Simultaneous Detection of Multiple Cytokines from Conditioned Media and Patient's Sera by an Antibody-Based Protein Array System

Ruo-Pan Huang,1 Ruochun Huang, Yan Fan, and Ying Lin
Department of Gynecology and Obstetrics, Emory University School of Medicine, 1639 Pierce Drive, Atlanta, Georgia 30322

Received January 9, 2001; published online May 31, 2001

We have developed a novel technique for high-throughput simultaneous screening of multiple cytokine expression based on a protein array system. Our method has the advantage of showing the specificity of enzyme-linked immunosorbent assays, sensitivity of enhanced chemiluminescence (ECL), and high-throughput of microspot. In this system, the cytokine array membranes were created by spotting capture antibodies onto the membranes. The membranes were then incubated with biological samples such as conditioned media and patient's sera. The bound proteins were then recognized by biotin-conjugated antibodies and detected by horseradish peroxidase-conjugated streptavidin coupled with ECL. Experiments demonstrated that 24 cytokines from conditioned media and patient's sera could be simultaneously detected using this new approach. This methodology should allow us to develop many high-density protein array systems to detect a variety of proteins. To validate and quantitate the expression of key molecules in a wide range of samples, we have developed conditioned medium arrays to evaluate hundreds and even thousands of samples from individual cells and patients in a single microarray. The combinations of protein arrays and conditioned medium arrays or serum arrays will provide a powerful tool to identify the protein expression profiles and rapidly validate their expression in many types and numbers of samples.

All cell functions, including cell proliferation, death, and differentiation, are controlled by many genes and signaling pathways. New techniques such as cDNA microarrays have enabled investigators to analyze global gene expression (1–3). However, almost all cell functions are executed by protein, which cannot be assessed by evaluation of DNA and RNA alone. Experimental analysis shows a clear disparity between the relative expression levels of mRNA and their corresponding proteins (4, 5). Furthermore, posttranslational protein modification, protein–protein interactions, and protein–DNA interaction, which are all vital for cellular activity, cannot be understood by studies of DNA and mRNA alone. Protein-based analyses are required to address these questions. Currently, two-dimensional SDS–PAGE coupled with mass spectrometry is the mainstream approach to analyze multiple protein expression (6, 7). However, the requirement of sophisticated devices and lack of quantitative measurement greatly limit its broad application. Surfaced-enhanced laser desorption and ionization coupled with mass spectroscopy can also be used in limited analysis of gene expression (8–11). Therefore, it is urgent to develop a simple, flexible, cost-effective, highly sensitive, and high-throughput approach. One of these directions is to develop an antibody-based protein array system (12). Arrays have the advantage of being scalable, flexible, and easy to perform. The nature of arrays allows a high-throughput screening using robotic, imaging, or analytical methods. Such antibody-based array systems have been proved mathematically by Ekins and Chu in the mid- to late 1980s (13–15). Since then, multiple attempts have been made. Among them, Silzel et al. demonstrated that multiple IgG2 sub-

1 To whom correspondence should be addressed at Department of Gynecology and Obstetrics, Emory University School of Medicine, 1639 Pierce Drive, Room 4219, Atlanta, GA 30322. Fax: (404) 727-8615. E-mail: rphuang@yahoo.com or rhuang@emory.edu.

2 Abbreviations used: ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assays; GROα, growth-regulated oncogene α; HRP, horseradish peroxidase; IL, interleukin-2; MCP-1, monocyte chemotactic protein-1; TNF, tumor necrosis factor; IgG, immunoglobulin G; G-CSF, granulocyte colony-stimulating factor;
classes could be detected by fluorescence imaging (16). Mendoza et al. at Genometrix explored the possibility of detecting multiple IgGs in a high-throughput format (17). Lueking et al. investigated the application of microarrays in the analysis of antigens (11). More recently, Tomlinson and colleagues developed an antibody array technique for high-throughput screening of recombinant antibodies (18, 19). Protein array technology has been further developed to examine protein–protein interaction (20, 21), to study yeast protein kinases (22), and to analyze autoimmune antibodies (23). However, to our best knowledge, no methodology in the literature is available for the simultaneous detection of multiple cytokines from physiological samples by protein array technology.

