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Establishment of a novel lectin–antibody ELISA system to determine core-fucosylated haptoglobin

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ABSTRACT

Background: Fucosylated haptoglobin (Fuc-Hpt) is a novel cancer biomarker that increases in various pathological conditions. We previously established a Fuc-Hpt lectin–antibody assay using *Aleuria aurantia* lectin (AAL), and applied this to diagnose several diseases, including various cancers. AAL recognizes both α1-3/1-4 and α1-6 fucosylation on *N*/*O*-linked glycans. These fucosylation types differ in biological function, and in regulation by different fucosyltransferases. Recently, we identified a novel lectin, *Pholiota squarrosa* lectin (PhoSL), which specifically recognizes α1-6 fucosylation (core-fucosylation).
Methods: We developed a lectin–antibody ELISA kit using PhoSL to determine core-Fuc-Hpt levels in sera from colorectal or pancreatic cancer patients.
Results: Serum levels of AAL-reactive Hpt are higher in pancreatic cancer patients, whereas those of PhoSL-reactive Hpt are higher in colorectal cancer patients. Mass spectrometry analyses of Hpt fucosylation levels were consistent with lectin–antibody ELISA results. Hpt-transfected colorectal cancer cell lines produced significant amounts of core-Fuc-Hpt, suggesting that colorectal cancer tissues produce core-Fuc-Hpt.
Conclusions: These differences in Fuc-Hpt patterns might depend on cancer cells and the surrounding cells, which produce Hpt.

1. Introduction

Fucosylation is one of the most important glycosylation types involved in cancer and inflammation [1]. Several types of fucosylated glycoproteins have been used as cancer biomarkers. Previously, we found that serum levels of fucosylated haptoglobin (Fuc-Hpt) were increased in the sera of patients with pancreatic cancer [2]. Mass spectrometry analysis reveals that the fucosylation on haptoglobin (Hpt) contains a variety of linkages such as α1-3/α1-4 and α1-6 fucose. To develop a more convenient assay system for measuring Fuc-Hpt, we have established a lectin–antibody enzyme-linked immunosorbent assay (ELISA) for Fuc-Hpt [3] and evaluated this ELISA system in various conditions [4]. In this lectin-ELISA system, we used AAL (*Aleuria aurantia* lectin), which recognizes all types of fucosylation. Our previous study demonstrated that the type of Fuc-Hpt that showed the greatest increase in the sera of patients with pancreatic cancer was the Lewis-type of fucosylation (α1-3/α1-4 fucosylation), while core fucose (α1-6 fucosylation) was only slightly increased in those sera [5]. In contrast, it is known that core-fucosylation has characteristic oligosaccharide functions in antibody-dependent cellular cytotoxicity [6] and growth factor signaling [7,8]. Previously, we reported that *Aspergillus oryzae* l-fucose-specific lectin (AOL) could specifically recognize core fucose [9]. However, AOL does not bind to α1-3/1-4 fucose, but binds slightly to α1-2 fucose.

Recently, we have identified PhoSL (*Pholiota squarrosa* lectin), which shows greater specificity towards core fucose [10]. Frontal chromatography revealed that PhoSL could only bind to glycans carrying core fucose. In this study, we investigated the ability of PhoSL to determine the presence of core-fucosylated haptoglobin using the lectin–antibody ELISA. We found that the positive rate of fucosylated haptoglobin was different between AAL ELISA and PhoSL ELISA. Here, we discuss the clinical significance of increases in core-fucosylated haptoglobin in cancer patients.

Abbreviations: Fuc-Hpt, fucosylated haptoglobin; PhoSL, *Pholiota squarrosa* lectin; AAL, *Aleuria aurantia* lectin; MEF, mouse embryonic fibroblast; Fut8, α1-6 fucosyltransferase.

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2. Materials and methods

2.1. Human serum samples

Seventy-one patients with colorectal cancer (stage I/II/III/IV; 9/18/24/20) and 55 pancreatic cancer patients (stage I/II/III/IV; 5/27/14/9) who underwent primary resection at Osaka University-related hospitals from 1995 to 2005 were enrolled in this study. Sera from these patients before surgery, and of 60 age-matched healthy controls who received health check-ups, were collected and kept frozen at −80 °C until use. Written informed consent was obtained from all subjects in this study. This study was approved by the ethics committee of Osaka University Hospital.

2.2. Flow cytometry

Mouse embryonic fibroblasts (MEFs) derived from α1-6 fucosyltransferase (Fut8)-deficient mice or wild-type mice were cultured in RPMI 1640 medium (Sigma, St. Louis) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. After harvesting with 1 mmol/l EDTA/phosphate-buffered saline (PBS), 3.0×10^5 cells were incubated in 0.1 μg/ml fluorescein isothiocyanate (FITC)-labeled-PhoSL/PBS on ice for 10 min. Cells were washed twice with 0.1% BSA/PBS, followed by flow cytometric analysis with a BD Accuri™ C6 Flow Cytometer and the C6 Flow Cytometer Starter Kit (BD Biosciences).

