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Identification of Cell Surface Markers to Differentiate Rat Endothelial and Fibroblast Cells Using Lectin Arrays and LC–ESI-MS/MS

Ji Eun Lee[†], **Shama P. Mirza**[‡], **Daniela N. Didier**[‡], **Mark Scalf**[†], **Michael Olivier**[‡], **Andrew S. Greene**[‡], and **Lloyd M. Smith**[†,*]
Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, Wisconsin 53706, and Biotechnology and Bioengineering Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

Abstract

Vascular endothelial cells located at the inner surface of blood vessels are a key component in angiogenesis and are employed as a primary cell type in the study of angiogenesis. These endothelial cells are, however, easily contaminated with fibroblast cells, which are located in proximity to the endothelial cells, during their isolation from tissue. It is thus important to find markers to distinguish the two cell types. In the present work, lectin arrays were prepared using aldehyde-terminated self-assembled monolayers (SAMs) and utilized to explore cell surface carbohydrate expression patterns on endothelial and fibroblast cells. It was found that the lectins *Griffonia simplicifolia* II (GS II) and *Ulex europaeus* agglutinin I (UEA I) selectively bind to rat fibroblast cells and not to rat endothelial cells. GS II-binding glycoproteins on fibroblast cells, which are potential cell surface markers to differentiate endothelial and fibroblast cells, were captured on a GS II lectin column and analyzed by LC–ESI-MS/MS. Six candidate cell surface glycoproteins were identified. Differential expression was confirmed by Western blot analysis for two of these proteins, lysosome-associated membrane glycoprotein-1 and transmembrane glycoprotein NMB.

Angiogenesis, the formation of new blood vessels from preexisting ones, plays critical roles not only in normal processes including wound healing and tissue development but also in many diseases such as hypertension and cancer.[1] Vascular endothelial cells located at the inner surface of blood vessels are a key component in the growth of new vessels, and have been employed as a primary cell type to better understand the mechanism of angiogenesis.[2–5] These endothelial cells are, however, easily contaminated with fibroblast cells, which are located in proximity to the endothelial cells, during their isolation from tissue.[6,7] Thus, multiple methods of isolating enriched populations of endothelial cells have been studied. These include sieving/filtration, manual weeding using phase-contrast microscopy, and density-gradient centrifugation.[8–11] Since these methods are laborious and experience is required to identify the endothelial cells on the basis of morphology, another approach using selective culture media is more widely employed for establishing pure endothelial cell cultures. The method is based upon the observation that the use of D-valine rich media inhibits the proliferation of the fibroblast cells, which do not have the D-amino acid oxidase necessary to convert D-valine to

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*Author to whom correspondence should be addressed..
†University of Wisconsin-Madison.
‡Medical College of Wisconsin.

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L-valine and thus cannot utilize D-valine.[7,12] Although this approach is useful to generate purified endothelial cell cultures, it is still important to find a quick and reliable method to evaluate the purity of endothelial cell cultures. Several endothelial cell markers including platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial-cadherin (VE-cadherin) have been identified for this purpose through antibody-mediated approaches such as immunohistochemical staining.[13–16] Unfortunately, these known markers are also expressed on fibroblasts based on our experimental results (data not shown), and thus are not useful for the detection of fibroblast cell contamination. Therefore, there is a need to find novel cell markers to differentiate the two cell types.

Mammalian cell surfaces are decorated with a dense layer of carbohydrates. The oligosaccharides present on both proteins and lipids play important roles in biological processes such as cell–cell interaction, cell recognition, and adhesion.[17,18] These cell surface carbohydrates display a structural diversity depending on cell type or differentiation state.[19,20] Thus, cell surface glycoproteins, which are membrane proteins with covalently bound carbohydrate side chains, are good candidate surface markers to distinguish different cell types.[21,22] For the identification of specific cell surface glycoproteins, the first step is to characterize the variations in cell surface carbohydrate expression patterns for different cell types of interest.

