Expression of Tumor-associated Epitopes on Epstein-Barr Virus-immortalized B-Cells and Burkitt's Lymphomas Transfected with Epithelial Mucin Complementary DNA

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ABSTRACT

Mucins are among the best described human tumor-associated antigens. At least 73 tumor-reactive anti-mucin antibodies have been described; in addition, we have previously demonstrated the existence of tumor-specific cytotoxic T-lymphotocyte epitopes on the mucin produced by breast and pancreatic tumors. To determine whether the appearance of tumor-associated mucin epitopes can be explained by altered post-translational modification of mucin in tumors, or whether the generation of these epitopes requires changes in the mucin gene itself, we studied four Burkitt's lymphomas and six Epstein-Barr virus-immortalized B-cell lines transfected with an expression construct containing the mucin complementary DNA. Transfected cell lines showed stable maintenance of the mucin gene, which comprises 20 or more tandem repeats of a 60-nucleotide sequence. Transfected cells expressed many tumor-associated mucin epitopes, suggesting that the changes in mucin synthesis seen in breast and pancreatic tumors are present in other malignant cell types as well. Furthermore, even though each cell line was transfected with the identical mucin construct, each expressed a different subset of tumor-associated mucin epitopes. This suggests that the specificity of these epitopes for tumors is not due to genetic alterations of the mucin gene in tumors. Incubating transfected cells with phenyl-N-acetyl-α-galactosaminide, an inhibitor of O-linked glycosylation, altered cell surface carbohydrate structures and resulted in increased expression of all tumor-associated epitopes, implicating incomplete glycosylation of mucin in the generation of these epitopes. These findings suggest that alterations in the posttranslational modification of normal gene products can result in the expression of novel epitopes. Furthermore, the ability to transfected cancer patients' Epstein-Barr virus-immortalized B-cell lines with mucin will provide an unlimited supply of autologous, mucin-bearing cells with which to study these patients' T-cell response to mucin.

INTRODUCTION

Mucins comprise perhaps the best described family of human tumor-associated antigens. Mucins are high molecular weight glycoproteins consisting of an extended polypeptide core which is highly glycosylated by O-linked carbohydrates. The gene encoding the mucin produced by breast and pancreatic epithelium consists of a variable number of tandem repeats (often more than 20) of a 60-nucleotide sequence, designated MUC1 (1). The MUC1 gene is found both in normal breast and pancreatic epithelial tumors and in tumors arising from these tissues. Tandem repeats of different sequences encode mucins produced by gastrointestinal epithelium (MUC2 and MUC3) (2, 3) and tracheobronchial epithelium (MUC4) (4). While mucins are produced by both normal and malignant epithelial cells, many of the more than 73 anti-mucin antibodies which have been generated have some degree of tumor specificity (1). In addition, our laboratory has shown a specific T-cell-mediated immune response to human mucin-producing breast and pancreatic tumors (5–7). Like some of the anti-mucin antibodies, these tumor-reactive CTL were able to distinguish between the mucins, produced by normal and malignant cells (5).

The appearance of tumor-associated mucin epitopes on malignant cells has been postulated to result from either (a) mutation of the mucin gene in tumors (8) or (b) aberrant processing (in particular glycosylation) of the mucin by malignant cells (9). Little direct evidence supports the mutation hypothesis. In fact, the MUC1 gene has been sequenced from both tumor (10) and normal peripheral blood lymphocytes (11) and is identical in both. In contrast, a large body of evidence exists suggesting that the appearance of tumor-associated epitopes on mucins is due to aberrant glycosylation of mucin by malignant cells (9, 12–14). This aberrant glycosylation is thought to result in both the formation of new carbohydrate epitopes and the expression of new epitopes on the mucin polypeptide core, which were formerly obscured by carbohydrate.

