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Chemotherapeutic Effect of CD147 Antibody-labeled Micelles Encapsulating Doxorubicin Conjugate Targeting CD147-Expressing Carcinoma Cells

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Abstract. *Background: CD147 (basigin/emmprin) is expressed on the surface of carcinoma cells. Materials and Methods: For studying the efficacy of CD147-targeting medicine on CD147-expressing cells, we studied the effect of anti-CD147-labeled polymeric micelles (CD147ab micelles) that encapsulated a conjugate of doxorubicin with glutathione (GSH-DXR), with specific accumulation and cytotoxicity against CD147-expressing A431 human epidermoid carcinoma cells, Ishikawa human endometrial adenocarcinoma cells, and PC3 human prostate carcinoma cells. Results: By treatment of each cell type with CD147ab micelles for 1 h, a specific accumulation of CD147ab micelles in CD147-expressing cells was observed. In addition, the cytotoxicity of GSH-DXR-encapsulated micelles against each cell type was measured by treatment of the micelles for 1 h. The cytotoxic effect of CD147ab micelles carrying GSH-DXR was 3- to 10-fold higher for these cells than that of micelles without GSH-DXR. Conclusion: These results suggest that GSH-DXR-encapsulated CD147ab micelles could serve as an effective drug delivery system to CD147-expressing carcinoma cells.*

Conjugates of monoclonal antibodies with anticancer drugs have been studied for many years as a potential approach to delivering these agents more specifically to cancer cells (1). For example, rituximab, a monoclonal antibody targeting pan-B cell marker CD20, was the first monoclonal neutralizing antibody developed for treatment (2-5). In addition, drug–monoclonal antibody conjugates are currently being developed for the treatment of various types of solid tumor (6).

The extracellular matrix metalloproteinase inducer CD147 (also known as basigin or emmprin) up-regulates matrix metalloproteinase expression in the surrounding fibroblasts and endothelial cells and promotes the invasion of cancer cells. CD147 is a 55-kDa molecule localized on the surface of tumor cells (7, 8) and expression of CD147 has been frequently detected in human tumors cells (9). CD147 is expected to potentially serve as a target for antitumor therapy. We prepared CD147 antibody, a murine monoclonal antibody specific for human ovarian cancer, by immunizing mice with the human ovarian germ cell line JOHYC-2 (10). This 12C3 epitope was in the extracellular region (11).

We previously found that conjugates of doxorubicin (DXR) and glutathione (GSH) (GSH-DXR) prepared for the purpose of overcoming drug resistance strongly induced apoptosis in many cancer cells at lower concentration than DXR (12-16).

Previous drug carriers were micro-(or nano)-particles, liposomes and non-micelle-forming polymeric carriers. Polymeric micelles have been developed as a new type of drug carrier. The study of polymeric micelle drug carriers started in the 1980s (17-19), and these carrier systems were recognized as one of the most potent drug carrier types in the 1990s (20-25). Then, in the 2000s, several significant related clinical trials (26-33) were carried out, while more and more research development projects were conducted.

A polymeric micelle is a macromolecular assembly composed of an inner core and an outer shell. The polymeric micelles can have a spherical or a cylindrical shape, depending on the chemical structure and chain length of the macromolecules. For the purpose of drug targeting, most polymeric micelle studies have dealt with the spherical shape, whereas a very limited number of the filamentous shape systems have been studied (34, 35). A spherical polymeric micelle structure is formed from block copolymers or graft copolymers (36).

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Key Words: Emmprin/CD147, BSG, basigin, drug delivery system, block copolymer, polymeric micelle.

In this study, we examined the therapeutic effect of CD147 antibody-carrying (CD147ab) micelles for the treatment of several cancer cell lines. In addition, CD147ab micelles encapsulating GSH-DXR were also tested for specific cytotoxicity against carcinoma cells.

Materials and Methods

Materials. DXR was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Anti-mouse IgG-alkaline phosphatase conjugate, GSH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazorium bromide (MTT), 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium tablet (BCIP/NBT), and plasmid of small/short hairpin RNA (shRNA) for human CD147 were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Dowex 50W x8 and glutaraldehyde were purchased from Nakarai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Cell lines. A431 human epidermoid carcinoma cell line, Ishikawa human endometrial adenocarcinoma cell line, and PC3 human prostate carcinoma cell line from Health Science Research Resources Bank (Tokyo, Japan) were cultured with RPMI 1640 containing 10% heat-inactivated fetal bovine serum (growth medium) under conventional conditions (37˚C, 5% CO_2).

