Detection of Nuclear Matrix Proteins in Serum from Cancer Patients

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ABSTRACT

Morphological characteristics of the cell nucleus have long been used by pathologists in the clinical diagnosis of cancer. The nuclear matrix of the cell, the structure that serves to organize the chromatin within the nucleus, is known to reflect these morphological characteristics with regard to cell and cancer type. Monoclonal antibodies were developed to extracted nuclear matrix proteins. These antibodies were used in two-site immunometric assays to detect soluble nuclear matrix proteins in the supernatants of two dying cell lines and 15 tumor tissues. Furthermore, nuclear matrix proteins were detected at elevated levels in the sera of cancer patients compared with normal patient sera. In one assay, 63.2% of cancer sera read above 95% of normal sera, and in another assay, 73.7% of cancer sera read above 95% of the normal sera. With the development of monoclonal antibodies with greater cancer specificity, the detection of circulating nuclear matrix proteins may become an important clinical tool in the diagnosis and monitoring of cancer.

INTRODUCTION

In the pathological diagnosis of cancer, the nucleus is the prime area of focus in the determination of malignancy (1). The nucleus of the cancer cell is enlarged and irregular in shape, and the organization of the chromatin and the nucleolus region have a characteristic appearance. The nuclear matrix, a structure first described by Berezney and Coffey (2), is thought to be the framework for the organization of the chromatin in the interphase nucleus. The composition of the nuclear matrix changes with cell type and cancer (3). Consequently, those changes at the morphological level may reflect changes in the proteins of the nuclear matrix at the molecular level.

Methods for measuring cytoplasmic and cell surface antigens have become important clinical tools for the detection and monitoring of cancer. Nuclear matrix proteins have not received as much attention because they make up the structural network of the nucleus which resists extraction except at elevated ionic strength. It was assumed that cell protein release occurred only through the shedding of cell surface antigens or active secretion of cellular products.

Here we describe the unanticipated finding that nuclear matrix proteins can be released from dying cells in a soluble form. Monoclonal antibodies to nuclear matrix proteins were used to develop two-site immunometric assays. These assays can detect nuclear matrix proteins in a quantitative fashion in supernatants of dying cell and tissue cultures. It is also demonstrated that certain nuclear matrix proteins can be detected at elevated levels in the serum of cancer patients.

MATERIALS AND METHODS

Isolation of Nuclear Matrix. Nuclear matrix proteins were isolated by a method based on that of Penman and Fey (4). Briefly, human cervical tumor cell lines ME-180 and CaSkii grown to confluence in Dulbecco's modified Eagle's medium:5% fetal bovine serum:5% Ser-

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2 The abbreviations used are: PBS, Dulbecco's phosphate-buffered saline solution 10x diluted to 1x (Irvine Scientific, Santa Ana, CA); PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene-bis(oxyethylenenitriilo)tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; NM, nuclear matrix; NMP, nuclear matrix protein.

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Monoclonal Antibody Characterization. The positive clones were evaluated for human tissue reactivity using cryostat-cut frozen tissue sections and the avidin/biotin peroxidase staining procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Monoclonal antibody 107-7 stains the nuclei of all cervical, breast, and bladder tumors tested and the nuclei of the epithelium of some normal tissues including cervical. Antibodies 302-29, 302-22, and 302-18 stain some of the nuclei of cervical tumors and weakly stain nuclei of some normal tissues tested and the nuclei of the epithelium of some normal tissues including cervical.

Immunoblot Detection of Nuclear Matrix Proteins. To determine if the epitopes of the monoclonal antibodies were present in nuclear matrix extracts and in the supernatants of dead and dying cell cultures, immunoblot experiments were done on nitrocellulose membranes (Schleicher & Scheull, Keene, NH) at 14,000 x g to remove cellular debris, and stored frozen.

Human tissue, both normal and tumor, that had been stored at -70°C was prepared in a similar fashion. Using aseptic techniques, the tissues were chopped into 0.1- to 0.3-cm cubes as they thawed and placed in a flask containing serum-free medium. Two to 4 g of tissue were used per 100 ml of medium. The samples were incubated for 7 days at 37°C with 5% CO₂. The medium was then collected, centrifuged at 14,000 x g to remove cellular debris, and stored frozen.

Reagents for the Two-Site Immunometric Assay. The monoclonal antibodies were isolated from mouse ascites by protein G purification (Genex, Gaithersburg, MD).

