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Secreted MUC1 Mucins Lacking Their Cytoplasmic Part and Carrying Sialyl-Lewis a and x Epitopes From a Tumor Cell Line and Sera of Colon Carcinoma Patients Can Inhibit HL-60 Leukocyte Adhesion to E-Selectin-Expressing Endothelial Cells

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Abstract A secreted MUC1 mucin from the spent medium of the colon carcinoma cell line COLO 205 carrying sialyl-Lewis a and x epitopes (H-CanAg) was purified by trichloroacetic acid precipitation and Superose 6 gel filtration. The purified H-CanAg inhibited adhesion of the leukocyte cell line HL-60 to E-selectin transfected COS-1 cells or interleukin-1β (IL-1β)–activated human umbilical vein endothelial cells. Sera from two patients with advanced colon carcinoma containing high concentrations of sialyl-Lewis a and x activity inhibited HL-60 cell adhesion to E-selectin–expressing COS-1 cells and IL-1β–activated endothelial cells. After affinity column absorption of the sialyl-Lewis a activity, the sera also lost most of their sialyl-Lewis x activity and at the same time their adhesion inhibitory effect. A large part of the sialyl-Lewis a/x activity in the two patients was found in fractions containing mucins having a MUC1 apoprotein, as shown by its size, and reactivity with the two anti-MUC1 apoprotein monoclonal antibodies, Ma552 and HMFG-2. The cell-adhesion inhibitory effect of the purified sialyl-Lewis a–carrying MUC1 mucin fraction from the sera of the two patients was stronger than that of smaller sized sialyl-Lewis a–carrying mucin-type glycoproteins also found in the patient sera. The MUC1 mucin fraction secreted by the COLO 205 cells and from the two sera were all shown to lack their C-terminal portion, in contrast to the MUC1 mucin from cells. It is hypothesized that sialyl-Lewis a– and/or x–containing mucins, especially MUC1, secreted by tumors can interact with E-selectin on endothelial cells and thus inhibit leukocyte adhesion.

Key words: glycoprotein, cell adhesion, COLO 205 cell line, affinity chromatography, MUC1 mucin

The cancer-associated carbohydrate epitope sialyl-Lewis a (NeuAcα2-3Galβ1-3[Fucα1-4]-GlcNAc-) was first detected by the 19-9 monoclonal antibody [Magnani et al., 1982] and its expression shown to be due to an induction of an α2,3-sialyltransferase. This enzyme made a precursor oligosaccharide that was fucosylated by the Lewis encoded enzyme [Hansson and Zopf, 1985]. The sialyl-Lewis a epitope is expressed in a tumor-associated way in colon and pancreatic cancers, although it is naturally present in bile and saliva [Zopf and Hansson, 1988]. Patients with gastrointestinal and pancreatic cancer have elevated levels of sialyl-Lewis a– and/or sialyl-Lewis x–containing mucins in their sera [Magnani et al., 1983; Kannagi et al., 1986].

The colorectal carcinoma cell line COLO 205 has been found to produce two mucin-type glycoproteins carrying the cancer-associated sialyl-Lewis a epitope. One of them, H-CanAg (heavy cancer antigen), had an estimated molecular mass of 600–800 kDa by SDS-PAGE and a high carbohydrate content (85%, by weight). The oligosaccharide chains had an average lengths of about 15 sugar residues and were rich in fucose and sialic acid. H-CanAg had an amino acid composition similar to MUC1 and an apoprotein reacting with antibodies directed to the MUC1

Abbreviations used: BSA, bovine serum albumin; IFMA, immunofluorometric assay; MAb, monoclonal antibody; TCA, trichloroacetic acid.

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apoprotein. It was concluded that the apoprotein was encoded by the *MUC1* gene [Baeckström et al., 1991, 1993]. Another mucin secreted by the COLO 205 cells, L-CanAg (light cancer antigen), has recently been shown to have CD43 as its apoprotein [Baeckström et al., 1995] and to bind to E-selectin [Zhang et al., 1994].

E-selectin or ELAM-1 [Bevilacqua et al., 1989] is one member of the selectin family [Lasky, 1992; Cummings and Smith, 1992] and recognizes carbohydrate ligands, such as sialyl-Lewis x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc-). It is an endothelial membrane protein containing an N-terminal lectin domain that is expressed on endothelial cells following induction by cytokines such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α). Its physiological function is to mediate the first adhesion of leukocytes to the endothelial vessel wall, resulting in a "rolling" phenomenon. Other cell-adhesion molecules mediate the following integrin-mediated adhesion and leukocyte extravasation [Lasky, 1992].

The sialyl-Lewis a epitope can also serve as a specific and as an effective ligand for E-selectin [Berg et al., 1991; Larkin et al., 1992; Takada et al., 1991]. A synthetic sialyl-Lewis a–protein conjugate can bind to E-selectin [Berg et al., 1991; Dejana et al., 1992], and liposomes containing sialyl-Lewis a–carrying glycosphingolipids can inhibit the adhesion of leukocytes and HL-60 cell to E-selectin–expressing cells, although leukocytes and HL-60 cells only express sialyl-Lewis x antigen (sialyl-Lewis a negative) [Takada et al., 1991].

The presence of membrane-bound sialyl-Lewis a and sialyl-Lewis x epitopes on cancer cells in the form of glycosphingolipids [Magnani et al., 1982] and mucins [Baeckström et al., 1991] has raised the question whether these epitopes could interact with E-selectin–expressing endothelial cells and thus promote vascular invasion and metastasis [Magnani, 1991]. A few reports have indicated that metastatic lesions contain more sialyl-Lewis x–positive cells than the original tumor [Matsushita et al., 1990; Matsusako et al., 1991] and that cancer sera can inhibit tumor cell adhesion to E-selectin, which is sialyl-Lewis x–dependent [Sawada et al., 1994].

