Original Article
Screening of surface markers on rat intestinal mucosa microfold cells by using laser capture microdissection combined with protein chip technology

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Abstract: Objective: The objective of this research was to investigate the possibility of screening surface markers on rat intestinal mucosa microfold cells (M cells) by using laser capture microdissection (LCM) combined with protein chip technology. Methods: We labeled rat intestinal mucosa microfold cells with Ulex europaeus agglutinin (UEA)-1 antibody and visualized these by immunofluorescence staining. Using the Proteome Profiler rat protein chip, we analyzed the protein expression profiles of LCM M-cells compared to lymph follicle-associated epithelial (FAE) cells, and we identified potential differences to screen for marker proteins. Results: M cells can be clearly distinguished from lymphoid FAE cells under the fluorescence microscope. We successfully cut, isolated, and obtained microfold and lymph FAE cells with more than 95% homogeneity. Six differentially expressed proteins were identified through comparison of the protein chip profiles of these 2 cell types. Among these, VEGF, LIX, CNTF, and IL-1α/IL-1F1 were found to be at significantly lower levels in M cells, IL-1ra/IL-1F3 and MIG/CXCL9 appeared in significantly higher levels in M cells (P < 0.05). Conclusion: The results presented here clearly demonstrate that the combined use of LCM and protein chip technology is effective in the screening of M cell surface markers with high sensitivity and specificity. This will facilitate isolation, identification, and establishment of M cell lines, allowing further characterization of their functional properties.

Keywords: Laser capture microdissection (LCM), proteomics, protein chip, M cells, marker

Introduction
Microfold cells (M cells) are specialized cells found mainly on the follicle-associated epithelium (FAE), and they are scattered among epithelial cells overlying the dome structures of lymphoid follicles in the small and large intestines. M cells are especially concentrated in lymphoid aggregates such as Peyer’s patches (PPs). They are also abundantly scattered in the distal colon and rectal mucosa [1]. M cells play crucial roles in antigen presentation and the initiation of immune responses. The infectious mechanisms of pathogens are dependent on interactions with mucosal epithelial cells. In recent years, there has been increasing interest in the feasibility of designing orally administered vaccines based on the functions of intestinal mucosa M cells [2, 3]. Therefore, the study of the specific surface proteins of M cells may help to elucidate their functional mechanisms with potential clinical applications.

The cellular morphology and structure of M cells varies greatly among species and also within species, where they can differ morphologically even within anatomic sites. The microfold structure is present only in human M cells, which lack microvilli, whereas there are abundant microvilli on the M cells of rabbits and mice. Moreover, the percentage and anatomical locations of M cells in FAE vary substantially among species, ranging from 5% to 10% in human and mouse PPs, yet they are present at levels of approximately 50% in rabbit and human caecum [4]. The surface markers of M cells also vary according to anatomical location. In previous studies using exogenous large molecule lectins to stain FAE in mouse PP, it was observed that intestinal mucosa M cells mainly
express α (1, 2)-linked fucose and can be detected with Ulex europaeus agglutinin (UEA)-1, which can identify an epitope of M cells that distinguishes them from intestinal epithelial cells; however, lectin UEA-1 cannot distinguish M cells in the mouse cecal colon.

Very few M cell-specific ligands and receptors have been identified, and the vast majority of those that have been found are not unique to M cells. Some important pathogen recognition receptors (PRRs) such as Toll-like receptor-4 (TLR-4), platelet activating factor receptor (PAFR), and α5β1 integrin are expressed on the surface of both human and mouse M cells. By interacting with pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, lipoteichoic acid, peptidoglycan, and bacterial flagellin, these PRRs can activate downstream pathways [5]. Although PRRs can be found in surrounding enterocytes, the expression of these receptors varies with different cells. For example, α5β1 integrin is dispersed on the lateral and basolateral surfaces of enterocytes, whereas in intestinal mucosa M cells, it is distributed only on the apical surface [3].

When screening mouse intestinal PP with UEA-1, a large amount of nonspecific binding occurs to tissue, and 40% of UEA-1 reacts strongly with goblet cells, with only 10% binding to intestinal mucosa M cells. These data illustrate that goblet cells may express the same UEA-1 immunogenic antigen as M cells; however, work is underway to develop novel M cell surface markers [6]. Malik et al. [7] developed an alginate-coated UEA-1 lectin ionic gel with an antigen desorption function. Research carried out on Balb/c mice indicated that this type of ionic gel can induce more efficient immune responses compared to a conventional vaccine. Moreover, Kim et al. [8] found that the complement C5a receptor (C5aR) is expressed on the apical surface of M cells and that the expression levels of C5aR mRNA in co-cultured Caco-2 cells was 6-fold higher than in mono-cultured Caco-2 cells. In addition to C5aR, GP2 protein was also detected on the surface of M cells and in mouse PPs.

