JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 381–385

An investigation of the growth of human dermal fibroblasts on poly-L-lactic acid *in vitro*

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A tissue engineered implant involving cells seeded on bioresorbable materials is an attractive proposition for the repair of soft tissue because in the long term the material is resorbed leading to a natural repair. The aim of this study was to assess the effect of poly-L-lactic acid (PLA) on the growth of human dermal fibroblasts *in vitro*. The results have been compared to other resorbable materials, polyhydroxybutyrate (PHB), polycaprolactone (PCL) and polyglycolic acid (PGA). Thermanox (Tmx) and tissue culture plastic (Tcp) were used as positive controls. PLA compared favourably with the other bioresorbable materials, as a substrate for fibroblast attachment and proliferation. No evidence of cytotoxicity was observed for cells cultured in polymer extracts. Colonies of adhered fibroblasts were observed on PLA which exhibited normal bipolar morphology when viewed using phase contrast microscopy and cryo-SEM. Cellular proliferation was measured by tritiated thymidine incorporation with total DNA as a baseline. Concerns associated with the use of PLA as the bioresorbable material utilized in tissue regeneration are primarily related to the effect of degradation products on the cellular component. No effect on the growth of dermal fibroblasts was observed in the presence of degradation products or lactic acid standards (0–1000 μg/ml/48 h). This study has demonstrated that human dermal fibroblasts proliferated and formed a confluent monolayer on PLA, which may prove to be a suitable scaffold for soft tissue repair systems, providing biomechanical and biodegradation requirements can be fulfilled.

1. Introduction

Presently there is considerable interest in the application of bioresorbable polymers for soft tissue reconstruction and repair [1–3]. In particular a tissue engineered implant consisting of a bioresorbable scaffold seeded with autologous cells would ultimately lead to an entirely biological repair once the scaffold has resorbed [4]. This would overcome the problems associated with traditional implants and grafts, such as adverse immunological response, donor site morbidity and wear particle responses [5]. An important factor to consider in the use of resorbable materials for tissue engineered implants is their effect on the proliferation rate of seeded cells [6–8]. There has been much debate over the effect of resorbable polymers on cell proliferation due to material characteristics including pH and the effect of degradation products. Good compatibility characteristics for polylactides have been reported, however, for both *in vitro* studies and *in vivo* studies [9, 10]. It must be appreciated that the biomaterial chosen for the scaffold matrix must also meet biomechanical and degradation rate criteria as well as being biologically compatible.

An extensive *in vitro* study of the growth of human dermal fibroblasts on polylactic acid (PLA) has been undertaken. Comparative data for fibroblasts cultured on other resorbable materials; polyhydroxybutyrate (PHB), polycaprolactone (PCL) and polyglycolic acid (PGA) has been included. Two non-resorbable materials in the form of Thermanox® (Tmx) discs and Tissue culture plastic (Tcp) have been used as positive control materials due to their optimized cell growth characteristics. It is important to confirm that no cytotoxic contaminants are present in the polymers. Thus, relative cytotoxicities of polymer extracts were assessed using cultured fibroblasts *in vitro*. This study was performed primarily to evaluate the cytotoxicity of any leachable substances, such as toxic impurities, in the test polymers.

The degradation of polylactic acid, PLA, is primarily a hydrolytic process. The products of hydrolysis are monomers and oligomers of lactic acid which may affect cellular responses [10, 11]. The effect of accelerated PLA degradation products and purified lactic acid on fibroblasts in culture has been assessed.

This paper was accepted for publication after the 1995 Conference of the European Society of Biomaterials, Oporto, Portugal, 10–13 September.

2. Materials and methods

2.1. Materials

Polymers, PLA, PCL, PHB and PGA, were compression moulded into films and cut into 15 mm diameter discs. These were sterilized by gamma irradiation (25 kGy).

Human dermal fibroblasts, (courtesy of Addenbrookes Hospital, Cambridge), passages 10–15, were used throughout the study and were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with L-glutamine (1% v/v), non-essential amino acids (1% v/v), penicillin/ streptomycin (1% v/v), foetal calf serum (10% v/v), Ascorbate (150 μg/ml) and HEPES buffer (20 mM).

2.2. Cytotoxicity testing

The cytotoxicity of extracts prepared from the polymers was assessed. Polyvinylchloride (PVC) containing 0.3% (w/w) tin was used as a toxic control material. Low density polyethylene (LDPE) was used as a non-toxic control. Polymer discs were incubated in 1 ml medium for 24 h at 37 °C/5%CO_2. Human dermal fibroblasts, grown to confluence on Tcp, were exposed to various dilutions of the extract for 24 h at 37 °C/5%CO_2. Cell viability was assessed by uptake of the vital stain, Neutral Red, and cell number was determined using Kenacid Blue.

