Epithelial Mucin-1 (MUC1) Expression and MA5 Anti-MUC1 Monoclonal Antibody Targeting in Multiple Myeloma


Introduction

Multiple myeloma (MM) is the second most common hematological cancer in the United States. It is typically incurable, even with myeloablative chemotherapy and stem-cell transplantation. The epithelial mucin-1 (MUC1) glycoprotein is expressed by normal and malignant epithelial cells but has also been shown to be expressed by MM cells. MUC1 is a useful antigenic target in solid tumors for clinical diagnostic and therapeutic monoclonal antibody (mAb)-based approaches. The MA5 mAb, as well as other anti-MUC1 mAbs reactive with the MUC1 variable number tandem repeat domain, exhibited moderate to strong reactivity with both MM cell lines and clinical samples. To explore the biochemical nature and potential of MUC1 as an antigenic target in MM, studies were performed to: (a) compare the mRNA and the MUC1 glycoprotein species between epithelial cancer and MM cell lines; and (b) develop and use a human MM tumor xenograft model system to study the biodistribution of the MA5 mAb. MA5 mAb was strongly reactive with six of eight human MM cell lines by flow cytometry. In seven of eight MM patient samples (bone marrow and/or peripheral blood) reactivity was found in 10–90% of the cells, whereas normal control (n = 5) and leukemia and lymphoma (n = 5) cells showed only 0–6% reactivity. 125I-labeled MA5 whole-cell binding studies showed quantitatively similar amounts of binding between strongly positive MM lines and high-MUC1-expressing breast carcinoma lines. mRNA expression was assessed by Northern blotting and reverse transcription-PCR. MM cell lines were positive by both methods, with strong similarity in the sizes of the mRNAs and cDNAs that were obtained. Finally, biodistribution experiments were carried out with 131I-labeled MA5 versus a nonbinding control 125I-labeled mAb in a s.c. MM xenograft model. Selective MM tumor uptake of the MA5 mAb was demonstrated, with a potential for delivering a tumor radiation absorbed dose of 8540 cGy/mCi of injected dose compared with 3099 cGy/mCi of tumor-absorbed dose delivered by nonspecific antibody.

Abstract

Multiple myeloma (MM) is the second most common hematological cancer in the United States. It is typically incurable, even with myeloablative chemotherapy and stem-cell transplantation. The epithelial mucin-1 (MUC1) glycoprotein is expressed by normal and malignant epithelial cells but has also been shown to be expressed by MM cells. MUC1 is a useful antigenic target in solid tumors for clinical diagnostic and therapeutic monoclonal antibody (mAb)-based approaches. The MA5 mAb, as well as other anti-MUC1 mAbs reactive with the MUC1 variable number tandem repeat domain, exhibited moderate to strong reactivity with both MM cell lines and clinical samples. To explore the biochemical nature and potential of MUC1 as an antigenic target in MM, studies were performed to: (a) compare the mRNA and the MUC1 glycoprotein species between epithelial cancer and MM cell lines; and (b) develop and use a human MM tumor xenograft model system to study the biodistribution of the MA5 mAb. MA5 mAb was strongly reactive with six of eight human MM cell lines by flow cytometry. In seven of eight MM patient samples (bone marrow and/or peripheral blood) reactivity was found in 10–90% of the cells, whereas normal control (n = 5) and leukemia and lymphoma (n = 5) cells showed only 0–6% reactivity. 125I-labeled MA5 whole-cell binding studies showed quantitatively similar amounts of binding between strongly positive MM lines and high-MUC1-expressing breast carcinoma lines. mRNA expression was assessed by Northern blotting and reverse transcription-PCR. MM cell lines were positive by both methods, with strong similarity in the sizes of the mRNAs and cDNAs that were obtained. Finally, biodistribution experiments were carried out with 131I-labeled MA5 versus a nonbinding control 125I-labeled mAb in a s.c. MM xenograft model. Selective MM tumor uptake of the MA5 mAb was demonstrated, with a potential for delivering a tumor radiation absorbed dose of 8540 cGy/mCi of injected dose compared with 3099 cGy/mCi of tumor-absorbed dose delivered by nonspecific antibody.

