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In vitro biocompatibility testing of polylactides

Part II *Morphologic aspects of different cell types*

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Four high-molecular weight polylactides, three poly-L-lactides of 100, 250 and 500 kDa and a poly-DL-lactide of 400 kDa, were tested qualitatively *in vitro*. Cells were cultured on polylactide films and with media based on the artificially aged polylactides, as well as with different concentrations of the final degradation products (monomers). Implant site-related cell types were selected. Three kinds of epithelial cells (middle ear, ear canal and nasal septum) as well as fibroblasts and osteosarcoma cells were used. Characteristic of all four polylactides was the normal morphology of cells when cultured on polylactide films and with the artificially ageing media. Although the polylactide films revealed still uncovered spots after 14 days culture, the control cultures were already confluent. The different concentrations of monomers revealed normal cell morphologies except for the 10 mg ml^{-1} concentration, which showed larger fibroblasts, and the nasal septum epithelium showed more signs of terminal differentiation for the 10 mg ml^{-1} D-monomer than was seen for the L-monomer even if the osmolarity was adjusted. The degradation products of polylactide are not expected to cause adverse reactions when implanted, since cell cultures with monomer concentrations up to 1 mg ml^{-1} resulted in normal morphologies. In the present study the cells were not able to cover the polylactide films completely.

1. Introduction

Polylactides are already used clinically in different disciplines such as maxillofacial surgery, dentistry and orthopaedics [1–3]. Research is still extending to improve the characteristics of the polymer. As a first approach of testing newly obtained polymers, *in vitro* studies should be used [4–6]. However, biocompatibility studies of polylactides are regularly performed *in vivo* but seldom *in vitro* [7, 8].

In vitro biocompatibility studies have the advantage that cell–material interactions can be studied without the predominant wound reaction that normally occurs after implantation. Such a wound reaction interferes with the events caused by the material itself. Besides the lack of an interfering wound and inflammatory reaction, the sensitivity of *in vitro* biocompatibility tests is known to be equal to or greater than that of *in vivo* studies [5, 9, 10]. In general, *in vitro* studies use quantitative measurements to determine an effect of the material on the cells, as described in [11]. Qualitative evaluations such as the morphology of cells are supplementary to quantitative measurements [12]. Normal phenotypic expression of cells is a major indication of its well-being. The cell surface appearance and intracellular accumulation of degradation products, for instance in lysosomes, are areas of interest that can be studied. Culture-dependent occurrences such as confluency, stratification and differentiation can also be observed.

In this study high-molecular weight polylactides, three poly-L-lactides of 100, 250 and 500 kDa and a poly-DL-lactide of 400 kDa, were tested qualitatively *in vitro*. Cells were cultured on polylactide films, as an approach to the *in vivo* situation shortly after implantation (degradation was hardly expected in the culture period of 14 days). Two other experimental settings were chosen in such a way that different stages of degrading implants were imitated *in vitro*. Therefore, the cells were either cultured with media based on the artificially aged polylactides, mimicking long-term implantation periods, or in different concentrations of the monomers resembling the release of final degradation products within a particular period. Implant site-related cell types were selected to evaluate the polylactides, because the sensitivity can depend on the cell type. The *in vivo* phenomenon that different cell types can respond differently towards the same material [13] was also demonstrated *in vitro* [14]. In the present experiments three kinds of epithelial cells, a stratifying squamous, a pseudostratified and a simple squamous epithelium, were used. Fibroblasts and osteosarcoma cells were also used. The osteosarcoma cells are derived from tumour cells and might, therefore, respond differently from non-neoplastic

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(bone-derived) cells. The cells were evaluated with light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Polylactides

The polylactides used in this study were supplied by Pural Biochem BV, Gorinchem, The Netherlands. Three poly-L-lactides with molecular weights of 100 000, 240 000 and 500 000 were used, and a poly-DL-lactide with a molecular weight of 400 000. Two lactic acid solutions were used as representatives of the final degradation products of the polylactides. D-Lactic acid and L-lactic acid [both 90% (w/v)] were supplied by Pural.

