Activation of Osteoblast-like MC3T3-E1 Cell Responses by Poly(Lactide)

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This study examined the osteoblast-like MC3T3-E1 cell responses to poly(ε-lactide) (PDLLA) and poly(l-lactide) (PLLA) with different weight average molecular weight (M.W.). Colony formation of MC3T3-E1 cells on the PLLA with M.W. 270000 or 1370000 was slightly lower than that on glass. The protein, DNA and hydroxyproline (HYP) content and alkaline phosphatase (ALP) activity for cells cultured on the PLLA (M.W. 270000 or 1370000) for 14 d were almost similar to those on glass. In contrast, the ALP activity of the cells cultured on low M.W. PLLA (M.W. 20000) increased. Osteoblast differentiation was stimulated by low M.W. PLLA but not by high M.W. PLLA. The addition of low M.W. PDLLA (M.W. 5000 or 10000), l-lactide or l-lactic acid into culture increased the protein, DNA and HYP content and ALP activity for cells at 100 μg/ml. Compared with four chemicals, PDLLA (M.W. 10000) had the strongest stimulation effect on the cell. The release of l-lactic acid from PLLA and PDLLA into aqueous solution during incubation only slightly affected cell activity. In a cell-free condition, in the presence of PDLLA, the ALP activity was maintained without inactivation, even after 24 h incubation. Such a phenomenon was not seen with l-lactide and l-lactic acid. This may be a reason why PDLLA has a stronger effect on osteoblast differentiation relative to l-lactic acid. These results suggested that increased osteoblast differentiation was induced by low M.W. PDLLA and PLLA, and these may be used as an effective material in the field of orthopedic and drug delivery systems for the treatment of bone diseases.

Key words poly(ε-lactide); poly(l-lactide); osteoblast; MC3T3-E1 cell; differentiation

Poly(lactide), including poly(ε-lactide) (PDLLA) and poly(l-lactide) (PLLA), are biodegradable materials, and their clinical applications are being widely studied. The mechanical properties and degradation rates of poly(lactide) depend on the molecular weight of the fabricated device. Poly(lactide) with a low molecular weight is used as a controlled drug release device, and with high molecular weight has been used as a screw pin and plate component for bone fixation in orthopedic surgery.1,2

There have been many reports on the biocompatibility of PLLA. B´es et al. reported that no acute chronic inflammatory reaction was observed from subcutaneous PLLA implantation into rats until 143 weeks.3 Ishaug et al. described that attachment, morphology, alkaline phosphatase (ALP) activity and collagen synthesis of rat calvarial cells cultured on PLLA films, with a molecular weight of 95800, was similar to those of tissue culture polystyrene.4 Otto et al. observed bone formation around a PLLA wire with a molecular weight 160 kD at 2 and 6 months after implantation.5 In contrast, Taylor et al. reported the toxicity of an extraction solution from PLLA.6 The molecular weight of PLLA used for the experiments was different, and the PLLA were molded by melt-pressed or hot extrusion, then sterilized after polymerization. Thermal treatment of PLLA decreased the molecular weight, and affected the cell activity.2,7,8 In the previous study, we expected that the low molecular weight PLLA produced by heat degradation would cause osteoblast differentiation.9

In this study, we further examined the response of mouse osteoblasts-like MC3T3-E1 cells cultured on several PLLA with different molecular weights to clarify which molecular weight of PLLA would induce osteoblast differentiation. These materials were not heat-treated after polymerization. In contrast to PLLA, there was no report on the stimulation of osteoblast activity by PDLLA. Even if they have a similar molecular weight, the physiological properties are fairly different between PDLLA and PLLA, so we examined the effect of PDLLA on the cells. The amount of l-lactic acid released from PDLLA and PLLA after incubation was determined to clarify the relationship between the degradation of test materials and cell responses. Further, the effect of test materials on ALP activity was investigated under a cell-free condition.

