A Unique Mucin Immunoenhancing Peptide with Antitumor Properties

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ABSTRACT

Implantation of DA-3 mammary tumor cells into BALB/c mice results in tumor growth, metastatic lesions, and death. These cells were transfected with genes encoding for either the transmembrane (DA-3/TM) or secreted (DA-3/sec) form of human mucin 1 (MUC1). Although the gene for the secreted form lacks the transmembrane and cytoplasmic domains, the 5′ sequences of these mucins are identical; however, the gene for the secreted mucin isoform ends with a sequence encoding for a unique 11 amino acid peptide. The DA-3/TM or DA-3 cells transfected with the neomycin vector only (DA-3.neo) have the same in vivo growth characteristics as the parent cell line. In contrast, DA-3/sec cells fail to grow when implanted in immunocompetent BALB/c animals. DA-3/sec cells implanted in nude mice resulted in tumor development verifying the tumorigenic potential of these cells. Pre-exposure of BALB/c mice to DA-3/sec cells afforded protection against challenge with DA-3/TM or DA-3.neo mammary tumors and the unrelated tumors K7, an osteosarcoma, and RENCA, a renal cell carcinoma. Partial protection against subsequent tumor challenges was also achieved by substituting the 11 amino acid peptide found only in the secreted MUC1 isoform, for the live DA-3/sec cells. Notably, the efficacy of this peptide is not strain restricted because it also retarded the growth of Lewis lung carcinoma cells in C57BL/6 mice. These findings reveal that a unique peptide present in the secreted MUC1 has immunoenhancing properties and may be a potential agent for use in immunotherapy.

INTRODUCTION

Mucin 1 (MUC1), a polymorphic type 1 transmembrane protein, which has a large, heavily glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic tail, is found on the apical surface of normal cells lining the lumen of ducts and glands (1, 2), normal epithelium (3), lymphocytes (4, 5), and dendritic cells (6, 7). The extracellular domain polymorphism is derived from the 20 amino acid repeat unit, which can vary from 20 to 100 repeats and has five potential O-linked glycosylation sites per repeat. MUC1 has been selected as a mediator of cell-cell interactions because of its proposed dual role as both an adhesive and antiadhesive molecule (8) and in signaling via the cytoplasmic tail (9, 10).

Breast tumors express very high levels of an altered form of the MUC1 glycoprotein (11) as do several other types of human cancers, including those of the pancreas, uterus, and colon (1), and this hypoglycosylated form of MUC1 is overexpressed on the entire surface of the transformed cells. The alteration of MUC1 in neoplasia makes it an ideal tumor antigen for use both diagnostically and in therapy, and several studies highlight the potential of the MUC1 molecule in the development of tumor-antigen–specific vaccines (12–14).

A secreted isoform of MUC1, MUC1/sec, was initially isolated from an infiltrating ductal breast carcinoma (15) and subsequently also found in patients with ovarian and cervical cancers. MUC1/sec is an alternative splice variant of the MUC1 gene, which includes the first 33 bp of intron 2, followed by a stop codon, and the protein therefore lacks the cytoplasmic and transmembrane domains. However, the extracellular portion of MUC1/sec, like that of MUC1, includes varying numbers of the tandem repeat sequences. Because MUC1/sec lacks both the transmembrane and cytoplasmic domains, it is secreted from the cells. Additionally, MUC1/sec contains an 11 amino acid peptide at its COOH terminus that is not found in other mucins.

To study immune responses against mammary tumors with a defined tumor antigen, we transfected DA-3 cells, a murine mammary tumor cell line developed in our laboratory, with the human transmembrane MUC1 isoform, DA-3/TM, the human secreted form, DA-3/sec, or the neomycin vector alone, DA-3.neo. Both the DA-3/TM and DA-3 neo cells produced viable tumors in intact BALB/c mice. However, the transfected DA-3/sec cells did not produce viable tumors in intact BALB/c animals but did grow in nude BALB/c animals. Furthermore, previous exposure to DA-3/sec cells conferred protection against challenge with the DA-3/TM or DA-3/neo cells and two other nonrelated tumors, a renal carcinoma and an osteosarcoma. The studies presented herein also indicate that the unique 11 amino acid peptide present in the secreted MUC1 isoform appears to be involved in the observed phenomenon and may serve as an immunoenhancing molecule.

MATERIALS AND METHODS

Mice and Tumors. BALB/c mice used in these studies were 8 to 12 weeks of age and bred and used in our animal facility at the University of Miami according to the guidelines of the NIH. BALB/c nu/+ were purchased from Taconic Laboratory (German, NY). The monoclonal DA-3 mammary tumor cell line, derived in our laboratory from the D1-DMBA-3 transplatable mammary adenocarcinoma, and the monoclonal transfecants were maintained as published previously (16). The renal carcinoma (RENSA) cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 50 mmol/L 2-mercaptoethanol. The K-7 osteosarcoma cells were grown in DMEM/high glucose with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 50 mmol/L 2-mercaptoethanol. The K-7 osteosarcoma cells were grown in DMEM/high glucose with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The RENCA and K-7 cell lines were maintained by Dr. Eduardo Sotomayor (Moffit Cancer Center, Tampa, FL). Lewis lung carcinoma cells, obtained from the American Type Culture Collection (Manassas, VA), were maintained per recommended conditions. Tumors were implanted by s.c. injection of 1 × 106 tumor cells, resulting in measurable tumors 7 to 10 days after implantation.