2.2. Cell types and culture conditions

The cell types used in the experiments were rat epithelial cells originating from the middle ear [15], the ear canal [16] and the nasal septum. Human fibroblasts and a human osteosarcoma cell line [17] were also used. The cells were originally obtained from explants in our laboratory [15], except the osteosarcoma cells, which were a gift from S. Rodan, Merck, Sharp and Dohme Laboratories, West Point, New York, USA. The culture conditions were described in [11]. In short, cells were cultured with Dulbecco's modified Eagles medium and F12 in a 3:1 ratio to which 5% foetal calf serum had been added. Epidermal growth factor (10 ng ml^{-1}) was added after 3 days culture. The medium was changed twice per week. The cells were cultured in 10% CO_2 at 37 °C. The epithelial cells were plated at a density of 1×10^5 cells (35 mm dish)$^{-1}$ together with lethally irradiated 3T3 feeder cells at the same density. The 3T3 cells were exposed to 3000 rad for 6.2 min by Co^{60} gamma-irradiation.

2.3. Experiments

The experimental conditions were described in detail in Part I [11]. In short, three types of experiments were used. In one experiment the cells were directly exposed to the polylactides by culturing on films. In another experiment the cells were cultured with artificial ageing media. These media were obtained by exposing polylactide films to elevated temperatures in a pseudo-extracellular fluid. The extraction fluids were used to prepare culture media. The third experiment investigated the effect of different concentrations (0.001, 0.01, 1 and 10 mg ml^{-1}) of D- and L-monomers. The monomers (the pH was adjusted with NaOH) were added to normal culture media as well as to media of which the osmolarity initially was lowered, after which the monomers were added. Routine culture medium served as a control as well as the medium to which extra NaCl was added. The osmolarities of the media obtained were determined (Table I). All experiments lasted up to 14 days.

TABLE I Osmolarities (mmol l^{-1}) of the media with the different monomer concentrations. Up to 1 mg ml^{-1} the osmolarities are hardly changed. The second values given for the 10 mg ml^{-1} concentrations are of media of which the osmolarities were initially lowered

Normal culture medium	337	
Control culture medium	378/508	
Normal culture medium + monomer (mg ml^{-1})	L-monomer	D-monomer
0.001	333	323
0.01	326	320
1	331	337
10	500/406	468/347

2.4. Preparation for morphological study

Cell cultures were fixed by immersion in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4 at 4 °C) for at least 2 h and post-fixed with 1% osmium tetroxide solution at room temperature for 30 min or overnight at 4 °C. The cell cultures, which were first used for light microscopy, were dehydrated, critical-point dried and gold sputter-coated for SEM or dehydrated, embedded in epon and cut into ultrathin sections for TEM.

The morphology of the cells was studied using phase-contrast light microscopy, SEM and TEM (SEM, Cambridge 180 Stereoscan or Philips SE 525; TEM, Philips EM 201).

Light microscopy was not possible for the cells cultured on the films as the films were generally too opaque. In general, the cultures from day 14 were used for extensive evaluation, the shorter culture periods being checked with light microscopy when possible, but not always with SEM and/or TEM.

3. Results

3.1. Normal morphology

By SEM flat cells with various amounts of microvilli were seen for all three types of epithelia. The cells also varied in size and form. Cell–cell contact was 100% for the middle-ear and nasal-septum epithelia, but less for the ear-canal cells (Fig. 1). The three kinds of epithelial cells possess some differences in culture-dependent occurrences such as confluency, stratification and differentiation. The cultures of the epithelia started by seeding epithelial cells with lethally irradiated feeder cells. Cultures of the three epithelial cell types revealed colonies of cells intermingled with lethally irradiated 3T3 feeder cells on day 1. The colonies grew until confluent cultures were present. The 3T3 feeder cells were pushed out of the cultures when the epithelial cells occupied more of the surface.

The middle-ear epithelium (a simple squamous epithelium) developed into a monolayer. Confluency was reached in 6 days. The nasal-septum epithelium (a pseudo-stratified epithelium) developed into a pseudo-multilayer. Confluency was reached within 6 days. The ear-canal epithelium showed clear signs of differentiation and cornification (Fig. 2). Confluency was reached within 10 days.