In this paper, we described a novel ELISA-based protein array system in which multiple cytokines can be simultaneously detected from an experimental model system, from tissue culture media, and from patient's sera with high sensitivity. After we identified the potential candidate proteins, the expression of these proteins from hundreds of biological samples can be analyzed quantitatively in a single array membrane. The experimental approach described here provides a powerful tool to study multiple protein expression and to rapidly validate the expression profile from hundreds or even thousands of samples simultaneously. To our best knowledge, this is the first practical protein array system developed so far to profile cytokine expression at physiological levels.

METHODS

Materials

All pairs of antibodies were purchased from BD Pharmingen (San Diego, CA). All cytokines except GROα and granulocyte colony-stimulating factor (G-CSF) were obtained from Peprotech (Rochy Hill, NJ). GROα and G-CSF were the products of BD Pharmingen. HRP-conjugated streptavidin was also purchased from BD Pharmingen.

Preparation of Array Membranes

A template of 504 spots (18 spots by 28 spots) covering an area of 48 cm² (6 × 8 cm) was generated from a computer. Hybond ECL membranes (Amersham Pharmacia Biotech, Piscataway, NJ) were placed on the top of the template. Through the white light box, the dark spots in the template were clearly visualized from the membranes and used as a guide to spot solution onto the membranes. Quantities of 0.25 μl of capture antibodies (200 μg/ml) and 0.5 μl of conditioned media were manually loaded onto membranes by a 2-μl Pipetman (Rainin, Woburn, MA) in duplicate. HRP-conjugated antibody was spotted onto membranes as positive control and identification of orientation of arrays. The strips were cut out for experiments.

Array Assay of Purified Cytokines

Membranes immobilized with capture antibodies were blocked with 5% bovine serum albumin (BSA)/TBS (0.01 M Tris–HCl, pH 7.6/0.15 M NaCl) for 1 h. Membranes were then incubated with a single or a combination of different cytokines (0.25 μg/ml) prepared in 5% BSA/TBS for 2 h at room temperature. After extensive wash with TBS/0.1% Tween 20 (three times, 5 min each) and TBS (two times, 5 min each) to remove unbounded cytokines, membranes were then incubated for 2 h individually or collectively with biotin-conjugated anti-cytokine antibodies (from 2.5 to 0.25 μg/ml). The membranes were washed and incubated with HRP-conjugated streptavidin (2.5 pg/ml) for 1 h at room temperature. Unbound materials were washed out with TBS/0.1% Tween and TBS. Finally, the signals were detected by an ECL system (Amersham Pharmacia Biotech).

Protein Array Assay for Detection of Cytokines from Conditioned Media and Sera

Ten milliliters of 50-fold-diluted conditioned media and 1 ml of 10-fold-diluted sera were incubated with cytokine array membranes. To prepare conditioned media, human glioblastoma cells U251 (24, 25) were plated in 35-mm tissue culture dishes at a density of 4 × 10⁴ cells per dish. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum for 24 h, after which the complete culture medium was replaced with serum-free DMEM. Cells were then stimulated for 48 h in the presence or absence of 50 ng/ml of recombinant human TNFα. The supernatants were collected, centrifuged at 10000 g, aliquoted, and stored at −80°C until testing. Patient's sera were obtained from the Department of Gynecology and Obstetrics, Emory University School of Medicine.

Conditioned Medium Arrays

Hybond ECL membranes were soaked with anti-MCP-1 capture antibody at a concentration of 20 μg/ml for 4 h at 4°C. Membranes were then air-dried. A quantity of 0.5 μl of conditioned media from different sources were spotted onto the membranes. After blocking with 5% BSA/TBS, membranes were incubated with biotin-conjugated anti-MCP-1 antibody. After washing, membranes were incubated with HRP-conju-

BSA, bovine serum albumin; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle medium.
gated streptavidin. The signals were visualized by ECL.

