Development of Targeted Therapy with Paclitaxel Incorporated into EGF-conjugated Nanoparticles

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Abstract. *Background: 2-Methacryloyloxyethyl phosphorylcholine (MPC) polymer is a suitable vehicle for paclitaxel (PTX) delivery. A new targeted therapy has been developed by conjugating epidermal growth factor (EGF) to MPC polymer and its growth inhibitory and antitumor effects on cancer cells overexpressing EGF receptors (EGFR) has been investigated. Materials and Methods: EGF was conjugated to poly [MPC-co-n-butyl methacrylate-co-p-nitrophenyloxycarbonyl poly (ethylene glycol) methacrylate] (PMBN) and mixed with PTX. The cytotoxicity of the resulting PTX incorporated into EGF-conjugated PMBN (EGF-PMBN-PTX) on EGFR-overexpressing and EGFR-deficient cell lines was compared with PTX incorporated into PMBN alone (PMBN-PTX) and PTX alone. Suspensions of the cells were injected into nude mice subcutaneously. EGF-PMBN-PTX, PMBN-PTX, PTX or NaCl solution was injected intraperitoneally. Results: The cytotoxicity and antitumor effect of EGF-PMBN-PTX were significantly greater than those of PMBN-PTX for EGFR-overexpressing cells but not for an EGFR-deficient line. Conclusion: These results suggest that EGF-PMBN-PTX may represent a more potent targeted therapy for tumors overexpressing EGFR.*

Paclitaxel (PTX) is effective in the treatment of breast, ovarian, lung and head and neck cancers (1). It is highly hydrophobic and almost insoluble in water (<0.3 μg/mL). For this reason, specific emulsifiers such as Cremophor EL (CrEL) are used to formulate PTX in commercially available injection solutions (2). However, CrEL has been reported to cause hypersensitivity reactions (HSRs) and neuropathy in some patients (1, 3). Premedication and infusions of excessively long duration (>3 h) are usually needed to prevent HSRs. To circumvent these undesirable effects resulting from the addition of CrEL efforts have been made to develop new taxane formulations that do not require CrEL as a solubilizer.

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with tyrosine kinase activity stimulated by the binding of growth factors, such as transforming growth factor (TGF)-α or epidermal growth factor (EGF), to the extracellular domain (4). Ligand-binding induces receptor dimerization and activates the intracellular kinase domains, resulting in autophosphorylation at their tyrosine residues, in turn activating downstream signaling pathways which regulate gene transcription and cell cycle progression (4, 5). EGFR overexpression is correlated with a poor prognosis in breast and esophageal cancer patients (6). Conventional therapies including surgery, radiation and chemotherapy are not always effective treatments for these patients. Thus, targeted therapies aimed at the EGFR are being currently explored. To this end, the EGFR-specific monoclonal antibody B4G7 was conjugated to gelonin (7) and peplomycin (8) in order to selectively target malignant cancer cells overexpressing the EGFR. Both immunoconjugates effectively lysed EGFR-hyperproducing squamous carcinoma cells in a dose-dependent and EGFR expression-dependent manner. However, practical clinical application of such conjugates faces several major problems as they are composed of plant or bacterial toxins and murine monoclonal antibodies.

The 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer has the same polar group (phosphorylcholine group) as biomembranes and possesses excellent biocompatibility, *i.e.*, lack of protein absorption and platelet adhesion (9). MPC polymers have been utilized as surface modifiers in many medical devices in order to improve biocompatibility (10, 11). The MPC polymer itself has an MPC unit and an n-

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Key Words: MPC polymer, paclitaxel, epidermal growth factor receptor, targeted therapy.

butyl methacrylate (BMA) unit, and because the former is extremely hydrophilic, copolymers with MPC can be dissolved in water. MPC polymers with hydrophobic monomer units could solubilize hydrophobic drugs and possibly enhance their water solubility (12, 13). Because MPC can stably incorporate hydrophobic materials, the possibilities of it being used as a transporter for PTX, which is very poorly soluble in aqueous media, were explored (14).

Poly [MPC-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycrabonyl poly (ethylene glycol) methacrylate (MEONP)] (PMBN) is a type of MPC polymer with active ester groups which can be conjugated to different proteins under mild physiological conditions (15). PMBN may be useful for targeted cancer therapy by conjugating ligands or antibodies.

In this paper, the growth inhibitory and antitumor effects of PTX incorporated into EGF-conjugated PMBN (EGF-PMBN-PTX) against cancer cell lines overexpressing the EGFR were investigated.

