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Preparation, characterization and *in vivo* pharmacodynamic evaluation of thymopentin loaded poly(lactide acid)/poly(lactide-*co*-glycolide acid) implants

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ABSTRACT

To avoid the clinical inconvenience of repeated injection of the immune modulator thymopentin (TP5), biodegradable implants comprising a mixed polymer matrix of poly(lactide acid) (PLA) and poly(lactide-*co*-glycolide acid) (PLGA) were produced using a simple extrusion method. Drug release from these TP5-loaded implants was characterized both *in vitro* and *in vivo*. Pharmacodynamic studies were carried out in immunosuppressed rats using the ratio of $CD4^+/CD8^+$ cells, determined by flow cytometry, as an index of immunity. The results indicated that the entrapment efficiency of the implants was greater than 98%, but the release rate of TP5 depended on the drug loading. Implants containing less than 10% TP5 showed consistent release over 30 days, with low burst-release both *in vitro* and *in vivo*. Improved immunity and survival rates were observed in rats treated by TP5 injection and in rats given middle-to-high dose implants. When the release of TP5 exceeded 0.1 mg/kg body weight/day the $CD4^+/CD8^+$ ratios increased in the 3 weeks after implantation, reaching a maximum (91.6% of the normal level) by the end of the third week. The TP5-loaded implants presented here provide a promising alternative to injections and the results support the further development of controlled-release TP5 formulations.

1. Introduction

Thymopentin (TP5) is a small synthetic molecular peptide consisting of five amino acids (H_2N-Arg-Lys-Asp-Val-Tyr-OH). The sequence is identical to that of residues 32–36 in endogenous thymopoietin II and it has a similar biological activity (Goldstein et al., 1979; Goldstein and Audhya, 1985). TP5 is used clinically to regulate abnormal immune responses, and to treat serious infections after surgical operations, type II diabetes mellitus, and various autoimmune diseases (Singh et al., 1998; Gonser et al., 1999). However, it has a very short half-life *in vivo* (Audhya and Goldstein, 1985) and is only available as a lyophilized powder in 1 mg dosages for intramuscular injection or intravenous infusion (usually given every day or every other day). This treatment regime is both inconvenient and stressful for patients, resulting in low compliance.

Many new drug delivery systems, such as oral bio-adhesive nanoparticles and powders for pulmonary delivery, have been proposed to avoid the frequent injection of TP5 (Yin et al., 2006; He et al., 2008; Gao et al., 2009; Wang et al., 2009). Orally administered nanoparticles significantly improve the gastrointestinal absorption of TP5 compared with conventional TP5 solutions (Yin et al., 2006; He et al., 2008). Even though TP5 nanoparticles offer a promising oral delivery system there are still some difficulties to overcome, such as low bioavailability, low entrapment efficiency and substantial levels of burst-release. A few reports have looked at injectable depots, but they only focused on the preparation of biodegradable microspheres (Conti et al., 1997; Bleich and Müller, 1996) and no *in vivo* evaluation of these long-acting formulations was undertaken. TP5 is a small hydrophilic molecule that is soluble in aqueous solutions (solubility > 10 g/l). This high hydrophilicity makes it difficult to trap TP5 in hydrophobic polyester microspheres resulting in low entrapment efficiency. Meanwhile, the initial burst-release from TP5-loaded microspheres is relatively high and the release duration is usually less than 2 weeks. Biodegradable implants represent a better alternative for the delivery of hydrophilic peptides because no aqueous medium is involved in their preparation. Therefore, complete entrapment could easily be achieved. The biodegradable polymers, poly(lactide acid) (PLA) and poly(lactide-*co*-glycolide acid) (PLGA) are biocompatible and provide a reliable matrix for implants (Anderson and Shive, 1997; Eperon et al., 2008; Jahno et al., 2007; Ramchandani and Robinson, 1998). Several long-acting preparations containing luteinizing hormone releasing hormone (LHRH) analogues are already on the market including Decapeptyl® and Zoladex®. To our knowledge, there are no published *in vivo* pharmacodynamic studies of long-acting TP5 preparations. Therefore, it is not known whether the pharmacological function of TP5 would be altered under controlled-release conditions.

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Table 1
Validation of the method for determining the entrapment efficiency of TP5 ($n = 3$). The recovery was tested by adding TP5 to blank implants and then treating them in the same way as the TP5-loaded implants.

