

Water-soluble Taxol Conjugates with Dextran and Targets Tumor Cells by Folic Acid Immobilization

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Abstract. *Background: Paclitaxel (taxol) is a very effective anticancer drug. However, the solution used to enhance the water solubility of paclitaxel, a mixture of polyoxyethylene castor oil (Cremphor EL) and dehydrated ethanol, causes irritation. Our aim is to develop a water-soluble taxol with a high anticancer effect. Materials and Methods: Taxol derivatives (conjugation with aminated dextran, Dex-TXL) were synthesized for the water-solubilization of taxol and folic acid (FA) was ionically and covalently conjugated with Dex-TXL. In addition, their anticancer activity was investigated in vitro with KB cells (a folate receptor-overexpressing carcinoma cell line of the oral cavity). Results: The high solubility of Dex-TXL conjugates (2700 times higher than that of intact taxol) needed no additional Cremophor EL or dehydrated ethanol. Dex-TXL showed 2-3 times greater anticancer effect when conjugated with FA. Conclusion: These results suggest that conjugation with dextran and FA could bring about an improvement in taxol anticancer therapy.*

Paclitaxel (taxol) is an anticancer drug used for lung, breast and ovarian tumors (1). Due to its poor water solubility, taxol is generally administered as a mixture with poly(oxyethylene)ed castor oil (Cremophor EL) and dehydrated ethanol (2). Cremophor EL causes serious side-effects, such as irritation, in approximately 30% of patients (3); therefore, a steroid drug is required prior to its use to suppress side-effects (4). To avoid the side-effects of Cremophor EL, the solubility of taxol is enhanced with poly(L-glutamic acid) (5) and albumin (6), and drug delivery systems such as liposome, polymer micelles, and nanoparticles have been used to enhance its water solubility and anticancer efficacy by systematic delivery (7, 8).

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Liposomes are microspheres consisting of lipid bilayer membranes that can hold a hydrophobic medicine in the interlayer and a water-soluble drug inside the spheres. In this way, the lipid bilayer provides a suitable microenvironment for solubilization of hydrophobic drugs. Liposome and polymer micelles have been widely investigated and some additional side-effects have been reported from their use (9).

Dextran is a natural polysaccharide that is highly compatible with the human body and has been clinically used as a substitute for blood plasma (10). Water-soluble taxol micelled by conjugation with dextran has enhanced permeability and retention (11). However, it is difficult to sterilize a micelled system and produce it in bulk quantities, so functional liposomed or micelled drugs, such as those that are pH or thermo-sensitive, require complicated synthesis and preparation protocols, and it is difficult to make these drugs in a uniform and reproducible manner (12). Conjugation of taxol with poly(ethylene glycol) (PEG) has been investigated to obtain highly water-soluble prodrugs; however, this procedure leads to a decrease in anticancer efficacy compared with intact taxol (13). Therefore, we explored an active drug targeting system (14).

Folic acid (FA), which is a water-soluble vitamin and plays an important role in cell proliferation, has also been used for targeting in micelle and liposome systems (15, 16). Overexpression of the FA receptor in some cancer cells such as ovary, brain carcinomas (17), and a human oral cancer cell line (KB) has been reported (18).

In this study, taxol was modified with dextran to enhance its water solubility and FA was immobilized for targeting by ionic adsorption and covalent bonding conjugation. The efficacies of the drugs against cancer cells were studied in detail.

Materials and Methods

Paclitaxel (taxol) was kindly provided by Samyang Genex Corporation (Korea). Dextran (MW 70,000) was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan). Folic acid (FA), dimethyl sulfoxide (DMSO), 1,1'-carbonyldiimidazole (CDI), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2,4,6-trinitrobenzenesulfonic acid