2.3. Morphology on resorbable materials

An investigation was conducted to determine whether the polymers were capable of supporting adherence and growth of fibroblasts. Human dermal fibroblasts were seeded on polymer discs at a density of 1×10^4 cells cm^{-2} and incubated for 3 days. The discs were removed from culture and processed for cryo-scanning electron microscopy (SEM). Comparison was made with a positive control of Tcp. In addition, the morphology of cells cultured on PLA and Tcp for up to 7 days was assessed using phase contrast microscopy.

2.4. Proliferation on resorbable materials

Cellular proliferation on the polymers was assessed using tritiated thymidine ([^{3}H]-TdR) incorporation. Human dermal fibroblasts were seeded on polymer discs at density of 1×10^4 cells cm^{-2} and incubated for 24 h. The medium was removed and replaced with fresh medium supplemented with [^{3}H]-TdR (1 μCi ml^{-1}), and the cells cultured for a further 24 h. The medium was removed and the cells were enzymatically lysed with papain (1 μl ml^{-1} in phosphate buffered saline (PBS) supplemented with 5 mM EDTA, 5 mM cysteine HCl) for 16 h at 60 °C. Total amounts of DNA were determined in the cell lysate by a DAPI fluorometric assay [12]. Tritiated thymidine incorporated into DNA was measured by trichloroacetic acid (TCA) precipitation of cell digest. The precipitate was vacuum filtered onto a porous membrane using the Millipore® multiscreen filtration system. Unprecipitated lysate containing the excess unbound radionucleotide was washed through the membrane. The radioactivity level was measured, following solubilization (0.01 M KOH), on a scintillation counter. In addition on the proliferation rate of cells cultured on PLA and Tmx was measured on days 1, 3 and 7 of culture using the same methods.

2.5. Degradation products

PLA degradation products were prepared by incubating PLA in distilled water at 80 °C for 5 days. Test solutions consisted of a range of concentrations of degradation products or lactic acid standards (0–1000 μg/ml) in culture medium. Human dermal fibroblasts were seeded on Tcp at a density of 1×10^4 cells cm^{-2} and incubated for 24 h at 37 °C/5%CO_2. The medium was removed and replaced with a solution containing degradation products or lactic acid. The cells were cultured for a further 48 h at 37 °C/5%CO_2. The cells were enzymatically lysed as above. Total amounts of DNA were determined in the cell lysate by a DAPI fluorometric assay.

3. Results

3.1. Cytotoxicity testing

Cytotoxicity was assessed by culturing cells in conditioned medium prepared from polymer discs. Cell viability data after culture for 24 h is presented in Fig. 1. Neither any of the polymers nor the non-toxic positive control extract, LDPE caused any alteration in cell viability at all concentrations. By contrast the toxic control extract, PVC + 0.3% tin was found to cause a marked decrease in cell viability when used undiluted in culture. This cytotoxic effect decreased as the extract was diluted.

3.2. Morphology on resorbable materials

Cryo-SEM indicated that by day 3, fibroblasts had attached and proliferated on all the materials. A confluent layer of cells was observed on Tcp after 3 days in

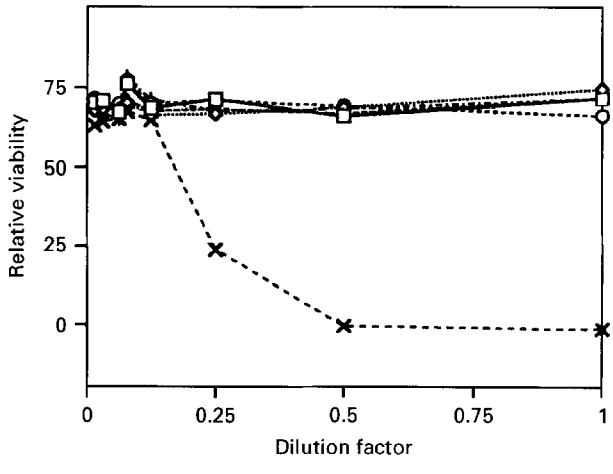

Figure 1 The relative viability of cells exposed to the test and control extracts, expressed as the neutral red absorbance divided by the kenacid blue absorbance (a dilution factor of 1 relates to the undiluted extract). Note that relative viability is expressed in arbitrary units and does not correspond to percentage viability. —□— PHB; —◇— PCL; —○— PLA; —△— PGA; —× PVC + tin; + —LDPE.

