Mucins Bearing the Cancer-associated Sialosyl-Tn Antigen Mediate Inhibition of Natural Killer Cell Cytotoxicity

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ABSTRACT

The sialosyl-Tn (STn) antigen is a mucin-associated carbohydrate antigen expressed by a variety of adenocarcinomas. In the colon, expression of this antigen has been associated with a poor prognosis, independent of tumor stage or histology. The present study was performed to determine whether this adverse clinical outcome might be due to an interaction between STn-positive mucin and natural killer (NK) cell cytotoxicity. Ovine submaxillary mucin (OSM), a mucin highly rich in STn antigen, partially inhibited NK cell cytotoxicity against K562 target cells, but only at high concentrations. Low concentrations of OSM were not inhibitory but became markedly inhibitory in the presence of ammonium ions. Two other STn-positive submaxillary mucins also markedly inhibited NK cytotoxicity when combined with ammonium ions. Removal of sialic acid from OSM reversed the OSM/ammonium-mediated inhibition of NK cell activity. Unlike the submaxillary mucins, two mucins derived from human breast and lung cancer cells which lack the STn antigen, did not inhibit NK cell activity in this system. Likewise, four other non-mucin glycoproteins which lack STn expression did not inhibit NK cells despite having levels of sialic acid that were, in some cases, comparable to submaxillary mucin. These results indicate that mucins bearing the cancer-associated STn antigen can effectively inhibit NK cell cytotoxicity in the presence of ammonium ions. While this NK cell inhibition is likely to be caused by ammonium, mucin markedly enhances this effect, thereby implicating a novel immunomodulatory property of mucin.

INTRODUCTION

Many tumor-associated antigens that are used clinically for diagnosis of epithelial-derived cancers are carbohydrate structures residing on mucin glycoproteins (1). However, little is known about whether such cancer-associated mucin antigens play a role in the biology of the cancer cell.

One antigen of interest is the sialosyl-Tn antigen (2). This structure is a disaccharide linked O-glycosidically to the apomucin polypeptide (Siaα2,6GalNAc-O-Ser/Thr) and as such represents one of the first steps in mucin glycosylation. In the colon, sialosyl-Tn antigen is not expressed in normal mucosa, whereas over half of premalignant adenomatous polyps and approximately 90% of colon cancer tissues express this determinant (3–6). Other adenocarcinomas of gastrointestinal and nongastrointestinal organs commonly express this antigen (6–9).

In colon cancer patients, the expression of sialosyl-Tn antigen by the tumor was found to be an independent factor associated with a poor prognosis (10). The association between sialosyl-Tn expression and an adverse outcome in colon cancer patients implies that this mucin antigen may be participating in the behavior of the colon cancer cell. One hypothesis to explain this finding is that sialosyl-Tn-positive mucin might interfere with immune detection and killing of cancer cell targets. Previous work by Van Rinsum et al. (11) lends support to this idea. In that study, among a panel of sialoligosaccharides that were tested for their ability to inhibit NK cell cytotoxicity of conventional K562 target cells, the sialosyl-Tn disaccharide was found to be the most potent inhibitor. This observation, along with experimental evidence implicating NK cells as important effector cells for controlling colon cancer metastasis (12), prompted us to pursue in depth the interaction between NK cells and sialosyl-Tn-positive mucin. As a prototype sialosyl-Tn-positive mucin we used OSM because virtually all of the carbohydrates on this mucin consist of the sialosyl-Tn antigen (13).

MATERIALS AND METHODS

Reagents

Mucins and Non-Mucin Glycoproteins. OSM was purified from sheep submaxillary glands by previously described methods (14). BSM and PSM were kindly provided by Dr. David Zopf (Bio Carb Inc., Gaithersburg, MD). Human mucins were purified from the ZR-75-1 breast carcinoma cell line (15) and the Calu-3 lung adenocarcinoma cell line (16), using previously described methods involving size exclusion and affinity chromatography. The major oligosaccharides of the mucin from breast cells have been characterized as the Thomsen-Friedenreich (Galβ1,3GalNAc) antigen and its mono- and disialylated derivatives (17). The lung mucin oligosaccharides also contain predominantly conjugated T-antigen in its un-, mono-, and disialylated forms (18). The non-mucin glycoproteins α1-acid glycoprotein, fetuin, ovalbumin, and thyroglobulin were purchased from Sigma Chemical Co. (St. Louis, MO).