Knockdown of CD147 expression in PC3 cells. Plasmid of shRNA for human CD147 was transfected into PC3 cells using the FuGENE 6 transfection reagent (Promega, Tokyo, Japan). The transfectants were selected by treatment with puromycin. A permanent CD147-knockdown clone (PC3/KD) was obtained.

Conjugation of DXR with GSH. GSH-DXR was prepared as described previously (13-16). In brief, the combination of 1 mg each of GSH and DXR in 0.15 M NaCl containing 0.1% glutaraldehyde was incubated at room temperature for 30 min. After incubation, GSH-DXR was purified using Dowex 50 W x8 (H^+ form). The concentration of DXR was measured by absorbance at 495 nm.

Preparation of immuno CD147ab micelles. Immuno CD147ab micelles were prepared by GSH-DXR incorporation process followed by conjugation with both CD147ab and green fluorescent protein (GFP). Poly(ethylene glycol)-β-poly(DXR-conjugated aspartic acid) {PEG-β-P[Asp(DXR)]} block copolymer was the starting material (20-22). The molecular weight of the polyethylene glycol polymer was 1.20×10^4, and the average number of aspartic acid units was 18.4. DXR molecules were conjugated to 67.7% aspartic acid units. For the antibody and GFP conjugation, an aldehyde-terminated block copolymer, CHO-{PEG-β-P[Asp(DXR)]} was used. In this CHO-terminated block polymer, the molecular weight of the poly(ethylene glycol) polymer was 1.20×10^4, and the average number of aspartic acid units was 18.6. DXR molecules were conjugated to 47.8% aspartic acid units. Murine IgG micelles were prepared using mouse IgG instead of CD147ab.

Incorporation of GSH-DXR. Owing to hydrophobic interactions between the chemically conjugated DXR moiety and GSH-DXR molecules, GSH-DXR was physically incorporated into polymeric micelles formed from PEG-β-P[Asp(DXR)] and CHO-PEG-β-P[Asp(DXR)] using the dialysis technique. PEG-β-P[Asp(DXR)], CHO-PEG-β-P[Asp(DXR)], and GSH-DXR were added to a solvent comprising water and N,N-dimethylformamide and dialyzed against water. The molar ratio of CHO-PEG-β-P[Asp(DXR)]: PEG-β-P[Asp(DXR)] was 1:9. After dialysis, the obtained micelles were purified by gel-filtration chromatography using Sephacryl S-300 with water as the eluent. The amount of GSH-DXR incorporated was measured by determining the absorption at 485 nm, based on the assumption that absorption at 485 nm was the sum of absorption of the polymer-bound-DXR and the physically bound GSH-DXR.

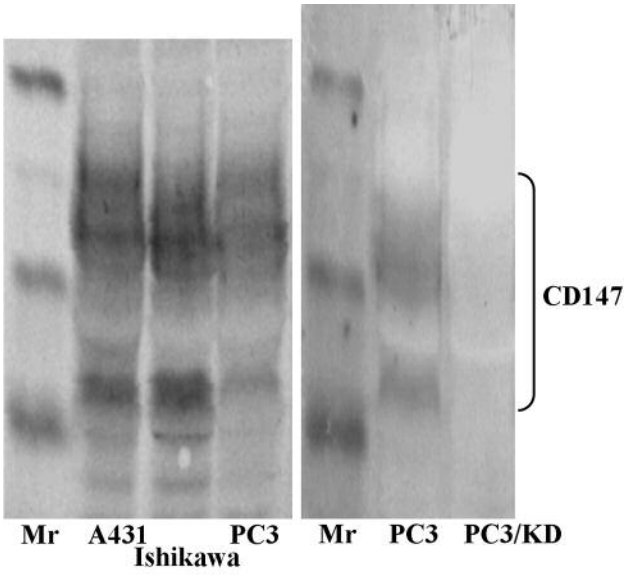

Figure 1. *Expression of CD147 in A431, Ishikawa, PC3 and CD147-knockdown PC3 (PC3/KD) cells detected by western blot analysis. Several bands of CD147 by binding of sugar chains were observed. Nevertheless, expression was not detected in PC3/KD. Mr, Protein marker.*