Transfection of DA-3 Cells. Stable transfecants expressing the various MUC1 isoforms were generated by transfecting an expression plasmid (pSV2neo) harboring either MUC1/TM or MUC1/sec DNA into DA-3 mouse mammary tumor cells as described previously (17). Control cells were transfected with the vector containing only the neomycin resistance gene.

Peptide Fragment and Antibody Preparation. The H23 monoclonal antibody (18) was used to detect the tandem repeat sequence common to both MUC1/TM and MUC1/sec. Antibody 1709, which recognizes only the secreted form of MUC1, was prepared by immunizing chickens with the MUC1/sec-specific peptide VSIGLSFPMLP conjugated to malamine-activated keyhole limpet hemocyanin (KLH). Free peptide concentrations and conjugations were maintained as published previously (16).
of at least 150 peptide molecules per KLH molecule were used for immunization. The MUC1/sec-specific peptide was prepared either conjugated to KLH or as a multiple antigenic peptide by Aves Laboratory (Tigand, OR). Synthesis of the scrambled version of the MUC1/sec peptide (VFGPSLMLPSI) in the context of a multiple antigenic peptide was also done by Aves Laboratory.

RNA Analysis. For the reverse transcription-PCR analysis, total RNA was isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). One microgram of total RNA per sample was reverse transcribed with a Primus thermocycler (MWG Biotech, High Point, NC) and the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) with oligo(dT)16 primers, according to the manufacturers’ instructions. The reaction was incubated for 60 minutes at 42°C, followed by inactivation of the murine leukemia virus reverse transcriptase at 99°C for 5 minutes. The cDNA products were subjected to PCR amplification for MUC1, MUC1/sec, and β-actin under the following conditions: 94°C for 10 minutes, followed by 35 cycles of 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 30 seconds, with a final extension of 10 minutes at 72°C. The primers were as follows: human MUC1 and human MUC1/sec sense, 5'-TGCACTAGGCTCAGCTTA-3'; human MUC1 antisense, 5'-GAAATGGCACATCATCAAGC-3'; human MUC1/sec antisense, 5'-GGAAGGAAAGGCGCATAC-3'; murine β-actin sense, 5'-TCGCCAGCACACCCTCATC-3', and murine β-actin antisense, 5'-GAGGAGGCCGTTGAAGGATG-3'. Fifteen microliters of the amplified DNA fragments were electrophoresed on a 1.6% agarose gel stained with ethidium bromide and visualized by UV transillumination.

Western Blot Analysis. Equal amounts of tumor cell supernatants were separated on 6% SDS polyacrylamide gels under reducing conditions and then transferred onto 0.45-μm pore size Protran nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) with a Trans-Blot electrophoretic blotting apparatus (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in 1× TBS-0.1% Tween 20, followed by 1 hour of incubation at room temperature with antibody against the tandem repeat portion of human MUC1. Blots were washed for 30 minutes with three changes of 1× TBS-0.1% Tween 20 solution, followed by 1 hour of incubation at room temperature with the horseradish peroxidase-conjugated antirabbit IgG antibody (Chemicon International, Temecula, CA). Blots were washed again for 30 minutes and incubated for 5 minutes with Supersignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). The results were visualized by exposing blots to BioMax autoradiographic film (Kodak, Rochester, NY).

Cytotoxicity Assays. Unseparated splenic cells used in the natural killer cell assay were prepared as described previously (19). Effector cells at a concentration of 25 × 10³/mL were added to microtiter plate wells in volumes ranging from 25 to 150 μL, depending on the final effector to target ratio desired. Twenty-five microliters of 5¹⁰⁶-labeled YAC-1 target cells at 1 × 10⁶ cells/mL were added to all wells so that the final volume in each well was 200 μL. After incubation for 4 hours at 37°C in 5% CO₂, 100 μL of medium were removed from each well and counted on a Packard MultiPrias 2 gamma counter. Percent cytotoxicity was calculated according to the following equation: 5¹⁰⁶(Total counts-Counts of target cell control)/Total counts, when the 0% control represented the amount of 5¹⁰⁶ released in cultures of CACACCTTCTAC-3

RESULTS

Expression of MUC1/sec Prevents Tumor Growth in DA-3 Cells. After transfection, reverse transcription-PCR was used to confirm the presence of MUC1 on the cells. Fig. 1A shows that DA-3/sec cells contained a band corresponding to the human form of MUC1/sec (Fig. 1A, Lane 2), and DA-3/TM cells express the MUC1-specific sequences (Fig. 1A, Lane 3). Untransfected DA-3 cells do not express any MUC1-related sequences (Fig. 1A, Lanes 4 and 5). Actin controls for each cell type are shown in Fig. 1A, Lanes 6 to 8. In additional studies, the DA-3/neo, DA-3/TM, and DA-3/sec, cells, as well as the parent cell line, were grown in vitro, and after 2 days, the culture supernatants were tested for the presence of MUC1 by Western blot analyses. As seen in Fig. 1B, with the H23 antibody specific for the human MUC1 tandem repeat, only the DA-3/sec cells secreted this mucin (Fig. 1B, Lane 5).

