Bar: 100 µm.
Figure 2 PLLA membrane before implantation showing polymer globular units (U) and pores (P). Bar: 100 µm.
Figure 3 PLLA membrane on second days post-implantation. Observe polymorphonuclear infiltrate (arrows), vascular edema (e) and fibrin network (f). Bar: 100 µm.
Figure 4 PLLA membrane seven days after implantation. Note capsule surrounding the membrane (c) and cellular infiltration within the polymer (arrowheads). Bar: 100 µm.
Figure 5 PLLA membrane 14 days after implantation showing giant cell (arrowhead) around the polymer fragment (U). Bar: 100 µm.
Figure 6 PLLA membrane 21 days after implantation. Observe blood vessel (v) of the capsule (c) within the membrane. Bar: 100 µm.
Figure 7 PLLA membrane 28 days after implantation. Note connective tissue infiltrate (*) within the polymer particles (p). Bar: 100 µm.
Figure 8 PLLA membrane 28 days after implantation under polarized light. Observe collagen fibers of the capsule (c), some of them penetrating the membrane (*). Note crystalline disposition of polymer units (p). Bar: 100 µm.

No auto-degradation of the globular units was seen. Cells and extracellular matrix components (mainly collagen fibrils) were present among the membrane units (Fig. 16).

Fourteen days after implantation, connective tissue had adhered to the surface of the membranes (Fig. 17), and by the 21st day a considerable amount of connective tissue was present into the pores of the polymer, and resulted in separation of the globular units (Fig. 18). Polymer degradation process started with the appearence