Introduction

MM is a B-cell malignancy that appears to result from the transformation and monoclonal expansion of a cell with characteristics of a plasma cell, i.e., a terminally differentiated B cell (1, 2). The expression by MM cells of certain non-B-cell antigens also raises the possibility of the transformation of an earlier, more multipotent lymphoid precursor cell. As with normal plasma cells, this degree of terminal differentiation is associated with the complete or partial loss of certain B-cell-associated antigens, such as surface immunoglobulin and CD8 (19–22). MUC1 is of particular interest because it is a diagnostic marker that exists as an integral membrane glycoprotein and as shed forms and because it is a therapeutic target for both cell-mediated and antibody-based immunotherapeutic strategies. Although no function has yet been determined for membrane-bound MUC1, a receptor/ligand-binding property has been associated with neoplastic progression and cellular adhesion (12). Although most of the work to date with this target antigen has been in breast and pancreatic cancers, a few reports have shown that MUC1 can be detected in the serum of MM patients and, thus, may also represent a surrogate tumor marker in MM (10).

Progress in the therapy of MM has been achieved over the past 25 years with the introduction of chemotherapy regimens, resulting in objective antitumor effects (1, 2, 13–15). Some additional therapeutic benefit has resulted from the more recent application of high-dose chemotherapy with autologous or allogeneic stem-cell rescue (14, 15). Nonetheless, MM remains fairly resistant to all of these approaches in the majority of cases. Long-term survival, in fact, has not improved much since the advent of chemotherapy with phenylalanine mustard (Melphalan) and glucocorticoids. Drug resistance mechanisms via multidrug resistance protein 1 and lung resistance-related protein have been described in MM and appear to be major mechanisms of resistance to therapy (16–18). Multidrug resistance protein 1 expression may be intrinsic to both malignant and nonmalignant
plasma cells because cell surface expression of this transporter protein was found in 42 of 43 samples, which included 19 patients with either monoclonal gammopathy of undetermined significance or amyloidosis (17). Lung resistance-related protein expression was shown to be an important factor in determining response to standard-dose Melphalan-prednisone (18). The expression levels of the antiapoptotic protein BCL-XL has also been shown to play a role in determining chemoresistance, with increased levels leading to relative drug resistance (19). BCL-2 may also play a role in chemoresistance in MM (20). Other factors, such as mutations in the ras oncogene or the p53 gene, are not common enough to play a major role in MM pathophysiology (21, 22). The tendency toward chemoresistance, along with the relative clinical radiosensitivity of MM (23–25), suggests that MM would be a favorable target for RAIT. The predominant involvement of the red marrow in MM lends further support to RAIT approaches because mAb uptake is both rapid and high in the bone marrow compartment. Indeed, preclinical experiments in immunotherapy and radioimmunotargeting in a MM model system have shown potential for these mAb-mediated approaches (26, 27). In addition, another approach has been developed to selectively deliver radiation doses to the red marrow in MM through the use of a bone-seeking mAb-mediated approaches (28).

Given the above background, we sought in the studies reported herein to address selected questions regarding MUC1 expression in MM that had not been fully addressed previously. These questions are: (a) What mRNA species are expressed by MM cell lines? (b) What is the general sequence structure of MUC1 cDNA in MM? (c) Is MUC1 a target antigen that can be effectively used in preclinical MM tumor xenograft biodistribution studies?

