Tumor-Associated Tn-MUC1 Glycoform Is Internalized through the Macrophage Galactose-Type C-Type Lectin and Delivered to the HLA Class I and II Compartments in Dendritic Cells

Chiara Napoletano,1 Aurelia Rugghetti,1 Mads P. Agervig Tarp,3 Julia Coleman,5 Eric P. Bennett,4 Gianfranco Picco,5 Patrizio Sale,12 Kaori Denda-Nagai,6 Tatsuro Irimura,4 Ulla Mandel,1 Henrik Clausen,5 Luigi Frati,1 Joyce Taylor-Papadimitriou,1 Joy Burchell,1 and Marianna Nuti1

1Department of Experimental Medicine, University of Rome "Sapienza", 2Department of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome, Italy; 3Department of Cell Biology, Department of Oral Diagnostics, University of Copenhagen, Copenhagen, Denmark; 4Breast Cancer Biology Group, King's College London School of Medicine, Guy's Hospital, London, United Kingdom; and 6Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan

Abstract

The type of interaction between tumor-associated antigens and specialized antigen-presenting cells such as dendritic cells (DCs) is critical for the type of immunity that will be generated. MUC1, a highly O-glycosylated mucin, is overexpressed and aberrantly glycosylated in several tumor histotypes. This results in the expression of tumor-associated glycoforms and in MUC1 carrying the tumor-specific glycan Tn (GalNAcc1-O-Ser/Thr). Glycopeptides corresponding to three tandem repeats of MUC1, enzymatically glycosylated with 9 or 15 mol of GalNAc, were shown to specifically bind and to be internalized by immature monocyte-derived DCs (iDCs). Binding required calcium and the GalNAc residue and was competed out by GalNAc polymer and Tn-MUC1 or Tn-MUC2 glycopeptides. The macrophage galactose-type C-type lectin (MGL) receptor expressed on iDCs was shown to be responsible for the binding. Confocal analysis and ELISA done on subcellular fractions of iDCs showed that the Tn-MUC1 glycopeptides colocalized with HLA class I and II compartments after internalization. Importantly, although Tn-MUC1 recombinant protein was bound and internalized by MGL, the glycoprotein entered the HLA class II compartment, but not the HLA class I pathway. These data indicate that MGL expressed on iDCs is an optimal receptor for the internalization of short GalNACs carrying immunogens to be delivered into HLA class I and II compartments. Such glycopeptides therefore represent a new way of targeting the HLA class I and II pathways of DCs. These results have possible implications in designing cancer vaccines. [Cancer Res 2007;67(17):8358–67]

Introduction

Cell transformation is often associated with glycan modifications occurring in the glycoproteins mostly present at the cell surface. Changes in glycan pattern are involved in oncogenic signaling and modulate cell-cell interactions, thus favoring tumor transformation, invasion, and metastatic spread (1).

In addition, altered glycosylation could affect the immunogenicity of membrane proteins and their interaction with the immune system, in particular, with antigen-presenting cells (2–4). A good example of a glycosylated tumor-associated antigen is the mucin, MUC1 (5). The MUC1 glycoprotein is a transmembrane epithelial mucin normally expressed on the apical surface of most simple glandular epithelial cells. Its extracellular domain is mainly made up by a repeated stretch of a 20-amino acid tandem repeated sequence (TR), enriched with S and T residues which are sites of attachment for O-linked glycans (6). In tumors, MUC1 is overexpressed and distributed all over the cell surface, aberrantly glycosylated, and shed in the tissue microenvironment. Although MUC1 expressed by normal mammary epithelial cells carries long, branched O-linked side chains, MUC1 expressed by breast cancer carries shortened O-glycans such as GalNAc1-O-Ser/Thr (Tn) or Galβ1-3GalNAc1-O-Ser/Thr (T, core 1), which may be sialylated to give sialyl-Tn or sialyl-T (6–8). Because MUC1 is highly expressed by a variety of epithelial and nonepithelial cancers, it represents an attractive tumor target for immunotherapy.

Several MUC1 formulations have been tested as immunogens in experimental models (9) and employed in clinical trials, using naked peptide/glycopeptide immunogens mainly based on the MUC1 TR (10–12). Synthetic MUC1 tumor-associated antigen glycoforms have been shown in experimental models to elicit cancer-specific anti-MUC1 antibody responses and override tolerance, whereas the unglycosylated form of the antigen has generally failed to produce effective immune responses (13, 14). It is therefore important to define the possible mechanisms of interaction between the different tumor-associated antigen glycoforms and the immune system which will also provide information for the generation of new immunogens for cancer vaccination.