If the expression of tumor-associated mucin epitopes on malignant epithelial cells and their absence in normal cells are results of aberrant glycosylation, then transfection and expression of the mucin gene in other transformed cell types might also result in differential expression of various mucin-associated epitopes. The glycosylation of the mucin (and therefore its antigenic profile) would depend on the complement of glycosyltransferases of the transfected cells and their activities; therefore, the expression of various tumor-associated epitopes might also vary between transfected cell lines. In contrast, if the expression of tumor-associated epitopes in malignant cells is due to mutations in the mucin gene, all cells transfected with the identical gene would be expected to produce a mucin expressing the same set of epitopes.

To test these predictions, we constructed an expression vector containing mucin cDNA (MUC1) cloned from a human pancreatic cancer cell line (10). This vector allows mucin production in Burkitt's lymphomas and EBV-immortalized B-cell lines. In the study reported here, we demonstrate high-level expression of mucin in ten different transfected cell lines. These transfected cells expressed a variety of mucin epitopes, including several which have been shown to be tumor associated. Furthermore, cells transfected with the same expression construct express different subsets of tumor-associated epitopes, consistent with the idea that the expression of these epitopes on tumors reflects different posttranslational modification of mucin in the various cells. Finally, some tumor-associated epitopes can be generated or increased in expression by incubation of Epithelial Mucin Complementary DNA.

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3 The abbreviations used in this paper are: CTL, cytotoxic T-lymphocyte(s); EBV, Epstein-Barr virus; phenyl-GalNAc, phenyl-N-acetyl-α-galactosaminide; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; mAb, monoclonal antibody.
transfected cells with an inhibitor of O-linked glycosylation, supporting the hypothesis that the inability of tumors to fully glycosylate mucin results in novel expression of tumor-associated antigenicity. Furthermore, the ability to transfect and express mucin in EBV-immortalized cells will provide an unlimited source of autologous mucin-producing cells with which to study individual cancer patients' T-cell responses to mucin.

MATERIALS AND METHODS

Culture of Cell Lines. EBV-immortalized B-cells and Burkitt's lymphomas were cultured in RPMI 1640 supplemented with 20% fetal calf serum (Flow Laboratories, McLean, VA), 2 mM glutamine (Gibco, Grand Island, NY), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Cells were grown in 25- or 75-cm² flasks (Nunc, Roskilde, Denmark); the pancreatic tumor cell lines MCF-7, BT-20, and SK-BR-3; and the pancreatic tumor cell lines CAPAN-1 and CAPAN-2 were obtained from the American Type Culture Collection, Rockville, MD; the pancreatic tumor lines PANC-89, HPAF, and QGP-1 were obtained from R. Metzgar, Durham, NC; and the Burkitt's lymphoma lines BJA.B and EB-3 were obtained from J. Dawson, Durham, NC. The EBV-immortalized B-cell lines CI58, XcXr, Ompa, LiSe, ArCo, and StBr were established in our laboratory.

Plasmid Constructions. To generate plasmids pDKOF.muc1 and pDKOF.muc1fs, a 4.4-kilobase EcoRI fragment containing the EBV origin of replication was isolated from the plasmid pH58 (15) and ligated to EcoRI-cut pHSAPR-1-neo (16) containing the β-actin promoter/enhancer. The ligation product was cut with Bal 31 nuclease for 5 min to eliminate the HindIII site within the pHEBo fragment, thereby ensuring that the HindIII site within the pHSAPR-1-neo fragment was unique for cloning purposes. The resulting plasmid, pDKOF, was cut with HindIII to accommodate a HindIII/EcoRI-linked 3.4-kilobase EcoRI fragment from pBS.4C1B (provided by R. Metzgar, Durham, NC), containing a nearly full-length MUC1 cDNA missing only the 5' 18 base pairs. For pDKOF.muc1, the HindIII/EcoRI linkers contained an in-frame translational start codon within a Kozak consensus sequence. For pDKOF.muc1fs, the start codon was out of frame from the downstream MUC1 gene.

