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In vitro biocompatibility testing of polylactides Part I Proliferation of different cell types

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Four polylactides, P-L-LAs 100 KD, 240 KD, 500 KD and a P-DL-LA 400 KD, were tested *in vitro* by using five cell types. Middle ear, ear canal and nasal septum epithelial cells were used as well as fibroblasts and osteosarcoma cells. The proliferation of cells was studied by culturing on polylactide films and by culturing with media based on artificially aged PLA. The fibroblasts and the osteosarcoma cells were also cultured with media containing *L*- or *D*-monomers in different concentrations. Significant differences in cell numbers of polylactide cultures and/or controls were observed. These differences varied per cell type and experimental setting. In general the biocompatibility of the PLAs was satisfactory. Some inhibitory effect on the proliferation of high (10 mg ml^{-1}) monomer concentrations in culture media was seen.

1. Introduction

Polylactide (PLA), a degradable polymer, has found a limited clinical application [1–4]. Polylactide is degraded by a random hydrolytic chain scission process; this is described both *in vivo* and *in vitro* [5, 6] and it has also been found that enzymatic degradation occurs *in vitro* [7] and *in vivo* [8]. The degradation rate of PLA polymers seems to depend on experimental setting, whereas the molecular mass and the composition of *L*- and/or *D*-enantiomers are the main determinants of the degradation rate [9–11]. Some studies revealed that after almost a year of implantation polylactide implants can still be present, hardly changed in shape or mass [9, 12–15]. Other implant studies show that a relatively high degradation could occur [16, 17].

Studying the effects of the degradation products on individual tissues *in vivo* encounters difficulties because implantation is attended with a wound reaction. *In vitro* cell culture studies have the advantage of relatively well-controlled variables. Most of the cell types normally present at the implantation site can be individually studied *in vitro*. It is known that *in vitro* cell testing is a very sensitive method for biocompatibility testing [18, 19]. Generally, *in vitro* toxicity is in concordance with *in vivo* results [20], but discrepancies were also observed [21, 22].

In the present study the effect of polylactides, with different degradation rates, on cell proliferation was quantitatively evaluated *in vitro*. Three poly-*L*-lactides were used as well as a poly-*DL*-lactide. Three distinct experiments were performed by using five different cell types. Three types of epithelial cells were used; middle ear, ear canal and nasal septum. An osteosarcoma cell-line with osteoblastic properties (representing a bony implantation site) and fibroblasts were used. The osteosarcoma cells were derived from tumour cells and might therefore respond differently from non-neoplastic (bone derived) cells. These five cell types were representatives for an ear, nose and throat implantation site. To test the polylactides, various experiments were undertaken. In the first, cells were directly exposed to the polylactides by culturing on PLA films. In the second, the long-term degradation of polylactide was simulated by exposure of cells to degradation products of artificially aged polylactides. The solution mimicking the long-term degradation was used as a basis for culture medium. Finally, in the third experiment the cells were given different concentrations of the end-products of PLA degradation. The *L*- and *D*-monomers were added in different concentrations to the culture media to investigate whether an effect on the proliferation could be defined.

2. Materials and methods

2.1. Polylactides

The polylactides examined in this study were supplied by CCA biochem bv, Gorinchem, The Netherlands. Three poly(*L*-lactides) with molecular masses of 100 000, 240 000 and 500 000 Da and a poly(*DL*-lactide) with a molecular mass of 400 000 Da were used and two lactic acid solutions, *D*- and *L*-lactic acid both 90% w/v, were supplied by CCA.

2.2. Cell types and culture conditions

The cell types used in the experiments were rat epithelial cells originating from the middle ear, the ear canal and the nasal septum. Human fibroblasts and a human osteosarcoma cell line [23] were also used. All

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the cultures, except the osteosarcoma cells, were originally obtained from explants in our laboratory [24]. The osteosarcoma cells are a gift from S. Rodan, Merck, Sharp and Dome Laboratories, West Point, USA.

The cells were cultured with Dulbecco's Modified Eagles Medium and F12 in a 3:1 ratio to which hydrocortisone (0.4 $\mu g\,ml^{-1}$), isoproterenol (10^{-6} M), penicillin (100 $U\,ml^{-1}$), streptomycin (100 $\mu g\,ml^{-1}$) and 5% foetal calf serum had been added. Epidermal Growth Factor (10 $ng\,ml^{-1}$) was added after three days of culture. The medium was changed twice a week. The cells were cultured in 10% CO_2 at 37 °C.