Various mechanisms of interaction between MUC1 and antigen-presenting cells have been described in normal tissues and in tumors. We have shown that MUC1 expressed by breast cancer cells is a counter-receptor for Siglec-1 (sialoadhesin) expressed by macrophages (15), and that sialylated MUC1 glycoforms present on erythroid cells during erythropoiesis can also bind Siglec-1 (16). Moreover, the tumor-associated MUC1 glycoform carrying sialyl-T has been shown to exert an immunosuppressive effect on antigen-presenting cells affecting differentiation and hampering their capacity to produce interleukin-12 (17).

In order to perform their function, dendritic cells (DCs) are equipped with a full array of highly specialized receptors, including
adhesion receptors and several pattern recognition receptors, such as lectin-like receptors and Toll-like receptors (18). The lectin-like receptors include C-type lectins that recognize specific carbohydrate structures in a Ca\(^{2+}\)-dependent manner. These include DC-SIGN, mannose receptor, Langerin, DEC205, and the macrophage galactose-type C-type lectin (MGL). In contrast to Toll-like receptors, the lectin-like receptors can take up antigen, with the cytoplasmic tail of C-type lectins containing signaling or internalization motifs for antigen processing (18, 19). Through their function in cell–cell adhesion, antigen recognition, and signaling, lectins can regulate Toll-like receptor signaling and influence the outcome of immune responses (18, 19). Moreover, C-type lectins can recognize endogenous self-antigens as well as pathogen-derived ligands, and this can influence the balance between tolerance and immunity (18, 20). Recent studies show that C-type lectin receptors participate in the detection of carbohydrate structures specifically expressed during tumor progression (21).

Here, we have investigated the interaction and the processing of MUC1-based glycopeptides carrying the tumor-associated Tn glycan with DCs. Our results showed that Tn-MUC1 selectively binds immature monocyte-derived DCs (iDCs), whereas the unglycosylated peptide does not. The binding requires cations and is competed out by the addition of GalNAc (N-acetylgalactosamine). The level of binding is correlated with the expression of MGL on DCs, and blocking and transfection experiments indicated that MGL is the receptor of the binding. Moreover, we could show that Tn-MUC1 glycopeptides bound to MGL can be internalized and eventually colocalized with HLA class I and II, whereas the Tn-MUC1 glycoprotein is found only in endosomal and HLA class II compartments.

### Materials and Methods

**Cell lines.** K562 cells and K562 MGL-transfected cell line (KG11; ref. 22) were cultured in RPMI 1640 (HyClone) supplemented with penicillin (Sigma Chemical Company) 100 units/mL, streptomycin 100 μg/mL (Sigma), 2 mmol/L 1-glutamine (Sigma), 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma) and 10% heat-inactivated FCS (Hyclone), 5% CO\(_2\) at 37°C. Neomycin (400 μg/mL; Sigma) was also added to KG11 cells as a selection marker.

**DC generation.** Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient (1,077 g/mL; Pharmacia LKB). Monocytes (CD14+) were cultured in RPMI 1640 (Hyclone) supplemented with penicillin (Sigma), 10 mmol/L MnCl\(_2\), 0.25% Triton X-100, and 2 mmol/L of UDP-GalNAc. Glycosylation was controlled using nanoscale reversed-phase columns (Poros R3, PerSeptive Biosystems) and MALDI-TOF mass spectrometry. The glycopeptides were purified by high-performance liquid chromatography on a Zorbax 300SB-C18 column (9.4 mm × 25 cm; Agilent Technologies) in a 1100 Hewlett Packard system using 0.1% trifluoroacetic acid and a gradient of 0% to 90% acetonitrile.

Quantification and estimation of yield of glycosylation reactions were done by comparison of high-performance liquid chromatography peaks by UV at 210 absorbance using 10-μg weighted peptides as standard. GalNAc glycosylation of peptides generally yielded 80% to 90% recovery. Purified glycopeptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE Pro MALDI-TOF mass spectrometer (PerSeptive Biosystems) equipped with delayed extraction. The MALDI matrix was 2,5-dihydroxybenzoic acid 10 g/L (Aldrich) dissolved in a 2:1 mixture of 0.1% trifluoroacetic acid in 30% aqueous acetonitrile. All mass spectra were obtained in the linear mode.

