Translymphatic Chemotherapy Targeting Sentinel Lymph Nodes Using a Novel Phospholipid Polymer–Paclitaxel Conjugate

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Abstract. Background: Although sentinel node (SN) biopsy has been applied for various types of solid tumors, its clinical significance is currently limited to the field of diagnostics. We developed a new translymphatic chemotherapy approach using a novel phospholipid polymer (PMB30W), which facilitates the dissolution of large amounts of paclitaxel. The purpose of the present study was to investigate the pharmacokinetics and antitumor effect of this conjugate (PTX–PMB30W) in a rat model. Materials and Methods: PTX–PMB30W was directly administered into the cecal submucosa or through the tail vein. The antitumor effect was compared between the two groups. Results: Paclitaxel concentrations in SNs remained constant over a 24-h period after local administration. Tumor growth was clearly suppressed by submucosal administration of PTX–PMB30W, resulting in a survival benefit compared to intravenous administration. Conclusion: Translymphatic chemotherapy targeting SNs via direct administration of PTX–PMB30W appears feasible, and this strategy may be applicable to the multi-disciplinary management of early solid cancer.

A sentinel node (SN) is defined as the first draining node from the primary lesion and the first possible site of cancer metastasis (1). SN biopsy has been developed as a new staging procedure for melanoma (2) and breast cancer (3) and is used worldwide. This technique can offer benefit to patients through preventing various complications caused by unnecessary prophylactic radical lymphadenectomy when SNs are negative for cancer metastasis (4). More recently, this concept has also been extended to a number of other solid tumor types (5, 6). However, in certain types of gastrointestinal (GI) cancers, there are some problems in performing SN navigation surgery. Invasive open surgery, such as thoracotomy in esophageal cancer, or surgical detachment and movement of the tissue, are essential to identify SNs because of the complicated lymphatic drainage system. Even in some cases of melanoma, SN distribution displays unpredictable patterns similar to GI cancer (7). These problems suggest the need for approaches that can directly target SNs.

We hypothesized that a new less-invasive treatment for targeting micrometastases in SNs without surgery could be developed. Firstly, we focused on a suitable size for the tracer in clinical SN navigation surgery, which is reportedly around 50 nm in diameter (8). We also paid attention to a novel phospholipid polymer using 2-methacryloyloxyethyl phosphorylcholine (MPC), whose size is nearly equal to the optimal diameter of tracers (9). MPC polymers possess the same polar phosphorylcholine group as biomembranes. The most effective phospholipid polymer, PMB30W, has a hydrophilic MPC unit and a hydrophobic butyl methacrylate unit as the co-monomer (10) (Figure 1). PMB30W forms stable polymeric lipid nanoparticles that completely dissolve large amounts of paclitaxel without the need for specific additives such as Cremophor EL. Although paclitaxel is particularly effective for treating various types of cancers (11), special solvents are required for its dissolution because of its low water solubility. These solvents can cause various clinical problems (12), while PMB30W alone did not have toxicity, anti-tumor activity, or severe side-effects (13). Submucosal administration of PTX–PMB30W around the primary cancer lesion appears to be a reasonable and feasible route for maintaining high concentrations in SNs without local reaction at the administration site. Recently, we reported our preliminary data of a PTX–PMB30W drug delivery system targeting...
SNs (14). In the current study, we verified the feasibility of translymphatic chemotherapy targeting SNs via submucosal administration of PTX−PMB30W in vivo.

Materials and Methods

Biochemical reagents. PMB30W was obtained from NOF Corporation (Tokyo, Japan). Paclitaxel and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Purified water (Nanopure deionization system, Barnstead/Thermolyne, Dubuque, IA, USA) was used for all aqueous solutions. All other reagents were of extra-pure grade, were commercially available and were used without purification.

Preparation of the PTX−PMB30W conjugate. A fixed amount of paclitaxel was dissolved in ethanol (50 mg/ml), and this solution was added to a 10% aqueous solution of PMB30W. The mixture was stirred, and ethanol was completely evaporated under reduced pressure to obtain the PTX−PMB30W conjugate. The final in vivo concentration of PTX−PMB30W was 5 mg/ml.