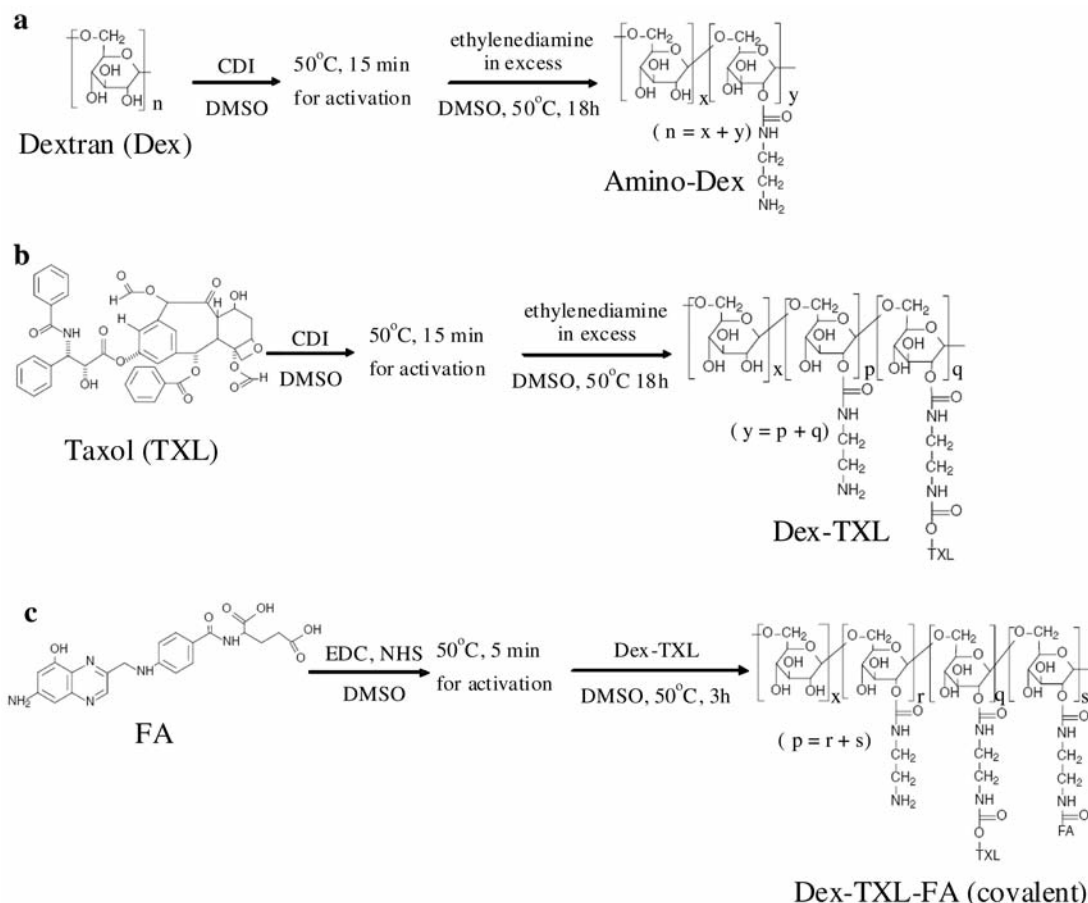


Figure 1. Synthetic scheme of (a) amino-Dex, (b) Dex-TXL and (c) Dex-TXL-FA (covalent).

(TNBS), ethylenediamine, and fluorescein isothiocyanate (FITC) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and used without further purifications. KB (DSP, 03-128), Caco2 (DSP, 04-037) and L929 (DSP, 03-439) cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). MA104 (RCB0994) cells were purchased from RIKEN Bioresource Center (Tsukuba, Japan). All cells were used for an experiment within five passages.

Synthesis of amino-Dex and TNBS assay. Dextran (10 g) was dissolved in 70 ml DMSO and mixed with a solution of 2 g CDI in 5 ml DMSO, and the activation reaction proceeded at 50°C for 15 min. Subsequently, 5 ml of ethylenediamine were added and the mixture was stirred at 50°C for 18 h as summarized in Figure 1(a). After dialysis against running water for 24 h and distilled water (3 L, 1.5 h × 2) with a dialysis membrane (cut-off molecular weight of 14,000 Da), amino-Dex was recovered by air and vacuum drying. The amino group content in the amino-Dex was determined by TNBS assay (19). Briefly, 0.3 ml of the 165 µg/ml aqueous amino-Dex solution, 2 ml of 4 w/v% NaHCO₃ containing 1 w/v% SDS (pH=9), and 1 ml of 0.1 w/v% TNBS aqueous solution were placed in a glass tube. After 2 h incubation at 37°C, the absorbance at 335 nm was measured using a UV spectrophotometer (HITACHI, U-2800A, Tokyo, Japan). Glycine was selected as the standard.