**Tn-MUC1 glycoprotein.** CHO-Id1 cells stably transfected with a soluble MUC1-murine-IgG\(_{2a}\) fusion construct containing 16 TRs (13) were cultured in Iscove's modified Dulbecco's medium (HyClone) with 600 μg/mL of neomycin. Exploiting the deficiency of UDP-Gal/UDP-GalNAc 4-epimerase in these cells (29), culturing with 1 mmol/L of GalNAc yielded cells expressing soluble Tn-MUC1. The presence of an enterokinase site between the MUC1 and the IgG\(_{2a}\)Fc allowed for the removal of the Fc region. MUC1 was purified from the culture supernatant by MoAb 5E5 (13) affinity chromatography after cleavage with enterokinase. Further details on the production and characterization of this recombinant glycoprotein will be published elsewhere.

**MUC1 binding and competition assay by flow cytometry analysis.** The binding of MUC1 to MGL-expressing cells was detected by flow cytometry. KG11 and DCs on day 4 of differentiation were incubated (2 × 10\(^4\)/sample) with biotinylated MUC1 peptide (MUC13TR) or MUC1 glycopeptides (Tn-MUC19Tn-3TR and Tn-MUC115Tn-3TR) or glycoprotein (Tn-MUC116Tn-3TR) coated onto red fluorescent fluorospheres NeutrAvidin-labeled microspheres (580/605 nm, 1.0 μm; Molecular Probes), with an efficiency of 98%, for 2 h on ice. Uncoated beads were used as negative controls. Cells were washed twice at 4°C with PBS with Ca\(^{2+}\)/Mg\(^{2+}\) + 0.5% bovine serum albumin (BSA; Sigma) or with PBS without Ca\(^{2+}\)/Mg\(^{2+}\) + 0.5% BSA + 5 mmol/L EDTA (MP Biomedical), and the binding was evaluated using a FACS Calibur flow cytometer (Becton Dickinson). For blocking experiments, after Fc receptor block (PBS + 10% HS for 30 min on ice), MoAb MLD-1 was added to DCs for 30 min on ice. After washing, DCs were incubated with the protein- or peptide-coupled beads for 2 h on ice. DCs were washed and the binding inhibition was analyzed by flow cytometry. For the competition assay, GalNAc and N-acetylgalcosamine (GlcNAc) polymers (40 μg/mL; GlycoTech), or unbiotinylated MUC19Tn-3TR and Tn-carrying MUC2 glycopeptides (40 μg/mL) were added to DCs for 30 min on ice. The peptide-coupled beads were added to DCs for 2 h on ice.
**Table 1. Peptide sequences and glycosylation sites**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence and glycosylation sites</th>
<th>Carbohydrate residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC13TR</td>
<td>VSAPDTPRPAGSTAPPAGH</td>
<td>None</td>
</tr>
<tr>
<td>Tn-MUC13TR</td>
<td>VTAPDTPRPAGSTAPPAGH</td>
<td>GalNAc α1-3-O-Ser/Thr</td>
</tr>
<tr>
<td>Tn-MUC15Tn-3TR</td>
<td>VTAPDTPRPAGSTAPPAGH</td>
<td>GalNAc α1-3-O-Ser/Thr</td>
</tr>
<tr>
<td>Tn-MUC2</td>
<td>PTTTPITTTTIVTPTITGQTPTPITTS</td>
<td>GalNAc α1-3-O-Ser/Thr</td>
</tr>
</tbody>
</table>

*α*Heterogeneous glycosylation ranging from 9 to 13 GalNAc residues (see Materials and Methods).

**MUC1 internalization and processing.** After binding in the presence of cations, DCs were washed twice and incubated for 2, 4, and 24 h at 37°C. DCs (8 × 10⁶/sample) were cytospun on SuperFrost Plus microscope slides (Menzel-Glaser) for 3 min at 500 rpm, and fixed with acetone/methanol (1:1; Carlo Erba Reagents) for 3 min at −20°C. After blocking of unspecific sites DCs were incubated with MoAbs HLA I (W6/32 clone) and HLA II (L243 clone) for 1 h at room temperature, then with Texas red–conjugated goat antiserum (H+L; Jackson ImmunoResearch Laboratories). DC acid vesicles were stained with Lyso Tracker red DND-99 (300 mmol/L; Molecular Probes) for 15 min at 37°C. FITC-streptavidin (Sigma) or the Tn-MUC1 MoAb, SE5, followed by FITC-conjugated goat antiserum I(ab), was used to visualize the biotinylated MUC1 glycopeptide. Following washes with PBS, the coverslips were mounted with VECTASHIELD mounting medium (Vector Laboratories, Inc.). Imaging was done by two-photon absorption fluorescence microscopy. A conventional confocal laser-scanning microscope (TE2000E, Nikon) and C1 Nikon Plus scanning head was converted for two-photon absorption use. Two-photon absorption fluorescence was excited by a titanium/sapphire ultrafast laser source (Mai Tai Laser 750-850; Spectra Physics) set at a wavelength of 750 nm and a power output of 850 mW, which corresponds to ~6 mW of average power in the focal plane. The fluorescence signal, collected by the Plan Apochromat 60×/1.40/0.21/oil objective (Nikon) and selected by the HQ335-50 filter (Chroma, Inc.) was fed to a multimode fiber directed to a photomultiplier (R928, Hamamatsu) in the C1 plus controller. Colocalization was done using SIV software (Scientific Volume Imaging).