2.3. Immunostaining

MEFs derived from Fut8-deficient mice or wild-type mice were cultured on 24-well glass plates. Under subconfluent conditions, cells were fixed with 4% paraformaldehyde/PBS (Wako). After blocking with 1% BSA/PBS for 30 min at room temperature, cells were stained with 0.1 μg/ml FITC-PhoSL/PBS for 1 h at room temperature. Cells were washed twice with PBS and stained with 4 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Stained cells were evaluated with confocal microscopy (Fluoview FV10i, Olympus).

2.4. Lectin–antibody ELISA for Fuc-Hpt

The Fab fragment of anti-human Hpt IgG (Dako) was coated onto the bottom of a 96-well ELISA plate, because IgG has the fucosylated oligosaccharide in its Fc portion. Coated plates were blocked with PBS containing 3% bovine serum albumin for 1 h, followed by washing with PBS containing 0.1% Tween 20 (PBS-T). A 50-μl aliquot of sera was placed into each well and incubated for 1 h at room temperature. The plate was washed three times with PBS-T, using Immuno Wash (Bio-RAD Model 1517, Bio-RAD, Tokyo, Japan). To detect Fuc-Hpt, 1/1000 diluted biotinylated AAL or PhoSL was placed into each well, followed by incubation at room temperature for 1 h. After washing plates three times with PBS-T, peroxidase-conjugated avidin was added to each well, followed by incubation at room temperature for 1 h. After washing 4 times with PBS-T, tetramethylbenzidine was added to each well and allowed to develop for 15 min. To stop the development, 1 mol/l sulfuric acid was added to each well. A standard curve for Fuc-Hpt was obtained as described elsewhere [11], using conditioned medium from the pancreatic cancer cell line PK8 transfected with an expression vector for Hpt, which was prepared in Immuno Biological Laboratories (Suppl. 1). The concentration of Fuc-Hpt was expressed in relative units to account for lot-specific differences.

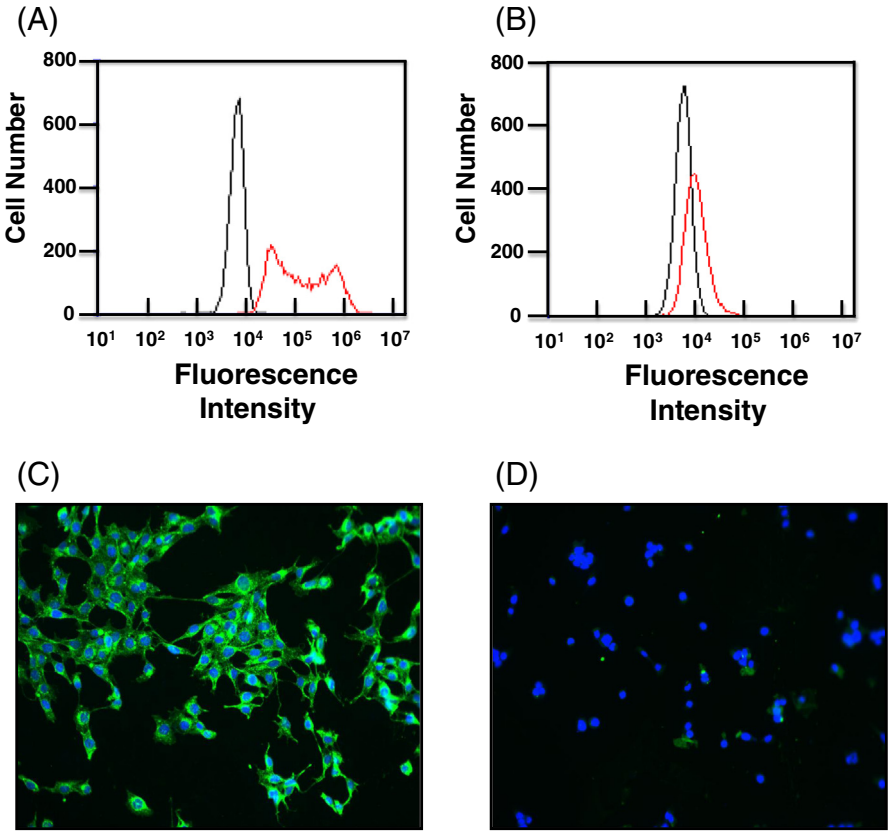

Fig. 1. Flow cytometry and immunofluorescent analysis of PhoSL staining in wild-type and Fut8 knockout mouse fibroblasts. (A) and (B) Approximately 1×10^4 cells were analyzed by flow cytometry using FITC-labeled PhoSL (A, wild-type MEFs; B, Fut8 knockout MEFs). The background signal in the absence of FITC-PhoSL is represented by the black line. PhoSL staining is represented by the red line. (C) and (D) Wild-type (C) and Fut8-knockout (D) MEFs were stained with FITC-labeled PhoSL. The detailed procedure is described in Materials and methods. No staining of PhoSL was observed in Fut8-knockout fibroblasts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)