Taxol conjugates with dextran (Dex-TXL). To activate the taxol OH group, a solution of 0.7 g taxol in 40 ml DMSO was added to a solution of 0.6 g CDI dissolved in 10 ml DMSO at 50°C for 15 min. Five grams of amino-Dex in 150 ml of DMSO were added to the solution, and the reaction proceeded at 50°C for 18 h. Dex-TXL was recovered by the same purification manner as described above. The yield of Dex-TXL was approximately 70%. The efficacy of introducing taxol into Dex-TXL was evaluated by UV absorption at 260 nm using a standard curve with intact taxol. The reaction is summarized in Figure 1(b).

Solubility of taxol conjugates in water. Taxol, and Dex-TXL were dissolved in DMSO at a concentration of 1 w/v% and 1 ml of these solutions was separately mixed with 9 ml of water to check their solubility in water.

FA adsorption onto amino-Dex. FA adsorption onto the amino groups in amino-Dex was evaluated using a dialysis membrane (cut-off molecular weight of 6,000-8,000). Ten ml of PBS with 0.5 g amino-Dex were put into a dialysis membrane and soaked in 40 ml of PBS containing 0.1 g FA at 25°C for 18 h. The FA concentration in the outer portion of the PBS was recorded by measuring the absorbance at 360 nm, and the amount of FA-adsorbed dextran was calculated.

Dex-FA uptake by KB cells. To investigate the uptake of amino-Dex by KB cells, FITC labelled amino-Dex (Dex-FITC) was prepared as follows. In one glass vial, amino-Dex and FITC were dissolved in DMSO at a concentration of 10 w/v% and 0.075 w/v%, respectively, and stirred at 50°C for 1 h, followed by reprecipitation of Dex-FITC with excess acetone and vacuum drying. Recovered Dex-FITC and FA were dissolved in phosphate buffered saline (PBS) at a concentration of 800 and 240 µg/ml, respectively. A solution of 0.5 ml Dex-FITC was simply mixed with 0.5 ml of FA and stirred at 25°C for 18 h to prepare FA-adsorbed Dex-FITC (Dex-FITC-FA).

KB (a human oral carcinoma cell line) cells were seeded onto 6-well tissue culture plates at 3×10^4 cells/well 24 h prior to adding the Dex-FITC-FA. After 4-day incubation at 37°C in FA-free medium, the cells were washed twice with PBS and fluorescent images were observed with a fluorescence microscope (KEYENCE, Biozero BZ-8000, Osaka, Japan) at wavelengths of 480 (excitation) and 520 nm (emission). The same experiment was conducted with a medium containing 1 mM of FA to determine FA receptor blocking.

Synthesis of covalently conjugated Dex-TXL-FA (Dex-TXL-FA [covalent]). A solution of 0.1 g FA and 0.15 g NHS in 20 ml DMSO was mixed with 0.07 g of EDC in 10 ml DMSO, and the activation reaction of the FA COOH groups proceeded at 50°C for 5 min. Subsequently, 0.5 g of Dex-TXL in 10 ml DMSO was added to the mixture and reacted at 50°C for 3 h. Dex-TXL-FA (covalent) was recovered after purification by dialysis against water and drying. FA content in the conjugation was determined by measuring absorbance at 360 nm. The solution was dialyzed against PBS (2 L, 8 h \times 6) to separate the adsorbed FA from the conjugation. Dex-TXL-FA (covalent) was recovered after further dialysis against distilled water and drying. FA content in the Dex-TXL-FA (covalent) was checked by measuring absorbance at 360 nm. The reaction scheme is given in Figure 1(c).

Cytotoxicity of taxol conjugates. KB, Caco2 (a human colon carcinoma cell line), MA104 (a monkey fetal kidney cell line) and L929 (a normal mouse cell line) were used for the evaluation, and showed different FA receptor expression. RPMI-1640 (without FA) supplemented with 100 µg/ml of penicillin-streptomycin and 10% fetal bovine serum was used for the culture of all cell lines. After seeding the cells onto 96-well culture plates at 10^3 cells/well in 0.1 ml medium, the cells were incubated at 37°C and 5% CO₂ for 24 h prior to adding 0.1 ml of medium containing the taxol conjugates. After 3-day incubation with different concentrations of conjugates, 0.1 ml of neutral red (NR, 150 µg/ml in medium) solution was added to each well and the cells further incubated for 6 h. After discarding the medium and fixing the cells with 1.0 v/v% glutaraldehyde aqueous solution containing 1.0 w/v% CaCl₂ for 1 min, the culture plates were rinsed with water and air dried. NR taken up by living cells was extracted with 0.1 ml of 1.0 w/v% acetic acid in a mixture of ethanol/water (50/50 by volume), and the absorbance was measured at 541 nm with a microplate reader (Versamax, Molecular Devices Co., Sunnyvale, CA, USA). The IC₅₀ was the drug concentration that caused 50% inhibition of the control growth rate.

