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Specific Targeting of Fluorescein Isothiocyanate with Ep-CAM Antibody (Specific targeting of FITC with Ep-CAM Antibody)

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The tetradecameric peptide (K47-K60) near the NH_2-terminal region of epithelial-cell adhesion molecule (Ep-CAM) was chosen as antigenic site and a polyclonal antibody was generated, which could recognize Ep-CAM from the mouse colon tissue or the colon cancer cell, CT-26, in Western blot analysis. Then, the fluorescein isothiocyanate (FITC), a fluorescence dye, was conjugated with the affinity purified Ep-CAM antibody using thiocyanate and the amino groups of FITC and antibody, respectively. The molar ratio of FITC to antibody was estimated approximately 1.86 to 1.00 by measuring the optical densities at 492 nm and 280 nm. Ep-CAM antibody-FITC conjugate was then used for immunohistochemistry of the CT-26 cells. Judging from the shapes formed by fluorescence, the Ep-CAM antibody could delivered FITC to the surface of cells in which Ep-CAM was expressed. This result implies that Ep-CAM antibody could be also used for the tissue-specific delivery of the photosensitizer to the target protein *via* antigen-antibody interaction.

key word: photosensitizer, photodynamic therapy, Ep-CAM antibody, FITC

INTRODUCTION

The photodynamic therapy (PDT) is one of the experimental approaches for cancer treatment. It is now becoming popular in the treatment for cancers especially in the region of head and neck, in which the local or systemic delivery of a photosensitizer is followed by tissue illumination by laser with an appropriated wavelength to induce the photo-oxidation damages in tumor sites. The properties of photo-sensitizers play a key role in PDT. Currently a half of all patients diagnosed with squamous cell cancers in the head and neck region recur after PDT. The reasons could be explained as follows: (i) the targeting is not specific enough for tumor cells to get the sufficient amounts of photosensitizer for treatment, or (ii) the binding of photosensitizer to tumor cells is not strong enough so that the photosensitizer tends to diffuse out before it gets illuminated. Although the conventional photosensitizers may have a tendency to be selectively accumulated in tumors, the increased tumor targeting could be obtained by use of macromolecular carriers that form complexes or covalent conjugates with photosensitizers [1, 2]. The targeting capability of these carriers may rely on physical and biological properties of them.

An antibody generated against epithelial-cell adhesion molecule (Ep-CAM) was used as a carrier protein in this study [3-5]. The Ep-CAM is a 40 kDa glycoprotein and a homophilic cell-to-cell adhesion molecule [6]. In most tissues, the enhanced expression of Ep-CAM is associated with active proliferation, whether normal or neoplastic. In squamous epithelia, *de novo* expression of Ep-CAM is related to neoplastic changes [7]. The gene for Ep-CAM, located in chromosome 4 in human, consists of 9 exons [8]. The exons 1, 2-6, and 7 encode the signal peptide, extracellular and the transmembrane domains of Ep-CAM, respectively, whereas the exons 8-9 encode the cytoplasmic domain. The extracellular domain can be a target region for the generation of antibody.

In this research the antibody of Ep-CAM, purified with protein A affinity column chromatography, was conjugated to a fluorescein isothiocyanate (FITC), the fluorescence dye, using a thiocyanate group and the molar ratio of FITC to immunoglobulin G (IgG) was estimated spectrophotometrically. Then, the IgG-FITC conjugate was used for the immunohisto-chemistry to investigate the tissue-specific targeting of photo-sensitizer to the tumor cells [9]. The results of this experiment could be beneficial to develop the tissue-specific delivery of photosensitizer in PDT in the near future [10, 11].

