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# Tumor cell MUC1 and CD43 are glycosylated differently with sialyl-Lewis a and x epitopes and show variable interactions with E-selectin under physiological flow conditions

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**The mucins secreted from the colon carcinoma cell line COLO 205 have the MUC1 and CD43 (leukosialin) as core proteins, where both carry sialyl-Lewis a and MUC1 sialyl-Lewis x epitopes. The adhesion of E-selectin expressing CHO cells to the coated mucins was analyzed in a flow system revealing that the MUC1 mucin adhered better than the CD43 mucin. One reason could be their different glycosylation, a difference that was explored by analyzing the biosynthesis of MUC1 and CD43 in COLO 205 cells. Both the MUC1 and CD43 mucins became sialyl-Lewis a reactive, but after different times as revealed by pulse-chase studies. However, only MUC1 became sialyl-Lewis x reactive. These differences suggest that MUC1 and CD43 are synthesized in different compartments of the cell. It was also observed that the mucins from colon carcinoma patients had MUC1-type mucins that carried both sialyl-Lewis a and x epitopes and CD43-type sialyl-Lewis a mucins with only low levels of sialyl-Lewis x epitopes. One could hypothesize that colon carcinoma derived MUC1 is decorated with potent E-selectin epitopes, and that this could be one of several reasons for the involvement of MUC1 in cancer development.**

***Keywords:*** **colon cancer, sialyl-Lewis a, sialyl-Lewis x, mucin, E-selectin**

***Abbreviations:*** **Si-Le$^a$, sialyl-Lewis a; Si-Le$^x$, sialyl-Lewis x.**

## Introduction

The cancer-associated carbohydrate antigen, sialyl-Lewis a (Si-Le$^a$; NeuAc$\alpha$2-3Gal$\beta$1-3[Fuc$\alpha$1-4]GlcNAc) was originally characterized as a glycosphingolipid [1], but is now known to be most abundant on molecules having biochemical properties of mucins [2,3]. The appearance of the Si-Le$^a$ epitope is due to the induction of an $\alpha$2,3-sialyltransferase synthesizing the precursor oligosaccharide for the Lewis-encoded enzyme [4]. This is the same principal pathway as for the biosynthesis of the corresponding type 2 isomer, making the sialyl-Lewis x epitope (Si-Le$^x$; NeuAc$\alpha$2-3Gal$\beta$1-4[Fuc$\alpha$1-3]GlcNAc). Patients with gastrointestinal and pancreatic cancers have elevated levels of both the Si-Le$^a$ and Si-Le$^x$ epitopes in their sera, a phenomenon used clinically as a serological marker for monitoring the progression of the disease [2,5–11].

Mucins are a group of large, highly *O*-glycosylated proteins that have been implicated in the development of cancer, especially the membrane-bound MUC1 mucin [12]. This mucin is overexpressed, differently glycosylated, and redistributed over the membrane in cancer cells. The cytoplasmic part of MUC1 interacts with several of the molecules (for example $\beta$-catenin), now known to be involved in tumor development [13]. We originally showed that two mucins, MUC1 and the leukocyte marker CD43 (leukosialin), were expressed and secreted from colon cancer cells [3,14].

The Si-Le$^x$ antigen is a natural ligand for E-selectin, but also the Si-Le$^a$ antigen has been shown to bind E-selectin [15], especially when clustered on mucin-like domains [16]. In previous studies [3,14,17,18], we could show that the MUC1 and CD43 Si-Le$^a$ mucin type molecules from the colorectal carcinoma cell line COLO 205 inhibited HL-60 leukocyte adhesion

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to E-selectin expressing COS-7 cells or endothelial cells. During these studies it was observed that the MUC1 mucin (originally called H-CanAg) was more efficient than CD43 (originally called L-CanAg) in inhibiting these interactions (at equivalent mass concentrations). We have now extended these observations with studies of the E-selectin interaction under flow conditions.