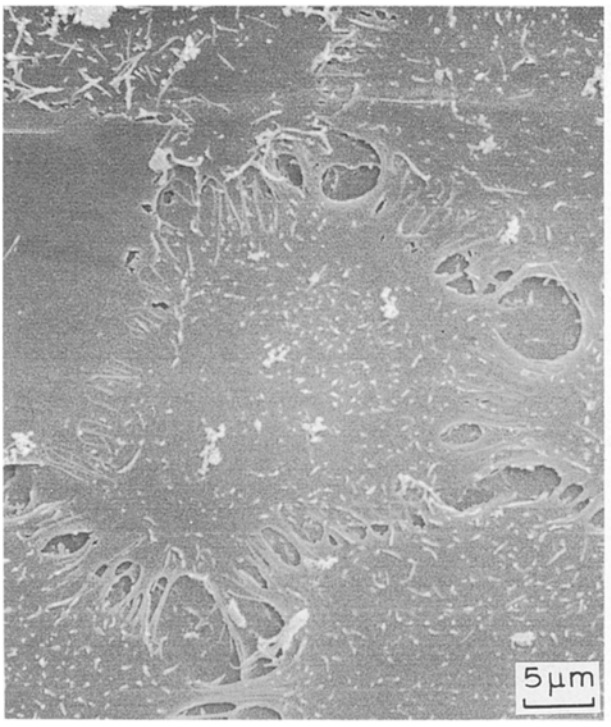

Figure 1 SEM micrograph of normal ear-canal epithelium after 6 days culture. Note the irregular cell shape and the partial cellular contact.

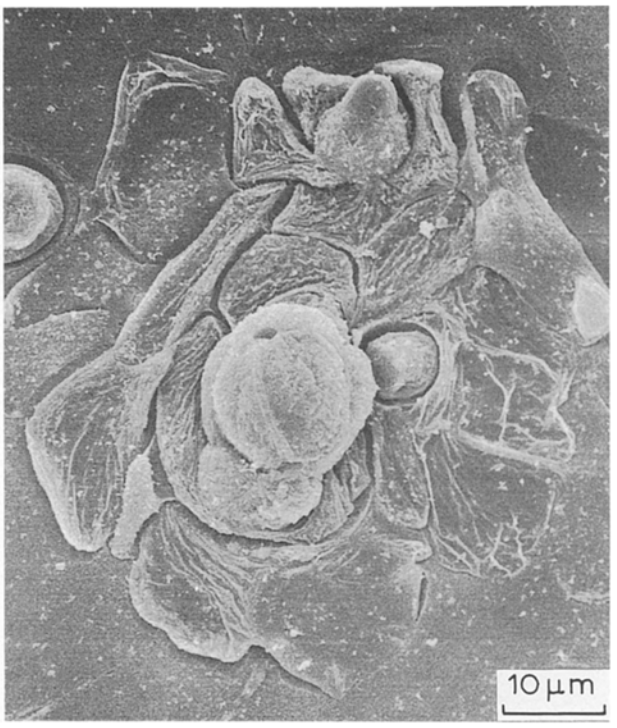

Figure 2 SEM micrograph of normal ear-canal epithelium after 6 days culture, already showing differentiation and cornification signs. Ruffled cell surfaces and a clod of cells.

In short, the osteosarcoma cells as well as the human fibroblasts developed into tracks of parallel-orientated cells while multilayering. The osteosarcoma cells had a spindle-like appearance, but flatter, rounder cells were also seen (Fig. 3). These flat, round cells were often seen at crossings of tracks. Some cells had microvilli and bleb-like structures on the cell surface. TEM revealed that these bleb-like structures were filled with the same cytoplasm as was seen for the rest of the cell, and no secretory vesicles were observed as might have been expected. The packing of the cells was loose.

The human fibroblasts were spindle-like in appearance and had hardly any microvilli on their surface. TEM often showed invaginations of the cell membrane, and no blebs were seen.

3.2. Culture on polylactide films

The first observation that was characteristic to all four polylactides was the normal morphology of cells cultured on polylactide films.

However, the three kinds of epithelial cells showed some differences in culture-dependent occurrences such as confluency, stratification and differentiation when cultured on films, compared with their control cultures. The epithelial cells cultured on the films never reached complete covering of the films during the culture period of 14 days. Cells surrounding uncovered parts of films were clustered and seemed unable to spread on the film (Fig. 4). Cornification of the ear-canal cells occurs normally after confluency is reached, but also in cultures grown on films that were not confluent (parts of the films were uncovered).

