Use of Two-Dimensional Gel Electrophoresis To Measure Changes in Synovial Fluid Proteins from Patients with Rheumatoid Arthritis Treated with Antibody to CD4

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Synovial fluid proteins from microliter volumes of synovial fluid were resolved by two-dimensional polyacrylamide gel electrophoresis and detected by silver staining to investigate the feasibility of using two-dimensional (2D) electrophoresis in the clinical research setting and provide global disease information of disease progression. Several hundred proteins could be resolved as spots, many of which displayed the characteristic pattern of plasma-derived glycoproteins. The lowest level of detection was approximately 0.2 ng from a total of 50 μg of protein loaded. Most of the proteins could be identified on the basis of pI and molecular weight when compared with plasma protein maps on the World Wide Web. Unknown proteins were characterized by mass spectrometry of tryptic digests and by comparison with peptide databases. Synovial fluids from patients with rheumatoid arthritis were analyzed using this technique. Each subject received a fixed dose of antibody to CD4 as part of a phase II clinical trial to determine the efficacy of this immunosuppressive treatment in modifying disease activity. Synovial fluid was removed at day 0, followed by administration of antibody. Subsequent removal of synovial fluid and additional administration of antibody were carried out at different times thereafter. Changes in levels of acute-phase proteins were quantified by densitometry of silver-stained 2D polyacrylamide gels. Other parameters of disease progression such as serum C-reactive protein and physician’s global assessment of clinical condition were used for comparison. In this way, changes in acute-phase proteins towards normal levels, as measured by 2D polyacrylamide gel electrophoresis, could be correlated with clinical improvement and conventional clinical chemistry measurements. Thus, the system can be used for quantitative analysis of protein expression in sites of autoimmune disease activity such as the synovial fluid of rheumatoid arthritis patients.

Since its original description independently but simultaneously by O’Farrell and Klose over 20 years ago, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used for many different applications where the high-resolution separation of proteins in complex mixtures is required (18, 22). During this time improvements to the methodology have been made, such as the introduction of immobilized pH gradients (5) for the isoelectric focusing dimension and increases in detection sensitivity (15). The introduction of mass spectrometry and database searches to identify proteins (26) has also made a major impact on the study of proteins and encouraged the emergence of the field of proteomics (28) to complement genomics research.

We have exploited these improvements in our study of the autoimmune disease rheumatoid arthritis (RA), in which the course of the disease was monitored by analyzing synovial fluid from the affected joints of a small number of patients in a dose escalation study. RA is one of a number of autoimmune diseases in which T lymphocytes are believed to be central to the etiology and pathogenesis (24). The main clinical feature of RA, however, is the presence of chronic cytokine-driven inflammation and resulting tissue destruction through the action of catabolic proteases (19). This has made the characterization of the underlying T-cell responses more difficult; however, antibodies specific for molecules on the surface of T cells such as CD4 have provided experimental tools and clinical reagents to test the hypothesis of T-cell involvement in RA. The work of Qin et al. (25), who demonstrated that a state of antigen unresponsiveness or tolerance could be induced in transplant rejection models by nondepleting anti-CD4 antibodies has led to the use of these reagents in humans. A recent dose escalation trial of a humanized antibody to CD4 is described in which clinical efficacy was observed at a dose of 300 mg per day. Synovial fluid specimens from some of these patients were available at different times after anti-CD4 treatment; it was thus possible to analyze biochemical changes in parallel to clinical responses by using small amounts of the fluid for the analysis of many proteins simultaneously. The study was intended to investigate the feasibility of using 2D-electrophoresis in the clinical research setting to provide global disease information of disease progression by analyzing what was available to us, namely, relatively small volumes of synovial fluid from a small number of patients in a dose escalation study. The value of these samples lies in the fact that they come from a clinical trial for novel biological therapy where clinical outcome and other parameters were measured, thus allowing the assessment

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of the feasibility of analyzing such samples using proteomics as an alternative to conventional independent assays. 2D electrophoresis technology, in conjunction with highly sensitive silver staining, can reveal more than 300 proteins in 0.8 µl of synovial fluid, having a sensitivity of approximately 0.2 ng per protein spot. The protein map compared well with that of plasma (1). Most of the proteins have been identified previously and mapped according to their mobility on gels (Swiss Institute of Bioinformatics proteomics server website http://www.expasy.ch). These include the acute-phase reactants whose expression is altered in acute and chronic inflammatory states, including RA. Indeed, the elevation of serum C-reactive protein (CRP) is one of the hallmarks of the disease, as is an elevated erythrocyte sedimentation rate (ESR) due to increased levels of acute-phase reactants (2, 11).