## Material and methods

### COLO 205 mucins and antibodies

The COLO 205 colon adenocarcinoma cell line (ATCC CCL 222) was maintained as described [19]. CHO cells stably transfected with full-length E-selectin, termed CHO-E cells [20] were kindly provided by Dr. R. Lobb (Biogen, Cambridge, MA). MAb C50, recognizing the Si-Le$^a$ epitope, MAb C241 against Si-Le$^a$, MAb CSLEX1, against Si-Le$^x$ (ATCC, HB 8580) and the rabbit antisera anti-GPEP18 against the nonphosphorylated cytoplasmic tail of CD43 were used as described before [3,17,18,21]. All fluoroimmunoassays were performed using the europium-labeled antibodies as described previously [3]. The MUC1 and CD43 mucins were purified from spent culture media from COLO 205 cells by trichloroacetic acid precipitation and gel filtration as described [22].

### Rolling of E-selectin expressing cells on MUC1 and CD43

The polystyrene plate, on which purified ligand was adsorbed, was assembled in a parallel plate laminar flow chamber as described previously [23,24]. The CHO-E cells were washed in H/H medium containing 5 mM EDTA, resuspended in H/H medium at $10^7$ cells/ml and stored at 4°C up to 1 hr before each flow experiment. Stored cells were resuspended in cell binding medium at $10^6$ cells/ml and perfused at room temperature through the flow chamber at desired flow rates, generated with an automated syringe pump (Harvard Apparatus, Natick, MA). Cellular interactions were visualized at two different fields of view (each one 0.17 mm$^2$ in area) using a 10× objective of an inverted phase contrast microscope (Diaphot 300, Nikon, Tokyo, Japan). Cell accumulation levels were determined as previously described [25]. Detachment assays were performed on cells accumulated on the substrates at a shear stress of 1.25 dyne/cm$^2$. The wall shear stress was then increased step-wise every 5 sec to a maximum of 30 dyn/cm$^2$. The number of cells remaining bound at the end of each 5 sec interval of shear increase was expressed relative to the number of cells accumulated originally on the ligand-coated substrates.

### Metabolic labeling and immunoprecipitation

COLO 205 (75% confluent) were starved for 1 hr before labeling with 150 $\mu$Ci Pro-Mix (Amersham Bioscienes) per 35 mm dish for 30 min followed by a chase for 1, 3 and 7 hr. The cells were lysed in 1 ml 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 1 $\mu$g/ml aprotinin (Bayer), 25 $\mu$g/ml leupeptin (Sigma), 0.7 $\mu$g/ml pepstatin (Roche), and calpain inhibitor I (Roche). The cell lysates were incubated overnight with 25 $\mu$l of preimmune rabbit or mouse serum at 4°C and precipitated with 150 $\mu$l of a 10% (w/v) suspension of fixed *Staphylococcus aureus* (Life Technologies). The immunoprecipitation was removed by centrifugation at 14,000 × g for 2 min and the specific antiserum (25 $\mu$l anti-GPEP18) or MAbs (25 $\mu$l hybridoma supernatant) were added and precipitated as described above. After washing the immunoprecipitation 5 times with 1 ml of 10 mM Tris-HCl, pH 7.5, 2 mm EDTA, 0.1% Triton X-100, and 0.1% SDS, bound immunocomplexes were released by 100 $\mu$l 2× SDS-loading buffer containing $\beta$-mercaptoethanol at 95°C for 5 min, analyzed by SDS-PAGE (3–15%) and fluorographed (Amplify, Amersham Biosciences).

### Patient sera

Sera from 20 colorectal carcinoma patients were taken on the day of primary surgery, but before start of the operation. About 5 ml serum was obtained after coagulation of peripheral blood and the sera were stored at −80°C. All patients gave their informed consent for involvement in this investigation, which was approved by the Ethics Committee of the Medical Faculty, Göteborg University. The sera were applied to a MAb C241 affinity column. After washing by 0.1 M Tris-HCl (pH 8.0); 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 1 mM EDTA and 0.5% Triton-X-100; 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl, 0.5% Triton X-100 and 0.1% SDS; 0.15 M NaCl containing 0.5% sodium-taurodeoxycholate; and 0.1 M Tris-HCl (pH 8.0) consecutively, the column was eluted with 50 mM diethylamine-HCl (pH 11.2) and the eluate was collected in 1 M Tris-HCl, pH 7.0, to neutralize pH. After dialysis and lyophilization, the samples were subjected to gel filtration on a 0.6 × 40 cm Superose 6 column (Amersham Biosciences) in 0.1 M ammonium acetate (pH 7.0).

