Nucleolar Proteins B23 and C23 as Target Antigens in Chronic Graft-Versus-Host Disease

By Józefa Węsińska-Gądek, Edward Penner, Eva Hitchman, Peter Kier, and Georg Sautermann

Previously we observed that sera from patients of allogeneic bone marrow transplant who developed extensive chronic graft-versus-host disease (GVHD) intensively stained the nucleolar region of target cells in indirect immunofluorescence microscopy. To identify the target antigens, immunoblotting experiments were performed using isolated nuclei, isolated nucleoli, and purified nuclear and nucleolar proteins as the antigen source. The nucleolar phosphoproteins B23 and C23 were identified as the main target antigens. Eleven of 19 extensive chronic GVHD sera reacted with these nucleolar phosphoproteins. In addition, four sera recognized histone H1, and two sera recognized the nuclear lamin A and C. Our patients reacting with the nucleolar proteins had symptoms resembling that of scleroderma or Sjögren’s syndrome.

A LLOGENEIC BONE MARROW transplantation (BMT) is an important therapeutic option in the treatment of hematologic malignancies and severe aplastic anemia. Long-term survivors of allogeneic transplantation often develop clinical complications defined as chronic graft-versus-host disease (GVHD). Sharing features common to systemic lupus erythematosus, Sjögren’s syndrome, and progressive systemic sclerosis (scleroderma), chronic GVHD clinically and immunologically resembles an autoimmune disorder. Several investigators have reported about distortions of humoral and cellular immune regulation in chronic GVHD.1,2,3

The occurrence of antibodies against nuclear constituents in sera of chronic GVHD patients has been suggested from observations in indirect immunofluorescence microscopy.3,4 Recently, we reported that sera of chronic GVHD patients strongly stained nucleoli in indirect immunofluorescence.5 In the present immunoblotting experiments the nucleolar phosphoproteins B23 and C23 were identified as the major target antigens.

MATERIALS AND METHODS

Cell Culture

HeLa S1 were grown at 37°C in monolayer cultures in Eagle’s Minimal Essential Medium supplemented with 10% fetal calf serum in an atmosphere of 5% CO2 in air.

Sera

Sera were obtained from 32 patients who underwent an allogeneic BMT. Nineteen of the 32 recipients had extensive chronic GVHD, three had limited chronic GVHD, and 10 had no evidence of chronic GVHD. From each patient serum samples were collected before and at different times after 130 days of BMT. Ten scleroderma sera displaying strong nucleolar staining were also included.

For control, sera from 48 healthy blood donors and from eight patients after autologous BMT were assayed.

Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Indirect Immunofluorescence Microscopy

Patients’ sera were tested by indirect immunofluorescence, using commercial HEp-2 human tissue culture preparation (Kallestad, Austin, TX) and HeLa cells grown on glass cover slips. The cells were washed in phosphate-buffered saline (PBS), fixed, and permeabilized as described previously.7 The cells were first incubated in appropriate dilution with patients’ sera and then with antihuman Ig coupled to fluorescein. As controls, sera from healthy specimens were used or incubation with human sera was omitted. These samples did not stain. Antibody titers were determined by serial dilution of patients’ sera.

Isolation of Nuclei and Nucleoli

PBS-washed HeLa cells were swollen and homogenized in 0.25% NP-40, 0.15% sodium deoxycholate in hypotonic buffer. Crude nuclei were suspended in 0.25 mol/L sucrose, 10 mmol/L MgCl2, 10 mmol/L Tris-Cl, pH 7.4, and centrifuged through a sucrose cushion. Nuclear proteins were prepared from isolated HeLa cell nuclei by modification of the methods of Muramatsu et al8 and Rothblum et al.9 The isolated nuclei were suspended in 0.34 mol/L sucrose, 5 mmol/L MgCl2, and sonicated until no nuclei remained intact. The sonicate was underlaid with 0.88 mol/L sucrose and centrifuged to sediment the purified nucleoli. All isolation steps were performed in the presence of protease inhibitors at 4°C.