The cells were harvested from 14 cm culture dishes in their 5th or 6th passage by trypsinization, except for the osteosarcoma cells [23] of which the number of passages is not known. The epithelial cells were plated at a density of 1×10^5 cells per 35 mm dish together with lethally irradiated 3T3 feeder cells at the same density. The 3T3 cells were exposed to 3000 rad (30 Gy) during 6.2 min by cobalt 60 gamma irradiation. The fibroblast and the osteosarcoma cells were plated at the same density but without the 3T3.

The test conditions in which the cells were studied were: (a) culture of cells on polylactide films, (b) culture of cells with media based on the artificially aged PLAs, (c) culture of cells with lactic acid monomers.

2.3. Exposure of the cells directly to the polylactides

All the cell types were cultured on polylactide films for up to two weeks. The films were prepared by a solvent casting method using 5% PLA (P-L-LA 100, 240, 500 KD or P-DL-LA 400 KD) in chloroform (w/w ratio). The initial thickness of the films was 500 μm. The films were cut into circular pieces to fit into 35 mm culture dishes (Greiner). They were then thoroughly rinsed in running tap water overnight and then washed in distilled water and air dried. The night before cell culturing the dishes with the films were sterilized by using ultraviolet light. The normal culture plastic (TCPS) served as a control.

2.4. Artificial ageing media

Polymers degrade faster at elevated temperatures and thereby liberating additives and toxic low molecular mass compounds, when introduced during processing and/or as a result of degradation itself. The additives and/or toxic low molecular mass compounds will diffuse out of the material into the extraction fluid. Parts of the same films as used in the first experiment were cut into pieces and exposed to 115 °C in a pseudo-extracellular fluid (PECF) for 60 h.

Ion concentration (meq l^{-1}) of PECF	
Na^+	154.5
K^+	5.4
Cl^-	118.5
HCO_3^-	44

The surface area of the polylactide films was 0.38 $cm^2\,ml^{-1}$ extrusion fluid. This method has been described in earlier publications [20, 25]. The heat-exposed solutions were used as a basis to prepare nutrient media for cell culturing. These media will be referred to as artificial ageing media. The heat-exposed pseudo-extracellular fluid without PLA served as a basis for the control medium.

2.5. Concentrations of *L*- and *D*-monomers

In the third experiment only nasal septum epithelial cells and human fibroblasts were cultured in medium containing 0.001, 0.01, 1, or 10 $mg\,ml^{-1}$ *L*- or *D*-monomers, also for two weeks. The lactic acid solutions were, after adjusting the pH with NaOH, added to routine culture medium in the different concentrations. Routine culture medium served as a control as well as routine medium to which sodium chloride ions (NaCl) where added. The amounts of sodium chloride were 2.18 $mg\,ml^{-1}$, resulting in an osmolarity of 378 $mOsm\,l^{-1}$. However, the concentrations of 10 $mg\,ml^{-1}$ increased the osmolarity to about 500 $mOsm\,l^{-1}$ (Fig. 1), because the monomers were added to media which already had osmolarities of about 337 $mOsm\,l^{-1}$. Therefore, in a subsequent experiment the media were prepared by adding ingredient after ingredient thereby reducing the amount of sodium chloride so that the monomers could be added, resulting in a limited increase of osmolarity (Fig. 2). Controls were made by adding normal amounts of sodium chloride to the media (resulting in medium similar to routine culture medium) and by adding 5 $mg\,ml^{-1}$ sodium chloride resulting in an osmolarity of 500 $mOsm\,l^{-1}$.