MATERIALS AND METHODS

Materials PLLA with a high weight average molecular weight (M.W.) of 1370000, and a medium M.W. of 270000, as calculated, were generously supplied by Gunze, Ltd. (Kyoto, Japan). PLLA with a M.W. of 20000 was supplied from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). PDLLA with a M.W. 5000 or 10000 and l-lactic acid were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Using information provided by the company, the PLLA (M.W. 270000 and 1370000) were synthesized by ring-opening polymerization of l-lactide using a tin compound as a catalyst.10,11 The resultant polymer was purified by precipitation into methanol from chloroform solution to remove the residual monomers. PLLA (M.W. 20000), PDLLA (M.W. 5000 and 10000) were produced by direct condensation without using polymerization catalysts, and were free from any contamination of heavy metals derived from polymerization catalysts (Wako technical bulletin, and personal communication). The PLLA and PDLLA were raw materials, not heat-processed after polymerization. All materials were utilized as supplied and were not further tested for residual monomer or other impurities. The melting points (mp) of PLLA (M.W. 270000 or 1370000) were analyzed by differential scanning calorimetry (Shimadzu DS-40 system, Kyoto, Japan), and were 170—180°C.

Coating of PLLA Coating of PLLA was carried out by the method described in a previous paper.12 Briefly, each

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PLL A was dissolved in dichloromethane at a concentration of 5 mg/ml. Two milliliters of the PLLA solution was added into a glass-dish or a glass-bottle 30 mm in internal diameter, and maintained at room temperature for 24 h to evaporate the solvent. The PLLA-coated dishes and bottles were placed in a vacuum drying oven at 25 °C for 24 h, then under atmospheric pressure at 35 °C for 2 d. For cell culture, the dishes were sterilized by exposure to ultraviolet light overnight.

**Gel Permeation Chromatography**

The molecular weight distribution and relative molecular weight of PLLA were determined by gel permeation chromatography (GPC). PLLA specimens were dissolved in chloroform at a concentration of 5 mg/ml, and 50 μl of the test solution was injected in a GPC apparatus (model LC10AT pump, Shimadzu Co., Kyoto, Japan) equipped with two GPC columns (TSKgel G5000HXL + TSKgel G4000HXL, each 7.8 mm i.d. × 30 cm, Tosoh, Tokyo, Japan), a refractive index detector (model RID-10A, Shimadzu Co.) and Class-LC Workstation GPC software (Shimadzu Co.). The flow rate of the chloroform mobile phase was 1.0 ml/min. Molecular weight values, including weight average (Mw), number average (Mn), and molecular weight distribution (Mw/Mn) for PLLA were measured by comparison with the calibration line which was made with polystyrene standards (Showa Denko, Tokyo, Japan).

**Determination of l-Lactic Acid**

The bottom of a glass bottle was coated with 10 mg of PLLA. Two milliliters of water was added into the bottle, and the bottle was incubated at 37 °C. After 2 weeks, the amount of l-lactic acid released into the water was determined. For PDLLA and l-lactide, these were first dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 mg/ml and diluted with water to a 100 μg/ml concentration. A bottle containing 2 ml of the solution was incubated at 37 °C for 1 week, and the supernatant collected was used for the determination of l-lactic acid. l-Lactic acid concentration was determined by using a diagnostics kit (Boehringer Mannheim GmbH, Mannheim, Germany). In principle, l-lactic acid reacted with nicotinamide adenine dinucleotide (NAD) in the presence of l-lactate dehydrogenase to form pyruvate and NADH. An increase in the absorbance at 340 nm due to NADH formation became a measure of l-lactic acid originally present. The detection limit of this kit was 0.3 μg of l-lactic acid.

**Cells**

Mouse osteoblast-like MC3T3-E1 cells were obtained from Riken Cell Bank (Tsukuba, Japan). Cells were grown in alpha minimum essential medium (α-MEM) (Gibco, Grand Island, NY, U.S.A.), and supplemented with 10% fetal bovine serum (FBS, Sanko Junyaku, Tokyo, Japan), 100 μg/ml penicillin and 100 μM/ml streptomycin (FBS-α-MEM) in a 37 °C humidified atmosphere of 5% CO₂. The cells were passaged with 0.05% trypsin and 0.1% ethylenediaminetetraacetic acid tetrasodium salts (EDTA) solution (Gibco Laboratories, Grand Island, NY, U.S.A.).