Materials and Methods

Cell Lines and Clinical Specimens. The MM cell lines RPMI 8226, U266, and MC/CAR were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). The MM cell line, JIN-3, was kindly provided by Dr. M. Kuehl, Navy-NCI Oncology Branch, Bethesda, MD. The KMS12-BM and KMS12-PE cell lines were generously provided by Dr. T. Otsuki, Kawasaki Medical School, Okayama, Japan, and the DUL4 cell line was kindly provided by the technical services department of Irvine Scientific (Santa Ana, CA). The MUC1 breast carcinoma cell lines, MCF7, MDA-MB468, T47D, and ZR-75-30, were obtained from ATCC for use as controls, as was the pancreatic carcinoma cell line, CaPan-1. The Ramos B-lymphoblast line was used as a control cell line and was also obtained from ATCC. The ovarian carcinoma cell line, 2008, was obtained from Dr. S. Howell (University of California, San Diego, CA). These cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS plus antibiotics, except for the MC/CAR line, for which the medium preparation recommended by ATCC was used. Clinical specimens consisted of either peripheral blood or bone marrow aspirates of patients with active MM and were obtained through St. Joseph’s Hospital, Paterson, NJ, and Dr. A. Lippman (Beth Israel Hospital, Newark, NJ). Mononuclear cells were isolated from bone marrow by density gradient centrifugation (Cell-Sep; Larex, Inc., St. Paul, MN).

RT-PCR and Northern Blotting. The starting template material for RT-PCR was total RNA, which was isolated from PBS-washed cells that were solubilized with a guanidine isothiocyanate-based buffer (Tri-Reagent; Sigma-Aldrich, St. Louis, MO). RNA was isolated according to a modification of the method of Chomczynski and Mackey (29). Total RNA (5 μg) was used as the template for cDNA synthesis, using the First Strand kit of Novagen (Madison, WI) according to manufacturer’s instructions, with 4% of the resulting cDNA product being used as template for each PCR reaction. Primers and dNTPs were added at standard concentrations, 0.5 and 200 μM, respectively. Thermostable DNA polymerase (0.5 μl; KlenTaQ; Ab Peptides, St. Louis, MO) was added to each tube, and 35 cycles of PCR were carried out under the following conditions: annealing temp of 65°C for 45 s, extension at 72°C for 30 s, and denaturation at 94°C for 30 s (initial denaturation was at 94°C for 2 min).

Total RNA (20 μg) from selected cell lines was denatured in a 50% formamide-2.2 M formaldehyde solution, fractionated by electrophoresis through a 0.8% agarose gel containing 0.66 M formaldehyde and transferred onto a nylon membrane (Hybond; Amersham Corp., Chicago, IL) by capillary blotting. RNA was fixed by baking at 80°C under vacuum. After prehybridization at room temperature in a solution containing 5× SSC buffer plus 0.2 M sodium phosphate (pH 6.8), 1× Denhardt’s reagent, 50% formamide, 100 μg/ml sheared salmon sperm DNA, 50 μg/ml yeast tRNA, and 20 μg/ml poly(A)+, the membrane was hybridized with the labeled MUC1 probe at 42°C for 14–16 h. The probe was labeled with [α-32P]dCTP by the nick translation method, according to the manufacturer’s instructions (Amersham). The specific activity of the probe was ~10^9 cpm/μg. This MUC1 probe was a cDNA insert cut from pBS 42TR FMUC1 vector via digestion with EcoRI and BamHI followed by gel purification of the desired fragment. This probe contained 42 VNTR sequences (30). The probe was incubated with the blot at a concentration of 1× 10^6 cpm/ml to 2× 10^6 cpm/ml; the membrane was subsequently washed under stringent conditions prior to exposure to film.

mAbs. The MA5 mAb was a murine IgG1, kindly provided by Immunomedics, Inc. (Morris Plains, NJ). This mAb has been characterized previously and has been studied clinically as a radioantibody imaging agent for detection of breast cancer (31, 32). The other anti-MUC1 mAbs that were used (mostly for flow cytometry) were also murine IgG1s. These were KC-4G3, H23 (obtained from ATCC), DF3-P (kindly provided by Dr. D. Kufe, Dana-Farber Cancer Institute, Boston, MA) and PAM4 mAb (33). The first three mAbs react with epitopes within the VNTR region, whereas PAM4 reacts with an epitope in the N-terminal, extracellular domain of MUC1.