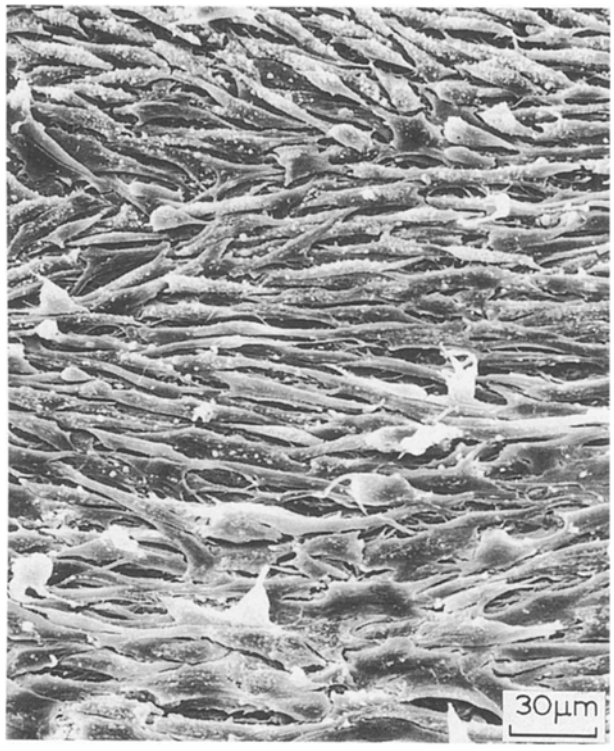

Figure 3 SEM micrograph of control culture of osteosarcoma cells after 14 days.

TEM revealed basal cells on which a few cell layers were present, and cornification (Fig. 5). Generally the cells cultured on the films were similar to those seen in the control cultures.

The fibroblasts detached easily from the films when being prepared for SEM or TEM. Whether full covering of the films had occurred could therefore not be

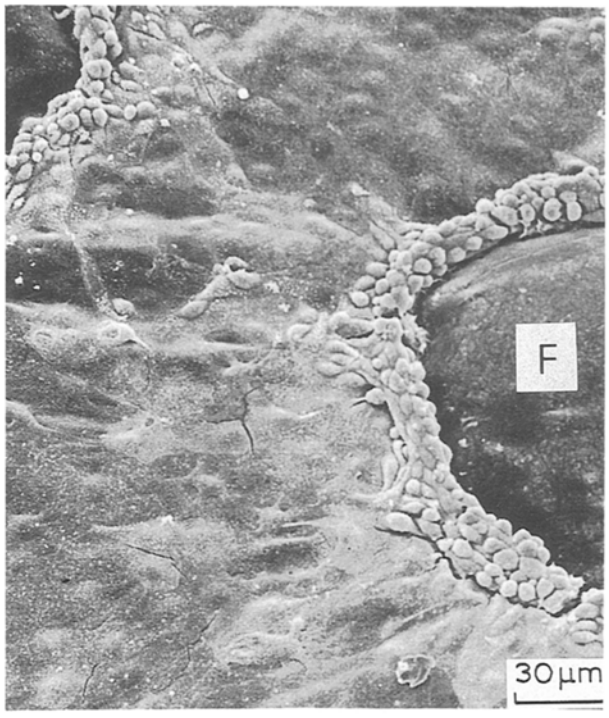

Figure 4 SEM micrograph of middle-ear epithelium cultured on the DL-polylactide film (F) for 2 weeks. Note the clustered cells around the bare spots