In the present work, lectin arrays were employed to profile the cell surface carbohydrate expression patterns of endothelial and fibroblast cells. Lectins are carbohydrate-binding proteins of nonimmune origin that can bind to multiple glycoproteins bearing the same carbohydrate motif and have been widely used to interpret the sugar codes on glycoproteins.[23–26] Nine different lectins were immobilized on aldehyde-terminated self-assembled monolayers (SAMs), and the binding patterns of the two cell types to each lectin were observed by phase contrast microscopy. *Griffonia simplicifolia* II (GS II) and *Ulex europaeus* agglutinin I (UEA I) lectins, which have binding specificity for nonreducing terminal *N*-acetyl glucosamine (GlcNAc) and α-1,2-fucose, respectively, generated different binding patterns for the two cell types. That is, fibroblast cells bound to GS II and UEA I, whereas the endothelial cells did not. This indicates that the binding partners of GS II and UEA I on fibroblast cells are potential cell surface markers to differentiate the two cell types. The identities of these fibroblast-specific cell surface markers were investigated by capturing them on a GS II lectin column followed by elution and analysis using LC–ESI-MS/MS. Six candidate cell surface glycoproteins were identified. Differential expression was confirmed by Western blot analysis for two of these proteins, lysosome-associated membrane glycoprotein-1 and transmembrane glycoprotein NMB.

EXPERIMENTAL SECTION

Materials

Concanavalin A (ConA), *Ricinus communis* agglutinin I (RCA I), wheat germ agglutinin (WGA), *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin I (MAA I), *Ulex europaeus* agglutinin I (UEA I), *Griffonia simplicifolia* II (GS II), agarose-bound *Griffonia simplicifolia* II (GS II), and *N*-acetylglucosamine were purchased from Vector Laboratories (Burlingame, CA). *Helix pomatia* agglutinin (HPA) was from MP Biomedicals (Solon, OH). *Griffonia simplicifolia* I (GS I), bovine serum albumin (BSA), trypsin-EDTA solution, and antibiotic-antimycotic solution were obtained from Sigma-Aldrich (Saint Louis, MO). Cellgro RPMI 1640 with L-glutamine and Dulbecco's phosphate-buffered saline (DPBS) were from Fisher Scientific (Pittsburgh, PA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Geneticin selective antibiotic solution, penicillin-streptomycin, and Colloidal Blue staining kit were obtained from Invitrogen (Carlsbad, CA). Gold-coated glass substrates 7.6 cm × 2.5 cm (1 nm chromium and 30 nm gold) were obtained from GenTel BioSciences, Inc. (Madison, WI). Econo-Pac columns and 4–15% tris-HCl gel were from Bio-Rad

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Laboratories (Hercules, CA). Protease inhibitor cocktail (EDTA free) was obtained from Roche Applied Science (Indianapolis, IN). Microcon YM-10 centrifugal filters were purchased from Millipore (Billerica, MA). Ultra MicroSpin columns (C_{18}) were from The Nest Group, Inc. (Southborough, MA).

Cell Culture

Vascular endothelial cells derived from Sprague–Dawley (SD)[27] and green fluorescent protein (GFP)-transgenic Dahl Salt-Sensitive[28] rats were cultured in RPMI 1640 with L-glutamine supplemented with 20% fetal bovine serum (FBS), 1% 100× antibiotic-antimycotic solution, and 0.4% gentamicin at 37 °C in a humidified incubator containing 5% CO_2. Rat1-R12 fibroblast cells originated from rat connective tissue were obtained from American type Culture Collection (ATCC) (Manassas, VA). Fibroblast cells were grown in DMEM supplemented with 20% fetal bovine serum (FBS), 1% penicillin-streptomycin solution, and 0.8% Geneticin selective antibiotic solution at 37 °C in a humidified incubator containing 5% CO_2.

Lectin Array Fabrication

Gold-coated glass substrates were briefly rinsed using deionized water and ethanol and then soaked in 1–10 mM di(11-undecanal) disulfide ($(CHO–(CH_2)_{10}–S–)_2$)/ethanol solution overnight.[29] The aldehyde-terminated substrates were washed thoroughly with ethanol and deionized water and dried gently with pure N_2 gas. Lectins were immobilized on the aldehyde-terminated gold substrates by depositing 0.7 μL by hand-spotting with a micropipet and incubation in a humid chamber overnight. Lectins were dissolved in HEPES buffer (10 mM HEPES, 1 mM $CaCl_2$, 0.1% NaN_3, pH 8.5) to 10 μM. $NaBH_3CN$ was added to the lectin solutions (50 mM final concentration) right before spotting on the gold substrate to reduce the imine product of reaction between the primary amine groups on the lectins and the aldehyde groups on the gold substrate to a more stable secondary amine.[30] After incubation overnight in a humid chamber at room temperature, the lectin-modified substrates were soaked in 1% BSA/Dulbecco's phosphate buffered saline (DPBS, without calcium and magnesium) for 30 min to block the remaining aminoreactive surface sites on the substrate. The substrates were kept on ice until use in cell-binding experiments.