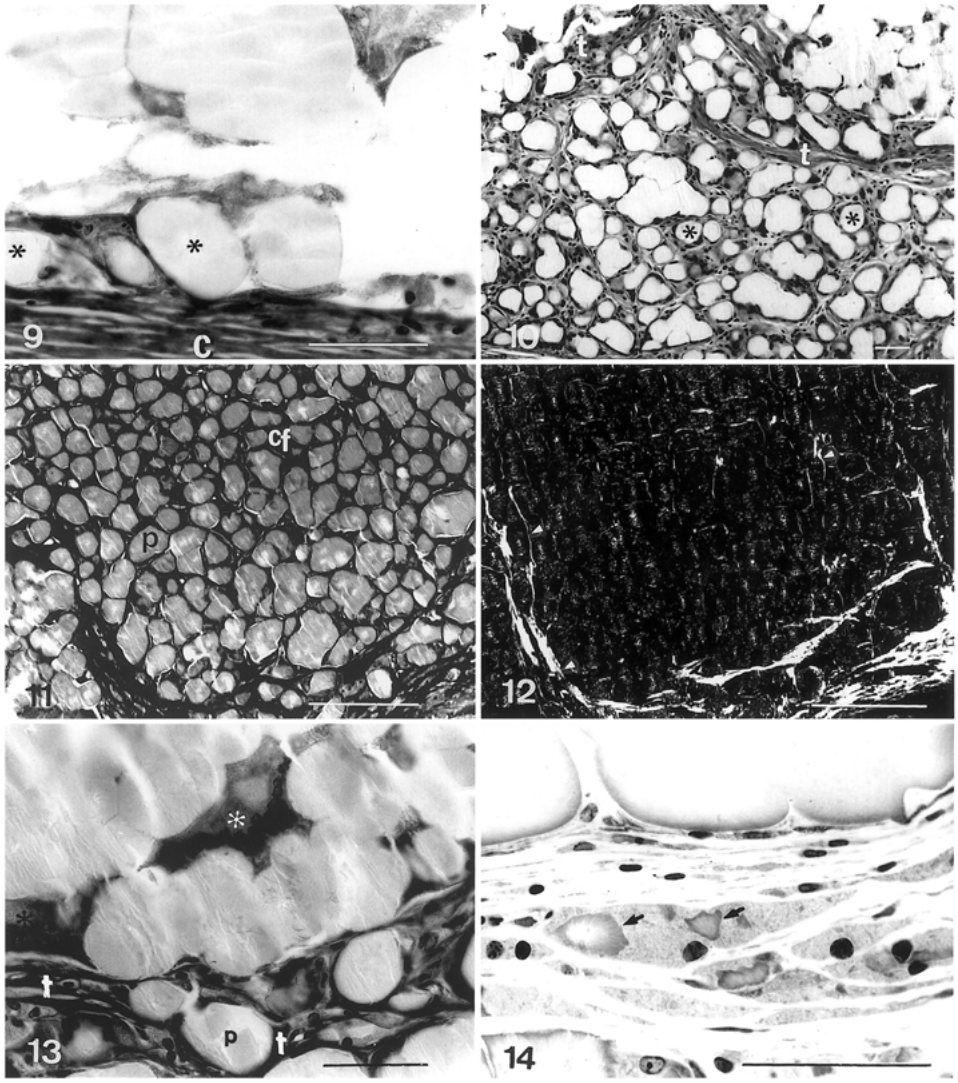

Figure 9 PLLA membrane 60 days after implantation showing polymer particles (*) isolated in the capsule (c). Bar: 100 μm.
Figure 10 PLLA membrane 90 days after implantation. Note intensive connective tissue infiltration (t) surrounding the polymer fragments (*). Bar: 100 μm.
Figure 11 PLLA membrane 180 days after implantation. Observe collagen fibers (cf) around the polymer particles group (p). Bar: 100 μm.
Figure 12 PLLA membrane 180 days after implantation under polarized light. Observe groups of collagen fibers (arrowheads) surrounding the membrane units and thereby separated them from each other. Bar: 100 μm.
Figure 13 PLLA membrane 180 days post-implantation. Note the presence of vascularized tissue (t) within the polymer (p) cointaining giant cell (*). Bar: 100 μm.
Figure 14 PLLA membrane 90 days after implantation. Observe cells of connective tissue with phagocyted polymer particles (arrows). Bar: 100 μm.

of centripetal fractures in the membrane units (Fig. 19) and involved cellular invasion.

By 60 days after implantation, most of the units had extensive fractures, indicating pronounced degradation (Fig. 20) that intensified so that 90 days after implantation the polymeric units were submerged in connective tissue (Fig. 21).

Finally, massive polymer degradation was observed 180 days after implantation, with the presence of membrane fragments within the connective tissue (Fig. 22).

Discussion

The first use of triethylcitrate as a plasticizer [14] was intented to provide a certain flexibility to tubular implants in order to allow the application of sutures to the implant surface.

The degradation time for membranes containing 7% of plasticizer was longer than for implants with 10% plasticizer [10]. The smaller amount of plasticizer reduced the porosity of the membrane and this apparently resulted in a lower invasion than that seen by Luciano [10].

Analysis of the samples obtained two days after the implantation revealed an inflammatory reaction with many neutrophils, macrophages, and more rarely eosinophils. Local trauma following surgical procedures provoked a strong inflammatory reaction for up to 7 days after the implantation. After this period, the strong inflammatory reaction gave place to a reaction against the implant [7, 15].

The presence of blood vessels within the samples obtained 21 days after implantation suggested that revascularization made the regeneration of damaged tissue possible. Padera *et al*. [3] claimed that porous materials have areas of low partial O_2 pressure. This