**Subcellular fractionation.** To detect Tn-MUC13TR, 5Tn-3TR in cytosolic and endocytic/lysosomal compartments of DC lysates, 40 μg/mL of glycopeptide was added to 15 × 10⁶ DCs for 2 h on ice. Cells were washed twice and incubated for 2 h at 37°C. After internalization, DCs were washed with ice-cold PBS and resuspended in an ice-cold isotonic buffer (0.25 mol/L mannitol, 10 mmol/L HEPES, 1 mmol/L EGTA, BSA 2%), supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin; all purchased from Sigma). Cells were mechanically homogenized, centrifuged at 2,600 rpm and again homogenized. DC suspension was centrifuged at 3,000 rpm to eliminate nuclei and unbroken cells (P1 fraction). The supernatant (S1 fraction) was further centrifuged at 11,000 rpm; the pellet (P10 fraction), containing endocytic/lysosomal compartments, was collected and stored at −80°C until use, whereas the supernatant (S10 fraction) was centrifuged at 33,000 rpm to isolate the cytoplasm (S20 fraction). The protein concentration of the two isolated fractions was measured by Bradford assay (Bio-Rad) with BSA (Sigma) as a standard according to the manufacturer's instructions.

**Western blotting.** P10 and S20 fractions (10 μg) were subjected to 10% SDS-PAGE and the resolved proteins were transferred electrophoretically to nitrocellulose transfer membrane (Schleicher & Schuell). After blocking with PBS + 5% BSA for 1 h at room temperature, membranes were incubated with mouse MoAb anti-α-tubulin (1:1,000; B-7 clone; Santa Cruz) or with anti-LAMP-1 (1:300; E-5 clone; Santa Cruz) for 1 h at room temperature, followed by peroxidase-conjugated goat antiserum IgG (1:20,000, H+L; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The protein bands were detected with enhanced chemiluminescence reagents (Amer sham Pharmacia) following the manufacturer’s instructions.

**ELISA assay.** Flat-bottomed 96-well EIA/RIA plates (Corning Incorporated) were precoated overnight with cytotoxic and endocytic/lysosomal fractions (5 μg/well) with or without Tn-MUC13TR (from 0.1 to 10 μg/mL) for the appropriate titration curve. After blocking with RPMI + 10% FCS for 2 h at 37°C, samples were incubated with mouse MoAb antihuman Tn-MUC1 (SE5 clone). Peroxidase-conjugated goat antiserum IgG (H+L; 1:5,000, Jackson ImmunoResearch) was added to the wells, followed by O-phenylenediamine substrate (Sigma-Aldrich). The absorbance was measured at 492 nm using a Labsystem Multiskan MS microplate reader (Lab Systems).

**Results**

**Tn-MUC1 glycopeptides bind iDCs.** The interaction between DCs and Tn-MUC1 glycoforms was studied using MUC1 glycopeptides (60mer corresponding to 3TR, carrying 9 and 15 mol of GalNAc) and non-glycosylated 60mer MUC1 peptide (Table 1). DCs were generated by peripheral blood monocytes using granulocyte macrophage colony-stimulating factor and IL-4. iDCs displayed the characteristic phenotype as shown in Fig. 1A (HLA II-DR*, CD86*, CD83*, and CD14−) and the addition of Tn-MUC1 glycopeptides did not alter this profile. Biotinylated MUC1 peptides were immobilized onto red neutravidin beads and incubated with DCs at day 4 of the culture. A discrete peak of fluorescence was observed when Tn-MUC13TR and Tn-MUC15Tn-3TR were added to DCs in the presence of Ca²⁺ (encircled area, 11% of positive cells), whereas no binding was observed using either uncoated beads or the glycosylated MUC13TR-coated beads (Fig. 1B). No MUC1 glycopeptide binding was observed in the absence of Ca²⁺. Confocal microscopy analysis done on cytosin preparations of DCs after binding confirmed that, in agreement with the flow cytometry data, Tn glycoform-binding occurred only for a small population of DCs, as detected by the red neutravidin beads (green, staining of HLA class I; Fig. 1C).

