CELL GROWTH ON BIODEGRADABLE POLY(DEPSIPEPTIDE-CO-LACTIDE) MATRIX RELEASING GROWTH FACTORS AS SCAFFOLD FOR TISSUE ENGINEERING

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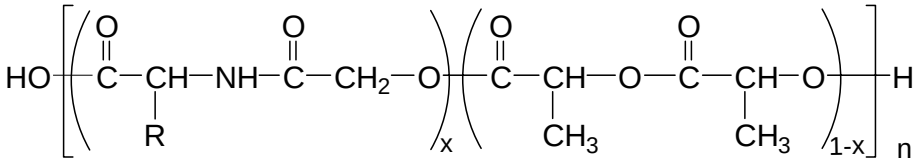
Introduction

The regeneration of damaged or lost tissue by tissue engineering has become an area of intense interest in biomedical materials science. Guided tissue regeneration (GTR) is an approach whereby cells are cultivated on temporary scaffolds at the site of damaged or lost tissue as a means of regeneration.[1] For this purpose, matrices with an appropriate biodegradation profile, excellent biocompatibility, cell attachment and safe degradation products are strongly desired. Recent studies have provided evidences that some growth factors improve the early healing process and regeneration of lost tissue. The biodegradable matrices are desired to show stable entrapment of growth factors, hydrophilic macromolecules, and sustained release of them during degradation process without deactivation of the growth factors.

Poly(α-hydroxy acid), such as polyglycolide (PGA), poly-L-lactide (PLLA) and their copolymers, have been frequently applied as implantable carriers for drug delivery systems and as surgical repair materials due to its good biodegradation properties, relatively high biocompatibility, high mechanical strength, and excellent shaping and molding properties. The degradation rates of poly(lactide-co-glycolide) can be modified by change of composition of lactide and glycolide. However, these polymers have no reactive side-chain groups and it has been difficult to introduce functionality to these polymers by the usual chemical modification methods. Moreover, these copolymers are usually hydrophobic. Such hydrophobic character is not suitable for efficient entrapment of growth factors.

Polydepsipeptides are copolymers containing amino acids and hydroxyl acids. We previously synthesized biodegradable poly(depsipeptide-*co*-lactide) with reactive side-chain groups by ring-opening copolymerization of L-lactide (LA) and cyclodepsipeptide consisting of glycolic acid (Glc) and aspartic acid (Asp) or lysine (Lys) to give poly[(Glc-Asp)-co-LA]: PGDLA and poly[(Glc-Lys)-co-LA]: PGKLA (Figure 1).[2] These poly(depsipeptide-co-lactide) obtained exhibited higher non-enzymatic degradability compared to PLLA, depending on the depsipeptide units content of the copolymer.[2] We also reported the preparation of microspheres with reactive surfaces from the copolymers, the chemical modification of those surfaces, and effective entrapment of hydrophilic drug model by electrostatic interaction.[3] Therefore, these copolymers are good candidates as scaffold for GTR. In fact, we demonstrated the films and sponges prepared from PGDLA and PGKLA showed faster and controllable degradation behavior, and better cell attachment and growth compared with PLLA film and sponges.[4-5]

In this study, we prepared films and sponges from PGDLA and PGKLA entrapping growth factors [epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF)] and model proteins. The release behavior of the growth factors from the matrices and cell growth on the matrices were investigated to evaluate the possibility of these copolymer as scaffold for GTR



R=CH_2COOH: Poly[(Glc-Asp)-*co*-LA]: PGDLA
R=$(CH_2)_4NH_2$: Poly[(Glc-Lys)-*co*-LA]: PGKLA

Figure 1. Structure of PGDLA and PGKLA.

