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Cell-Specific Targeting of Texas Red with Anti-Ep-CAM Antibody

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The polyclonal antibody was generated against the peptide fragment of 62 amino acid residues (D181-T242) near the COOH-terminal region of the extracellular domain of epithelial-cell adhesion molecule (Ep-CAM) and shown to be able to recognize Ep-CAM in competitive ELISA. Then, sulforhodamine 101 acid chloride (so called Texas red), a fluorescence dye, was conjugated to the affinity-purified anti-Ep-CAM antibody utilizing the reaction between the aliphatic amines of antibody and the sulfonyl chloride of Texas red. The molar ratio of Texas red to antibody was estimated to be approximately 1.86 by measuring optical densities at 280 nm and 596 nm, implying that the two molecules of Texas red at most were conjugated to antibody. The anti-Ep-CAM antibody-Texas red conjugate was then used for immunohistochemistry of CT-26 murine colon carcinoma cells. Based upon the fluorescence microscope images, anti-Ep-CAM antibody is able to deliver Texas red specifically to the surface of CT-26 cells on which Ep-CAM was actively expressed. This result indicates that anti-Ep-CAM antibody could be useful for the tissue-specific delivery of photosensitizers via antigen-antibody interaction.

key words: photosensitizer, cell-specific targeting, Anti-Ep-CAM antibody, Texas red, photodynamic therapy

INTRODUCTION

The photodynamic therapy (PDT), called photoradiation therapy, phototherapy, or photochemotherapy, involves the use of a drug called a photosensitizer, which is activated by being exposed to light [1-4]. Depending on the part of the body being treated, the photosensitizer can be either injected into the bloodstream or applied to the skin. After the photosensitizer is absorbed by cancer cells, a light source is applied to the area to be treated. The illumination by laser with an appropriated wavelength causes the photosensitizer to react with oxygen, which in turn generates chemically active oxygen radicals that actually kill the cancer cells. It is now becoming popular to treat various cancers with PDT especially in the region of head and neck, in which the properties of photosensitizers play a key role. However, currently a half of all patients diagnosed with squamous cell cancers in the head and neck region experience recurrence after PDT. The two possible reasons are follows: (i) the targeting is not specific enough for tumor cells to get the sufficient amounts of a photosensitizer for treatment, or (ii) the binding of a photosensitizer to tumor cells is not strong enough, causing the photosensitizer 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 [5, 6]. The targeting capability of these carriers may depend on their physical and biological properties [7-9].

The antibody generated against epithelial-cell adhesion molecule (Ep-CAM) was used as a carrier protein in this study. Ep-CAM is a 40 kDa glycoprotein and a homophilic cell-to-cell adhesion molecule [10]. 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 [11]. The gene for Ep-CAM, located in chromosome 4 in human, consists of 9 exons [12]. The exons 1, 2-6, and 7 encode the signal peptide, extracellular and the transmembrane domain of Ep-CAM, respectively, whereas the exons 8-9 encode the cytoplasmic domain. The extracellular domain can be a target region for the tissue-specific delivery of photosensitizers.

In this research anti-Ep-CAM antibody was generated, purified with protein A affinity column chromatography, and conjugated to sulforhodamin 101 acid chloride (Texas red), a fluorescence dye. The molar ratio of Texas red to the purified antibody (IgG) was estimated spectrophotometrically. Then, the IgG-Texas red conjugate was used for immunohistochemistry to investigate the tissue-specific targeting of photosensitizers to the tumor cells [13]. The results of this experiment could be applied to the development of the tissue-specific delivery of photosensitizers in PDT [14, 15].

MATERIALS AND METHODS

Generation of Ep-CAM Antibody

The hydropathy plot analysis of the extracellular domain of Ep-CAM was carried out and the fragment of 62 amino acid

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residues spanning from D181 to T242 near the C-terminal region was chosen as an immunogenic site because of its hydrophilic property (Fig. 1). The DNA fragment encoding 62 amino acid residues was cloned to pGEX-4T-1, the expression vector, after reverse transcriptase-polymerase chain reaction (RT-PCR), and then expressed as a fusion protein of glutathione *S*-transferase (GST). The fusion protein carrying the peptide fragment of 62 amino acid residues of Ep-CAM was directly purified with the immobilized glutathione affinity column chromatography and analyzed onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). 300mg of the affinity-purified immunogen premixed with Freund's complete adjuvant (Sigma, USA) was directly injected into subcutaneously as an antigen into New Zealand white rabbit. The next injections were performed every 2 weeks with Frueund's incomplete adjuvant. The titer of antibody was measured with enzyme linked immunosorbent assay (ELISA).