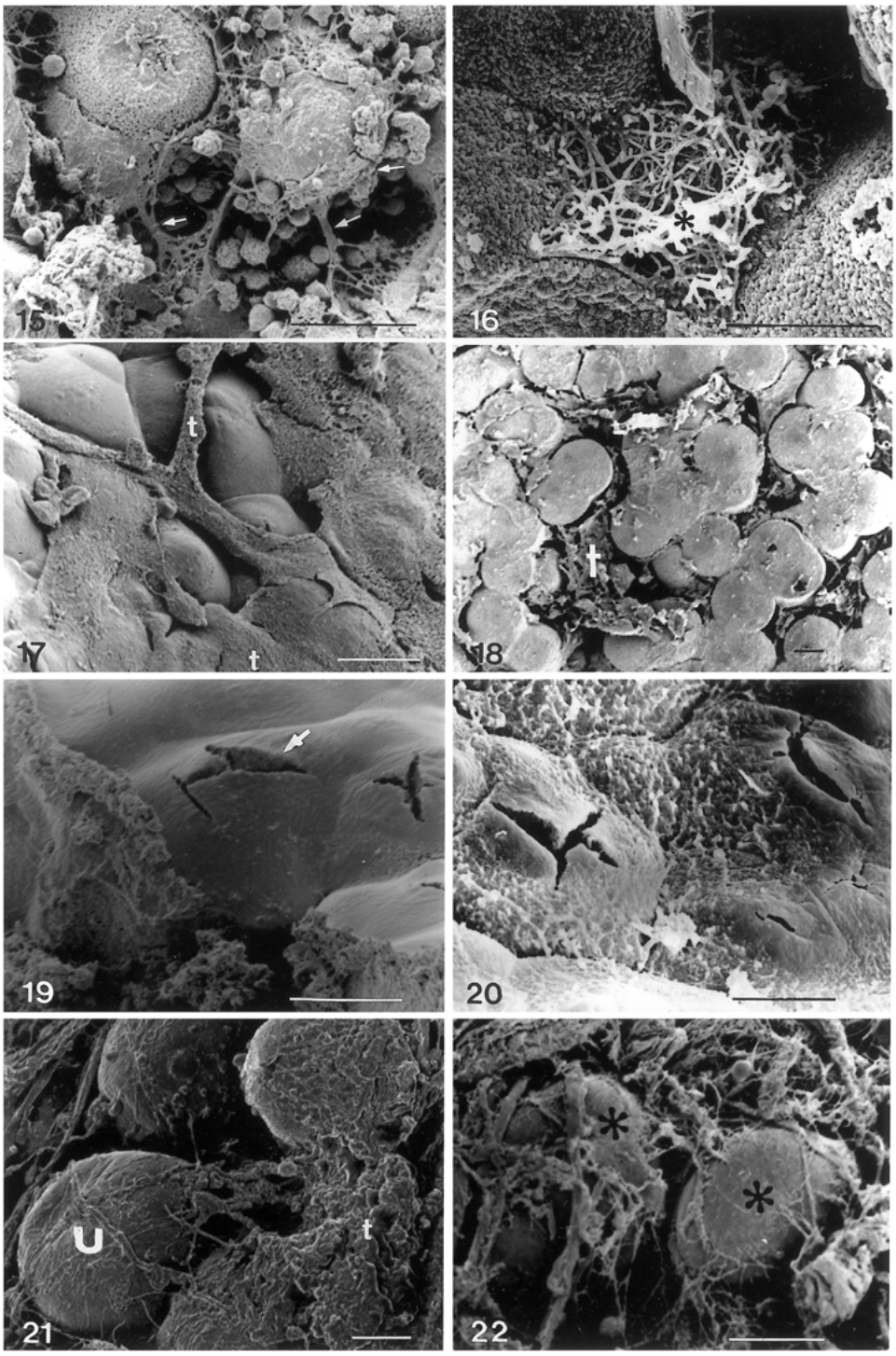

Figure 15 PLLA membrane on second day after implantation. Note great number of cells and fibrin network (arrows). Bar: 20 µm.
Figure 16 PLLA membrane seven days post-implantation. Observe tissue fibril elements (*) within the pore. Bar: 20 µm.
Figure 17 PLLA membrane 14 days after implantation. Note tissue (t) adhered to membrane face. Bar: 20 µm.
Figure 18 PLLA membrane 21 days post-implantation. Tissue invasion (t) can be noted. Bar: 20 µm.
Figure 19 PLLA membrane 28 days after implantation. Observe the presence of centripetal fractures of the globular units (arrow). Bar: 20 µm.
Figure 20 PLLA membrane 60 days after implantation showing extensive fractures. Bar: 20 µm.
Figure 21 PLLA membrane 90 days post-implantation. Note polymer fragments (U) submerged in the tissue (t). Bar: 20 µm.
Figure 22 PLLA membrane 180 days after implantation. Observe massive degradation process with polymer particles (*) within the connective tissue. Bar: 20 µm.

hypoxia should stimulate the secretion of angiogenic factors by macrophages to act directly on endothelial cells. Some of these substances, such as angiotropine and tumor necrosis factor, stimulate the migration of endothelial cells, while others such as epidermal growth factor, promote mitosis of these cells.

The presence of giant cells was observed from the seventh day onwards. According to Lam *et al*. [16], the giant cells observed in foreign body reactions originate from the fusion of macrophages, a process induced by cytokines such as interleukin 4 and gamma-interferon. These cells showed a large number of mitochondria and

this may be associated with the elimination (degradation) of PLLA via the tricarboxylic acid cycle to generate carbon dioxide. Brady *et al*. [17] used implants of D,L-poly(lactic acid) labeled with ^{14}C in the abdominal wall of mice. After 168 days, 36.8% radioactivity had been eliminated, most of this through exhaled CO_2 (29.4% through respiration, 4.6% through the urine, and 2.8% through the feces).

Multinucleated giant cells containing phagocytosed PLLA membrane particles in their cytoplasm were seen in samples collected 90 and 180 days after implantation. The products of the enzymatic degradation of these fragments could enter the tricarboxylic acid cycle of mitochondria, which could provide an additional means of degrading PLLA besides hydrolysis.

