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In vivo efficacy of paclitaxel-loaded injectable in situ-forming gel against subcutaneous tumor growth

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ABSTRACT

Injectable in situ-forming gels have received considerable attention as localized drug delivery systems. Here, we examined a poly(ethylene glycol)–b-polycaprolactone (MPEG–PCL) diblock copolymer gel as an injectable drug depot for paclitaxel (Ptx). The copolymer solution remained liquid at room temperature and rapidly gelled in vivo at body temperature. In vitro experiments showed that Ptx was released from MPEG–PCL copolymer gels over the course of more than 14 days. Experiments employing intratumoral injection of saline (control), gel-only, Taxol, or Ptx-loaded gel into mice bearing B16F10 tumor xenografts showed that Ptx-loaded gel inhibited the growth of B16F10 tumors more effectively than did saline or gel alone. Further, intratumoral injection of Ptx-loaded gel was more efficacious in inhibiting the growth of B16F10 tumor over 10 days than was injection of Taxol. A histological analysis demonstrated an increase in necrotic tissue in tumors treated with Ptx-loaded gel. In conclusion, our data show that intratumoral injection of Ptx-loaded MPEG–PCL diblock copolymer yielded an in situ-forming gel that exhibited controlled Ptx release profile, and that was effective in treating localized solid tumors.

1. Introduction

During the course of cancer treatment, almost all patients with cancer receive some form of surgery to remove as much of the tumor as possible (Benotti and Steele, 1992). However, the risk of recurrence stemming from residual cancer cells remains, and may be averted through administration of local radiotherapy or systemic chemotherapy. In systemic chemotherapy, the antitumor activity of anticancer drugs may be enhanced by changing the manner of drug administration, with particular focus on direct intratumoral injection (Goldberg et al., 2002; Ta et al., 2008; Rossi et al., 2003). Such targeted delivery would be expected to provide a high local concentration of the anticancer drug, reducing systemic drug levels and thereby decreasing the incidence of side-effects commonly observed with systemic therapy (Chvapil, 2005).

Paclitaxel (Ptx) is one of the most effective naturally occurring antineoplastic drugs discovered in recent decades (Spencer and Faulds, 1994). The drug interacts with tubulin dimers in the G2 phase of the mitotic cell cycle to promote microtubule polymerization; the resulting formation of highly stable microtubules prevents cell division and accounts for the cytotoxic properties of Ptx (Singh et al., 2008). Ptx has excellent therapeutic efficacy against a variety of solid tumors, including breast cancer, ovarian carcinoma, lung cancer, head-and-neck carcinoma, and acute leukemia (de Bree et al., 2006; Singla et al., 2002; Fjällskog et al., 1993). However, Ptx is a hydrophobic molecule that is poorly soluble in water. Currently, the only commercial available formulation in clinical use is Taxol, which consists of a solution of Ptx prepared using a mixture of the polyethoxylated castor oil Cremophor EL, and ethanol (Terwogt et al., 1997; Chun et al., 2009; Ding et al., 2005). Taxol must be repeatedly administered and thus causes serious side-effects, particularly hypersensitivity reactions, some of which are life-threatening. Efforts to eliminate these problems have focused on developing new drug delivery systems for Ptx that achieve site-specific delivery, prolong action periods, and improve patient compliance (Cheng et al., 2007).

During the last decade, injectable in situ-forming gels have attracted considerable attention as polymeric drug carriers (Vintiloiu and Leroux, 2008; Park et al., 2008). Among the advantages of such gels are their ability to conform to any shape at a specific site, and the fact that they can be introduced using minimally invasive non-surgical procedures. Several block copolymers consisting of polyethylene glycol (PEG) and biodegradable polyesters, such as poly(L-lactic acid) (PLLA), poly(glycolic acid)

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(PGA), or their copolyesters (PLGA), and the pluronic series, have been prepared and examined as candidate injectable in situ-forming gels (Sasatsu et al., 2008; Ong et al., 2009; Bajpai et al., 2008).