**Colony Formation Assay**

Two milliliters of MC3T3-E1 cell suspension (50 cells) were seeded into 35 mm tissue culture dishes (Corning, NY, U.S.A.). After 4 h, the media were replaced with 2 ml of culture medium containing various concentrations of each test chemical, and further incubated for 7 d. In another experiment, the cells were seeded into glass dishes coated with 10 mg of PLLA, and incubated for the same period. After incubation, the cell cultures were washed once with PBS, fixed with 10% formalin solution, and stained with Giemsa staining. The number of colonies formed on each dish was recorded.

**Cell Culture for Assays of Cell Activity and for Staining**

MC3T3-E1 cell suspension (5 × 10⁵ cells in 2 ml of culture medium) was added into glass dishes coated with PLLA, and cultured for 2 weeks. The medium was exchanged once a week.

The effects of PDLLA, l-lactide and l-lactic acid on the cells were examined by the addition of each test chemical into the culture. Test chemicals were dissolved in DMSO to a concentration of 50 mg/ml or 5 mg/ml, and sterilized by filtration through a 0.22 μm filter. The chemical solution was then diluted with the culture medium and sonicated without adjustment of pH, just before use. The cells (5 × 10⁴ cells) were added into 35 mm tissue culture plastic dishes (Corning, NY, U.S.A.), and incubated for 4 h in order to attach them to the bottom of the dishes. Then, the media were replaced with 2 ml of culture medium containing 10 or 100 μg/ml of each test chemical, or culture medium containing an equal amount (2 μl) of DMSO (control). The cells were cultured for 2—4 weeks. The medium was changed once a week with a freshly made culture medium containing each chemical at the same concentration.

**Preparation of Cell Lysates for Assay**

Cell lysates were prepared according to the method of Hakeda et al. After removal of the culture medium from the dishes, cells were washed twice with phosphate-buffered saline (PBS). The cells were recovered by trypsinization from the dishes, then washed twice with PBS by centrifugation at 1200 rpm for 5 min. The residues were resuspended in 1 ml of 0.2% Nonidet P-40 solution, and then stored frozen at −20 °C until measurement. After thawing, the cells were sonicated in an ice bath for 2 min using an ultrasonic processor (model VC-50T Sonics & Materials Inc., Danbury, CT, U.S.A.). These cell lysates were used as sample solutions for the measurements of protein, DNA and HYP content and ALP activity.

**Protein and DNA Content**

The protein content of the cell lysates was determined by the method of Lowry et al. using bovine serum albumin (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) as a reference standard. DNA content was determined by fluorescence assay using Hoechst 33258 dye (Wako Pure Chemicals Industries Ltd., Osaka, Japan). Briefly, a 100 μl aliquot of 1 μg/ml Hoechst 33258 solution, 2 ml of PBS and 20 μl of the cell lysate were mixed and kept at room temperature for 30 min. The fluorescence of the reaction solution was then read at an excitation wavelength of 356 nm and an emission wavelength of 458 nm. Calf thymus DNA (Sigma Chemical, St. Louis, MO, U.S.A.) was used as a reference standard.

**Hydroxyproline Content**

To quantify the accumulation of the collagenous matrix deposited by MC3T3-E1 cells, colorimetric analysis of the hydroxyproline (HYP) content of the cell layer was performed. HYP content was determined according to the method of Huszar et al. The chloramine T solution and aldehyde-perchloric acid solution used for the assay were prepared as follows: Chloramine T (sodium N-chloro-p-toluene-sulfonamide) of 1.41 g was dissolved in 10 ml of 1-propanol, then 10 ml of water and 80 ml of pH 6.6 buffer (citric acid monohydrate 25 g, acetic acid 6 ml, sodium acetate trihydrate 60 g, sodium hydroxide 17 g to 500 ml)
were added. Aldehyde-perchloric acid solution was freshly made: fifteen grams of \( p \)-dimethylaminobenzaldehyde was dissolved in 62 ml of 1-propanol, and 26 ml of perchloric acid was added slowly.

