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Technical note

A monoclonal antibody recognizes undifferentiation-specific carbohydrate moieties expressed on cell surface of the human dental pulp cells

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ABSTRACT

Human dental pulp cells are obtained from dental pulp tissue, and have the ability to form dentin and a pulp-like complex. Although adult stem cells have been identified from the primary culture by using specific cell surface markers, the identity of surface markers for the purification of stem cells within the dental pulp population are still unclear. Previously, we had constructed monoclonal antibodies against the undifferentiated cell-specific surface markers of human dental pulp cells (hDPCs) by performing decoy immunization. Among them, a monoclonal antibody against the cell surface antigen of the undifferentiated hDPCs (named UPSA-1) was purified and its heavy and light chain consensus regions were analyzed. The cell surface binding affinity of UPSA-1 mAb on the undifferentiated hDPCs was stronger than that on the differentiated cells. When tunicamycin was applied to hDPSCs during culture, the cell surface binding affinity of the antibody was dramatically decreased, and dentinogenic differentiation was reduced. The purified UPSA-1 antigen band resulting from immunoprecipitation disappeared or shifted down on the SDS-PAGE by deglycosylation. These data suggested that glycosylation on the cell surface might be a marker of an undifferentiated state, and that UPSA-1 mAb might be useful for identifying the carbohydrate moiety on the cell surface of undifferentiated pulp cells.

1. Introduction

Adult stem cells have been identified in various tissues, such as bone marrow and skin within tissue-specific niches. However, they are found in small amounts in adult tissues and show limited self-renewal potential in culture. These properties, together with the heterogeneous feature of some adult stem cell types, make it difficult to study them at the molecular level. This heterogeneity has evolved as a mechanism that enables stem cells to respond to differentiation-inducing signals during self-renewal (Graf and Stadtfeld, 2008). Human dental pulp cells (hDPCs) are a source of multipotent stem cells for pulp and dentin regeneration (Gronthos et al., 2002). hDPCs are obtained from the primary culture of dental pulp tissues, and the primary cell population are predicted to be a mixture of differentiated cells, progenitors, and stem cells. Cell surface markers have been used to select different subsets of DPCs displaying different differentiation potentials (Kawashima, 2012). STRO-1 identifies a subgroup of cells with dentinogenic properties from dental pulp cells (Yang et al., 2009). Cells that are positive for CD34 and CD117 and negative for CD45 are highly clonogenic (Laino et al., 2005). Other markers expressed by dental pulp primary cells are CD29 and CD44 (Jo et al., 2007), as well as CD73 and CD105 (Pivoriuunas et al., 2010). Some of these markers are detected in perivascular, cell rich zone, odontoblastic layer and central pulp core (Machado et al., 2016). In addition to membrane proteins as the cell surface antigens of stem cells, oligosaccharide moieties of cell surface glycoproteins and glycolipids play important roles in the differentiation, adhesion, migration and growth of stem cells (Chen et al., 2015; Gu et al., 2012; Lau et al., 2007; Zachara and Hart, 2002). Cell surface glycans help cells communicate with their extracellular environment, encounter other cells and ligands, and facilitates cell adhesion by proper protein-protein interaction (Haltiwanger and Lowe, 2004; Rudd et al., 1999). Due to their lineage-specific nature in different cell types, glycan structures can be used as promising targets for the identification and isolation of stem cell markers (Lanctot et al., 2007; Tateno et al., 2007; Varki, 2006). Typical embryonic stem cell markers, glycolipids SSEA-3 and SSEA-4, were identified from monoclonal antibodies recognizing oligosaccharide epitopes, which are glycosphingolipids (Lanctot et al., 2007; Muramatsu and Muramatsu, 2004). Polysialylation of neuronal cell adhesion molecules and various N-glycosylation

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patterns are promising candidate markers to detect and discriminate neural stem cells (Close et al., 2003; Kim et al., 2012) and adipogenic progenitors (Hamouda et al., 2013), respectively. Although many markers expressed by fibroblastic cells show cross-reactivity with hDPCs, there are no specific and strict protein markers or carbohydrate epitopes characterizing the subset of stem cells within the hDPC population. In order to identify potential markers for stem cells in the hDPC population, we previously screened surface antigenic molecules from undifferentiated hDPCs, which would contain stem cells, and collected monoclonal antibodies against surface antigens (Hwang et al., 2015b). One of those antibodies recognizes the cell surface glycan structure of undifferentiated hDPCs, potentially providing a new glycan-based surface marker for stem cells in the hDPC population.

2. Materials and methods

2.1. Cell culture and chemical treatment

For primary culture of human dental pulp cells (hDPCs), periodontal ligament cells (PDLCs), gingival fibroblasts (GFs) and follicle cells (FCs), human third molars were obtained from adult patients (17–28 years of age), and supernumerary teeth (s-PDLC & s-DPC) were extracted from children patients (4–6 years of age) under guidelines approved by the IRB of the Dankook Dental Hospital. Human dental tissues were chopped and digested by 3 mg/ml collagenase type-I (Millipore), 4 mg/ml dispase (Sigma) for 1 h at 37 °C (Choi et al., 2015; Min et al., 2011). Isolated single cell suspension was incubated with α-MEM (Hyclone) containing 20% fetal bovine serum (FBS, Hyclone) and antibiotics (Lonza) at 37 °C in 5% CO_2. The basal medium for hFOB osteoblast (ATCC) was a 1:1 mixture of Ham's F12 and DMEM with 10% FBS (Hyclone), and cells were cultured at 34 °C in 5% CO_2. The osteocarcinoma cell lines such as MG63, SAOS2, KHOS, U2OS, HOS, RD-ES and SJSA1 were cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2. In order to induce mineralization, cells were cultured with medium containing 100 μM ascorbic acid, 100 nM dexamethasone, and 5 mM β-glycerophosphate for 14 days.