Cell Binding to Immobilized Lectin Substrates

Endothelial cells and fibroblast cells were separately trypsinized and centrifuged at 1000*g* for 10 min. The cell pellet from each cell type was resuspended in DPBS (without calcium and magnesium) followed by another 10 min centrifugation. The supernatant was removed and the cell pellet was resuspended in DPBS (with calcium and magnesium) to a concentration of 3.3×10^5 cells/mL for binding experiments. A total of 3 mL of cell suspension from each cell type was separately added to the lectin-modified substrates and then incubated for 30 min on ice. After incubation, the substrates were dipped into 6 mL of DPBS (without calcium and magnesium) solution in a well of a six-well plate and promptly removed in order to wash off nonspecifically bound cells. After four such washes, the cells bound to the lectin-modified substrates were observed and imaged using a 4× magnification objective on an Olympus IX81 phase contrast microscope with a Hamamatsu camera for taking phase contrast images or a Nikon Eclipse 600 microscope with a PI MicroMax camera for taking fluorescence images (excitation, 494 nm; emission, 518 nm).

Membrane Protein Preparation

A total of 5×10^8 fibroblast cells were lysed in cell lysis buffer A (200 mM mannitol, 70 mM sucrose, and 10 mM HEPES adjusted to pH 7.4, and protease inhibitor cocktail without EDTA) by homogenizing with a 23 gauge needle attached to a 1 mL syringe. The cell lysate was centrifuged at 7700*g* for 10 min, and the supernatant (soluble protein fraction) was discarded.

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The remaining cell pellet was resuspended using cell lysis buffer A and centrifuged at 830*g* for 10 min. The resulting pellet (insoluble protein fraction) was delipidated by chloroform extraction as described[31] and solubilized using binding buffer A (1% NP-40, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.4) followed by filtration (0.22 μm filter). The solubilized membrane protein solution was then diluted with the same volume of equilibration buffer (150 mM NaCl, 10 mM Tris-HCl, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4).

Griffonia simplicifolia II (GS II) Bound Protein Analysis

A volume of 1.5 mL of agarose-bound *Griffonia simplicifolia* II (GS II) lectin was loaded onto an Econo-Pac column and packed using gravity. The GS II lectin column was washed using binding buffer B (150 mM NaCl, 10 mM Tris-HCl, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4), and the delipidated membrane proteins were loaded onto the column. After the column was washed using binding buffer B to remove the unbound materials, the GS II bound proteins were eluted using *N*-acetylglucosamine solution (0.4 M, dissolved in binding buffer B). The eluted sample was concentrated using a Microcon centrifugal filter device (10 kDa MW cutoff) followed by buffer exchange with 31 mM Tris-HCl and 0.1% SDS solution (pH 7.4). The sample was then run on a SDS-PAGE gel (4–15% Tris-HCl gel) for analysis. The gel was stained using a Colloidal Blue staining kit to visualize the GS II bound proteins. Five of the stained gel bands were excised and subjected to in-gel digestion. Briefly, the proteins were reduced with 100 mM dithiotreitol (DTT) in 25 mM NH_4HCO_3 for 30 min at 56 °C and alkylated with 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 for 30 min at room temperature followed by trypsin digestion overnight. Peptides were then extracted with 0.1% trifluoroacetic acid (TFA) and 70% acetonitrile/5% TFA in water, respectively. Extracts were dried in a Speedvac and subsequently acidified to 0.1% TFA. The samples were desalted using an Ultra MicroSpin column (C_{18}).

Mass Spectral Analysis (LC–ESI-MS/MS)