For biotinylation, the antibodies were dialyzed overnight against 0.1 M sodium bicarbonate (pH 8.4), and the immunoglobulin G concentration was adjusted to 1.0 mg/ml. To 5 ml of antibody solution, 0.5 ml of bovine serum albumin in DMSO were added. After a 2-h room temperature incubation in the dark, 0.5 ml of 2 M Tris-HCl (pH 8.0) were added and incubated for a further 30 min.

Western Blots of Nuclear Matrix Proteins. Nuclear matrix preparations ME-180 and CaSki were used for Western blotting with the four monoclonal antibodies used in the immunonassays. Two of the monoclonal antibodies showed reactivity (Fig. 2). The nuclear matrix proteins were electrophoresed according to the method of Laemmli (7). The proteins in the gels were then electrophoretically transferred to methanol-activated Immobilon-P transfer membranes (Millipore, Bedford, MA) at 100 V for 4 h (8). The immunostaining of the Western blot followed the procedure described above for the immunoblot.

Differential labelling was confirmed by immunofluorescent staining of ME-180 cultures and supernatants of cancer cells and tissues. Row A, monoclonal antibody 107-7; Row B, antibody 302-29; Row C, antibody 302-18; Row D, antibody 302-22; Row E, anti-keyhole limpet hemocyanin antibody. The nuclear matrix proteins with molecular weights of approximately 150,000 and shows minor reactivity with a M, 110,000 protein. Antibodies 302-18 and 302-29 showed no reactivity.

Preparation of Antigen from Dead and Dying Cells. The human cervical tumor cell line ME-180 was grown to confluence by standard culturing techniques. The medium was replaced with serum-free medium, and the cells were placed in a 37°C incubator with 5% CO₂ for 7 days. The medium was then collected, centrifuged at 14,000 x g to remove cellular debris, and stored frozen.

Immunoblot analysis of reactivity of anti-nuclear matrix monoclonal antibodies with nuclear matrix and supernatants of cancer cells and tissues. Row A, monoclonal antibody 107-7; Row B, antibody 302-29; Row C, antibody 302-18; Row D, antibody 302-22; Row E, anti-keyhole limpet hemocyanin antibody. Column 1, ME-180 nuclear matrix; Column 2, T47-D (a breast tumor cell line) culture supernatant; Column 3, T47-D nuclear matrix; Column 4, lung tumor supernatant; Column 5, ovarian tumor supernatant; Column 6, breast tumor supernatant; Column 7, breast tumor supernatant.
Fig. 3. Immunofluorescence microscopy of the reactivity of anti-nuclear matrix monoclonal antibodies with ME-180 cells. A, 4′,6-diamidino-2-phenylindole DNA staining; B, 302-22 immunofluorescent staining (× 100); C to E, immunofluorescent micrographs of 302-18 (C), 302-29 (D), and 107-7 (E) (× 63).

An equal volume of 0.1% PBS-merthiolate containing 1% bovine serum albumin was added, and the mixture was dialyzed 3 times against the PBS-merthiolate.

For iodination of streptavidin, 2 μg of chloramine-T trihydrate (Sigma, St. Louis, MO) were added to a mixture containing 10 μg of the binding protein and 1 mCi of 125I in 20 μl of 0.25 M sodium phosphate (pH 7.4). After incubating for 25 s, the reaction was stopped by adding 50 μl of a solution containing 800 μg/ml of Cysteamine (Sigma) and 100 μg/ml of potassium iodide in 0.05 M sodium phosphate (pH 7.4). After the addition of 0.5 ml of 1% bovine serum albumin in PBS (pH 7.4), the material was fractionated on a 10-ml Sephadex G-100 column (Pharmacia, Sweden). The specific activity of the streptavidin fraction routinely fell between 85 and 100 μCi/μg. The mid fractions of the protein peak were used in the sandwich immunoassay.

Two-Site Immunometric Assay. Microtiter plates (Immulon II; Dynatech, Chantilly, VA) were coated with purified capture antibody at 5 μg/ml in PBS at pH 7.4 overnight. The plates were washed 3 times with 100 μl of PBS, then blocked with 200 μl of blocking buffer (10% normal goat serum: 1% normal mouse serum: 0.1% bovine γ-globulin) for 1 h at room temperature, and washed 3 times with PBS. The samples, standards (supernatants of dead ME-180 cell cultures calibrated off the first master supernatant standards of known NMP activity), or sera were measured by incubating 100 μl in the well for 2 h at room temperature. After washing on a plate washer 3 times, 5 μg/ml of biotinylated tracer antibody diluted in blocking buffer were added to each well and incubated for 1 h at room temperature. After three more washes, the bound biotinylated antibody was detected by adding 100 μl of the 125I-streptavidin at 0.25 ng/ml and incubating for another hour at room temperature. After washing out unbound 125I-streptavidin, the bound fraction was detected by counting the remaining radioactivity in an LKB gamma counter. The NMP concentration of samples was determined by comparing the counts obtained against the reference standards.