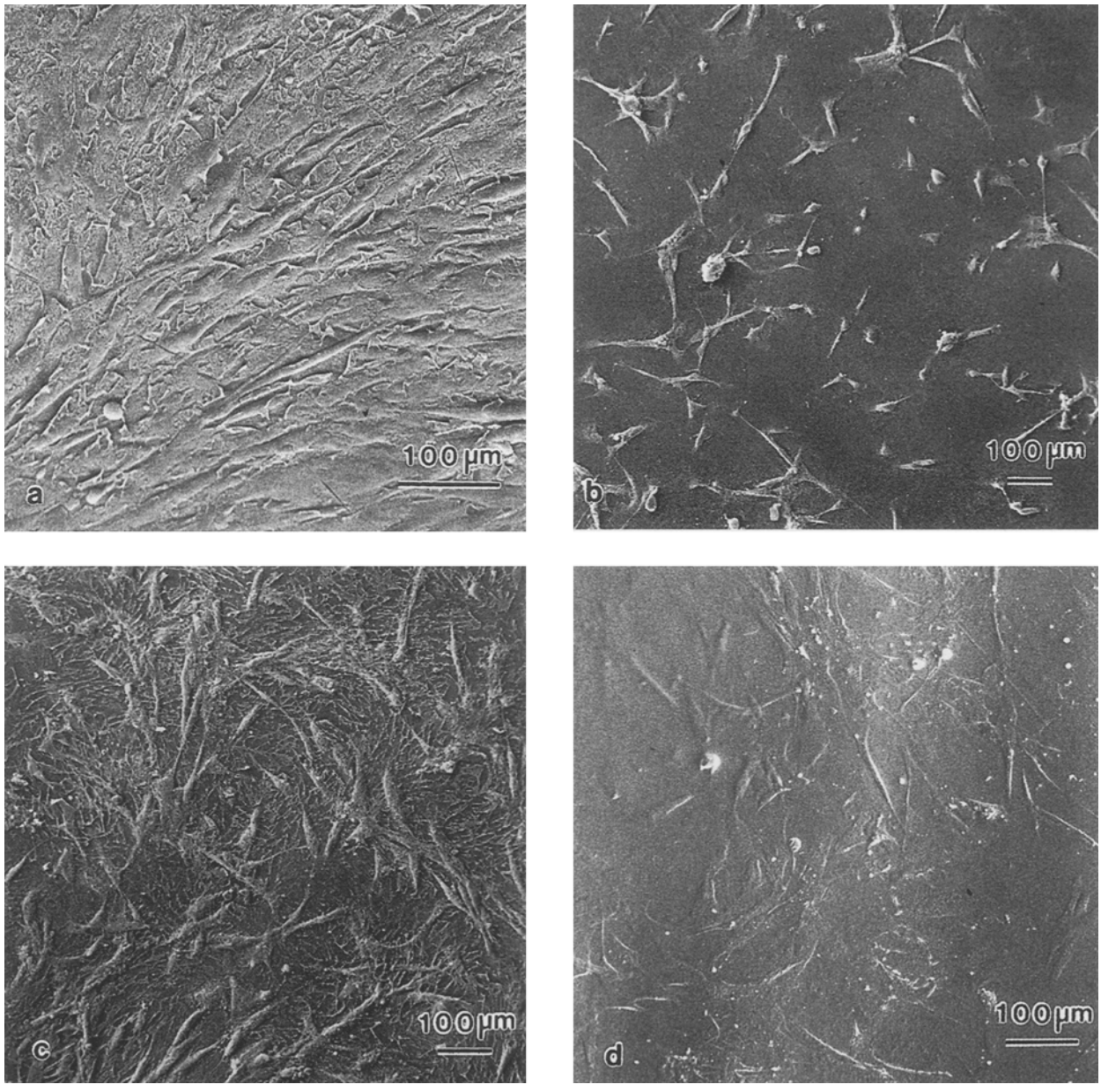

Figure 2 Scanning electron micrograph of fibroblasts cultured on bioresorbable materials for up to 3 days in culture (a) Tcp, (b) PHB, (c) PCL (d) PLA.

culture (Fig 2a). PHB, PCL and PLA all demonstrated sub-confluent monolayer of cells (Fig. 2b, c, d). Phase contrast microscopy indicated that fibroblasts cultured on Tmx (Fig. 3a) for 24 h had a greater density than those grown on PLA (Fig. 3b). By day 7, however, cells on both PLA and Tmx had reached confluence (Fig. 3c, d).