The tryptic peptides generated from the five stained gel bands were analyzed using an ion trap LTQ mass spectrometer interfaced with a nano-HPLC system (Thermo Electron) using a C_{18} capillary column which was packed with 5 μm C_{18} particles (Luna $C_{18,}$ Phenomenex). The solvents A and B used for chromatographic separation of peptides were 5% acetonitrile in 0.1% formic acid and 95% acetonitrile in 0.1% formic acid, respectively. The capillary column were run at 200 nL/min with the following gradient conditions: 0–30 min 0–5% B, 30–180 min 5–35% B, 180–240 min 35–65% B, 240–250 min 65–100% B. The condition 100% B was held for 10 min, then switched to 100% A and held for another 40 min. The ions eluted from the column were electrosprayed at a voltage of 1.7 kV. The capillary voltage was 45 V, and the capillary temperature was 200 °C. No auxiliary or sheath gas was used. Helium was used in the trap as a collision gas for ion fragmentation. The target value to fill the ion trap was 3×10^4 ions in the MS scan mode and 1×10^4 in the MS/MS mode. A full scan mass spectrum (400–2000 *m*/*z*) was followed by fragmentation of the six most abundant peaks from the full scan MS, using 35% of the normalized collision energy for the MS/MS spectra. Dynamic exclusion was enabled with a repeat count of 2, an exclusion duration of 3 min, and a repeat duration of 30 s. The chromatographic and mass spectral functions were controlled by the Xcalibur data system (ThermoFinnigan, Palo Alto, CA). The raw data generated by MS was transferred into a peak list by Extract_msn (Bioworks 3.2) software. The MS data and MS/MS were searched against Uniprot Rodent database v49.1 having 16 570 protein entries using TurboSEQUEST (v.27; revision 12).[32] The search was limited to only tryptic peptides with two missed cleavages allowed. The search criteria were set to a mass tolerance of 2.5 Da for MS data and 1.0 Da for MS/MS data with variable modifications of methionine oxidation (+16 Da) and carbamidomethylation of cysteine (+57 Da). The peptide identifications were filtered from the search results using the Epitomize program.[33] The Xcorr versus charge state filter used was set to Xcorr values of 1.8, 2.5, and 3.0 for charge states +1, +2, and +3, respectively.

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These filter values are similar to others previously reported for SEQUEST analyses.[34] Peptide probability score values are set at 0.6 or more based on the peptide false discovery rate (FDR) calculations of the data. The FDR was calculated by searching the data against a custom decoy database,[35] and those that have a FDR of 5% or less are considered to be correct identification. The peptide probability score of >0.6 (5% FDR) was taken as the threshold for accepting the individual MS/MS spectrum. All proteins were identified by two or more peptides. Finally, the peptides listed were manually verified for correct identification by comparing the experimental spectra with the theoretical b and y ion spectra. Identification was based on proteins identified by peptides unique to the proteins that are reported. In cases in which the same protein was identified in multiple species, redundancy was eliminated by selecting the proteins from rat, which is the source of the sample.

Western Blot Analysis

Cultured endothelial and fibroblast cells were rinsed three times with DPBS (without calcium and magnesium). Cells were scraped, pelleted, and resuspended in cell lysis buffer B (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Trizma base, pH 8.0). Cells were sonicated and assayed for total protein (DC protein assay, BioRad). A total of 25 mg of protein was separated on a 8% Tris-HCl SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane, which was blocked overnight in 5% nonfat dry milk or 0.3% bovine serum albumin (BioRad), both diluted in Tris-buffered saline (50 mM Tris and 750 mM NaCl, pH 8) with 0.08% Tween 20 (BioRad). The blots were then incubated with a monoclonal antibody against lysosome-associated membrane glycoprotein 1 (LAMP-1, 1:1000 dilution, Santa Cruz Biotechnology clone LY1C6) or a polyclonal antibody to transmembrane glycoprotein NMB (GPNMB, 1:1000 dilution, Santa Cruz Biotechnology clone K-16). All LAMP-1 and GPNMB antibodies were diluted in 2% nonfat dry milk or 0.3% bovine serum albumin, respectively, and incubated for 1 h at room temperature. Washed blots were incubated with appropriate secondary antibody conjugated to horseradish peroxidase (BioRad) at a dilution of 1:2000 and then detected using the SuperSignal West Dura Chemiluminescence Substrate (Pierce) detection system. Membranes were exposed to CL-XPosure Film (Pierce) for 5~10 s and developed using a Kodak X-Omat 2000 processor.

RESULTS

Cell Surface Carbohydrate Characterization Using Lectin Arrays

Nine lectins (ConA, RCA I, WGA, SNA, MAA I, HPA, GS I, GS II, and UEA I) were immobilized on the aldehyde-terminated SAMs on gold substrates. The lectins were covalently attached to the SAMs by reaction between the primary amine groups present on the lysine residues of the proteins and the aldehyde groups of the SAMs. The endothelial and fibroblast cells were then separately captured on the lectin-modified substrates through the lectin and cell surface–carbohydrate interaction. The binding patterns of the two cell types to each lectin spot were observed by phase contrast microscopy (see Figure 1). Both endothelial and fibroblast cells bound to ConA, RCA I, WGA, SNA, MAA I, HPA, and GS I, which indicates that both cell types have α-Man, terminal β-Gal, β-GlcNAc, sialic acid, α-GalNAc, and α-Gal expressed on their cell surfaces as seen in Table 1.[36] In contrast, endothelial cells clearly did not bind to the lectins GS II and UEA I, which recognize nonreducing terminal GlcNAc and α-1,2-fucose, respectively, whereas fibroblasts clearly did bind. This observation indicates that the fibroblast cells express terminal GlcNAc and α-1,2-fucose, whereas the endothelial cells do not.