All four DA-3 cell lines were used for in vivo experiments to determine tumor incidence and time of appearance in BALB/c mice. Implantation of DA-3, DA-3/neo, and DA-3/TM mammmary tumor cells into mice gave rise to palpable tumors of approximately equal size by 7 days, and by 15 days, essentially all animals had measurable tumors (Table 1). In sharp contrast, the DA-3 cells transfected with the secreted isoform of MUC1, DA-3/sec failed to produce viable tumors. Remarkably, these animals remained tumor free > 12 months after implantation, whereas animals with the other three DA-3 cell types failed to survive past 100 days (Fig. 1C). In other studies, mice implanted with 1, 2, or 10 × 10⁶ cells DA-3/sec cells showed no tumor growth even at the highest tumor cell concentration. Inoculation of 20 × 10⁶ DA-3/sec cells resulted in initial tumors at 3 days, which began to regress at day 7, and completely disappeared by day 10 (data not shown).

One explanation for these results was that the transfection process selectively impaired the basic growth potential of the DA-3/sec cells. However, the in vitro growth kinetics of the four DA-3 tumor cell types were similar, indicating that the transfection process did not alter their growth potential (Fig. 1D).

Immunocompromised Mice Support the Growth of DA-3/sec Cells. To determine whether the DA-3/sec cells had lost in vivo tumorigenic potential, the four DA-3 cell lines were implanted in BALB/c mice after in vivo treatment with a standard 4-hour 5¹⁰⁶ Cr release assay (21).

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HeLa, and Lanes 8: C/H11003. A total of 10^6. At 24, 48, 72, and 96 hours after initial seeding, the appropriate wells were trypsinized and counted using trypan blue to exclude dying cells. DA-3 cells were transfected with plasmids encoding either the full-length MUC1 gene (DA-3/TM) or the MUC1/sec gene (DA-3/sec). A. Subsequent to antibiotic selection, RNA was isolated to generate cDNA, and PCR was done for the presence of either MUC1 or MUC1/sec message. Lanes 1: molecular weight marker; Lane 2: DA-3/sec cDNA amplified with MUC1/sec primers; Lane 3: DA-3/TM cDNA amplified with MUC1 primers; Lane 4: DA-3 cDNA amplified with MUC1/sec primers; Lane 5: DA-3 cDNA amplified with MUC1 primers; Lanes 6–8: DA-3/sec, DA-3/TM, and DA-3 cDNA amplified with β-actin primers. B. One hundred micrograms of total protein from supernatants from tumor cell lines grown 3 days in culture were analyzed by Western blot analysis with the H23 antibody specific for MUC1 tandem repeats. Only DA-3/sec cell supernatant is positive for H23 staining, consistent with MUC1/sec secretion. Lanes 1: HeLa, Lanes 2: CELL2, Lanes 3: DA-3, Lanes 4: DA-3/TM, Lanes 5: DA-3/sec, 6: DA-3/neo, Lanes 7: HeLa, and Lanes 8: DA-3/sec. Lanes 7 and 8 incubated with secondary antibodies only. C. A total of 1 × 10^6 DA-3, DA-3/neo, DA-3/TM, or DA-3/sec cells was s.c. injected into 8- to 12-week old BALB/c mice to assess their ability to form tumors in vivo. Animals were monitored for survival over a several month period. Mice receiving DA-3/sec cells remained healthy over the course of the entire experiment. D. In vitro growth kinetics of DA-3 cells and transfectants. Cells were seeded in 6-well tissue culture plates at an initial density of 2 × 10^4. At 24, 48, 72, and 96 hours after initial seeding, the appropriate wells were trypsinized and counted using trypan blue to exclude dying cells. DA-3 cells transfected with MUC1/sec have faster in vitro growth kinetics than the other cell types.

BALB/c nu/nu mice, and tumor incidence and size were recorded. Fig. 2A shows that the DA-3, DA-3/neo, and DA-3/TM cells caused tumors by 7 days after implantation in nude BALB/c mice. In contrast to the results shown for intact BALB/c in Table 1 and Fig. 1C, 30% of the nude mice that received DA-3/sec tumor cells developed tumors by 14 days after inoculation, and by day 25, all of the nude animals had tumors (Fig. 2A). Thus, the failure of DA-3/sec cells to produce tumors in the intact BALB/c mice appears to be immunologically controlled because implantation of this tumor in nude mice did result in 100% tumor development, albeit with altered kinetics. To confirm these findings, two additional DA-3/sec cell transfectants were implanted into both nude and intact BALB/c animals. Although none of the DA-3/sec transfectants grew in the immunocompetent BALB/c mice, all three, i.e., DA-3/sec, DA-3/sec11, and DA-3/sec22, grew in BALB/c nude mice, although the growth kinetics of the three cell lines were somewhat different (Fig. 2B). Interestingly, implantation of cells from DA-3/sec tumors that grew in nude BALB/c animals did not result in tumors in immunologically intact BALB/c mice but did grow when re-inoculated into other nude BALB/c animals (data not shown).