Flow Cytometry. Cell lines were washed and set up at ~0.5 × 10^6 cells/tube and incubated with 10 μg/ml control and test mAbs for 30 min at 4°C in flow buffer (PBS-3% FBS-0.1% NaN3). After washing with the same buffer, the second step reagent, pretitrated F(ab’)2 goat antimouse IgG-fluorescein isothiocyanate (Biotech International, San Diego, CA), was added, and cells were incubated for an additional 30 min at 4°C.
Table 1  MA5 (anti-MUC1) reactivity with cell lines (immunofluorescent flow cytometry).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>MA5 positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI8266</td>
<td>Myeloma</td>
<td>98.3</td>
</tr>
<tr>
<td>ARH77</td>
<td>Myeloma</td>
<td>7.4</td>
</tr>
<tr>
<td>DUL4</td>
<td>Myeloma</td>
<td>4.0</td>
</tr>
<tr>
<td>H929</td>
<td>Myeloma</td>
<td>87.8</td>
</tr>
<tr>
<td>JN-3</td>
<td>Myeloma</td>
<td>97.9</td>
</tr>
<tr>
<td>U266</td>
<td>Myeloma</td>
<td>84.3</td>
</tr>
<tr>
<td>Ramos</td>
<td>B-cell lymphoma</td>
<td>0.5</td>
</tr>
<tr>
<td>Jurkat</td>
<td>T-cell lymphoma</td>
<td>0.8</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast carcinoma</td>
<td>57.4</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast carcinoma</td>
<td>96.6</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Breast carcinoma</td>
<td>98.5</td>
</tr>
</tbody>
</table>

*This value represents the percentage of cells positive for reactivity with MA5 after subtraction of value for binding of nonspecific antibody (Ag8).

Table 2  MA5 (anti-MUC1) reactivity with clinical specimens (immunofluorescent flow cytometry).

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Diagnosis</th>
<th>Specimen type</th>
<th>MA5 positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Myeloma</td>
<td>Bone marrow</td>
<td>25.5</td>
</tr>
<tr>
<td>6</td>
<td>Myeloma</td>
<td>Bone marrow</td>
<td>14.3</td>
</tr>
<tr>
<td>1</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>12.3</td>
</tr>
<tr>
<td>2</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>64.2</td>
</tr>
<tr>
<td>3</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>18.9</td>
</tr>
<tr>
<td>4</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>14.9</td>
</tr>
<tr>
<td>5</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>7.6</td>
</tr>
<tr>
<td>7</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>24.0</td>
</tr>
<tr>
<td>8</td>
<td>Myeloma</td>
<td>Peripheral blood</td>
<td>32.2</td>
</tr>
<tr>
<td>2</td>
<td>Lymphoma/leukemia</td>
<td>Peripheral blood</td>
<td>0.0-6.0</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>Peripheral blood</td>
<td>0.0-0.2</td>
</tr>
</tbody>
</table>

*This value represents the percentage of cells positive for reactivity with MA5 after subtraction of value for binding of nonspecific antibody (Ag8).

Bone marrow mononuclear cells were isolated via density gradient centrifugation.

 Buffy coats were isolated, and erythrocytes were lysed with 10 mM potassium bicarbonate buffer containing 154 mM ammonium chloride and 0.1 mM EDTA.

The cells were then fixed with 1.5% paraformaldehyde in PBS and analyzed on a Becton Dickinson (Mountain View, CA) FACSscan.