Selective Binding of GS II to Fibroblast Cells

The specificity of fibroblast cell binding to GS II lectin was tested in the presence of endothelial cells. Endothelial cells derived from transgenic Dahl Salt-Sensitive rats, which express GFP, [28] were used because they permit endothelial cell binding to the lectin to be readily determined

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by fluorescence microscopy (excitation, 494 nm; emission, 518 nm). To test the feasibility of this approach, the GFP-endothelial cells (1×10^6 cells) were added to GS I-modified substrate, and the binding was examined by fluorescence microscopy as well as phase contrast microscopy (see Figure 2A). As seen in the figure, the binding image generated by fluorescence microscopy was comparable to that obtained by phase contrast microscopy. Next, the same number (5×10^5 cells) of fibroblast cells and the GFP-endothelial cells were mixed and added to GS I- and GS II-modified substrates, respectively. The binding patterns were monitored by both phase contrast microscopy and fluorescence microscopy (see Figure 2B,C). As seen in the bottom panel of the cell binding to GS I (Figure 2B), about 50% of the cells bound to GS I were from the endothelial cells, which coincides with the results obtained from the individual binding experiments of the endothelial and fibroblast cells. In contrast, there is no detectable binding of the endothelial cells to GS II as seen in the bottom panel of Figure 2C. This indicates that GS II can selectively capture fibroblast cells in the presence of endothelial cells.

GS II Bound Protein Analysis

The cell surface glycoproteins bound to GS II lectin on fibroblast cells were isolated for identification. Crude membrane protein fractions were prepared from 5×10^8 fibroblast cells and were delipidated using chloroform extraction to remove the lipid bilayer. The total amount of delipidated protein obtained using a BCA (bicinchoninic acid) protein assay was 1.89 mg. The proteins were then loaded onto a GS II lectin column to capture glycoproteins containing *N*-acetyl glucosamine on the nonreducing terminus, and the bound materials were eluted using *N*-acetyl glucosamine.

GS II Bound Protein Identification

The eluted proteins from the GS II lectin column were run on a SDS-PAGE gel, and five of the stained gel bands were visualized after colloidal blue staining (see Figure 3). The five gel bands were excised, and ingel tryptic digestion was performed in order to identify the proteins. The digested peptides were analyzed by nano-LC tandem mass spectrometry, and a total of 14 proteins were identified (see Table S-1 in the Supporting Information). A total of 11 proteins out of the 14 proteins identified were classified as glycoproteins based on the SWISS-PROT database (http://ca.expasy.org/sprot/) and literature searches. Of these 11 glycoproteins, 5 were identified as being associated with other cellular locations (see Supporting Information, Table S-1), while 6 proteins were identified as being located on the plasma membrane, making them candidate cell surface glycoproteins for interacting with the GS II lectin present on fibroblast cell surfaces (See Table 2). Lysosome-associated membrane glycoprotein 1 and 2 (LAMP-1, 2) are single-pass type I membrane proteins and highly glycosylated proteins containing 18 and 16 potential N-glycosylation sites, respectively. LAMPs cycle between lysosomes in the cytoplasm and the cell surface, being expressed on both plasma membrane and lysosome membrane.[37–40] It has been suggested that LAMPs could be involved in cell adhesion to extracellular matrix components.[41] Transmembrane glycoprotein NMB (GPNMB) is also a single-pass type I membrane protein and has 11 potential N-glycosylation sites. This protein was originally found in lowmetastatic melanoma cell lines, but the protein expression is not restricted to cells of the melanocytic lineage.[42–44] Tyrosine-protein phosphatase nonreceptor type substrate 1 (SHPS-1) is a single-pass type I membrane protein with 13 potential N-glycosylation sites. SHPS-1 interacts with the SH2 domain containing proteins (SHP-1 and SHP-2) which function in both growth factor- and cell adhesion-induced cell signaling.[45] α-2-Macroglobulin receptor-associated protein (α_2MRAP) is a glycoprotein anchored to the cell surface by the hydrophobic region in the NH_2-terminal region.[46–48] RNA-binding protein Ewing Sarcoma (EWS) was previously known to be expressed in the nucleus and cytosol; however, recent findings show that the protein is also found on cell surfaces and contains an *N*-acetylglucosamine moiety.[49,50]

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Western Blot Analysis

Two of these proteins, LAMP-1 and GPNMB, were selected for further verification of the mass spectral results by Western blot analysis. We performed Western blot experiments on both endothelial cells and fibroblast cells using antibodies to LAMP-1 and GPNMB (see Figure 4). The four different blot images for the two proteins were generated from identical samples (technical replicates). Both LAMP-1 and GPNMB expression in fibroblast cells were consistently higher than in endothelial cells, validating their utility as cell surface markers to differentiate the two cell types.