Added (μg/ml)	Recovered (μg/ml)	Recovery rate ±SD (%)	Mean recovery rate ±SD (%)
25.22	24.57	97.42 ± 0.54	
98.05	98.11	100.06 ± 1.10	99.10 ± 0.99
251.00	250.52	99.81 ± 1.32	

The aim of this study was to develop and evaluate a long-acting TP5 formulation with high encapsulation and low burst-release. The TP5-loaded biodegradable implants were composed of PLA and PLGA and prepared using an extrusion method. TP5 release from the implants was evaluated *in vitro* at different levels of drug loading and *in vivo* by analyzing the residual drug content. The immunomodulatory effects of TP5 released from these biodegradable implants was evaluated by monitoring the ratio of $CD4^+/CD8^+$ cells in an immunocompromized rat model. These pharmacodynamic data provide the experimental evidence needed to support the continued development of other controlled-release TP5 formulations.

2. Materials and methods

2.1. Materials and animals

Poly (lactic-glycolic acid) (PLGA, 75:25) with a molecular weight (MW) of 60 kDa was a kind gift from Shanghai Modern Pharmaceutical Engineering Research Center (Shanghai, China). Poly (DL-lactide acid) (PLA), MW 30 kDa, was purchased from Shandong Medical Equipments Research Center (Shandong, China). TP5 was purchased from Shanghai Zillion Pharmaceuticals (Shanghai, China). Cyclophosphamide was obtained from Jiangsu Hengrui Medicine Co. Ltd. (Zhejiang, China). Red blood cell lysis buffer was purchased from BD Biosciences (San Jose, USA). PE-conjugated anti-rat CD8a and FITC-conjugated anti-rat CD4 were purchased from BioLegend (San Diego, USA). Bovine serum albumin (BSA) was purchased from Shanghai Bio Life Science & Technology Co. Ltd. (Shanghai, China). Tween 80 was supplied by Hong Kong Farco Chemical (Hong Kong, China). All other reagents were of analytical or chromatographic grade.

Male Sprague-Dawley rats (250 ± 10 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and kept in a temperature-controlled room at clean level with a 12 h light–dark cycle. Food and tap water were provided *ad libitum*. All animal experiments were approved by the Animal Ethical Committee of Fudan University and complied with the guideline for Care and Use of Laboratory Animals.

2.2. Preparation of biodegradable TP5 implants

The biodegradable implants were prepared using a simple extrusion method. Briefly, TP5 microparticles (mean particle size = 5 μm) were obtained using an air flow pulverizer (Jetpharma CH-6828, Switzerland) and dispersed in 2 ml of methylene chloride solution containing a mixture (400 mg/ml) of PLGA and PLA (the w/w ratio of PLGA to PLA was 1:3 for all the formulations). The micronized TP5 was homogeneously distributed throughout the polymer solution by magnetic agitation. The methylene chloride was then allowed to evaporate at ambient temperature until a paste was obtained. The resulting PLGA/PLA-TP5 mixture was extruded from a home-made extruder and any residual organic solvent was removed from the cylindrical rods under vacuum at room temperature for 48 h. The material was then cut into implantable rods (2 mm in diameter and 10 mm in length, and weighing about 27 mg). Finally, the implants were sterilized using ultraviolet radiation at 254 nm for 3 h before implantation. After sterilization, no TP5 degradation was observed, which was determined by HPLC as described below.

2.3. Determination of entrapment efficiency

The amount of TP5 trapped within the PLGA/PLA implants was determined using an extraction method. Briefly, the TP5-loaded implants (each weighing 10 mg) were dissolved in 0.5 ml methylene chloride and the TP5 extracted into 4 ml of phosphate buffer solution (50 mmol/l, pH 7.0) by vortexing for 1 min. The supernatant was filtered through a 0.22 μm microporous membrane, and a 20 μl aliquot injected onto a high performance liquid chromatography column (HPLC, Agilent 1100 series, Waldbronn, Germany). A C18 column (Dikma Diamonsil, Beijing, China; 4.6 mm × 200 mm; 5 μm) was used with a mobile phase consisting of 50 mmol/l PBS (pH 7.0) and methanol (85:15) and at a detection wavelength of 275 nm. The flow rate was 1 ml/min. Coefficients of variation for intra-run and inter-run of TP5 determination were 0.81% and 0.98%, respectively, and limit of quantification (LOQ) was lower than 10 μg/ml at a signal-to-noise level of 10. The entrapment efficiency of the implant was calculated relative to its weight by comparing the total amount of TP5 used to prepare the implant with the amount present in the aqueous supernatant.

This method of recovery was tested by adding differing amounts of TP5 to blank implants (each weighing 10 mg) and then extracting it. Recovery rates ranged from 97.42% to 100.06% and the results are shown in Table 1.