Radiotracer Binding to Cell Lines. MA5 mAb was iodinated using Na125I (New England Nuclear, Boston, MA). The 125I was activated with precoated Iodogen tubes (Pierce, Rockford, IL), according to the method of Chizzonite et al. (34). Briefly, 100 µl of 0.2 M sodium phosphate buffer (pH 7.4) was added to a 1 mCi stock vial of Na125I. The entire solution volume was transferred to a precoated Iodogen tube and activated for 7 min at room temperature, after which the Na125I solution was transferred to a 1.5-ml tube containing 20 µg of MA5 mAb in 25 µl of PBS. The iodination reaction was allowed to proceed for 7 min at room temperature, after which bound and free iodine were separated on a PD-10 column (Pharmacia, Piscataway, NJ), which was equilibrated and eluted with PBS containing 0.2% gelatin. Radiolabeled mAb was analyzed by both instant TLC and gel filtration high-performance liquid chromatography, with radioactive and absorbance monitoring (human serum albumin, the carrier protein used, served as internal standard). 125I-labeled MA5 routinely showed <3% unbound iodide and >95% monomeric IgG with a specific activity of 444–925 MBq/mg (12–25 mCi/mg). For cell-binding experiments, 1 × 10⁶ cells/tube in 100 µl of buffer were used. To each tube, either binding buffer (RPMI 1640 supplemented with 10% FBS) or a 150-fold molar excess of unlabeled MA5 was added. Tubes were incubated for 15 min at 4°C, after which the 125I-labeled MA5 was added to a final concentration of 0.75 µg/ml (saturating binding conditions). The binding reaction was carried out for 90 min at 4°C, after which cell-bound label was separated from free label by centrifuging the cells (7000 × g for 75 s) through a 200-µl cushion of 80% dibutyl phthalate-20% olive oil (Sigma-Aldrich, St. Louis, MO) in a 0.4-ml polyethylene tube. The tips of the tubes were excised and counted in a gamma counter.

Western Blotting. After washing with PBS, approximately 10⁷ cells were solubilized with buffer consisting of 0.2% Zwittergent 3-12 in 0.5 M Tris-HCl (pH 8.0) containing 0.15 M NaCl, 2 mM phenylmethylsulfonyl fluoride, and 0.5 mM N-tosyl-phenylalanyl chloromethyl ketone. After solubilization at 4°C for 60 min, nuclei and debris were pelleted at 10,000 × g for 5 min. Aliquots of these whole-cell lysates were loaded onto a 4% SDS-PAGE gel and separated. The gel was then electroblotted (24 V for 16 h) onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After the membrane was blocked with blocking/incubation buffer (PBS containing 0.5% BSA and 0.2% Tween-20), MUC1 was detected by incubating...
the membrane successively with 5 μg/ml MA5 mAb in blocking buffer for 1 h at room temperature, followed by three washes with blocking buffer. The blot was then incubated for 1 h with a 1:5000 dilution of antimouse IgG-peroxidase (Jackson Labs, West Grove, PA). The blot was then developed with the SuperSignal system (Pierce), according to the manufacturer’s instructions, and exposed to X-ray film.

**MM Xenograft Model and Biodistribution Studies.**

s.c. tumors were established by two approaches. One of these was to expand the JJN-3 cell line in vitro, wash and resuspend the cells in fresh medium (RPMI 1640 + 10% FBS), and inject the cells s.c. into the flanks of immunodeficient mice (10 × 10^6 cells/0.3 ml) or (15 × 10^6 cells/0.3 ml injected into each mouse). The mouse strains that were used were C.B-17 SCID and Cr:NIH(S) nu/nu. The other approach for establishing s.c. tumors was to excise established JJN-3 tumors (0.6–1 cm^3) and disect viable regions into individual pieces of ~40 mg. These pieces were then implanted s.c. into anesthetized mice of the same strain followed by a skin-closing suture. When tumors derived from either approach reached a size of 0.3–0.6 cm^3, tumor-bearing mice were co-injected with a mixture of 25 μCi of 123I-labeled MA5 and 10 μCi of a nonbinding control mIgG [either P3X63Ag8 (MOPC-21) or anti-α-fetoprotein] labeled with 125I. Groups of five mice were injected and sacrificed at 1, 4, and 7 days post injection, at which time tumors and normal tissues were harvested and counted. mAb uptake values were determined and expressed as the %ID/g. Localization indices were calculated according to the formula: localization index = (uptake of 123I-labeled MA5 in tumor/uptake of 123I-labeled MA5 in blood) + (uptake of control mAb in tumor/uptake of control mAb in blood), with uptake units being %ID/g. Radiation dose estimates were also determined from the biodistribution data. Calculations were performed by first integrating the trapezoidal regions for tumors or the exponential regions for the normal tissues, as defined by the time-activity data. To avoid overestimation of the cumulative radiation absorbed dose to tumor, a zero time value of zero was assumed for this trapezoidal fit method. For normal tissues, the zero time point was extrapolated from the exponential curve. The resulting integral for each organ was converted to cGy/mCi (cGy/MBq) using S values appropriate for the radionuclide and organ weight. These S values were derived by assuming uniformly distributed activity in small, unit-density spheres (35).