Cancer cell preparation. The AH130 rat hepatoma cell line derived from the ascites of rats and having a high potential to metastasize to lymph nodes (LNs) and liver was kindly provided by Dr. Nagata (Department of Internal Medicine, Keio University School of Medicine, Tokyo). AH130 cells were maintained by serial intraperitoneal implantation into male Donryu rats every seven days, and ascites were used in the experiments on the seventh day of passage. The cells in the ascites were washed three times with RPMI-1640 supplemented with 10% Fetal Calf Serum. AH130 cells were collected by gently aspirating the suspension. The final cell concentration was 1×10^7 cells/ml of RPMI-1640 in the animal experiments.

Colorimetric cell viability assay. AH130 cells (2×10^4/well) were seeded in 50 µl of medium per well in 96-well plates. Twenty-four hours after seeding, 50 µl of DMEM containing dissolved paclitaxel was added to each well. The cells were exposed to serially-diluted agents. Cell viability was measured after 24 h using the Cell Counting Kit-8 (Peptide Institute Inc., Osaka, Japan). To calculate cell viability, the absorbance of each well was measured using a microplate reader at a test wavelength of 450 nm and a reference wavelength of 630 nm.

Animal preparation. Male Donryu rats (CLEA Japan Co., Tokyo, Japan) weighing 200-320 g were used in this study. All animal protocols were approved by the Keio University Animal Research Committee (no.030022). The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and the abdomen was opened via a small midline incision. The cecum was exteriorized promptly, allowing for easy identification of LNs. Approximately 0.05 ml of the cancer cell suspension (1×10^7/ml of AH130 cells) was inoculated into the cecal submucosa using a microsyringe with a 30-gauge needle. This experimental model of metastasis has been established by Nagata et al. (15).

Lymphatic mapping using isosulfan blue. Isosulfan blue (1% lymphazurin; US Surgical, Norwalk, CT, USA) has been the dye of choice for SN mapping in melanoma and breast cancer. To assess the feasibility of the SN concept in an animal model, approximately 0.05 ml of isosulfan blue was administered into the rat cecal submucosa using the same method described above.

Administration of PTX−PMB30W and agent distribution. The PTX−PMB30W conjugate solution and paclitaxel solution containing Cremophor EL were directly administered to the cecal submucosa of the experimental metastasis model rats by the mesocecal technique as mentioned previously. Paclitaxel was administered at a concentration of 6 mg/kg to each rat in each group. Local reaction at the administration sites and body weight changes were compared between the groups for assessing the safety of PTX−PMB30W. Furthermore, in order to evaluate the drug delivery system, PTX−PMB30W (3 mg/kg) was also directly administered into the cecal submucosa (SM group, n=15) or through the tail vein (IV group, n=15) of Donryu rats. At various time points after administration, the rats were sacrificed via an ether overdose, and LNs around the primary tumor were removed and weighed to measure paclitaxel concentrations. LNs excluding the SN were defined as secondary or distant LNs based on their anatomical positions (Figure 2A). After the specimens were crushed homogeneously with adding 1.0 ml of 1/15 M phosphoric acid, centrifugation was performed at 13,000 g for 5 min. The supernatant was measured by high-performance liquid chromatography with a UV detector (absorbance=227 nm). Paclitaxel concentrations were determined by comparing the specimens with the standard concentrations. Limit of detection and limit of quantification were 10 ng/ml and 50 ng/ml, respectively.

Antitumor effect in vivo. After AH130 cells were implanted into the cecal submucosa of 18 Donryu rats, the rats were divided into three groups so that the mean weight of each group was similar. On the next day, all rats underwent another surgery in the same wound via minilaparotomy, and the cecum with AH130 tumors was exteriorized. Each group was administered PTX−PMB30W under one of the following regimens: (a) submucosal administration of 6 mg/kg paclitaxel around the inoculated site (SM group); (b) intravenous administration of 6 mg/kg paclitaxel in the tail vein (IV group); and (c) no additional therapy (control). In the SM and IV groups, the rats underwent administration as described for the distribution analysis, whereas exteriorized cecum in the control group was returned to the abdominal cavity without additional
treatment. On day 7 after inoculation, the mesocecal LNs were isolated to compare their weights and pathological findings. Moreover, the survival rates of the three groups were analyzed in other rats (n=18) treated using the same protocol but with a prolonged observation of 30 days.

**Statistical analysis.** Student’s t-test was performed using SPSS Statistical Software version 17.0 (SPSS, Inc., Chicago, IL, USA). All data are shown as the average and standard deviation at each time point. A \( p \)-value less than 0.05 was considered statistically significant. Survival curves were plotted according to the Kaplan–Meier method, and the survival rate was compared using the log-rank test.