2.6. Quantitative evaluation

For all three test conditions cells were counted on day 1, 4, 6, 10, and 14. For the second and third experiments, cells were also counted on day 3. Three dishes for each sample were used. Statistical analysis was performed for each cell type per experiment by using multi-analysis of variance (MANOVA) of the logarithmic transformed cell numbers at a confidence level of 95%. On the basis of the calculations made by the MANOVAs, multiple range tests using the Sheffé

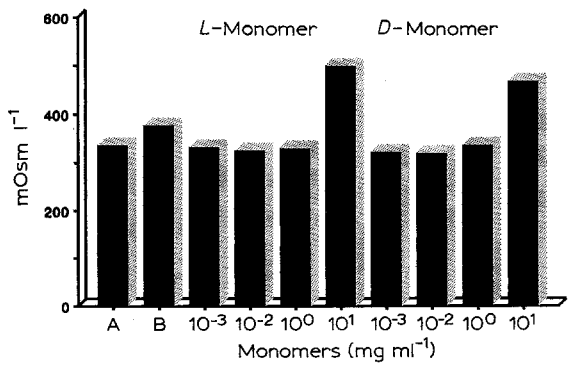

Figure 1 The osmolarities of media ($mOsm\,l^{-1}$) to which different concentrations of L- or D-monomer were added. Control A is routine culture medium, Control B is routine culture medium + 2.18 $mg\,ml^{-1}$ NaCl. The L- and D-monomers are added in the concentrations 0.001, 0.01, 1 and 10 $mg\,ml^{-1}$.

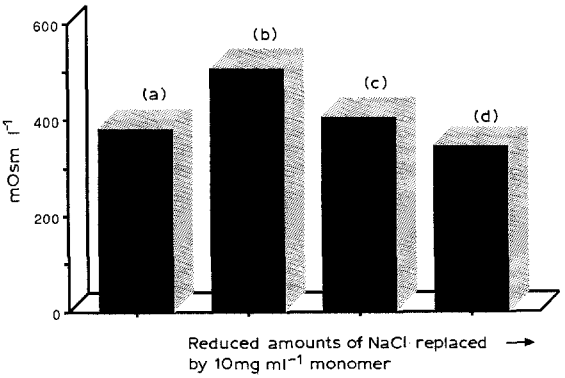

Figure 2 The osmolarities of media (mOsm l^{-1}) to which L-monomer (c) and D-monomer (d) were added (10 mg ml^{-1}) without increasing the osmolarity. Medium with extra NaCl (b) (5 mg ml^{-1}) served as a control as well as medium with normal amounts of NaCl (a).

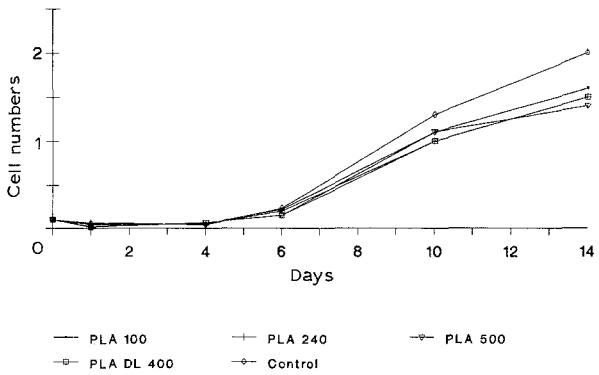

Figure 3 Growth curves of human fibroblasts cultured on polylactide films.

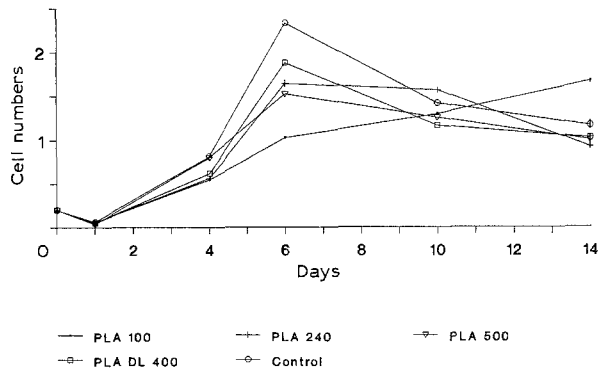

Figure 4 Growth curves of ear canal epithelial cells cultured on polylactide films.

method were performed. Multiple range testing is useful to obtain an impression of which condition(s) was or were significantly different. The multiple range test ranks the cell numbers. Statistical calculations were performed with the software package STAT GRAPHICS.

2.7. Light microscopy

The cell cultures were observed by using a phase contrast microscope. Cell cultures were fixed in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 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.