3.3. Proliferation on resorbable materials

The relative cellular proliferation rates on the bioresorbable materials is illustrated in Fig. 4. Significant cellular proliferation was observed on both PCL and Tcp, the latter acting as a positive control. Slower proliferation rates were observed on PHB and PLA relative to the Tcp value, while PGA exhibited the slowest cellular proliferation rate on the materials tested. Relative cellular proliferation rates on PLA and Tmx were determined on days 1, 3 and 7 of culture (Fig. 5). On day 1, proliferation on PLA was markedly lower than on Tmx. The proliferation rate on Tmx decreased throughout the culture period, whereas the proliferation rate on PLA increased between days 1 and 3. By day 7 the rates on the two materials were similar.

3.4. Degradation products

The effect of exposure of fibroblasts to PLA degradation products and lactic acid was determined over 48 h at a range of concentrations up to 1 mg ml^{-1} lactic acid (Fig. 6). No reduction in the DNA content of the cultures was observed.

4. Discussion

The object of this study was to investigate the growth of human dermal fibroblasts on PLA and a variety of other resorbable polymers. The information gained, in

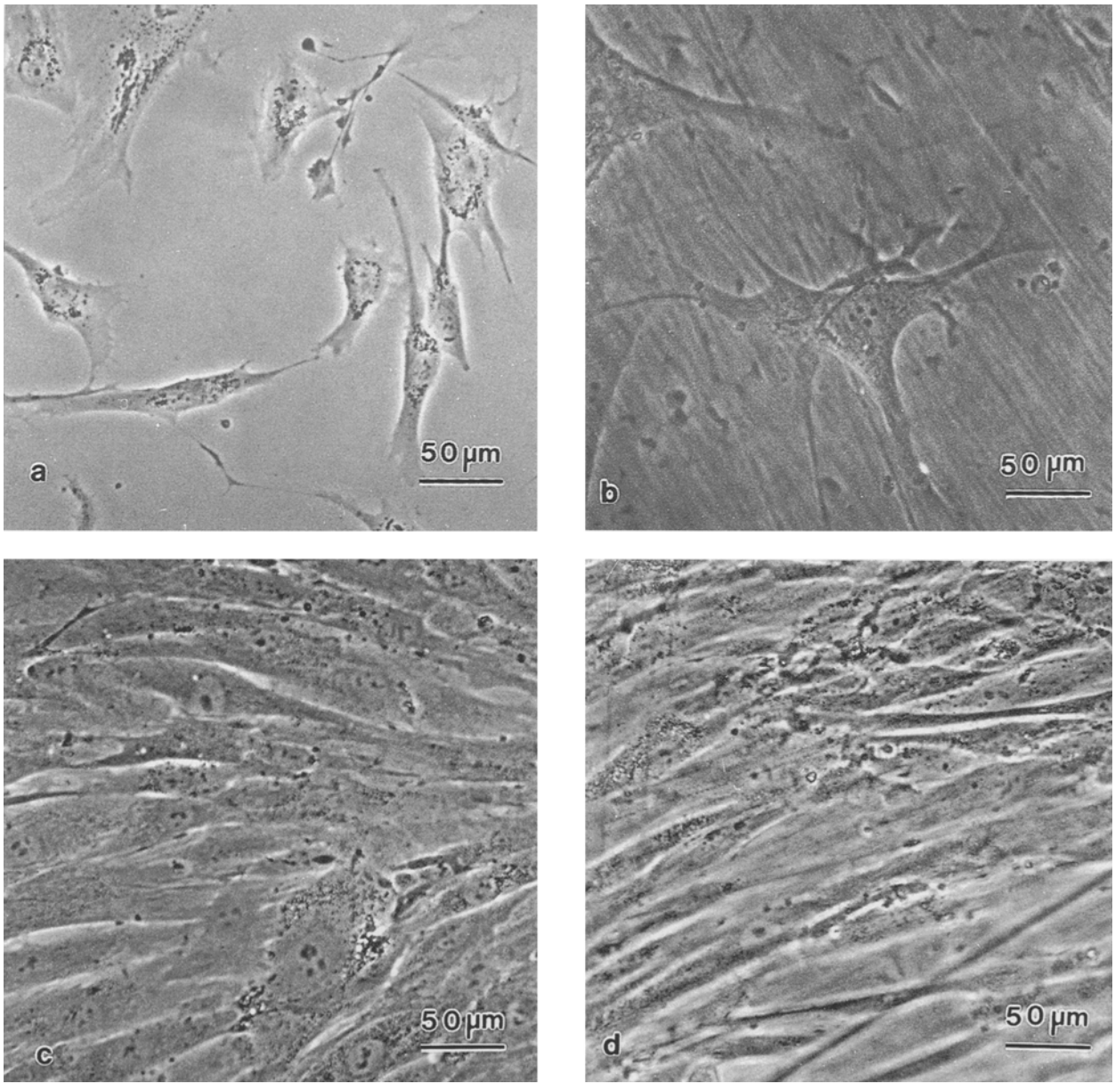

Figure 3 Light micrograph (300 ×) of fibroblasts cultured on PLA and Tmx. (a)Tmx day 1; (b) PLA day 1; (c) Tmx day 7; (d) PLA day 7.

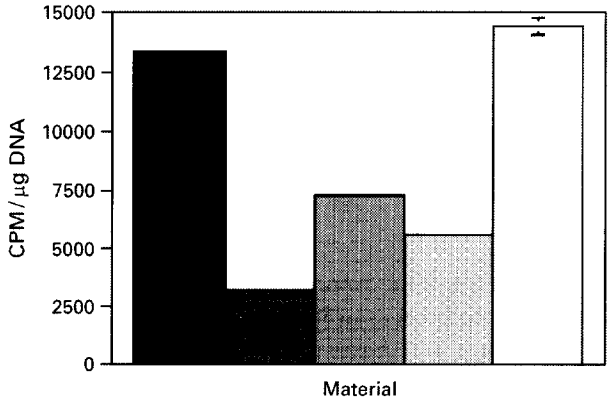

Figure 4 Relative cellular proliferation rates of fibroblasts cultured on bioresorbable materials. Thymidine uptake is expressed as (cpm/µg DNA) (mean ± SD, $n = 6$). ■ PCL; ■ PGA; ▨ PHB; ▨ PLA; □ TCP.

