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Inhibition of Galβ1,4GlcNAc α2,6 sialyltransferase expression by antisense-oligodeoxynucleotides

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Abstract Treatment of human colorectal carcinoma cells HT29 with specific antisense oligodeoxynucleotides led to a decreased Galβ1,4GlcNAc α2,6 sialyltransferase activity on the level of protein expression as well as on the mRNA level. Antisense treatment did not effect cell viability or cell growth. Oligodeoxynucleotides which were complementary to the region upstream of the initiation codon were particularly effective in inhibition of enzyme expression. No such inhibition was found by treatment of cells with oligodeoxynucleotides complementary to the region downstream of the initiation codon or by treatment of cells with scrambled controls or sense oligodeoxynucleotides.

Key words: Galβ1,4GlcNAc α2,6 sialyltransferase; Antisense-oligodeoxynucleotides; Cell surface glycoconjugates; Colorectal carcinomas; Metastasis

1. Introduction

Tumor-associated alterations of cell surface glycosylation play a crucial role for adhesion and metastasis of carcinoma cells. Of particular interest are the sialyltransferases, a group of anabolic enzymes which transfer the negatively charged carbohydrate sialic acid onto different glycoproteins and glycolipids. Human Galβ1,4GlcNAc α2,6 sialyltransferase (α2,6ST), which is only slightly expressed in normal colonic mucosa cells, shows an enhanced activity in metastasizing colorectal carcinomas [1–3]. Hypersialylation due to enhanced enzyme activity may lead e.g. to reduced cell-cell adhesion [4] and/or increased adhesion of tumor cells to the basal membrane [5] and may hamper immune response mechanisms [6]. As a first step towards an understanding of the functional relevance of α2,6ST, we tried to inhibit enzyme expression by treatment of human colorectal cancer cells with specific antisense oligodeoxynucleotides (ODN).

2. Material and methods

2.1. Oligodeoxynucleotide (ODN) synthesis

ODNs were synthesized in 40 nm lots using an Applied Biosystems 380B automated DNA synthesizer and HPLC purified (BioTez, Berlin-Buch). Sequences were complementary to the region next to the initiation codon of human 2,6ST-cDNA [7,8] (Table 1). All ODNs were synthesized as phosphorothioates. Instead of thymidine C-5 propynyl-2′-deoxyuridine was used as described [9].

2.2. Treatment of HT29 human colorectal carcinoma cells with ODNs

Cells (5×10^4/ml) were seeded into 6-well tissue culture plates and grow until just confluent in DMEM (Biochrom, Germany), 7% FCS, 5% CO_2. Approximately 1.5×10^6 cells per well were counted. The cell monolayer was carefully washed with 2×5 ml DMEM without FCS, then 2 ml (10 μg/ml) Lipofectin (GIBCO) in DMEM without FCS was added and cells were incubated for 2 h at 37°C. ODNs were heated 20 min at 70°C and renatured 15 min at 22°C before dilution. Then 2 ml of a ODN solution in DMEM containing Lipofectin as described above was added to the cells. After a 4 h incubation at 37°C, 4 ml medium containing 14% FCS was added and cells were incubated for another two days at 37°C, 5% CO_2. Cell growth was determined using the MTT (3-[4,5-dimethylthiazol-2yl])-2,5-diphenyltetrazolium bromide) assay (Sigma) according to standard protocols. Each ODN experiment was repeated at least three times in duplicates.

2.3. Fluorometric sialyltransferase assay

Monolayers of cells, untreated or treated with 2 μM, 1 μM ODN in 6-well plates were washed with ice-cold phosphate-buffered saline (PBS), pH 7.3, at 4°C for a total period of 5 min to remove unbound ODN. Washes were completely removed by aspiration. The remaining cell sheet was incubated on a rocking plate for 30 min at 4°C in 500 μl 0.25 M sodium cacodylate buffer (Roth, Germany), pH 6.5, containing 0.4% Triton-X-100 (Sigma). Supernatants were centrifuged at $20\,000\times g$, 4°C for 20 min. Protein content in diluted supernatants was determined by BIO-RAD (München) protein assay using bovine serum albumin as standard. Enzyme activity was measured fluorometrically according to [10]. Briefly, 5 μl enzyme extract containing 5 μg protein was incubated with 5 μl of 0.25 M sodium cacodylate buffer, pH 6.5, 1.3 μl acceptor HPLC-purified asialo-α1-acid glycoprotein (25 mg/ml) (Sigma), 1.4 μl 350 μM CMP-9-fluoresceinyl-NeuAc (Boehringer-Mannheim) and 7.3 μl H_2O for 30 min at 37°C. Reaction was stopped by addition of 2.7 μl CTP (Sigma). Ten microliters of the assay mixture were applied to a liquid chromatography system. Fluorescence was detected at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Separation of fluorescently labeled glycoprotein from donor substrate was performed on an column (12.5×0.4 cm) of Sephadex G50 (Pharmacia) operated isocratically with 0.1 M Tris/HCl, pH 8.5 at 0.5 ml/min. As control experiments, similar assays were performed with 0.5 μU to 0.025 μU rat liver α2,6 sialyltransferase (Boehringer-Mannheim). Endogenous activity (after incubation without acceptor asialo-α1-acid glycoprotein) was subtracted.