CD147ab and GFP conjugation: The GSH-DXR-incorporated micelles thus obtained were mixed with both CD147ab and GFP at a molar ratio of 23:1:0.5 in 0.2 M Tris-HCl buffer at pH 6.5, and the solution stirred at room temperature for 24 h. The reaction mixture was then purified by gel-filtration chromatography using Sephacryl S-300 in water for removal of unconjugated antibody. Complete removal of the unconjugated antibody and GFP was confirmed by gel-permeation chromatography using a TSK gel 4000PWXL-equipped HPLC system (Tosoh Corporation, Tokyo, Japan). The reaction ratio of the antibody molecule was found to be 52% upon calculation of the decrease in the antibody's peak area (refractive index) in the HPLC graphs obtained after and before the conjugation reaction.

Western blot analysis. CD147 in cells extracted with 1% Triton X-100 was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide) and analyzed by western blot using 1 μg/ml of CD147ab (12C3) as the primary antibody and 1 μg/ml of alkaline phosphatase-labeled anti-mouse IgG as the secondary antibody, and this amount was assayed semi-quantification.

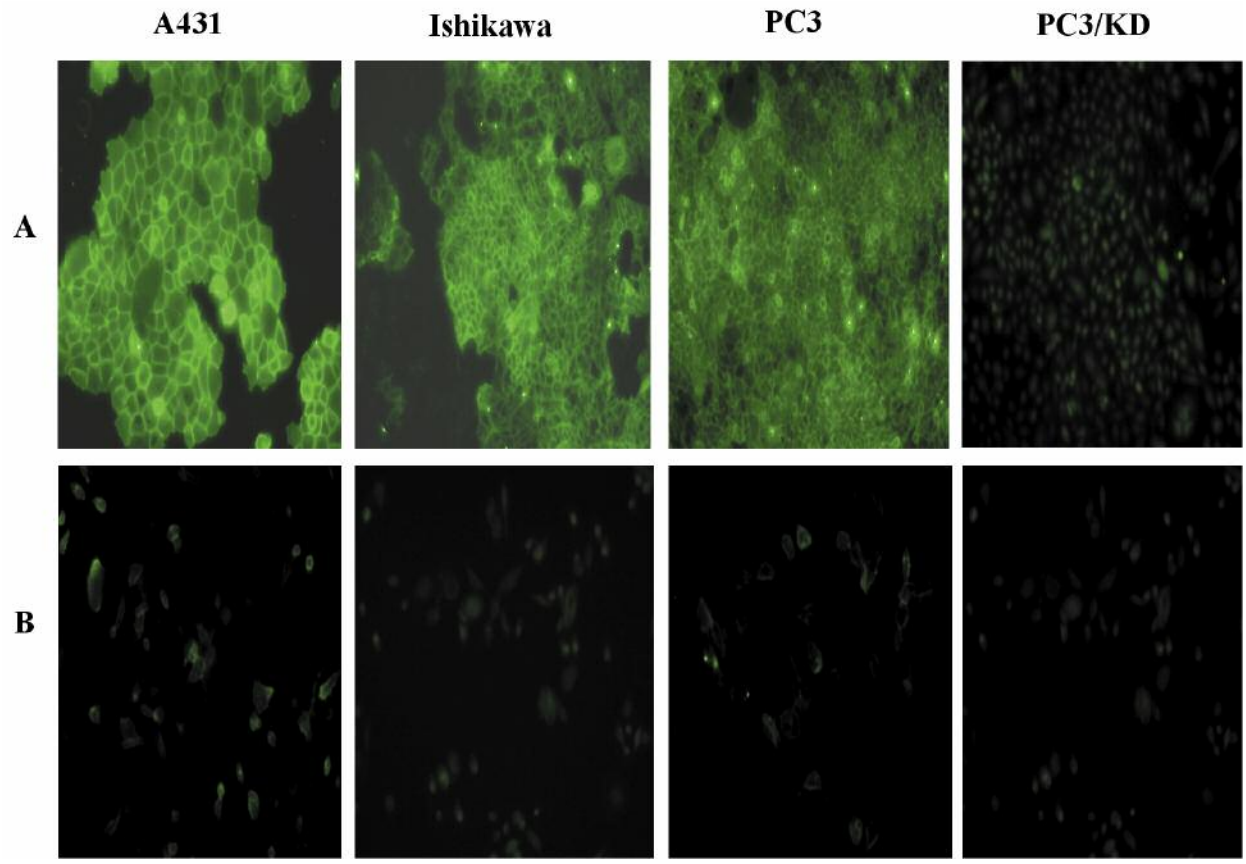

Figure 2. *Accumulation of a conjugate of doxorubicin with glutathione (GSH-DXR)-encapsulating CD147ab- and green fluorescent protein (GFP)-double-labeled micelles (GSH-DXR-CD147ab/GFP micelles) in A431, Ishikawa, PC3 and CD147-knockdown PC (PC3/KD) cells. Treatment of these cells with GSH-DXR-CD147ab/GFP micelles for 1 h (A), and with the GSH-DXR-CD147ab/GFP micelles and excess CD147ab (B). Specific accumulation of GSH-DXR-CD147ab/GFP micelles by CD147-expressing cells was observed. Accumulation of the micelles after 1h exposure in each cell line was observed by fluorescent microscopy.*