conjunction with mechanical and degradation data, may be useful in the selection of resorbable scaffold materials for tissue engineered soft tissue repair systems.

Cytotoxicity studies indicated no alteration in the viability of fibroblasts cultured in medium containing extracts from any of the polymers when compared to a non-toxic control (LDPE). Extracts from the toxic control (PVC + 0.3% tin), however, induced a dose-dependent reduction in cell viability. Thus, none of the leachable substances from any of the polymers were found to be toxic to fibroblasts under these conditions.

Scanning electron microscopy demonstrated that by day 3 the cells had not reached confluence on any of the materials, as observed for cells on Tcp. Cell density was greatest on PCL, with similar but lower densities on the other two polymers. However, light microscopy studies indicated a confluent monolayer of cells on PLA similar to that of Tmx at day 7. Thus, although the cells are able to attach and spread on the polymers, there is evidence to suggest that their rate of proliferation is slower on the polymers than on Tcp or Tmx.

Cellular proliferation was quantified by measuring [^{3}H]-TdR incorporation into DNA, with total DNA

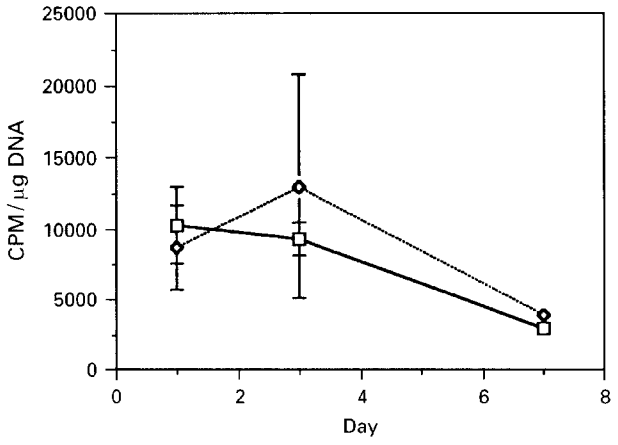

Figure 5 Cellular proliferation rate of dermal fibroblasts cultured for up to 1, 3 and 7 days on Tmx (—□—) and PLA (—◇—). Thymidine uptake is expressed as cpm/μg DNA (Mean ± SD, $n = 4$).