Characterization of Antibody Generated against Ep-CAM

The anti-Ep-CAM antibody was purified with HiTrap™ protein A HP column (Amersham, Sweden) and ÄKTA purifier 10 (Pharmacia, USA) after a fractional precipitation with 50% ammonium sulfate. One column volume of the sample was loaded onto HiTrap™ protein A HP column, pre-equilibrated with phosphate-buffered saline (PBS), and washed with 3 column volumes of PBS. The antibody was then eluted with 0.1M Glycine-HCl buffer, pH 2.7, and one tenth volume of 1M Tris-HCl buffer, pH 9.0 was added to the eluent in order to maintain pH.

The binding affinity of the purified antibody was measured by an indirect ELISA. The expressed fusion protein of the peptide fragment of 62 amino acid residues of Ep-CAM with GST was digested with thrombin, which cleaved the thrombin

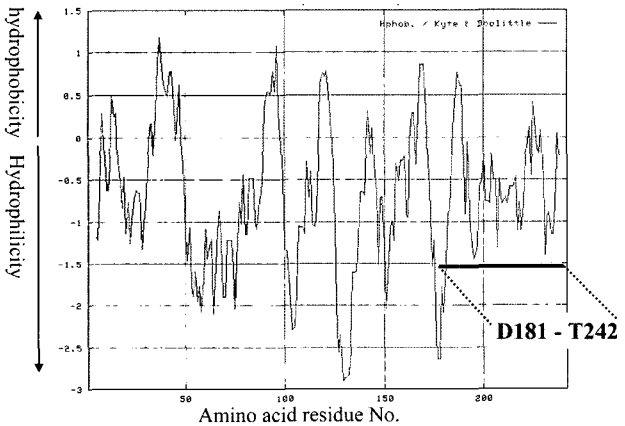

Figure 1. Hydropathy analysis of the extracellular domain of the mouse Ep-CAM. The y and x axes indicate the hydrophobicity/ hydrophilicity values and the amino acid residue number of Ep-CAM, respectively. The hydropathy index for each amino acid residue is adapted from Kyte and Doolittle, 1982 [23].

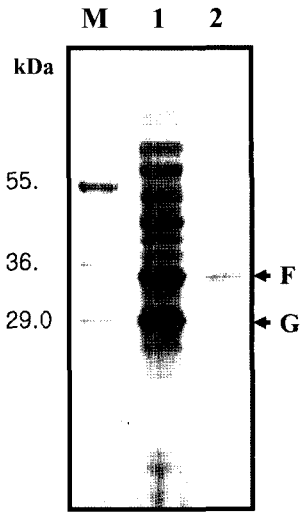

Figure 2. SDS-PAGE analysis of the induced glutathione *S*-transferase fusion protein from the cell lysate and the affinity purified protein. The lanes are as follows: the lane M is the protein size marker II (purchased from Tefco, Japan); the lane 1 is the cell lysate broken up by sonication; and the lane 2 is the affinity purified glutathione *S*-transferase (GST) fusion protein. The letters 'F' and 'G' indicate the GST fusion protein and GST itself, respectively.

site (NH_2-LVPR-COOH) located between the antigenic peptide and GST. The antigenic peptide was coated on the bottom of 96 well plates, washed with PBS with 0.05% Tween 20 (PBST), and blocked with 0.1% casein. The purified antibody, which had been diluted with PBS serially 10^{-2} to 10^{-5} times, was added to the wells and incubated for 1 hour at room temperature. On the other hand, the binding specificity was also measured by a competitive ELISA. The serially diluted antibody was preincubated with various concentrations of the immunogen at 37°C for 30 min and added to 96 well plate coated with the antigenic peptide. The goat-anti-rabbit IgG antibody conjugated with horse raddish peroxidase was used for amplification of the signal and the color reaction to quantitate the binding affinity and specificity.