Recently, we reported on a novel biodegradable diblock copolymer, MPEG–PCL, formed from PEG and poly(caprolactone) (Ahn et al., 2009; Hyun et al., 2007; Kim et al., 2006a,b). MPEG–PCL copolymer solutions exhibited sol-to-gel transitions at body temperature. In addition, we found that the subcutaneous injection of a MPEG–PCL copolymer solution resulted in in situ gel formation without inside-needle gelation. It is suitable for long-term drug delivery system because of its low degradation rate. Over the course of a series of investigations, our laboratory has formulated an injectable in situ-forming MPEG–PCL gel for various biomedical applications.

The overall aim of the current study was to develop a simple and generally applicable intratumoral injection strategy for systemic chemotherapy using Ptx. Accordingly, we sought to evaluate an injectable in situ-forming gel prepared with MPEG–PCL diblock copolymer in the context of the following specific questions: (1) does the MPEG–PCL diblock copolymer act as a suitable drug depot for Ptx in vitro and in vivo? (2) Does intratumoral injection of MPEG–PCL result in in vivo gel formation? (3) Do Ptx-loaded MPEG–PCL gels exert a growth inhibitory effect against tumors? Resolving these issues will have a significant impact on the application of intratumoral injections for systemic chemotherapy with Ptx, holding the promise of achieving prolonged action periods and thus facilitating patient compliance and comfort.

2. Materials and methods

2.1. Synthesis of MPEG–PCL diblock copolymers

MPEG–PCL diblock copolymer (750–2400) was prepared using a block copolymerization method reported previously (Kim et al., 2006a,b).

2.2. Viscosity measurements

The MPEG–PCL diblock copolymer was dissolved in 5 ml vials at 80 °C with deionized water to yield 15, 20, and 25 wt% concentrations, and was stored at 4 °C. After 15 h, viscosity was measured using a circulating bath with a programmable controller (TC-502P, Brookfield Engineering Laboratories, Middleboro, MA) and a Brookfield DV-III ultraviscometer equipped with a programmable rheometer. The viscosity of each polymer solution was investigated using a T–F spindle rotating at 0.2 rpm between temperatures of 10 and 60 °C, raised in increments of 1 °C.

2.3. In vitro Ptx release

One-milliliter solutions of 15, 20, and 25 wt% diblock copolymer containing 1 mg/ml Ptx and 20 wt% diblock copolymer containing 0.5, 1 and 2 mg/ml Ptx in PBS were dispensed into 5 ml vials and immersed in a water bath at 80 °C to dissolve block copolymer above the melting temperature of PCL. The solutions were then ultrasonically agitated for 20 min and left overnight (15 h) at 4 °C. For Ptx release experiments, the diblock copolymer/Ptx mixture was incubated at 37 °C for 1 h to form a gel. Then, 4 ml of PBS at 37 °C was added to each gel, and the vial was shaken at 100 rpm and 37 °C. At specified sample collection times, 1 ml of solution was removed from the vial and replaced with 1 ml of fresh PBS at 37 °C. The amount of Ptx in the supernatant solution was calculated by reference to a standard calibration curve prepared from solutions containing known concentrations of Ptx in PBS and methanol (80/20, v/v). The amount of Ptx was analyzed using a high-performance liquid chromatography (HPLC), Agilent 1200 series LC Systems system equipped with detection at 220 nm using a Diode Array Detector (Agilent Technologies, Inc., Santa Clara, USA). A Sunfire C18 column (4.6 mm × 150 mm, 5 μm) was used. The mobile phase consisted of a distilled water:acetonitrile:methanol (41:48:11, v/v) mixture, and the column was eluted at a flow rate of 1.0 ml/min. Three independent release experiments were performed for each gel composition.

2.4. Cytotoxicity studies

For control or Taxol experimental, 1.5×10^5 cells/well were plated in a 48-well plate (BD Bioscience, Bedford, MA) and incubated for 1 day at 37 °C in a humidified incubator containing 5% CO_2. After 24 h, the cells were treated with saline or Taxol (0.2 mg Ptx/well). For experiments using gels only or Ptx-loaded gels (0.2 mg Ptx/well), a 20 μl aliquot of MPEG–PCL diblock copolymer solution was pipetted into a clean 48-well plate and maintained at 37 °C in a humidified incubator for 1 h to create a gel. Each gel was then seeded with 1.5×10^5 cells/well and maintained at 37 °C in a humidified incubator containing 5% CO_2. After 1, 4, and 10 days, the in vitro cytotoxicity of various injection formulations toward B16F10 cancer cells was compared using the WST-1 assay (Roche, Germany). Briefly, 100 μl of WST-1 reagent was added to each well, the plates were incubated at 37 °C for 4 h, and the samples were then shaken for 1 min. A 100-μl aliquot from each well was transferred to a 96-well plate, and absorbance at 450 nm was measured with a microplate reader (EL808 ultramicroplate reader; Bio-Tek Instrument, USA). All experiments were performed three times.