3. Results

3.1. Exposure of the cells directly to the polylactides

Proliferation of the cells on the polylactide films was studied by counting cell numbers after 1, 3, 6, 10 and 14 days of culture. The cell numbers were statistically evaluated and the shapes of the growth curves were commented upon; not all data are shown in figures. The cell numbers of the controls (tissue culture polystyrene) were the highest during the two weeks of culture for all cell types, except for the middle ear epithelium. The middle ear epithelium and the fibroblasts revealed no significant differences in cell numbers for the control and the polylactides. The growth curves of the human fibroblasts are shown in Fig. 3. The nose epithelial cells and the osteosarcoma cells showed only significant differences between the control and polylactide 100 KD. The ear canal epithelial cells showed only a significant difference between the control and polylactide 500 KD.

In general, the proliferation of the cells on the polylactide films as compared with the control revealed a similar pattern although some retardation could be observed. The ear canal epithelium revealed a differently shaped growth curve for cells cultured on the polylactide film 100 KD as compared with the others. The cell number increased throughout the two weeks whereas the others already revealed a slight decrease or a plateau after six days of culture (Fig. 4).

Statistical calculations revealed differences between the polylactides and/or the control. A barchart of the ranking order of the materials per cell type is shown in Fig. 5. One case excepted, the control had the highest ranking numbers whereas the polylactide films revealed variation in ranking numbers.

Proliferation of cells on the films was difficult to examine by light microscopy because the films were in general insufficiently transparent. With increasing culture time the films became more opaque.

3.2. Artificial ageing media

Cell proliferation was studied by counting cell numbers after 1, 3, 4, 6, 10 and 14 days of culture. The cell numbers were statistically evaluated and the shapes of the growth curves were commented upon; not all data are shown in the figures. Statistical calculations showed that the osteosarcoma cells proliferated less in the artificial ageing media based on the polylactides 100 and 240 KD than in the other artificial ageing media (Fig. 6). The other cell types did not show this phenomenon.

In general, the proliferation pattern of the cells cultured with the PLA artificial ageing media were similar to the control artificial ageing medium. The artificial ageing medium based on polylactide 240 KD displayed a slightly different growth curve for the middle ear epithelium. A plateau was not reached but

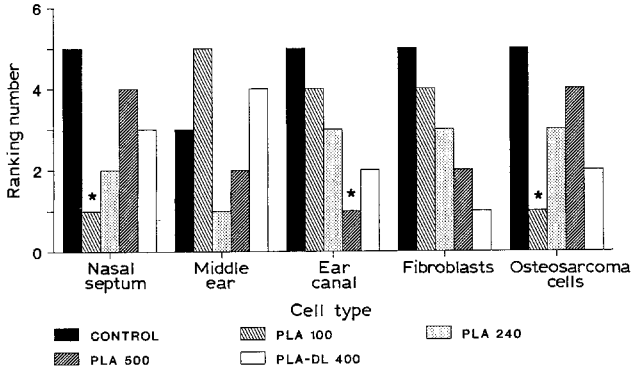

Figure 5 The polylactides and the control with the corresponding ranking numbers per cell type are shown. Ranking number 1 indicates lowest cell number. * Significantly different from the control ($p < 0.05$).

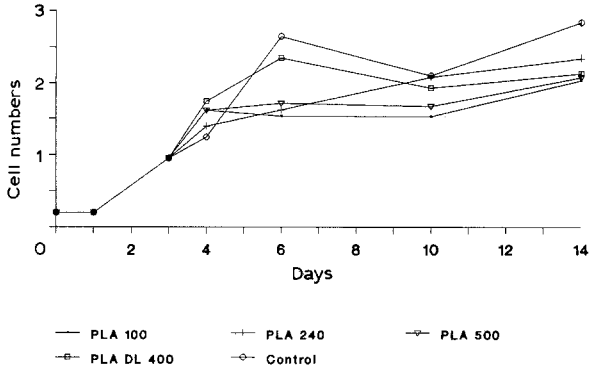

Figure 7 Growth curves of middle ear epithelial cells cultured with artificial ageing media.

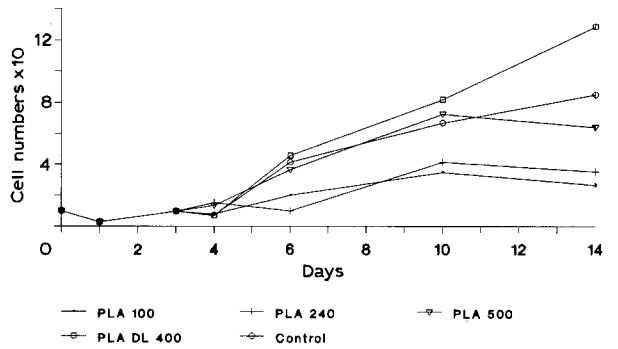

Figure 6 Growth curves of osteosarcoma cells cultured with the artificial ageing media.