Materials and Methods

Biochemical reagents. Poly [MPC-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl poly (ethylene glycol) methacrylate (MEONP)] (PMBN) was constructed from an approximately 40 mol% MPC unit, a 50 mol% BMA unit and a 10 mol% MEONP unit, yielding a molecular weight of ca. 47,000. The procedure for preparing PMBN has been described elsewhere (15). Human recombinant EGF was purchased from Biosource International (Camarillo, CA, USA). PTX and CrEL-free PTX (pure PTX) were obtained from Bristol-Myers Squibb (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines. The BT-20 breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The A431 squamous carcinoma and H69 small cell lung cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). BT-20 and A431 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 μg/mL amphotericin B and 100 μg/mL kanamycin. H69 was grown in RPMI-1640 containing the same supplements as above. All cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37˚C.

Preparation of PTX incorporated into EGF-conjugated PMBN. Briefly, 4 mg of PMBN and 0.04 mg of EGF were dissolved in 10 mL phosphate-buffered saline (PBS), pH 7.4 (Invitrogen, Carlsbad, CA, USA), and agitated for 48 h at 4˚C. The solution of EGF-conjugated PMBN was then dialyzed using a 10,000 mw cutoff dialysis cassette (Pierce Biotechnology, Inc., Rockford, IL, USA) for 48 h, thus removing p-nitrophenol and free EGF. Given amounts of PTX dissolved in 1.0 mL ethanol were then added to the PBS solution containing the EGF-conjugated PMBN and the ethanol was removed by evaporation under reduced pressure to obtain EGF-PMBN-PTX.

The reaction rate of EGF with PMBN was determined by the absorption spectra of *p*-nitrophenol (wavelength 404 nm). PMBN in water released *p*-nitrophenol by hydrolysis. EGF and PMBN in water released more *p*-nitrophenol correlating with the conjugation of EGF. PMBN in NaOH (0.2 mol/mL) reacted with all ester groups releasing *p*-nitrophenol. The reaction rate of EGF with PMBN was calculated by the formula: reaction rate (%)=(A_b-A_a) /A_c ×100, where A_a, A_b and A_c are the absorbances of *p*-nitrophenol released from PMBN in water (a), EGF and PMBN in water (b) and PMBN in NaOH (c), respectively (Figure 1). The absence of *p*-nitrophenol and the free EGF in the solution after dialysis were confirmed by their absorption spectra (404 nm and 270 nm, respectively) (data not shown). The diameter of this aggregate was determined by measuring dynamic light scattering (DLS) with a Nicomp Model 370 (Particle Sizing Systems, Santa Barbara, CA, USA).

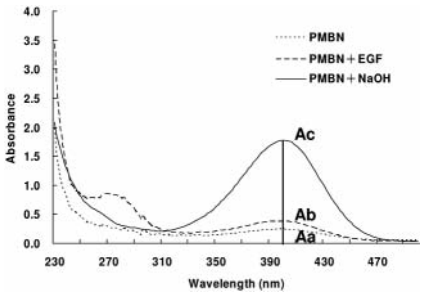

Figure 1. *Absorption spectra of reagents. The reaction rate of EGF with PMBN was determined by the absorption spectra of p-nitrophenol (wavelength 404 nm). The reaction rate of EGF with PMBN was calculated by using the absorbances of a (Aa), b (Ab) and c (Ac).*

Cytotoxicity. Cytotoxic activity was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were seeded into a 96-well plate (1×10^5 cells/well) and incubated overnight at 37˚C, prior to addition of PTX alone, PTX incorporated into PMBN (PMBN-PTX) or EGF-PMBN-PTX. The concentration of EGF and PMBN was 10 nM and 1.0 μM, respectively. PTX was titrated from 0.01 to 100 nM. Seventy-two hours after addition of these agents, cells were washed with PBS and a fresh mixture of MTT (0.4% in PBS) and sodium succinate (0.1 M in PBS) was added to each well to dissolve the MTT formazan. The absorbance of each well was measured in a microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Animals. Four week-old female *BALB/c, nu/nu*, athymic mice, 15 to 20 g, were purchased from CLEA Japan Co. (Tokyo, Japan) and maintained under specific pathogen-free conditions in a laminar air flow rack. They were given a minimum acclimation period of 1 week and received autoclaved food and water. All animal protocols were approved by the Animal Experimentation Ethics Committee of Keio University and procedures were according to the guidelines defined by the United Kingdom Co-ordinating Committee on Cancer Research (16).