RESULTS

Immunoblot studies of the supernatants of dead tissue culture cells, using monoclonal antibodies to the nuclear matrix proteins, indicate that these proteins survive cell death and are released from dying cells in a soluble form. All anti-NMP antibodies reacted with the supernatant of dying cells and isolated nuclear matrix. The anti-keyhole limpet hemocyanin antibody used as a negative control showed no reactivity with the spent supernatant, confirming the specificity of the antibody reaction (Fig. 1).

Two anti-nuclear matrix monoclonal antibodies were used to develop a two-site immunometric assay. Assay 29-7 consists of an anti-ME-180 nuclear matrix antibody, 107-7, used as the capture and of antibody 302-29, an anti-CaSki antibody, used as the tracer. The supernatant of the dying cultures of the cell line ME-180 was used as the source of the ligand and demonstrated a dose response, an increase in signal as the concentration of dead cell protein increases (Fig. 4). The units of released 29-7 antigen recapitulate the protein concentration of the master batch of dying ME-180 cell supernatant standards. The dose-response result demonstrates that the assay was able to detect and quantify its antigen in the tissue culture supernatant of dying cells.

Spiking studies using two different nuclear matrix preparations isolated by the Penman and Fey method (4) demonstrated a linear response in the assay that uses standards prepared from the supernatants of dying cells (Table 1). Interestingly, the concentration of the antigen in the isolated nuclear matrix preparations is approximately the same as found in the dead cell supernatants (1:1 for the CaSki samples and 2:1 for the ME-180 samples). The degree of parallelism was determined...
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Fig. 4. Dose-response curve of a representative immunoradiometric assay for the 29-7 matrix protein. Standard curve represents antigen released from the cervical adenocarcinoma cell line, ME-180. Antigen dose, shown on abscissa, is defined as matrix antigen units/ml as explained in the text.

Table 1 Parallelism study of two nuclear matrix preparations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Observed dose (units/ml)</th>
<th>Dilution, expected</th>
<th>Recovery (% of observed/expected) units/µg</th>
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<tr>
<td>CaSki NM</td>
<td>100.0</td>
<td>103.0</td>
<td>1.03</td>
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<tr>
<td>50.0</td>
<td>50.0</td>
<td>51.50</td>
<td>97.1</td>
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<tr>
<td>25.0</td>
<td>23.6</td>
<td>25.75</td>
<td>91.7</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>14.4</td>
<td>12.88</td>
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<td>Mean</td>
<td></td>
<td></td>
<td>100.2</td>
<td>1.03</td>
</tr>
<tr>
<td>ME-180 NM</td>
<td>132.0</td>
<td>431.0</td>
<td>3.27</td>
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<tr>
<td>66.0</td>
<td>212.9</td>
<td>215.50</td>
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<tr>
<td>33.0</td>
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<td>107.75</td>
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<tr>
<td>16.5</td>
<td>55.8</td>
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</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>98.1</td>
<td>3.22</td>
</tr>
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Table 2 Detection of two nuclear matrix protein antigens

Supernatants from 15 different dying tumor and normal tissues were run in immunometric assays 29-7 and 22-18. Results are expressed units/g of tissue.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Sample</th>
<th>Concentration (units/g)</th>
<th>29-7 NMP (units/g)</th>
<th>22-18 NMP (units/g)</th>
<th>Recovery (expected)</th>
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<tr>
<td>Breast cancer</td>
<td>256</td>
<td>7,333</td>
<td>8,600</td>
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<tr>
<td>Breast normal</td>
<td>252</td>
<td>2,705</td>
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<tr>
<td>Breast normal</td>
<td>247</td>
<td>500</td>
<td>1,250</td>
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<tr>
<td>Breast normal</td>
<td>264</td>
<td>0</td>
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<tr>
<td>Breast normal</td>
<td>254</td>
<td>1,513</td>
<td>2,789</td>
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<tr>
<td>Liver cancer</td>
<td>999</td>
<td>0</td>
<td>932</td>
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<td>451</td>
<td>131</td>
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<tr>
<td>Lung cancer</td>
<td>121</td>
<td>12,000</td>
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<td>Lung cancer</td>
<td>95</td>
<td>5,357</td>
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<td>Lung normal</td>
<td>107</td>
<td>4,166</td>
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<tr>
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<td>650</td>
<td>1,200</td>
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<td>Metastasized liver</td>
<td>403</td>
<td>300</td>
<td>1,133</td>
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<td>Ovarian cancer</td>
<td>291</td>
<td>10,909</td>
<td>14,454</td>
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<tr>
<td>Ovarian cancer</td>
<td>999</td>
<td>6,517</td>
<td>2,760</td>
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DISCUSSION