## Results

### Rolling of E-selectin expressing cells on MUC1 and CD43 coated surfaces

The MUC1 and CD43 mucins were purified from spent COLO 205 culture media and coated in a flow chamber where they were analyzed for accumulation of E-selectin expressing CHO cells (CHO-E) under flow conditions. All adhesive interactions between these CHO-E cells could be abolished by EDTA or an antibody against E-selectin (not shown). The MUC1 mucin supported up to 10-fold higher levels of E-selectin-dependent CHO cell accumulation per field than CD43 mucin coated at identical input (Figure 1, shear stress 1.75 dyne/cm$^2$). Furthermore, the resistance of adherent cells to detachment by increasing shear forces was significantly higher for CHO-E cells accumulating on MUC1 than for identical cells on CD43. The number of CHO-E cells that remained bound to the coated MUC1 was

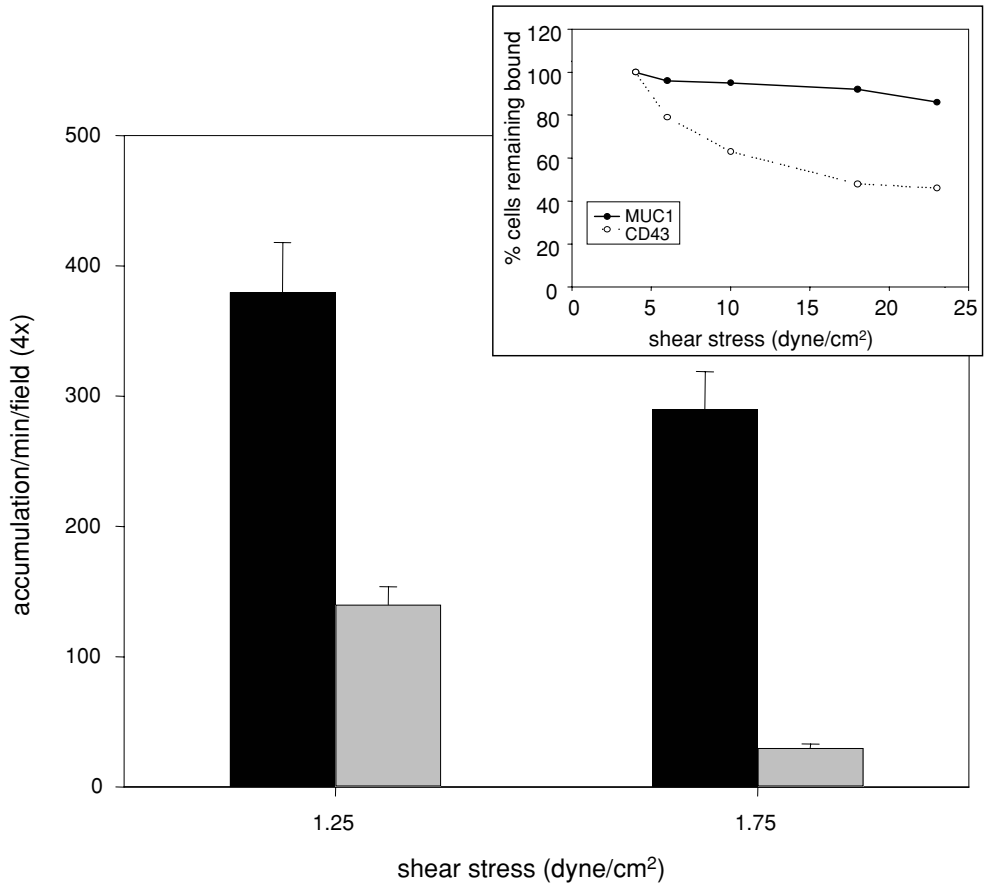


**Figure 1.** Accumulation of E-selectin-expressing CHO cells on substrates coated with MUC1 or CD43 under physiological shear flow. Cells ($10^6$/ml) were perfused at the indicated shear stresses for 1 min over substrates coated each with 0.1 $\mu$g/ml of the COLO 205 MUC1 mucin (black) or CD43 mucin (grey). The number of cells accumulated and their resistance to detachment by increasing shear stresses (inset) has been determined as described in Materials and Methods. Values are mean $\pm$ range of two determinations. One representative experiment out of three.