Antitumor Immune Cell Effector Functions. Natural killer (NK) cells are known to exert cytotoxic activity against tumor targets. We evaluated whether unseparated splenocytes from mice implanted with transfectants of the different MUC1 isoform have innate cytotoxic activity against the NK target cell YAC-1. Fig. 3A shows that splenocytes from BALB/c mice implanted with DA-3/sec cells 3 or 10 days before the assay had a higher level of NK-type cytotoxic activity than those from either control mice or those implanted with DA-3/TM cells.

In additional studies, we looked for the presence of specific cytotoxic T cells in mice implanted with either DA-3/TM or DA-3/sec cells. Splenic cells were expanded in vitro in the presence of either

![A UNIQUE MUC1/SEC-DERIVED IMMUNOENHANCING PEPTIDE](https://example.com/image.png)

Fig. 1. MUC1/sec-transfected cells remain viable yet fail to grow in vivo. DA-3 cells were transfected with plasmids encoding either the full-length MUC1 gene (DA-3/TM) or the MUC1/sec gene (DA-3/sec). A. Subsequent to antibiotic selection, RNA was isolated to generate cDNA, and PCR was done for the presence of either MUC1 or MUC1/sec message. Lanes 1: molecular weight marker; Lane 2: DA-3/sec cDNA amplified with MUC1/sec primers; Lane 3: DA-3/TM cDNA amplified with MUC1 primers; Lane 4: DA-3 cDNA amplified with MUC1/sec primers; Lane 5: DA-3 cDNA amplified with MUC1 primers; Lanes 6–8: DA-3/sec, DA-3/TM, and DA-3 cDNA amplified with β-actin primers. B. One hundred micrograms of total protein from supernatants from tumor cell lines grown 3 days in culture were analyzed by Western blot analysis with the H23 antibody specific for MUC1 tandem repeats. Only DA-3/sec cell supernatant is positive for H23 staining, consistent with MUC1/sec secretion. Lanes 1: HeLa, Lanes 2: CELL2, Lanes 3: DA-3, Lanes 4: DA-3/TM, Lanes 5: DA-3/sec, 6: DA-3/neo, Lanes 7: HeLa, and Lanes 8: DA-3/sec. Lanes 7 and 8 incubated with secondary antibodies only. C. A total of 1 × 10^6 DA-3, DA-3/neo, DA-3/TM, or DA-3/sec cells was s.c. injected into 8- to 12-week old BALB/c mice to assess their ability to form tumors in vivo. Animals were monitored for survival over a several month period. Mice receiving DA-3/sec cells remained healthy over the course of the entire experiment. D. In vitro growth kinetics of DA-3 cells and transfectants. Cells were seeded in 6-well tissue culture plates at an initial density of 2 × 10^4. At 24, 48, 72, and 96 hours after initial seeding, the appropriate wells were trypsinized and counted using trypan blue to exclude dying cells. DA-3 cells transfected with MUC1/sec have faster in vitro growth kinetics than the other cell types.

Table 1 Incidence and time of appearance of DA-3, DA-3/neo, DA-3/TM or DA-3/sec tumors in BALB/c mice

<table>
<thead>
<tr>
<th>Tumor cell inoculum*</th>
<th>Day of tumor appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>DA-3</td>
<td>84/100</td>
</tr>
<tr>
<td>DA-3/neo</td>
<td>68/100</td>
</tr>
<tr>
<td>DA-3/TM</td>
<td>81/100</td>
</tr>
<tr>
<td>DA-3/sec</td>
<td>0/100</td>
</tr>
</tbody>
</table>

* 10^6 cells of each type of tumor were injected s.c. in BALB/c mice.
DA-3/TM or DA-3/sec cell stimulators and tested against DA-3–transfected cell targets. The data presented in Table 2 show that splenocytes from mice that received injections of DA-3/sec cells and stimulated in vitro with DA-3/sec cells were capable of efficiently lysing DA-3/sec target cells. Additionally, splenocytes from animals implanted with DA-3/sec and DA-3/TM cells mixed in a 1:1 ratio and stimulated in vitro with DA-3/sec cells were also capable of lysing DA-3/sec targets. In contrast, the same effector cells with the same in vitro treatment did not lyse DA-3/TM target cells. Furthermore, splenic cells from mice injected with the mixed DA-3/sec:DA-3/TM cells did not lyse either DA-3/TM or DA-3/sec targets after in vitro activation with DA-3/TM tumor cells. These results suggest that antitumor activity is induced by DA-3/sec cells but not DA-3/TM cells.