Other Reagents. Monoclonal antibody TKH2 (mouse IgG1) which specifically recognizes sialosyl-Tn antigen was a generous gift of Dr. Sen-itaroh Hakomori (Biommrence Institute, Seattle, WA). Peroxidase-labeled rabbit anti-mouse IgG + IgA + IgM and ABTS substrate for ELISA assays were purchased from Zymed Laboratories (South San Francisco, CA). Human erythroleukemia cell line K562, obtained from American Type Culture Collection, was used as the target cell in the cytotoxicity assay. These cells are routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hazleton, Lenexa, KS), glutamine (2 mg/l), penicillin (50 units/ml), and streptomycin (50 µg/ml).

Cytotoxicity Assay

NK cytotoxicity was measured by a 4-h 51Cr-release assay. Heparinized blood from a healthy donor was mixed with an equal volume of Dulbecco's PBS and overlaid onto Ficoll-Paque (Pharmacia). The tube was centrifuged at 650 g for 30 min at room temperature and the mononuclear cells were recovered from the interface. The cells were then washed by centrifugation once with PBS and twice with RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine,

3 The abbreviations used are: NK, natural killer; OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; PSM, porcine submaxillary mucin; Sia, sialic acid; GalNAc, N-acetylgalactosamine; Ser, serine; Thr, threonine; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2'-azino-di(3-ethylbenzothiazoline sulfonic acid).
penicillin (50 units/ml), and streptomycin (50 μg/ml) (assay medium) and were used as NK effector cells.

Two million K562 cells were pelleted, resuspended in 50 μl of assay medium, and labeled with 100 μCi of 51Cr (259 mCi/mg chromium, ICN Radiochemicals, Irvine, CA) for 1 h at 37°C in a 7% CO2 atmosphere. The labeled cells were washed twice with assay medium and used as target cells in all cytotoxicity assays.

Cytotoxicity assays were performed in multiwell tissue culture plates with U-shaped wells (Flow Laboratories, Inc.). In each well, prelabeled K562 cells (5 × 10⁴ cells in 100 μl of assay medium/well) were mixed with various numbers of mononuclear lymphocytes in 100 μl of the same medium to make different effector/target cell ratios. The plates were then centrifuged at 100 × g for 3 min and incubated for 4 h at 37°C. After the incubation the plates were again centrifuged in the same way and 100 μl of the supernatant from each well were harvested for determination of 51Cr counts released in the medium.

All assays were performed in quadruplicate and included control wells for maximal release in 5% Triton X-100 and for spontaneous release in the absence of effector cells but with test compounds for inhibition. The results were expressed as the percentage of specific lysis calculated from the following formula, using mean values of quadruplicates:

\[
\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100
\]

Spontaneous release was always less than 10% of maximal release. Standard deviation values of the quadruplicates did not exceed 10% in any case.

Inhibition of NK Cytoxicity

To examine the inhibitory effect of certain mucins and glycoproteins, each compound was dissolved in 0.2 M ammonium acetate buffer, lyophilized, and then used in the cytotoxicity assay by suspending 51Cr-labeled K562 cells (5 × 10⁴ cells/ml) in the solutions. In each case, controls were dissolved in water and lyophilized prior to using in the cytotoxicity assay. Percentage of inhibition was calculated according to the following formula:

\[
\left(1 - \frac{\% \text{ of specific cytotoxicity (control)}}{\% \text{ of specific cytotoxicity (experimental)}} \right) \times 100
\]

Pronase Digestion

Mucins and glycoproteins were dissolved in 0.2 M ammonium acetate buffer, pH 7, containing 1 mM CaCl₂ at 3 mg/ml. Pronase (1 mg/ml) was added to the solution and the reaction mixtures were incubated at 37°C for 2 days. After the incubation, mixtures were heated in boiling water for 5 min and lyophilized. The lyophilized material was redissolved in assay medium and used in cytotoxicity assays.

Removal of Sialic Acid from OSM

OSM was dissolved in sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂ at 10 mg/ml and digested with neuraminidase (0.1 unit/ml, *Vibrio cholerae*; Calbiochem, La Jolla, CA) for 22 h at 37°C. The digestion in combination with OSM inhibited NK cytotoxicity. As shown in Table 1, in the case of intact (untreated) OSM, a concentration of 1.25 mg/ml was not inhibitory, but at 5 mg/ml there was 30% inhibition of NK cell function. In contrast, Pronase-digested OSM at both concentrations inhibited NK activity almost completely. Curiously, in control samples of OSM that received Pronase buffer alone but no Pronase, there was a dramatic and nearly complete inhibition of NK activity. However, without OSM, the ammonium acetate buffer alone, followed by lyophilization, was not inhibitory. These results suggested that the buffer used for Pronase digestion in combination with OSM inhibited NK cytotoxicity.