2.2. Antibody purification

The mAb was purified by column chromatography from the hybridoma culture media. Briefly, culture media were pooled and loaded on the column of Protein G agarose (Incospharm). After washing with PBS, the bound IgG was eluted with 100 mM glycine buffer (pH 2.8). Then, 1 M Tris-HCl (pH 9.0) was quickly added in the eluted fractions in ratio of 10:1 for neutralization. After dialysis in PBS, antibody was quantified.

2.3. Antibody gene sequencing

The primers for antibody sequencing were synthesized and used as reported by Wang et al. (Table 1) (Wang et al., 2000). Hybridoma cells (~1×10^6 cells) were pooled and total RNA was extracted from cells by using Easy-spin™ total RNA extraction kit (Intron). Then, cDNA was synthesized by ONE-STEP™ RT-PCR premix (Intron) as followed: 30 min at 45 °C for cDNA synthesis, and 1 cycle of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C and the final cycle of 5 min at 72 °C for PCR reaction. The cDNA products were cloned into pBluescript KS (+) vector, and sequencing was performed. Consensus domains of antibody were estimated by using the IMGT/V-QUEST program (version 3.2.21).

Table 1
DNA sequences of the primers used for reverse transcription PCR (RT-PCR) of mouse IgG V_L and V_H gene. Mixed base codes were indicated as followed; R = a & g, Y = c & t, M = a & c, K = g & t, S = c & g, W = a & t, V = a, c & g, and N = a, c, g & t.

Mouse heavy chain constant region primers, VH primers	
IgG	5′-ATA GAC AGA TGG GGG TGT CGT TTT GGC-3′
5′ MH1	5′-SAR CTN MAG CTG SAG SAG TC-3′
5′ MH2	5′-SAR GTN MAG CTG SAG SAG TCW GG-3′
Mouse kappa chain constant region primers, VL primers	
5′ Mk	5′-GAY ATT GTG MTS ACM CAR WCT MCA-3′
Primer 6	5′-GAC ATT GTG CTG ACC CAA TCT CCA GCT TCT-3′
Primer 7	5′-GAC ATT CAG CTG ACC CAG TCT CCA-3′

2.4. Flow cytometry

Cells were detached by dissociation buffer (Millipore) for 10 min at room temperature (RT). 1×10^6 cells were incubated with 2.5 μg of the purified monoclonal antibody for UPSA-1 (undifferentiated pulp stem cells surface antigen-1), anti-Stro-1 antibody (2.5 μg, R&D system), PE-conjugated CD24, CD44, CD73 and CD90 antibodies (1:100, BD Biosciences) in PBS containing 1% BSA for 1 h on ice. In case of cells treated with UPSA-1 mAb and anti-Stro-1 antibody, PE-conjugated anti-mouse IgG (1:100, Santa Cruz) was added as the secondary antibody for 1 h on ice. After washing, cells were analyzed by FACSCalibur™ (BD Biosciences). Quantification of antibody binding affinity was analyzed by using Cell Quest and WinMDI program.

2.5. Inhibition of glycosylation and deglycosylation analysis

For inhibition of glycosylation in cells during culture, tunicamycin (Sigma) was directly treated in medium to 1 mg/ml for indicated time. For chemical deglycosylation, 10 mM sodium periodate was treated in the purified product by immunoprecipitation for 1 h at room temperature (Raju and Davidson, 1994). For enzymatic deglycosylation, purified samples were treated with Protein Deglycosylation Mix™ (NEB) including *O*-glycosidase, Neuraminidase, β(1-4)-Galactosidase, and β-*N*-acetylglucosminidase under denaturating and non-denaturating condition as the procedures of the provider. For denaturating reaction, 1% NP-40 was added to the reaction mixture, following heating at 100 °C for 10 min.

2.6. Immunoprecipitation, SDS-PAGE, and western analysis

In order to identify surface antigens, cells were labeled by EZ-Link Sulfo-NHSLC-Biotin (Thermo Scientific) for 30 min at 4 °C, and lysed by treatment with 1% NP-40 buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, pH 8.0, 2 mM EGTA, pH 8.0, 1% NP-40, protease inhibitors). Cell extract was incubated with 10 μg of anti-UPSA-1 antibody for 16 h at 4 °C, followed by incubation with Protein G agarose (Incospharm) for 3 h at 4 °C. The immunoprecipitants were separated on SDS-PAGE, transferred to a PVDF membrane (Millipore), and then probed with the anti-UPSA-1 antibody, followed by treatment with streptavidin-HRP (GE Healthcare). The antigen signals were visualized by using ECL Western Blotting Detection Kit (GE healthcare), and exposed under X-ray film. Glycoproteins on SDS-PAGE were detected by sugar-specific staining with Pro-Q Emerald 300 (Invitrogen). Images of the Pro-Q Emerald 300 stained gel under an UV transilluminator at 300 nm (Syngene, GBOX-EF) were compared with the gel stained with Coomassie Brilliant Blue.

2.7. Immunofluorescence analysis

Immunostaining of cell surface antigen was performed as previously mentioned (Hwang et al., 2015b). Briefly, cells were cultured on a cover-slip, and were treated with 10% horse serum for blocking. Without additional fixation, cells were incubated with the primary antibody at 4 °C for 16 h, followed by treatment with FITC-conjugated anti-mouse IgG (1:100 dilutions, Santa Cruz). Nuclei were detected by staining with 4,6-diamidino-2-phenylindole (DAPI).