In other studies, splenic CD4+ and CD8+ T cells were isolated from mice that received injections 2 weeks prior of DA-3/sec cells. These T-cell subsets, which were over 90% pure, were activated in vitro for 5 days with mitomycin C-treated DA-3/sec cells. Fig. 3B shows that CD4+ T cells had no cytotoxic activity against the DA-3/sec cells. In contrast, CD8+ T cells had higher cytotoxic levels against these targets than the unseparated T cells. These results point to CD8+ T cells as the major effectors of DA-3/sec mediated lysis.

Prior Exposure of BALB/c Mice to DA-3/sec Cells Confers Protection against Several Tumor Types. We evaluated whether previous exposure of BALB/c mice to DA-3/sec cells conferred protection against the MUC1-expressing tumor DA-3/TM or tumor cells containing the selection vector only, DA-3/neo. Before challenge, experimental groups of six animals were inoculated s.c. twice with 1 x 10^6 DA-3/sec cells 2 weeks apart. The challenge consisted of either 1 x 10^6 DA-3/TM or DA-3/neo cells mixed with 1 x 10^6 DA-3/sec cells. Six control animals received the tumor challenge alone without previous exposure to DA-3/sec cells.

By 2 weeks after implantation, all of the animals in the control groups had tumors. Importantly, however, the animals that received DA-3/TM tumor cells mixed with DA-3/sec cells after prior exposure to DA-3/sec cells (Fig. 4A, left panel) showed a delay in the time of tumor appearance as compared with the control group, and a substantial number of the animals failed to develop any tumors. Furthermore, this protection did not appear to be due to a recognition of the MUC1 molecule because a similar effect was seen in animals exposed to DA-3/sec cells and challenged with a mixture of DA-3/neo and DA-3/sec mammary tumor cells (Fig. 4A, right panel).

A series of studies were done to evaluate whether pre-exposure to DA-3/sec cells also conferred protection against the growth of tumors other than those of the DA-3 mammary tumor background. The renal cell carcinoma, RENCA, and the K7 osteosarcoma, both syngeneic to BALB/c mice, were used. The BALB/c mice pre-exposed twice, 2 weeks apart, to the DA-3/sec cells, followed by a challenge with a mixture of RENCA cells and DA-3/sec cells showed a 40% reduction in the number of animals developing tumors as compared with the untreated control mice receiving the same inoculation (Fig. 4B, left panel). As was the case with DA-3/TM and DA-3/neo cells, the growth of RENCA cells was not impaired unless they were mixed with the DA-3/sec cells at the time of challenge, even if the animals had been previously exposed to DA-3/sec cells. Similar results were obtained in experiments with the K7 cells (Fig. 4B, right panel), where 60% of the animals failed to develop tumors.

In another experiment, animals that were pre-exposed twice to DA-3/sec cells and then challenged with 1 x 10^6 DA-3/TM cells but not DA-3/sec cells showed only a short time delay in time of tumor appearance, i.e., 13 of 22 animals had palpable tumors at 2 weeks, whereas, in the control group of untreated animals, inoculated with 1 x 10^6 DA-3/TM cells, 13 of 14 had tumors at 1 week. However, we found that implantation of a lower number of DA-3/TM cells (5 x 10^3) mixed with 10^6 DA-3/sec cells appeared to afford protection against growth of the tumor even when the mice had not been pre-exposed to DA-3/sec cells (Fig. 4C). These results indicate that mixing of challenge with DA-3/sec is important for overall protection against tumor formation and that pre-exposure of mice to DA-3/sec

**Table 2.** Cytotoxic activity of splenic cells from mice implanted with DA-3/TM, DA-3/sec, or both against tumor targets after in vitro stimulation

<table>
<thead>
<tr>
<th>Effector cells*</th>
<th>Stimulator cells†</th>
<th>% cytotoxicity against tumor targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:1</td>
<td>10:1</td>
<td>5:1</td>
</tr>
<tr>
<td>DA-3/TM</td>
<td>DA-3/TM</td>
<td>2.80</td>
</tr>
<tr>
<td>DA-3/TM</td>
<td>DA-3/sec</td>
<td>1.70</td>
</tr>
<tr>
<td>DA-3/TM + DA-3/sec</td>
<td>DA-3/TM</td>
<td>2.40</td>
</tr>
<tr>
<td>DA-3/TM + DA-3/sec</td>
<td>DA-3/sec</td>
<td>1.90</td>
</tr>
<tr>
<td>DA-3/sec</td>
<td>DA-3/sec</td>
<td>1.50</td>
</tr>
</tbody>
</table>

NOTE. Error in all cases was <2% of the value shown.