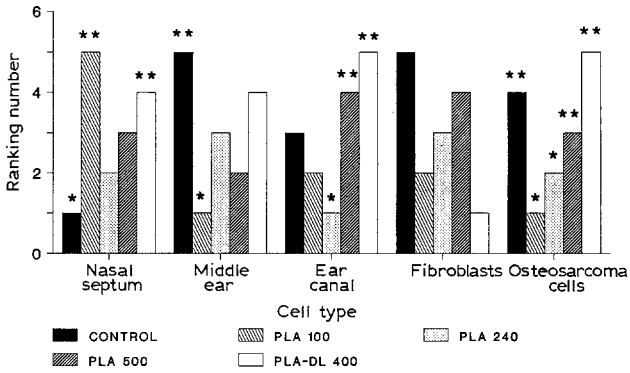

Figure 8 The polylactides and the control with the corresponding ranking numbers per cell type are shown. Ranking number 1 indicates lowest cell number. * and ** are significantly different ($p < 0.05$) from each other.

an increase in cell number up to day 14 was seen (Fig. 7).

The cultures could be well observed by using light microscopy with the first experiments where the films hindered observation. The proliferation of cells cultured with the PLA-based artificial ageing media was similar to those cultured with the control artificial ageing medium. The increase of cell numbers was generated from colonies eventually resulting in confluent cultures. Confluent cultures were already present after six days of culture, except for the ear canal epithelial cells, which were confluent after 10 days of culture. The fibroblasts and the osteosarcoma cells developed a stratification of cells combined with a parallel orientation of cells into tracks.

Statistical calculations revealed differences between the polylactides and/or the control. A barchart of the ranking order of the materials per cell type is shown in Fig. 8. A variation in ranking numbers is seen for all conditions.

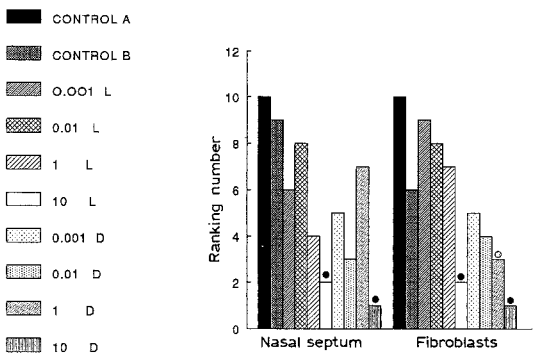

Figure 9 Ranking numbers of both cell types for each culture condition. Ranking number 1 indicates lowest cell number. (●) Significantly ($p < 0.05$) lower cell numbers than the other concentrations and the control. (○) Significantly different from the L-monomer concentrations and the controls but not from the other D-monomer concentrations.

3.3. Monomer concentrations

In this experiment we investigated the effect of different monomer concentrations on cell proliferation. The cells were counted after 1, 3, 4, 6, 10 and 14 days of culture. Of the used concentrations only the highest concentrations, both *L*- and *D*-monomers, revealed an apparent retardation in proliferation. Fig. 9 shows the ranking numbers of both cell types for each culture condition. Some discrimination in the ranking order is present for the human fibroblast. All the *D*-monomer concentrations have lower cell numbers as compared with the corresponding *L*-monomer concentrations. Statistically, however, besides the highest concentrations, only the 1 mg ml^{-1} *D*-monomer was significantly lower from the other *L*-monomer concentrations. For the nose epithelial cells such a deviation was not observed.