MATERIALS AND METHODS

Generation of Ep-CAM Antibody

The hydropathy plot analysis of the extracellular domain of Ep-CAM was carried out and the peptide fragment spanning K47 to K60 near the N-terminal region was chosen for the antigenic determinant based on the hydrophilic property (Fig. 1). The 14mer peptide was then ordered for synthesis to the company named Peptron, and purified by High Performance Liquid

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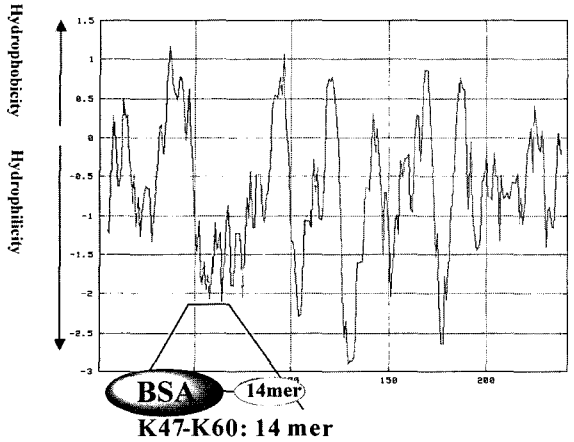

Figure 1. Hydropathy analysis of the extracellular domain of the mouse Ep-CAM. The y and x axes indicate the hydrophobicity value and the amino acid residue number of Ep-CAM, respectively. The hydropathy index of each amino acid residues are adapted from Einsenberg *et al.*, 1982.

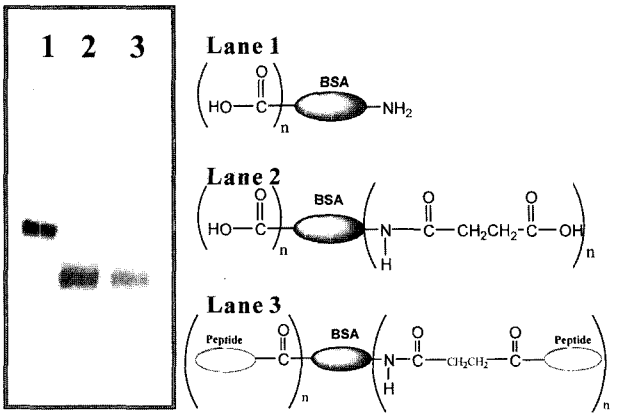

Figure 2. Non-denatured polyacrylamide gel electrophoresis analysis fo BSA-peptide conjugate. The lanes are as follows: the lanes 2 and 3 are BSA-SA conjugate and BSA-SA-Peptiede conjugate, whereas the lane 1 is BSA as a control.

Chromatography (HPLC : HP 1100 series LC/MSD). The chemically synthesized peptide as an antigen was con-jugated to bovine serum albumin (BSA), the carrier protein. The carboxylic group of BSA was first modified with succinic anhydride (Fig. 2, the lane 2) to enhance the coupling efficiency for 2 hrs at room temperature and the peptide was conjugated with the modified BSA for 4 hrs at room temperature while stirring (Fig. 2, the lane 3). The precipitate was removed by centrifugation and the unbound succinic anhydride was also dialyzed out. The final product, BSA-peptide conjugate, was separated and analyzed onto 10% Non-denatured polyacryl-amide gel electrophoresis (Native-PAGE). Three hundreds micrograms of BSA-peptide conjugate were injected sub-cutaneously into New Zealand white rabbit with Freunds adjuvant (complete). The next injections were performed every 2 weeks with Frueunds adjuvant (incomplete). The titer of antibody was measured with enzyme linked immunosorbent assay (ELISA).

Characterization of Antibody Generated against Ep-CAM

The protein extracts from both mouse colon tissue and CT-26 colon cancer cell were separated onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the apparatus, Xcell II™ (Novex, USA). The stacking and separating gels contained 4% and 12% of SDS, respectively. The separated proteins were visualized with Coomassie Brilliant Blue staining. Western blot immunoassay was performed. The proteins separated onto SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane for 90min at 125mA. The PVDF membrane was then incubated with the blocking buffer (5% non-fat dry milk) for 1hr at room temperature and with the peptide-directed Ep-CAM antibody for 2 hrs at room temperature. The