* Splenic cells from animals implanted 2 weeks prior with 1 x 10^6 of the tumor cell listed.
† Mitomycin C-treated tumor cells.
‡ E:T ratio in cytotoxicity assay done as detailed in Materials and Methods.
§ Representative average value of triplicate cultures from one of three experiments.
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D.A. background. A total of 10^6 K7 osteosarcoma or RENCA cells was injected into weeks after the third injection, mice were challenged with 10^6 of either DA-3/TM or RENCA cells mixed with 10^6 DA-3/sec cells before injection. RENCA-challenged mice, treated (P<0.003). Protective effect of DA-3/sec cells is not limited to cells of the same strain. Fig. 5B, right panel, when the peptide-KLH developed tumors (Fig. 5B). In both experiments, 100% of the control mice receiving no prior exposure to peptide-KLH developed tumors within 2 weeks (Fig. 5B). Importantly, in the peptide-KLH-protected animals, the survivors showed no signs of tumor 6 months after challenge with either DA-3/TM or DA-3/neo cells. KLH alone had no effect on tumor growth because 100% of the mice that received a challenge of 10^6 DA-3/TM or DA-3/neo cells formed tumors in the presence or absence of KLH.

These results were not due to a toxic effect of the peptide, which we have named immunoenhancing peptide, as direct addition of this molecule to cultured DA-3, DA-3/TM, DA-3/neo, or DA-3/neo tumor cells did not affect their in vitro growth (Table 3). Furthermore, culturing the cells with peptide for 4 days before implantation did not affect their potential to produce tumors in BALB/c mice. Tumor cells treated in this manner grew with the same in vivo kinetics as cells that had never been exposed to this molecule. In additional studies, we evaluated the in vivo protective effect of peptide pretreatment against two other tumors. Fig. 5C, left panel, shows that pre-exposure to immunoenhancing peptide-KLH afforded only minimal protection against challenge of RENCA cells mixed with peptide-KLH in BALB/c mice. However, as seen in Fig. 5C, right panel, when the immunoenhancing peptide-KLH was used twice as pretreatment in another mouse strain, C57BL/6, and the animals were subsequently challenged with Lewis lung carcinoma cells mixed with peptide, 50% of the animals were protected against tumor development. These results suggest that the effect of MUC1/3/sec-derived peptide is not limited to one particular strain of mouse.

In addition to the peptide-KLH conjugation, we synthesized immunoenhancing peptide as a multiple antigenic peptide (multiplic antigenic peptide). Either immunoenhancing peptide or a scrambled sequence was conjugated to a branched lysine core with four peptides per molecule. The multiple antigenic peptide configuration has several advantages over a KLH conjugation, including higher immunogenicity, smaller volumes of peptide to inject, and, importantly, we have found that no adjuvant is required.

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**Fig. 4.** DA-3/sec cells promote tumor immunity against multiple tumor cell types. A. Mice previously exposed to DA-3/sec cells can reject a challenge of DA-3/3TM or DA-3/neo cells mixed with DA-3/sec cells. BALB/c mice received two s.c. injections of 10^6 DA-3/sec cells 2 weeks apart with no tumors appearing after each inoculation. Two weeks after the third injection, mice were challenged with 10^6 of either DA-3/3TM or DA-3/neo cells mixed with 10^6 DA-3/sec cells (treated). Controls consist of naive mice injected with challenge. Percentages of mice developing tumor over time represent the results of two independent experiments of six mice per group. DA-3/3TM-challenged mice, controls versus treated (P<0.003); DA-3/neo-challenged mice, controls versus treated (P=0.003). B. Protective effect of DA-3/sec cells is not limited to cells of the same strain. A total of 10^6 K7 osteosarcoma or RENCA cells was injected into BALB/c mice that previously received two injections of DA-3/sec cells. Both naive (control) and DA-3/sec-injected (treated) mice were challenged with either 10^6 K7 or RENCA cells mixed with 10^6 DA-3/sec cells before injection. RENCA-challenged mice, controls versus treated (P=0.062). K7 osteosarcoma-challenged mice, controls versus treated (P=0.013). C. Effective antitumor activity can be detected without previous exposure to DA-3/sec cells when a lower number of DA-3/3TM cells is mixed with DA-3/sec cells at the time of challenge. Results represent data from two experiments with five animals per group. Mice receiving the mixture of DA-3/3TM and DA-3/sec were protected compared with DA-3/3TM only controls (P<0.0005).
BALB/c mice were pre-exposed to either the multiple antigenic peptide-immunoenhancing peptide or multiple antigenic peptide-scrambled as described in the Materials and Methods. Subsequently the mice pre-exposed to the corresponding peptide were challenged with DA-3/TM or DA-3.neo (Fig. 5D) tumor cells mixed with either multiple antigenic peptide-immunoenhancing peptide or multiple antigenic peptide-scrambled. Interestingly, the multiple antigenic peptide-immunoenhancing peptide successfully protected 50% of mice challenged with either DA-3/TM or DA-3.neo. In contrast, the multiple antigenic peptide-scrambled form failed to elicit any protection against tumor growth. These results suggest that immunoenhancing peptide can be used to delay and/or prevent tumor cell growth in vivo.

