Cell Surface Sialomucin and Resistance to Natural Cell-mediated Cytotoxicity of Rat Mammary Tumor Ascites Cells

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ABSTRACT

MAT-B1 and MAT-CI ascites sublines of the 13762 rat mammary adenocarcinoma both contain sialomucin as a major cell surface component and are resistant to cytolysis by normal rat spleen lymphocytes [3 ± 2% (SD) and 0 ± 1%, respectively]. Susceptibility to lysis did not increase following treatment of cells with neuraminidase, fucosidase, or α- or β-galactosidase. Treatment with trypsin significantly increased the susceptibility of MAT-B1 (14 ± 3%) but not MAT-C1 (5 ± 2%). Following 1 month in culture, the sialomucin content of MAT-B1 cells dropped from 30% to 8% (determined by glucosamine labeling) and natural cell-mediated cytolysis increased to 16 ± 4%, whereas the sialomucin content and susceptibility of MAT-C1 cells did not change. The results indicate that the relatively minor changes associated with removal of cell surface sialic acid or fucose residues do not result in increased susceptibility of the ascites cells to cytolysis. However, susceptibility of MAT-B1 cells to lysis by normal rat spleen lymphocytes was inversely correlated with the amount of major glycoprotein (r = -0.96).

INTRODUCTION

NK1 and NC are thought to play a significant role in arresting tumor cell growth and preventing metastasis (1-3). Although NK and NC activities differ primarily in that targets used in NK assays are of lymphoid origin, it is unclear whether there is any difference in the mechanism of cytolysis displayed by NK and NC (4). Furthermore, the factors which determine whether tumor cells are susceptible to NK- or NC-mediated cytotoxicity are poorly understood. NK resistance (5) and metastatic ability (6) have been correlated with cell surface sialic acid content and NK susceptibility has been associated with expression of asialoglycoprotein receptors [GalNAcβ1→Galβ1→GlcN-ac-α-mannose] (7). A variety of monosaccharide phosphates have been shown to inhibit NK activity (8, 9) as well as some N-linked oligosaccharides (10). Furthermore, some cell surface glycoproteins have been shown to inhibit the binding of NK to YAC-1 targets (11). Several studies have shown that susceptibility to NK cytolysis increases on culturing (1), although the molecular basis for this is not understood.

MAT-B1 and MAT-CI ascites sublines of the 13762 rat mammary adenocarcinoma both contain a major cell surface glycoprotein which has been extensively characterized (12-14). This glycoprotein is a sialomucin containing approximately 70% by weight of O-linked oligosaccharides from 3 to 6 residues long (13). The structures of the major oligosaccharides have been determined (14), and differences in the type and content of sialic acid have been demonstrated between the two cell lines (15). Furthermore, previous work has shown that MAT-B1 cells, when maintained for some time in culture, fail to express the major glycoprotein (16).

Both MAT-B1 and MAT-C1 cells are resistant to natural cell-mediated cytolysis by normal rat spleen lymphocytes (17). The goal of this work was to examine the role of sialomucin in resistance to cytolysis by examining the properties of glycosidase-treated, trypsin-treated, and cultured cells.

MATERIALS AND METHODS

All chemicals were reagent grade unless otherwise specified. d-1-14C Glucosamine hydrochloride (45-60 mCi/mmol) and d-6-3H Glucosamine hydrochloride (10-30 Ci/mmol) were purchased from New England Nuclear. 51Cr (150 mCi/mg) was from ICN. β-Galactosidase (Aspergillus oryzae), α-galactosidase (green coffee beans), α-fucosidase (bovine epidymis), trypsin (1-tosylamido-2-phenylethyl chloromethyl ketone-treated bovine pancreas), and trypsin inhibitor (soybean) and Histopaque were obtained from Sigma; neuraminidase (Vibrio cholerae) was a product of Worthington. RPMI 1640, fetal bovine serum, glutamine, and antibiotics were from Gibco. Complete media consisted of RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine, and 25 mM N-2-hydroxy-ethylpiperazine-N'-ethanesulfonic acid.

Rat Mammary Tumor Ascites Cells. MAT-B1 and MAT-C1 refer to ascites sublines of 13762 rat mammary adenocarcinoma which arose by passage of the MAT-B and MAT-C lines (Mason Research Laboratories, Worcester, MA) in the laboratory of K. L. Carraway, Tumor-bearing rats were kindly provided by K. L. Carraway, University of Miami School of Medicine. Cells were maintained by weekly transfer in 2-3-month-old female Fischer 344 rats and through frozen stocks. The properties of these cell lines have been described previously (12). To initiate culturing of the ascites cells, cells were removed from the peritoneal cavity aseptically with a syringe and were suspended in complete medium at a concentration of 1 x 10⁵ cells/ml. Cells were maintained at 37°C in a 5% CO₂/95% air humidified atmosphere and subcultured twice weekly.

