MUC1-specific Cytotoxic T Lymphocytes Eradicate Tumors When Adoptively Transferred in Vivo

Pinku Mukherjee, Amelia R. Ginardi, Teresa L. Tinder, Christopher J. Sterner, and Sandra J. Gendler
Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259

Abstract
We have previously reported that MUC1 transgenic mice with spontaneous tumors of the pancreas (designated MET) naturally develop MHC class I-restricted, MUC1-specific CTLs as tumors progress (P. Mukherjee et al., J. Immunol., 165: 3451–3460, 2000). From these MET mice, we have isolated, expanded, and cloned naturally occurring MUC1-specific CTLs in vitro. In this report, we show that the CTL line is predominantly CD8+ T cells and expresses T-cell receptor Vβ chains 5.1/5.2, 11, 13, and 2 and Vα chains 2, 8.3, 3.2, and 11.1/11.2. These CTLs recognize several epitopes on the MUC1 tandem repeat with highest affinity to APGSTAPPA. The CTL clone, on the other hand, is 100% CD8+ cells and expresses a single Vβ chain of 5.1/5.2 and Vα2. It recognizes only the H-2Db class I-restricted epitope of MUC1, APGSTAPPA. When adoptively transferred, the CTLs were effective in eradicating MUC1-expressing injected tumor cells including mammary gland cells (C57mg) and B16 melanomas. These results suggest that MUC1-specific CTLs are capable of possibly preventing, or at least substantially delaying, MUC1-expressing tumor formation. To our knowledge, this is the first evidence that demonstrates the naturally occurring MUC1-specific CTLs isolated from one tumor model has antitumor effects on other MUC1-expressing tumors in vivo. Therefore, our data confirm that MUC1 is an important tumor rejection antigen and can serve as a target for immunotherapy.

Introduction
Human cancer remains a major health problem, and novel therapeutic approaches are necessary to advance patient care. The recent molecular identification of tumor antigens recognized by CTLs derived from cancer patients has initiated a new era in tumor immunology. Thus far, nearly all of the defined therapeutic approaches are necessary to advance patient care.

on the potential of the synthetic peptides to mimic endogenously produced antigenic peptides. Synthetic peptides have been used successfully to expand in vitro tumor-reactive CTLs in blood samples from cancer patients (1). This approach is being exploited to generate large numbers of autologous tumor antigen-specific CTLs for adoptive transfer immunotherapy. MUC1 is one such tumor antigen that is being used by several investigators for the generation of CTLs.

MUC1 is a cell-associated mucin that is developmentally regulated and aberrantly expressed by carcinomas, which makes it an important marker in malignancy (2–6). MUC1 is a particularly attractive target for immunotherapy because the expression of MUC1 is altered in cancer. For example, MUC1 is overexpressed, aberrantly glycosylated, and ubiquitously distributed all around the cell rather than being restricted to the apical surfaces of ducts and glands. The recent description of MUC1 as a target for CTLs has raised interest in using this protein as a target for immunotherapy (7, 8). It is expressed by most adenocarcinomas of the breast, lung, stomach, pancreas, colon, prostate, ovary, and cervix and elicits humoral and cellular immune responses in humans. In 1997, cancers that expressed MUC1 accounted for ~72% of new cases and for 66% of deaths (9). However, the presence of these responses in patients with progressive disease suggests that immune recognition is not followed by eradication of tumors. We hypothesize that tumors can be recognized in vivo by antigen-specific T cells, but that such encounters occur late in tumor progression and ultimately lead to exclusion, deletion, or dysfunction of responding T cells such that the antitumor response is interrupted. Understanding this phenomenon is essential for the development of effective immunotherapy strategies to restore antitumor immunity in cancer patients. Until recently, the mouse has not been a suitable preclinical model for testing MUC1-specific immune responses because human MUC1 differs substantially in sequence from mouse Muc1 and is strongly antigenic in the mouse.