Transfection of EBV-immortalized B-Cells and Burkitt's Lymphomas. Cells were transfected with plasmid DNA using the lipofection technique (17). Briefly, 5 × 10⁴ to 1 × 10⁵ cells were seeded at 3 × 10⁵ cells/ml. The next day, the cells were washed in phosphate-buffered saline and resuspended in 200 μl OPTI-MEM (Gibco). Two μg plasmid DNA were diluted in 0.5 ml OPTI-MEM and then added to a mixture of 5 μl Lipofectin (BRL, Gaithersburg, MD) in 0.5 ml OPTI-MEM. After 10 min the cells were added to this mixture and incubated at 37°C for 24 h. The cells were then resuspended in 15 ml growth medium. Forty-eight h later, aliquots of cells were transferred to 200, 400, 600, 800, and 1000 μg/ml Geneticin (Gibco) to select for transfectants. The cultures were cleared of dead cells and debris once or twice a week by centrifugation through a Ficoll-Hypaque gradient (Organon Technica, Durham, NC). Long-term cell lines were maintained in medium containing the maximum tolerated concentration of Geneticin.

DNA Analysis. DNA was extracted as described (18). DNA was analyzed by Southern blot hybridization as described (18) using an oligo-labeled 3.4-kilobase mucin gene fragment from the plasmid pBS.4C1B. Hybridization was performed in 50% formamide-5 X Denhardt's solution-1% SDS-0.5 mg/ml salmon sperm DNA-5 X SSPE at 37°C for 24 h. Two washes were performed in 2 X SSPE, 0.1% SDS at room temperature for 20 min each, followed by three washes in 0.1 X SSPE-0.1% SDS at 52°C for 20 min each.

Antibodies and Flow Cytometry. The tumor-reactive, mucin-specific antibodies used in this study are listed in Table 1. Indirect immunofluorescence was performed as described (5). Cells were analyzed by flow cytometry using either an Ortho Cytofluorograph 50H or a Becton-Dickinson FACScan.

Inhibition of Mucin Glycosylation. Mucin-producing transfecants were plated 3 × 10⁵/well of a 96-well plate in 5% phenyl-N-acetyl-D-galactosamine (Sigma) (28) dissolved in 200 μl growth medium. After 36 to 42 h, cells were spun down, washed, and analyzed by immunofluorescence.

RESULTS

Introduction of the MUC1 Gene into EBV-immortalized B-Cells and Burkitt's Lymphomas. Transfection and expression of the MUC1 gene encoding the breast/pancreatic mucin (1) in EBV-immortalized B-cells and in Burkitt's lymphomas were achieved using the vector pDKOF (Fig. 1A). The EBV origin of replication allows maintenance of the plasmid in EBV-immortalized B-cells and Burkitt's lymphomas, and the human β-actin promoter (16) drives high-level transcription of downstream sequences. Two derivative plasmids containing the MUC1 gene were constructed as described in "Materials and Methods." The expression plasmid pDKOF.muc1 contains 22 repeats of the 60-nucleotide tandem repeat MUC1 sequence. The frame-shift control plasmid pDKOF.muc1fs contains 24 of the tandem repeats but out of frame from the translational start site. Southern blot analysis of DNA from transfected Raji and CI58 cell

Table 1 Monoclonal antibodies to mucin

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>Epitope</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-3</td>
<td>IgG1</td>
<td>T</td>
<td>PDTR</td>
<td>19</td>
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<tr>
<td>HMFIGF-2</td>
<td>IgG1</td>
<td>T</td>
<td>DTR</td>
<td>20</td>
</tr>
<tr>
<td>B72.3</td>
<td>IgG1</td>
<td>T</td>
<td>Sialyl-Tn</td>
<td>21</td>
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<tr>
<td>DUPAN-2</td>
<td>IgM</td>
<td>N/T</td>
<td>LsTa</td>
<td>22</td>
</tr>
<tr>
<td>BC-1</td>
<td>IgG3</td>
<td>N/T</td>
<td>APDTR</td>
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<tr>
<td>DF-3</td>
<td>IgG1</td>
<td>T</td>
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<td>24</td>
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<td>13944</td>
<td>IgG1</td>
<td>T</td>
<td>Protein core</td>
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<tr>
<td>201E9</td>
<td>IgM</td>
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<td>IgG1</td>
<td>N/T</td>
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<td>HMPV</td>
<td>IgG1</td>
<td>T</td>
<td>APDTR</td>
<td>27</td>
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</table>

a The antibodies have varying degrees of tumor preference: T, preferential binding to tumors; N/T, binding to both normal and tumor cells.