Our results show that levels of synovial fluid proteins may be directly quantified from 2D gels and that changes in acute phase protein levels correspond to clinical improvement. Thus, in principle it should be possible to monitor the expression of any protein which can be resolved by 2D-PAGE and which is expressed at levels greater than 200 ng/ml in unfraccionated synovial fluid.

MATERIALS AND METHODS

Antibody to CD4. 4162W9/4, a humanized antibody to human CD4, does not mediate complement lysis and has weak antibody-dependent cellular cytotoxicity activity (12). Antibody to CD4 (30, 100, or 300 mg/day) was administered intravenously over a period of 2 h in the outpatient clinic on 5 consecutive days.

Clinical samples. Patients with clinically active RA were recruited from out-patient clinics at King’s College and Lewisham Hospitals, London, United Kingdom. Written informed consent was obtained from each patient prior to enrollment. Synovial fluid samples were obtained just prior to antibody administration and at different times thereafter and were stored at −80°C.

Clinical assessment. Arthritis activity was assessed using American College of Rheumatology composite scoring criteria (8). Serum ESR and CRP levels were measured using standard clinical chemistry procedures.

2D-PAGE. (i) Isoelectric focusing dimension. 2D-PAGE was carried out essentially as described by Billeciqut et al. (8) and detailed on the Swiss 2D-PAGE database (http://expasy.ch/ch2d).

Synovial fluid samples (6.5 µl) were incubated at 100°C for 5 min with 10 µl of 10% (wt/vol) SDS–2.3% (wt/vol) dithiothreitol (DTT) and then diluted to 500 µl in 8 M urea–4% (wt/vol) 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate (CHAPS)–40 mM Tris base–65 mM DTT containing a trace amount of bromophenol blue.

Sample volumes of 65 µl, equivalent to 0.8 µl of plasma, and containing 50 µg of total protein, were loaded in sample cups at the cathodal end of 18-cm immobilized nonlinear pH 3–10 gradient strips (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Samples were focused for a total of 99.9 kVh in a Bio-Rad Protean xi apparatus until the bromophenol blue marker reached the bottom of the gel.

(ii) Second dimension electrophoresis using SDS-PAGE. Approximately 2 cm was cut from the cathodal end of the strip, and the arrow tip was cut from the anode end. Strips were transferred to the top of an 18-by-16-cm, 1.5-mm-thick, 9–16% polyacrylamide gel and held in position with molten 0.5% (wt/vol) agarose in cathode buffer containing trace bromophenol blue. Anode buffer was 0.375% (wt/vol) Tris-acetate, pH 8.8, and the cathode buffer was 192 mM glycine Tris (pH 8.3)–0.1% (wt/vol) SDS as recommended by Herbert et al. (14).

Gels were run at 10 mA/igel for the first hour followed by 40 mA/igel and at a constant temperature of 10°C in a Bio-Rad Protean xi apparatus until the bromophenol blue marker reached the bottom of the gel.

Samples were run in triplicate.

Detection of separated proteins by silver staining. Gels were routinely stained using ammoniacal silver as described (6, 15) and scanned using an Epson GT9000 flatbed scanner and Adobe Photoshop version 3.0 at a scanning resolution of 200 dots per inch. Gels for analysis by mass spectrometry were stained as described by Betts and Smith (3) using a modification of the method of Shevchenko et al. (27).

Phoretix analysis. Spot analysis was carried out using Phoretix software version 3.51 (Phoretix International, Newcastle upon Tyne, United Kingdom).

MS. (i) Sample preparation for MS. Protein spots of interest were excised from the gel, reduced, carboxymethylated, and digested in situ with trypsin overnight at 37°C as described (16). Gel digests were centrifuged, and an aliquot of the supernatant was taken for analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS).

(ii) MALDI MS. MALDI MS was performed on a TofSpec SE time-of-flight mass spectrometer equipped with a delayed-extraction ion source (Micromass, Manchester, United Kingdom). Samples were prepared as described (16). Spectra were internally calibrated using the matrix ion at m/z 1060.10 and trypsin autolysis peaks at m/z 2163.06 and m/z 2289.15. Monoisotopic masses were assigned, and proteins were identified by peptide mass fingerprinting using Peptide Search software (20) and a mass accuracy of 0.1 Da.