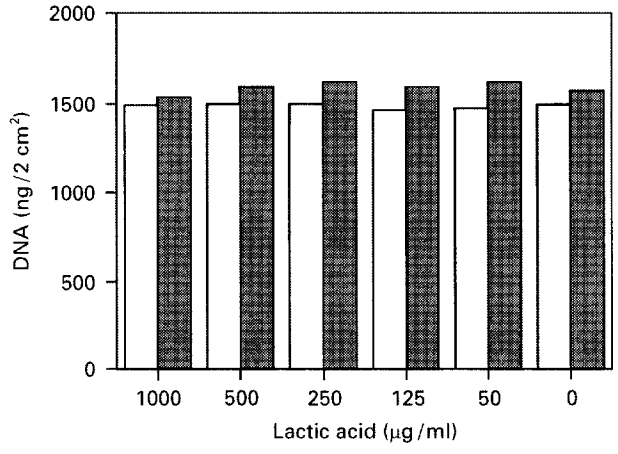

Figure 6 DNA content of dermal fibroblast culture following 48 h incubation with polylactic acid degradation products and lactic acid standards (Mean ± SD, $n = 4$). □ PLA deg. prod.; ▩ standard.

as a baseline. [^{3}H]-TdR incorporation could be measured in all cases, indicating that fibroblasts will proliferate on all of the materials tested. The qualitative morphological data correlates well with the quantitative biochemical analysis. The day 1 differences between the [^{3}H]-TdR incorporation for cells cultured on Tmx compared to PLA are probably due to the formation of a confluent monolayer of cells on Tmx which will result in contact inhibition of cell proliferation. Indeed, by day 7 rates on both materials were indistinguishable as both groups had reached confluence.

PLA degradation products may alter cellular responses due to molecular components or to reduced pH conditions which are induced. The presence of PLA degradation products or lactic acid was not found to alter the DNA content of the cultures, suggesting that the viability and proliferation of the cells is normal. Thus there is no evidence to suggest any detrimental effect of degradation products on fibroblasts at the concentrations tested. Nor is there any evidence to suggest a difference in cellular response to solutions containing oligomers of PLA (in the degradation products) when compared to lactic acid monomers under these conditions.

5. Conclusions

In this study we have demonstrated that human dermal fibroblasts will adhere and proliferate on PLA. No evidence of cytotoxicity was detected and the cells had normal morphological characteristics. The fibroblasts proliferated and formed a confluent monolayer after approximately 7 days. It would appear that PLA would be a suitable material for use in tissue engineered soft tissue repair systems. While there were slight variations in the rate of proliferation between PLA and the other resorbable materials tested, all will support the attachment and proliferation of fibroblasts. Therefore other factors such as the mechanical properties and degradation profile of the material may be of equal importance when selecting a material for repair systems.

Acknowledgement

This project is funded by EPSRC, DOH, Smith & Nephew Group Research under a DTI Link initiative.

References

1. A. U. DANIELS, *J. Appl. Biomater.* **5** (1994) 51–64.
2. J. B. KING, *Clin. Orthop. Rel. Res.* **196** (1984) 139–53.
3. M. BERCOVY, D. GOUTALLIER, M. C. VOISIN, D. GEIGER, D. BLANQUAERT, A. GAUDICHET and D. PATTE, *Clin. Ortho. Rel. Res.* **196** (1985) 159–68.
4. J. F. HANSBOROUGH, M. L. COOPER, R. COHEN, R. SPIELVOGEL, G. GREENLEAF, R. L. BARTEL and G. NAUGHTON, *Surgery* **111** (1992) 438–46.
5. N. P. RHODES, *J. Mater. Sci. Mater. Med.* **5** (1994) 666–70.
6. J. L. RICCI, A. G. GONA and H. ALEXANDER, *J. Biomed. Mater. Res.* **25** (1991) 651–66.
7. K. M. MILLER, *J. Biomed. Mater. Res.* **23** (1989) 911–30.
8. D. A. LEE, *Osteoarthritis and Cartilage* **1** (1993) 137–46.
9. O. LAITINEN, *Arch. Ortho. Trauma Surg.* **112** (1993) 270–74
10. A. van SLIEDREGT, J. A. van LOON, J. van der BRINK, K. de GROOT and C. A. van BLITTERSWIJK, Biomaterials **15** (1994) 251–56.
11. F. R. ROZEMA, *J. Mater. Sci. Mater. Med.* **5** (1994) 575–81.
12. J. KAPUSCINSKI, B. SKOCZYLAS, *Anal. Biochem.* **83** (1977) 252–57.