ELISA

Conventional ELISA was performed according to manufacturer’s instruction (BD PharMingen). Essentially 96-well ELISA plates were coated overnight at 4°C using 50 μl of 8 μg/ml capture antibodies. One percent BSA/PBS was used as a blocking buffer. One-hundred microliters of conditioned media and twofold-diluted patients’ sera and different concentrations of standard cytokines were added to each well in duplicate. Unbound materials were washed out with PBS/0.05% Tween. One-hundred microliters of 1 μg/ml of the appropriate biotinylated anti-cytokine detection antibody was added to each well. The plates were incubated for 1 h at room temperature. After washing, 100 μl of streptavidin–HRP-conjugated antibodies was added to the wells and incubation was continued for 30 min at room temperature. Following by extensive washing, color development was done by incubation with substrate solution containing ethylbenzthiazoline sulfonate (Sigma, St. Louis, MO). OD at 405 nm was determined by a microplate reader. Standard curves were generated by Sigma plot and the concentrations of different samples were determined from the standard curves.

Densitometry

The intensities of signals were scanned and quantitated by densitometry (Bio-Rad, Hercules, CA).

RESULTS

Specificity of Protein Arrays to Detect Cytokines

To develop a practical protein array system with high specificity and sensitivity, we took advantage of the specificity of ELISA, sensitivity of ECL, and high-throughput of microspot. A pair of antibodies, which recognizes two different epitopes of the same antigen were used. One antibody was spotted and functions as a capture. Another corresponding antibody was labeled with biotin and functions as a detector. The signals were then developed by an ECL system. Previously, we have demonstrated that up to six different cytokines and eight different antibodies can be simultaneously detected in this system (manuscript in press). Here we examined whether this approach could be extended to detect as many as 24 cytokines simultaneously, the membranes immobilized with different antibodies were incubated with different combinations of cytokines and corresponding antibodies as indicated in Fig. 2. Specific signals were detected as expected.

Detection of Cytokine Expression from Conditioned Media and Patient’s Sera

The most challenging question in antibody-based microarray is whether it can be used to directly detect protein expression product from conditioned media, patient’s specimen, crude cell lysate, or crude tissue lysate.

The conditioned media collected from different sources were tested for their cytokine expression. As shown in Fig. 3, several cytokines are differentially secreted from human glioblastoma U251 cells treated with or without tumor necrosis factor α (TNFα). As control, no significant levels of cytokines were detected from cell-free medium alone. Densitometry showed that the MCP-1 level increased 2.5-fold in TNFα-treated U251 cells compared with the untreated cells and IL-8 level increased 17-fold. The results were independently confirmed by conventional ELISA as shown in Table 1. Previous studies demonstrated that TNFα up-regulated MCP-1 expression (26), consistent with our protein array data. Furthermore, our result also suggests that TNFα can up-regulate IL-8 expression in human glioblastoma U251 cells, providing new information on the regulation of mediators by TNFα.

We also tested the detection of cytokines in patient’s sera. Sera from several patients who had abnormal pap
FIG. 1. (A) Detection of cytokines in array format showing high specificity. Hybond ECL membranes immobilized with different captured antibodies against different cytokines were incubated subsequently with individual cytokine or control as indicated in figure and then with corresponding biotin-conjugated anti-cytokine or control as described under Materials and Methods. The signals were visualized by ECL. (B) Detection of cytokines in array format showing high sensitivity. Cytokine array membranes were incubated with different concentrations of MCP-1 and IL-2 as indicated. The intensities of signals were detected by densitometry and plotted against the concentrations of MCP-1 or IL-2.
results in gynecological examination were screened for their cytokine expression. As indicated in Fig. 4, specific cytokines were detected in different patient samples. The relative expression levels of MCP-1, IL-8, and TNF\(\alpha\) were determined by densitometry (Table 1) and agreed well with ELISA results.