2.4. In vitro release studies

Implants were placed in 1.5 ml Eppendorf tubes and 1 ml of PBS (50 mmol/l, pH 7.4) containing 0.02% Tween-80 and 0.02% NaN_3 was added (release medium). The tubes were incubated at 37 °C for 4 weeks. The release medium was replaced by fresh buffer every day for the first 5 days for the implants loaded with 10% and 25% TP5 (or every other day for the implants loaded with 1% TP5) and then every 5 days until more than 90% of the encapsulated TP5 was released. The TP5 concentration in the medium was determined by HPLC as described above.

2.5. In vivo release studies

Implants formulated using PLGA/PLA at a ratio of 1:3 (w/w) and a theoretical drug loading of 1% were selected for the *in vivo* release studies. Twelve male rats were randomly divided into six groups and 1% TP5 implants sited subcutaneously (two implants per rat, equal to a dosage of 0.536 mg TP5; 2.144 mg/kg). Briefly, rats were anesthetized by intraperitoneal injection of 30 mg/kg pentobarbital sodium, and two separate 1 cm incisions made in the left and right dorsal areas. Subsequently, a 1% TP5 implant was embedded into the subcutaneous tissue and the incision was sutured. The animals were allowed to recover in a heated chamber and then kept in their home cages until they were sacrificed. The implant residues were removed at the designated time points (1, 3, 10, 15, 20 and 28 days), immediately washed in saline solution and the fibrous tissues wrapped around them removed. After desiccation, the TP5

content of the implants was determined and calculated according to the method described above. The amount of TP5 released from the implants was calculated using the following equation:

$$\textit{In vivo}\ \text{drug release percent}\,(\%) = \left(1 - \frac{\text{amount of TP5 in the residue}}{\text{amount of TP5 in the original implant}}\right) \times 100\%$$

2.6. Scanning electron microscopy

Morphologies on the cross-section of the implants were imaged by scanning electron microscope (TESCAN 5136MM, Czech Republic). The implants were solidified in liquid nitrogen and then cut off with a scalpel. Prior to image scanning, the samples were coated with gold vapor under argon atmosphere.

2.7. In vivo pharmacodynamic studies

2.7.1. Implant administration

Twenty-five male rats were randomly divided into five groups for the pharmacodynamic study. The $CD4^+/CD8^+$ ratio in the peripheral blood of each rat was determined as an "internal control" before implantation. The rats were immunosuppressed by intraperitoneal injection of cyclophosphamide (CTX) at a dose of 50 mg/kg every 5 days. After 10 days of CTX treatment the $CD4^+/CD8^+$ ratio was determined to confirm immunosuppression. The first group of rats was subcutaneously injected every day for 4 weeks with TP5 solution at a dose of 0.072 mg/kg/day. In the second group each rat was given one blank dorsal implant as described above. The other groups were given one to three 1% TP5-loaded implants as follows: group 3 was given one 1% TP5 implant (1.072 mg/kg, low dose), group 4 was given two 1% TP5 implants (2.144 mg/kg, middle dose), and group 5 was given three 1% TP5 implants (3.216 mg/kg, high dose). Throughout the whole experimental period the rats were intraperitoneally injected with CTX every 5 days. A 200 μl aliquot of blood was collected into a heparinized tube via the caudal vein 7, 14, 21, and 28 days after implantation and stored in 4 °C. All the samples were analyzed by flow cytometry within 12 h.

2.7.2. Flow cytometric analysis of peripheral blood

The lymphocyte populations in the peripheral blood were analyzed using dual-color flow cytometry. Blood samples (100 μl) were washed in PBS by centrifugation at 1500 rpm for 10 min and then mixed with 10 μl PE-conjugated anti-rat CD8a and 10 μl FITC-conjugated anti-rat CD4. After incubation in the dark at room temperature for 30 min, 500 μl of red blood cell lysis buffer was added into the blood sample (1:10 dilution), incubated in the dark at room temperature for 10 min, and washed twice with PBS by centrifugation at 1500 rpm for 10 min. The sample was stained with polyoxymethylene and analyzed using a FACScan flow cytometer (FACSCalibur, Becton Dickinson, USA) equipped with an argon laser tuned to 488 nm. More than 1×10^4 cells were collected for each sample. Data were analyzed using CELLQuest software and represented as dual-parameter density plots (Fig. 1). The $CD4^+/CD8^+$ ratios were obtained by calculating the respective amounts of the labeled $CD4^+$ and $CD8^+$ in the blood samples, and were used to evaluate the effectiveness of the TP5 released from the implants. The mean values ± standard deviations (SD) of the recorded results were used to compare the pharmacodynamic effects of the TP5 formulations. A Student's *t*-test was used to calculate the *p* values for statistical significance: a *p*-value of <0.05 was regarded as significant and a *p*-value of <0.01 as highly significant.