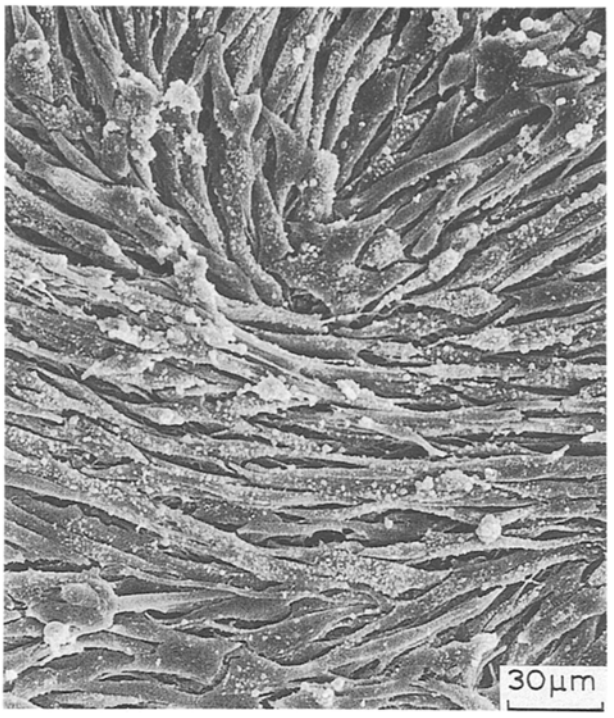

Figure 6 SEM micrograph of osteosarcoma cells cultured on polylactide film (molecular weight 250 000)

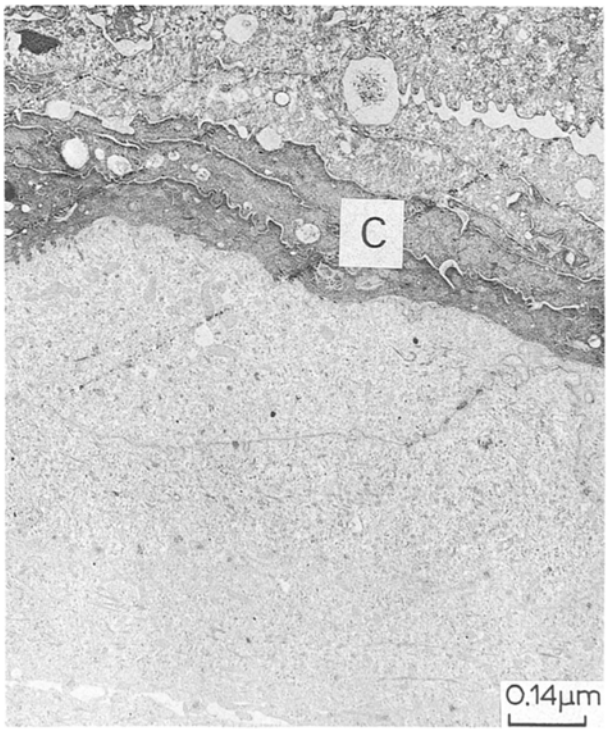

Figure 5 TEM micrograph of ear-canal cells cultured on polylactide film (molecular weight 250 000). Cross-section of the multilayer, cornification (C) is visible.

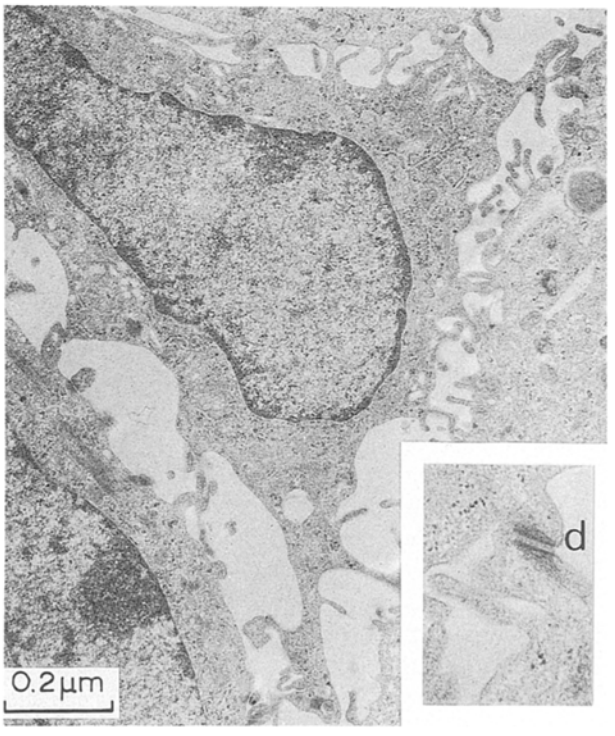

Figure 7 TEM micrograph of nasal-septum epithelium cultured with control artificial ageing medium for 14 days. The inset shows a desmosome (d).

concluded. The cells that could be evaluated were similar to those of control cultures. The osteosarcoma cells revealed uncovered spots of the films, but the morphology was normal (Fig. 6).

