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Short communication

Extracellular and transmembrane region of a podocalyxin-like protein 1 fragment identified from colon cancer cell lines

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Abstract

Regulated intramembrane proteolysis of membrane proteins has been shown to play an important role in cell differentiation and in the pathogenesis of diseases. The aim of the present study was to identify novel peptides generated by intramembrane proteolysis. The peptides were identified in serum-free cultured (SFC) media from various cell lines by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). A 2315-Da peptide found only in medium from SFC colon cancer cell lines was identified and shown to consist of a portion of both the extracellular and transmembrane regions of human podocalyxin-like 1. This protein fragment was not found in lung or pancreatic cancer cell lines by immunoprecipitation-SELDI tests using an antibody specific to this fragment, suggesting that this human podocalyxin-like protein 1 fragment may be unique to colon cancer cell lines.

Keywords: Serum-free cultured (SFC) medium; Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS); Colon cancer cell lines; Podocalyxin-like protein 1 (Podocalyxin-1) fragment

1. Introduction

The extracellular domain of ∼2% of membrane-anchored cellular proteins can be released from the cell surface by an endogenous proteolytic cleavage process known as extracellular domain shedding (Arribas and Borroto, 2002). Proteolysis is conducted by enzymes which are either released into the pericellular space, or anchored to the plasma membrane. This process can potentially regulate most cellular functions mediated by transmembrane proteins and, therefore, has attracted the attention of cell biologists focused on problems such as cell adhesion and signal transduction, and on certain pathologies such as Alzheimer's disease (AD) and cancer (Arribas and Borroto, 2002).

AD-associated β-amyloid peptides (βA) are generated by the sequential processing of amyloid precursor protein (APP) by different proteolytic activities (collectively known as α-, β- and γ-secretases). βA peptides 40 or 42 amino acids length (βA40 and βA42) are generated by γ-secretase proteolysis of the transmembrane region of APP (Arribas and Borroto, 2002; Lichtenthaler et al., 2002; Yu et al., 2001). The identification of such transmembrane proteolytic processes can reveal physiological functions for extracellular domain shedding, alternative pathways in cell signaling, cell regulation, and protein processing.

Abbreviations: SELDI-TOF-MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight; LMW, low molecular weight; IB, immunoblotting; IP, immunoprecipitation.

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The presence of βA40 and βA42 in cell lines and in biopsy tissue isolated from Alzheimer's disease patients led us to suspect that low molecular weight (LMW) peptides (approximately 4 kDa in the case of AD) are difficult to detect by conventional techniques such as current proteomics-based approaches. In contrast, mass spectrometric techniques have been used to obtain cellular expression patterns of small peptides with masses less than 10 kDa. A peptidomic-based approach is useful for finding small molecules that may lead to the discovery of new peptides; towards this end, serum-free cultured (SFC) medium represents the simplest material for isolating endogenous substances originating from cells (Sasaki et al., 2002; Sato et al., 2001, 2002) since it contains secretory peptides, as well as peptide fragments from large proteins (Sasaki et al., 2002).

The present study was performed to identify LMW peptides in SFC media from various carcinoma cell lines by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). A 2315-Da peptide found only in colon cancer lines was shown to consist of a portion of both the extracellular and transmembrane region of human podocalyxin-like protein 1 (podocalyxin-1).

This is the first report of the identification of a fragment of human podocalyxin-1 in colon cancer cell lines.

2. Materials and methods

2.1. Cell lines

Colon cancer cells (Caco-2) and lung adenocarcinoma cells (LC-2/ad) were obtained from the Riken Cell Bank (Ibaraki, Japan). Colon cancer cells Colo320DM, Lovo and HCC56, and lung adenocarcinoma cells PC-3, RERF-LC-MS and RERF-LC-Ad2, were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). Colon cancer cells SW48, SW1116, RKO, HT29 and LS123, lung adenocarcinoma cells H23, H1975 and H1650, and Tera-1 embryonal carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Lung adenocarcinoma cell line A549 was obtained from the Cell Resource Center for Biomedical Research at Tohoku University (Miyagi, Japan). Pancreatic adenocarcinoma cells Capan-1, BxPC-3, PK-8, KP1N, KLM-1, PSN-1, PK-1 and AsPC-3 were obtained as described previously (Sasaki et al., 2002; Sato et al., 2001, 2002).