NanoES MS. Prior to nanoelectrospray (NanoES) analysis, peptides were extracted from the gel pieces as described (17). Dried digest mixtures were desalted prior to NanoES analysis (PerSeptive Biosystems, Framingham, Mass.). Peptides were dissolved in 0.5% (vol/vol) formic acid, loaded on to pulled-glass capillaries packed with approximately 5 µl of POROS R2 sorbent, and washed with 5 µl of 0.5% (vol/vol) formic acid. Samples were eluted with approximately 2 µl of 1% (vol/vol) formic acid, 50% (vol/vol) methanol, and 1 µl of the eluate inserted into the spraying needle. Needles for electrospraying were made as described (30, 31). Electrospray mass spectra were acquired on an API III triple quadrupole machine (Perkin-Elmer Sciex, Ontario, Canada) equipped with a NanoES ion source (30, 31). Proteins were identified by the sequence tag approach using Peptide Search software (20).

RESULTS

2D-PAGE gel separation of synovial fluid proteins. Synovial fluid from RA patient 22 (0.8 µl) was subjected to 2D-PAGE and proteins visualized by silver staining as described in Materials and Methods. Gels were done in triplicate to minimize gel-to-gel variation. Spot variability was measured in pilot experiments and found to be as follows (coefficient of variation = standard deviation/mean × 100): serum retinol binding protein (SRBP), 29%; haptoglobin, 5%; transthyretin, 14%. These are very comparable to the values quoted by Bini et al. (4). Figure 1 shows the entire gel in which approximately 300 individual proteins ranging in molecular mass from 200,000 to 10,000 Da with pIs between 3 and 9.5 could be resolved. The most abundant proteins were albumin and immunoglobulins, and the overall profile was very similar to that obtained with plasma (not shown). Of note are the many trains of spots, which are known to have the same primary structure but different degrees of glycosylation, leading to a progressive change in the pI and molecular weight (e.g., α1-antitrypsin [23]). Thus, the total number of spots which were resolved by this gel was on the order of 1,000.

Staining sensitivity was estimated at 0.2 ng/spot following reduced one-dimensional SDS-PAGE of the humanized monoclonal antibody anti-CD4, stained using the silver staining method of Hochstrasser and colleagues (6, 15). This gave visible bands at 0.3 ng of light chain and a more strongly stained band at 0.6 ng of heavy chain (Fig. 2). Background staining was far higher in this system than in that developed by Hochstrasser and colleagues (6, 15), which uses a cross-linker piperazine diacylamide. Bands had a width of 8 mm, causing us to estimate that some of the small visible spots on 2D-SDS-PAGE had protein contents of approximately 0.2 ng. On 2D-PAGE of synovial fluid, the staining saturation for
albumin had obviously been reached (Fig. 1). Due to the nonlinear nature of the stain this was necessary to detect less abundant proteins, highlighting the need for prefractionation of samples and the development of stains with high sensitivity and a linear dynamic range. The sample spots analyzed were normalized with respect to each other in order to reduce variability due to differential staining, a procedure used by others (4, 7).
Identification of acute-phase proteins. Acute-phase proteins are either positively or negatively regulated in inflammatory diseases and are therefore either enhanced or decreased in these conditions. Figure 1 shows the positions of spots on 2D-PAGE gels which correspond to the following proteins: haptoglobin-α2 (positive), serum retinol binding protein, and transthyretin (negative). These could be readily identified and quantified using densitometry and image analysis. Only two novel spots were seen to change over the time course of the study that correlated with the physician’s global assessment of clinical improvement. These were identified by mass spectrometry as fragments of CRP (Fig. 1). These were detected below apolipoprotein A-1 in gels of synovial fluid from RA patients and diminished in staining intensity as anti-CD4 treatment progressed in good responders (Fig. 3). These were not present in the gel database. Six identical spots from three gels were reduced and carboxymethylated as described above. MALDI MS analysis of the protein spot of interest resulted in a weak spectrum from which no identification could be obtained. The remaining digest mixture was therefore further analyzed by NanoES MS. This resulted in sequence data being obtained for two peptides, ESDTSYVSLK and GYSIFSYATK, matching human CRP (P02741). This protein was not used further since it was not visible in all of the clinical samples used in this study.