2.5. In vivo tumor growth

Hairless mice were housed in sterilized cages with sterile food and water and filtered air, and were handled under a laminar flow hood using aseptic techniques. To establish the tumor model, approximately 2×10^5 cells in a 0.2 ml suspension were inoculated subcutaneously into the back of each animal, previously anesthetized using a ketamine–rompun mixture (1:1 ratio, 1.5 ml/kg). The time at which the volume of solid tumors reached ~140 mm^3 was defined as day 0. Twenty-four hairless mice (6-weeks old), divided randomly into four groups of six mice each, were used in the animal tests. Because some saline and gel-only controls died after 10 days, the analysis of animal treatments was based on a 10-day experimental duration. On day 0, 100 μl of one of four solutions was subcutaneously injected into the tumor using a 26-gauge needle and disposable (1 ml) syringe. The four experimental groups were (1) normal saline, (2) gel-only, (3) Taxol-only (0.2 mg Ptx), or (4) Ptx-loaded gel (0.2 mg Ptx). The tumor diameters were measured in two dimensions every other day with vernier calipers. The tumor volume (V) was calculated according to the following formula: $V = [\text{length} \times (\text{width})^2]/2$ (Devalapally et al., 2007). The resulting tumors were then allowed to develop and were biopsied in vivo at 1, 4, and 10 days. All animal treatments and surgical procedures followed approved protocols and were performed in accordance with the Korea Research Institute of Chemical Technology's Council on Animal Care Guidelines.

2.6. Histological analysis

On days 1, 4, and 10 after injection, mice were killed and the tumors were individually dissected and removed from the subcutaneous dorsum. The tissues were immediately fixed with 10% formalin and embedded in paraffin. The embedded specimens were sectioned (4 μm) along the longitudinal axis of the

tumor, and the sections were stained with hematoxylin and eosin (H&E).

2.7. Statistical analysis

Cytotoxicity data were obtained from independent experiments in which each of the four treatment conditions were tested in triplicate. Tumor sizes were evaluated in independent experiments with $n = 5$ for each data point. All data are presented as means ± standard deviations (SD). The results were analyzed by one-way ANOVAs using the Prism 3.0 software package (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Preparation of an injectable in situ-forming gel

Our previous work suggested that aqueous solutions of MPEG–PCL diblock copolymers could exhibit sol-to-gel phase transitions as a function of temperature (Kim et al., 2006a,b). Block copolymer solutions of MPEG (MW = 750) and PCL (MW ≈ 1400–3000) were liquid at room temperature and exhibited a sol-to-gel phase transition as the temperature was increased. On the basis of previous results, we chose a diblock copolymer solution containing 750 g/mol MPEG and 2400 g/mol PCL.

An aqueous solution of the MPEG–PCL diblock copolymer was prepared in PBS at 80 °C at concentrations of 15, 20, and 25 wt%. At ambient temperature, the diblock copolymer solution existed in a translucent emulsion–sol state. As shown in Fig. 1, the viscosity of the 15, 20, and 25 wt% solutions were 1 cP at 20–30 °C, but increased at 37, 32 and 31 °C, respectively. This indicates that the onset temperature for phase transition rose as the concentration decreased. The viscosities of the 15, 20, and 25 wt% solutions at body temperature were 4.4×10^4, 17.5×10^4, and 3×10^5 cP, respectively. In addition, MPEG–PCL diblock copolymers showed good mechanical strength and rapid (<10 s) sol-to-gel phase transitions.