Immunoreaction of CD147-labeled micelles. A431, Ishikawa and PC3 cells were incubated with GSH-DXR-encapsulated CD147ab- and GFP-double-labeled micelles (GSH-DXR-CD147ab/GFP micelles) for 1 h and washed with phosphate-buffered saline. The fluorescence of GFP was detected by fluorescent microscopy, and these amounts were assayed semi-quantification.

Cytotoxicity of GSH-DXR-CD147ab/GFP micelles. The cytotoxicity of GSH-DXR-CD147ab/GFP micelles against A431, Ishikawa, PC3 and PC3/KD cells after a 1-h treatment was measured after 96 h by an MTT assay (12-16). The cell death rate due to GSH-DXR-CD147ab/GFP micelles and GSH-DXR-encapsulating murine IgG/GFP micelles (0.1 μM GSH-DXR) was expressed relative to that induced by CD147ab/GFP micelles and mouse IgG/GFP micelles without GSH-DXR, respectively (100% survival rate).

Protein determination. Protein concentration was assayed by a Bio-Rad protein assay kit (Bio-Rad Corporation, Tokyo, Japan) using bovine serum albumin (Wako, Japan) as the standard.

Results

Expression of CD147 in A431, Ishikawa, PC3 and PC3/KD cells by western blot analysis. The expression of CD147 (55-kDa) in A431, Ishikawa and PC3 cells at the protein level was observed by western blot analysis using 12C3 as a primary CD147 antibody (Figure 1). Several bands of CD147 by binding of sugar chains were observed. Nevertheless, expression was not detected in PC3/KD cells.

Accumulation of CD147ab/GFP micelles in A431, Ishikawa, PC3 and PC3/KD cells. After treatment of A431 cells with GSH-DXR-encapsulating CD147ab- and GFP-double-labeled micelles (GSH-DXR-CD147ab/GFP micelles) for 1 h, we found that the GSH-DXR-CD147ab/GFP micelles accumulated in the cells (Figure 2A). Moreover, co-treatment