Here we report that purified H-CanAg can inhibit the adhesion of sialyl-Lewis x expressing HL-60 promyelocytic cells to either E-selectin transfected COS-1 cells or IL-1β–activated human umbilical vein endothelial cells. It is also shown that sera from two colon carcinoma patients containing high concentrations of sialyl-Lewis a– and x–carrying MUC1 mucins could inhibit HL-60 cell adhesion to E-selectin–expressing cells.

MATERIALS AND METHODS

Mouse monoclonal antibody against E-selectin, BBA1, was from British Bio-technology (Oxon, UK). C50, a monoclonal IgM antibody recognizing the sialyl-Lewis a and sialyl-lactotetra epitopes, MAb C241 against sialyl-Lewis a, isotype IgG, and Ma552, a MAb reacting with the MUC1 apoprotein [Baeckström et al., 1993], were gifts from CanAg Diagnostics (Gothenburg, Sweden). HMFG-2, a MAb recognizing the MUC1 apoprotein, and the CT-1 rabbit antiserum against C-terminal MUC1 [Pemberton et al., 1992], and the corresponding CT-1 peptide were kind gifts from Dr. Joyce Taylor-Papadimitriou and Dr. Sandra Gendler (ICRF, London, UK). Superose 6 was from Pharmacia (Uppsala, Sweden), NHS-d-biotin, papain, L-cysteine, and iodoacetamide from Sigma (St. Louis, MO), and streptavidin-fluorescein from Amersham (Amersham, UK). Bovine serum albumin (BSA) and human endothelial cell growth factor were obtained from Boehringer Mannheim (Mannheim, Germany). Preparation of europium-labeled C50 antibody and europium-labeled goat-antimouse antibody has been described previously [Baeckström et al., 1991]. MAb BBA1 was labeled with NHS-d-biotin according to the supplier's description. Iscove's modified Dulbecco's medium, RPMI 1640 medium, 199 medium, fetal calf serum, and trypsin were obtained from Gibco (Paisley, UK). Nu-serum was from Collaborative Research (Bedford, MA). F-8 microtiter strips with a plastic surface for tissue culture (Nunclon) were specially prepared by Nunc (Roskilde, Denmark). COS-1 cells (CRL 1650; American Type Culture Collection, Rockville, MD) were grown in Iscove's modified DMEM medium containing 10% fetal calf serum, and HL-60 cells (CCL 240; American Type Culture Collection) were grown in RPMI 1640 medium with 10% fetal calf serum. Human umbilical vein endothelial cells were obtained from Dr. Bo Risberg (East Hospital, Gothenburg, Sweden) and were grown in 199 medium supplemented with 20% fetal calf serum, heparin, and human endothelial cell growth factor. The expression

vector pELAM-1 (membrane-bound E-selectin) and its host strain MC1061/p3 were kind gifts from Dr. Brian Seed (Harvard Medical School, Boston, MA) [Bevilacqua et al., 1989]. Plasmid DNA was prepared by alkaline lysis of the bacteria, and the plasmid DNA was purified by two consecutive CsCl gradient centrifugations.

Preparation of Monoclonal Antibody CSLEX1

The hybridoma CSLEX1 (HB 8580; American Type Culture Collection) was grown in RPMI 1640 medium supplemented with 15% fetal calf serum. The spent culture supernatant (about 20 μg/ml of CSLEX1 antibody) was precipitated by 50% saturated ammonium sulphate. The precipitates were thoroughly dialyzed against 2 mM sodium phosphate (pH 6.5) and subjected to a Sephacryl S-300 column for gel filtration with 20 mM sodium phosphate, pH 6.5, as elution buffer. Fractions were analyzed for mouse immunoglobulin activity using immunofluorometric assay (with rabbit-antimouse immunoglobulin as catching antibody and europium-labeled goat-antimouse as detecting antibody). The mouse immunoglobulin fractions were combined and subjected to an antimouse IgM affinity column (AbZorb; PIERCE, Rockford, IL). After washing, the column was eluted with buffer containing 0.1 M glycine and 0.5 M NaCl, pH 2.4, and the eluates were collected in 2.0 M potassium phosphate, pH 7.4, to neutralize pH. The antibody was dialyzed against PBS, concentrated by ultrafiltration, and used as purified antibody in the subsequent experiments.

Patient Serum

Serum A was from a patient with a sigmoidal colon cancer removed 1 year before the serum was taken. The patient died 1 week later and had both lung and liver metastases. Serum B was taken from a patient with a right side colon carcinoma with a diffuse tumor growth in the abdomen 2 months before the serum was taken. The patient died 11 days later. Both patients gave their informed consent for involvement in this investigation, which was approved by the Ethics Committee of the medical faculty, University of Göteborg.

Preparation of Purified H-CanAg

H-CanAg was purified from colon carcinoma cell line COLO 205 (grown in Iscove's medium with 10% fetal calf serum) culture supernatant as described before [Zhang et al., 1994]. Briefly, the culture supernatant was precipitated by the addition of trichloracetic acid (TCA) to a final concentration of 0.1 g/ml and immediately centrifuged (20,000*g*, 10 min). The TCA soluble sample was quickly neutralized by addition of 1 M Tris-HCl, pH 8.0, and 5 M NaOH and concentrated again by ultrafiltration. The yield of C50 activity at TCA precipitation was over 50%. After dialyzing and lyophilizing, the sample was subjected to gel filtration on a Superose 6 column using 0.5 M NH_4Ac, pH 7.0, as eluent. Fractions were analyzed for CA50 activity using C50/C50 immunofluorometric assay (IFMA, MAb C50 as both catching and detecting antibody) and Ma552/C50 IFMA (MAb Ma552 as catching antibody and MAb C50 as detecting antibody) [Baeckström et al., 1991, 1993]. The peak fractions (13–19) were combined, concentrated by ultrafiltration, dialyzed against water, lyophilized, and used as purified H-CanAg. H-CanAg from COLO 205 cells was prepared as described previously [Baeckström et al., 1991].