The results obtained from nine donors are summarized in Fig. 1D. Although there was variability among individuals, the binding occurred only with the Tn-MUC1 glycopeptides and was dependent on the presence of Ca²⁺ (Tn-MUC13TR, Tn-MUC15Tn-3TR, P < 0.01; Tn-MUC13TR, P < 0.05). The amount of GalNAc per repeat seemed to slightly influence the level of binding in favor of the more heavily glycosylated peptide (Tn-MUC15Tn-3TR). The difference was, however, not significant.

**Tn-MUC1 glycopeptides bind the C-type lectin MGL.** One receptor expressed by iDCs, and that has been described as recognizing GalNAc residues, is the C-type lectin MGL (30). Using...
MGL-specific antibody, MGL expression was detected in DCs from day 2 in vitro differentiation, was maximum between days 3 and 4, and was down-regulated by day 5 in the culture conditions used in this study (Fig. 2A). In all donors tested for MUC1 binding, the variability of MGL expression was identified and directly correlated to Tn-MUC1 glycopeptide binding. Donors could be divided into two groups according to MGL expression on iDCs (high expression, MFI > 20; low expression, MFI < 20) and the results of the MUC1 glycoform-binding plotted according to this variable (Fig. 2B). Tn-MUC1glycoform-binding ranged between 11% and 18% in MGL high expression iDCs, whereas it was significantly decreased in iDCs with low MGL expression (P < 0.001).

These results were highly indicative of MGL being the receptor responsible for the binding of the MUC1 glycopeptides. The binding of the glycopeptides to K562 cells transfected with MGL (KG11 cells) was therefore investigated. As can be seen in Fig. 2B, Tn-MUC1glycoform-binding in a Ca²⁺-dependent manner to KG11 cells expressing MGL, but not to the parental untransfected cell line. Thus, MUC1 glycopeptides carrying the tumor-associated Tn glycoform could specifically bind MGL. To confirm that the MUC1 glycopeptides were binding to MGL expressed on iDCs, binding studies were carried out in the presence of MoAb MLD-1, a blocking antibody to MGL. (Fig. 3A). This antibody blocked 80% of the binding of the Tn-MUC1glycoform and 60% of the binding of the Tn-MUC1glycoform. No inhibition of binding was seen with a control antibody (MOPC21).

Moreover, competition experiments employing either carbohydrate polymers (GalNAc and GlcNAc) or Tn-carrying glycopeptides (Tn-MUC1glycoform and Tn-MUC2; Fig. 3B and C) showed that the...
binding of MUC1 glycopeptides was out-competed by the presence of the free GalNAc or the unbiotinylated Tn-MUC19Tn-3TR or a MUC2 glycopeptide carrying Tn (Fig. 3B and C). In contrast, the GlcNAc polymer did not affect the binding of the MUC1 glycopeptides (Fig. 3B). Taken together, these data show that MUC1 glycopeptides carrying the tumor-associated Tn glycoform bind to MGL expressed by iDCs.

Throughout all experiments, inhibition of Tn-MUC19Tn-3TR binding was significantly stronger than the inhibition of Tn-MUC15Tn-3TR binding (MLD-1, P < 0.01; GalNAc, P < 0.01; Tn-MUC19Tn-3TR, P < 0.05; Tn-MUC2, P < 0.001), suggesting that the number of carbohydrate structures was important in determining the avidity of the glycopeptide for the receptor.