Aliquots of cell lysate (100—500 \( \mu l \), not over 10 \( \mu g \) HYP) were pipetted into 2 ml polypropylene tubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and dried in an oven at 100 °C. Fifty microliters of 4 \( \times \) NaOH was added into the tube and the tube was autoclaved at 121 °C for 10 min. After cooling to room temperature, 50 \( \mu l \) of citric acid was added into each tube. One milliliter of chloramine T solution was added into each tube, and the mixture was kept at room temperature for 20 min. After the addition of 1 ml of aldehyde-perchloric acid solution, the tubes were vortexed and incubated in a water bath at 65 °C for 15 min. The absorbance of the reaction solutions obtained was read at 550 nm.

**Alkaline Phosphatase Activity** (ALP) activity was determined by a modification of the methods of Lowry et al.\(^{18}\) and Hakeda et al.\(^{13}\) The reaction mixture consisted of 0.25 ml of 0.1 M carbonate buffer (pH 10.2), 0.25 ml of 4 mm MgCl\(_2\) in buffer, 0.5 ml of 0.2 M p-nitrophenylphosphate solution as a substrate, and an appropriate amount of the cell lysate (50—100 \( \mu l \)). The solution was incubated at 37 °C for 30 min. The enzymatic reaction was stopped by adding 2 ml of 0.25 \( \times \) NaOH, and the absorbance at 410 nm of p-nitrophenol liberated was measured. Calibration curves for ALP activity were made from the absorbance measurement of various concentrations of ALP standard solution (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), which was prepared in a similar manner as above. The enzymatic activity (mU) of the cell lysate was calculated from the standard curve.

**ALP Stain** After incubation, cell cultures were washed twice with PBS, then fixed with 10% formalin solution. The cells were then washed with water and stained with Naphthol AS-MX phosphoric acid disodium salt (Sigma) and a fast red violet LB salt (Sigma) mixture prepared as follows: Ten mg of Naphthol AS-MX was dissolved in 20 ml of 0.056 M 2-amino-2-methylpropanol solution (pH 9.8), then 10 mg of fast red violet LB salt was added with stirring. After 10 min, the insoluble materials were removed by filtration. This staining solution was prepared just before its use. Two milliliters of the solution were added into the culture dish and incubated at room temperature for over 30 min, then rinsed with water.

**Alizarin Red S and von Kossa’s Stain** To determine the mineralization (calcium deposition) of the cell culture, two methods were used. For Alizarin red S stain, after washing and fixing, the cell cultures were covered with an Alizarin red S solution (0.5 g alizarin red S in 50 ml distilled water mixed with 0.1 ml ammonium solution in 100 ml distilled water, pH 6.36—6.40) for 5—10 min.

For von Kossa’s stain, the fixed cultures were stained with 1 ml of 5% AgNO\(_3\) solution at room temperature overnight. After being washed twice with water, the cells were treated with 5% \( \text{Na}_2\text{S}_2\text{O}_3\) solution for 5 min.

**Statistical Analysis** Values of cell activity were expressed as means ± standard deviation (S.D.) of at least three dishes. When comparing different samples, results were considered to be statistically different when \( p < 0.05 \) using Student’s \( t \)-test for unpaired samples.

<table>
<thead>
<tr>
<th>Material</th>
<th>Colony formation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass (control)</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>PLLA (M.W. 270000)</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>PLLA (M.W. 137000)</td>
<td>89 ± 11</td>
</tr>
</tbody>
</table>

Fifty MC3T3-E1 cells were cultured on PLLA films for 7 d, and the colony number formed on each film was counted. Values are mean ± standard deviation (S.D.) of 4 dishes.