Direct measurement of acute-phase protein expression in synovial fluid of RA patients undergoing therapy with 4162W94. Synovial fluids from three patients (subjects 11, 16, and 22) who were receiving daily doses of 30, 100, or 300 mg of antibody to CD4, respectively, were analyzed by 2D-PAGE before and during the treatment period. Levels of haptoglobin-α2, SRBP, and transthyretin were determined following densitometry of the silver-stained gels. Each value was normalized to the total intensity of staining, and the values compared with serum CRP and ESR values as well as the physicians’ global assessment of patient response to treatment. Figure 4 shows the results expressed relative to the day 0 value (taken as 1) for each parameter. Higher levels of disease activity, as measured by increased ESR, physician’s global assessment, and elevated serum CRP could be correlated with an increase in haptoglobin-α2 and decrease in SRBP and transthyretin and vice versa. This was particularly striking with subject 22, whose disease activity was significantly reduced over time.

DISCUSSION

This study has demonstrated under the conditions described the potential of proteomics in analyzing the protein complement of synovial fluid in the clinical research setting, thus offering a suitable alternative to conventional measurements of disease progression, particularly where the collection of multiple samples from the same patient is possible. Since Choy et al. (6a) have used a nondepleting antibody to CD4 in a dose escalation study for RA, we have exploited this to evaluate changes in the protein composition of synovial fluids during treatment using this powerful analytical tool. Significant advances in 2D-gel electrophoresis technologies over the last few years have provided a system for the resolution and detection of many proteins in cell extracts or biological fluids which is superior to earlier systems. Since the pioneering studies of Sanchez et al. on human plasma maps (26), several studies have identified changes in serum glycoproteins in patients with hepatocarcinoma (21) and other liver diseases (13, 10). Variations on the technique have been used to identify insulin-like growth factor binding proteins by binding radiolabeled ligands to serum proteins separated by 2D-PAGE (29).
Synovial fluids have been analyzed by 2D-PAGE in a previous study (9), but no differences could be detected between RA and control groups, and the electrophoretic patterns were poor. Since the introduction of the immobilized pH gradients, improved silver staining techniques, and MS used in our study, it is possible to identify and quantify specific known markers of the inflammatory process. These acute-phase proteins have been studied extensively using more conventional biochemical techniques, such as immunoassays, and more recently, using similar electrophoretic methods (4, 7). In the latter studies, the acute-phase response of acute-phase reactants in serum was measured in individuals with acute bacterial or viral infection (4) or inflammation due to typhoid vaccination or rheumatoid arthritis (7). Of interest was the observation that different stimuli invoked specific changes in acute-phase protein expression, no doubt reflecting a particular proinflammatory cytokine response (e.g., interleukin 1 [IL-1], tumor necrosis factor, IL-6). Unfortunately, the dynamic range of the protein stain is such that we could theoretically detect other “interesting” markers, but only after extensive work on developing a prefractionation strategy allowing enrichment of low-abundance proteins, which was not possible due to the limited number and volume of the available samples. The dynamic range of the stain thus limited analysis to the more abundant acute-phase proteins, which are known markers of inflammatory disease including RA. However, the IL-6 and tumor necrosis factor levels were clearly reduced, as determined using conventional assays, in parallel with a normalization of the acute-phase response seen on 2D-PAGE.

Although the proteins measured using 2D-PAGE have been described in the RA literature (2), they may be considered forerunners of more novel markers of disease activity and progression. Many more proteins are present in synovial fluids, but at lower concentrations. For example IL-6 levels could be detected in patient’s synovial fluid at levels of 15 ng/ml or more by immunoassay, but were undetectable by 2D-PAGE as described. Achieving the level of detection commensurate with immunoassay will only be possible on 2D-PAGE using prefractionated synovial fluid in which the major serum proteins (albumin and immunoglobulins) are substantially reduced, but not at the expense of the nonspecific depletion of minor proteins.

When this occurs, it will be possible to identify many subtle

**FIG. 3.** Silver-stained SDS-PAGE gel (9–16% polyacrylamide gradient) showing 50 μg of synovial fluid proteins from subject 22 on day 0 prior to administration of 300 mg of antibody to CD4. Proteins were separated in the first dimension on a nonlinear pH 3-10 immobilized pH gradient (IPG) strip. Zoomed areas highlighting changes in acute-phase proteins and decrease in CRP fragment are shown on days 4, 7, 15, and 28.
changes in protein expression, permitting protein enrichment to determine the mass spectrum and hence identity prior to development of specific immunoassays. From the perspective of the rheumatologist, it will be important to identify reliable markers of bone and cartilage destruction as well as chronic inflammation, and this could be a realistic prospect in the near future.

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