Tn-MUC1 glycopeptides are internalized by iDCs via MGL receptor and localize in HLA class I and class II compartments. Several C-type lectins on DCs are able to internalize upon ligand binding (19). Following internalization, exogenous antigens are transported into early/late endosomes and lysosomes. Endocytic vesicles, containing degraded protein fragments, fuse with MIIC vesicles (MHC class II compartments), where binding of peptides to HLA class II occurs. DCs are also able to cross-present exogenous antigens in association with HLA class I. We therefore investigated the ability of MGL to internalize Tn-MUC19Tn-3TR. Following 2 h of binding, the cells were washed and incubated at 37°C for 2, 4, and 24 h. The cells were analyzed by confocal microscopy after staining with anti-HLA I, anti-HLA II MoAbs, and with Lyso Tracker reagent to label the acid vesicles (endosomes and lysosomes). Figure 4 reports results observed at 2 and 24 h (A and B, respectively). MUC1 glycopeptide was visualized by green fluorescence using FITC-conjugated streptavidin (second column). Similar results were also obtained when MUC1 glycopeptide was revealed by anti-MUC1 MoAb staining followed by FITC-conjugated antimouse antibody (data not shown). HLA class I, HLA class II, and endosomal/lysosomal compartments were identified by red fluorescence (first column, rows 1, 2, and 3, respectively). The staining merge and colocalization are shown in column 3 and 4, respectively. After 2 h at 37°C, Tn-MUC19Tn-3TR was found internalized and associated with both class I and II compartments (Fig. 4A, row 1), whereas the glycopeptide was observed colocalized with HLA class II in defined compartments (Fig. 4A, row 2) and with the Lyso Tracker compound in the acid vesicles (Fig. 4A, row 3). Similar results were obtained at further time points tested (4 and 24 h). Figure 4B shows the results obtained at 24 h. Tn-MUC19Tn-3TR was still visible in both HLA class I and class II compartments (Fig. 4B). As expected, the unglycosylated peptide was not internalized and was therefore not present in the different compartments analyzed (Fig. 4C, all rows).

To validate the presence of the MUC1-glycosylated peptide in the cytosol and in the acid vesicles, cell fractions corresponding to the cytoplasm and to endosomal/lysosomal compartments were isolated by differential centrifugation from DCs lysates after binding and 2 h of internalization. Western blotting using antibodies against specific cell compartment markers (LAMP-1 for endosomal vesicles and α-tubulin for the cytoplasm) was used
to confirm the origin of the cell fractions. Fractions were analyzed by ELISA with an antibody that is specific for the Tn-glycoform on MUC1 (MoAb 5E5; Fig. 4D). Tn-MUC116TR was present in both compartments (S20 corresponding to cytoplasm and P10 for the acid vesicles), although it seemed to be more concentrated in the HLA class II fraction (P10).

Recombinant Tn-MUC116TR glycoprotein is internalized through MGL but remains confined to the DC endocytic compartment. Aberrantly glycosylated MUC1 carrying Tn is expressed by the vast majority of breast cancers, and MUC1 can be secreted by these and other carcinomas (7). To determine if MGL could bind and internalize glycoproteins in addition to glycopeptides, recombinant MUC1 carrying the Tn glycan was analyzed. The extracellular domain of MUC1 fused to the Fc region of mouse IgG2a was transfected into CHO-IdlD cells. These cells are deficient in UDP-galactose 4'-epimerase and therefore cannot synthesize O-linked glycans (26) without the addition of exogenous sugars. Growth of the MUC1-Ig-transfected cells in the presence of GalNAc resulted in the secretion of MUC1 carrying only GalNAc (full characterization of this product will be published elsewhere).

After cleavage of the Fc tail, the purified MUC1 glycoprotein was biotinylated, coupled to the fluorescent beads and then incubated with iDCs. Tn-rMUC116TR glycoprotein bound to 17.5% of the iDCs and the binding was abrogated by the addition of the blocking antibody to the MGL receptor (Fig. 5A), indicating that it specifically bound to MGL. Moreover, when cells were incubated at 37°C, the protein was internalized (Fig. 5B and C). However, unlike the MUC1 glycopeptides that could be found in both HLA class I and II compartments, the green fluorescence label associated with the Tn-rMUC116TR protein was found colocalized only with HLA class II and with the endosomal/lysosomal compartments both at 2 (Fig. 5B) and 24 h (Fig. 5C). No association with HLA class I could be observed. These results sustain the hypothesis that the MUC1 antigen in the form of a large glycoprotein with 16 TR is unable to proceed towards HLA class I processing.

Discussion

The development of effective cancer vaccines is one of the major challenges facing immunologists (31). Our increased understanding in the changes of gene expression that occurs with malignancy has lead to the definition of potential targets that can be used for immunotherapeutic intervention (32). Moreover, it has now become clear that changes in posttranslational modifications, particularly glycosylation, play an important role in tumorigenesis and in the immune response to tumor antigens. One of the most promising tumor-associated antigens proposed as a potential target is the MUC1 glycoprotein that is overexpressed and aberrantly glycosylated in the majority of epithelial cancers (33). Thus, whereas MUC1 expressed by normal mammary epithelial cells carries long and branched O-linked glycans, the glycans carried by MUC1 expressed by breast carcinomas are much shorter and include the simple monosaccharide Tn (8).