**RESULTS**

**Colony Formation** Fifty MC3T3-E1 cells were cultured on the PLLA films for 7 d, and the colony number formed was counted. A small but not significant decrease in colony formation was observed on the PLLA compared with a glass dish (Table 1). The addition of PDLLA, \( l \)-lactide and \( l \)-lactic acid showed cytotoxicity at a concentration of 1000 \( \mu g/ml \), and no colony was formed. At lower concentrations, none of the chemicals affected colony formation (Fig. 1).

**Cell Activity Cultured on PLLA with Various Molecular Weights** There was no morphological change in cells cultured on PLLA for 2 weeks compared with those on a glass dish. Protein, DNA and HYP content and ALP activity for cells cultured on the PLLA (M.W. 270000) or PLLA (M.W. 1370000) were almost similar to those of cells cultured on glass (Fig. 2). No activation of osteoblastic differentiation by these PLLA was observed. In contrast, protein, DNA and HYP contents and ALP activity of cells cultured on PLLA (M.W. 20000) were higher than those on glass (Table 2). The ALP activity per DNA of cells on the PLLA (M.W. 20000) significantly increased.

**Cell Activity Cultured with Low Molecular Weight PDLLA, \( \alpha \)-Lactide and \( \alpha \)-Lactic Acid** MC3T3-E1 cells were cultured with a concentration of 10 or 100 \( \mu g/ml \) of PDLLA (M.W. 5000 and 10000), \( \alpha \)-lactide and \( \alpha \)-lactic acid for 2 weeks, and the cell activities were measured (Table 3). The amount of protein, DNA and HYP of the cells increased by the addition of each chemical. Among these parameters, ALP activity was significantly increased at a concentration of 100 \( \mu g/ml \) of the test chemical. The ALP/DNA value of the cell lysates by culture with PDLLA (M.W. 10000) or PDLLA...
(M.W. 5000) was significantly higher compared with that with t-lactide, t-lactic acid and the control. In comparing the four chemicals, PDLLA (M.W. 10000) had a strongest stimulation effect on the differentiation of MC3T3-E1 cells.

The cultures were stained to examine the presence of ALP in cells. The cells cultured with PDLLA (M.W. 10000 and 5000) were stained in greater concentration than those cultured with t-lactic acid, t-lactide or the control (Fig. 3). After 4 weeks, the cell cultures were stained with an alizarin red S stain or a von Kossa’s stain for evaluating tissue mineralization (calcium deposition). The mineralization in cell culture was particularly enhanced by the addition of PDLLAs, but not by t-lactic acid or t-lactide (Fig. 4).

**Change in Molecular Weight and t-Lactic Acid Release**

To examine the rate of hydrolysis of the test chemicals during incubation, the molecular weight and the amount of t-
lactic acid released was measured. The incubation period was set based on the culture period for 2 weeks on the material. The results of Mw, Mn and Mw/Mn for PLLA determined by GPC were given in Table 4. After incubation in distilled water for 14d, there was little decrease in the molecular weight of PLLA. In the case of incubation with MC3T3-E1 cells in culture medium, the molecular weight of PLLA was dramatically decreased, and a wide molecular weight distribution was shown.

Release of L-lactic acid released from 10 mg of PLLA into aqueous solution was determined following 14d of incubation (Table 5). The maximum value of 11 µg of L-lactic acid was obtained from PLLA (M.W. 20000), and the amount has a tendency to increase with decreasing M.W. of PLLA.

Table 6 showed the L-lactic acid concentration in PDLLAs and L-lactic acid solution after 7d of incubation. The incubation time was decided based on the exposure and medium exchange period in the cell culture. In a 2 ml solution of 100 µg/ml L-lactide, 55 µg/ml L-lactic acid was detected. In contrast, the amount of L-lactic acid in the PDLLA (M.W. 5000 and 10000) solution was small.

Effect of the Presence of Test Chemical on ALP Activ-

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (µg/ml)</th>
<th>L-lactic acid produced (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lactide</td>
<td>10</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>55.0</td>
</tr>
<tr>
<td>PDLLA (M.W. 5000)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.4</td>
</tr>
<tr>
<td>PDLLA (M.W. 10000)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Test chemicals were dissolved in dimethylsulfoxide at a concentration of 50 mg/ml, and then diluted with water to each concentration. Two ml of test chemical solution was added into a glass tube, and incubated at 37°C for 7d. The amount of L-lactic acid produced in the solution was determined. The data were from a single experiment.