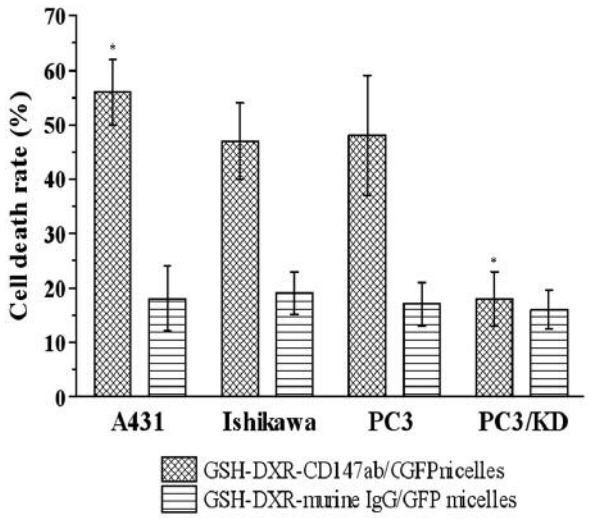

Figure 3. *Cytotoxicity against A431, Ishikawa, PC3 and CD147-knockdown PC3 (PC3/KD) cells of conjugate of doxorubicin with glutathione (GSH-DXR)-encapsulating CD147ab- and green fluorescent protein (GFP)-double-labeled micelles (GSH-DXR-CD147ab/GFP micelles) (0.1 μM of GSH-DXR, for 1-h exposure) and GSH-DXR-murine IgG/GFP micelles (0.1 μM GSH-DXR, for 1-h exposure). MTT assay was carried out 96 h after treatment. The cell death rate was expressed relative to that of CD147ab/GFP micelles and murine IgG/GFP micelles without GSH-DXR (100% survival). Data are the mean±SD (three independent experiments, *p<0.01).*

of the cells with GSH-DXR-CD147ab/GFP micelles and excess CD147ab (100 μg/ml) competitively suppressed the accumulation of the micelles (Figure 2B). Therefore, specific accumulation of GSH-DXR-CD147ab/GFP micelles was observed on CD147-expressing cells. However, accumulation was not observed in PC3/KD cells (Figure 2).

Cytotoxicity of GSH-DXR-CD147ab/GFP micelles against A431, Ishikawa, PC3 and PC3/KD cells. Cytotoxicity of GSH-DXR-CD147ab/GFP micelles against A431, Ishikawa, PC3 and PC3/KD cells treated for 1 h was measured after 96 h (Figure 3). The cell death rate for GSH-DXR-CD147ab/GFP micelles (0.1 μM GSH-DXR) and control GSH-DXR-murine IgG/GFP micelles (0.1 μM GSH-DXR) was found to be 56% and 18% for A431, 47% and 20% for Ishikawa, 48% and 17% for PC3, and 18% and 16% for PC3/KD, respectively (Figure 3). It is suggested that the cytotoxicity of GSH-DXR-CD147ab/GFP micelles is dependent on the expression of CD147.

Discussion

By western blot analysis, high levels of CD147 protein expression were observed in various carcinoma cells but not in normal tissues (10, 11). Considering that the anticancer drug was effective at an early stage, it was expected that CD147 could have sufficient ability in targeted therapy.

Various physiological processes, such as proliferation and differentiation of epithelial cells (37-39), fertilization (40), differentiation and activation of immune cells (41-43), integrin-mediated adhesion of myocytes of the left heart ventricle to basement membrane components (44), selective transport processes in endothelial cells to maintain blood–brain barrier function (45), erythrocyte maturation (46) and wound healing (47) might potentially be influenced by anti-CD147 therapy (9). Thus, potential side-effects of CD147-targeted conjugate therapy should be carefully evaluated.

We previously demonstrated that GSH-DXR exhibited potent cytotoxicity compared to DXR (14-16). The 50% growth-inhibitory concentrations of DXR and GSH-DXR for rat ascites hepatoma AH66 cells were 300 nM and 5 nM, respectively. The effect of GSH-DXR delivery to chemotherapy targets was examined using CD147ab/GFP micelles. CD147ab/GFP micelles specifically accumulated in CD147-expressing cells but not in CD147-deficient PC3/KD cells (Figure 2). Moreover, GSH-DXR-encapsulated CD147ab/GFP micelles exhibited a potent cytotoxicity against CD147-expressing cells (Figure 3). No cytocidal effect was observed for CD147ab alone, and the cell death rate of CD147ab (100 μg/ml) was 3% or less in all cells used (data not shown). We also demonstrated in previous work that the effect of GSH-DXR was reversed in multidrug-resistant cells in which AH66/DR cells overexpressed P-glycoprotein and showed resistance to 10 μM DXR (12-14). Therefore, it was expected that targeted chemotherapy of GSH-DXR would be effective for both drug-sensitive and drug-resistant tumor cells.

In this study, encapsulation of GSH-DXR into aCD147ab/GFP micelles was also examined and resulted in specific cytotoxicity against CD147-expressing carcinoma cells. We previously demonstrated that the micelles have an extremely long circulation time in blood (21) and could be expected to have a therapeutic effect in *in vivo* targeted chemotherapy.

Further studies will attempt to confirm the specific cytotoxicity using a three-dimensional cell culture system using a bio-reactor.

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