Recent advances in immunology have prompted vaccine-oriented researchers to focus attention on the mechanisms underlying innate and adaptive immune responses (34). DCs play a critical role in this scenario, devoted to the detection of exogenous antigens carried by pathogens or vaccines, and in dictating the strength, duration, and quality of T and B cell responses. The uptake and processing of aberrantly glycosylated MUC1 by DCs is an important issue to be investigated. It is now becoming clear that specific tumor-associated glycoforms of MUC1 can interact differently with the immune system. We have previously described that the recombinant MUC1 protein carrying the sialyl-T affects DC differentiation and maturation (17), whereas MUC1 purified from tumor cells induced differentiation of DCs, but prevented the development of an efficient Th1-type response (35). We observed that Tn-MUC1 glycoforms did not affect DC differentiation or their ability to produce IL-12p70 (data not shown). Moreover, we show that Tn-MUC1 (MUC1 carrying GalNAc), can

---

**Figure 3.** A, blocking studies of MUC1 glycopeptide binding to DCs using mouse MoAb antihuman MGL (MLD-1; white columns) and MOPC 21 (black columns) as a control. B, competition studies of Tn-MUC1 glycopeptide binding to DCs using GalNAc (white columns) and GlcNAc (black columns) polymers. C, competition studies done using Tn-MUC116TR (black columns) and Tn-MUC2 (white columns) glycopeptides as binders. The results of all experiments were plotted as a percentage of binding inhibition and were done in nine donors. P < 0.05, statistically significant differences.
specifically bind the C-type lectin MGL expressed by iDCs and this binding results in the internalization of the glycopeptides and their delivery into HLA class I and II compartments. In contrast, although recombinant MUC1 protein carrying the Tn glycan binds and can be internalized through MGL, it only seems to be delivered into the HLA class II pathway and not into the cytoplasm and the HLA class I pathway.

The C-type lectin MGL has been shown to be an endocytic receptor (30, 36, 37) and using carbohydrate profiling the specificity of MGL for α- and β-linked GalNAc has been described (37). MGL...
can also recognize terminal GalNAc carried on glycans expressed by the human helminth parasite, *Schistosoma mansoni*, confirming its role in innate immunity as a pattern recognition receptor (38, 39). However, the binding observed with intact tumor adenocarcinoma cells points out the possible role of this receptor in driving immune responses to tumor-associated antigens (38). Tn-MUC1 is the most tumor-specific glycoform and is expressed by the vast majority of breast carcinomas, including ductal carcinoma *in situ*, but not by normal mammary epithelial cells (8). MUC1 glycopeptides carrying Tn can overcome humoral tolerance (13) and designer peptides carrying Tn have been shown to induce antibodies and protect mice from tumor challenge (40).

Recent studies have suggested that a Tn-MUC1 can bind the MGL receptor (41). However, the role of GalNAc was not fully characterized as the glycoprotein was produced by the use of exoglycosidases, which may not yield a pure product and the presence of the Fc region in the fusion protein may have influenced binding. Here, we report studies into the binding of the MGL receptor using enzymatically glycosylated MUC1 glycopeptides and recombinant MUC1 glycoprotein, both carrying the GalNAc (Tn) glycan exclusively, which provide further insight into the binding properties of MGL. Thus, binding of Tn-MUC1 to iDCs could be inhibited by an antibody against MGL and competed out by coincubation of GalNAc carried on synthetic polymers, or a MUC2 peptide carrying GalNAc residues. These results unequivocally show that binding of the receptor is determined by the presence of GalNAc, and that MGL has no requirement for a particular peptide backbone. Binding competition experiments with GalNAc polymers or different Tn-glycopeptide designs suggested that MGL has little if any requirement for a particular pattern or density of Tn structures provided by the peptide backbone. We did find that the Tn-MUC1 glycopeptide with high glycan density (15 Tn residues) seemed to show stronger interaction with MGL than Tn-MUC1 glycopeptide with low glycan density (9 Tn residues). Despite the homogeneous expression of MGL on DCs, Tn-MUC1 glycopeptide binding was observed only in a small group of DCs, as visualized by flow cytometry analysis of Tn-rMUC116TR binding to DCs in the presence and in absence of Ca²⁺, and blocking studies of glycoprotein binding to DCs using MoAb anti-MGL. Biotinylated Tn-rMUC116TR was coated onto red neutravidin beads and was added to DCs at day 4 of the culture. The percentage of positive cells is gated in M1. These results are representative of a single donor. Intracellular localization of Tn-rMUC116TR after binding followed by 2 h (B) or 24 h (C) of internalization. Green, Tn-rMUC116TR, red, cell compartments marked with MoAbs anti-HLA I, anti-HLA II, and with Lyso Tracker red DND 99 for the acid vesicles. White, the colocalization of red and green fluorescence (last column); third column, fluorescence overlap (original magnification, ×60; bar, 10 μm).

