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Original article

Establishment of a fluorescence-based method to evaluate endocytosis of desialylated glycoproteins *in vitro*

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ABSTRACT

Insufficient sialylation can result in rapid clearance of therapeutic glycoproteins by intracellular degradation, which is mainly mediated by asialoglycoprotein receptors (ASGPRs) on hepatic cells. In contrast, for glycoproteins, a long half-life is often related to high level of terminal sialic acid. These could be extremely important for insufficient sialylated biomedicines in clinic, and development of therapeutic glycoproteins in laboratory. However, how the desialylated glycoproteins are removed and how to evaluate the ASGPRs mediated endocytosis *in vitro* needs further investigate. Herein we described an integrative characterization of ASGPRs *in vitro* to elucidate its endocytosis properties. The endocytosis was determined by a fluorescence-based quantization method. The results showed that the ASGPRs could bind to poorly sialylated glycoproteins including asialofetuin and low sialylated recombinant Factor VIIa with a relatively higher ASGPRs binding affinity, and induce a more rapid endocytosis *in vitro*. Moreover, the mechanism under the internalization of ASGPRs was also investigated, which was found to depend on clathrin and caveolin. Utilizing the relative fluorescence quantification can be suitable for measurement of insufficient sialylated glycoprotein endocytosis and quality control of therapeutic glycoproteins, which could be useful for the understanding of the development of therapeutic glycoproteins.

1. Introduction

Due to the development of recombinant DNA biotechnology [1,2], the use of recombinant therapeutic proteins have increased over the past few years [3]. Most of them are glycoproteins produced in mammalian cell lines like Chinese-hamster-ovary-cells (CHO) [4–7] and Human-embryonic kidney 293 cells (HEK 293) [8–10]. Glycosylation, a most common post-translational modification (PTMs) in mammalian cells [11,12], is critical for the function (*e.g* protein-protein/target interaction) and physiological properties (*e.g* stability, solubility) of glycoproteins [3,13]. Glycosylation refers to the attachment of sugar moieties to the protein surface. Among the glycans attached, N-linked (occurs in endoplasmic reticulum) and O-linked (occurs in endoplasmic reticulum, Golgi, cytosol and nucleus) glycosylation are the most commonly investigated [14–16]. The N-linked glycan types include high mannose, hybrid and complex, which share a consensus sequence of Asn-X-Ser/Thr where X can be any amino acid except Pro[17,18]. Whereas, the O-linked glycan types are more complicate and no particular sequence motif has been identified.

The short circulatory half-life due to rapid elimination through proteolytic, glomerular filtration and receptor-mediated clearance is a common inferiority for all protein-based drugs [19,20]. Glycoprotein receptors mediated specific endocytosis in the surface of hepatocytes (*e.g* asialoglycoprotein receptors, ASGPRs) leads the clearance of glycoproteins, which was depending on their terminal sugar residues of glycoproteins *in vivo* [13,21,22]. Desialylated glycoprotein with Gal or GalNAc terminal could be recognized by ASGPRs, which leads to a rapid removal from serum [23–25]. The endocytosis of ASGPRs is considered to reduce half-life of therapeutic glycoproteins in circulatory, thus resulting in poor pharmacokinetics. Previous reports used animal model to evaluate the pharmacokinetic properties of different sialylated glycoproteins, and it is always a challenge to explain the ASGPRs mediated endocytosis process. Meanwhile, how to investigate the ASGPRs induced endocytosis *in vitro* could be urgently-needed, which could provide a more convenient way to better understand

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the removal of insufficient glycoproteins. In the present study, We used asialofetuin as ligand of ASGPRs [26], and introduced an integrative characterization of endocytosis and intracellular degradation, and further investigate the endocytosis of recombinant Factor VIIa.

2. Materials and methods

2.1. Cell lines and cell culture

CHO-K1 was cultured in F-12/DMEM medium (Gibico, China), supplemented with 10% fetal bovine serum (Gibico, New Zealand). HepG2 was cultured in high glucose dulbecco's modified eagle's medium (Gibico), supplemented with 10% fetal bovine serum (Gibico). L-02 was cultured in RPMI-1640 medium (Gibico) supplemented with 10% fetal bovine serum (Gibico). All cells were incubated in T25 flasks or 96-well plates (Corning, China) at 37 °C, 5% CO_2.

2.2. FITC labeling assay

Fluorescein isothiocyanate (FITC) conjugations were carried out according to the manufacturer's instructions (Sigma, China). Briefly, FITC power was dissolved in DMSO at 2 mg/50 μL per reaction within 5 min before the conjugation. 4 mg/200 μL asialofetuin (Sigma) solution were prepared in fresh 0.1 M carbonate bicarbonate buffer (pH = 9.0) respectively. Add 50 μL FITC dropwise and stir quickly. Incubate the reactions for 4 h at room temperature. Dialyze the labeled mixture with PBS buffer for 24 h, change PBS every 6 h. Determine the absorbance at both 280 nm and 495 nm to calculate the fluorescein/protein molar ration (F/P) according to the equation [27]: $\frac{F}{P} = \frac{A_{495} \times C}{A_{280} - 0.35 \times A_{495}}$, $C = \frac{MW \times E_{280}}{389 \times 195}$, E_{280} is the absorption at 280 nm of asialofetuin at 1.0 mg/mL. Conjugated samples were then applied to SDS-PAGE to analyze the purity.