Ascites cells were washed 3 times in PBS, pH 7.4, prior to any other treatment. Glycosidase digestions were performed by incubating 1 x 10⁷ cells in 0.4 ml of 0.025 m sodium citrate, pH 5.5-0.10 m NaCl containing β-galactosidase (0.25 mg), α-galactosidase (0.5 unit), α-fucosidase (0.02 unit), or neuraminidase (0.1 unit) for 30 min at 37°C. Trypsin (0.05 mg) treatment was performed in PBS, pH 7.4 at 37°C for 30 min, and trypsin inhibitor (0.1 mg) was added following incubation. Cells were pelleted, 50-μl aliquots of the supernatants were taken for sialic acid analysis, and cells were washed twice with PBS, pH 7.4. The viability following these treatments was typically >80%. Sialic acid was determined by the thioacetamide assay (18); supernatants from trypsin-treated cells were hydrolyzed with 0.05 m H₂SO₄ at 80°C for 1 h prior to analysis.

51Cr Release Assay. Spleen lymphocytes from normal 2-3-month-old female Fischer 344 rats were prepared as described by Reynolds et al. (19). Briefly, mononuclear cells in Hanks' balanced salt solution were separated by density gradient centrifugation over a cushion of Ficoll-Hypaque. Cells were washed, counted, and resuspended in complete media. YAC-1 cells, MAT-B1 cells, or MAT-C1 cells (5 x 10⁶ cells) were labeled with 100 μCi 51Cr by incubation for 1 h at 37°C. The cells were washed 3 times, counted, and resuspended at 1 x 10⁶ cells/ml. Labeled target cells (10⁴ cells) were incubated with spleen lymphocytes at effector:target ratios of 100:1 to 12.5:1 in triplicate wells of round-bottomed microtiter plates in a total volume of 200 μl. Following incubation for 8 h, soluble 51Cr was determined by gamma counting of
100-µl samples of all cultures. Spontaneous release was measured in the absence of effector, and total releasable label was determined by treating the target cells with detergent. NK activity is expressed as % of specific $^{51}$Cr release

$$\text{% of specific }^{51}\text{Cr release} = 100 \times \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}}$$

Glycosamine Labeling of Ascites and Cultured Cells. Cultured or washed ascites cells ($5 \times 10^6$ cells) were incubated with 5 µCi [³⁵S]-glucosamine (cultured) or 50 µCi [³H]glucosamine (ascites) in a total of 1 ml complete media at 37°C for 4 h. Cells were washed twice with PBS and suspended in 0.5 ml PBS. Absolute ethanol (1.1 ml) was added, and samples were centrifuged at 10,000 x g for 10 min. The pellet was suspended in 0.5 ml 4 M guanidine-HCl, and 50 µl were layered onto a CsCl gradient containing 4 M guanidine-HCl and analyzed as described previously (13). Fractions of approximately 0.5 ml were collected, and the entire fraction was mixed with 5 ml Instagel for scintillation counting. Densities were determined from the differences in filled and dry weight of a calibrated 100-µl pipet. Previous work has demonstrated that the major glycoprotein is effectively recovered from mannosamine-labeled cells by ethanol precipitation followed by cesium chloride density gradient centrifugation. The glycoprotein bands at a density of approximately 1.4 g/ml and accounts for approximately 50% of the total cpm in the ethanol-precipitable fraction of mannosamine-labeled cells (15).

In a separate density gradient separation, fractions with a density between 1.36 and 1.44 g/ml were pooled, dialyzed/concentrated against distilled water, incubated for 16 h at 45°C with 0.05 M NaOH-1 M NaBH₄, and neutralized with acetic acid. The sample was fractionated on a Bio-Gel P-4 column equilibrated with 0.1 M pyridine acetate, pH 6.0.

RESULTS

Susceptibility of Ascites Cells to Cytolysis. Previous work has shown that normal Fischer 344 rat spleen lymphocytes are cytotoxic toward YAC-1 targets in a $^{51}$Cr release assay. In our hands, normal rat spleen cells resulted in 70 ± 7% cytotoxicity of YAC-1 cells during an 8-h incubation at an effector:target ratio of 100:1. For all assays described below, the cytotoxicity toward YAC-1 cells was determined to ensure the activity of the spleen lymphocyte preparation.

Both MAT-B1 and MAT-C1 cells were resistant to lysis by preparations of normal rat spleen lymphocytes in an 8-h $^{51}$Cr release assay (Fig. 1). Nevertheless, MAT-B1 and MAT-C1 cells were resistant to lysis by preparations of normal rat spleen lymphocytes in an 8-h $^{51}$Cr release assay (Fig. 1). However, trypsin-treated MAT-B1 cells did show a reproducible increase in cytotoxicity (Fig. 1; Fig. 2B).

The amount of sialic acid released during neuraminidase or trypsin treatment (Fig. 1) was compared with values of total cellular sialic acid (18 and 56 nmol/10⁷ cells for MAT-B1 and MAT-C1, respectively) to determine the percentage released. Neuraminidase results in a loss of 90% (MAT-B1) versus 40% (MAT-C1) of cellular sialic acid, whereas trypsin results in a loss of 44% (MAT-B1) versus 16% (MAT-C1). Clearly, the exposure of mucin-type galactose residues by removal of sialic acid does not result in cytolyis in this system. Since the major glycoprotein ASGP-1 accounts for greater than 80% of the total cellular sialic acid, it is assumed that trypsin treatment, particularly of MAT-B1 cells, results in a significant loss of this glycoprotein. Increased cytotoxicity is observed only when a substantial portion of the entire glycoprotein is removed. Since more cell surface material is released from MAT-B1 (44%) versus MAT-C1 (16%) following trypsin treatment, it is not surprising that the susceptibility of trypsin-treated MAT-B1 cells is higher than that of MAT-C1.