Results

Synthesis of amino-Dex and TNBS assay. The concentration of the amino group introduced into dextran was evaluated by the TNBS method. The degree of introduction was easily

controlled by the CDI concentration, which activates OH groups in dextran, and the higher the concentration of CDI, the higher the conversion. For instance, the degree of introduction was 1.1, 4.2, 6.6, and 10.0% in dextran sugar units (DSU) when the CDI concentration was 2.9, 11.4, 17.1, and 28.6 mg/ml in the reaction, respectively. The reaction conditions produced 0.5 mol of OH groups in dextran for each mol of CDI added, and the low efficacy might be due to the impurity of CDI, dextran, or DMSO. In this reaction, the maximum degree of introduction was 10.0% and adding more CDI caused the dextran to form a gel. Therefore, 10% amino-Dex was selected for taxol conjugation.

Taxol conjugation with amino-Dex (Dex-TXL). The taxol introduction into dextran was evaluated by the UV absorption of taxol. The degree of introduction was 1.2, 2.3, and 5.1% DSU when the taxol concentration was 3.5, 7.0, and 14.0 mg/ml in the reaction, respectively. Compared with adding the amino group into dextran, taxol conjugation with amino-Dex using CDI had a lower efficiency, and only 50% of the taxol could be introduced into the amino-Dex even when 4.5 times excess (molar ratio) CDI was added to taxol. Seven replications of the taxol introduction at a concentration of 3.5 mg/ml produced an average concentration of $1.2 \pm 0.2\%$ DSU, suggesting high reproducibility for Dex-TXL synthesis. Furthermore, the Dex-TXL synthesis required a minimum number of reagents and short reaction times, which made it easy to prepare on a large scale. Syringe filter of 0.2 µm was easily used to sterilize the Dex-TXL.

Solubility of Dex-TXL in water. Dex-TXL was dispersed in water, and the solubility results are shown in Figure 2. Taxol was highly hydrophilic, colorless, and transparent when conjugated with amino-Dex. In contrast, there was a white precipitate when intact taxol was mixed with water. Dex-TXL dissolved not only in distilled water but also in PBS and culture medium without DMSO, which was used only to disperse intact taxol. Furthermore, 2.3 and 5.1% Dex-TXL also dissolved in these media (data not shown).

FA adsorption onto amino-Dex. Figure 3 shows FA adsorption onto amino-Dex as a function of introducing amino groups into dextran. FA adsorption was strongly dependent on the amino group concentration that was introduced, and a higher amino group concentration resulted in higher adsorption. In addition, almost one mol of FA was adsorbed onto one mol of amino groups when 6.6% amino groups were introduced, suggesting that one FA carboxylic group was ionically adsorbed onto one dextran amino group. Of course, adsorption is an equilibrium event, and the degree of adsorption or desorption is directly affected by many environmental conditions such as FA and amino-Dex concentrations, pH, and