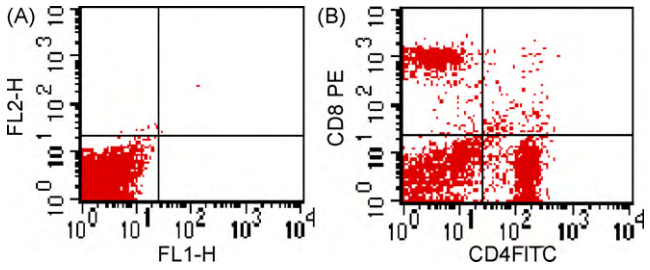

Fig. 1. A typical dual-parameter flow cytometry histogram from a rat blood sample: (A) control blood. (B) Peripheral blood incubated with FITC-conjugated CD4 and PE-conjugated CD8a antibodies.

3. Results

3.1. Biodegradable implants

The extrusion technique yielded cylinder-shaped implants with smooth surfaces. Scanning electron micrographs showed that the implants had a dense inner structure (Fig. 2). Micronized TP5 was evenly distributed throughout implant (arrow a) and many micropores, which may have formed during the evaporation of the methylene chloride, were evident in the solidified polymer matrix (arrow b).

The biodegradable TP5 implants were loaded with differing amounts of drug ranging from 1% to 25%, and the entrapment efficiencies are shown in Table 2. The entrapment efficiency of TP5 was greater than 98% in all cases, and there was no significant difference in the entrapment efficiency as drug loading increased. These results indicate that extrusion is a feasible approach to the preparation of a biodegradable implant with improved entrapment efficiency for hydrophilic peptides without destroying their structure.

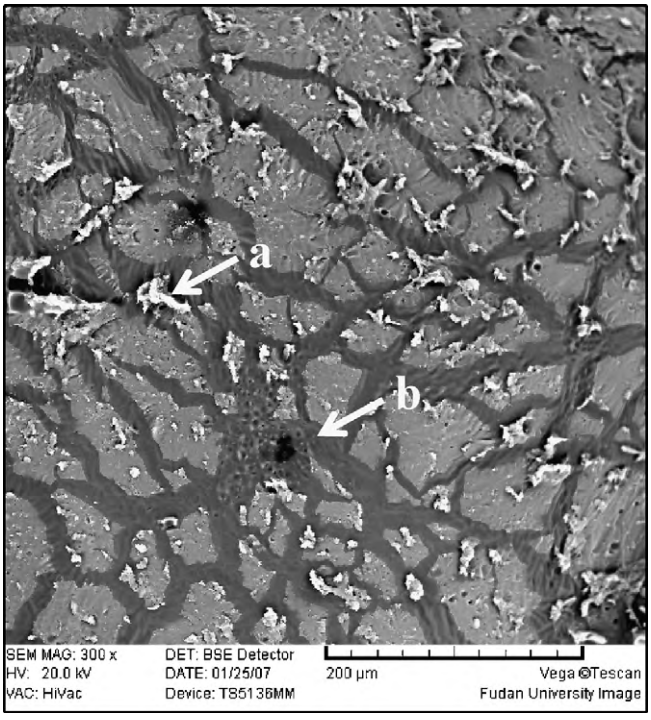

Fig. 2. Scanning electronic micrograph showing a cross-section of the implant containing 1% TP5 (magnification ×300). The entire scale bar represents a distance of 200 μm. Both the micronized TP5 within the implant and the micropores formed during solvent evaporation are indicated by arrows (a and b, respectively).

Table 2
Entrapment efficiency of the TP5 implants with different drug loadings (mean ± SD, $n = 3$).

Formulation	1% TP5 implant[a]	10% TP5 implant	25% TP5 implant
PLGA (MW 60 kDa) (%, w/w)	24.75%	22.5%	18.75%
PLA (MW 30 kDa) (%, w/w)	74.25%	67.5%	56.25%
Theoretic loading (%, w/w)	1%	10%	25%
Actual loading (μg TP5/mg implant)	10.1 ± 2.2	106.5 ± 5.0	239.0 ± 4.5
Entrapment efficiency (%)	98.9 ± 1.0	99.4 ± 0.3	99.3 ± 0.2

[a] Actual loading and entrapment efficiency of 1% TP5 implants were calculated based on scale-up batches, which were prepared according to the process as described in Section 2.2. The obtained implants were used in the *ex/in vivo* release and pharmacodynamic studies.