**Results**

**Cytotoxicity in vitro.** In order to evaluate the therapeutic potential of paclitaxel, *in vitro* cytotoxicity assays were performed. These assays revealed that paclitaxel inhibited the growth of AH130 cells in a concentration-dependent manner (Figure 3). Paclitaxel was highly cytotoxic towards AH130 cells, and the 50% inhibitory concentration (IC\(_{50}\)) was approximately 10 ng/ml.

**Lymphatic mapping.** SNs were defined as LNs with reproducibility in their position within the mesocecum, which was stained blue immediately after administration of isosulfan blue into the cecal submucosa. Figure 2B shows lymphatic drainage from the administration sites of the cecum. In the meso-cecal LNs corresponding to the blue nodes, blue staining was washed out gradually after 20 min. Hence, mesocecal LNs were defined as SNs in this study.

**Local reaction after administration into the cecal submucosa.** Direct administration of PTX−PMB30W into the cecal submucosa caused no local reaction. However, the development of a local necrotizing reaction was confirmed a few days after administration of Cremophor EL-containing paclitaxel (data not shown). Furthermore, body weights decreased significantly in rats administered Cremophor EL-containing paclitaxel (\( p < 0.001 \), Figure 4).

**Drug delivery system of PTX−PMB30W.** In the SM group, the mean paclitaxel concentration in SNs 6 h after administration was 6.5-fold higher than that in the IV group (\( p < 0.05 \), Figure 5A). Paclitaxel concentrations were maintained at least 154.7 ng/g for 24 h in the SM group, whereas paclitaxel was undetectable in the IV group. Conversely, time-dependent changes in paclitaxel concentrations in the liver were comparable between these groups (Figure 5B). Mesenteric...
LNs were divided into three groups based on their anatomical positions along the vessel (Figure 2A). Paclitaxel concentrations in these LNs are shown at various time points for the SM group in Figure 5C. In particular, paclitaxel concentrations in SNs were significantly higher than those in the other LNs within 12 h of cecal administration.

Antitumor effect. Figure 6A shows the weights of SNs on day 7 after administration in the three groups. Although weights of SNs in the SM group were significantly lower than those in the other LNs within 12 h of cecal administration.

Figure 3. Colorimetric cell viability assay for AH130 cells. The growth of AH130 cells treated with paclitaxel (PTX) for 24 h is shown. Paclitaxel inhibited the proliferation of AH130 cells in a concentration-dependent manner.

Figure 4. Comparison of body weight changes with the use of different additive agents. Adverse effects of submucosal administration of PTX–PMB30W and Cremophor EL-containing paclitaxel (PTX) are compared. Significant differences were observed in body weights between the two groups. The body weights of rats administered PTX–PMB30W did not decrease after treatment.

Figure 5. Paclitaxel concentrations in the sentinel node (SN), liver, and lymph nodes (LNs). Chronological changes in paclitaxel (PTX) concentration in SNs (A) and liver (B) after submucosal (SM) or intravenous administration (IV) of PTX–PMB30W are shown. A: Paclitaxel concentrations in the SNs remained higher than 150 ng/g for 24 h continuously in the SM group, whereas that in the IV group concentrations were much lower at 6 and 12 h after inoculation. B: There was no significant difference in paclitaxel concentrations in the liver between the SM and IV groups. C: Comparison of paclitaxel concentrations among LNs in the SM group revealed that significant amounts of paclitaxel remained in SNs within 12 h after inoculation.
in the other groups, there were no differences in weights between the IV and control groups. On day 7 after tumor inoculation, SNs in the control group were markedly larger than those in the SM group (Figure 6B and C). In addition, we extended the observation period to 30 days under the same therapeutic protocol in the SN metastatic model. The survival rate of rats in the SM group was significantly higher than that of rats in the IV and control groups (Figure 6D).

**Discussion**

To the best of our knowledge, this is the first study to describe translymphatic chemotherapy targeting SNs. This strategy is unique, since a novel biocompatible nanoparticle containing an anticancer agent was administered directly around the primary tumor under the same procedure used for clinical SN mapping. Our study revealed that local...
administration of PTX−PMB30W led to an antitumor effect because high paclitaxel concentrations were maintained in SNs without any necrotic reaction at the administration sites.