Beumer *et al*. [7] used double-layered implants with no added plasticizer. During degradation, mono- and multinucleated macrophages (foreign body giant cells) containing polymeric fragments were observed at the implant–tissue interface in the 13th week after implantation, indicating intracellular implant degradation.

In agreement with the above authors, we observed the formation of vascularized fibrous tissue with the presence of collagen, indicating a rebuilding of damaged tissue following implantation. However, there was no cellular reaction to a foreign body.

No neoplasic areas were observed up to 180 days after implantation. The addition of triethylcitrate minimized the rigidity of the polymer and reduced its degradation time, thereby limiting the risks of a neoplasic reaction in the area of the implant. Our results differ from those by Nakamura *et al*. [18], which reported that the implantation of PLLA membranes with no added plasticizer induced the formation of neoplasic areas at the implantation site. The authors attributed this response to the constant mechanical irritation by the polymer at the site of implantation, which in turn resulted from the extremely long degradation time of the polymer.

Based on the results obtained here, we conclude that the presence of a subcutaneous PLLA implant not only provokes an inflammatory reaction, but also helps to rebuild the damaged tissue. A knowledge of the appropriate percentage of plasticizer for each implantation site is thus important so that the degradation time of the polymer can be made compatible with the proliferation of new tissue.

Acknowledgments

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References

1. K. A. ATHANASIOU, G. G. NIEDERAUER and C. M. AGRAWAL, *Biomaterials* **17** (1996) 93.
2. S. L. ISHAUG, M. J. YASZEMSKI, R. BIZIOS and A. G. MIKOS, *J. Biomed. Mat. Res.* **28** (1994) 1445.
3. R. F. PADERA and C. K. COLTON, *Biomaterials* **17** (1996) 277.
4. A. G. MIKOS, M. D. LYMAN, L. E. FREED and R. LANGER, *ibid.* **15** (1994) 55.
5. L. CALANDRELLI, B. IMMIRZI, M. MALINCONICO, M. G. VOLPE, A. OLIVA and F. DELLA RAGIONE, *Polymer* **41** (2000) 8027.
6. A. R. SANTORS JR, S. H. BARBANTI, E. A. R. DUEK, H. DOLDER, R. S. WADA and M. L. F. WADA, *Artifical Organs* **25** (2001) 7.
7. G. J. BEUMER, C. A. VAN BLITTERSWIJK and M. PONEC, *Biomaterials* **15** (1994) 551.
8. T. H. BARROWS, in ''Synthetic Bioabsorbable Polymers'' (Technomic Publishing Company, 1991) p. 243.
9. M. VAN DER ELST, A. R. A. DIJKEMA, C. P. A. T. KLEIN, P. PATKA and H. J. TH. M. HAARMAN, *Biomaterials* **16** (1995) 103.
10. R. M. LUCIANO, in '' Síntese, Caracterização e Degradação de Membranas de Poli(ácido lático), um polímero bioabsorvível''. UNICAMP, Masters dissertation. 1997.
11. H. A. VON RECUM, R. L. CLEEK, S. G. ESKIN and A. G. MIKOS, *Biomaterials* **16** (1995) 441.
12. B. S. KIM and D. J. MOONEY, *J. Biomed. Mat. Res.* **41** (1998) 332.
13. R. M. LUCIANO, in ''Proceedings of the European Medical & Biological Engineering Conference'', Vienna, November 1999, edited by P. Peregrius (Published for International Federation for Medical & Biological Engineering, 1999) p. 214.
14. C. F. SILVA, R. MADISON, P. DIKKES, T. CHIU and R. L SIDMAN, *Brain Res.* **342** (1985) 307.
15. J. E. BERGSMA, F. R. ROZEMA, R. R. M. BOS, G. BOERING, W. C. DE BRUIJN and A. J. PENNINGS, *J. Biomed. Mat. Res.* **29** (1995) 173.
16. K. H. LAM, J. M. SCHAKENRAAD, H. ESSELBRUGGE, J. FEIJEN and P. NIEUWENHUIS, *ibid.* **27** (1993) 1569.
17. J. M. BRADY, D. E. CUTRIGHT, A. MILLER and G. C. BATTISTONE, *ibid.* **7** (1973) 155.
18. S. NAKAMURA, S. NINOMIJA, Y. TAKATORI, S. MORIMOTO, I. KUSABA and T. KUROKAWA, *Acta Orthop. Scand.* **64** (1993) 301.

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