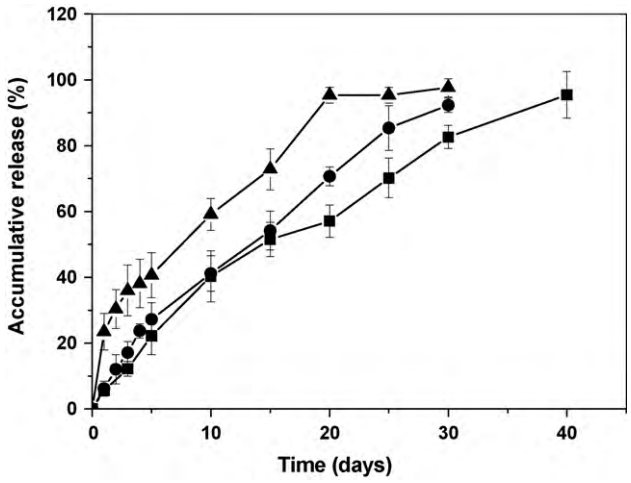

Fig. 3. Effect of TP5 loadings on *in vitro* drug release by the TP5 implants ($n = 3$). Drug loadings for the TP5 implants were 1% (-■-), 10% (-●-), and 25% (-▲-), respectively.

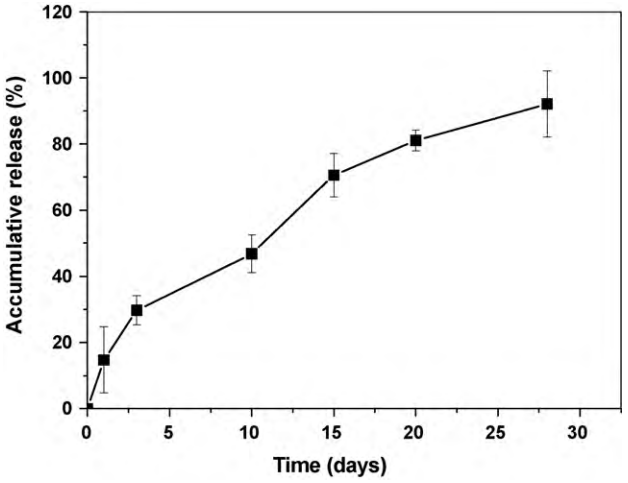

Fig. 4. *In vivo* drug release profiles of the implant containing 1% TP5 ($n \geq 3$).

3.2. In vitro release

Fig. 3 shows the *in vitro* cumulative release profiles obtained from TP5 implants with different drug loadings expressed as the percentage of TP5 released with respect to the total amount of TP5 entrapped. A burst-release (23.42%) is evident from the release curve of the implant containing 25% TP5 on the first day, followed by a slow, continuous release over the next 20 days. The initial release from the implant with the lower drug-loadings was significantly less than that of highest drug-loading (6.17% for the 10% TP5 implant and 5.61% for the 1% TP5 implant). The release of intact TP5 from both formulations lasted for a period of 30 or 40 days, during which no pulse-release phase was observed, and both cases fitted zero-order release kinetics ($r_{10\%} = 0.9916$ and $r_{1\%} = 0.9833$). The release rates of TP5 from the implants with 1% and 10% drug loadings were about 7.8 μg/day and 88.4 μg/day, respectively in the period of time of zero-order release kinetics. These results suggest that both the intensity and duration of TP5 release from the implants correlates with the level of drug loading.

3.3. In vivo release

To gain further insight into the *in vivo* performance of the implants, the formulation containing 1% TP5 (which contained the equivalent amount of TP5 as a daily injection for 1-month) was used in the *in vivo* release experiments and subsequent pharmacodynamic studies. The cumulative amount of TP5 released on the first day was 14.77%, which was double that seen in the *in vitro* release studies. Fig. 4 shows a near-consistent release over the following 4 weeks with no obviously second pulse-release phase. More than 95% of the TP5 was released from the implant within 1-month. After 3 days of implantation, the implants became wrapped in fibrous tis-