b One letter amino acid code identifies the epitope along the tandemly repeated mucin polypeptide core: VTSAPDTRPAPGSTAPPAGH. Antibodies B72.3 and DUPAN-2 recognize the carbohydrate epitopes indicated.

c p.c., personal communication.
for this blot. The difference in size between the pDKOF.muc1 and pDKOF.muc1fs inserts reflects the presence of 2 more tandem repeats in pDKOF.muc1fs. Most importantly, the proper insert size shows that the entire gene is retained, in spite of its tandemly repeated structure, which has led in retroviral and vaccinia expression systems to homologous recombination between repeat units and deletion of all but a few repeats (29).

Differential Expression of Tumor-associated Epitopes on Transfected EBV-immortalized B-Cells and Burkitt's Lymphomas. To test whether cells stably transfected with the entire mucin cDNA were in fact expressing mucin on their surface, we performed immunofluorescence staining using a number of anti-mucin antibodies (see Table 1). Some antibodies were chosen which recognize the mucin polypeptide core, while others recognize mucin carbohydrate side chains. In addition, some antibodies chosen were highly tumor specific, while others recognized mucin from both normal and malignant cells. As controls, untransfected parental cells and cells transfected with the control frame shift construct were stained with anti-mucin antibodies. None of these cells expressed mucin (data not shown). In contrast, each of the ten in-frame-transfected cell lines expressed a unique combination of the various mucin epitopes. Fig. 2 compares transfected cell lines to each other and to breast and pancreatic tumor cell lines. Although each cell line expressed its own unique combination of mucin epitopes, the transfected cell lines could be grouped into two very broad categories based on their patterns of mucin epitope expression. Group 1, consisting of the transfected EBV-immortalized B-cell lines XxCr,muc1, OmPa.muc1, CISm.muc1, and LiSe.muc1 and the transfected Burkitt's lymphoma line Raji.muc1, expressed high total surface levels of mucin as determined by staining with antibody BC-3, which binds mucin from both normal and malignant cells. Furthermore, these cells expressed high levels of a number of tumor-associated epitopes such as HMFG-2, 139H2, DF3, and 115D8. The pattern of staining of group 1 cells was most similar to that of the breast tumor cell lines MCF-7 and BT-20 and the pancreatic tumor lines CAPAN-1 and QGP-1. In contrast, group 2, consisting of the
transfected EBV-immortalized B-cell lines ArCo.muc1 and St-Br.muc1 and the transfected Burkitt's lymphomas BJA.B.muc1, Daudi.muc1, and EB-3.muc1, expressed lower total levels of mucin as determined by BC-3 binding and did not express most of the other, more tumor-specific epitopes. These group 2 cells did, however, express detectable levels of the epitopes recognized by antibodies 115D8, BC-1, and BC-2. The pattern of staining of group 2 cells was more similar to that of the breast tumor cell line SK-BR-3 and the pancreatic tumor lines HPAF, CAPAN-2, and PANC-89. These findings suggest that whatever the aberrations in mucin synthesis that cause the expression of tumor-associated epitopes on breast and pancreatic tumors, similar changes are found in other transformed cell types as well. These findings also suggest that there may be a specific change or set of changes which accounts for the increased expression of many tumor-associated epitopes in group 1 cells as compared to group 2 cells. Furthermore, since all cell lines were transfected with the identical mucin gene, the presence or absence of a particular tumor-associated mucin epitope does not require a genetic change in the mucin gene itself.