**Results**

**Anti-MUC1 VNTR-Region mAbs React with MM Cell Lines and Clinical Specimens.** A panel of cell lines, consisting of both hematopoietic cell lines and breast carcinomas, was analyzed by flow cytometry for reactivity with anti-MUC1 mAbs (Table 1). Six of eight MM cell lines tested demonstrated strong reactivity with the MA5 mAb as well as with other anti-MUC1 mAbs with anti-VNTR specificity, including the DF-3P mAb. The latter mAb is notable for the fact that it was raised against a MUC1 VNTR-peptide immunogen. Testing of clinical samples (bone marrow and/or peripheral blood) from MM patients with active disease showed moderate to strong reactivity with the MA5 mAb in 88% (seven of eight), whereas none of five normal control blood specimens showed reactivity with this antibody (Table 2). In addition, five leukemia and lymphoma patient specimens (bone marrow and/or blood) gave only weak (1–6%) positive cell responses. Representative histograms are shown in Fig. 1. On a semi-quantitative basis, the degree of fluorescence shift (i.e., the mean channel) was similar to that observed with the breast carcinoma cell lines.

**Radiolabeled MA5 Shows Specific Binding to MM Cell Lines.** To confirm the findings from flow cytometry, MM lines as well as other MUC1+ and MUC1− cell lines from the flow cytometry panel were tested for the binding of 123I-labeled MA5 at a saturating mAb concentration. A good correlation was found between the mean fluorescence intensity in flow cytometry and absolute specific radiolabeled antibody binding. The specific 123I-labeled MA5 cpm bound varied between MM lines. Those with the highest levels of binding had levels similar to those observed in high-expressing MUC1+ breast carcinoma cell lines (Fig. 2).
**MUC1 mRNA Is Present As Assessed by Both RT-PCR and Northern Blotting.** Total RNA was isolated from specimens and was then analyzed by both methods. RT-PCR was performed initially with primers designated 262-forward (5'-TTG-AAT-GCT-CAC-AGC-CCC-GGT-TCA-GGC-TCC-3') and 1017-reverse (5'-TTT-GAA-TTC-CTA-TTC-AGA-AAT-GTG-TCT-CTG-3'), designed from GenBank accession no. J05582, human pancreatic mucin mRNA, complete coding sequence. These sequences are outside the VNTR and are far enough 5' and 3', respectively, of the flanking VNTR-degenerate sequences of MUC1 to allow for amplification of one predominant PCR product (Fig. 3). This amplicon was 1.8 kb and was present in all of the MUC1+ breast, ovarian, pancreatic, and MM cell lines tested. Other hematopoietic cell lines with no protein expression by flow cytometry and/or mAb tracer-binding studies were also negative by RT-PCR. In addition, PCR reactions were performed with a primer pair (both 20-mers) spanning the 5' region of MUC1 from the translation initiation codon up to nucleotide 262. These PCR reactions likewise showed the presence of a single amplicon of the correct size with cDNA derived from the same cell lines (data not shown).