Anti-nuclear matrix monoclonal antibodies were used to demonstrate that these proteins are released by dying cell lines and tissues. The amount of released proteins was quantified by two-site immunometric assays. The assays were used to measure the concentration of these ligands in nuclear matrix prepared by the method developed by Fey and Penman (3, 4) and by the method of Stuurman et al. (10). The concentration of the ligand is similar in the supernatants of both dead cells and nuclear extracts.

An additional discovery that nude mice bearing human tumors produced anti-nuclear antibodies specific for the matrix (by Western blot analysis and immunofluorescence, data not shown) demonstrated that some of the proteins of the nuclear matrix survive the death of tumor cells in vivo long enough to induce an immune response. The immune response raised the possibility that nuclear matrix proteins of tumor cells are released at the time of cell death into the circulation.

Tumor markers have been classified into several categories: oncofetal antigens (carcinoembryonic antigen and α-fetoprotein); ectopic hormones (adrenocorticotropic hormone, antidiuretic hormone, parahormone, and calcitonin); placental proteins (human chorionic gonadotropin, human placental lactogen, and Regan isoenzyme of alkaline phosphatase); enzymes (prostatic acid phosphatase and lactic dehydrogenase); and cell surface and cytoplasmic antigens (CA-125, TA-4, and tissue polypeptide antigen) (11). The fact that two immunoassays for nuclear matrix proteins detect levels of circulating nuclear matrix proteins above normal in more than half of the cancer sera indicates that nuclear matrix proteins of tumors are released into the circulation or that nuclear matrix proteins of normal tissues are released in response to invasion by cancer cells. The specificity and sensitivity of the two assays for cancer
signify that the proteins of the nuclear matrix belong to a new, distinct class of monoclonal antibody-defined tumor markers.

Pathologists rely primarily upon well-defined changes observed within the nucleus as morphological markers for the diagnosis of cancer. All of the current serum assays for the clinical diagnosis of cancer are based upon antibodies to antigens which are not responsible for morphological markers used by the pathologists. The nuclear matrix is the nonchromatin structural protein and ribonucleoprotein network that remains in the nucleus after extraction with detergents and high-ionic-strength buffers. Different components of the matrix include lamina proteins defining the surface of the nucleus, hundreds of low abundance nuclear proteins, heterogeneous nuclear RNA, and matrix-associated DNA regions (2, 3, 10–18). It is believed that one of the functions of the matrix is to organize the genome within the nucleus, through the attachment of DNA loop domains to specific regions of the nuclear matrix/chromosome scaffold (17). Studies establishing a functional relationship between DNA replication and nuclear matrix protein structures (17–19) support the premise that proteins associated with cancer-related DNA changes can become clinically useful for specific types of cancer. The organizational and regulatory role of the nuclear matrix in the cell would predict that the nuclear changes observed by the pathologists would correlate to changes in the matrix at the molecular level.

Since nuclear matrix proteins are the insoluble fraction of nuclear extracts of living cells, it was not obvious that they could be used as cancer markers in serum assays. It was not predicted that they would be released from dying cells in a soluble form and that when released they would be capable of surviving in the circulation long enough and at a high enough concentration to be detected. The ability of the two immunoassays that use monoclonal antibodies to human cervical cell line nuclear matrix to objectively quantitate different circulating nuclear matrix proteins demonstrates the potential of producing additional nuclear matrix immunoassays with greater cancer and tissue specificity and sensitivity. In the future, immunoassays that use more appropriate cancer and tissue type antibody-defined tumor markers could be utilized in the evaluation of the cancer patient from the perspective of detecting the possible presence of cancer, monitoring recurrence or monitoring therapies.

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