Inhibition of tumor growth in athymic mice. Suspensions of 1.0×10^6 cells of A431 and H69 in 150 μL of PBS were subcutaneously (*s.c.*) injected into athymic mice (n=4 per group) in the bilatero-abdominal region. After the tumor volume reached 100 mm^3, EGF-PMBN-PTX, PMBN-PTX, PTX alone or 0.9% NaCl solution was injected intraperitoneally for 5 consecutive days. Fifteen mg/kg of

PTX were administered to each group except the untreated controls. The dose of PMBN and EGF was 10 mg and 4.5×10^{-1} mg for each animal, respectively. Tumor volume was calculated by the formula: $W\ (mg)=0.5\times LS^2$, where L is the major axis (mm) and S is the minor axis (mm). Tumor growth was monitored up to 21 days after the first treatment.

Statistical analysis. The data on growth inhibitory effects were analyzed by Student's *t*-test. The data on antitumor effects and body weights were analyzed by the Wilcoxon rank sum test. All data are shown as the mean value at each time point. All tests were two-sided and *p*-values <0.05 were considered statistically significant. Experiments *in vitro* were performed in triplicate and repeated on at least three separate occasions. Statistical analysis was carried out using Stat View 5.0 (SAS Institute, Inc., Cary, NC, USA).

Results

Characteristics of PTX incorporated into EGF-conjugated PMBN. Two mg of PTX could be completely dissolved in 1.0 mL of polymer solution containing 100 mg/mL of PMBN (data not shown). The reaction rate of EGF with PMBN in this study was 8.4% according to measurements of *p*-nitrophenol absorption (Figure 1). The diameter of the EGF-PMBN-PTX particles was 50-75 nm according to the DLS measurements (data not shown).

Cytotoxicity of PTX incorporated into EGF-conjugated PMBN. The cytotoxicity of EGF-PMBN-PTX for A431 cells was significantly greater than that of PMBN-PTX (*P*-values at 0.01 nM, 0.1 nM, 1.0 nM and 10 nM of PTX were <0.001 and at 100 nM, 0.026). The 50% inhibition concentration (IC_{50}) of EGF-PMBN-PTX was also lower than that of PMBN-PTX (0.9 nM and 2.4 nM, respectively). Similarly, EGF-PMBN-PTX-mediated cytotoxicity for BT-20 cells was significantly greater than PMBN-PTX (*P*-values at 0.1 nM, 1.0 nM and 10 nM of PTX were <0.001; at 0.01 nM, 0.038; and at 100 nM, 0.029). The IC_{50} of EGF-PMBN-PTX was also lower than that of PMBN-PTX (1.1 nM and 8.1 nM, respectively) (Figure 2A and 2B). On the other hand, there were no significant differences in the growth inhibitory effects of EGF-PMBN-PTX and PMBN-PTX on the H69 cell line (Figure 2C).

Antitumor effects in vivo of PTX incorporated into EGF-conjugated PMBN in mice. In agreement with the *in vitro* data, growth of A431 but not H69 tumors was inhibited to a greater extent by EGF-PMBN-PTX than PMBN-PTX in mice ($p<0.001$) (Figure 3A and 3B). No adverse effects such as weight loss were observed in either group (Figure 4).

Discussion

In the present study, EGF was conjugated to PMBN incorporating PTX in order to develop a targeted therapy against tumor overexpressing EGFR. The cytotoxicity and antitumor effects of EGF-PMBN-PTX for cancer cells overexpressing the EGFR were significantly greater than that of PMBN-PTX *in vivo* and *in vitro*. In contrast, there was no difference between them for EGFR-deficient cancer cells.