**Figure 5.** A, flow cytometry analysis of Tn-rMUC116TR binding to DCs in the presence and in absence of Ca²⁺, and blocking studies of glycoprotein binding to DCs using MoAb anti-MGL. Biotinylated Tn-rMUC116TR was coated onto red neutravidin beads and was added to DCs at day 4 of the culture. The percentage of positive cells is gated in M1. These results are representative of a single donor. Intracellular localization of Tn-rMUC116TR after binding followed by 2 h (B) or 24 h (C) of internalization. Green, Tn-rMUC116TR, red, cell compartments marked with MoAbs anti-HLA I, anti-HLA II, and with Lyso Tracker red DND 99 for the acid vesicles. White, the colocalization of red and green fluorescence (last column); third column, fluorescence overlap (original magnification, ×60; bar, 10 μm).
flow cytometry and confocal microscopy. Such a binding pattern could be due to the level of lectin oligomerization that seems to influence C-type lectin binding (19).

We also monitored the fate of the Tn-MUC1 antigen upon internalization. The results indicate that the glycopeptide, as detected by the specific MoAb 5E5 recognizing the Tn-MUC1 epitope, is found in both HLA class I and II compartments from 2 h and up to 24 h after internalization at 37°C. However, the Tn-MUC1163TR glycoprotein was only found to be associated with the endosomal and HLA class II compartments, and was not associated with the class I pathway. Hitlhold et al. have shown that MUC1 glycoforms present in the ascites of cancer patients can be taken up by DCs via the mannose receptor (42). However, in contrast to Tn-MUC1 endocytosed via MGL, these glycoforms of MUC1 were blocked in early endosomes and did not enter the class II pathway. Our results provide convincing evidence to conclude that glycopeptides and glycoproteins can be delivered into the HLA class II pathway. However, it should be kept in mind that glyco-sylation of particular serines and threonines within the tandem repeats of MUC1 have been shown to inhibit the processing of this glycoprotein (43, 44), although others have shown that glycosylated antigens can be presented in HLA class II (45, 46). Evidence for the presentation of naturally occurring glycopeptides by the HLA class I pathway is less convincing. Certainly, CTL can be induced by designer glycopeptides (47, 48) and MUC1 mers carrying Tn (49), but in the case of the MUC1 glycopeptide, CTL killing of cells expressing endogenous processed glycoprotein could not be shown (49).

The findings reported here are highly significant because they imply that MGL-mediated internalization of GalNAc-carrying glycopeptides in DCs, cells in which cross-priming has been well established, are delivered into cell compartments that are associated with the induction of Th1 immunity. Although this may not be relevant for the immune response to endogenous MUC1 as the recombinate glycoprotein was not found in the class I pathway and glycans can interfere with antigen processing, these data certainly have strong implications for cancer vaccine design. Thus, the demonstration that glycopeptides carrying Tn could efficiently bind MGL, and could be internalized and delivered to the HLA class I and class II pathways, makes this an extremely attractive mechanism for the targeting of DCs and the delivery of tumor-associated epitopes for presentation via HLA class I and class II.

Acknowledgments


Grant support: EC 5th Programme: QLK3-CT-2002-02010, Italian Ministry of Health and Italian Association for Cancer Research (M. Nuti) and Cancer Research U.K. (J. Taylor-Papadimitriou, J. Coleman, G. Picco, and J. Burchell), and the Danish Medical Research Council (M.P. Agervig Tarp). C. Napoletano is supported by a fellowship from Consorzio Interuniversitario Trapiantati d’Organo.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We are most grateful to Thomas Noll and Jochem Gattgens (Institute of Biochemistry Research Center, Germany) for their invaluable contribution of large-scale culture of CHO-ID1 cells and to Dr. P.O. Livingston (Memorial Sloan-Kettering Cancer Center, New York, NY) for kindly providing the Tn-MUC2 glycopeptide.

References

23. Kinga DM, Kozarsky KE, Hobbie L, Krieger M.


Tumor-Associated Tn-MUC1 Glycoform Is Internalized through the Macrophage Galactose-Type C-Type Lectin and Delivered to the HLA Class I and II Compartments in Dendritic Cells

Chiara Napoletano, Aurelia Rughetti, Mads P. Agervig Tarp, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/17/8358

Cited articles
This article cites 49 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/17/8358.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/17/8358.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.