Purification of Sialyl-Lewis a–Carrying Mucins From Colon Carcinoma Patient Sera

Patient serum was applied to an affinity column (MAb C241 covalently coupled to protein A-Sepharose). After washing, the gel was eluted at pH 11.2. The eluate was collected in 1 M Tris-HCl, pH 7.0, to neutralize pH as described before [Baeckström et al., 1991]. The affinity purification gave a yield of over 70% as measured with the C50/C50 assay. After dialysis against water and lyophilization, the samples were subjected to gel filtration on a Superose 6 column and eluted with 0.1 M ammonium acetate, pH 7.0. Fractions were analyzed for sialyl-Lewis a activity with C50/C50 IFMA. The first sialyl-Lewis a peak in the void volume was further fractionated by gel filtration on a S-500 column and analyzed for sialyl-Lewis a activity with C50/C50 IFMA or MUC1 with Ma552/C50 IFMA.

Deglycosylation

Samples were subjected to trifluoromethanesulfonic acid treatment as described previously, a procedure leaving significant amounts of GalNAc attached to the protein core [Baeckström et al., 1991].

Fluoroimmunoassay

Fluoroimmunoassay was performed as described before [Baeckström et al., 1991; Zhang et al., 1994]. Briefly, samples diluted with assay buffer were incubated in microtiter plates coated with MAb C50, CSLEX1, or Ma552. The bound antigens were detected by incubation with europium-labeled antibodies and measured as fluorescence of released europium ions. C50 activity was determined by a homologous fluoroimmunometric assay using C50 both as solid phase-bound catching antibody and as europium-labeled tracer. The antigenic activity was expressed in arbitrary units (U), defined in relation to a reference [Johansson et al., 1991]. One microgram of protein of H-CanAg corresponds to about 22 kU [Baeckström et al., 1991].

Assay for the Detection of C-Terminal MUC1 Protein

Microtiter plates coated with MAb C50 were incubated for 2 h with serial dilutions of H-CanAg (prepared from COLO 205 cell extracts or from cell culture supernatant) or MUC1 mucin fraction purified from patient sera (starting concentration of 100 U/100 μl). The plates were incubated for 2 h with the polyclonal rabbit antiserum CT-1 directed against the C-terminal, cytoplasmic MUC1 protein. The bound antiserum was detected by europium-labeled antirabbit antibody and measured as fluorescence of released europium ions. The peptide concentration used to inhibit CT-1 antiserum binding to cellular H-CanAg was 0.1 mg/ml.

Preparation of F(ab')$_2$ Fragment From MAb C241

The F(ab')$_2$ fragment of MAb C241 was prepared as described previously [Zhang et al., 1994]. Briefly, MAb C241 was cleaved by incubation with papain at 37°C for 24 h, and the reaction was stopped by adding iodoacetamide. The sample was subjected to gel filtration on a 0.6 × 40 cm Superose 6 column using 0.1 M NH_4Ac, pH 7.0, as eluent. F(ab')$_2$ fractions were collected and concentrated by ultrafiltration.

Preparation of COS-1 Cells Transiently Expressing Membrane-Bound E-Selectin

COS-1 cells were cultured and transfected with expression vector pELAM-1 [Bevilaqua et al., 1989] as described before [Zhang et al., 1994]. Briefly, COS-1 cells grown in a 10 cm petri dish were transfected with 5 μg plasmid DNA in the presence of 10% Nu-serum, 400 μg/ml DEAE-Dextran, 100 μM chloroquine for 2–3 h and treated with 10% dimethyl sulfoxide for 2 min at room temperature. One day after transfection, COS-1 cells were transferred to plastic microtiter wells after trypsinization, and they were ready for assay 40–48 h posttransfection. The transfection efficiency was between 40 and 60% (as revealed by biotin-labeled MAb BBA1 and streptavidin-fluorescein staining and examined under a fluorescent microscope).

Adhesion of HL-60 Cells to E-Selectin Transfected COS-1 Cells

HL-60 cells were radiolabeled and applied to E-selectin transfected COS-1 cells as described before [Zhang et al., 1994]. Briefly, E-selectin transfected COS-1 cells grown in microtiter wells were preincubated with H-CanAg for 30 min at room temperature, and ^{51}Cr-labeled HL-60 cells were added. They were incubated with shaking for 20 min at room temperature and washed with RPMI 1640 medium. The adherent cells were lysed in 1 M NaOH (30 μl/well), and the radioactivity was determined. For some wells, H-CanAg was first incubated with F(ab')$_2$ fragments of MAb C241 at room temperature for 30 min; then the mixture was applied to transfected COS-1 cells for preincubation. The incubation buffer lacking Ca^{2+} and containing 20 mM EDTA was included as negative control of selectin binding.

Adhesion of HL-60 Cells to Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells grown in microtiter strip wells were maintained in Eagle 199 medium supplemented with 20% fetal calf serum, 10 IU/ml heparin, and 20 μg/ml endothelial cell growth factor. To keep the same condition, only passage 2 endothelial cells were used in the experiments. After confluence, the endothelial cells were stimulated with 40 units/ml interleukin-1β (IL-1β) for 4 h at 37°C in an atmosphere of 5% CO_2, and they were preincubated with samples for 30 min at room temperature. Then ^{51}Cr-labeled HL-60 cells were applied to the endothelial cells, and they were incubated, washed, and counted as mentioned above. The incubation buffer lacking Ca^{2+} and containing 20 mM EDTA was included as negative control of selectin binding.