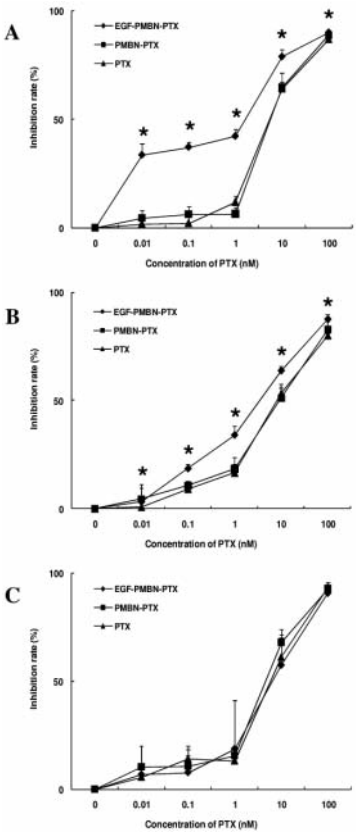

Figure 2. *Growth inhibitory effects of PTX incorporated into EGF-conjugated PMBN. A, Cytotoxicity of EGF-PMBN-PTX was significantly greater than that of PMBN-PTX for A431 cells (P-values at 0.01 nM, 0.1 nM, 1.0 nM and 10 nM of PTX <0.001; at 100 nM, 0.026). B, Cytotoxicity of EGF-PMBN-PTX was significantly greater than that of PMBN-PTX for BT-20 (P-values at 0.1 nM, 1.0 nM and 10 nM of PTX <0.001; at 0.01 nM, 0.038; and at 100 nM, 0.029). C, No significant difference in growth inhibitory effects of EGF-PMBN-PTX or PMBN-PTX on the H69 cell line. *p<0.05, significant difference compared with PMBN-PTX (Student's t-test).*

PTX has become an important agent in chemotherapy of breast, ovarian, head and neck and non-small cell lung cancers, as well as sarcoma. One problem associated with its clinical use has been the frequent presence of HSRs and neuropathy because of the content of CrEL (17, 18). Therefore, to prevent these undesirable side-effects, efforts have been made to develop new PTX formulations that do not require CrEL as a solubilizer. Over the past few years, significant progress has been made in the development of alternative formulations of PTX. The approaches used thus far include cosolvents, liposomes and various conjugates such as a polymer and albumin.

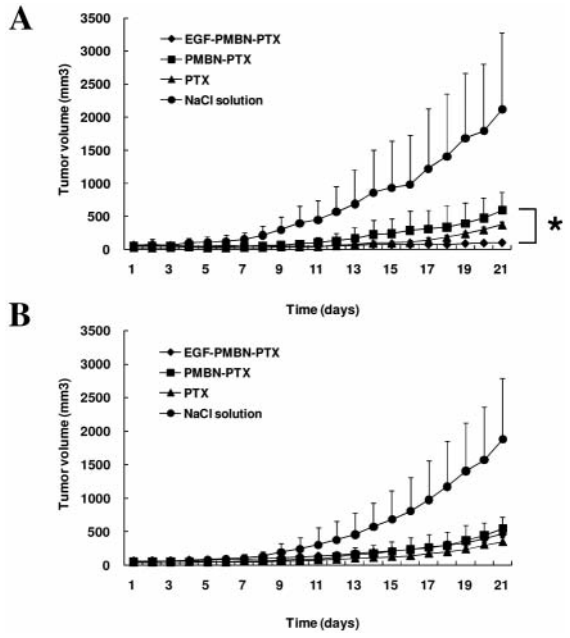

Figure 3. *Antitumor effects of PTX incorporated into EGF-conjugated PMBN in vivo in mice. A, Tumor growth suppression of EGF-PMBN-PTX was significantly greater than that of PMBN-PTX for A431 tumors. B, No significant difference in antitumor effects between EGF-PMBN-PTX and PMBN-PTX on H69 tumors. *p<0.001, significant difference compared with PMBN-PTX (Wilcoxon rank sum test).*

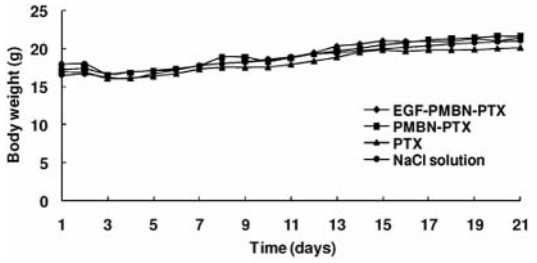

Figure 4. *Changes in body weight. Weight losses were not observed in any group.*

Liposomal PTXs have common features of favorable toxicity profiles and targeted drug delivery to tumor sites (19-21). Results with liposomal PTX were encouraging with respect to reducing toxic side-effects. PTX encapsulated in cationic liposomes improved antitumor efficacy by selectively targeting tumor vessels. Genexol-PM, a polymeric micelle-formulated PTX free of CrEL has also been administered safely, without HSRs and with a favorable toxicity profile (22). ABI-007, a human albumin-conjugated PTX, was also well tolerated and mediated some antitumor responses, even in patients who had had prior PTX therapy (23). Compared with conventional PTX, ABI-007 has been reported to be more effective in terms of antitumor activity in metastatic breast cancer (24). In phase I studies, Genexol-PM and ABI-007 displayed a 3-fold increase in the MTD (maximum tolerated dose) and a significantly increased antitumor efficacy (22, 23).