As current domestic and overseas research on M cells mainly focuses on experimental rat models, SD rats were used in this study. Using UEA-1, an antibody known to recognize M cells, we screened and marked rat intestinal M cells. M cells and lymph follicle epithelial cells were purified using LCM combined with protein chip technology, and we investigated novel M cell membrane proteins as potential specific markers, in order to advance future research into the biological characteristics of these cells.

**Material and methods**

**Animals and treatment**

Sprague-Dawley rats (weighing approximately 100-120 g; male and female) were provided by Shanghai Slack Animal Center (Shanghai, China), acclimatized for 2 days, and fasted for 24 hours before experimentation. After anesthesia via intraperitoneal injection with 3% pentobarbital sodium (40-45 mg/kg), rats were sacrificed and whole small intestines collected into 0.9% balanced saline solution, scoured carefully, secretions and excess fecal residue removed, sheared, and the tissue containing the PPs were located (an average of 8-12 PPs in each rat), collected into a tube, and stored at -80°C, after freezing and transport in liquid nitrogen.

**Experimental materials**

All reagents used in this study were domestic, unless otherwise specified. FITC-labeled UEA-1 was purchased from Sigma (Munich, Germany); PEN-coated slides were purchased from Leica, and the cryotome (Leica CM1850), LCM machine (Leica LMD6500), ECLTM CHEMI Reagent, SuperRX medical X-ray film, BCA protein quantification kit, and Proteome ProfilerTM Arrays Rat Cytokine Array Panel A protein chip kit were purchased from R & D company.

**Preparation and staining of frozen sections**

Intestinal tissue was examined to ensure that it contained intestinal mucosa FAE and selected specimens embedded in optimal cutting temperature (OCT) Tissue-Tek embedding medium. It was then dissected into 10-μm-thick serial sections, subjected to improved H&E and immunofluorescence staining, and then placed on PEN-coated slides. Improved HE staining was performed by staining with hematoxylin for 1 s; ice-water flushing for 10 s; eosin for 10 s; dehydration in 85%, 95%, and 100% ethanol for 10 s each; and clearing in xylene for 2 min.
For immunofluorescence staining, the samples were fixed in anhydrous alcohol that was pre-cooled at -20°C for 5 min, rinsed 3 times with PBS-1% Triton X-100, and blocked with PBST-0.05% BSA in a wet box at 4°C for 50 min. After discarding the blocking solution, FITC-labeled lectin UEA-1 (5 μg/ml) was added, and samples were incubated in wet boxes overnight at 4°C, washed 3 times with PBS, and stored at -80°C.

**Laser capture microdissection**

PEN-coated slides were placed face down after staining, and a 0.2-ml Eppendorf tube cap was placed on the microdissection machine dedicated tubing rack. Real-time monitored samples were prepared for separation on a computer screen by using a CCD camera, the target tissue (M cells and FAE cells) were selected using a computer. M cells were marked by UEA-1 fluorescent antibody around the lymphoid follicle epithelium. After the selected region was sketched, its area was calculated using the computer software and stored in a document form. The microdissection process was automatically controlled and completed by the software. Dissected M and FAE cells were transferred into 2 separate Eppendorf tubes. Protein samples were processed within 2 h to prevent degradation. For data collection, the photographs captured before and after cutting were compared. If adhesion phenomena were observed after microdissection, further dissections were manually performed. After removal of Eppendorf tubes, the dissected membranes and samples were attached to the adherent tube cap.