only marginally decreased (down to 92%) when the shear force was increased from 1.25 to 23 dyne/cm$^2$, whereas the number of cells bound to CD43 decreased by 55% (Figure 1, inset). Nevertheless, at lower shear stresses the differences in accumulation of E-selectin CHO cells or resistance to detachment observed on the two glycoproteins became smaller (Figure 1 and inset). These results show that MUC1 binds the E-selectin CHO cells stronger than CD43 and can withstand a much higher shear force than CD43, in particular under stringent force conditions. That MUC1 binds stronger to E-selectin was previously suggested in a static cell adhesion assay where these mucins were used as inhibitors of adhesion of HL-60 or polymorphonuclear leukocytes to E-selectin [26]. The MUC1 was then estimated to be about 5–10 fold more potent than CD43 based on mass or 20–40 fold on molar concentrations. The flow chamber assays used cannot provide an exact quantitative estimation of the relative potencies as differences in coating density of the MUC1 and CD43 cannot be excluded. However, at the low coating densities used, it was assumed that both glycoproteins coated at similar efficiencies due to their similar nature. Assuming similar coating of the test substrates, the 10-fold difference in accumulation potential of E-selectin expressing cells observed between MUC1 and CD43 matched the 5–10 fold difference between the inhibitory capacities of the two mucins in the static assays. As these two assays, done in different ways, are pointing in the same direction make it likely that the difference in potency of the two mucins is about a magnitude. One reason for this could be the previous observation that CD43 carry less Si-Le$^x$ epitopes compared to MUC1 [22]. To explore this further, the biosynthesis of MUC1 and CD43 in COLO 205 cells was studied.

## Differences in the biosynthesis of MUC1 and CD43 in COLO 205 cells

COLO 205 cells were metabolically labeled, chased for 1, 3, and 7 hr and the cell lysates immunoprecipitated with the anti-Si-Le$^x$ MAb CSLEX1, the anti-Si-Le$^a$ MAb C241 and the polyclonal antiserum anti-GPEP18 reacting with the cytoplasmic domain of CD43 followed by analysis by SDS-PAGE and fluorography (Figure 2). Several differences in the appearance of the bands were observed. The CD43 band appeared after one hour and this band was also stained with the anti-Si-Le$^a$ MAb.