DISCUSSION

In this study, lectin arrays were prepared using nine different lectins (ConA, RCA I, WGA, SNA, MAA I, HPA, GS I, GS II, and UEA I) and used to evaluate cell surface carbohydrate expression patterns of rat endothelial and fibroblast cells by observing the cell binding patterns to the lectins. Cell surface carbohydrates are mainly in the form of oligosaccharides covalently attached to proteins forming glycoproteins, and to a lesser extent covalently attached to lipids forming glycolipids. The saccharides usually found in core structures of mammalian cell glycoproteins are α-Man, GlcNAc, and GalNAc.[20] This agrees with our cell binding results showing that both endothelial and fibroblast cells bind to Con A, WGA, HPA, and GS I. A variety of carbohydrate structures such as Gal, sialic acid, fucose, and GlcNAc can be attached to the core structures of glycoproteins, forming complex carbohydrate structures. From the cell binding results generated using RCA I, SNA, and MAA I, both endothelial and fibroblast cells express similar levels of terminal Gal and sialic acid on their cell surfaces. In contrast, no binding of endothelial cells to GS II and UEA was observed, whereas strong binding of fibroblast cells to both these lectins was evident. Although both GS II and WGA bind to GlcNAc, GS II specifically binds to nonreducing terminal GlcNAc residues, while WGA recognizes internal GlcNAc residues as well. Therefore, the cell binding results indicate that the rat fibroblast cells express nonreducing terminal GlcNAc, whereas the rat endothelial cells do not. The binding results obtained from UEA I also indicate that rat endothelial cells do not express α-1,2-fucose while rat fibroblast cells do. While the rat endothelial cells do not express α-1,2-fucose as observed in the present study, several reports have shown that human endothelial cells do express this carbohydrate epitope.[51,52] This indicates that rat and human endothelial cells differ in their cell surface carbohydrate expression profiles.

The selectivity of the fibroblast cell binding to GS II was further tested using a mixture of endothelial and fibroblast cells. As seen in Figure 2C, GS II selectively captured fibroblast cells in the presence of endothelial cells. This confirms that our lectin array format with GS II can be employed as a method for identifying fibroblast cell contamination of the endothelial cell population. Further work was performed to identify the binding partner of the GS-II lectin on fibroblast cells to discover potential cell surface markers that differentiate endothelial and fibroblast cells. In order to perform the mass spectrometric identification experiments, 5×10^8 cells were required. The endothelial cells used in this study are primary cells directly derived from Sprague–Dawley rats. These cells have a limited lifespan, allowing only four passages from the derived cells, resulting in a total of approximately 1×10^7 cells. Therefore, it was not possible to use the endothelial cells as a control in the mass spectrometric experiments. Instead, Western blot analysis was used to verify the mass spectral results (see Figure 4).

It is possible that cell surface glycolipids as well as glycoproteins could contribute to the differential binding of the endothelial and fibroblast cells to GS II lectin. However, in this study our main focus was to identify the cell surface glycoproteins that differentiate the endothelial and fibroblast cells. Recently, we have found that identification of membrane-bound and membrane-associated proteins is greatly improved by removing the lipid bilayer during

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membrane protein preparation.[31] In this study, the delipidation method was used to enrich the membrane proteins, which could increase the efficiency of purifying the cell surface glycoproteins interacting with GS II on fibroblast cell surfaces. The enriched membrane proteins were loaded onto a GS II column and eluted using *N*-acetylglucosamine. From mass spectral analysis of tryptically digested GS II bound proteins, six cell surface glycoproteins (LAMP-1, -2, GPNMB, SHPS-1, α_2MRAP, and EWS) were identified.

Two of the proteins (LAMP-1 and GPNMB) identified with high confidence were subjected to further validation studies by Western blot analysis. The Western blot results (Figure 4) show that both proteins are expressed at higher levels in fibroblast cells than in endothelial cells and can therefore be used as markers to differentiate the two cell types. We note that there could also be changes in the level or complexity of glycosylation between the endothelial and fibroblast cells that could affect the selective binding of the cells to the lectin arrays.