3.2. In vitro Ptx release

The release behavior of Ptx from MPEG–PCL diblock copolymer gels prepared at 15, 20, and 25 wt% and different concentrations of Ptx (0.5, 1 and 2 mg/ml) was examined. Fig. 2 shows the percentage cumulative release profiles of Ptx over 14 days. In the first 12 h, an initial burst around 10% of release was observed in all Ptx-loaded gels. After the initial burst, Ptx was slowly released over the next 14 days. Even though less than 25% of total Ptx was released, we chose 20 wt% MPEG–PCL diblock copolymer solution with Ptx 1 mg/ml as a candidate to form instantaneous gels at body temperature.

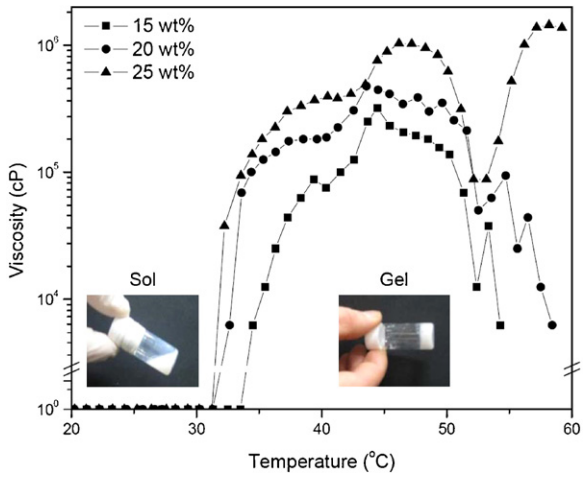

Fig. 1. Viscosity versus temperature curve of MPEG–PCL diblock copolymer solutions at 15, 20, and 25 wt% concentrations.

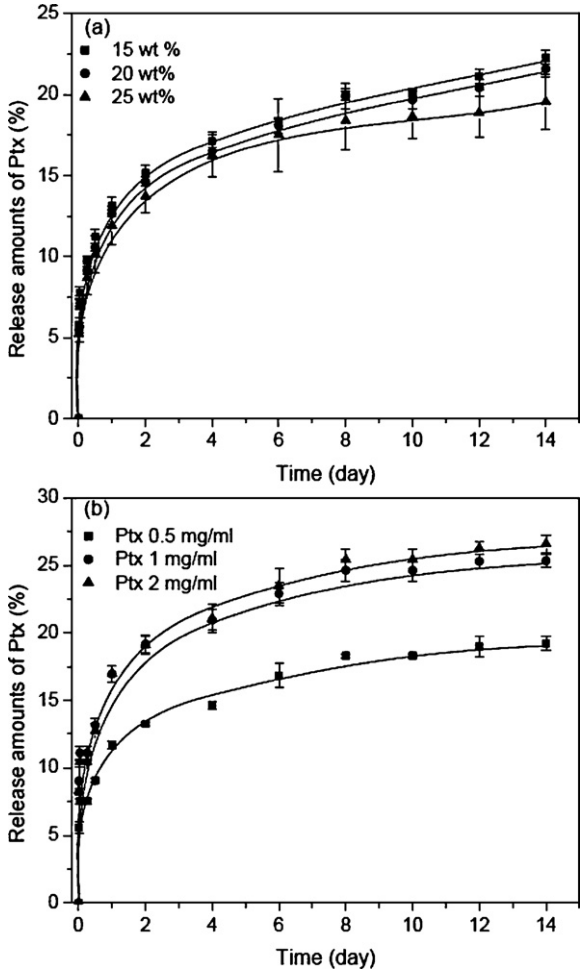

Fig. 2. In vitro release of Ptx from gels prepared with (a) MPEG–PCL concentrations of 15, 20 and 25 wt% with 1 mg/ml Ptx, (b) 20 wt% MPEG–PCL with 0.5, 1 and 2 mg/ml Ptx.

3.3. Anti-proliferative effects of treatments

Saline, gel-only, Taxol-only, and Ptx-loaded gels were examined for their anti-proliferative activities against B16F10 cancer cells (Fig. 3). The population of cells increased as a function of culture time after addition of saline, and proliferation was only slightly inhibited by addition of gel-only. Both Taxol and Ptx-loaded gels exerted a significant inhibitory effect on cell proliferation. On day 1, the effect of Taxol was greater than that of Ptx-loaded gels, but inhibition of cell proliferation by Ptx-loaded gels was higher on day 10.