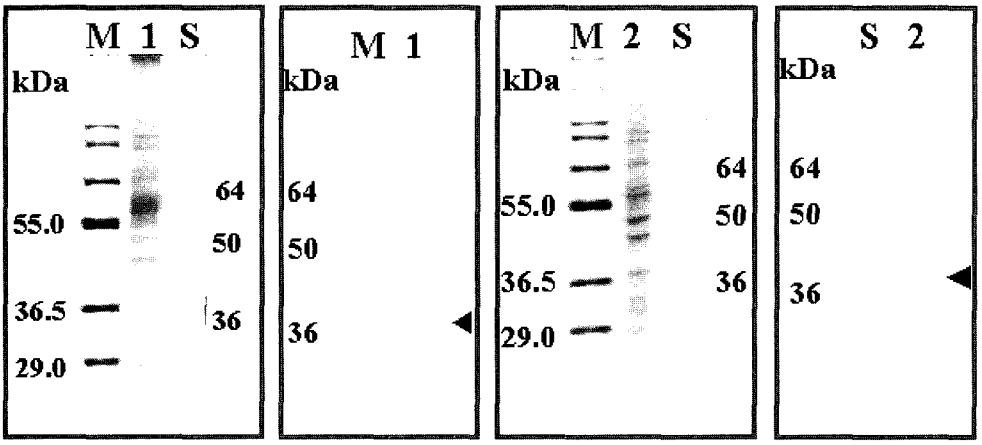

Figure 3. SDS-PAGE and Western blot analyses of the polyclonal antibody generated against BSA-Peptide. The lanes are as follows; M : protein size marker II (Tefco. Japan); S : SeeBlue™ Plus 2 Pre-Stained marker (Invitrogen, USA); lane 1 : colon tissue extracts (A); lane 2 : CT-26 colon cancer cell extracts (B); black arrowhead : Ep-CAM.

PVDF membrane was washed 3 times in a solution containing 0.1% (v/v) Tween 20, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8mM KH_2PO_4, pH 7.3, and incubated with the secondary antibody, a goat anti-rabbit antibody conjugated with horseradish peroxide (HRP) (diluted 1:10,000; KBL, USA) for 1hr at room temperature. The color development reaction was done with a 20 mg of 3,3-diaminobenzidine (Sigma, USA) and 0.015% hydrogen-peroxide in 50ml of the phosphate buffered saline (PBS).

Conjugation of FITC to Ep-CAM Antibody

The antibody generated against Ep-CAM was conjugated to FITC (Sigma, USA) according to Harlow and Lanes method [12]. The amino group of antibody reacted with the thiocyanate group of FITC. At least 2 mg/ml of anti-peptide antibody and 1 mg/ml of FITC were dissolved in 0.1 M sodium carbonate, pH 9.0, and in dimethyl sulfoxide (DMSO), respectively. Then, 500 µl of the FITC solution was added slowly to every 1ml of the antibody solution and the reaction was performed in the dark for 8 hrs at 4°C. Fifty milimoles of NH_4Cl were added and incubated for another 2 hrs at 4°C. The unbound FITC was removed by dialysis. The molar ratio of FITC to antibody was estimated by measuring the absorbance at 280nm and 492nm.

Immunohistochemistry of the Colon Cancer Cell with Ep-CAM Antibody-FITC Conjugate

The glass slide for coating was soaked for 10 seconds in a working solution containing 5ml of 3-aminopropyltriethoxysilane and 250 ml of dry acetone, and washed 5 times in distilled water. The glass slide was then dried at room temperature and examined according to the Wood and Elliss method [13]. The harvested colon cancer cell, CT-26, cohesive to the coated glass slide, was incubated for 15 min at 37°C, fixed with 100 % methanol (-20°C) for 15 min, and subsequently air-dried. The fixed cells were blocked with 5% casein in PBS for 1 hr at room temperature. The cell was washed for 30 min in PBS and then the Ep-CAM antibody-FITC conjugate was incubated for 1hr at room temperature. After washing in PBS for 30 min, the fluorescence image of FITC was observed with the fluorescent microscope.