RESULTS

Purification of Mucins From COLO 205 Cells and Patient Sera

The MUC1 mucin (H-CanAg) secreted into the culture medium of COLO 205 cells was purified with trichloroacetic acid precipitation and gel filtration [Zhang et al., 1994]. Two patients with advanced colorectal cancer had a very high concentration of sialyl-Lewis a and x activity in their sera, as measured by C50/C50 (C50 as catching antibody and europium-labeled C50 as detecting antibody) and CSLEX1/CSLEX1 (CSLEX1 as catching antibody and europium-labeled CSLEX1 as detecting antibody) immunofluorometric assay (IFMA). One had 20 kU/ml of sialyl-Lewis a activity (called patient A) and the other 12 kU/ml (called patient B), where the normal range is set to below 20 U/ml [Johansson et al., 1991]. Using the assay for sialyl-Lewis x, patient A serum had an activity of 290 ($\times 10^3$ cps) and patient B 44 ($\times 10^3$ cps) as compared to normal controls that had about 11 ($\times 10^3$ cps). Using sialyl-Lewis a as a marker, the mucins containing this epitope were purified from the sera of the two patients. In the first step, the patient sera were purified by affinity chromatography using the MAb 241 specific against sialyl-Lewis a epitope attached to Sepharose. The sample bound to the column was eluted by high pH and the nonabsorbed sera analyzed for CA50 and CSLEX1 activity. This column retained almost all sialyl-Lewis a activity (the CA50 activity decreased from 20 kU/ml to 14 U/ml after the affinity chromatography for patient A), but about 7% and 29% of the CSLEX1 activity remained. The eluted samples were separated by gel filtration on Superose 6. The fractions were assayed for CA50 activity (Fig. 1), and the reactive fractions in the void volume were combined and lyophilized. The sialyl-Lewis a active fractions eluted after the main peak were also combined and used in subsequent steps as the "smaller mucin" fraction. When the large and small mucin fractions were analyzed with the CSLEX1/CSLEX1 assay and in a combined CSLEX1/C50 assay (CSLEX1 as catching and europium C50 as detecting antibody), only the larger fractions showed sialyl-Lewis x reactivity (not shown). In the third step, the void volume fractions were separated on a Sephacryl S-500 column. All fractions were assayed for MUC1 mucin using a combined Ma552/C50 IFMA or for CA50 activity (not shown). Both

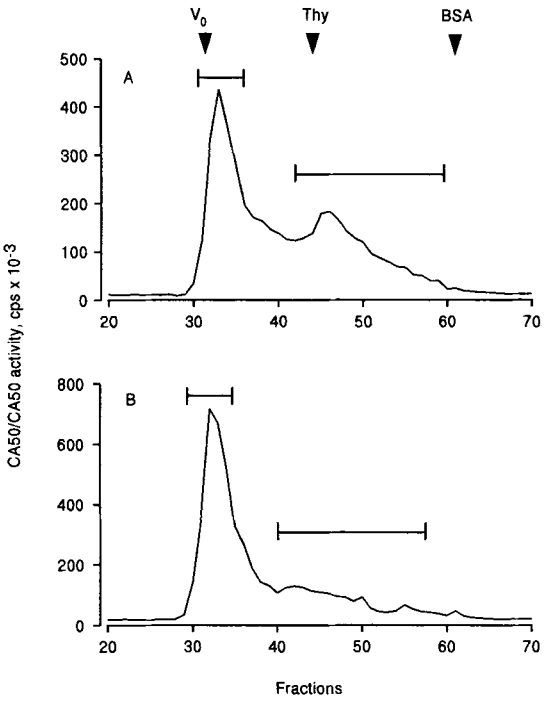

Fig. 1. Separation of sialyl-Lewis a–expressing glycoproteins from patient sera by gel filtration. After affinity chromatography, the samples were fractionated on a 0.6 × 40 cm Superose 6 column using 0.1 M NH_4Ac as eluent. Fractions were analyzed for CA50 activity by fluoroimmunoassay using C50/C50 IFMA (MAb C50 as catching antibody and europium-labeled MAb C50 as detecting antibody). The bar indicates the fractions combined and used in the following experiments. **A:** Patient A. **B:** Patient B. Indicated are void volume (v_0), retention volumes of thyroglobulin (Thy), and bovine serum albumin (BSA).

assays showed one homogenous peak at fraction 30–38, that was in the included volume of this column, for patient A and B. The MUC1 reactivity coincided with the sialyl-Lewis a reactivity and with H-CanAg analyzed in parallel on the same column. The sialyl-Lewis a reactive peaks from the two patients were combined, lyophilized, and used for the subsequent experiments.

Presence of MUC1 Mucin in the Serum of Patients With Colon Cancer

To test if the above proposed presence of MUC1 mucins in the crude patient sera could be detected directly, these were analyzed using Ma552/C50 IFMA. The results showed that this assay detected MUC1 apoprotein in both patient sera (Fig. 2A1–C1). To further investigate and prove this, mucins were purified from patient sera as described above. The sialyl-Lewis a fractions from the Sephacryl S-500 column that showed MUC1 reactivity with Ma552 was degly-

cosylated with trifluoromethanesulfonic acid. The samples were coated into microtiter strips and assayed for MUC1 mucin using MAb HMFG-2, reacting with the tandem repeat of the MUC1 apoprotein. The results showed that this MAb reacted strongly with the partially deglycosylated samples of the major sialyl-Lewis a peak from both patients but not the intact samples (Fig. 2A2–C2).