Conditioned Medium Microarrays for High-Throughput Molecular Profiling

Through protein array techniques, we can simultaneously detect multiple cytokines and identify the key molecules important in specific cases. To further investigate the role of these key molecules, one usually needs to examine their expression in many samples. This would be a costly and time-consuming process, if one repeats protein arrays for all samples. Since at this stage of investigation a limited number of protein molecules are to be examined, it would be much more efficient to screen as many samples as possible at one time. One of the difficulties in performing this type of experiment is the sensitivity of protein detection when sample volume is significantly scaled down. Another potential problem is the variable efficiency in detecting different antigens since different antigens have considerable distinct binding properties to membranes.

**FIG. 2.** Simultaneous detection of multiple cytokines in an array format. The combinations of multiple cytokines as indicated in the figure were incubated with cytokine array membranes. The membranes were then processed and developed as described under Materials and Methods.

**FIG. 3.** Detection of multiple cytokine expression from conditioned media. The cytokine array membranes were incubated with 50-fold-diluted conditioned media from human glioblastoma cells U251 treated with or without TNF\(\alpha\). The membranes were then incubated with a cocktail of biotin-labeled antibodies against all 24 cytokines and the signals were detected by ECL.
To overcome these two major obstacles, we coated membranes homogenously with specific antibody against corresponding antigen. For example, to detect MCP-1 levels in different samples, membranes were coated with capture anti-MCP antibody. Different samples were then spotted onto the membranes. The expression levels of MCP-1 were then detected by biotin-conjugated anti-MCP-1 and HRP-conjugated streptavidin. The signals were then detected by ECL. The intensities of signals derived from standard MCP-1 were determined by densitometry and plotted against concentrations of MCP-1. Membranes were coated with anti-MCP-1 (20 μg/ml) or BSA (20 μg/ml) for 4 h. 0.5 μl of purified human MCP-1 was spotted onto the anti-MCP-1-coated membranes. After incubation with biotin-conjugated anti-MCP-1 and HRP-conjugated streptavidin, the membranes were subjected to ECL.

<table>
<thead>
<tr>
<th>Table 1: Comparison of Protein Levels by Protein Arrays and ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-8</strong></td>
</tr>
<tr>
<td><strong>INFγ</strong></td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
</tr>
</tbody>
</table>

Note. The relative expression levels in protein arrays were determined by densitometry. The actual amounts of protein levels were quantitated by ELISA. Fold change represents cytokines differentially expressed between TNFα-treated/untreated conditioned media and between patient sera Nos. 4 and 11.

**FIG. 4.** Detection of cytokine expression from patient’s sera. 10-fold-diluted patient’s sera were incubated with cytokine array membranes. The signals were detected as described under Materials and Methods.

**FIG. 5.** Conditioned medium arrays. (A) Conditioned medium array design. (B) 0.5 μl of conditioned media from different sources was spotted onto the anti-MCP-1-coated membrane. The membrane was then incubated with biotin-conjugated anti-MCP-1 and HRP-conjugated streptavidin. The signals were then detected by ECL. (C) The intensities of signals derived from standard MCP-1 were determined by densitometry and plotted against concentrations of MCP-1. Membranes were coated with anti-MCP-1 (20 μg/ml) or BSA (20 μg/ml) for 4 h. 0.5 μl of purified human MCP-1 was spotted onto the anti-MCP-1-coated membranes. After incubation with biotin-conjugated anti-MCP-1 and HRP-conjugated streptavidin, the membranes were subjected to ECL.
with specific antibody increased detection sensitivity at least 100-fold (Fig. 5D).

DISCUSSION

Global analysis of protein expression is vital for our understanding of cell functions, the maintenance of health state of human being, and the development of disease, particularly cancer. To develop a flexible, simple, and high-throughput assay system for simultaneous detection of multiple protein expression, we took advantage of the specificity of ELISA, high sensitivity of ECL, and high-throughput of microspot. As a first step toward this goal, we established a method for simultaneously detecting multiple cytokine expression. This technology can be used to directly detect cytokines from conditioned media, from patient’s sera, and other sources. The principles described here can be extended to detect other types of protein from a variety of sources.