Effect of Buffer Components on NK Cell Activity. To further elucidate which component of the buffer was actually exerting this inhibitory effect, sodium acetate and ammonium chloride were individually tested (Fig. 2). The results demonstrated that ammonium ions, but not acetate ions, were responsible for inhibition of NK activity. Even at 10 mM, ammonium chloride inhibited NK activity almost completely, whereas 40 mM sodium acetate was only slightly inhibitory (24% inhibition). A

\[
\text{Analytical Methods}
\]

Protein concentration was determined by the method of Lowry et al. (19). Sialic acid content of mucins and glycoproteins was assayed by the thiobarbituric acid method (20) following hydrolysis of samples in 0.1 M H₂SO₄ at 80°C for 1 h.

\[
\text{RESULTS}
\]

Effect of Ovine Submaxillary Mucin on NK Activity. Fig. 1 depicts the effect of OSM on NK lytic activity. OSM at 5 and 10 mg/ml inhibited the specific lysis of 51Cr-labeled K562 cells by approximately 30 and 70%, respectively, whereas lower concentrations of this mucin were not inhibitory. To determine whether the sialosyl-Tn antigen (as opposed to the peptide backbone or whole mucin molecule) was responsible for this inhibition, OSM was digested with Pronase. The digests were lyophilized, dissolved in the assay medium, and tested for inhibition of NK activity. As shown in Table 1, in the case of intact (untreated) OSM, a concentration of 1.25 mg/ml was not inhibitory, but at 5 mg/ml there was 30% inhibition of NK cell function. In contrast, Pronase-digested OSM at both concentrations inhibited NK activity almost completely. Curiously, in control samples of OSM that received Pronase buffer alone but no Pronase, there was a dramatic and nearly complete inhibition of NK activity. However, without OSM, the ammonium acetate buffer alone, followed by lyophilization, was not inhibitory. These results suggested that the buffer used for Pronase digestion in combination with OSM inhibited NK cytotoxicity.

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Table 1 Effect of Pronase digestion of OSM on NK activity

<table>
<thead>
<tr>
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<th>% of specific cytotoxicity of OSM*</th>
</tr>
</thead>
</table>
|                | 1.25 mg/ml | 5 mg/ml
| Control (medium without mucin) | 50.7       | 50.7       |
| Intact OSM     | 54.3       | 35.5       |
| OSM + Pronase  | 0.8        | 1.1        |
| OSM + buffer (ammonium acetate) | 10.5      | 2.2        |

* All assays were performed by using effector target ratio of 50:1.

Percentage of specific cytotoxicity with ammonium acetate buffer alone (without OSM) after lyophilization was 52.2%.

Free sialic acid at 5 mM inhibited NK cytotoxicity by 8%. However, OSM with this much sialic acid (i.e., 6.5 mg/ml) inhibited NK activity by 40–45%. Although increasing the concentration of free sialic acid to 25 mM can inhibit the NK activity by about 25%, at this concentration many other monosaccharides such as galactose, fucose, mannose, galactosamine, glucosamine, N-acetylgalactosamine, and N-acetylglucosamine, also exhibit 12–31% inhibition (data not shown).

Effect of Other Mucin Molecules and Glycoproteins on NK Activity. To determine whether NK inhibition was specific for OSM, other mucins and nonmucin glycoproteins were dissolved in 0.2 M ammonium acetate buffer, lyophilized, and

neuraminidase was omitted from the incubation mixture. Neuraminidase-digested OSM showed only 13.9% inhibition of NK cytotoxicity in contrast to 91.9% inhibition by the control sample. This suggests that sialic acid residues on OSM are necessary for the interaction between ammonium ions and the mucin.

Fig. 2. Dose-response curve of the effect of ammonium chloride or sodium acetate on NK cell cytotoxicity. Effector:target ratio, 50:1.

![Graph](image)

Fig. 3. Effect of OSM and ammonium chloride on NK cell cytotoxicity. □, OSM alone (2.5 mg/ml); ○, ammonium chloride alone (2.5 mM); △, OSM (2.5 mg/ml) dialyzed against 2.5 mM ammonium chloride. E:T, effector:target.

![Graph](image)

Fig. 4. Effect of removing sialic acid from OSM on NK cell cytotoxicity. ■, OSM alone (2.5 mg/ml); □, OSM dialyzed against ammonium acetate (Am Ac) and lyophilized; ○, OSM digested with neuraminidase and then dialyzed against ammonium acetate and lyophilized. For details of neuraminidase digestion, see "Materials and Methods." Effector:target ratio, 50:1.