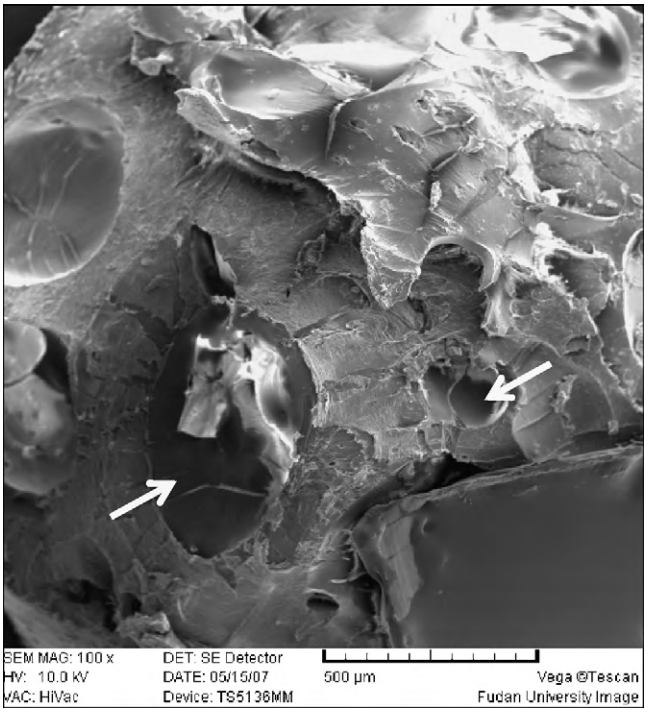

Fig. 5. Scanning electronic micrograph showing a cross-section of the recovered implant containing 1% TP5 after 28 days of *in vivo* release (magnification ×100). The entire scale bar represents a distance of 500 μm. The arrows indicate the expanded pores inside the implant due to the degradation of the polymer matrix.

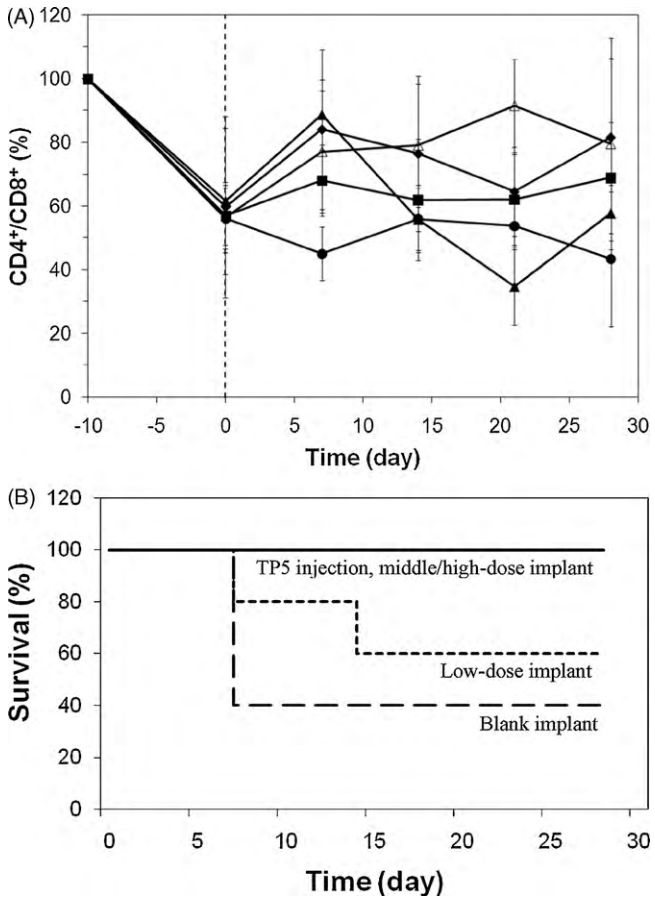

Fig. 6. Effects of TP5 on immunity after administration to rats ($n = 5$). Rats were immunosuppressed by intraperitoneal injection of CTX at a dose of 50 mg/kg every 5 days. (A) The $CD4^+/CD8^+$ ratio in peripheral blood varied as a function of time. -■-: TP5 injection, -●-: blank implant, -▲-: low-dose implant (1.072 mg/kg TP5), -♦-: middle-dose implant (2.144 mg/kg TP5), and -Δ-: high-dose implant (3.216 mg/kg TP5). (B) Survival curve of rats after implantation. Solid line = TP5 injection, middle- and high-dose implants; dotted line = low-dose implant; dashed line = blank implant.

sue, and these fibrous capsules became thicker over time. Both the shape and appearance of the implants remained intact after 28 days, however, they swelled slightly and became more porous due to the degradation of the polymer matrix. Fig. 5 shows a cross-section from an implant recovered after 1-month. The electron micrograph clearly shows the breakdown in the polymer structure, resulting in increased porosity.