2.4. Radiometric sialyltransferase assay

Sialyltransferase was extracted as shown above. Standard assay (100 μl) was performed with 50 μg protein extract and 1.25 mg N-acetyllactosamine (Dextra, UK) in 50 mM sodium cacodylate, pH 6.4, containing 375 μg bovine serum albumin (BSA), 0.25 mM CDP-choline, 0.25 mM $MnCl_2$, 0.1% Triton-X-100 and 0.5 mM PMSF. Transfer was initiated by addition of 10 nmol CMP-NeuAc (Boehringer-Mannheim), containing CMP-[3H]-NeuAc (Du Pont) with an activity of 3700 Bq (70 000 cpm). After incubation for up to 300 min at 37°C, the sample was boiled for 5 min at 95°C, centrifuged ($20\,000\times g$) and filtered. Separation of 6′- and 3′-sialyl-N-acetyllactosamine isomers on a NH_2-Lichrosorb column (Merck, Darmstadt, Germany) was done as described [2]. Briefly, chromatography was run isocratically with 83/17 (v/v) acetonitrile/15 mM KH_2PO_4 for 40 min, then the proportion of the mixture was changed to 78/22. Fractions of 3 ml were collected and radioactivity counted by liquid scintillation spectrometry in Ultima Gold. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol product/min.

2.5. RT-PCR for α2,6ST mRNA

Monolayers of cells, untreated or treated with 2 μM, 1 μM ODN in

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6-well plates were washed with ice-cold PBS, pH 7.3, at 4°C for a total period of 5 min to remove unbound ODN. Washes were completely removed by aspiration. RNA was extracted from the remaining cell sheet using guanidine thiocyanate-phenol-chloroform (GIBCO) according to [11] and treated with RNase-free DNase (Boehringer-Mannheim). Five micrograms of total cellular RNA were reverse transcribed by M-MLV reverse transcriptase (ProMega) with random hexamers (Pharmacia). CDNA was amplified using α2,6ST-specific primers: forward primer ST181, CTGCCCAAGGAGAGCATTAG; forward primer ST379, AAAAACCTTATCCCTAGGCTGC; reverse primer STrev, TGGTAGTTTTTGTGCCCACA. β-Actin was co-amplified within the same tube using forward primer actin1, TGACGGGGTTCACCCACACTGTGCCCATCT; reverse primer actin2, AGTCATAGTCCGCCTAGAAGCATTTGCGGT. Amplification was done using hot start PCR (5 min at 94°C). Cycling conditions were 1 min at 94°C, 1 min at 57°C, 2 min at 72°C for 30 cycles. PCR reaction mixture contained 1.5 μl PCR-buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ with 0.1% gelatine (w/v)), 5 μl cDNA, 1.5 μl 2 mM of dATP, dCTP, dGTP, dTTP (Pharmacia), 50 pmol α2,6ST-specific primers and 0.4 pmol β-actin primers, 0.1 μl Taq Polymerase (Perkin-Elmer) and H_2O ad 15 μl. PCR-products were electrophoresed in 3% agarose (Roth, Germany) containing ethidium-bromide (GIBCO). For fluorescence measurements FluorImager SI (Molecular Dynamics) was used. The coefficient of variation among several measurements of the same sample was less than 3%.

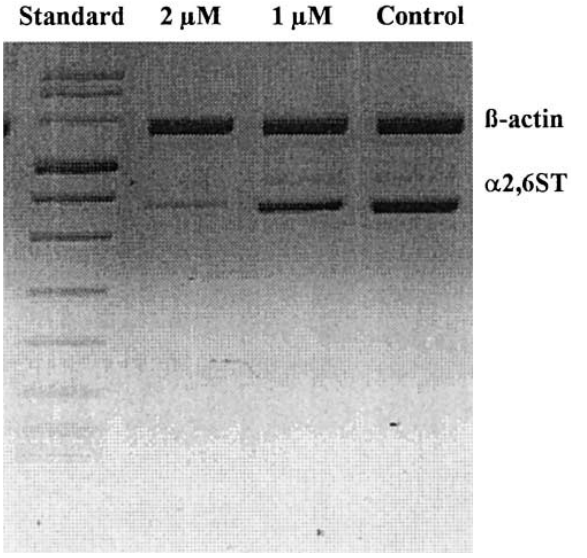

Fig. 1. RT-PCR of α2,6ST mRNA in HT29 cells treated with AS 7. HT29 cells were incubated with 2 μM, 1 μM antisense ODN or remain untreated (control). Images were obtained by fluorescence scanning of electrophoretically resolved, ethidium bromide-stained PCR products derived from co-amplification of β-actin (upper lanes) and α2,6ST (lower lanes) target sequences.