<table>
<thead>
<tr>
<th>Material</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA (M.W. 20000)</td>
<td>11.7</td>
<td>11.5</td>
</tr>
<tr>
<td>PLLA (M.W. 270000)</td>
<td>4.7</td>
<td>4.8</td>
</tr>
<tr>
<td>PLLA (M.W. 1370000)</td>
<td>2.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Ten milligrams of PLLA were coated on the bottom of a glass bottle. Two milliliters of distilled water was added into the bottles and incubated at 37°C for 2 weeks. The amount of l-lactic acid released was determined.

Table 4. Change in Molecular Weight and Its Distribution for PLLA after Incubation

<table>
<thead>
<tr>
<th>Material</th>
<th>Before incubation</th>
<th>After immersion in distilled water</th>
<th>After incubation with cells in culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mw</td>
<td>Mn</td>
<td>Mw/Mn</td>
</tr>
<tr>
<td>PLLA (M.W. 270000)</td>
<td>270000</td>
<td>170000</td>
<td>1.59</td>
</tr>
<tr>
<td>PLLA (M.W. 1370000)</td>
<td>1370000</td>
<td>660000</td>
<td>2.08</td>
</tr>
</tbody>
</table>

The bottom of a glass bottle was coated with 10 mg of PLLA. Two ml of distilled water or culture medium with cells was added into the bottle and incubate at 37°C for 2 weeks. Cell culture medium was exchanged once a week, and water in bottles was not exchanged during incubation. After incubation, PLLA specimens were recovered, dried, and then dissolved in chloroform for measurement of molecular weight by gel permeation chromatography. The molecular weight values, including weight average (Mw), number average (Mn), and molecular weight distribution (Mw/Mn) for PLLA were measured according to comparison with the calibration by which was made with polystyrene standard.
showed that the proliferation of fibroblasts and osteosarcoma cells was inhibited by culture on PLLA (M.W. 100000, 240000 and 500000). A slight decrease in colony formation of MC3T3-E1 cells was observed on PLLA (M.W. 270000 and 1370000) compared with glass (Table 1). The early attachment and growth of cells on PLLA were thought to be somewhat lower than that on glass. The protein and DNA content of cells cultured on the PLLA (M.W. 270000 and 1370000) for 2 weeks were almost at the same level as that on the tissue culture plastic dishes or glass dishes (Fig. 2). A decrease in the M.W. of PLLA, from 270000 to 170000, was observed after incubation with cells in culture medium, and about 5 μg of l-lactic acid was released (Tables 4 and 6). These changes in the chemical property of PLLA during incubation did not affect the cell activity. The PLLA with a M.W. of over 200000 provided a suitable substrate for MC3T3-E1 cells, but the stimulation of osteoblast differentiation was thought to be weak.

The ALP activity per DNA of cells cultured on PLLA (M.W. 20000) was higher than those on glass (Fig. 2). In our previous study, the M.W. of PLLA (M.W. 1000000) decreased to below 20000 by heating at 250 °C, and the cells cultured on the heated PLLA showed high ALP activity and produced a large amount of HYP. Otto et al. also reported that PLLA (M.W. 20000) conversely increased ALP activity, and stimulated osteoblast differentiation in vitro. Our results in this study corresponded to them. The maximum release of l-lactic acid from 10 mg PLLA (M.W. 20000) into aqueous solution was lower than 12 μg after 2 weeks incubation (Table 6). As shown in Table 3, l-lactic acid induced increases in the amount of protein, DNA and HYP produced, and ALP activity at a concentration of 100 μg/ml, but not at 10 μg/ml. Therefore, the concentrations of l-lactic acid detected were not able to affect osteoblast differentiation. From these results, the cell activation on PLLA (M.W. 20000) was apparently due to PLLA itself but not to l-lactic acid. The release rate of degradation products from crystalline and amorphous substrates is different, and these products affect cell behavior. The degree of crystallinity of PLLA is thought to be considerably different between M.W. 1000000 and 20000, so a similar influence may be applicable for osteoblasts. Park and Cima reported that the morphology and differentiated function of hepatocytes or fibroblasts were affected by the crystallinity of PLLA. In any case, low M.W. PLLA is important in the stimulation of bone formation. Mechanical strength decreases with decreasing M.W. of the fabricated devices. In order to produce useful orthopedic devices that stimulate bone formation, it is needed to make the M.W. of PLLA lower only on the surface. For example, electron beam irradiation may be effective.