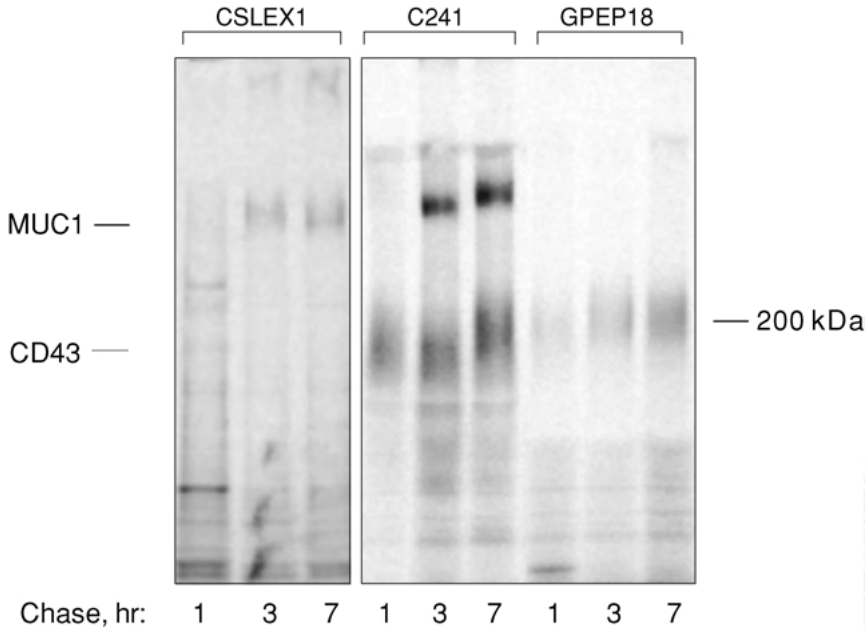


**Figure 2.** Pulse-chase studies on the appearance of Si-Le$^a$ and Si-Le$^x$ epitopes on MUC1 or CD43 produced in COLO 205 cells. The cells were pulse-labeled with 150 $\mu$Ci of [$^{35}$S] Pro-Mix for 30 min and chased for the time periods indicated. Lysates of the cells were precipitated once with nonimmune rabbit or mouse antiserum before immunoprecipitating with the anti-Si-Le$^a$ MAb C241, the anti-Si-Le$^x$ MAb CSLEX1 and the rabbit anti-CD43 antiserum anti-GPEP18. Eluted immunoprecipitates were separated on a 3–15% SDS-PAGE gel under reducing conditions and fluorographed.

The intensity of these CD43 bands increased up to 7 hr. Only weak staining was found with the anti-Si-Le$^x$ MAb of the CD43 band. In contrast, the MUC1 band was found to carry both the Si-Le$^x$ and Si-Le$^a$ epitopes, but these were not revealed until after 3 hr. The results suggest that MUC1 and CD43 are, despite their similarities, differently processed in the secretory pathway of the cell.

### Sialyl-Lewis a and x on mucin-type molecules in the sera of colon cancer patients

Due to the small amounts of mucins in the sera of colon carcinoma patients, these had to be enriched by anti-Si-Le$^a$ affinity purification before gel filtration on Superose 6 [18]. The amount of Si-Le$^a$ mucins varied with the patient and severity of the disease, but chromatograms similar to the media from the COLO 205 were often obtained (not shown). One example is shown in Figure 3. The large Si-Le$^a$-containing peak, appearing just after the void volume, is eluting at the same position as MUC1 from COLO 205 cells and reacted with MUC1 antibodies after deglycosylation (not shown). A second, broad Si-Le$^a$ reactive peak was found eluting in the fractions 45–55. This is the position for CD43 from COLO 205 cells, but it has not been possible to confirm the CD43 nature of this peak. The first Si-Le$^a$ peak containing MUC1 reacted strongly with the anti-Si-Le$^x$ MAb, but the second CD43-like peak did not (Figure 3). The presence of Si-Le$^a$ and Si-Le$^x$ epitopes in MUC1-type mucins and low levels of Si-Le$^x$ in CD43-type mucins is thus similar as for the mucins from the colon carcinoma cell line COLO 205.

## Discussion

Facts on the MUC1 and CD43 mucins secreted from COLO 205 cells have been gathered in Table 1 [3,17,22]. The glycans on MUC1 and CD43 have a similar mean chain length (15 and

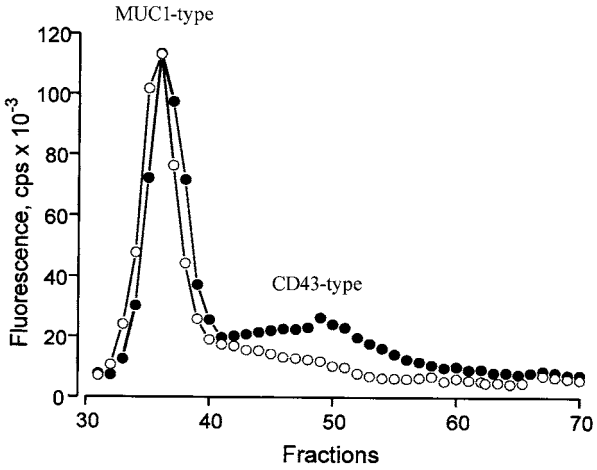


**Figure 3.** Analysis of mucin-type molecules in the serum from a colon cancer patient. The Si-Le$^a$-mucins from serum was separated by gel filtration on a Superose 6 column and the fractions analyzed in a sandwich-Delphia for Si-Le$^a$ (-●-) and Si-Le$^x$ (-○-) expression. The microtiter plates were coated with MAb C50 (anti-Si-Le$^a$) or CSLEX1 (anti-Si-Le$^x$), incubated with the fractions, bound material detected with Eu-labeled MAb C50 (anti-Si-Le$^a$) and the fluorescence measured.