Unmasking of Tumor-associated Epitopes by Inhibition of Mucin Glycosylation Using Phenyl-N-acetyl-α-galactosaminide. Since alterations in the mucin gene apparently do not account for the tumor specificity of some anti-mucin antibodies, we examined whether alterations in the glycosylation of the transfected mucin could change mucin epitope expression. Phenyl-N-acetyl-α-galactosaminide is an inhibitor of O-linked glycosylation. Phenyl-GalNAc acts as a competitive inhibitor of UDP-Gal:GalNAc-β1,3-galactosyltransferase, one of the earliest glycosyltransferases in the mucin glycosylation pathway (28). Incubation of transfected cells with phenyl-GalNAc resulted in marked alteration of cell surface carbohydrates (not exclusively mucin) as initially tested by staining with the lectins peanut agglutinin, soybean agglutinin, and succinyl-concanavalin A (data not shown). In the specific case of the transfected mucin molecules, treatment with phenyl-GalNAc increased the expression of the tumor-associated polypeptide core epitopes SM-3, HMF-2, DF-3, 201E9, and 139H2 (Fig. 3). This increase most likely reflects unmasking of segments of the mucin polypeptide core which were formerly obscured by carbohydrate. Inhibition of glycosylation affected the expression of mucin epitopes to different degrees on different cell lines. These findings suggest that aberrations in mucin glycosylation in transformed cell lines can be responsible for the expression of tumor-associated mucin epitopes. Furthermore, the variable effect of inhibition of glycosylation reflects the heterogeneity of specific glycosyltransferase defects from cell line to cell line.

DISCUSSION

Many authors have suggested that the appearance of tumor-associated mucin epitopes on malignant cells is the result of aberrant glycosylation of mucin by these cells (9, 12—14). A limited amount of biochemical analysis supports this hypothesis. The carbohydrate side chains of the normal mucin form long, highly branched structures (30), while the carbohydrate side chains of tumor mucins tend to be short and unbranched (31). In addition to the new carbohydrate epitopes formed, the less bulky nature of these side chains presumably allows the unmasking of formerly cryptic epitopes on the mucin polypeptide core. The glycosylation of mucin is performed by cellular glycosyltransferases, enzymes which also glycosylate a number of other cellular products. Cellular glycosyltransferase activity is known to vary with several factors, including malignant transformation (13, 32), state of differentiation (33, 34), and certain disease states (35, 36).

The data presented in this paper support the growing body of work implicating aberrant glycosylation of mucin in tumors as the cause of the expression of tumor-associated mucin epitopes and show that mutation of the mucin gene is not required for tumor-associated epitope expression. The marked heterogeneity of mucin epitope expression between transfected cell lines reflects the variety of factors which can affect cellular glycosyltransferase activity. The expression of certain of these mucin epitopes can be increased by incubation of the transfected cells with phenyl-GalNAc, an inhibitor of O-linked glycosylation. Specifically, phenyl-GalNAc acts as a competitive inhibitor of UDP-Gal:GalNAc-β1,3-galactosyltransferase, which is one of the earliest glycosyltransferases in the mucin glycosylation pathway (28). The premature termination of certain carbohydrate side chains in the presence of this inhibitor allows the expression of some mucin polypeptide core epitopes, which were formerly obscured by carbohydrate. The modest increases of some of these epitopes are probably due to the incomplete inhibition of mucin glycosylation with phenyl-GalNAc (28), and also to the fact that only those mucin molecules synthesized and glycosylated after the introduction of inhibitor are affected. Mucin molecules synthesized and glycosylated before the 24- to 36-h incubation period with the inhibitor are not affected by inhibitors of glycosylation. In fact, in similar experiments using a vaccinia expression system, where all mucin synthesis occurs...
during a short, 12- to 16-h time period, incubation of the infected cells with phenyl-GalNAc during the mucin synthesis period resulted in a much larger increase in tumor-associated mucin epitopes over controls (data not shown).