A Northern blot was performed using total RNA that was isolated from MM and other selected cell lines. This blot showed bands at ~7, 4.4, and 2.1 kb in both the MM and MUC1+ epithelial cell lines, with no hybridization signal observed in cell lines that were negative by flow cytometry (Fig. 4). The mRNA species that were observed were very similar in size to those described previously for MUC1.

**Western Blot Analysis of MUC1 from MM Revealed Species of Similar Molecular Mass to Those Expressed by Epithelial MUC1+ Cell Lines.** MM and control cell lines were detergent solubilized, and aliquots of the soluble extracts were separated by SDS-PAGE followed by electroblotting onto nitrocellulose membranes for immunodetection with the MA5 mAb (Fig. 5). In all of the breast, ovarian, and MM cell lines tested thus far, a predominant immunoreactive band of ~400 kDa was observed, whereas cell lines that were negative for MUC1 mRNA expression or cell surface protein expression (by flow cytometry) were also negative by this method. The pancreatic carcinoma cell lines tested had strong signals in Northern blotting and RT-PCR, but these cell lines were nonreactive with MA5 in flow cytometry and in Western blot analysis.

**MA5 mAb Showed Selective Uptake into MM Xenografts in Nude Mice.** A s.c. MM model using the JJN-3 cell line growing as a xenograft in the athymic nude mouse was used in these experiments. Tissue uptake of radiolabeled antibody, expressed as %ID/g, is shown in Fig. 6 for day 4, at which time point the maximum tumor uptake of MA5 (11.8% ID/g) was observed. Tumor/nontumor ratios for MA5 were always greater than for the nonspecific control P3×63Ag8 mAb. A tumor localization index of 3.2 was observed for 131I-labeled MA5 at day 4, with nontumor tissue localization indices in the
3070s MA5 Anti-MUC1 mAb Targeting of Multiple Myeloma

Fig. 6 MA5 uptake into JIN-3 (MM) xenografts in nude mice. The %ID/g for 131I-labeled MA5 and 125I-labeled nonspecific control isotype/sub-class-matched Ag8 mAb in JIN-3 tumors and normal organs at 4 days post injection are shown.

Discussion

Consistent with previous reports using other anti-MUC1 mAbs, we showed reactivity of the MA5 anti-MUC1 mAb with MM cell lines and clinical specimens. Because this mAb reacts with the VNTR repeat sequence of MUC1, we considered it formally possible that this sequence may be shared by a related gene that is expressed in MM and possibly other cancer types. We therefore proceeded to characterize both the MUC1 mRNA and glycoprotein species from MM and compared it to the corresponding MUC1 species in carcinomas, such as breast, ovarian, and pancreatic.

In earlier efforts to generate anti-epithelial tumor mAbs using tumor-derived immunogen preparations, MUC1 proved to be very antigenic. Thus, many anti-MUC1 mAbs have been generated. The majority of these mAbs recognize epitopes in the VNTR; Ref. 31). Subsequent cloning and sequencing of MUC1 cDNAs from different cell sources by several groups showed that the protein-coding sequence consists of three general regions, a 5′ upstream region, a central VNTR region, and 3′ downstream region (36–40). Cloning and sequencing of MUC1 from breast and pancreatic sources shows a very high level of sequence identity (>99%; Ref. 40). The VNTR region contributes substantially to the size and the properties of the MUC1 glycoprotein. It is composed of repeating units of 60 nucleotides, with from ~40–90 such units being present in the mature protein. This variation in molecular mass stems mostly from allelic variation in the number of VNTR units. MUC1 alleles are usually expressed codominantly, which results in the presence of two mRNA species of between 4 and 7 kb and two corresponding glycoprotein species with molecular masses of 300 to >1000 kDa. This additional size heterogeneity of the mature glycoprotein species results from heterogeneous glycosylation, mostly of the O-type (41, 42).