We then examined the effect of PDLLA with various molecular weights on MC3T3-E1 cell differentiation as an experiment related to PLLA characterization. Since large M.W. PDLLA is not generally produced, we used low M.W. PDLLA in the study. The PDLLA was suspended into the culture medium and exposed to cells, as well as to l-lactide and l-lactic acid. Compared with the four chemicals, PDLLA (M.W. 10000) had the strongest effect on cell response, increase in ALP production and mineralization (Table 3, Figs. 3, 4). Hydrolysis of PDLLA to l-lactic acid during incubation was slight (Table 5). These results mean that low M.W.

**DISCUSSION**

If PLLA stimulates bone formation, the products made with this material will be useful as a device of bone fixation. There are similar reports on bone formation around PLLA devices in vivo. The PLLA specimens were molded by melt-pressed or hot extrusion, and sterilized after polymerization; the M.W. of PLLA used for each experiment was different. In a previous study, we reported that low M.W. PLLA produced by heat degradation induced osteoblast differentiation. However, it was not clear yet what range of M.W. of PLLA stimulates osteoblast differentiation. In this study, we further examined the effects of three PLLA with high, middle and low M.W. of 1370000, 270000 and 20000, respectively, on MC3T3-E1 cells activity in vitro.

Ishaug et al. reported that the attachment of rat calvarial cells cultured on PLLA films (M.W. 95800) was similar to those of tissue culture polystyrene. Van Sliedregt et al.
PDLLAs themselves enhanced bone formation.

Osteoporosis usually strikes old people. Estrogen is used for the treatment of this disease, but it is distributed to other tissues besides the bone, causing several side effects.\(^{20}\) Drug delivery systems to targeted tissues have been developed to decrease the side effects of a drug. Kasugai et al. indicated that a small peptide conjugate which has an affinity to hydroxyapatite (a major inorganic component in hard tissues) is effective for selective drug delivery to bone.\(^{21}\) Yoshioka et al. reported that the release profile of progesterone from PDLLA was easily altered by gamma-irradiation.\(^{22}\) Because PDLLA itself has a stimulatory effect on bone formation, if the surface of PDLLA is treated so as to selectively bind to bone, PDLLA is thought be an effective carrier for drug delivery to bone.

It is unknown why low M.W. PDLLA stimulates MC3T3-E1 cell differentiation stronger than l-lactic acid or \(\alpha\)-lactide. We expected that each chemical might not only increase the production of ALP, but also affect the stability of ALP produced by cells. With a cell free condition, the activity of ALP standard solution was decreased to about 47% of the initial activity after 24 h incubation. The presence of \(\alpha\)-lactic acid and \(\alpha\)-lactide could not inhibit the decrease, but PDLLA could maintain the initial activity (Fig. 5). The persistence of ALP activity may be one reason the PDLLA has a higher differentiation potential compared with \(\alpha\)-lactic acid and \(\alpha\)-lactide.

In the present study, we examined the effects of PLLA and PDLLA with various M.W. on the proliferation and differentiation of osteoblast-like MC3T3-E1 cells. The increase in osteoblast differentiation was caused by PLLA and PDLLA with low M.W. of 5000—20000. PLLA and PDLLA are already used as medical devices, but the activities of these materials observed could lead to a new therapeutic method in orthopedic surgery.

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