Purification of Proteins

Nucleolar proteins. Isolated nucleoli were treated overnight with 4 mol/L urea, 3 mol/L LiCl.10 After centrifugation at 27,000 g for 20 minutes, the supernatant was dialysed against buffer A (6 mol/L urea, 20 mmol/L Tris-Cl, pH 6.0, 1 mmol/L EDTA, 1 mmol/L dithiothreitol [DTT] and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]), centrifuged and applied to a diethyl aminoethyl (DEAE)-cellulose column (DE-52, Whatman, Maidstone, Kent, UK) preequilibrated with buffer A. The proteins were stepwise eluted with increasing NaCl concentrations. The fractions containing the nucleolar proteins B23 or C23 were dialyzed and lyophilized.

H1 histones. H1 histones were isolated as described previously.11

Nuclear lamina-pore complex fraction. The nuclear lamina-pore complex fraction was prepared from isolated nuclei of HeLa tumor cells as described before.9

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Electrophoretic Separation of Proteins

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab gels as described by Laemmli except that 6 mol/L urea was included in the sample buffer and 4 mol/L urea in the gel solution. Two-dimensional PAGE was performed according to O'Farrell, with the following alterations. In the lysis buffer and the gel mixture, Nonidet P-40 was replaced by CHAPS; Bio-Rad, Richmond, CA). To achieve good resolution of proteins in the pH range 5 to 8 of the isoelectric focusing (IEF) gels, ampholines pH 3.5 to 10 and pH 5 to 8 (LKB, Bromma, Sweden) were mixed at a ratio of 1:4. In the second dimension samples were run on 10% SDS polyacrylamide slab gels. For calibration IEF protein markers (BDH, Poole, UK), PI range 4.7 to 10.6, were applied. Proteins were electrophoretically transferred to nitrocellulose sheets. Proteins were detected by Coomassie blue or silver staining of the gels, or by Ponceau S staining of nitrocellulose.

Immunoblotting

The blots were preincubated with 3% bovine serum albumin in PBS. The blots were then exposed to appropriate concentrations of patients' sera, extensively washed, and incubated with 125I-protein A (Amersham, Buckinghamshire, UK). The reactive antigens were detected by autoradiography, using X-Omat S film (Kodak, Rochester, NY).

RESULTS

Sera of 32 patients who had received allogeneic bone marrow grafts were screened for the presence of autoantibodies by indirect immunofluorescence. Samples were collected before and at different times after transplantation. Interestingly, the majority of sera (12 of 19) obtained from patients with extensive chronic GVHD strongly stained the nucleoli of HEp-2 and HeLa cells. In contrast, no positive reaction was observed in either three patients with limited or 10 patients without chronic GVHD. Likewise, 32 sera collected before BMT, eight sera of control patients after autologous BMT, and 48 sera of the healthy blood donors did not stain (Table 1).

As seen in Fig 1, the positive sera displayed three different staining patterns. In most cases, only the nucleoli showed fluorescent staining. In some cases, additional diffuse nuclear staining was seen. In rare cases, a rim-like nuclear staining was observed in addition to nucleolar staining.

To identify the reactive antigens, immunoblotting experiments were performed, using total nucleolar proteins as the antigen source. Figure 2 shows that two proteins at about 100 Kd and at 37 Kd, respectively, were recognized predominantly by antinucleolar antibody-positive sera. A few sera reacted solely with one of these antigens (lanes d and e). Sera giving additional nucleoplasmic staining in immunofluorescence reacted additionally with a double band at approximately 30 Kd (lane c).

After two-dimensional separation of nucleolar proteins, two spots were recognized by the sera, one at 100 Kd, PI 5.5, and the other at 37 Kd, PI 4.9 to 5.2 (Fig 3). The positions of the spots coincided with those characteristic for the major nucleolar proteins C23 and B23, prominently visible after Ponceau red staining (not shown).