Since conditioned media, patient’s sera, or any biological fluids can be directly used in our protein arrays without any purification step, the present methodology provides for a highly efficient way to detect cytokine levels compared to cDNA microarrays. Cytokines are traditionally detected by ELISA; however, our approach has several advantages over ELISA. First and foremost is that our approach can simultaneously detect multiple cytokines. Second, the microarray format substantially reduces the cost and labor since very small amounts of antibodies are required and the whole experiment can be done within several hours. Furthermore, the sensitivity is high. As low as 4 pg/ml of MCP-1 can be detected in the protein array format. In contrast, at least, 40 pg/ml of MCP-1 is required to produce an unambiguous signal in ELISA assays. Furthermore, the detection range is much greater than ELISA. For example, the detection range of IL-2 varied from 25 to 250,000 pg/ml, whereas the range varies only within 100- to 1000-fold in a typical ELISA. Thus, the detection range is at least 100-fold greater in the protein array compared with ELISA. The variability is also lower than ELISA. As determined by densitometry, the variation between two spots ranged from 0 to 10% in duplicated experiments. In contrast, we routinely have much higher variation (about 20%) in ELISA. Finally, our approach is much quicker and is much easier to adapt to high-throughput format.

Simultaneous detection of multiple cytokines will undoubtedly provide a powerful tool to study cell function. Cytokines play important roles in innate immunity, apoptosis, angiogenesis, and cell growth and differentiation (27, 28). Aberrant cytokines have been implicated in most disease processes including cancer and cardiac diseases. The interaction of cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli and positive as well as negative regulatory loops are complex and often involve multiple cytokines. Our human cytokine array system will undoubtedly provide a new approach to investigate these complex questions.

In addition to being sensitive, specific, and quantitative, this approach is intrinsically parallel and readily scalable to the monitoring of a much larger numbers of protein expression. Although it is not possible to mix thousands of detection antibodies together, it is possible to get around this problem by utilizing motif-specific antibodies which recognize dozens, even hundreds and thousands, of different proteins. In such cases, only very limited number of detection antibodies would be required to detect large numbers of antigens in protein arrays. By applying microarray printer, scanning CCD imager, and analysis software, the antibody-based protein arrays should become an increasingly important tool to profile protein expression patterns, postmodification, and interactions.

Protein array technology allows us to simultaneously detect multiple proteins from the same sample and identify the key molecules. To validate and further extend the findings from protein array, one needs to analyze hundreds or even thousands of samples at the same times. Here we demonstrated that such a task could be accomplished by cell culture medium arrays and serum arrays, a methodology adapted from tissue arrays (29, 30). In theory, thousands of samples could be easily spotted onto single membranes to survey a particular protein expression. By precoating membranes with capture antibody, we routinely increased the detection levels at least 100-fold compared to uncoated membranes. In this system, 0.5 μl of sample with a concentration of 125 ng/ml was detected. This means that levels as low as 60 pg of total protein can be detected. The approach described here is particularly important in clinical settings to establish the diagnostic, prognostic, and therapeutic importance of each of the emerging molecules identifying in protein arrays.

ACKNOWLEDGMENTS

This work was supported by NIH/NCI Grant CA89273 (R.P.H.) and ACS Grant RPG-99-164-01-CNE (R.P.H.). We also express our thanks for support by the Helen Dyar King Fund at the Arizona Community Foundation for Cancer Research. We are grateful for Drs. Rajeshwar Tekmal and Elizabeth Bonney, Emory University School of Medicine, for providing patient’s sera in this study. We also thank Dr. Neil Sidell for his critique of the manuscript.

REFERENCES

2. Carulli, J. P., Artinger, M., Swain, P. M., Root, C. D., Chee, L., Tulig, C., Guerin, J., Osborne, M., Stein, G., Lian, J., and Lo-