C-Terminal Portion of the MUC1 Mucin From COLO 205 Cells and Patient Sera

Equal amounts (measured as CA50 activity) of H-CanAg purified from COLO 205 cells, of H-CanAg purified from COLO 205 spent culture supernatant, and of MUC1 mucin fraction purified from two patient sera were immobilized in wells coated with MAb C50 and examined for binding by a rabbit antiserum (CT-1) against the cytoplasmic C-terminal portion of the MUC1 protein. The results showed that the antiserum reacted with H-CanAg purified from cell extracts, a reactivity that was inhibited by a synthetic CT-1 peptide (Fig. 3). In contrast, there was almost no reactivity with H-CanAg from spent culture supernatant or the MUC1 mucin fraction from the patient sera. This indicated that secreted MUC1 mucins from cells and patient sera lacked their cytoplasmic part and proposed that the extracellular part had been cleaved off from the cell membrane.

Inhibition of HL-60 Cell Adhesion to E-Selectin–Transfected COS-1 Cells

In order to test if the H-CanAg mucin can inhibit E-selectin–mediated leukocyte adhesion, ^{51}Cr-labeled HL-60 cells were applied to E-selectin transfected COS-1 cells which had been preincubated with H-CanAg. As shown in Figure 4, like MAb BBA1 against E-selectin [Zhang et al., 1994], H-CanAg can inhibit HL-60 cell adhesion to E-selectin transfected COS-1 cells in a partially concentration-dependent way. This inhibition can be blocked by preincubating H-CanAg with the F(ab')$_2$ fragment of the MAb C241, directed against the sialyl-Lewis a epitope.

The two patients having a high concentration of sialyl-Lewis a and x activity in their sera may have concentrations of sialyl-Lewis a and x mu-

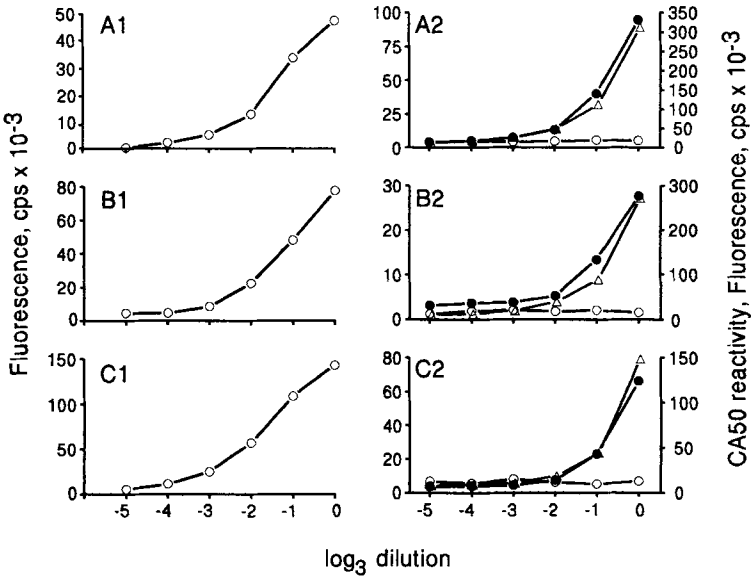

Fig. 2. Fluoroimmunoassay for the presence of sialyl-Lewis a–carrying MUC1 mucin in the sera of the two patients (A and B). **A1–C1:** Serial dilutions (20 μl in 100 μl assay buffer for starting well) of crude sera (A and B for patient A and B) were incubated in microtiter strips coated with MAb Ma552 directed against intact MUC1 mucin and the bound MUC1 mucin was detected by europium-labeled MAb C50 (○). Purified H-CanAg mucin (1 kU/100 μl for the starting well) was analyzed as a comparison (C). **A2–C2:** The combined major sialyl-Lewis a fractions from Sephacryl S-500 were deglycosylated with trifluoromethanesulfonic acid and coated into microtiter strips in serial dilution (CA50 activity: 40 units in the starting well) and examined for MUC1 mucin reactivity using MAb HMFG-2 directed against MUC1 tandem repeat and an europium-labeled secondary antibody (●). The same intact samples (same amount in CA50 activity) were analyzed in the same way (○) or detected by europium-labeled C50 (△). As a comparison, purified H-CanAg was deglycosylated and examined (CA50 activity: 10 units for the starting well) in the same way (C).

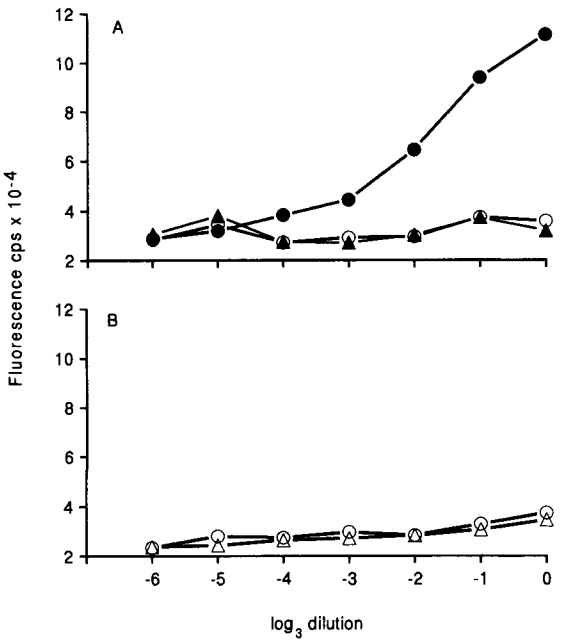

Fig. 3. Analysis of the C-terminal portion of sialyl-Lewis a–carrying MUC1 mucin fraction by fluoroimmunoassay. **A:** Serial dilutions (starting concentration 100 U/100 μl) of H-CanAg prepared from COLO 205 cell extracts (●/▲) or H-CanAg from spent cell culture supernatant (○) were incubated for 2 h in microtiter strips coated with MAb C50 and tested for binding of a polyclonal rabbit antiserum (CT-1) against the C-terminal portion of the MUC1 protein in the absence (●) or presence (▲) of the synthetic CT-1 peptide. **B:** The MUC1 mucin fraction purified from the two patient sera (○ and △) was tested in the same way. The bound CT-1 antiserum was detected by europium-labeled antirabbit antibody and measured as fluorescence of released europium ions.