2.3. In vitro *fluorescence microscopy analysis*

Fluorescence microscopy analysis was performed for the endocytosis of FITC-asialofetuin to investigate the internalization of glycoprotein receptors in the cell surface. 1×10^4 cells per well (96 well plate, Corning) were seeded 24 h before endocytosis assays. Wash twice with pre-heated Krebs'-HEPES buffer, and then FITC-asialofetuin was added and incubated for the required time at 37 °C in dark. Endocytosis reactions were stopped by discarding the incubated solution, cells were washed twice with Krebs'-HEPES buffer and fixed in 50 μL 4% paraformaldehyde immediately for 10 min in dark. For permeabilization, cells were washed with BD cytofix/cytoperm buffer (BD, USA). For EEA1 detection, 50 μL/well of mouse anti-EEA1 primary antibody (Abcam, USA, 1:50 in PBS with 3% BSA) was added, 4 °C for 1 h and 50 μL Alexa Fluor 555-labeled donkey anti-mouse IgG (Beyotime, China, 1:1000 in PBS with 3% BSA) was used as the secondary antibody. For detecting lysosome, cells were incubated with Lyso-Tracker Red (Beyotime, 1:10000) at 4 °C for 1 h. For nucleus staining, cells were stained with DAPI (50 μL per well, Beyotime). Images were captured using an inverted fluorescence microscope (OLYMPUS, Japan) and analyzed using Image pro plus software.

2.4. Relative endocytosis quantification

To quantify the intracellular endocytosed glycoprotein, cells were seeded into a black plate with clear bottom. Required procedures were taken for endocytosis according to the microscopy analysis section. Then cells were lysed with 100 μL RIPA lysis buffer (Beyotime) to extract intracellular FITC, and the fluorescence was measured by SAFIRE2 (Tecan, Switzerland). Meanwhile, the initial fluorescence intensity of FITC-asialofetuin without cell incubation was detected as blank control parallelly.

2.5. Inhibition assays of endocytosis

Endocytosis inhibition assays were base on specific inhibitors. ASGPRs (1:1000 in Krebs'-HEPES buffer, Santa Cruz, USA) antibodies was used to pre-block ASGPRs at 4 °C for 30 min. Dynamin function was inhibited by pre-incubation with dynasore (100 μM, Sigma) [28,29]. The activity of clathrin was disrupted by pre-added chlopromazin (10 μg/mL, Sigma) [30], and 100 μg/mL Filipin (Sigma) [31] was used to inhibit caveolae-mediated-endocytosis. Pinocytosis was blocked through inhibition of actin polymerization by cytochalasin D (2 μM, sigma) [32]. ATP metabolism was inhibited to determine passive diffusion process by 2-deoxyglucose (50 mM, Sigma) and NaN_3 (w/v 1%, Sigma).

2.6. Flow cytometry analysis

Intracellular endocytosed fluorescence was also assessed using flow cytometry. HepG2 and L-02 were washed twice and resuspended in Krebs'-HEPES buffer, incubated with indicated FITC-asialofetuin at 37 °C in dark. Then cells were washed twice and fixed by paraformaldehyde for 10 min. Cell surface fluorescence was quenched by 4% trypan blue, cells were collected by BD FACS Calibur (BD) and analyzed using Flowjo software (Treestar, USA).

2.7. Western blot

Cell samples were collected and lysed with RIPA lysis buffer containing protease inhibitor cocktail (Thermo scientific, USA). Total proteins were quantified and adjusted to the same concentration. Cell lysates were resolved by SDS-PAGE (10%) and transferred to PVDF membranes (Millipore, USA). The ASGPRs and β-actin expression in cell lysates were detected by their specific antibodies respectively using an ECL Detection System.

2.8. MST assays

MicroScale Thermophoresis (MST, NanoTemper, Germany) was used to detect the affinity of asialofetuin, fetuin, rFVIIa and $rFVIIa^{lowsa}$ with Human ASGPR1. Before the MST assays, ASGPR1 was labeled with RED-NHS-647 protein labeling kit according to the manufacture's instructions (Nano Temper). 10 μL labeled receptor was mixed with 10 μL gradient glycoproteins, and filled into capillaries to measure the fluorescence intensity in a Monolith NT.115 system. In the measuring, 20% IR-laser power was chosen. The binding data were fit using the Hill equation in Prism (Graphpad, USA)

2.9. Statistics

Student's *t*-test was used to calculate statistical significance. In all figures, error bars denote standard deviation.

3. Results

3.1. Asialofetuin was endocytosed in a time- and dose-dependent manner

After the asialofetuin was well labeled with FITC (Fig. S1, Table S1), two hepatocytes cell lines were chosen to perform the endocytosis. Significant differences in the intensity of intracellular