3.3. Artificial ageing media

The same cell types were also cultured with artificial ageing media. These media mimicked the release of materials of long-term implanted polylactide. The same characteristic was found as was seen for the culture on films; all cell types cultured with the artificial ageing media revealed normal morphologies. Culture-dependent occurrences such as confluency, differentiation and cornification were similar to those in control cultures (Fig. 7).

3.4. Culture with different L- and D-monomer concentrations

The nasal-septum epithelium and the fibroblasts were cultured with different concentrations of monomers. The cells cultured with these media proliferated well compared with the control cells, except for the highest concentrations (10 mg ml^{-1}).

The fibroblasts had a normal appearance but developed into less-dense tracks for the 10 mg ml^{-1} concentrations. Cross-sections (light microscopy and TEM) revealed that cell development into multilayers occurred, as was seen for the other concentrations and the controls. The TEM morphology of the cells had a similar appearance to that seen for the control cells. The nasal-septum epithelial cells revealed more variation in cell size for the 10 mg ml^{-1} concentration, and sometimes more vacuoles were seen in the cytoplasm. The cells were already confluent at the beginning of the experiment (at day 4) when they were cultured with routine culture medium and showed no decrease in confluency after adding the experimental media.

When both cell types were cultured again with 10 mg ml^{-1} monomer concentration but with an osmolarity correction, the morphologies were more like those of control cultures. An exception was the nasal-septum epithelium, which revealed more-manifest cornified envelopes with 10 mg D-monomer ml^{-1}. This was seen best with phase-contrast light microscopy (Fig. 8).

When the cells were cultured with media to which 500 mmol l^{-1} extra NaCl was added the fibroblasts were big and flat, and the nasal-septum epithelium showed the same deviations as was seen earlier with the 10 mg ml^{-1} monomer without osmolarity corrections (Figs 9 and 10).

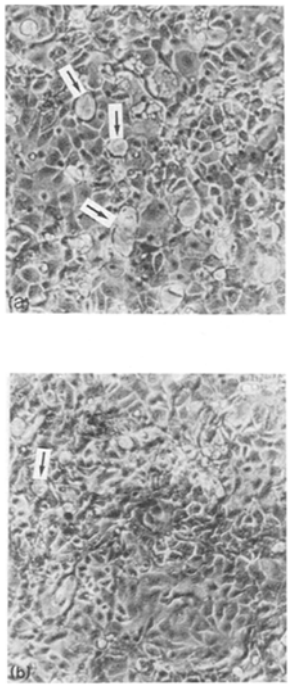

Figure 8 Phase-contrast light micrograph of nasal-septum epithelium cultured with (a) 10 mg D-monomer ml^{-1} medium for 14 days. Note the more cornified envelopes as is seen for the 10 mg L-monomer ml^{-1} medium (b).

4. Discussion and conclusions

The four high-molecular weight polylactides were tested *in vitro* using cell cultures, and the morphology was evaluated. The experimental setting in which cells were cultured on polylactide films can be regarded as a short-term real-time experiment. The results of culturing on films indicated that polylactide has no negative effect on individual cells, but some unfavourable potentials are encountered when used as a substrate for cell culture. The inability of cells to cover the whole films might be caused by local impurities. However, in a study by Cima *et al.* [18] a similar inability to cover was seen. Hepatocytes were cultured on films of poly-DL-lactic acid–co-glycolic acid (PGLA) and on a blend of PGLA and poly-L-lactic acid, as a control served a coating of collagen. The hepatocytes cultured on the polymers were globular at the edge of cell colonies, whereas they were fully spread on the control cultures. In the present study all four polylactides showed parts uncovered by cells, and no clear difference in covering related to molecular weight or type (L versus DL) was seen.

In a previous article [11] the proliferation was described for the same cells cultured on polylactide films. Of these five cell types used, four types revealed lower cell numbers for cultures on films than for the control, but the differences between the controls and the cultures on films were minor. Apparently the proliferation was not inhibited, but were adherence- and spreading-inhibited on some parts of the films. This is in concordance with the study of Cima *et al.* [18] who found a similar inability of covering and an unaffected proliferation as indicated by the deoxyribonucleic acid synthesis levels were the same for cultures on the polymers as for the collagen-coated controls.