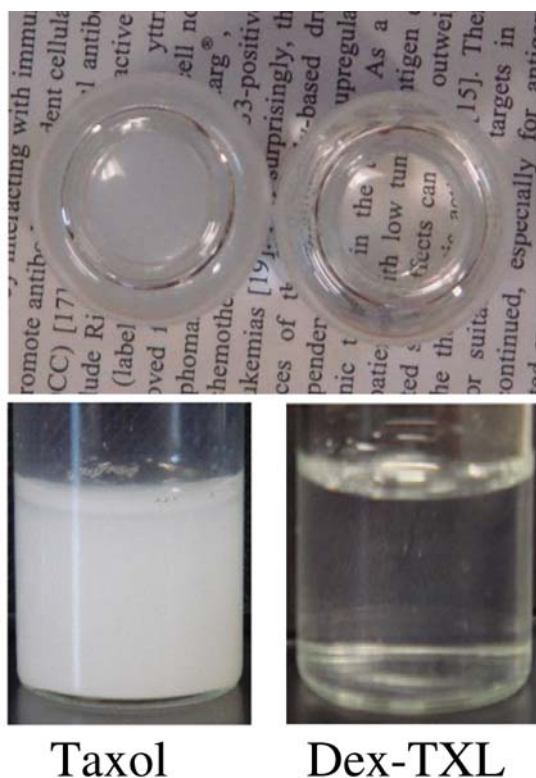


Figure 2. Solubility of Dex-TXL in water. Taxol and Dex-TXL were dissolved in dimethyl sulfoxide at a concentration of 1 w/v%, and 1 ml of the solution was mixed with 9 ml of distilled water. Dex-TXL with 1.2% taxol in dextran sugar units was used for this experiment.

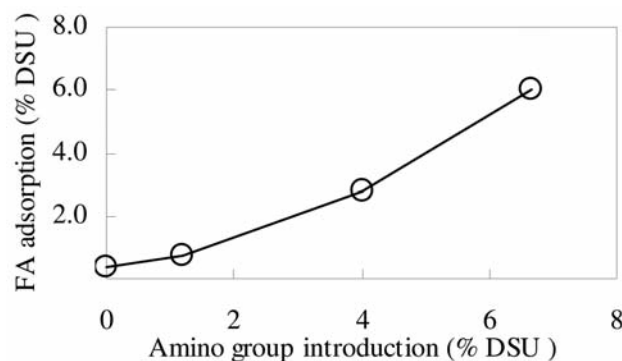


Figure 3. FA adsorption onto amino-Dex, in which the amino-Dex and FA concentration were 0.62 and 4.52×10^{-3} M, respectively. DSU, Dextran Sugar Unit.

temperature of the solutions. Only the concentrations of FA and amino-Dex in PBS were quoted as the content of the mixed solution in cell culture experiments.

Dex-FA uptake by KB cells. The uptake of FA-adsorbed Dex-FITC by KB cells was observed with a fluorescence microscope, and the results are given in Figure 4. Cell nuclei were clearly stained by FITC when cultured with Dex-FITC-FA (Figure 4B). In contrast, there was no FITC staining in the Dex-FITC (Figure 4A) and Dex-FITC-FA (Figure 4C) in which 1 mM FA was added before adding the Dex-FITC-FA, indicating that FA markedly enhanced the uptake of dextran molecules.