In this project, we have used a MUC1 transgenic mouse (MUC1.Tg) expressing human MUC1 as a self-molecule crossed with an oncogene-expressing mouse to generate mice that spontaneously develop MUC1-expressing (MUC1+) tumors of the pancreas (MET mice). As tumors progress in the MET mice, low level anti-MUC1 cellular and humoral immune responses develop that are not effective against the spontaneous pancreatic tumors. However, we were able to isolate MUC1-specific CTLs from these mice and develop CTL lines and clones in vitro. These CTLs were effective in eradicating MUC1+ tumors in vivo when adoptively transferred, suggesting that anti-MUC1 immune responses can be effective against tumors if they occur early during tumor progression. Moreover, these CTLs are effective against MUC1+ tumors and have no effect on MUC1- tumors. These CTLs are reactive only against MUC1 as expressed by the tumors and do not cause any autoimmune destruction of organs that normally express MUC1.
Materials and Methods

Mouse Models. MUC1 transgenic (MUC1.Tg) mice were developed in our laboratory and have been described previously. These mice express human MUC1 in a developmentally regulated and tissue-specific fashion because the endogenous MUC1 promoter was used to drive expression. Mice transgenic for this protein develop B- and T-cell compartment tolerance and are refractory to immunization with the protein encoded by the transgene (10). MET mice were generated as described previously (11). MUC1 transgenic mice are bred with oncogene-expressing mice that spontaneously develop tumors of the pancreas (ET mice) and are designated as MET. The ET mice were obtained from Dr. Judith Tevethia (Pennsylvania State University, Hershey, PA) (12) and express the first 127 amino acids of SV40 large T antigen under the control of the rat elastase promoter. Fifty % of the animals develop life-threatening pancreatic tumors by ~21 weeks of age (12). The spontaneous tumors in MET mice arise naturally in an appropriate tissue background and in the context of a viable immune system. The tumors develop more slowly than injected tumors, giving the host immune system time to respond. All mice are on the C57BL/6 background. Mice were bred and maintained in specific pathogen-free conditions in the S.C. Johnson Medical Research Building animal facility at Mayo Clinic Scottsdale. All experimental procedures were conducted according to Institutional Animal Care and Use Committee guidelines.

PCR Screening. PCR was used to routinely identify MUC1.Tg- and ET-positive mice in the colony. PCR was carried out as described previously (10, 12). The primer pairs for MUC1.Tg were 5'-CTTGCCAGGACCAGATCCAGCAACG-3' (745–765 bp) and 5'-CTCCACGTGGAGCGATTGATG-3' (1086–1065 bp); for ET, 5'-GCTCCTTTAACCCACCTG-3' (745–765 bp) and 5'-CTCCACGTGGAGCGATTGATG-3' (1086–1065 bp); for ET, 5'-GCTCCTTTAACCCACCTG-3' (745–765 bp) and 5'-CTCCACGTGGAGCGATTGATG-3' (1086–1065 bp). The PCR product of each reaction was analyzed by size fractionation through a 1% agarose gel. Amplification conditions for MUC1 were the same, except the annealing temperature was 61°C. PCR products were sequenced to rule out contamination.

Flow Cytometry. Tumor cell lines were analyzed by immunofluorescence for surface expression of MHC class I and MUC1. The MUC1-specific CTL line and TR clone 1 were analyzed by two-color immunofluorescence for surface expression of CD8α and TCR Vα1 chains or TCR Vβ chains as per the manufacturer's recommendations. All fluorescently labeled antibodies were purchased from PharMingen (San Diego, CA). The antibody used for MUC1 staining was HMFG-2, a monoclonal antibody (2). The minimum epitope recognized by HMFG-2 is the immunodominant MUC1 TR epitope, DTR (13). MHC class I H-2D^b tetramers containing MUC1 TR APGSTAPPA peptides were created by Dr. Larry Pease at Mayo Clinic Rochester. CTLs were stained with 0.1 mg/ml MUC1 tetramers (directly conjugated to phycoerythrin) for 1 h on ice. The irrelevant tetramer used was Thielers' virus epitope D^b:VP2_121–130 (FHA GSL FLF VM; Ref. 14). The cells were then stained with anti-CD8 antibody conjugated to FITC for 15 min prior to analysis by two-color flow cytometry. Flow cytometric analysis was done on a Beckon Dickinson FACScan using the Cell Quest program.