CONCLUSION

Vascular endothelial cells, which are a primary cell type employed in the study of angiogenesis, are easily contaminated with fibroblast cells located in proximity to endothelial cells. In this study, cell surface markers that differentiate rat endothelial and fibroblast cells were identified using lectin arrays in conjunction with mass spectrometry. The lectin arrays were employed to rapidly screen cell surface carbohydrate structures on both cell types. It was found that GS II and UEA I lectins selectively bind to rat fibroblast cells and not to rat endothelial cells, providing a useful means to evaluate the purity of endothelial cell culture. The identity of the fibroblast-specific cell surface markers was further investigated by lectin affinity capture and mass spectrometry. Glycoproteins on fibroblast cells interacting with the GS II lectin were captured on a GS II lectin column and separated by gel electrophoresis, and mass spectrometry of the gel-purified material by LC–ESI-MS/MS revealed several cell surface glycoproteins as candidates for the fibroblast-specific markers. This study illustrates the utility of lectin arrays in conjunction with mass spectrometry for the rapid determination of cell-type specific markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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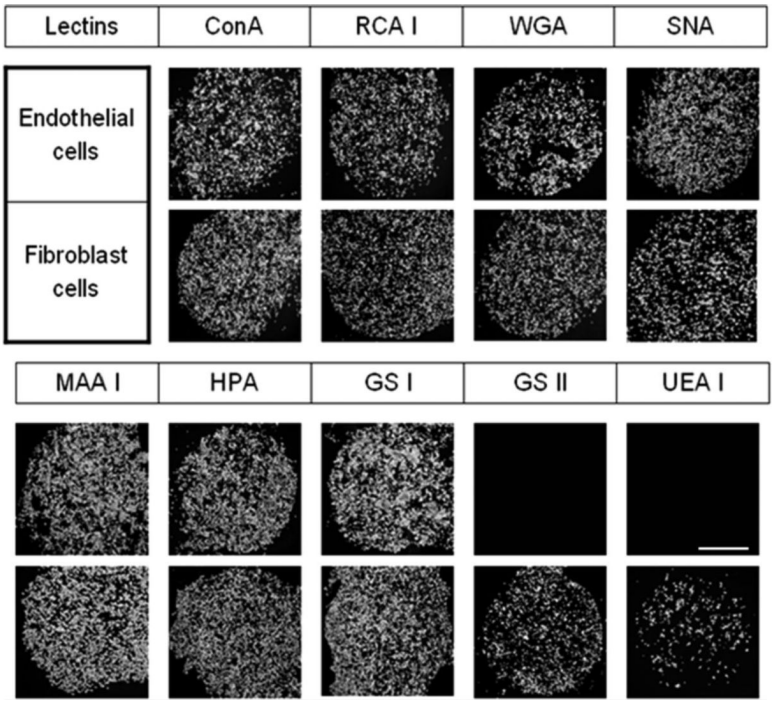

Figure 1.
Optical images of endothelial and fibroblast cell binding to the lectin-modified substrates by phase contrast microscopy. A total of 1×10^6 endothelial cells and fibroblast cells, respectively, were added to lectin-modified substrates and captured by each lectin depending on the carbohydrate expression on the cell surfaces. Bar: 500 μm, 4× magnification objective lens used.

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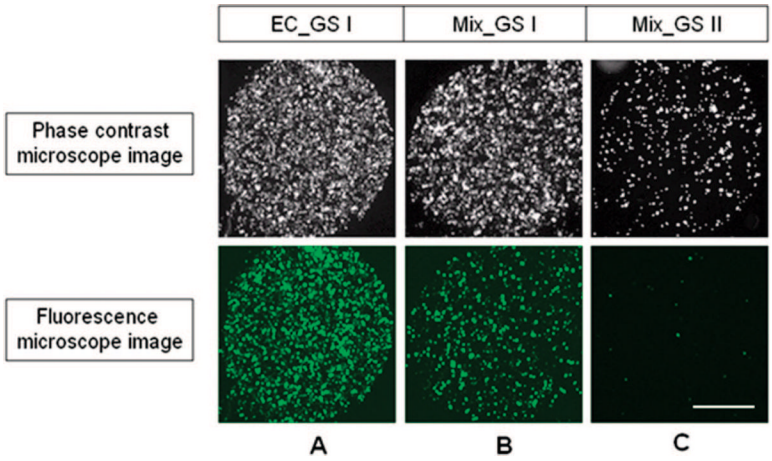

Figure 2.