To further prove the identity of the two target antigens, the nucleolar proteins B23 and C23 were extracted and chromatographically purified on DEAE-cellulose under conditions defined by Michalik et al. The separated proteins were used in the following immunoblotting experiments. Again, chronic GVHD sera reacted with each of the fractionated proteins (Fig 4), thus confirming their identity as nucleolar proteins B23 and C23.

Sera exhibiting diffuse nuclear in addition to nucleolar staining (Fig 1b) were screened for possible reactions with other antibodies. While not recognizing DNA in the enzyme-linked immunosorbent assay (ELISA), the antibodies reacted with a double band at the position of H1 histones. This was seen in immunoblots of total nuclear proteins and of total histones isolated from HeLa tumor cells (not shown). Figure 5 illustrates that the antibodies indeed recognized isolated H1 histones.

From our experience, the rim-like staining exhibited by two sera in immunofluorescence (Fig 1c) was indicative for the presence of antibodies against constituents of the nuclear envelope. Accordingly, immunoblots were performed using a “nuclear lamina-pore complex”-defined fraction highly enriched in nuclear lamins as the antigen source. Figure 6 documents that two chronic GVHD sera recognized the nuclear lamins A and C (lanes c and d). The identity of the nuclear lamins A and C was unequivocally proven by reaction with a specific monoclonal antibody (lane b).

Table 1. Prevalence of Antinucleolar Antibodies in Chronic GVHD Sera

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>Nucleolar</th>
<th>Nuclear Homogenous</th>
<th>Rim-Like</th>
<th>Nucleolar Recognized</th>
<th>Antigens Recognized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic BMT</td>
<td>32</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>11</td>
<td>B23, C23, H1, Lamin A/C</td>
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<tr>
<td>Extensive c-GVHD</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Limited c-GVHD</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No c-GVHD</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autologous BMT</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Controls</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Abbreviation: c-GVHD, chronic GVHD.
Fig 1. Nucleolar staining by chronic GVHD sera in indirect immunofluorescence. HEp-2 cells. (a) Strong nucleolar staining; (b) nucleolar plus homogenous nuclear staining; (c) nucleolar plus rim-like nuclear staining.
In chronic GVHD sera, nucleolar antigens recognized by these sera were electrophoresed, blotted, and exposed to sera. Lanes a through e, chronic GVHD sera displaying nucleolar staining in immunofluorescence; lane f, control serum from a bone marrow recipient with no evidence of chronic GVHD, serum negative in immunofluorescence; lane M, marker proteins. The apparent molecular mass is indicated by triangles.

The incidence of antinucleolar antibodies is given in Table 1. Only patients with extensive chronic GVHD produced antibodies against nucleolar proteins, with B23 and C23 being the major targets. Thus, 11 of 19 extensive chronic GVHD sera reacted with these nucleolar phosphoproteins. To show the relationship between the immunofluorescence patterns and the antigens detected by immunoblotting, the sera of the extensive chronic GVHD patients are individually listed in Table 2. In all cases, the intracellular localization of the antigens correlated with their identity, as found in the immunoblotting experiments.

Clinically, most of the extensive chronic GVHD patients producing antinucleolar antibodies showed symptoms resembling those of scleroderma or Sjögren's syndrome. Therefore, the chronic GVHD sera were also tested for the presence of antibodies against topoisomerase I, the autoantigen characteristic for scleroderma. However, none of the chronic GVHD sera reacted with topoisomerase I (not shown).