Large particulates (>1 μm in diameter) are rapidly cleared by the reticuloendothelial system (RES) (25), but smaller particles have prolonged half-lives in the circulation and an increased number of passes through the tumor vasculature, which facilitates more effective targeting. In addition, the tumor vasculature exhibits an enhanced permeability and retention (EPR) effect (26). Polymeric aggregate delivery systems such as ABI-007 and Genexol-PM are considered to be additionally advantageous because of their nanoscopic sizes (ABI-007, 130 nm; Genexol-PM, 10-200 nm) and preferential tumor distribution (27, 28). On this basis, MPC polymer might also be expected to have an EPR effect and be available for selective delivery by passive targeting to tumor sites since the size of EGF-PMBN-PTX particles is 50-75 nm. These would not be taken up by RES similarly to ABI-007 and Genexol-PM.

Owing to the presence of the active ester group, PMBN is available for conjugation to any materials with ester bonds. Interleukin-2 (IL-2) has been previously conjugated to MPC polymer. The inhibitory effect of the PMBN with IL-2 incorporating PTX was higher than that of the unconjugated version for adult T-cell leukemia cells expressing high affinity IL-2 receptors (29). PMBN offers the possibility of active targeting, unlike liposomal PTXs, ABI-007 or Genexol-PM. PMBN is superior to other solvents because of these two excellent properties (both active and passive targeting).

In addition, cationically modified MPC polymer is suitable for incorporating genes (30). A nonviral vector has also been developed, which is a cationically modified MPC polymer conjugated to hepatitis B surface (HBs) antigen. By using MPC polymer conjugated to HBs-containing plasmid (*sFlt-1* or *GFP*), specific expression of the *sFlt-1* or *GFP* was achieved in human hepatocellular carcinoma cells (HepG2) *in vitro* and *in vivo* (31).

It has previously been reported that the PTX incorporated into MPC polymer displayed a >3-fold increase of the MTD compared with the conventional PTX and that 100% of mice survived after administering a dose as high as 100 mg/kg, whereas the same dose of conventional PTX was uniformly lethal. S.c. injection of PTX incorporated into MPC polymer did not cause any macroscopic or microscopic changes in the skin (32). These advantages might be attributed to the exclusion of CrEL and the controlled release of PTX. PTX incorporated into MPC polymer might enable rapid intravenous bolus injection without HSRs.

The presence of EGFR in normal cells might result in injury of normal organs. However, this possibility is very unlikely for the following reasons: i) the EGFR levels of

cancerous tissues are much higher than that of the adjacent normal tissues (33) and thus the conjugate is expected to target mainly the former while having little effect on the latter; ii) the occurrence of intracellular fenestrations in capillaries has been noted in many tumors (34), and the conjugate might more easily penetrate these vessels because of its small size; iii) the intercellular adhesion of cancer cells is impaired, compared with normal cells (35), allowing the conjugate to easily reach the cancer cell surface receptor; iv) early clinical trials of a TGFα-*Pseudomonas* exotoxin conjugate have already demonstrated the feasibility of targeting in this way (36).

Here it is reported that EGF stimulates the growth of A431 tumor cells *in vivo* but inhibits proliferation of the A431 cell line *in vitro* (37). The inhibitory effect of EGF on A431 proliferation was also observed in this study. ANOVA analysis revealed a synergy in the cytotoxicity mediated by EGF and PTX against A431 cells (data not shown). It has also previously been reported that there is a positive correlation between the effect of EGFR-targeted therapy and EGFR expression (8, 38, 39), as well as the growth inhibitory effect of RNase-EGF fused proteins and their internalization into cells overexpressing EGFR, which was visualized by fluorescence microscopy (39). These results suggested that PTX was internalized into the cell because EGF-PMBN-PTX binds the EGFR and the EGF-EGFR complex is internalized into the cell in the normal manner.

In conclusion, EGF-PMBN-PTX showed a selective growth inhibitory effect against EGFR-overexpressing cancer cells *in vitro* and *in vivo*, which is considered to be receptor-mediated. These results suggest that EGF-PMBN-PTX may represent a novel potent targeted therapy for tumor overexpressing the EGFR. MPC polymer might be exploited in various targeted therapies by conjugating and incorporating different agents.

Acknowledgements

The authors thank Otoe Suzuki for her assistance with experimental data acquisition.

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Received August 18, 2008
Revised December 2, 2008
Accepted January 26, 2009