Several of the specific mucin epitopes analyzed in this study deserve special attention. The epitope recognized by mAb SM-3 was of particular interest to us, since we have previously shown the ability of this antibody to inhibit lysis of mucin-bearing tumors by tumor-reactive CTL (5, 6). The SM-3 epitope was not detectable on any of the transfected cell lines. It was expressed, however, if cells were incubated with phenyl-GalNAc, an inhibitor of O-linked glycosylation. The epitope recognized by mAb DF.3 has been reported to be associated with breast tumors having a relatively favorable clinical prognosis. One postulated reason for this is that DF.3 detects only better differentiated tumors (37). Alternatively, we would suggest that the expression of the DF.3 epitope may result in a more effective antitumor immune response. Since the DF.3 epitope is expressed at high levels on only a subset of transfected cells, subsequent experiments will allow us to distinguish between these possibilities. Finally, the epitope recognized by mAb B72.3 was not expressed on any of the transfected cell lines. B72.3 has been used in the immunohistological study of tumor sections to aid the pathological diagnosis of breast cancers (38) and is known to react with the antigen sialyl-Tn (39). However, B72.3 does not bind to most cultured human tumor cell lines. Those tumor cell lines that do stain with B72.3, such as the breast tumor line MCF-7 or the colon tumor line LS174T, do so only very poorly, with less than 10% of the cells staining positive. While we see about 10% staining of MCF-7 (Fig. 2), the lack of B72.3 reactivity on mucin-transfected cells indicates that these transfectants are similar to most cultured tumors in their lack of expression of the B72.3 epitope.

The demonstration that differential posttranslational modification of gene products can lead to expression of novel antigens may have implications for other disease processes in addition to cancer. Aberrations in glycosylation in otherwise normal cells might lead to expression of antigens to which the immune system is not tolerant, thereby inducing autoimmune disease. One such autoimmune process may be ulcerative colitis, which has been suggested to result from an anti-mucin immune response (40). Differential O-linked glycosylation in response to inflammatory stimuli has been demonstrated for macrolin, a macrophage-restricted membrane sialoprotein (41). Expression of novel antigens due to aberrant glycosylation may also occur on still other O-linked glycoproteins, such as leukosialin and CD45, which are expressed by a wide variety of cell types (42).

One particular use of mucin-transfected cells in the future will be in elucidating the exact relationships between cellular glycosyltransferase activity, carbohydrate side chain structure, and mucin antigenicity. We are also interested in the relationship of oncogenic transformation to mucin antigenicity and are presently cotransfusing mucin transfectants with various oncogenes to determine whether this transformation will alter the antigenic profiles of the cells. We are particularly interested in transfusion of individual cancer patients' EBV-immortalized B cells with mucin. This provides an unlimited supply of autologous, mucin-producing cells with which to study these patients' T-cell responses to mucin. Important in this regard is the observation that mucin-transfected cells retain a highly multivalent form of the mucin gene. T-cell recognition of the mucin apparently requires the multiple identical antigenic repeats found on the complete mucin (5, 6). This suggests that mucin-transfected cells will be superior for T-cell studies to vaccinating or retrovirally infected cells, which contain no more than a few repeats. In ongoing studies, we have used transfected cells to establish bulk T-cell cultures and clones from cancer patients. These T-cells demonstrate specific cytotoxic activity against mucin-transfected cells but not against control untransfected cells. A detailed description of these bulk cultures and clones will be presented separately.

Finally, several authors have postulated that the breast/pancreatic mucin (MUC1) might form the basis for an effective antitumor vaccine (5, 29). The findings in this paper demonstrate that the glycosylation state of the mucin is critically important to its immunogenicity in terms of antibody reactivity. The glycosylation state of the mucin probably also affects the expression of the CTL epitope as well, since the epitope recognized by mAb SM-3, which is closely associated with the CTL epitope, is profoundly affected by glycosylation changes. Therefore, any attempt at inducing T- and B-cell mediated antitumor immunity must take into account the glycosylation state of the mucin immunogen.

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