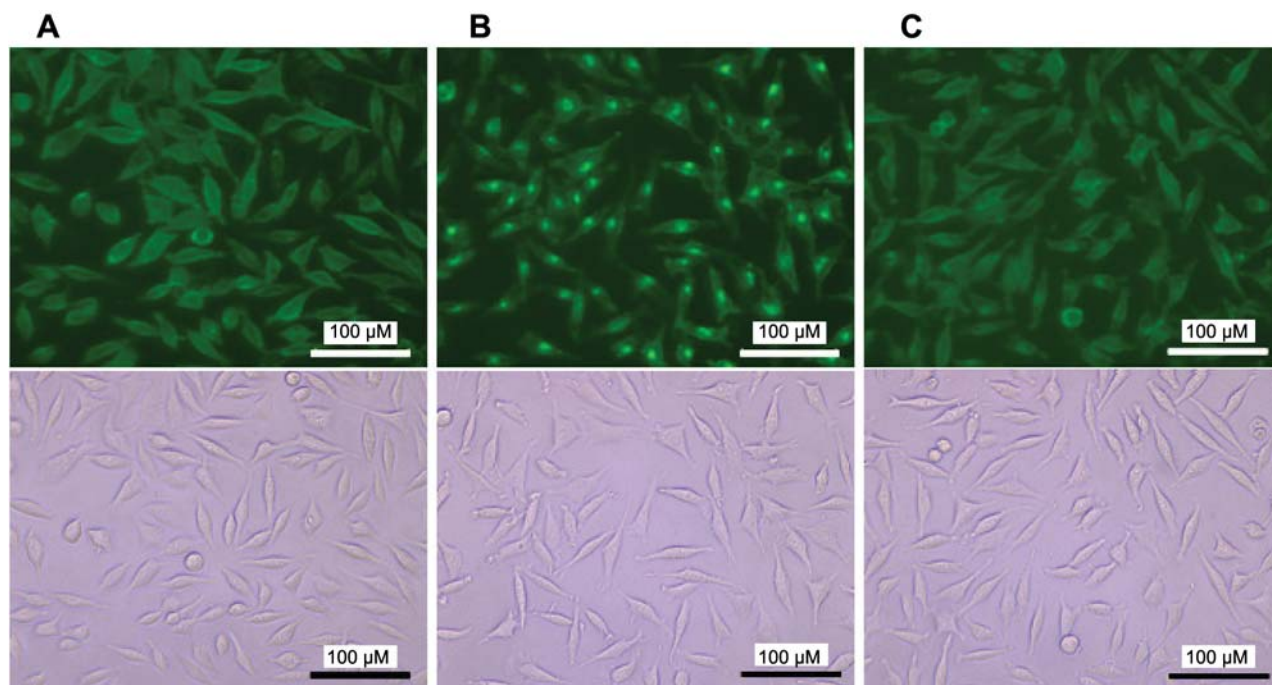


Figure 4. Uptake of FA-adsorbed Dex-FITC by KB cells. KB cells were cultured in (A) Dex-FITC, (B) Dex-FITC-FA, and (C) Dex-FITC-FA with 1 mM of FA.

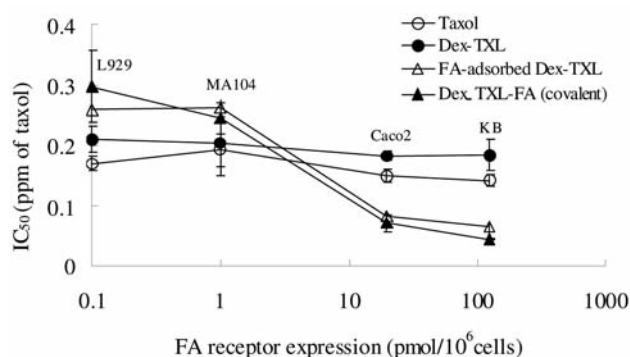


Figure 5. Effect of FA receptor expression in various cell lines on Dex-TXL cytotoxicity. Dex-TXL contained 1.2% of taxol in dextran sugar units. FA-adsorbed Dex-TXL contained 1.2% of FA and Dex-TXL-FA (covalent) was conjugated with 1.0% of FA.

Synthesis of Dex-TXL-FA [covalent]. Dex-TXL-FA (covalent) was prepared to compare its anticancer effect with that of FA-adsorbed Dex-TXL. The introduction of FA into Dex-TXL was evaluated by measuring the UV absorption of FA. The degree of introduction was about 1.5% DSU when the FA concentration was 2.5 mg/ml in the reaction mixture. FA concentration in the dialysis tube containing Dex-TXL (covalent) leached to a constant value of around 1.0 mg/ml after 48 h when the initial FA concentration in the tube was 1.7 mg/ml and the outer solution was PBS (2 l). In contrast, in the case of FA-adsorbed Dex-TXL, the concentration rapidly decreased almost zero within 24 h due to the desorption of FA from dextran in PBS. These results suggest that FA was successfully conjugated with Dex-TXL *via* a covalent bond. Dex-TXL-FA (covalent) was easily sterilized with 0.22- μ m syringe filter.

Cytotoxicity of Dex-TXL to KB cells. Dex-TXL cytotoxicity to KB cells was evaluated by NR assay, and the results are given in Table I A. Dex-TXL (1.2 and 2.3%) showed the same level of toxicity as that of intact taxol. However, introducing additional taxol into the dextran resulted in lower cytotoxicity, which might have been due to a difficulty in conjugate uptake (5.1% introduction).

FA adsorption onto Dex-TXL and cytotoxicity. Dex-TXL containing 1.2% of taxol and FA were separately dissolved in PBS, and the same volume of each solution was mixed and stirred at 25°C for 18 h. When the FA concentration was 7.13×10^{-3} , 3.56×10^{-2} , and 3.56×10^{-1} mM, the FA added was 0.2, 1.2, and 11.6% DSU, respectively. The cytotoxicity of FA-adsorbed Dex-TXL is summarized in Table I B. The highest cytotoxicity was observed when 1.2% FA was added. A lower concentration seemed to be ineffective for KB cells to recognize the FA (0.2%). In contrast, additional FA

Table I. Cytotoxicity[†] of Dex-TXL and FA-adsorbed Dex-TXL to KB cells.