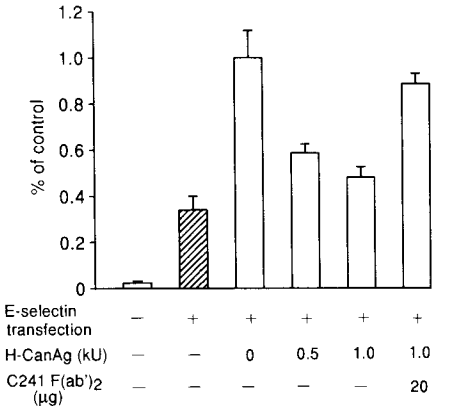

Fig. 4. Effects of H-CanAg on HL-60 cell adhesion to E-selectin transfected COS-1 cells. E-selectin transfected COS-1 cells were preincubated with H-CanAg for 30 minutes. For some wells H-CanAg was first incubated with the $F(ab')_2$ fragment of MAb C241 at room temperature for 30 min; then the mixture was applied to the cells. ^{51}Cr-labeled HL-60 cells were added, and the incubation was continued with shaking for 20 min. The microtiter wells were washed with RPMI 1640 medium. The adherent cells were lysed in 30 μl/well 1 M NaOH and the radioactivity measured. The inhibition of adhesion was expressed as percent cell adhesion relative to blank control (mean ± SD). *Hatched bar,* negative control in the absence of Ca^{2+} and in the presence of EDTA. Data represent two experiments with triplicates each. The y-axis is $\times 10^{-2}$.

cins high enough to inhibit E-selectin–mediated adhesion of leukocytes. To test this, the undiluted patient sera were preincubated with E-selectin–transfected COS cells before the addition of ^{51}Cr-labeled HL-60 cells. The results in Figure 5 show that the crude patient sera could inhibit HL-60 cell adhesion to E-selectin. The inhibitory effect was 67% for the patient (A) with the highest amount of sialyl-Lewis a activity (20 kU/ml) and 45% for the patient (B) with 12 kU/ml (the background for nontransfected cells was subtracted). Sialyl-Lewis a affinity extraction of the sera removed most of the sialyl-Lewis x reactivity as well as almost all of the inhibitory effect of the sera on leukocyte adhesion (Fig. 5).

Inhibition of HL-60 Cell Adhesion to Human Endothelial Cells

To investigate if H-CanAg had any effect on HL-60 cell adhesion to naturally occurring E-selectin, interleukin-1β (IL-1β)–activated human umbilical vein endothelial cells (HUVEC) were examined. Also in this system, H-CanAg could partially inhibit HL-60 cell adhesion to IL-1β–stimulated endothelial cells in a concentration-dependent way (Fig. 6).

The crude sera from the two patients also partially inhibited adhesion of HL-60 cells to IL-1β–stimulated endothelial cells (Fig. 7). The inhibitory effect was 85% for patient A and 61% for patient B (the background subtracted). The sera lost their inhibitory effect after sialyl-Lewis a affinity absorption. However, the background adhesion to nonstimulated endothelial cells was relatively high. This may be due to components in serum mediating HL-60 cell adhesion in an E-selectin–independent way or the presence in the serum of IL-1β and other cytokines that could stimulate endothelial cells or leukocytes.

Furthermore, the purified sialyl-Lewis a active fractions from the sera of the two patients were also compared for inhibitory effect by preincubation with IL-1β–stimulated endothelial cells. In both samples the major sialyl-Lewis a mucin peak, containing MUC1, could inhibit HL-60 cell adhesion to the endothelial cells by

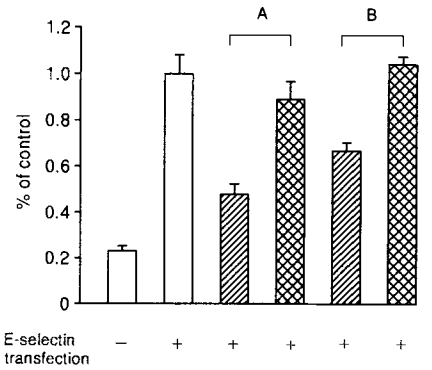

Fig. 5. Effects of crude patient sera on HL-60 cell adhesion to E-selectin transfected COS-1 cells. E-selectin transfected COS-1 cells were preincubated with patient sera (50 μl) for 30 min. The experimental procedure was the same as in Fig. 4. The inhibition of adhesion was expressed as percent cell adhesion relative to control serum (mean ± SD). By adding purified H-CanAg up to 50 kU/ml to the control serum, a 50% inhibition was obtained. Ten additional colon cancer sera with C50 activity in the normal range (below 20 units/ml) were analyzed in the same way. These gave an 80–114% inhibition relative to control serum. A, patient A; B, patient B; *open bar,* control serum with sialyl-Lewis a activity below 5 U/ml; *hatched bar,* patient serum; *cross-hatched bar,* patient serum after affinity absorption of sialyl-Lewis *a activity.* The y-axis is $\times 10^{-2}$.