RESULTS AND DISCUSSION

It is very important to deliver the photosensitizer specifically to target cells or tissues in PDT, which can be achieved by use of antigen-antibody interaction that is very strong and specific. Two factors have to be considered: (i) selection of the antigenic protein actively expressed in the cancer cell and (ii) development of the conjugation method of the photosensitizer to antibody. Ep-CAM is one of the tumor-associated marker proteins, actively expressed in the proliferating human malignant neoplastic tissues such as carcinoma of small intestine, colorectal adenocarcinoma, lung carcinoma, and adenocarcinoma of cervix. The extracellular domain of Ep-CAM is exposed to the extracellular space so that it could interact with its antibody if the space is allowed for. Therefore, the relatively hydrophilic segment, the peptide spanning Arg-47 to Arg-60 near the amino terminal region of the extracellular domain of Ep-CAM, was selected as an antigenic determinant to enhance the probability of interaction between antigen and antibody.

The measured titer of the polyclonal antibody generated against Ep-CAM was 0.43 at 10^3 dilution in ELISA. This value seems to be relatively low compared to that from the protein-directed antibody. This could be due to the property of antigen itself or the way of immunoassay measured. In other words, the tetradecameric peptide as an antigen might not be long enough to stimulate the immune system effectively and the directly coated peptide in the ELISA plate was not held properly to cause inaccurate measurement of the antibody titer. In order to increase the titer, the antibody was purified with the affinity column chromatography technique. This purified Ep-CAM antibody was able to recognize the Ep-CAM molecule in Western blot analysis (Fig. 3A), whose molecular weight was approximately 40 kDa, from the mouse colon tissue extract and the CT-26 colon cancer cell extract, implying that the antibody generated in this study can be used for the tissue-specific delivery of photosensitizer.

It may not be sometimes easy to evaluate the tissue-specific delivery of photosensitizer by the peptide-directed Ep-CAM antibody. This is the case for Photogem, one of the hematoporphyrin-derived photosensitizers that can be frequently used in PDT. Therefore, the fluorescence dye, FITC, was used instead of Photogem in this experiment. Fluorescence molecule can be used as sensitive marker in elucidating a variety of cell functions a through *in situ* hybridization. Especially FITC is one of the most commonly used fluorescence maker. It exhibits a good quantum yield, although conjugation reduces its fluorescence by as much as 50%. Excitation and emission occur at 494 and 520 nm, respectively. The fluorescence and absorbance of FITC is very pH sensitive. The thiocyanate group of FITC was used for conjugation to the amino group of antibody and the antibody-FITC conjugate was used for immunohistochemistry. The molar ratio of FITC to antibody was estimated approximately 1.86 to 1.00 by measuring the optical densities at 492 nm and 280 nm. The fixed CT-26 cells were probed with the peptide-directed Ep-CAM antibody-FITC conjugate (Fig. 4). The most intensely fluorescenced areas indicated by arrow heads could be the interaction sites of Ep-CAM and its antibody. Judging from the shapes formed by fluorescence, the Ep-CAM antibody is likely to bind to the surface of the cell, implying that it could deliver FITC to the target protein, Ep-CAM, *via* antigen-antibody interaction. Therefore, this experimental result suggests that antibody generated against the tumor marker protein could be used for the tissue-specific delivery of photosensitizer in PDT.

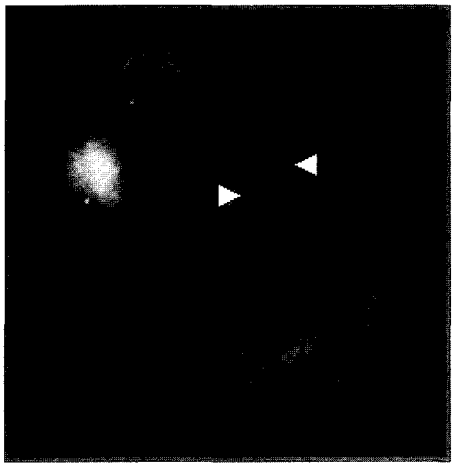

Figure 4. Localization of Ep-CAM in the CT-26 colon cancer cells with the peptide-directed Ep-CAM antibody-FITC conjugate. The cells are probed with the Ep-CAM antibody-FITC conjugate. The most intensely fluorescenced areas indicated by arrow heads could be the sites that Ep-CAM antibody interaction occurs.

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