An experimental model of mesoceleal LN metastasis is appropriate for investigating various reactions in SNs. Nagata et al. reported a well-defined time course of changes in cancer cell growth at early stages of metastasis in this model (15). We clearly demonstrated a correspondence between mesoceleal LNs and SNs via an influx of lymphatic mapping agent. Most important for this system is determining how to promptly transport anticancer agents into SNs and maintain their stable concentrations. We attempted to solve these problems by using PMB30W as a transporter. The diameter of this conjugate is suitable for identifying SNs (8). PMB30W has a great advantage as it enhances the water solubility of paclitaxel without the need for Cremophor EL. Paclitaxel concentrations in the PMB30W aqueous solution reached 5.0 mg/ml, although its solubility in an aqueous medium is less than 0.3 μg/ml (10). Furthermore, PMB30W can form a stable biocompatible nanoparticle with a phospholipid polar group. MPC polymer is utilized for coating medical devices such as artificial blood vessels (16-18). This safety aspect supports the finding that PTX−PMB30W directly administered into the cecal submucosa induced no local reaction despite containing high paclitaxel concentrations. Reportedly, PTX−PMB30W has no adverse effects even when administered intravenously or subcutaneously (10, 13).

In contrast, administration of paclitaxel with Cremophor EL induced necrotizing changes at the administration sites and significant weight loss in our study. These results suggest that the direct administration of PTX−PMB30W into the submucosal layer around the primary tumor may be an applicable clinical approach similar to endoscopic techniques.

Pharmacokinetic studies demonstrated that paclitaxel concentrations in SNs after sub-mucosal administration of PTX−PMB30W were significantly higher than that after intravenous administration. In addition, paclitaxel uptake was limited to SNs specifically along the lymphatic basin. Importantly, paclitaxel concentrations in SNs were maintained for 24 h after direct submucosal administration of PTX−PMB30W, which is much higher than the IC_{50} of paclitaxel in AH 130 cells. These findings suggest that translymphatic chemotherapy targeting SNs in a rat model is feasible.

It has been reported that the presence of cancer cells in the medulla of SNs was clearly confirmed from 6 to 48 h after inoculation in the SN metastasis model (15). In our study, there was a significant difference in macroscopic findings of SNs on day 7. Tumor growth was clearly suppressed by submucosal administration of PTX−PMB30W, resulting in significantly lighter SNs than those obtained using an intravenous or a non-therapeutic approach. In addition, when the observation period was extended to 30 days, the survival rate in the SM group was significantly higher than that in the other groups. Thus, we conclude that translymphatic chemotherapy targeting SNs via direct administration of PTX−PMB30W around primary tumors is effective because of the accumulation of higher paclitaxel concentrations than those observed after intravenous administration.

Some studies reported the development of other carriers to transport anticancer agents in LNs by other approaches. Despite their efficacy, these procedures cannot be applied for standard treatment because they are not performed as clinical approaches in daily practice, for example, direct administration of the popliteal LN or replacement pleural cavity (19, 20). By contrast, our translymphatic approach via direct administration into the submucosal layer around the primary tumor is a realistic approach for daily clinical practice similar to endoscopic techniques, such as endoscopic mucosal resection or endoscopic submucosal dissection. These techniques have been established as standard therapy for early GI cancer in Japan (21), and they are increasingly used in Asian and Western countries in current practice (22).

These minimally-invasive techniques allow for safe and efficacious treatment in situations that would otherwise require major surgery (23). In other words, the probability of metastasis to SNs is the most important factor for selecting therapy, major surgery, or endoscopic resection/dissection. This study suggests the feasibility of a newer hybrid therapy positioned between surgery and endoscopic techniques. Therefore, translymphatic chemotherapy targeting SNs combined with endoscopic resection/dissection may become useful for refusal or high-risk cases of major surgery. This strategy would be applicable for the multi-disciplinary management of superficial GI cancer with micrometastases limited to SNs distant from the primary lesion.

**Conclusion**

The present study provides pre-clinical evidence that translymphatic chemotherapy targeting SNs using a novel biocompatible polymer can safely increase the antitumor effect of paclitaxel compared to the one obtained using the traditional intravenous procedure. This new less-invasive strategy could help solve some problems in performing SN navigation surgery. Our results suggest that this may be a promising non-surgical approach for treating patients with lymph node micrometastases associated with solid tumors.

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References