For comparison, sera were collected from nontransplanted scleroderma patients. The 10 selected sera extensively staining the nucleoli in immunofluorescence did not react with the nucleolar protein C23 (Table 1), but did recognize topoisomerase I as an antigen (not shown). Only one of the scleroderma sera reacted with the phosphoprotein B23 (Table 1). Thus, it appears that the autoantigen patterns recognized by chronic GVHD and scleroderma sera are different.
Fig 6. Nuclear lamins A and C recognized by chronic GVHD sera. The immunoblot was performed using the nuclear lamina-pore complexes fraction isolated from HeLa tumor cells as antigen source. Lane a, Coomassie blue staining; lane b, immunodetection by monoclonal antinuclear lamin A/C antibodies; lanes c and d, detection by sera giving nucleolar plus rim-like nuclear staining in immunofluorescence.

DISCUSSION

We have previously shown that sera of chronic GVHD patients predominantly stain the nucleolar structure of HeLa and HEp-2 cells in indirect immunofluorescence.\textsuperscript{6} The results of the present immunoblotting experiments show that the sera contain antibodies directed against the nucleolar phosphoproteins B23 (nucleophosmin) and C23 (nucleolin). In the rare cases exhibiting a differing staining accentuation, histone H1 and the nuclear lamins were identified as additional antigens. The occurrence of antihistone H1 antibodies coincided with diffuse nuclear staining, and the occurrence of antilamin A/C antibodies coincided with rim-like nuclear staining.

The nucleolus is an intranuclear organelle in which preribosomal RNA is synthesized and processed, and in which the precursors of the ribosomal subunits are assembled.\textsuperscript{17,18} The 37-Kd phosphoprotein B23 and the 100-Kd phosphoprotein C23 are major components of the nucleolus, B23 being located in the granular and C23 in the fibrillar compartment of the nucleolus. The proteins are believed to be involved in ribosome biogenesis and intranuclear transport of preribosomal particles.

Antibodies reacting with nucleolar constituents have been observed in certain overlap connective tissue diseases and, most frequently, in progressive systemic sclerosis.\textsuperscript{19,20} In scleroderma autoantibodies to DNA topoisomerase I, RNA polymerase I, PM-Scl, fibrillarin, and 7-2 RNP have been found in 10% to 70% of the patients.\textsuperscript{18-21} Reaction with phosphoprotein B23 was reported to occur in 1 of 11 cases.\textsuperscript{22}

In the present study, the incidence of antibodies to the nucleolar protein B23 was very high, especially when compared with the above cited data. Thus, 10 of 19 extensive chronic GVHD sera recognized protein B23.

Furthermore, 6 of the 19 sera recognized the nucleolar protein C23. To our knowledge, C23 has not been previously found to be a target antigen in autoimmune disorders.

Clinically, it is of interest that the reaction against nucleolar components occurred only in patients with extensive chronic GVHD and coincided with or preceded the occurrence of clinical symptoms.\textsuperscript{6} In our experience, detection of antinucleolar antibodies by immunoblotting techniques was more sensitive than the detection by indirect immunofluorescence. The clinical use of these observations remains to be determined.

| Table 2. Relationship Between Immunofluorescence and Immunoblotting Reactivity of Extensive Chronic GVHD Sera |
|---|---|---|---|---|
| Patient | Immunofluorescence Pattern | Antigens Recognized | Lamins A/C |
| | Nuclear Homogenous | Nucleolar | Rim-Like | H1 | B-23 | C-23 |
| P.K. | − | + | − | − | + | + |
| K.H. | − | + | − | − | + | + |
| P.E. | − | + | − | − | + | + |
| H.H. | − | + | − | − | + | + |
| H.E. | − | + | − | − | + | + |
| S.F. | + | + | − | + | + | + |
| L.A. | + | + | − | + | − | − |
| O.B. | + | + | − | + | − | − |
| H.B. | + | + | − | + | + | + |
| B.R. | − | + | − | − | + | − |
| S.L. | − | + | − | − | + | − |
| W.J. | − | + | − | − | + | − |
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Nucleolar proteins B23 and C23 as target antigens in chronic graft-versus-host disease

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