Generation of the CTL Line and Clone. CTLs were obtained from splenocytes of MET mice, 18 weeks of age, and expanded on IFN-γ-treated, irradiated B16.MUC1 cells (20,000 rads for 2 consecutive days). The irradiated B16.MUC1 cells served as feeders. CTLs were maintained on the feeders with IL-2 (100 units/ml) and IFN-γ (150 pg/ml) for 2 weeks prior to removing them from antigenic stimulus and growing on irradiated splenocyte feeders with IL-2 (100 units/ml) for another 2–3 weeks until cells reached confluency. CD8-expressing (CD8^+ cells were selected (98% of the CTLs were CD8^+) by magnetic activated cell sorting and further maintained on irradiated splenocyte feeders with IL-2 (100 units/ml). When cells reached confluency, their lytic activity was determined by an in vitro CTL assay using B16.MUC1 as target cells. These cells were then maintained as a CTL line in DMEM complete medium supplemented with IL-2 (100 units/ml). A portion of these CTLs was used to generate CTL clones by limiting dilution procedure, such that each well in a 96-well plate (containing splenic feeders) received a single cell. We have generated 550 clones with this procedure, and in this report, we present studies completed with the CTL line and one clone (TR clone 1).

Magnetic Activated Cell Sorting. Lymphocytes were stained for 30 min on ice with anti-CD8 antibody conjugated to microbeads (Miltenyi Biotechnologies, Auburn, CA). CD8α cells were positively selected on an RS-type magnetic column using the Vario MACS magnetic device, following the protocol provided by the manufacturer (Miltenyi Biotechnologies). Purity of the selected cells was verified by flow cytometry and ranged from 92 to 95%. Cells were further cultured for in vitro CTL assay and adoptive transfer experiments.

RPMI 1640 supplemented with 10% HAT (Life Technologies, Inc.).
MET Mice Naturally Develop MUC1-specific CTLs. We have shown previously that by 15 weeks of age when the MET mice already have a large tumor burden in the pancreas, they develop MUC1-specific CTLs that peak at 18 weeks of age and drop off by 21 weeks. We have also shown that these CTLs recognize the immunodominant TR region of MUC1, and that they are MHC class I restricted (11). One of the major goals of immunotherapy is to generate and target CTLs that can effectively eliminate tumor cells. Although the MET mice do develop CTLs, the CTLs develop late in tumor development, when the tumor is of substantial size (1.5–2.0 g) and therefore have no detectable effect on the spontaneous pancreas tumor (11). Thus, we hypothesized that the presence of MUC1-specific CTLs earlier during tumor progression may have an effect against the tumors. To test this hypothesis, we have developed a MUC1-specific CTL line and 550 CTL clones from MET mice 18 weeks of age. Prior to using tumor target cells in a CTL assay to assess lytic activity of the CTL line and TR clone 1, we determined the expression levels of MUC1 and MHC class I on target cells. By FACS analysis, we show that 58% of B16.MUC1 and 44% of B16.neo cells express low levels of MHC class I, and 100% of B16.MUC1 cells express intermediate levels of MUC1 (as assayed by fluorescence intensity). As expected, none of the B16.neo cells expressed any MUC1 (Fig. 1). Similarly, 43% of C57mg.MUC1 cells expressed low levels of MHC class I, and 68% expressed intermediate levels of MUC1 (Fig. 1). The data presented in Fig. 1 are representations of the staining pattern in these cells. This pattern may change with a new batch of cells, time in culture, and with number of passages; we have observed that MUC1 as well as MHC class I levels drop when cells are maintained in culture for extended periods of time (data not shown).

In Fig. 2, we show the in vitro lytic activity of the MUC1-specific CTLs against tumor target cells that express MUC1, including B16.MUC1 with 70% lysis and C57mg.MUC1 with 27% lysis at an E:T ratio of 100:1. The CTLs had no effect on tumor cells (B16.neo) that did not express MUC1, showing 7% lysis (Fig. 2A), suggesting that the CTLs are specifically directed against the MUC1 antigen. The reason for lower lysis of C57mg.MUC1 cells as compared with B16.MUC1 is not clear, but we do know that C57mg.MUC1 cells grow slower in culture and do not take up 51Cr as effectively as the B16.MUC1 cells (data not shown).