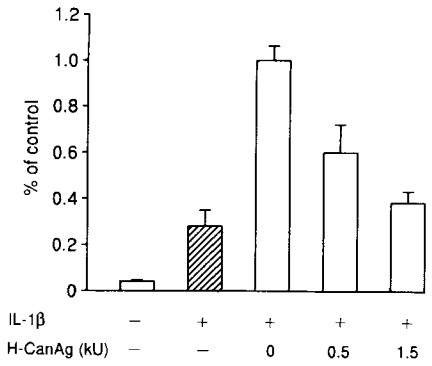

Fig. 6. Effects of H-CanAg on HL-60 cell adhesion to IL-1β–stimulated human umbilical vein endothelial cells. H-CanAg was preincubated with IL-1β–activated endothelial cells for 30 min at room temperature. ^{51}Cr-labeled HL-60 cells were added, incubated, and detected in the same way as in Fig. 4. The inhibition of adhesion was expressed as percent cell adhesion relative to blank control (mean ± SD). Hatched bar, negative control as in Fig. 4. The y-axis is $\times 10^{-2}$.

76% (patient A) and 50% (patient B) when the same amount (measured as CA50 units) of sialyl-Lewis a–carrying mucins was used. The effect of the MUC1 mucin fraction was larger than the corresponding "smaller" sialyl-Lewis a mucins when identical amounts of sialyl-Lewis a activity were tested (Fig. 8).

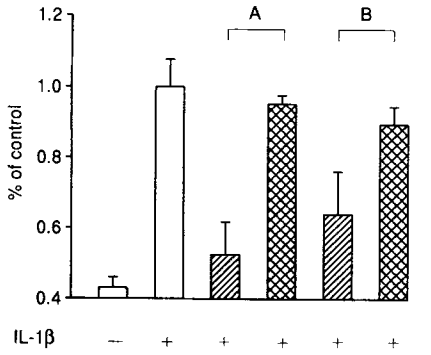

Fig. 7. Effects of patient sera on HL-60 cell adhesion to IL-1β–stimulated human umbilical vein endothelial cells. Patient sera (50 μl) were preincubated with IL-1β–activated endothelial cells for 30 min at room temperature. The experiments were performed as in Fig. 4. The inhibition of adhesion was expressed as percent cell adhesion relative to control serum (mean ± SD). A, patient A; B, patient B; *open bar,* control serum; *hatched bar,* patient serum; *cross-hatched bar,* patient serum after affinity extraction of sialyl-Lewis a activity. The y-axis is $\times 10^{-2}$.

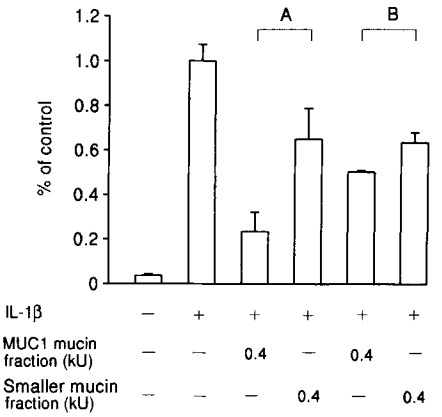

Fig. 8. Effect of purified mucins from patient sera on HL-60 cell adhesion to IL-1β–stimulated human endothelial cells. The purified mucins (400 C50 units) from the patient sera were preincubated with IL-1β–activated endothelial cells for 30 min at room temperature. ^{51}Cr-labeled HL-60 cells were added, incubated, and detected in the same way as in Fig. 4. The inhibition of adhesion was expressed as percent cell adhesion relative to blank control (mean ± SD). A, patient A, B, patient B. The y-axis is $\times 10^{-2}$.

DISCUSSION

The present study showed that the purified MUC1 mucin, H-CanAg, from COLO 205 spent culture supernatants could inhibit HL-60 cell adhesion to E-selectin expressed on transfected COS-1 cell or IL-1β–stimulated human umbilical vein endothelial cells. This inhibition is concentration-dependent but does only reach just above 50%. This is probably due to the increased

nonspecific adhesiveness observed for transfected COS cells [Zhang et al., 1994; Aruffo, 1992], or may be due to other adhesion molecules which were not studied here. The inhibitory effect of H-CanAg on HL-60 adhesion to transfected COS-1 cells could be blocked by $F(ab')_2$ fragments of monoclonal antibody (C241) directed against sialyl-Lewis a epitope.

Two patients with advanced colon carcinoma having very high concentrations of sialyl-Lewis a and x activity in their sera were studied. The major mucin fraction expressing sialyl-Lewis a and x was proved to contain MUC1 apoprotein, as shown by its reactivity with two anti-MUC1 apoprotein monoclonal antibodies, Ma552 and HMFG-2, in intact or deglycosylated form. The size of this fraction, as estimated from gel filtration chromatography, was similar to that of MUC1 mucins like H-CanAg. However, the presence of additional mucins of similar size or complexes of mucins cannot be excluded. Also, large mucin macromolecules as these would be expected to carry multiple oligosaccharide chains, some of which might be ligand structures for selectin besides the ones analyzed here. Depletion of sialyl-Lewis a activities would automatically remove also other epitopes expressed on the same macromolecule. Ideally the MUC1 mucin should have been affinity-purified, but presently available antibodies do not allow this for MUC1 mucins from colon cancer. The limited amount of sera also precluded gel filtration under denaturing conditions, as this demands dialysis to remove guanidinium chloride before the immunoassays. The crude sera from these patients could inhibit HL-60 cell adhesion to E-selectin on transfected COS-1 cells or IL-1β–stimulated human umbilical vein endothelial cells. The inhibitory effect was over 60% on the adhesion to COS-1 cells or above 80% on the endothelial cell adhesion for patient A when compensating for adhesion to nontransfected or nonstimulated cells, respectively. The inhibitory effect was slightly lower for patient B, probably partly due to the lower amount of sialyl-Lewis a and x mucins in this sera. Both sera lost most of their inhibitory effects when the sialyl-Lewis a activity was removed by affinity absorption using a specific sialyl-Lewis a antibody. The purified sialyl-Lewis a– and x–carrying MUC1 mucin fraction from the two patients inhibited HL-60 cell adhesion to E-selectin expressed on IL-1β–stimulated human umbilical vein endothelial cells. Although the same amount (measured as CA50 units) of sialyl-Lewis a mucin was used, the effect of the MUC1 mucin fraction from the patient A was higher than that from patient B, an observation that we cannot currently explain. One possibility is the higher expression of sialyl-Lewis x activity in patient A, or perhaps it is due to other unknown selectin ligand structures besides sialyl-Lewis a and x. The smaller sialyl-Lewis a–carrying glycoproteins also showed inhibitory effect, although at a lower level than the MUC1 mucin fraction when identical levels of sialyl-Lewis a (in terms of CA50 units) were used. These "smaller" glycoproteins were present in lower amounts than the MUC1 mucin when measured as sialyl-Lewis a activity. These data suggest that the inhibitory effect of the patient sera is largely due to the MUC1 mucin fraction. But the different inhibitory potentials between the larger and smaller mucins might also be a consequence of different recognition of them by E-selectin or due to other additional ligands, besides the sialyl analogues, expressed on the larger mucin. This is an important point for further studies, although difficult due to the small amount of patient material that is available.