Experimental

Materials

L-Lactide (LA) was supplied by Wako Pure Chemical Co. (Tokyo, Japan). Organic solvents were purified by the usual distillation methods. Mouse fibroblast NCTC clone 929 (L929, IFO50409) cells were obtained from the Health Science Research Resources Bank (HSRRB). Rat neural pheochromocytoma (PC12, JCRB0733) cell were obtained from Japan Collection Research Bioresources (JCRB). Fetal calf serum (FCS) was purchased from JRH Bioscience. Eagle's minimum essential medium (E-MEM) was purchased from Nissui Pharmaceutical Co. Tin 2-ethylhexanoate and other chemicals used were commercial grade and used without purification. Insulin, lysozyme and lactoferrin , EGF, FGF and NGF were also supplied by Wako Pure Chemical Co. (Tokyo, Japan).

Synthesis PGDLA and PGKLA

Cyclodepsipeptides consisting of glycolic acid (Glc) and aspartic acid (Asp) or lysine (Lys), cyclo[Glc-Asp(OBzl)] and cyclo[Glc-Lys(Z)], were synthesized as reported previously.9 The synthesis of PGDLA and PGKLA was carried out by ring-opening copolymerization of LA with the cyclodepsipeptides in bulk using tin 2-ethylhaxanoate as an initiator according to the method reported previously.10 with minor modification as follows. Cyclodepsipeptide, LA and freshly prepared solution of tin 2-ethylhaxanoate in dry tetrahydrofuran (THF) were placed in a glass tube with a stopcock. After removing the THF under vacuum and purging with nitrogen, the tube was sealed in vacuo. The sealed tube was heated at 160 °C for 2 min and then held at 135 °C for 24 h when cyclo[Glc-Asp(OBzl)] was used as a cyclodepsipeptide. In the case of cyclo[Glc-Lys(Z)], the sealed tube was heated at 115 °C and held at that temperature for 24 h. The reaction mixture obtained was dissolved in a small amount of chloroform, and poured into a large quantity of diethyl ether to precipitate PGDLA or PGKLA having protected side-chain groups. The molar ratio of tin 2-ethylhaxanoate to the monomers (cyclodepsipeptide + LA) was adjusted to 1000. The feed ratio of cyclodepsipeptide/LA was 5/95. The deprotection of side-chain groups of PGDLA or PGKLA was carried out by treatment with a mixture of trifluoromethane sulfonic acid, thioanisol and trifluoroacetic acid, or 25% hydrogen bromide/acetic acid solution, respectively, as reported previously,10 to give PGDLA or hydrogen bromide salt of PGKLA. The depsipeptide unit content in the copolymers was estimated from ^{1}H-NMR spectra (JEOL GSX-400) in $CDCl_3$. The molecular weights of the copolymers were estimated by size-exclusion chromatography (SEC) (column, Toso TSK Gel-Multipore HXL-M × 2; eluent, THF; detector, refractive index (RI); standard, polystyrene).

Preparation Protein-Loaded Films and Sponges.

Protein-loaded films were prepared by a W/O emulsion solvent evaporation method. Insulin, lysozyme and lactoferrin were chosen as model proteins for EGF, bFGF and NGF, respectively, considering their molecular weights and isoelectric points and used for release test. Model proteins (4.5mg) were dissolved in ultra pure water (90μL). This aqueous solution was added into chloroform (2.9mL) or benzene (4.96mL) containing polymer [PLLA, PGDLA or PGKLA] (180mg) and sonicated at room temperature with stirring. The sonication was carried out with a bath-type sonicator (Bransonic 220, Yamato) for 5 min and then probe-type sonicator (UD-200, Tomy) at 100W for 5 min. The emulsion obtained in chloroform were cast on a glass dish (ϕ = 30mm), and dried in air overnight at room temperature to obtain films. The emulsion obtained in benzene was freeze-dried after freezing by liquid nitrogen to obtain sponges.

In Vitro Release Behavior of Proteins.