3.4. Intratumoral injection

Fig. 4a shows the average tumor size of 140 mm^3 on day 0. Saline, gel-only, Taxol, or Ptx-loaded gel was administered to the center of the tumor tissue by intratumoral injection (Fig. 4b). Fig. 4c shows the tumor after intratumoral injection. The tumors were removed after 1, 4, and 10 days, and excised tumors were photographed (Fig. 4d). The change in tumor volume from the initial volume (~140 mm^3) was monitored for 10 days after intratumoral injection (Fig. 5). After injection of saline or gel-only, the

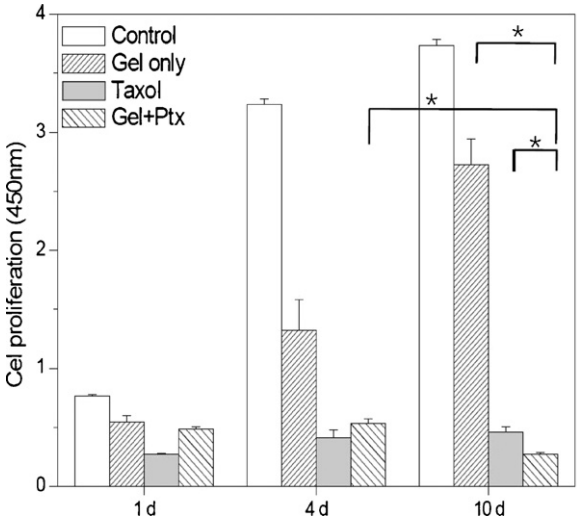

Fig. 3. In vitro cytotoxicity of saline, gel-only, Taxol and Ptx-loaded gels toward B16F10 cancer cells measured by WST-1 assay. Statistical analyses were performed using one-way ANOVAs with Bonferroni's multiple comparison (*$p < 0.01$).

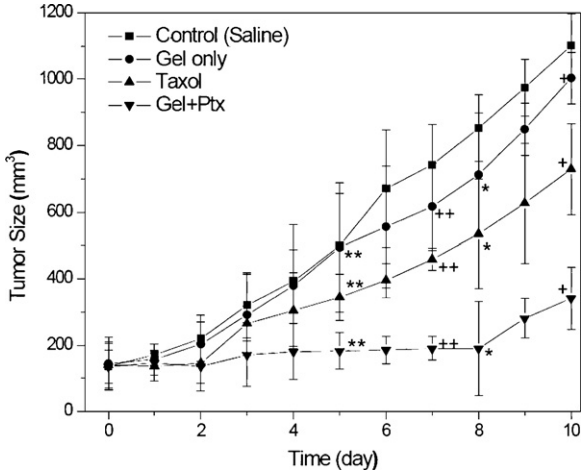

Fig. 5. Inhibition of tumor growth by intratumoral injection of saline, gel-only, Taxol or Ptx-loaded gels. Dorsal subcutaneous implantation of B16F10 cancer cells into mice was followed by administration of each solution after tumors had reached a volume of ~140 mm^3. Statistical analyses were performed using one-way ANOVAs with Bonferroni's multiple comparison ($p < 0.001$ versus Ptx-loaded-gel injection group at 5 [**], 7 [++], 8 [*] and 10 [+] days).

size of excised tumors steadily increased as a function of time after intratumoral injection. Intratumoral injection of Taxol slowed tumor growth early after administration (days 1 and 2), maintaining tumor volume at approximately the initial level. Three days after Taxol administration, however, tumor volume increased abruptly and then continuously increased with a slope similar to that of the saline and gel-only controls, over 10 days. Overall, however, injection of Taxol reduced tumor growth rate compared with injection of saline or gel-only. Strikingly, almost no tumor growth was observed in animals injected with Ptx-loaded gel, during the entire experimental period. Ptx-loaded gels maintained tumors at their initial volume for 8 days, after which there was a slight increase in tumor volume. There were no significant changes in the body weight of mice following treatment (data not shown).