3.4. Pharmacodynamics

After two intraperitoneal injections of CTX, the $CD4^+/CD8^+$ ratio in the peripheral blood decreased significantly (to 57.1%, 56.0%, 61.6%, 59.8%, and 56.7% of normal levels in groups 1–5, respectively) indicating immunosuppression.

Both the $CD4^+/CD8^+$ ratio and the survival rates of the rats varied as a function of time and are summarized in Fig. 6. After the blank implants had been embedded for 1-week, the $CD4^+/CD8^+$ value was decreased by approximately 11% and three animals died. During the following 3 weeks, the $CD4^+/CD8^+$ ratio in this group remained low, ranging from 56% to 43% of the normal value. In contrast, the $CD4^+/CD8^+$ ratio in the TP5-treated groups, including the injection (group 1) and implant groups (groups 3–5), had increased by the end of the first week (by 11.0%, 27.2%, 24.3% and 20.5%, respectively). The $CD4^+/CD8^+$ ratio in the low-dose implant group (group 3) increased at the beginning of the experiment, but then rapidly decreased. By the end of the third week a difference of up to 27.3% was seen in the $CD4^+/CD8^+$ ratio between the low-dose implant group and the injection group suggesting that 1 TP5 implant had only a limited effect on immunity. One animal in the low-dose group died in the first week of the experiment and another died during the second week. By the end of the first week a significant ($p < 0.05$) increase in the $CD4^+/CD8^+$ ratio was seen in both the injection group and the group implanted with high-dose TP5 (group 5). Moreover, the $CD4^+/CD8^+$ ratio in groups 4 and 5 was also higher than that in the injection group. The $CD4^+/CD8^+$ ratio in group 5 continued to increase, and was significantly higher at the end of second and third weeks than before implantation ($p < 0.05$ and $p < 0.001$, respectively), reaching a maximum value of 91.6% of the normal level. No animals in the injection group or groups 4 and 5 died during the pharmacodynamic study.

4. Discussion

As a synthetic pentapeptide, TP5 is a promising immune modulator currently used in the clinic to treat autoimmune and immune deficiency diseases that require repetitive administration via injection. To improve both therapeutic quality and patients' compliance, many studies have focused on the development of novel TP5 formulations, such as sustained-release biodegradable microspheres and orally deliverable nanoparticles. However, the low entrapment efficiency coupled with rapid drug release *in vitro*, mainly due to the small molecular size and high hydrophilicity of TP5, has been problematic. To obtain a slow and sustained release of TP5, we used a simple extrusion method to produce implants. In our previous studies, it is found that the release of a hydrophilic, small molecule from biodegradable implants composed of PLGA only completed in less than 2 weeks with significant bursts. However, the release was slowed down when PLA was added into the formula, and a zero-order release was observed when the ratio of PLGA to PLA raised to 1:3 (w/w) (unpublished data). Therefore, in this work we used a mixture of PLGA and PLA at a ratio of 1:3 (w/w) as the matrix of biodegradable implants. Since no aqueous phase was involved in the preparation process, the entrapment efficiency of TP5 within the implants was much higher (>98%) than that of microspheres and nanoparticles. The entrapment efficiency of TP5 within PLGA microspheres and nanoparticles prepared using the classic double-emulsion method is reported to be less than 85% (Conti et al., 1997) and 30% (Yin et al., 2006), respectively. Although the entrapment efficiency of the implants in the present study was not affected by drug loadings ranging from 1% to 25%, the rate of *in vitro* release did increase in parallel with the drug loading. The initial release from the 25% TP5 implant reached 23.42% within the 1 day, much higher than that from the 10% or 1% TP5 implants. The rapid release observed with higher drug loading could be attributed to the existence of hydrophilic channels (shown in Fig. 2) within the PLA/PLGA matrix (Zhou et al., 1998). As these channels are filled with hydrophilic peptide and expand as the drug loading is increased, the entrapped TP5 could easily diffuse out of the polymer matrix. This may explain why the 25% TP5 implant exhibited a rapid release accompanied by a significantly increased burst-dose.