**Protein extraction and determination of dissected samples**

Eppendorf tubes were centrifuged to collect the dissected adherent tissue at the bottom of the tube and left on ice for future manipulation. Protein extraction was performed using pre-cooled protein extraction buffer (RIPA), containing protease inhibitors and PMSF. Samples in the extraction buffer were placed on ice and centrifuged (12000 ×g, 10 min, 4°C), after vigorous mixing using a vortex mixer for 30 s, every 5 min. The supernatant contained the total tissue proteins. Extracts from several supernatants were pooled in a fresh Eppendorf tube and stored at -80°C. Electrophoretic analyses were carried out by SDS-PAGE after determination of protein concentrations using a BCA protein quantification kit. Gels were prepared by addition of TEMED (10 μl) to 10 ml of 10% separation gel, which was mixed, and 8 ml of it was poured slowly and allowed to set for 20-30 min. Next, 10 ml of 10% stacking gel was prepared, TEMED (5 μl) was added to it, mixed, and immediately poured. The comb was then inserted, and the gel was allowed to completely polymerize (approximately 30 min). The glass plates were then fixed to the electrophoresis equipment and placed in the electrophoresis tank. Protein standards (10 μl) were measured along with 20 μl M cell and lymph follicle epithelial cell samples. Sample buffer (3 times the volume) was added to give a final volume of 30 μl. Before gel loading, samples were denatured in boiling water for 2-5 min. The tanks were filled with buffer, power was switched on, and the positive and negative poles were connected; electrophoresis was thus conducted at a stabilized voltage of 120 V and an initial constant current of 15 mA, which was changed to 30 mA once the samples entered the separation gel. Electrophoresis was stopped when bromophenol blue was 5 mm apart from the gel margin. The gel plate was then placed into a culture dish, and incubated for approximately 1 h in the staining solution. The gel was then thoroughly rinsed with distilled water after staining until the protein bands were clearly visible.

**Protein chip array analysis**

M cells (n = 4) and FAE cells (n = 2) were added to PBS containing protease inhibitors, treated with Triton X-100 (final concentration, 1%) after equilibration, and centrifuged (10000 ×g, 5 min) after freezing/thawing. Buffer 4 (0.5 ml) was added to the extracted tissue supernatants, followed by the addition of buffer 6 to a final volume of 1.5 ml. Following the addition of 15 μl of reconstituted Detection Antibody Cocktail, samples were incubated at room temperature for 1 h. For chip processing, 2 ml of buffer 6 was added, and the samples were sealed and incubated at 20°C for 1 h. Samples were then loaded onto the chip and incubated overnight; each chip was washed with the wash buffer at a dilution of 1:50 (3 washes, 10 min each wash); then, the wash buffer was added to chips at a dilution of 1:2000 and these were allowed to rock gently at room temperature for 30 min. The film was then washed further (3
washes, 10 min each), incubated in ECL reagent, and placed into a cassette; finally, the film was rinsed.

Data processing and statistical analysis

The greyscale data of each well of the protein chip arrays was detected using the ImageJ software. Briefly, peak values of gray dots obtained by film scanning were measured, and the Wilcoxon test was used to analyze the data and study on antlers of protein grayscale level.

Results

Laser capture microdissection results

FAE cells can be clearly visualized in H&E stained tissues and intestinal epithelial cells and lymph follicle epithelial cells can be well distinguished based on their organizational structure. In the immunofluorescence specimens, some M cells in the mucosa follicular epithelium, and goblet cells in the intestinal epithelium were fluorescently labeled at an excitation wavelength of 490-495 nm (Figure 1A and 1B). We were, therefore, able to distinguish intestinal epithelial cells from lymphoid FAE cells in H&E stained slides, and we could also identify M cells in immunofluorescence-stained slides (Figure 1C and 1D).

Protein extraction and protein chip results

The concentrations of proteins extracted from M and lymph FAE cell samples were determined using BCA protein quantification kits in 96-well plates. Protein concentrations of lymph follicle epithelial and M cells were 36.2 µg/µl and 16.4 µg/µl, respectively. Differences in concentrations of 6 proteins were detected from a total of 6 samples of M and lymph follicle epithelial cells by protein chip screening, VEGF, LIX, CNTF, and IL-1α/IL-1F1 were found at lower levels in M cells than lymph follicle epithelial cells, whereas IL-1ra/IL-1F3 and MIG/CXCL9 were observed.
Screening of surface markers on microfold cells

Figure 2. Polyacrylamide electrophoresis results.

Figure 3. Protein chip well template.

at relatively higher levels (P < 0.05). Figure 2 shows the results of polyacrylamide gel electrophoresis. The volume of both samples was 10 μl; group 1 contains FAE cells, group 2 contains M cells, 13% SDS-PAGE gels were used. The Rat Cytokine Array Panel A protein chip template is shown in Figures 3 and 4. Table 1 indicates protein grayscale levels for each protein chip and differences between the 2 groups.