We tested one of the MUC1-specific CTL clones (TR clone 1) for its lytic activity against MUC1. The targets used in this assay were DCs pulsed with either MUC1 TR 25-mer STAPPAH or 9-mer APGSTAPPA peptide. The results in Fig. 2B show 50% lysis of DCs pulsed with STAPPAH and 44% lysis of DCs pulsed with APGSTAPPA at an E:T ratio of 100:1. Further characterization of the TR clone 1, using 9-mer over-
lapping peptide sequences of the entire MUC1 TR region, suggested that the TR clone 1 exclusively recognized APGSTAPPA epitope and was indeed clonal (data not shown). 4

**MUC1-specific CTL TR Clone 1 Expresses a Single TCR Vβ Chain and a Vα Chain on Its Surface.** To further determine the clonality of the CTL TR clone 1, we determined the surface expression of TCR Vα and Vβ chains on the CTL line and TR clone 1. Using a panel of 15 anti-TCR Vβ and 4 anti-TCR Vα antibodies and FACS analysis, we have determined the expression of Vβ and Vα chains of the CTLs. Although the CTL line expresses four TCR Vβ chains 5.1/5.2, 11, 13, and 2 and four TCR Vα chains 8.3, 3.2, 11.1/11.2, and 2, the TR clone 1 expressed a single TCR Vβ chain of 5.1/5.2 and a single TCR Vα chain 2 chain (Fig. 3).

**The MUC1-specific CTL Line Is MHC Class I Restricted.** To confirm our previous finding that the MUC1-specific CTLs that naturally develop in the MET mice are MHC class I restricted (11), we tested our CTL line for class I restriction. In this study, we used L cells transfected with either H-2D<sup>b</sup> (L-H-2D<sup>b</sup>) or H-2K<sup>b</sup> (L-H-2K<sup>b</sup>) MHC class I molecules and pulsed them overnight with MUC1 TR peptides STAPPAHGV and SAPDTRPAP as target cells. These peptides were chosen because they are known to specifically bind MHC class I H-2D<sup>b</sup> and H-2K<sup>b</sup> molecules, respectively (15). The CTL line lysed 88% of L-H-2D<sup>b</sup> cells pulsed with STAPPAHGV and only 5% of L-H-2K<sup>b</sup> cells pulsed with the same peptide. On the other hand, the line showed 30% lysis of L-H-2K<sup>b</sup> cells pulsed with SAPDTRPAP as compared with 3% lysis of L-H-2D<sup>b</sup> cells pulsed with the same peptide (Fig. 4). This result suggests that the MUC1-specific CTL line recognizes STAPPAHGV presented in context with H-2D<sup>b</sup> and SAPDTRPAP presented in context with H-2K<sup>b</sup>.

**The MUC1-specific CTL Line and TR Clone 1 Bind to the H-2D<sup>b</sup> Tetramer Containing APGSTAPPA.** Because we had high lysis of DCs pulsed with APGSTAPPA (Fig. 2B) and L-H-2D<sup>b</sup> cells pulsed with STAPPAHGV (Fig. 4), we were interested in developing H-2D<sup>b</sup> tetramers containing one of these peptides. However, previous studies have shown that H-2D<sup>b</sup> optimally binds the APGSTAPPA peptide (15); thus, we obtained the H-2D<sup>b</sup> tetramer containing the APGSTAPPA peptide, generated by Dr. Larry Pease (14). We show by FACS

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4 Manuscript in preparation.
One of the major goals of immunotherapy is to generate and direct CTLs that can effectively eliminate tumor cells. Much of the research has focused on identifying and characterizing proteins expressed on tumor cells that may serve as potential tumor-specific antigens for recognition by CTLs (16). Some of the most promising candidates represent conventional cellular proteins that are expressed in both normal and transformed cells.
expression. There is an increase in the amount of MUC1 expressed on cells and in circulation. Its distribution is no longer restricted to the apical surface of the ducts and glands but is found throughout the tumor mass and on the surface of tumor cells. Importantly, glycosylation is altered. Oligosaccharides are shorter and fewer in number, revealing immunodominant peptide sequences that on normal cell surfaces would be sequenced by glycosylation. Thus, MUC1 peptides may be used in immunotherapy strategies to activate the immune system to kill tumors expressing these epitopes.