DISCUSSION

The present study identifies an 11 amino acid peptide derived from the COOH terminus of MUC1/sec as an immunostimulatory molecule capable of preventing tumor formation in murine models of cancer. The failure of DA-3 mammary adenocarcinoma cells transfected with MUC1/sec to grow in vivo led us to additionally explore this protein. MUC1/sec, an alternative splice variant of MUC1, is secreted because it lacks both the transmembrane and cytoplasmic tail of MUC1, and the 3’-end of the MUC1/sec transcript encodes for a unique 11 amino acid peptide. The fact that DA-3 cells transfected with full-length MUC1 grow with similar kinetics as parental tumors in vivo suggests that unique properties of MUC1/sec contribute to its inability to establish tumors in vivo.

Both humoral and adaptive immune responses against MUC1 have been detected in various model tumor systems, as well as in cancer patients (25). MUC1, which is expressed by a variety of epithelial tissues, is highly glycosylated on normal cells masking the antigenic epitopes of the core protein. In contrast, in cancer cells of various origins, MUC1 is overexpressed and hypoglycosylated. This allows the immune system to recognize the normally masked antigenic mucin sites on tumor cells without major targeting of normal cells.

Earlier studies by Finn et al. (26) with lymphocytes from pancreatic patients showed that MUC1 is recognized by both CD4+ and CD8+ human T cells as a cell surface molecule directly through the T-cell receptor but independent of MHC class I or class II molecules. Similar results were later observed by these investigators in other tumor types (27). Thus, cytotoxic T cells from malignant ovarian tumors were found to recognize polymorphic epithelial MUC1 core peptides (28).

Table 3. Effect of the MUC1/sec unique peptide (IEP) on in vitro tumor growth

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Media only</th>
<th>+ IEP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA-3</td>
<td>3.7 ± 0.08</td>
<td>3.5 ± 0.08</td>
</tr>
<tr>
<td>DA-3/neo</td>
<td>3.9 ± 0.33</td>
<td>4.0 ± 0.22</td>
</tr>
<tr>
<td>DA-3/TM</td>
<td>3.1 ± 0.40</td>
<td>3.0 ± 0.31</td>
</tr>
<tr>
<td>DA-3/sec</td>
<td>2.9 ± 0.18</td>
<td>3.1 ± 0.28</td>
</tr>
</tbody>
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* 50 μg/well.
† Total cells after 4 days in culture.
‡ Results expressed as ×10^6 cells + SD.

Abbreviation: IEP, immunoenhancing peptide.
Interestingly, cytotoxic T cells in mice immunized with human MUC1 are MHC restricted (27).

In our system, NK and CD8 T cells may be playing an in vivo role in DA-3/sec tumor cell lysis. Uninfused splenocytes from BALB/c mice implanted with DA-3/sec tumor cells have higher NK activity against YAC-1 targets than normal BALB/c mice, and this increased cytolytic activity is enhanced upon longer in vivo exposure to DA-3/sec cells. NK cells are classic antitumor effector cells and can be activated through the ligation of multiple surface receptors and through cytokine stimulation (29). Exposure to DA-3/sec cells may induce activation of NK cells through direct cell-cell interactions, or, alternatively, indirect activation may occur by stimulating cytokine secretion by antigen-presenting cells. The ability of DA-3/sec-primed CD8 T cells to lyse DA-3/sec tumor cells in vitro was also showed (Table 2). In contrast, DA-3/TM-primed effector cells could not lyse DA-3/TM targets, suggesting that the DA-3/sec tumor cells may be more immunogenic or have an increased susceptibility to lysis than parental or MUC1-transfected DA-3 cells. T-Cell immunity is thought to be a major driving force in the rejection of tumor cells (25). Indeed, many tumor vaccination models, including those aimed at using the MUC1 antigen, attempt to specifically invoke Th1 immunity in the hopes of generating tumor-specific T cells (12, 30, 31). It is clear that DA-3/sec cells elicit T-cell immunity; however, what role the MUC1/sec-derived peptide plays in the generation of Th1 immunity is still unclear.

Several immunostimulatory molecules and adjuvants can inherently induce Th1 immunity. For example, bacterial oligodeoxynucleotides containing specific motifs centered on a CpG dinucleotide (CpG-ODN) have been routinely used to induce CTL effectors (32, 33). Several mechanisms are induced upon CpG-ODN stimulation, including local cytokine and chemokine expression that result in activation of components of the innate immune system such as NK cells. CpG-ODN stimulates a nonspecific antitumor response by binding Toll-like receptor 9, resulting in the activation of antigen-presenting cells through enhanced processing of antigen and secretion of cytokines (34, 35). A recent study, however, has revealed repeated injections of CpG in mice result in multifocal liver necrosis and immunosuppression (36). Cytokines, such as granulocyte macrophage colony-stimulating factor, have also been used as immunostimulatory agents to stimulate antitumor immunity (37). As with CpG-ODN and many cytokines, the MUC1/sec-derived peptide, immunoenhancing peptide, clearly enhances antitumor activity. Although the precise mechanism of immunoenhancing peptide is currently unclear, preliminary data suggest that neither immunoenhancing peptide nor DA-3/sec tumor cells do not stimulate the functional maturation of bone marrow-derived dendritic cells in vitro, nor do they stimulate proinflammatory cytokine stimulation from dendritic cells,3 in contrast to CpG-ODN and cytokine adjuvants. However, mice inoculated with DA-3/sec cells have increased NK activity, and currently, we are investigating whether immunoenhancing peptide alone can also elicit this response. Although many adjuvants can have substantially negative side effects, no adverse effects, including weight loss or autoimmune responses, were observed in immunoenhancing peptide-treated animals.