Optical images representing fibroblast cell binding specificity for GS II. Endothelial cells (1×10^6) expressing green fluorescent protein (GFP) were captured on GS I-modified substrate and imaged using both phase contrast microscopy and fluorescence microscopy (excitation, 494 nm; emission, 518 nm). (A). Fibroblast cells (5×10^5) and endothelial cells (5×10^5) expressing GFP were mixed and captured on (B) GS I and (C) GS II-modified surfaces. Bar: 500 μm, 4× magnification objective lens used.

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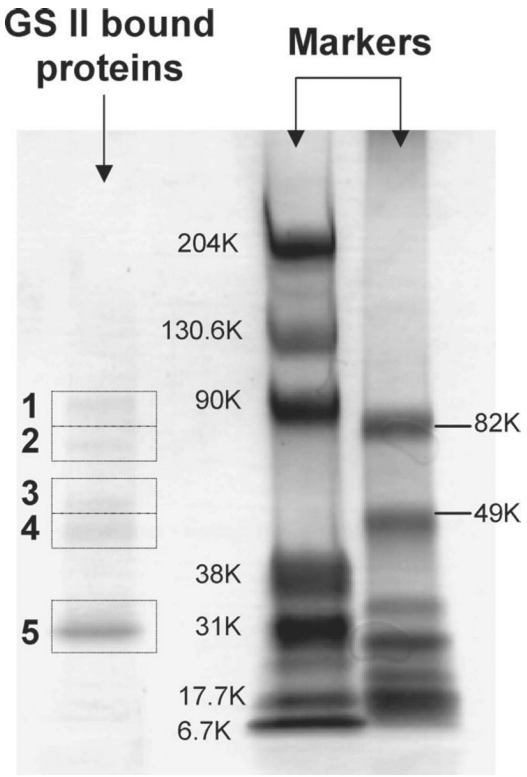

Figure 3.
SDS-PAGE gel image of GS II bound proteins obtained from the fibroblast cell membrane protein fraction. A delipidated membrane protein fraction was obtained from a 5×10^8 fibroblast cell lysate and loaded on a GS II lectin column followed by elution of the bound proteins using GS II inhibiting sugar.

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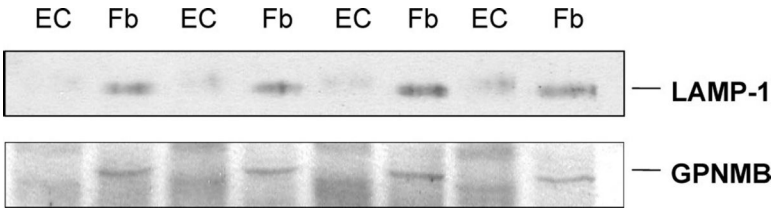

Figure 4.
Western blot analysis for LAMP-1 and GPNMB (EC, endothelial cells; Fb, Fibroblast cells).

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Table 1

Carbohydrate Specificities of Lectins Used in This Study[a]

lectins	carbohydrate specificity
concanavalin A (ConA)	α-Man
Ricinus communis agglutinin I (RCA I)	terminal β-Gal
wheat germ agglutinin (WGA)	β-GlcNAc, sialic acid
Sambucus nigra agglutinin (SNA)	α-2,6 sialic acid
Maackia amurensis agglutinin I (MAAI)	α-2,3 sialic acid
Helix pomatia agglutinin (HPA)	α-GalNAc
Griffonia simplicifolia I (GS I)	α-Gal, α-GalNAc
Griffonia simplicifolia II (GS II)	nonreducing terminal α- or β-GlcNAc
Ulex europaeus agglutinin I (UEAI)	α-1,2 fucose

[a] More detailed specificity information can be found in ref 32. Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

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Table 2

Candidate Cell Surface Glycoproteins Interacting with GS II on Fibroblast Cells

protein name	accession No	unique peptides	sequence coverage %	max Xcorr
lysosome-associated membrane glycoprotein 1 (LAMP-1)	P14562	7	17	5.3518
transmembrane glycoprotein NMB (GPNMB)	Q6P7C7	3	5	5.6157
tyrosine-protein phosphatase nonreceptor type substrate 1 (SHPS-1)	P97710	3	6	4.1961
α-2-macroglobulin receptor-associated protein (α_2MRAP)	Q99068	2	9	5.4104
RNA-binding protein EWS	Q61545	2	6	6.3497
lysosome-associated membrane glycoprotein 2 (LAMP-2)	P17046	2	5	3.3625