2.2. Sample preparation and analysis on SELDI arrays

SFC media from cell lines were prepared as previously described (Sato et al., 2001) and concentrated using a 3 M Empore™ C18 Cartridge 4315 standard density (GL Sciences, Tokyo, Japan) for SELDI analysis (Ciphergen Biosystems, CA, USA). The SFC medium was acidified to a final concentration of 0.02 N HCl, permitting the capture of analytes on the cartridge. The cartridge was washed with 10% acetonitrile (ACN)–0.1% trifluoroacetic acid (TFA), and then the captured analytes were eluted with 60% ACN-0.1% TFA. Finally, SFC media samples for SELDI analysis were concentrated 25-fold, and a sample aliquot (1 μl) was analyzed on a reverse-phase (H4) ProteinChip array. The mass spectrometer was externally calibrated with single and doubly-charged bovine insulin β chain (3495.9 Da) ions, allowing m/z values of each molecule bound on the array surface to be accurately determined. All SELDI tests were performed on a H4 reverse-phase ProteinChip array as described below.

2.3. Peptide identification

Concentrated SFC medium was applied to a C18 column (Cadenza CD-C18, column size 150 × 2 mm, Imtakt, Kyoto, Japan) equilibrated with 0.1% TFA at a flow rate of 200 μl/min. The 2315-Da peak was isolated using a linear gradient from 10–60% ACN/0.1% TFA (first step), followed by a 25–35% ACN/0.1% TFA (second step) gradient over 20 min. The amino acid sequence of the 2315-Da peak was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS (Ultraflex-TOF-TOF, Bruker Daltonics, Bremen, Germany).

In order to identify the 2315-Da peak, we searched the NCBInr sequence database using the Sequence-Query search engine in Mascot. The 2315-Da peak was determined to be a fragment (amino acids 415–435; LGDQGPPEEAEDRFSMPLIIT) of human podocalyxin-1 precursor (accession number: O00592 in NCBInr).

2.4. Preparation of monoclonal antibodies to the 2315-Da peptide

Monoclonal antibody (clone: 7G8; subclass: IgG1) was generated from female Balb/c mice immunized with a synthetic version of the 2315-Da peptide (NH_2-LGDQGPPEEAEDRFSMPLIIT-COOH) corresponding to amino acid residues 415–435 of human podocalyxin-1.

2.5. Immunoprecipitation-SELDI (IP-SELDI) tests

Five micrograms of 7G8 and 30 μl of Sepharose slurry coupled to Protein G (Pharmacia) were mixed for 60 min at 4 °C. After several washings with PBS, concentrated SFC medium was neutralized (pH 7.5), added to the Sepharose, and then incubated overnight at 4 °C. After several washings with PBS, the Sepharose was immersed in 100 μl of 0.2% TFA to release the bound peptides. Five-microliter aliquots of the analyte were analyzed by immunoprecipitation-SELDI (IP-SELDI) tests.

2.6. Immunoblotting (IB)

SFC medium was concentrated using an Amicon Ultra limited filter 30-K (Millipore). Protein concentrations were determined using a BCA assay kit and BSA as the standard (Pierce, Rockford, IL, USA). Proteins in filtered SFC media (15 μg total proteins) were separated on a 10% SDS–PAGE gel, then transferred to polyvinylidene fluoride 0.45 μm membranes. The transferred membrane was blocked using 3% BSA and 3% polyvinylpyrrolidone in 50 mM Tris–HCl buffered saline with 0.05% Tween-20 (TBST). The membrane was incubated with monoclonal antibody 3D3, which recognizes the extracellular domain of human podocalyxin-1 (Kershaw et al., 1997; Schopperle et al., 2003) (Zymed Laboratories, San Francisco, CA, USA), or with 7G8. Washed membranes were incubated with goat polyclonal anti-mouse IgG (Fc γ) labeled with horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc, PA, USA) and the signal was detected using Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

3. Results

3.1. Differential profiling on SELDI-TOF-MS using SFC media from carcinoma cell lines

Fig. 1 shows representative MS spectra of SFC media in the mass range *m/z* 2000–2500. A 2315-Da peak was detected in the colon cancer cell lines Caco-2, Lovo, HCC-56 and LS123, but not in other colon cancer cell lines. In contrast, this peak was not found in the SFC from any of the lung and pancreas cancer cell lines analyzed.

3.2. Purification of the 2315-Da peak and determination of its sequence

The 2315-Da peak was partially purified by RP-HPLC using SFC medium from Caco-2 cell line since this culture