The tumor growth rate and tumor volume doubling times (DTs) are listed in Table 1. The Ptx-loaded gel formulation produced significant inhibitory effects against the tumor compared with control and gel-only. Administration of Taxol alone also significantly inhib-

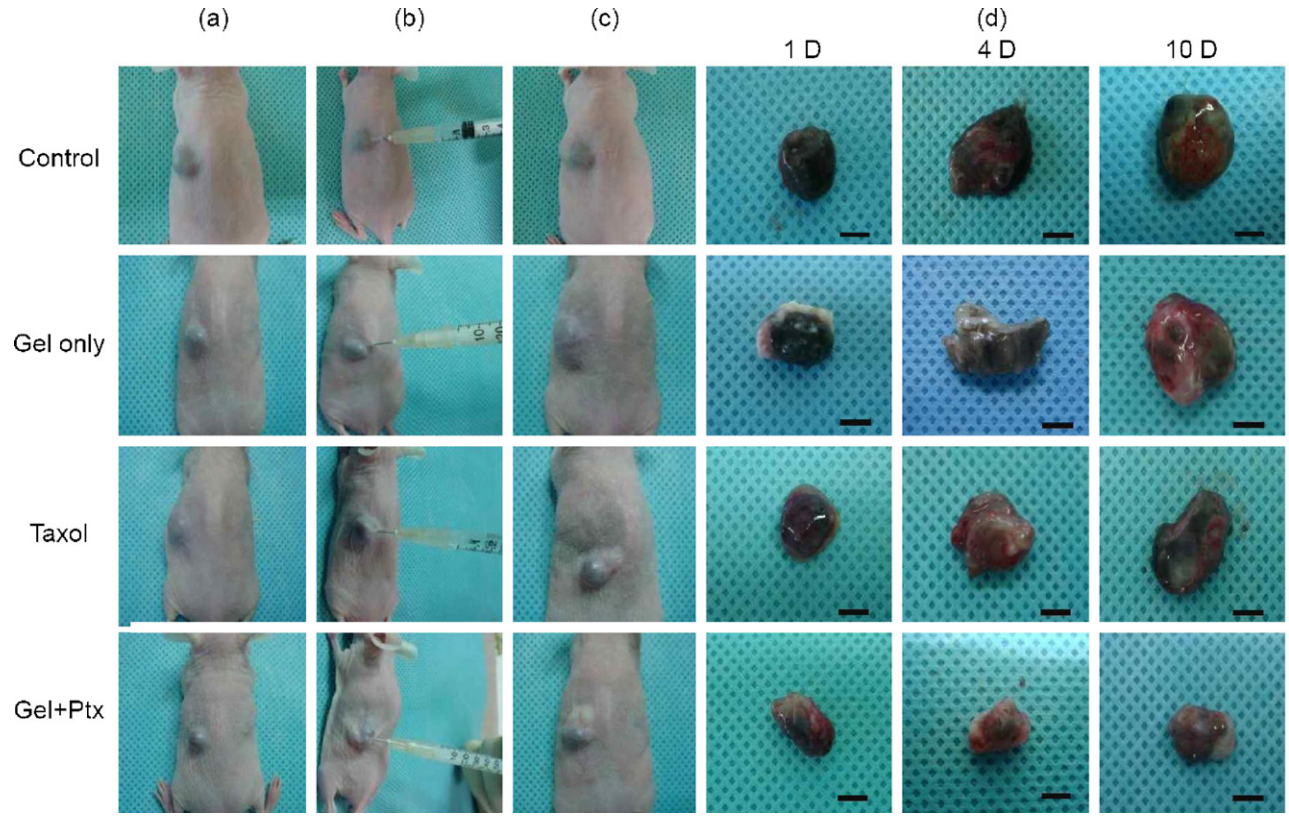

Fig. 4. The formed tumor (a), intratumoral injection (b), the injection site 1 day after injection (c), and the excised tumor removed after 1, 4, and 10 days (d). Scale bars represent 0.5 cm.

Table 1
Tumor growth rate and tumor volume doubling times after intratumoral injection of saline, gel-only, Taxol or Ptx-loaded gel.