The artificial ageing experiment can be regarded as an accelerated-time experiment. The degradation of the polylactides was expected to be accelerated in the pseudo-extracellular fluid when heated [19, 20]. The products released are supposed to be similar to the released products when polylactide is implanted for longer periods. Cells cultured with the artificial ageing media were similar to control cultures. This means that degradation products of polylactide are expected to be biocompatible at longer intervals.

Cells cultured with the different monomer concentrations showed that up to 1 mg monomer ml^{-1} medium did not cause any alterations in morphology or culture characteristics. Although this experiment needs further research to exclude the effects of osmolarity- and/or ion-changes [21], it has shown that the toxicity levels are probably not reached even at 10 mg ml^{-1} concentration. However, the proliferation was slightly inhibited [11].

The *in vitro* biocompatibility of the polylactides seems to be good. In these experiments the pH was adjusted and maintained within the physiological range. An extrapolation to the *in vivo* situation is difficult since the exact pH deviations in the environment of a degrading polylactide implant are not known.

In this study different cell types were used to extend the information obtained from just one or two cell

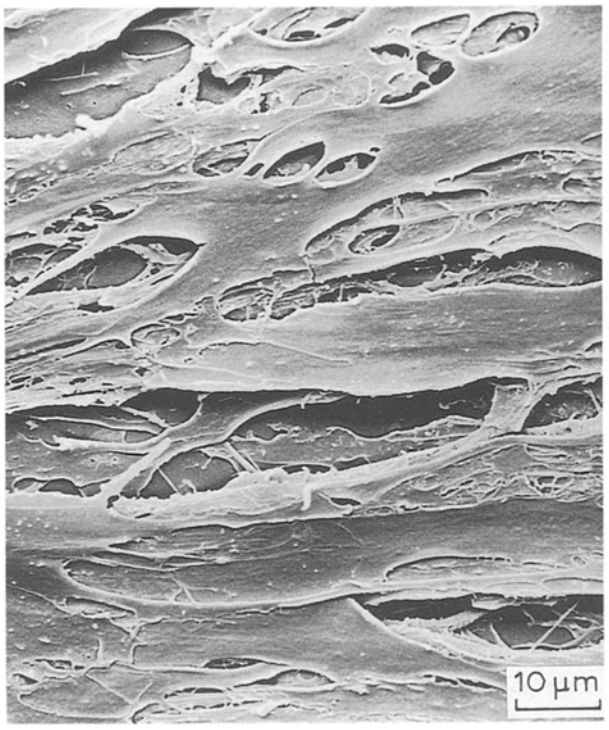

Figure 9 SEM micrograph of human fibroblasts cultured for 14 days (control culture)

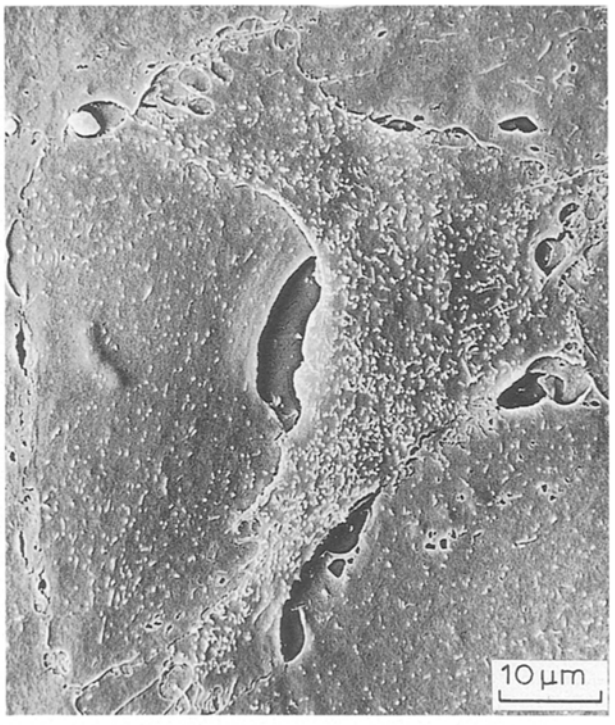

Figure 10 SEM micrograph of human fibroblasts cultured with medium with an osmolarity of 500 mmol l^{-1}. The shape of the cells is different from that seen in the control cultures.

types. From the results it is concluded that the use of five different cell types did not add supplementary information as compared to the use of one or two cell types.

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