Conjugation of Sulforhodamine 101 Acid Chloride to Anti-Ep-CAM Antibody

The antibody generated against Ep-CAM was conjugated to sulforhodamin 101 acid chloride, Texas red (Sigma, USA) [16]. The amine group of antibody was reacting with the sulfonyl group of Texas red. 200 μg/ml of Texas red in acetonitrile was added to at least 2 mg/ml of the purified antibody in 0.15M NaCl and 0.2M sodium bicarbonate, pH 9.0, incubated on ice for 2 hours, and dialyzed in 0.2M sodium carbonate and 0.1M Tris-HCl, pH7.4 to remove the unbound Texas red. The molar ratio of Texas red to antibody was estimated by measuring the absorbance at 280nm and 596nm.

Immunohistochemistry of Colon Cancer Cells with Anti-Ep-CAM Antibody-Texas Red Conjugate

The glass slide for coating was soaked for 10 seconds in a working solution containing 5 ml 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 Ellis's method [17]. The harvested colon cancer cells, CT-26, cohesive to the coated glass slide, were incubated for 15 min at 27°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 hour at room temperature. The cells were washed for 30 min in PBS and then the anti-Ep-CAM antibody-Texas red conjugate was incubated for 1hr at room temperature. After washing with PBS for 30 min, the fluorescence image of Texas red was observed with the fluorescence microscope.

RESULTS AND DISCUSSION

Since currently available photosensitizers leave people very sensitive to light for a time because of their nonspecificities, it is very important to deliver the photosensitizer specifically to target cells or tissues in PDT, achievable by a very strong and specific antigen-antibody interaction. In this respect, the critical point of the experiment could be how to generate the target-specific antibody. In order to manufacture the specific antibody, at least two factors have to be considered: (i) selection of the antigenic protein actively expressed in the cancer cell and (ii) 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. Thus, the extracellular domain of Ep-CAM could be used as a site for the antigen-antibody interaction. Therefore, the relatively hydrophilic segment, the peptide fragment spanning from Asp-181 to Thr-242 near the carboxyl terminal region of the extracellular domain of Ep-CAM, was selected as a immunogenic determinant to increase the probability of interaction between antigen and antibody and the anti-Ep-CAM antibody was generated.

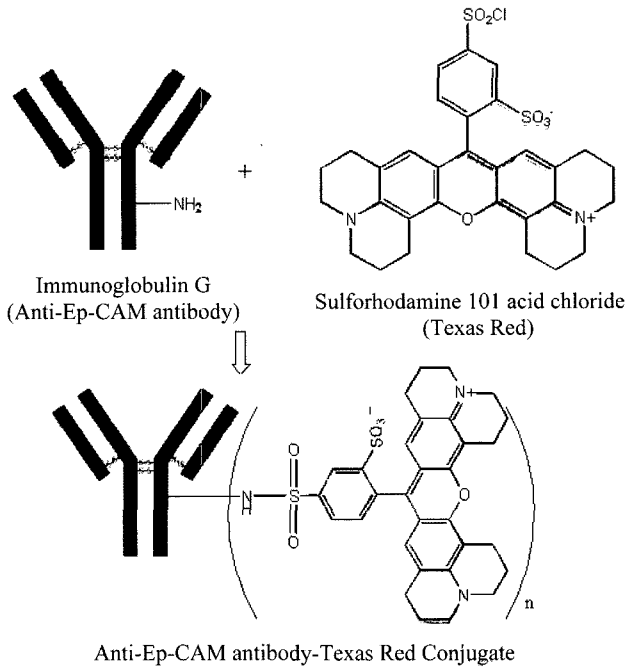

Figure 3. Schematic diagram of the conjugation method of sulforhodamine 101 acid chloride to anti-Ep-CAM antibody. The amine group of antibody was used for the reaction to sulfonyl group of Texas red, resulting in anti-Ep-CAM antibody-Texas red conjugate.

The measured titer of the polyclonal antibody generated against the peptide fragment of Ep-CAM was 0.98 in an indirect ELISA when the antibody was diluted to 10^{-3} times (data not shown). This value is at least two times higher than that from the peptide-directed antibody against Ep-CAM previously prepared [18], implying that the binding affinity is enhanced as the size of the antigenic determinant increased. The binding specificity of antibody was also measured with a competitive ELISA. The purified antibody was diluted and incubated with various concentrations of antigen, the fusion protein of the peptide fragment of Ep-CAM with GST. If the antibody can react with antigen specifically, the titer will be decreased in the competitive inhibition assay as the concentration of the inhibitory antigen increases. The concentration of antigen needed to cause the inhibition of antigen-antibody interaction was shown to be approximately 1 μg/mL (data not shown) and the degree of inhibition increased as the concentration of competitive antigen increased. This result implies that anti-Ep-CAM antibody interacts specifically with its antigen, the peptide fragment of 62 amino acid residues of Ep-CAM.