Interestingly, the immunostimulatory activity is encoded within a mucin protein. Although this is a unique property, another mucin, MUC7, has been shown to encode a 20 amino acid peptide that has potent antimicrobial properties (38, 39). Because the mucin proteins are found at many interfaces between the host cells and external environments, it is logical to conclude that this protein family, while serving as physical barriers, may also encode properties capable of stimulating an immune response.

Here, we show that mice previously exposed to MUC1/sec-expressing cells or a MUC1/sec-derived peptide are able to slow or, in some cases, prevent subsequent growth of several tumor types. One caveat of these experiments is the fact that, to date, the challenge must be co-injected with either DA-3/sec cells or the MUC1/sec-derived peptide for the antitumor effect to be substantial. Work on alternative vaccine protocols and therapeutic applications are ongoing in an attempt to solve this problem.

The total protection or tumor retardation observed in mice previously exposed to DA-3/sec cells and then challenged with DA-3/TM cells could be attributed to previous exposure to the entire MUC-1 molecule. However, animals challenged with DA-3/neo cells were equally protected, indicating that the effect was not that of a classic antigen recognition vaccination protocol. This conclusion was further strengthened when similar effects were observed in mice pre-exposed to DA-3/sec cells and challenged with the RENCA renal carcinoma and the K7 osteosarcoma. Although these tumors are syngeneic to BALB/c mice, they are unrelated to the DA-3 mammary tumor background. One could hypothesize that the unique peptide of MUC1/sec may be functioning, in part, as an immunoadjuvant because, at the time of challenge, the tumor cells had to be mixed with DA-3/sec cells to observe the antitumor effect. Indeed, the hydrophilic nature of the peptide suggests that it may be highly immunogenic. Thus, the presence of these 11 amino acids in the cells transfected with MUC1/sec may be crucial for the antitumor effects observed.

The immunologic mechanisms involved in the absolute protection against tumor growth observed when the DA-3/sec cells are initially inoculated in untreated BALB/c mice may not be identical to those observed in the vaccination-like protocols with the peptide. There appears to be no antigenic specificity afforded by the MUC-1/sec in the latter, although the MUC1 molecule has been used in various classic antigen-specific studies (40–43). Indeed, there may be additional contributions from other domains of the secreted mucin molecule, such as the tandem repeats, conferring protection against the initial growth of DA-3/sec cells in immunocompetent mice. However, in vivo protocols, with the peptide in the absence of other portions of the secreted MUC1, results in retarded tumor appearance or absence of growth, strongly suggesting that the unique peptide can per se play a significant role in the antitumor process. Ongoing experiments include the generation of DA-3 cells that express MUC1/sec lacking the tandem repeat portion of the gene to additionally assess the role of the MUC1/sec protein in tumor development.

Determinant spreading is the process in which antigens that are distinct and non cross-reactive with the stimulating antigen become additional targets of the induced immune response. The process of determinant spreading has been observed during clearance of infectious diseases, in response to allotransplants, and during human immunotherapy trials (44). The MUC1/sec-derived peptide (immunoenhancing peptide) is capable of stimulating an immune response against multiple tumor targets across MHC backgrounds, highlighting the possibility that immunoenhancing peptide may be an immunodominant epitope resulting in the lysis of tumor cells that are presenting immunoenhancing peptide. The resulting lysis would then lead to an environment in which antigen presenting cells are stimulated to present tumor-specific antigens that ultimately lead to the prevention of tumor cell growth.

As mentioned previously, the tumor challenge needed to be mixed with immunoenhancing peptide or DA-3/sec cells to have a significant decrease in growth. This may suggest that the mechanism of action of the MUC1/sec protein or immunoenhancing peptide occurs within the tumor cell itself and not on the host immune system. Alternatively, the

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3 I. Grosso, unpublished observations.
MUC1/sec or immunoenhancing peptide may act on the surrounding tissue, including epithelial cells, inducing secretion of proinflammatory cytokines and other factors that would promote antitumor responses. We are currently characterizing the cell types and mechanisms involved in the immunoenhancing potential of immunoenhancing peptide.

The results of the present study suggest, however, that this peptide may be helpful alone or in combination with other molecules against not only a wide variety of tumors but also in cases when immunostimulation against various pathogens is desirable.

REFERENCES

A Unique Mucin Immunoenhancing Peptide with Antitumor Properties

Lynn M. Herbert, Joseph F. Grosso, Mantley Dorsey, Jr., et al.

*Cancer Res* 2004;64:8077-8084.

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