As mentioned before, of the concentrations used only the highest concentrations, both *L*- and *D*-monomers, revealed an apparent retardation in proliferation. However, the osmolarities were increased to a level of approximately 500 mOsm l^{-1}. To investigate whether this was caused by the high osmolarities or the high monomer concentrations, the cells were also cultured with routine culture medium to which sodium chloride was added to the same level of 500 mOsm l^{-1} and compared to cells cultured with media to which 10 mg ml^{-1} monomer was added without increasing the osmolarity considerably (by lowering the amount of sodium chloride and replacing it by high concentrations of monomer). The osmolarities were 406 mOsm l^{-1} for the *L*-monomer and 347 mOsm l^{-1} for the *D*-monomer. The cell numbers of the cultures with high monomer concentrations again revealed retardations which were not significantly different from the medium of which the osmolarity was increased to a level of 500 mOsm l^{-1} (Fig. 10). This was observed for both cell types. The cells cultured with the routine culture medium had significantly higher cell numbers than cells given media with the monomers or high sodium chloride.

Both cell types used, nose epithelium and fibroblasts, showed a normal proliferation pattern using light microscopy. The nose epithelium was confluent already after four days of culture.

4. Discussion and conclusions

The polylactides tested in the present study showed differences in cell proliferation. The differences between the polylactides and/or the control were dependent on cell type and experimental condition. A prominent retardation or stimulation caused by the polylactides was not observed. This was seen for the exposure of cells directly to the films as well as for the exposure to the artificial ageing media. Although *in vitro* cell testing is a very sensitive method for biocompatibility testing, to discriminate toxic from non-toxic materials seems to be easier than to determine the degree of toxicity [20, 25–28]. Since dissimilarity in reactions of distinct cell types towards the same material occurs *in vitro* [29, 30] as well as *in vivo* [31], it is necessary to test with the cell types present at the implantation site. Epithelial cells will normally not be in contact with an implant. However, diffusion of toxic compounds out of the implant could eventually reach epithelial cells. Therefore epithelial cells should be included in testing materials which will not be in contact with epithelium *in vivo*.

The experiment in which the cells were cultured with the monomers of polylactide revealed that the amount of cells grown in media containing 10 mg ml^{-1} was significantly decreased as compared to lower concentrations. Cells cultured with media of which the osmolarity was increased less by reducing the amount of NaCl demonstrated that the cell numbers were not significantly different from those cultured with media with an osmolarity of 500 mOsm l^{-1}. Some effect caused by the monomer concentration is therefore likely. Further investigation on this topic is required to exclude an effect of osmolarity [32]. The question is whether the concentration of 10 mg ml^{-1} is a realistic concentration for *in vivo* situations. It is possible to imagine that a polylactide block of 1 cm^3 (virtually 1 ml) is implanted and degrades suddenly for 1% into its monomers causing a 10 mg ml^{-1} release. However, the *in vitro* results of the present study should not be extrapolated directly to the *in vivo* situation since, for instance, in these *in vitro* experiments a pH effect is excluded.

From the present results it can be concluded that the tests were not sufficiently sensitive to discriminate between the polylactides differing in molecular mass or composition in a way that a specific polylactide could be described as an inhibitor or stimulator of cell proliferation. The polylactides tested by culturing cells on films and by culturing cells with artificial ageing media (based on the degradation products) revealed a satisfactory biocompatibility. Significant differences were observed, but these varied per cell type and/or experimental setting. The monomer experiments need further investigation.

Quantitative testing, although very useful in evaluating large numbers of cell types under various conditions, is a restricted approach to biocompatibility testing. Qualitative aspects also need to be considered. In Part II (in preparation) the morphology of the cells using light microscopy, transmission and scanning electron microscopy will be described.

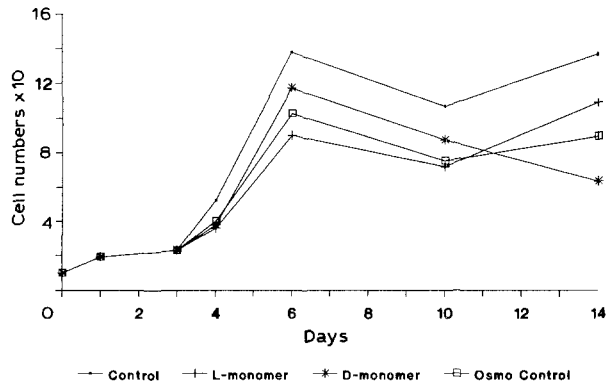

Figure 10 Growth curves of nasal septum epithelial cells cultured with media containing 10 mg ml^{-1} L- or D-monomer, medium to which 5 mg NaCl ml^{-1} was added resulting in a 500 mOsm l^{-1}, and a routine culture medium.

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