Sometimes the evaluation of the tissue-specific delivery of photosensitizers by antigen-antibody interaction is difficult. This is the case for Photogem®, one of the hematoporphyrin-derived photosensitizer that is a challenging and perspective high performance photodynamic drug for cancer [19-21]. In order to overcome this problem, the fluorescence dye, Texas red, was used instead of Photogem® in this experiment as a sensitive marker in elucidating a variety of cell functions through *in situ* hybridization. Texas red is most commonly used especially on Fluorescence activated cell sorter (FACS) machine with a dye laser (excitation 595-605 nm). Sulforhodamine 101 acid chloride has a molar extinction coefficient of 139,000M^{-1} cm^{-1} at 576 nm [22], making it a relatively bright reagent, allowing for discrimination of weakly-expressed antigens, while exhibiting a good quantum yield. The sulfonyl group of Texas red was used for conjugation to the amine group of antibody and the antibody-Texas red conjugate was used for immunohistochemistry. The molar ratio of Texas red to

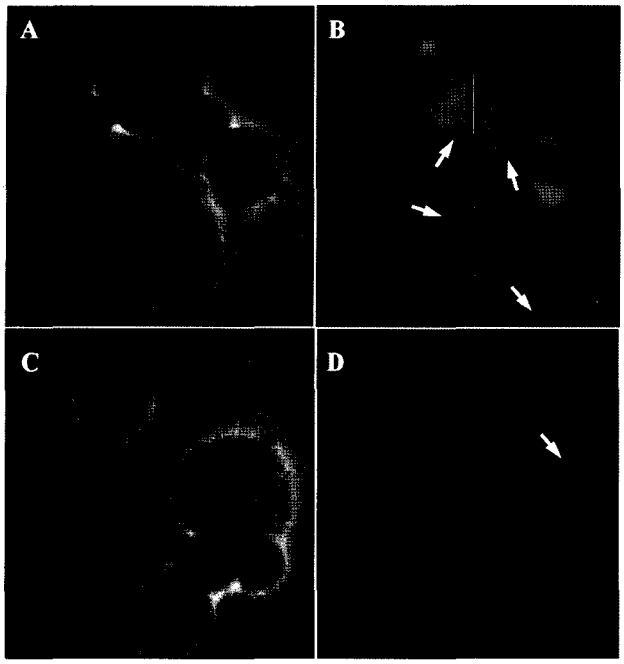

Figure 4. Localization of Ep-CAM in CT-26 cells with anti-Ep-CAM antibody-Texas red conjugate. The CT-26 murine colon carcinoma cells were probed with Texas red conjugated to anti-Ep-CAM antibody [B] or non-specific antibody [D]. The cells were also stained with Hematoxylin-Eosin [A and C]. The most intensely fluorescenced areas indicated by arrow heads in the panel B could be the sites that the specific interaction between antigen and antibody has occurred based upon Hematoxylin-Eosin staining.

antibody was estimated to be approximately 1.86 to 1.00 by measuring the optical densities at 280 nm and 596 nm, implying that the two molecules of Texas red at most were conjugated to antibody. The fixed CT-26 murine colon carcinoma cells were probed with Texas red conjugated to anti-Ep-CAM antibody (Fig. 4B) or non-specific antibody (Fig. 4D). By comparisons with the Hematoxylin-Eosin stained images (Fig. 4A and C), the most intensely fluorescenced areas indicated by arrow heads in the panel B could be the sites in which the interaction between antigen and antibody has occurred. This kind of interaction must be specific because there's no signal when non-specific antibody was used instead of anti-Ep-CAM antibody (Fig. 4D). Judging from the shapes formed by fluorescence, Ep-CAM antibody is likely to bind to the surface of the CT-26 cell, implying that it could also deliver Photogem® to the target tissue via antigen-antibody interaction. Therefore, this experimental result indicates that anti-Ep-CAM antibody could be useful for the tissue-specific delivery of photosensitizer via antigen-antibody interaction in PDT.

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