Cytotoxicity and Expression of Major Glycoprotein of Cultured Cells. Following 2 months of culturing, the lytic susceptibility of MAT-B1 cells increased from 3 to 16%, whereas
MAT-C1 susceptibility ranged between 0 and 3% (Fig. 1; Fig. 2C). Fig. 3 shows the effect of culturing on expression of the major glycoprotein, ASGP-1. This glycoprotein has a density of 1.4 g/ml in CsCl-4 M guanidine-HCl gradients and comprises 35–40% of the [3H]glucosamine label incorporated by MAT-B1 or MAT-C1 cells. Cells cultured for 1 week incorporated 25–30% of the total [14C]glucosamine into ASGP-1. Following 2 months, MAT-C1 cells still formed ASGP-1 (25% of total 14C) whereas MAT-B1 cells incorporated less than 10% of the [14C]glucosamine into fractions with densities between 1.35 and 1.45 (Fig. 3).

Both the expression of the major glycoprotein and susceptibility were determined for cultured cells at various times following the initiation of culturing. The expression of ASGP-1 was determined by density gradient analysis of [14C]glucosamine-labeled cells. The percentage of ASGP-1 was calculated by dividing the dpm recovered in the peak (density, 1.35–1.45 g/ml) by the total dpm recovered from the entire gradient. A negative correlation was observed between cytotoxicity and expression of ASGP-1 for MAT-B1 cells (Fig. 4; r = -0.96). The loss of ASGP-1 occurred between 6 weeks and 2 months of culturing. Neither the cytotoxicity nor the expression of ASGP-1 appeared to change for MAT-C1 cells following 3 months in culture.

In order to determine whether low levels of ASGP-1 were still being synthesized in MAT-B1 cells following 6 weeks of culture, the small amount of [14C]glucosamine-labeled material with density between 1.35 and 1.45 g/ml from cultured cells was compared with 3H-labeled ASGP-1 from ascites cells. These samples were dialyzed, treated with alkaline borohydride, and chromatographed on Bio-Gel P-4. The results demonstrate that the 14C-labeled material produced by cultured MAT-B1 cells is not degraded by alkaline borohydride as is 3H-labeled ASGP-1 (Fig. 5). Thus MAT-B1 cells, following 6 weeks in culture, are apparently devoid of the major glycoprotein.

DISCUSSION

Terminal galactose residues have been shown to be critical for binding of cells and glycoproteins to receptors on hepatocytes (20) and macrophages (21). The findings that susceptibility to NK lysis is negatively correlated with cell surface sialic acid (5) and is associated with the presence of asialo-GM2 (7) suggested that galactose might also play a role in recognition by NK. Our results demonstrate that exposure of galactose residues associated with mucin-type glycoproteins does not result in increased susceptibility to NC-mediated cytotoxicity. Neuraminidase treatment was also not effective in increasing NK susceptibility of an NK-resistant cell line (22). In our system, susceptibility increased only under conditions where substantial amounts of the sialomucin were removed from the cell surface, through either trypsin treatment or culturing.

Previous work has demonstrated that susceptibility to NK cytolysis can be increased following culturing (1), although the molecular basis for this change has not been examined. Our results indicate that the loss of ASGP-1, a major cell surface sialomucin, is correlated with susceptibility to cytolysis in
MAT-B1 cells. Whether the sialomucin prevents binding of cytolytic cells to the tumor cells or renders the tumor cells resistant to cytolytic factors (23) cannot be determined on the basis of these studies. Factors other than the sialomucin may also play a role in the resistance to lysis, since the cytotoxicity of cells devoid of the sialomucin (cultured MAT-B1 cells) is still fairly low (16%). Our results have not eliminated the possibility that the increased susceptibility of MAT-B1 cells during culturing may be due to the emergence of a minor NC-sensitive clonal population of MAT-B1 cells.

Several other types of tumors have been shown to contain cell surface sialomucins, including human (24) and mouse (25) melanomas, mouse mammary adenocarcinomas (26), and rat hepatomas (27). The sialomucin epiglycanin has been implicated in the masking of histocompatibility antigens in the allotransplantable mouse mammary tumor TA3-Ha (26). ASGP-I is very similar to epiglycanin except that the structures of the O-linked oligosaccharides are not identical (14, 28). The relationship between sialomucin and NK resistance has not been reported previously. Since resistance to natural cell-mediated cytotoxicity has been correlated with increased tumorigenicity (3) and metastatic ability (2) and since resistance of MAT-B1 cells to lysis is inversely correlated with expression of sialomucin, these cell surface glycoproteins may play a critical role in NC resistance and in the evasion of the immune response by tumor cells.

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REFERENCES

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