	Control	Gel-only	Taxol	Ptx-loaded gel
Tumor growth Rate (mm^3/day)	96.4 ± 3.4	85.7 ± 0.3	58.9 ± 9.4[a]	20.6 ± 6.1[a,b]
Tumor volume doubling time (days)	3.3 ± 0.2	3.6 ± 0.221	4.3 ± 0.7[a]	8.7 ± 3.9[a,c]

[a] $p < 0.05$, versus control at the same time-point.
[b] $p < 0.05$, versus Taxol at the same time-point.
[c] $p = 0.07$, versus Taxol at the same time-point.

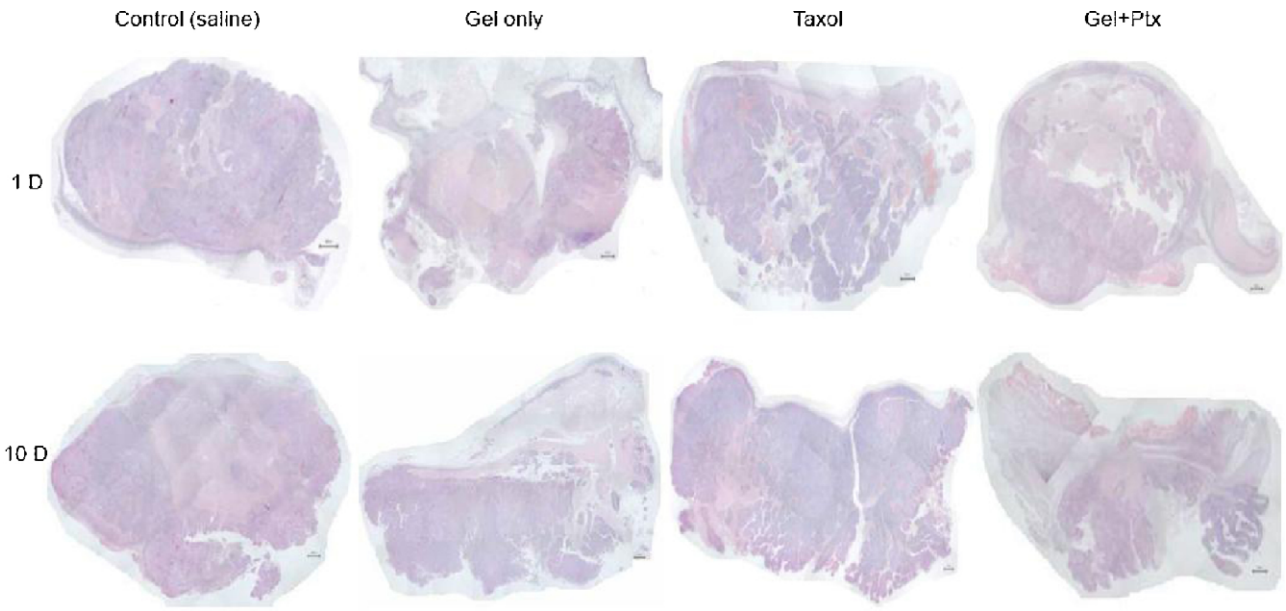

Fig. 6. H&E-stained histological sections of tumors treated with saline, gel-only, Taxol or Ptx-loaded gels on days 1 and 10 after administration.

ited tumor growth, although the inhibitory activity was less than that of Ptx-loaded gels, which showed a marked effectiveness in decreasing tumor propagation and extending the DTs. Intratumoral injection of Ptx-loaded gel resulted in the lowest tumor growth rate and the longest DT compared with saline and gel-only controls and Taxol alone. The inhibitory effects at each time-point were significantly different.

3.5. Histology studies

Fig. 6 shows H&E-stained histological sections of saline-, gel-only-, Taxol-, and Ptx-treated tumors on days 1 and 10 after administration. All tumors exhibited some level of necrosis, observed as variably sized regions containing necrotic tissue interspersed between regions of viable tumor. On day 1, necrosis in tumors treated with Taxol or Ptx-loaded gels was observed to extend radially from the tumor center. Tumors from the gel-only group exhibited a lower proportion of necrotic cells than did tumors from Taxol and Ptx-loaded gel groups, and tumors from saline controls showed no necrosis. After 10 days, tumors from gel-only and Taxol groups showed few necrotic regions, whereas tumors injected with Ptx-loaded gels contained a much larger proportion of necrotic regions.