A Dex-TXL

Sample	% Introduction of taxol*	IC ₅₀ (ppm) [‡]
Intact taxol		0.165±0.009
Dex-TXL	0	>1.0×10 ⁴
	1.2	0.177±0.007
	2.3	0.151±0.031
	5.1	0.330±0.051

B FA-adsorbed Dex-TXL[‡].

FA added*	IC ₅₀ (ppm)
0	0.141±0.016
0.2	0.135±0.015
1.2	0.042±0.004
11.6	0.158±0.028

*in dextran sugar units. [†]data are average±S.D. (n=8); [‡]Dex-TXL of 1.2% introduction was used. IC₅₀: 50% inhibitory concentration.

adsorption caused an increased desorption of FA, which was easily recognized by KB cells, leading to a low anticancer effect (11.6% FA added).

FA receptor expression and cytotoxicity of Dex-TXL. Four different cell lines (L929, MA104, Caco2, and KB) with different degrees of FA receptor expression were selected, and the effect of FA on Dex-TXL cytotoxicity was evaluated. The results are shown in Figure 5 as a function of FA receptor expression. Without FA, there was no clear difference in cytotoxicity among the cell lines, and the IC₅₀ was constant at approximately 0.2 ppm (taxol and Dex-TXL). In contrast, the extent of FA receptor expression considerably affected cytotoxicity of taxol when coupled with FA (adsorbed and covalent). Higher FA receptor expression resulted in higher taxol cytotoxicity, and the cytotoxic effects on KB cells were approximately 2 (adsorbed) and 3 times (covalent) greater than without FA.

Discussion

Many studies aimed at avoiding the use of Cremophor EL in taxol solutions due to a side effect (20). The solubility of taxol in water was ~80 mg/ml for Dex-TXL (5.1% introduction), which is approximately 2,700 times higher than that of intact taxol (21) and 14 times higher than that of clinical taxol containing Cremophor EL (6 mg/ml) (2). In addition, the solubility of Dex-TXL was far higher than that of taxol in an organic solvent or prodrug delivery system (2,

22). Recently, PEG was used as a linker molecule with several drugs and other polymers (23). Dex-TXL showed a high dissolubility in comparison with PEG which was a popular material. Generally, PEG has one or two OH groups in its terminal, which limits the number of medicines that can be conjugated in a molecule. In contrast, dextran has many OH groups, which makes it easy to enhance the water solubility of drugs and produce a drug-targeting effect.

The cancer targeting effect of the FA improved with anticancer activity for the taxol uptake by the cancer cells. In general, taxol taken up into Caco2 cells is released by a P-glycoprotein pump function in the cell membrane (24). Results of the effect of FA on Dex-TXL cytotoxicity suggest FA-adsorbed or FA-covalent Dex-TXL effectively escaped the pump function system, perhaps due to FA receptor mediated endocytosis (25, 26). There was almost no FA effect in the L929 and MA104 cells due to their poor FA expression. The difference in toxicity between FA-adsorbed and FA-covalent Dex-TXL might be due to the difference in the amount of desorbed FA, as shown in Table I B. These results suggest that the extent of FA receptor expression required for FA targeting was approximately 20 pmol/10⁶cells.

Direct conjugation of taxol with FA and its lower toxicity and poor targeting effects have been reported (13). In addition, amino-Dex (0% taxol) itself induced almost no cytotoxicity, whereas some drug carrier micelles cause toxicity (27). And it was reported that taxol toxicity decreased 50 times when conjugated with PEG (28). Adsorbed drug systems similar to FA-adsorbed Dex-TXL have been reported (29). For example, methotrexate (MTX) is an FA antimetabolite and anticancer drug with a similar structure to FA (30). The positive charge of gold nanoparticles (AuNP) and the negative charge of the MTX carboxyl group produces MTX-adsorbed AuNP, which has enhanced anticancer effects *in vitro* and *in vivo* (29).

In summary, taxol conjugated with dextran produced a highly water soluble taxol with no decrease in anticancer efficacy following conjugation. FA conjugation with taxol *via* adsorption and covalent bonding showed excellent targeting effects.

In our next study, we will apply FA conjugated Dex-TXL to *in vivo* tumor models.

The enhanced permeability and retention effect (31) have been already determined by using Dex conjugation with drug (32). As for the accumulation in the cancer organization, molecular size is important, and Dex-TXL showed approximately 100nm by the particle size measurement by the dynamic light scattering method (DLS).

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