![Graph](image)
tested for inhibition in the NK assay (Table 2). BSM and PSM in the presence of ammonium were as potent as OSM in inhibiting NK cytotoxicity, showing virtually 100% inhibition. However, the breast and lung cancer mucins had no inhibitory effect. In addition, the four non-mucin glycoproteins in the presence of ammonium also had no inhibitory effect (fetuin, thyroglobulin, and ovalbumin), or only a minimal one (12.6% inhibition by \( \alpha_1 \)-acid glycoprotein).

Since sialic acid was apparently involved in OSM/ammonium inhibition of NK function, the other mucins and glycoproteins were analyzed for their sialic acid content (Table 2). This analysis revealed that the 3 submaxillary mucins had sialic acid levels between 107 and 240 \( \mu \)g/mg, whereas the breast and lung mucins had very low levels of sialic acid. Of the non-mucin glycoproteins, only fetuin and \( \alpha_1 \)-acid glycoprotein had appreciable levels of sialic acid. However, as shown in Table 2, despite comparable sialic acid contents between PSM and \( \alpha_1 \)-acid glycoprotein, PSM completely suppressed NK activity, whereas the non-mucin \( \alpha_1 \)-acid glycoprotein inhibited NK function by only 12.6%. These results suggest that despite comparable degrees of sialylation, non-mucin glycoproteins are incapable of inhibiting NK cytotoxicity.

Expression of Sialosyl-Tn Antigen on Mucins and Glycoproteins. While sialic acid seems to be important in this system, the preceding experiments indicate that the inhibitory effect is not simply due to the presence of sialic acid in general. Rather, the requirement might be for sialosyl-Tn expression in particular. To test for the expression of sialosyl-Tn antigen on mucins and glycoproteins ELISA was performed with the use of monoclonal antibody TKH2. As shown in Fig. 5, only the submaxillary mucins (OSM, BSM, PSM) expressed sialosyl-Tn antigen, whereas the breast cancer and lung cancer mucins, as well as the four non-mucin glycoproteins which did not inhibit NK function, did not express sialosyl-Tn antigen.

DISCUSSION

Qualitative differences between normal and cancerous colonic mucin have been recognized for quite some time, but little is known about the role of mucin in tumor cell biology or immunology. Several lines of evidence support an active role for mucin in colon cancer behavior. First, highly mucinous colon cancer cells are more metastatic than those with less mucin production, and by inhibiting mucin synthesis, metastatic capability can be impaired (21). Second, expression of total sialomucins by histologically normal colonocytes at surgical resection margins of colon cancer specimens correlates with a greater risk of recurrent cancer (22, 23). Furthermore, expression of mucin-associated sialosyl-Tn antigen by colon cancer tissues has been correlated with an adverse outcome (10).

Table 2 Sialic acid content and NK inhibitory activity of glycoproteins

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>% of inhibition of cytotoxicitya</th>
<th>Sialic acid content (( \mu )g/mg)</th>
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</thead>
<tbody>
<tr>
<td>Mucins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSM</td>
<td>99.5</td>
<td>240</td>
</tr>
<tr>
<td>BSM</td>
<td>99.5</td>
<td>140</td>
</tr>
<tr>
<td>PSM</td>
<td>98.8</td>
<td>107</td>
</tr>
<tr>
<td>Lung mucin</td>
<td>0.4</td>
<td>11</td>
</tr>
<tr>
<td>Breast mucin</td>
<td>4.8</td>
<td>3</td>
</tr>
<tr>
<td>Non-mucin glycoproteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha_1 )-Acid glycoprotein</td>
<td>12.6</td>
<td>115</td>
</tr>
<tr>
<td>Fetuin</td>
<td>3.9</td>
<td>50</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>-4.8</td>
<td>0</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>-6.3</td>
<td>0</td>
</tr>
</tbody>
</table>

To extend our understanding of the biological role of mucin, the present study focused on one particular arm of the human immune response, namely NK cells. Using OSM as a mucin that is highly rich in sialosyl-Tn antigen, we noted that this mucin by itself could inhibit NK cell function by 29.2% at 5 mg/ml, and by 68.5% at 10 mg/ml; lower concentrations, however, were not inhibitory. This confirms a previous report by van Rinsum et al. (11) in which 5 mm OSM (which corresponds to approximately 6.5 mg/ml, based on sialic acid concentration) inhibited NK cytoplastic activity by 47.8%.