The MUC1 mucin was first observed in the mammary gland as several monoclonal antibodies produced against breast cancer cells reacted with it [Hilkens et al., 1984]. It is normally found in the mammary gland, and the basis for its tumor-associated reactivity with different antibodies is its lower glycosylation in cancer cells [Gendler et al., 1988]. The MUC1 apoprotein has a cytoplasmic, a transmembrane portion, and a large extracellular portion made up of a variable number of a 20 amino acids long tandem repeat [Ligtenberg et al., 1990; Gendler et al., 1990]. This extracellular part is heavily glycosylated with O-linked oligosaccharides. MUC1 mucins have also been found in conjunction with other types of cancer as pancreatic and colorectal cancers [Baeckström et al., 1991; Lan et al., 1990]. In both these cases the MUC1 mucins carry tumor-associated carbohydrate epitopes.

Although MUC1 mucin is a membrane-bound glycoprotein, it can be shed from the cells or cleaved off from the plasma membrane [Ligtenberg et al., 1992] and enter the circulation [Metzgar et al., 1984; McGuckin et al., 1994]. A cleavage site has been localized to a region between 71 and 53 amino acids from the trans-

membrane domain in MUC1 produced by a mammary cell line [Ligtenberg et al., 1992; Hilkens and Buijs, 1988]. The protein is probably cleaved very early during biosynthesis, but the two parts remain attached to each other. How and when these can be separated is not known. In the case of cultured COLO 205 cells the MUC1 mucin (H-CanAg) largely remains membrane-bound, probably via its membrane-spanning region as this H-CanAg reacted with a C-terminal–specific antiserum. H-CanAg was also found in the spent media, but now it lacked reactivity with the CT-1 antiserum against the C-terminal portion of MUC1 protein. This H-CanAg probably lacks both the membrane-spanning and cytoplasmic domains. The MUC1 mucins from the patient sera were also unreactive with the CT-1 antiserum and thus probably contain only the extracellular portion of MUC1. The basis for the release of the extracellular MUC1 in tumor patients is currently not known, but it might be similar to the phenomenon discussed above. However, the very high levels of MUC1 mucin in the sera of some patients may suggest a high proteolytic activity at a site different from the one observed by Ligtenberg et al. [1992] or a mechanism for releasing the already cleaved extracellular portion. In addition to high tumor burden, a high degree of release from tumor cells might be an explanation for the very high levels of circulating MUC1 mucin in the serum of some patients.

Patients with gastrointestinal and pancreatic cancer often have elevated levels of sialyl-Lewis a– and/or sialyl-Lewis x–containing mucins in their sera. Thus, one may raise the question whether the elevated level of sialyl-Lewis a– and x–carrying mucins in patient serum can interfere with the recruitment of leukocytes to the tumor locations by competing or blocking the binding sites of E-selectin. Our studies support such a hypothesis, as both H-CanAg from COLO 205 cells and MUC1 from tumor sera can inhibit the adhesion of HL-60 cells (like most leukocytes expressing sialyl-Lewis x only) to E-selectin–expressing COS cells or activated endothelial cells. However, the levels of sialyl-Lewis a mucins in the systemic circulation is usually relatively low (below 100 CA50 units/ml for most patients with colorectal carcinoma) [Johansson et al., 1991], but the local concentration of sialyl-Lewis a–expressing mucins may be relatively high in and around the tumor producing these mucins. One may hypothesize that this high local concentration of secreted sialyl-Lewis a mucins can inhibit leukocyte extravasation to the tumor and could be at least partly responsible for the often noticed low leukocyte infiltration found in cancer tissues of patients with poorer prognosis [MacCarty, 1922; Kikuchi et al., 1979; Shimokawara et al., 1982; Sadanaga et al., 1994].

In the present study, two advanced colorectal patients were shown to have such high levels of sialyl-Lewis a–expressing mucins, especially MUC1 mucin, that the crude sera directly inhibited HL-60 leukocyte adhesion to E-selectin–expressing COS cells and endothelial cells. This suggests that the amount of mucins produced by the cancer cells and released into the circulation could be sufficient to impede leukocyte attachment and extravasation to a significant level in all organs of the body. This may be one of the explanations for the immunodepressed state often found in cancer patients [Rolston and Bodey, 1993; Yamamura and Azuma, 1983] with advanced disease. A further understanding of the relation between the membrane-bound and secreted sialyl-Lewis a– and/or x–expressing mucins and their biochemical background in relation to the clinical outcome of a cancer disease will be of great importance.

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