**Table 1.** Differences of MUC1 and CD43 from COLO 205 colon carcinoma cells

| | *(H-CanAg) MUC1* | *(L-CanAg) CD43* |
|---|---|---|
| Sialyl-Lewis a epitopes[a] | + + + | + + + |
| Sialyl-Lewis x epitopes[a] | + + + | (+) |
| Kinetics of sialylation[c] | slow | fast |
| Mean length of glycosylation[b] | 15 residues | 13 residues |
| Inhibition of leukocyte adhesion to E-selectin expressing cells[a] | Good | Weak |
| Retardation of E-selectin cells[c] | Good | Weak |

[a]From Zhang et al. [22].
[b]From Baeckström et al. [3].
[c]This work.

13 residues, respectively) and both carry high levels of Si-Le$^a$ epitopes. However, when the levels of Si-Le$^x$ were measured, it turned out that CD43 had much lower levels than MUC1. This was not expected as their oligosaccharides are long and these peripheral epitopes are usually shared with different glycoconjugates. In the pulse-chase studies, the initial biosynthesis was similar for the two sialyl-Lewis epitopes on MUC1. For the Si-Le$^a$ reactive CD43mucins, these appeared earlier than the MUC1 mucins and only small amounts of Si-Le$^x$ CD43 mucins were formed (Table 1). Thus there are several lines of evidence for different processing and glycosylation of the MUC1 and CD43 mucins. The reasons for this are not known, but the differences in glycosylation kinetics suggest that these relatively similar molecules are passing different paths through the secretory pathway. This is probably not a specific phenomenon for COLO 205 cells, as the patient sera remind of the COLO 205 mucins with both Si-Le$^a$ and Si-Le$^x$ epitopes on the mucins over the MUC1 peak and low Si-Le$^x$ in the Si-Le$^a$ carrying CD43-type mucins. It is thus possible that this is a general phenomenon due to different processing of these molecules in the cell.

In the present and previous studies, MUC1 has been found to be more efficient than CD43 in binding E-selectin. Only a static adhesion assay was used before and it has been argued that the differences were not physiological. To address this, we tested the MUC1 and CD43 mucins form COLO 205 cells in a flow system revealing a 10-fold difference also with this method. This suggests that MUC1 could be about a magnitude more potent in binding E-selectin than CD43. One reason for this could be their differences in Si-Le$^x$ expression, but also the presentation of the E-selectin epitopes should be considered. One important factor is the organization of the epitopes along the saccharide chain. There are more and more evidence accumulating that the E-selectin recognition under flow depends on clustering of adjacent Si-Le$^x$ or Si-Le$^a$ ligands (R. Alon, unpublished). Thus the higher potency of MUC1 to inhibit E-selectin as a soluble ligand as well as to promote adhesion to immobilized molecules could be the organization of these epitopes.

It is presently not possible to finally determine why these two mucins show this difference in binding potency to E-selectin, but it is obvious that the intracellular processing and glycosylation of MUC1 and CD43 are different. It is thus tempting to hypothesize that the biosynthesis of MUC1 give this molecule an interesting potency and that this could be a selective advantage for the cell during tumor progression. The importance of MUC1 in this context is supported by the extensive studies on this mucin in relation to cancer and the suggestions that it can be involved in tumor progression and metastasis [12,13]. In contrast to this, there are fewer observations supporting a role of CD43 in tumor progression, except for the observation of CD43 expression in some cancer cells as well as in colon adenoma [27]. This could reflect a true functional difference and participation in tumor development. The present findings suggest that colon carcinoma derived MUC1 is processed and decorated with E-selectin binding epitopes, making it into a potent E-selectin ligand.

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