Unexpectedly, while investigating whether Pronase-digested OSM might be inhibitory to NK cells, we disclosed a striking inhibitory potential of OSM in the presence of ammonium acetate, even at low concentrations of OSM which were not otherwise inhibitory. Further clarification of this phenomenon revealed that it was the ammonium ion, not the acetate moiety, which was responsible, and that this inhibition was not due to impaired viability of the effector NK cells.

It is known that ammonium is an effective inhibitor of NK cell function (24). In our experiments, more than 5 mm ammonium chloride was required to show inhibition, whereas medium containing 2.5 mm ammonium chloride did not inhibit NK cell function. However, when the latter concentration was combined with 2.5 mg/ml OSM (which was in itself also not inhibitory), a marked inhibition was observed. In assays that used ammonium acetate buffer followed by lyophilization, it is difficult to know what concentration of ammonium is in the final assay mixture, since this buffer is volatile. Without mucin, however, ammonium acetate buffer after lyophilization had no effect on NK activity. We therefore believe that the ammonium ion is responsible for the NK cell inhibition in this system, but the mucin acts as a concentrator and carrier of ammonium to enhance this effect. This novel function of mucin as a carrier of cations is supported by recent studies documenting that normal intestinal mucin is involved in the transport of iron and other cations (25).

The ability of OSM to inhibit NK cells in the presence of ammonium ion was dependent upon sialic acid, since removal of sialic acid by neuraminidase abolished the inhibitory
capability of OSM. Since virtually all of the sialic acid residues on OSM occur as the sialyl-Tn antigen, this raised the question of whether sialylation in general, or sialyl-Tn antigen specifically was responsible for inhibiting NK cytolitic function in the presence of ammonium. To address this issue, other glycoproteins and mucins were tested in the assay in the presence of ammonium. Of four non-mucin glycoproteins tested, none was inhibitory. The two glycoproteins which lack sialic acid (ovalbumin and thyroglobulin) had no inhibitory capacity, but even the other two compounds (α1-acid glycoprotein, fetuin) which had levels of sialic acid comparable to that of mucin failed to inhibit NK cell function. This suggests that non-mucin glycoproteins, regardless of sialic acid content, are incapable of mediating NK inhibition. Of the five mucins studied, those from breast and lung were not inhibitory, and they contain little measurable sialic acid (Table 2) and no sialyl-Tn antigen (Fig. 5). Only the three submaxillary mucins inhibited NK cell activity. These submaxillary mucins share several features in common, including relatively simple oligosaccharide structures, similar degrees of sialylation, and the presence of sialyl-Tn antigen.

Thus, our data indicate that sialomucins bearing sialyl-Tn antigen, in the presence of ammonium ion, are very effective inhibitors of NK cell function. Other studies have described an inhibitory effect of sialomucins on NK-mediated lysis of target cells (11, 26). Moriarty et al. (26) observed that rat mammary tumor cells which are resistant to NK-mediated lysis express elevated levels of sialomucin. Van Ronsum et al. (11) isolated O-linked and N-linked sialooligosaccharides from glycoproteins and observed that the sialyl-Tn disaccharide was the most potent inhibitor of NK cell function. Although neither study specifically looked for a role of ammonium ion in the NK assay, it is intriguing that in the first study, freshly harvested tumor cells were treated with ammonium chloride to lyse RBC (26), and in the second study, ammonium acetate buffer was used to elute many of the oligosaccharides (11). This raises the possibility that even in these studies, ammonium ion may have contributed to the inhibitory effect seen with the sialomucins or sialooligosaccharides. In the present study, the procedure for purifying OSM does not involve the use of ammonium, so any effect seen with OSM alone is not due to ammonium.

The results of our experiments suggest that sialomucins, particularly in the presence of ammonium ion, are able to interfere with NK cell cytotoxicity of tumor targets. Whether a similar mechanism may be occurring in vivo can only be speculated upon at present. It is possible that this phenomenon might be occurring at the level of the cell surface or in the circulation, since mucins often circulate in cancer patients' sera (27, 28). In either case, we hypothesize that in the presence of elevated levels of sialyl-Tn-positive mucin, even a small amount of ammonium ion (perhaps physiological concentrations) may be enough to impair NK cell function, thereby providing immune escape for the cancer cell. As such, mucin may be playing a "cytoprotective" role for the cancer cell, allowing it to escape detection or killing by the immune system. Such a cytoprotective role has recently been described for the human breast mucin antigen DF3, which protects Schistosoma mansoni organisms from antibody-dependent cytotoxicity by eosinophils (29). Further investigation will help to define the role of mucins as immunomodulating agents, and whether they interfere with other cytotoxic effector cell mechanisms.

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REFERENCES


MUCIN INHIBITION OF NK CELL CYTOTOXICITY

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