The release behavior of model proteins (insulin, lysozyme, or lactoferrin) from films was investigated in vitro. The films (5×10mm) were soaked in 2.5mL of 1/15M phosphate buffer solution (PBS) (pH = 7.4) and incubated at 37˚C. After certain period, small aliquot of the solution was taken and replaced with an equal volume of fresh buffer solution. Then, the sample solution was investigated by UV spectrometry (281nm) to determine the amount of proteins.

Cell Culture

L929 cells were subcultured in E-MEM with 10% (v/v) FCS with harvesting by [PBS(-)] (Nissui Pharmaceutical Co.) containing 0.025% (w/v) trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA). The medium was exchanged every 3 day.

L929 Cell Proliferation on Growth Factor-Loaded Matrices

Growth factor-loaded films and sponges were prepared by the same method as described above. Cell proliferation was investigated after 1 to 14 days. Films or sponges were placed culture glass dishes. The suspension of L929 cells (2.0 mL, 1.5 x 105 cells/mL) in E-MEM containing 10% FCS was distributed in the glass dishes and cultured in a humidified atmosphere containing 5% CO2 at 37 °C. After incubation for certain period, the films on the glass dishes were rinsed 3 times with 1 mL of E-MEM to remove non-attached cells. The number of cells proliferation on the films was measured by 3-(4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide (MTT) assay. E-MEM (2.19 mL) and aqueous MTT solution (438 mL) were added to the glass dishes, and incubated at 37 ˚C for 4 h. The formazan precipitate was then dissolved by the addition of 2.19 mL of 0.04N-HCl/isoproipanol solution containing 10% Triton-X. Aliquots of the supernatant of the solution in the dishes were transferred into 96-well micro-plates. The absorbance at 630 nm was measured using a microplate reader (MTP-120 Corona Electric Co.).

Results and Discussions

Poly(depsipeptide-co-lactide)s having functional groups, carboxyl or amino groups, were synthesized by copolymerization of LA with cyclodepsipeptide having protected side-chain groups, cyclo[Glc-Asp(OBzl)] or cyclo[Glc-Lys(Z)], and subsequent removal of benzyl (Bzl) or benzyloxycarbonyl (Z) group. The yields of the copolymerizations was over 70%. The yields of the deprotection of the side-chain groups were 70-85%. It was confirmed by SEC analysis before and after the deprotection reaction that no degradation of the polymer main-chain occurred under the deprotection

condition. When the cyclodepsipeptide unit in feed is given by X [cyclodepsipeptide (mol) / total monomer (mol) × 100], the copolymer obtained is expressed by PGDLA(X/(100-X)) or PGKLA(X/(100-X)). The depsipeptide units contents in the copolymers for PGDLA (5/95), PGDLA (10/90), PGKLA (5/95) and PGKLA (10/90) were estimated to be 2.0, 4.2, 2.2 and 5.8 mol%, respectively, from the 1H NMR spectra. The release behaviors of model proteins (insulin, lysozyme, and lactoferrin) from these copolymer and PLLA films and sponges were investigated in PBS (pH = 7.4) at 37˚C. The release rates of the proteins from the PGKLA and PGKLA matrices were faster than those from PLLA. It took about 14 days for releasing 50% of protein from the PGKLA and PGKLA films, and after 28days the release (%) is around 80%. On the other hand, the release (%) of the proteins from PLLA film did not reach 50% after 28 days. These results can be explained by the fact that the biodegradation rates of copolymers were faster than that of PLLA.10 The release behavior of the proteins from sponges showed similar tendency. These results mean these copolymer matrices show the sustained release of the protein for a month. L929 cell growth on the copolymer and PLLA films was investigated after 1 to 14 days. As a control, growth factor-unloaded firms were prepared, and the growth factors were added on these films and sponges when medium was exchanged. Cell proliferations on growth factor-loaded films and sponges were higher than the experiments without growth factors, as well as growth factor added condition. These results mean that the growth factors released from the copolymer films and sponges were not denaturated and kept their activity. These all results show PGDLA and PGKLA are good candidates for scaffold for guided tissue regeneration.

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