Discussion

M cells are associated with antigen presentation. In the gut lumen, M cells transport antigens or pathogens across the epithelium to the subcutaneous lymphoid tissues, triggering mucosal immune responses or immune tolerance, and it has been postulated that this represents the first step in the induction of mucosal immune responses. Many substances are transported via M cells, and this further stimulates the body's immune response. In recent years, some M-like cell lines, including the most commonly used colorectal cancer Caco-2 cells and human Raji B cells, have been established [9]. Furthermore, much research has focused on surface markers, antigen absorption, oral drug, and vaccine presentation mechanisms of M-like cells in vitro [10, 11]; however, no actual M cell line has been established to date.

Therefore, a critical problem in current proteomic research is to develop an appropriate method for M cell separation and purification. LCM is the best way to obtain highly homogeneous tissue cells, as it dissects and obtains target cells outlined by the system using a laser beam; of course, it is first necessary to locate target tissues by immunohistochemical or immunofluorescence methods. However, in view of the complicated LCM operation process and expensive consumables, including hats and cutting slides, and because microdissection is the only source of M cell tissue protein and requires a considerable number of samples, traditional 2D electrophoresis technology and bio-mass spectrometry have obvious deficiencies in their abilities to separate of infinitesimal and extremely complex protein mixtures extracted from cells obtained using LCM technology. Therefore, subsequent analysis of cells obtained by LCM needs to be performed by using methods with high sensitivity and specificity and requiring small sample quantities, such as electrospray ionization mass spectrometry (ESI-MS/MS), liquid chromatography mass spectrometry combined technology (LC-MS/MS), and protein chips (or protein microarray). Similar to DNA chips, protein chip technology has developed rapidly in recent years, leading to a new type of proteomics detection technology with 4 primary characteristics; high-parallelism, high-throughput, miniaturization, and atomization. The amount of information a protein chip could process is far greater than that possible using 2D gel electrophoresis, and even low abundance substances can be detected, provided they are able to bind to a surface probe. Protein chips contain 2 key factors: molecules spatially arranged in a fixed format that enable the discrimination of protein...
components (often antibodies), and a system that enables detection of interactions between individual proteins and their corresponding identifier in a mixture [12]. Therefore, a protein chip could capture a specific target protein in a sample through antigen-antibody interactions, and then qualitative or quantitative analysis of target proteins can be performed using a detector. Probe proteins on protein chips can be chosen according to various aims, and they can be assayed by biological activity with high specificity and affinity by using molecules such as antibodies, antigens, receptors, and enzymes [13]. Von Eggeling and colleagues [14] have previously used the combination of microdissection techniques and protein chips for clinical research to compare the protein expression spectrums of normal and tumor tissues: first, using microdissection to obtain the pure tumor and normal tissues, extracting tissue proteins, applying these to the protein chip, and washing off unbound proteins; second, for determining the protein expression spectrum through analysis of the chip. It is not possible to perform such an analysis by using available samples sizes by 2D gel electrophoresis.

In this study, M cells samples in the small intestine PPs of SD rats were selected by using LCM. By this method, 4 replicate M cell and 2 paired lymph follicle epithelial cell samples, not containing M cells, were prepared, with each containing approximately 12000-15000 cells. We carried out Proteome Profiler rat cell protein microarray analysis of these 6 samples, and we detected 6 differentially expressed proteins by comparing the chip profiles of M cells with those of lymph follicle epithelial cells. VEGF, LIX, CNTF, and IL-1α/IL-1F1 were observed at significantly lower levels than IL-1ra/IL-1F3 and MIG/CXCL9 in M cells, compared to their levels in lymph follicle epithelial cells (P < 0.05). However, given the limitations of protein chip technology, we were unable to identify further unknown rat cell surface proteins.

The purpose of this study was to screen for specific surface antigens of M cells and only MIG/CXCL9 and IL-1ra/IL-1F3 were found to be highly expressed specifically in these cells. MIG/CXCL9 is an IFN-γ-induced chemokine, which has chemotactic effects on activated and tumor infiltrating lymphocytes [15], and its receptor CXCL, is a 7-transmembrane domain G-protein-coupled receptor. CXCL9-induced chemotaxis is mainly mediated together with CXCR3, and CXCR3 and CXCL9 can stimulate Src phosphorylation and Src kinase activity, while simultaneously increasing activity of phosphatidylinositol 3 kinase and its downstream (AKT) pathway. Through antigen presentation, CXCL9 also plays an important role in recruiting T cells to peripheral inflammation sites, and enhances interactions among T, B, and dendritic cells in lymphoid organs to some extent, thus regulating physical responses to pathogens [16]. IL-1ra/IL-1F3 is an endogenous inhibitor of IL-1; it can bind to IL-1RI and IL-1RII, but it does not initiate signal transduction and thus functions as a competitive inhibitor of the activity of IL-1 through IL-1R. A large number of animal studies have shown that IL-1ra can reduce the occurrence of low blood sugar levels generated by colony stimulating factor due to endotoxemia, as well as strengthen organic tolerance [17].