From a spontaneous MUC1-expressing pancreatic tumor mouse model (MET), we have successfully isolated, expanded, and cloned naturally occurring MUC1-specific CTLs in vitro. These CTLs are CD8+ T cells that express TCR Vβ5.1/5.2 and Vα2 (Fig. 3) and recognize a H-2Db MHC class I-restricted immunodominant epitope of MUC1 (STAPPAHGV or APGSTAPPA; Figs. 4 and 5). These CTLs can eradicate MUC1+ tumor cells when adoptively transferred in vivo (Figs. 6 and 7). It is important to note that the CTLs characterized in this report differ from those originally described by Dr. Olivera Finn’s laboratory (7, 8, 29, 30). The CTLs obtained from the MET mice are class I-restricted CTLs, and we have not seen MHC unrestricted CTLs in these animals. Class I-restricted CTLs have, however, been described recently in humans (31, 32).

Data presented in this study suggest that immunotherapeutic regimens using adoptive transfer of MUC1-specific T cells can eradicate, delay, or substantially reduce the incidence of MUC1+ tumors, which suggest that tumors are somewhat susceptible to immune intervention, at least at early stages. This is the first evidence that demonstrates that the naturally occurring MUC1-specific CTLs isolated from one tumor model has anti-tumor effects on other MUC1-expressing tumors in vivo. Moreover, these CTLs are effective only against MUC1+ tumors and have no effect on MUC1− tumors. Another important observation is that these CTLs are only reactive against tumor-expressed MUC1 and do not cause any autoimmune destruction of organs that express normal MUC1. Therefore, our
data confirm that MUC1 as expressed on tumors is an important tumor rejection antigen and can serve as an excellent target for immunotherapy.

Although all of the adoptive transfer data presented here are on injectable tumor models, we are most interested in determining whether the CTLs can be as effective in mice that develop spontaneous tumors. Two models of spontaneous tumors that were developed in our laboratory are under study: (a) the MET mice from which the CTLs originated; and (b) mice that develop spontaneous MUC1-expressing tumors of the mammary gland (designated MMT). Preliminary data suggest that the CTLs may be effective in delaying tumor onset in the MMT mice (data not shown). These studies provide us with the opportunity to develop clinical trials using MUC1-specific, cell-based vaccines.

Human cancers frequently express abnormal or altered self-proteins that are potentially immunogenic and trigger immune recognition. For example, low-level humoral and cellular immune responses to several antigens, including MUC1, HER2/neu, p53, and prostate-specific antigen, are present in a significant proportion of early- and late-stage cancer patients (33). It is important to determine how to obtain an effective immune response, because native immune responses fail to eradicate tumors. Understanding why these low-level immune responses are not effective against the growing tumor is key in developing improved and novel immunotherapeutic strategies for cancer. However, to be successful, these strategies ultimately need to induce reversal of self tolerance without autoimmune destruction and bypass any immune evasion mechanisms used by the tumor cells. The creation of mice bearing transgenic TCRs specific for tumor antigens have revolutionized the study of: (a) immune evasion mechanisms by tumors; (b) effects of self tolerance on the host’s ability to eliminate tumor cells expressing self epitopes; and (c) autoimmune destruction. These mice can provide an unlimited source of tumor-specific T cells for in vivo studies. Thus, our future direction is to develop transgenic mice that develop CD8+ CTLs that express TCRs specific for the immunodominant epitope of MUC1 so that we may study the above mechanisms as well as the effector functions of CTLs specific for MUC1. Once we identify some of these crucial mechanisms, our ultimate goal is to develop effective immunotherapeutic strategies aimed at preventing and treating human cancers.

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