Our RT-PCR and Northern blotting results show that there is both sequence and structural relatedness of MUC1 mRNA from MM and MUC1+ epithelial cell lines. Whereas DNA sequence data from the PCR amplicons obtained from MM lines are not yet available, our data show that the resulting PCR fragments resulting from both the 5′ and the trans-VNTR primer pairs were very similar in MM and pancreatic, breast, and ovarian carcinomas. Northern blot analysis showed that the mRNA species of MM and carcinoma cell lines were of very similar molecular weights when probed with a MUC1 VNTR probe. Likewise, Western blot analyses showed similarity of the MUC1 glycoprotein species between MM and epithelial cell lines, using the MA5 mAb as probe. Quantitative estimation of MUC1 expression showed that some of the MM lines showed absolute specific binding of 125I-labeled MA5 of a similar magnitude as high-expressing MUC1+ carcinoma cell lines at saturating MA5 mAb protein concentrations. Mean fluorescence channel values were, likewise, similar between the high MUC1-expressing MM and the carcinoma cell lines. Thus, high-level expression of apparently canonical MUC1 mRNA and glycoprotein species were seen in both MM cell lines as well as clinical specimens.

Several prior reports have shown that MUC1 may be expressed on lymphoid cell populations, particularly MM cell lines (8–11). Another report showed that MM cells and clinical samples react with a mAb, MUSE11, which is VNTR-reactive. It was also shown that MM cells appeared to release MUC1 because levels above the cutoff value for normal controls were observed in 12 of...
25 (48%) MM serum samples tested (10). Circulating MUC1 was compared between patients with MM and breast and pancreatic carcinoma. MM and breast carcinoma showed predominantly one band of ~300 kDa, whereas serum MUC1 from pancreatic carcinoma showed multiple bands of ~300–450 kDa. The latter finding is consistent with the tendency of pancreatic carcinoma-derived MUC1 to have a higher degree of O-glycosylation compared with breast carcinoma (39). Another major finding of this report was that a normal T-lymphocyte bulk cell line could be generated from repeated in vitro stimulation of peripheral blood mononuclear cells from a MM patient with MUC1+ tumor cell lines. This cell line, which exhibited a CD3+/TCR αβ+/CD8+ phenotype, possessed an ability to lyse both MUC1+ MM cell lines as well as one of four breast carcinoma cell lines tested while having no effect on MUC1– cell lines (including the natural killer cell-sensitive K562 cell line). These results are consistent with our findings with respect to the epitope reactivity in breast cancer and MM lines displayed by the anti-MUC1 mAbs used. In addition, our results indicate very similar MUC1 glycoprotein species between breast carcinoma and MM cell lines. Our results extended these findings with respect to comparisons of MUC1 mRNA and RT-PCR species (both trans-VNTR and 5'-region amplicons).

The expression of MUC1 at high levels on a majority of myeloma specimens may provide a new target and approach for therapy, namely RAIT. Radiolabeled antibodies have been used with some success in the detection, diagnosis, staging, and therapy of many different types of malignancies, including MUC1-expressing tumors. However, to the best of our knowledge, this treatment modality has not been explored in MM patients. Our studies have demonstrated the potential for applying radiolabeled MA5 as a therapeutic agent for this disease. At least in this tumor model (JIN-3 in athymic nude mice), 131I-labeled MA5 has demonstrated the potential to provide an effective radiation dose to the tumor. Although a high radiation dose was also shown for the blood, with the current widespread use of autologous or allogeneic stem cells myelotoxicity can be significantly reduced. Hepatotoxicity would be the next major concern; however, the radiation dose potentially delivered by 131I-labeled MA5 to this organ was only 2355 cGy/Mbq; thus, the total liver dose was 706 cGy at the murine maximum tolerated dose) of administered dose, which is well below the hepatotoxic level of ~1500 cGy usually observed in mice (this dose threshold is 3000–3500 cGy in humans). We are currently performing RAIT studies to examine the antibody form and radionuclide (131I versus 90Y) to provide conclusive evidence of a therapeutic potential in MM and the rationale for a clinical study.

References

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Epithelial Mucin-1 (MUC1) Expression and MA5 Anti-MUC1 Monoclonal Antibody Targeting in Multiple Myeloma


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