4. Discussion

Primary clinical treatment of tumors is usually achieved by surgery or radiation (Parvez, 2008; Cho, 2008). However, local recurrence of tumors generally occurs near the site of the previous surgical excision. Intratumoral injection of anticancer agents has been proposed by several investigators as one treatment option for preventing this phenomenon (Pawar et al., 2004; Ranganath and Wang, 2008; Springate et al., 2008; Lee et al., 2009). An important feature of a successful engineering solution employing intratumoral injection is the development of a drug depot at the injected site.

In our present work, we examined injectable, in situ-forming, MPEG–PCL diblock copolymer gels as the basis for intratumoral injection of the anticancer drug, Ptx. An aqueous solution of MPEG–PCL undergoes a sol-to-gel transition in response to temperature, remaining liquid at room temperature and rapidly forming a gel at body temperature (Kim et al., 2006a,b). The gel exhibits structural integrity and maintains its mechanical strength for more than 1 month.

If a gel is to be successfully used as a depot for an anticancer drug, the gel should exhibit a desirable release profile of the drug under physiological conditions. To test this, we loaded Ptx into the copolymer and tested drug release from the resulting gels in vitro. The Ptx-loaded copolymer solutions were easily prepared and remained as solutions at room temperature. At body temperature, the Ptx-loaded copolymer solutions formed gels and maintained adequate structural integrity. Prolonged release of Ptx from the Ptx-loaded gel was observed for more than 14 days, with the 15 wt% MPEG–PCL copolymer gel exhibiting a slight higher release of Ptx than did the 20 and 25 wt% copolymer gels. This behavior reflects the mechanical strength of the gel. The released amounts of Ptx were also dependent on the initial dose of Ptx. Even though the amount of Ptx released from all Ptx-loaded gels is not much after burst release probably due to the very low solubility of Ptx under in vitro condition, however, Ptx-loaded gel can be compared with a Taxol formulation prepared using a solution of Cremophor EL and ethanol (Terwogt et al., 1997; Chun et al., 2009; Ding et al., 2005), which could not form a drug depot and was unable to maintain an

effective concentration of Taxol for more than 1–2 days under any experimental condition tested.

The Ptx-loaded copolymer solutions were easily injected into the tumor center of rats using a 26-gauge needle. The MPEG–PCL diblock copolymer formed a drug depot at the tumor site, rather than spreading or dissipating, which should allow the gel to slowly release Ptx. Consistent with such a slow local release mechanism, intratumoral injection of Ptx-containing gels more effectively inhibited tumor growth than did Taxol, increasing the average tumor volume doubling time to 9 days, compared with 4.3 days in the Taxol-treated group and 3.3 days in the saline control group. Tumor volume was maintained at a similar level by intratumoral administration of Taxol or Ptx-loaded copolymer for the first 2 days, but tumor growth rebounded rapidly thereafter in the Taxol-treated group, whereas tumor volume was maintained at the original volume for at least 8 days in the Ptx-loaded gel group. Tumor histology was similar on day 1 under the two treatment paradigms, but there was no pronounced effect of Taxol at later times when an increase in necrotic tissue was clearly evident in tumors injected with Ptx-loaded gels. These observations support the idea that Ptx-loaded gels, in contrast to injected Taxol alone, form in vivo drug depots that slowly release Ptx. This mode of delivery may produce treatment effects that are different from standard Taxol treatment.

5. Conclusion

We have explored the potential clinical utility of a Ptx-containing drug depot using an in situ gel-forming MPEG–PCL diblock copolymer/Ptx solution. Ptx in vitro release profiles from Ptx-loaded gels demonstrated controlled delivery for more than 1 month. The present findings show that an MPEG–PCL diblock copolymer gel can act as injectable drug depot that maintains structural integrity under physiological conditions. Animals that received intratumoral injections of Ptx-loaded gels displayed marked inhibition of tumor growth. The Ptx-loaded MPEG–PCL diblock copolymer gel described here would be ideal for future use as an effective treatment for localized solid tumors. Further experiments investigating tumor growth inhibition in large-animal models over a longer experimental period using various anticancer drugs embedded in MPEG–PCL diblock copolymer are currently underway.

Acknowledgements

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