Since the half-life of TP5 is short (approximately 30 s) (Audhya and Goldstein, 1985) it is difficult to quantify the plasma drug concentration *in vivo*. Therefore, the amount of residual TP5 within the implants was determined after implantation and used to evaluate the amount of *in vivo* release. The dose of TP5 used in the *in vivo* release study was equivalent to a clinical adult dose of 30 mg per 70 kg bodyweight and was calculated based on the body surface area of humans and rats. Figs. 3 and 4 show that as the amount of TP5 initially released *in vivo* increased as the duration of release shortened. This is consistent with the results from a study

of biodegradable drug delivery systems (Negrín et al., 2004) and is usually attributed to the *in vivo* attachment of lipids and other physiological active substances to the hydrophobic polymer surface, which accelerates the penetration of moisture and causes the biodegradable matrix to swell (Menei et al., 1993; Tracy et al., 1999). Under this situation, TP5 was rapidly released from the interior of the implants. Additionally, immune responses at the implantation site and the acidic microenvironment caused by the degradation products may also increase the rate of *in vivo* degradation and TP5 release by the implants (Ali et al., 1994).

PLA and PLGA are biocompatible materials approved by U.S. Food and Drug Administration (FDA). They are widely used in the preparation of biodegradable microspheres and implants, and are proved to be safe by clinical application. Furthermore, formation of fibrous capsule (fibrosis) is a common phenomenon of chronic inflammatory response after the microspheres or implants were administrated (Anderson and Shive, 1997), so we did not perform histological evaluation on the surrounding tissues during the *in vivo* release studies.

The amino acid sequence of TP5 is identical to that of the active motif of naturally secreted thymopoietin. It also has identical immune-modulating properties. TP5 was reported to be safe at relatively high doses (Friedmann, 1985). TP5 enhances the production of thymic T cells and may help to restore immunity in immunosuppressed individuals (Singh et al., 1998). Cellular immunity in mammals is mediated by CD4 and CD8, both of which are expressed at high levels on the surface of the peripheral blood T lymphocytes (Janeway, 1992). The immune state of both humans and rats is indicated by the ratio of $CD4^+/CD8^+$ T cells; a lower ratio implies impaired immunity (Veys et al., 1981; Afeltra et al., 1991). Therefore, the $CD4^+/CD8^+$ ratio was chosen as the index for *in vivo* pharmacodynamic studies (Yin et al., 2006). Multicolor flow cytometry is the method of choice for the quantitative detection of $CD4^+$ and $CD8^+$ cells in peripheral blood because of its high sensitivity and precision (Islam et al., 1995). The ratio of $CD4^+$ to $CD8^+$ cells, as well as their absolute values, can be obtained directly from the dual-parameter histograms (Fig. 1).

The effect of the biodegradable implants on immunity was evaluated by treating control rats with blank implants. Three out of the five rats in this group died after implantation. The $CD4^+/CD8^+$ ratio in the surviving rats continued to decrease during the first week after implantation but increased in the following week, returning to pre-implantation levels. This suggests that biodegradable implants composed of PLA and PLGA cause a degree of immunosuppression during the initial period after implantation. Notably, this impaired immunity was restored within 2 weeks. The $CD4^+/CD8^+$ ratio in the groups treated with the various TP5 formulations (injections and implants) increased during the first week. The increase in $CD4^+/CD8^+$ ratio seen in the first week was followed by a persistent decrease over the following 2 weeks in both the low- and middle-dose implant groups, indicating that the amount of TP5 released (less than 0.1 mg/kg body weight per day) was not sufficient to consistently evoke a positive immunological effect. In other words, these implants failed to improve immune function over the 1-month period. In contrast, an increased $CD4^+/CD8^+$ ratio was maintained for 3 weeks post-implantation in the high-dose group (group 5). The survival rates of the rats correlated with the $CD4^+/CD8^+$ data, with death only being recorded in the low-dose group (group 3; 1 death in Week 1 and another in Week 2). This clearly illustrates the immunomodulatory effect of the TP5 implants. The results of these pharmacodynamic experiments may be useful in the design of other long-acting TP5 delivery systems, such as biodegradable microspheres.

In conclusion, biodegradable TP5 implants were produced using a simple extrusion method to provide an alternative delivery system for TP5. Highly hydrophilic peptides, like TP5, are theoretically very difficult to encapsulate into biodegradable microparticles. However, our implants had high entrapment efficiency, which is crucial for the development of a long-lasting formulation. When the drug loading was less than 10%, the implants showed a low burst-release both *in vitro* and *in vivo*, followed by a constant, steady release over 30 days. Pharmacodynamic evaluation of the implants in immunosuppressed rats showed that, compared with conventional TP5 injections, the implants containing higher doses were more effective in reconstituting impaired immune responses. These results show that it is feasible to design a controlled-release delivery system for TP5 for the treatment of immunodeficiency diseases.

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