In conclusion, the combined technologies of protein chips and LCM were effective in preliminary screening of the surface proteins of M cells. However, given the high cost of LCM and delays in devising rat cell protein chips, we failed to detect suitable markers to distinguish peripheral lymphoid follicular epithelial cells from intestinal mucosal cells, or to verify dissected M cells. Further work needs to be done to improve methodology, as well as to seek for better technical support.
Screening of surface markers on microfold cells

Table 1. Protein chip protein grayscale levels

<table>
<thead>
<tr>
<th>Dot location</th>
<th>Target/Control</th>
<th>Lymph follicle epithelium grayscale</th>
<th>M cell grayscale</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3, A4</td>
<td>CINC-1</td>
<td>286.506 ± 13.727</td>
<td>N.A.</td>
<td>N.A.</td>
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<tr>
<td>A9, A10</td>
<td>CNTF*</td>
<td>1536.792 ± 119.490</td>
<td>331.911 ± 52.083</td>
<td>0.029</td>
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<tr>
<td>A11, A12</td>
<td>Fractalkine/CX3CL1</td>
<td>1873.561 ± 193.206</td>
<td>1734.501 ± 217.281</td>
<td>0.498</td>
</tr>
<tr>
<td>A15, A16</td>
<td>sICAM-1/CD54</td>
<td>2043.740 ± 467.153</td>
<td>2562.714 ± 120.563</td>
<td>0.354</td>
</tr>
<tr>
<td>B3, B4</td>
<td>IL-1α/IL-1F1*</td>
<td>2608.571 ± 135.467</td>
<td>1213.240 ± 493.286</td>
<td>0.020</td>
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<tr>
<td>B5, B6</td>
<td>IL-1β/IL-1F2</td>
<td>1004.518 ± 51.260</td>
<td>909.963 ± 75.168</td>
<td>0.165</td>
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<tr>
<td>B7, B8</td>
<td>IL-1α/IL-1F3*</td>
<td>1358.558 ± 129.278</td>
<td>2527.150 ± 127.680</td>
<td>0.008</td>
</tr>
<tr>
<td>C7, C8</td>
<td>IP-10/CXCL10</td>
<td>232.634 ± 125.524</td>
<td>599.183 ± 341.473</td>
<td>0.130</td>
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<tr>
<td>C9, C10</td>
<td>LIX*</td>
<td>2103.421 ± 63.739</td>
<td>650.983 ± 207.686</td>
<td>0.001</td>
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<tr>
<td>C11, C12</td>
<td>L-Selectin</td>
<td>3049.029 ± 36.309</td>
<td>3349.277 ± 230.477</td>
<td>0.159</td>
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<tr>
<td>C13, C14</td>
<td>MiG/CXCL9*</td>
<td>935.272 ± 19.139</td>
<td>2485.801 ± 176.506</td>
<td>0.001</td>
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<tr>
<td>C15, C16</td>
<td>MIP-1α/CCL3</td>
<td>307.789 ± 184.262</td>
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<td>/</td>
</tr>
<tr>
<td>C17, C18</td>
<td>MIP-3α/CCL20</td>
<td>2623.747 ± 382.992</td>
<td>2612.157 ± 318.469</td>
<td>0.974</td>
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<tr>
<td>D3, D4</td>
<td>RANTES/CCL5</td>
<td>3203.682 ± 328.586</td>
<td>3292.163 ± 321.604</td>
<td>0.768</td>
</tr>
<tr>
<td>D5, D6</td>
<td>Thymus Chemokine/CXCL7</td>
<td>2818.979 ± 82.670</td>
<td>2917.012 ± 66.032</td>
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<td>D7, D8</td>
<td>TIMP-1</td>
<td>2923.988 ± 543.885</td>
<td>2923.937 ± 269.532</td>
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<tr>
<td>D9, D10</td>
<td>TNF-α/TNFSF1A</td>
<td>221.220 ± 86.656</td>
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<td>/</td>
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<tr>
<td>D11, D12</td>
<td>VEGF*</td>
<td>2312.020 ± 135.740</td>
<td>1512.012 ± 289.920</td>
<td>0.024</td>
</tr>
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</table>

*P < 0.05.

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Disclosure of conflict of interest

None.

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Screening of surface markers on microfold cells