3. Results

3.1. Effects of ODN treatment on cell growth

Incubation of HT29 cells with ODN *Scramble 2* (Table 1) in concentrations of up to 4 μM did not effect cell growth as detected by MTT-Test. Treatment of HT29 cells with a 4 μM ODN solution of *Scramble 2* decreased the number of cells to

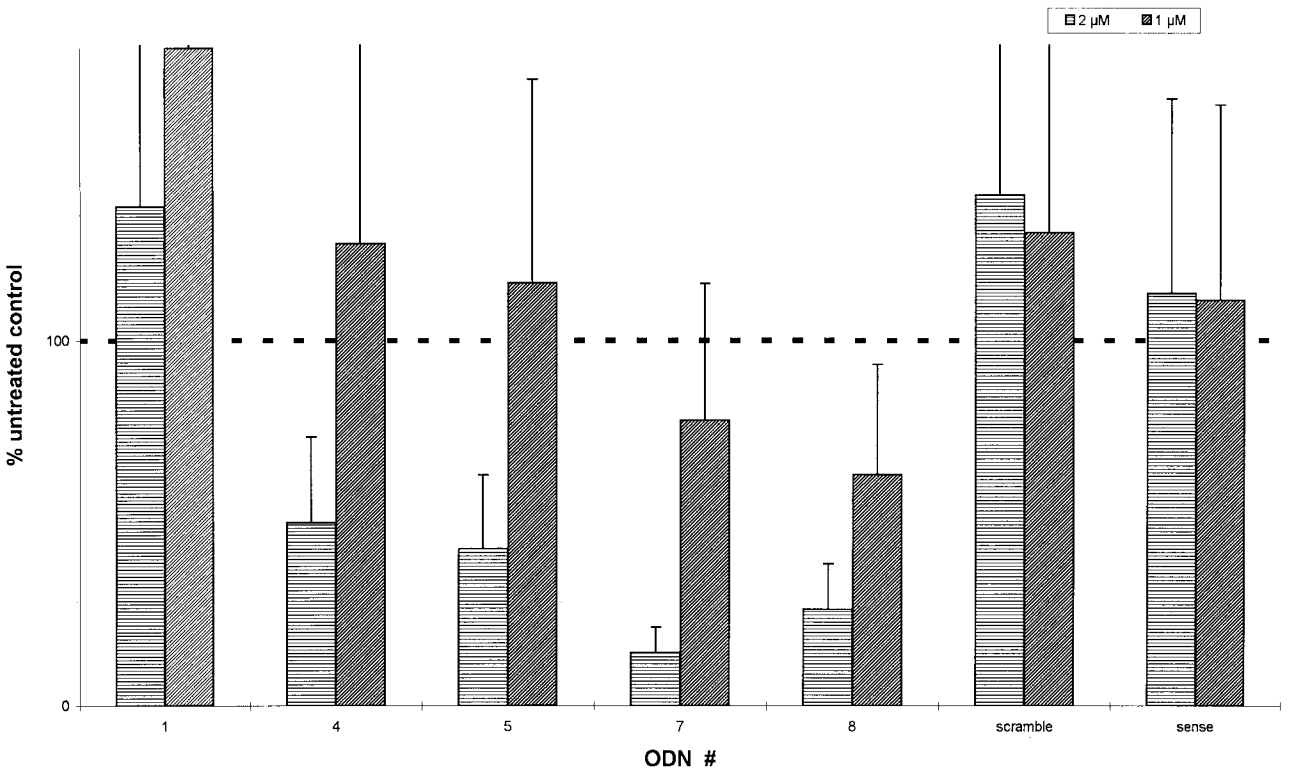

Fig. 2. Effect of antisense-ODN treatment on α2,6ST mRNA level. HT29 cells were incubated with 2 μM, 1 μM antisense ODN or remain untreated (control). α2,6ST mRNA was detected by RT-PCR with specific primers. Fluorescence of electrophoretically resolved, ethidium bromide-stained PCR products of β-actin and α2,6ST target sequences was determined for each treatment. Semi-quantitative analysis was done by comparison with β-actin co-amplified within the same tube. For each ODN experiment, α2,6ST mRNA level was compared with co-amplified β-actin and expressed as percent of the ratio of the untreated control (= 100%). Untreated controls showed 25 ± 12% of β-actin fluorescence. Each value represents the mean ± SD of at least three determinations.

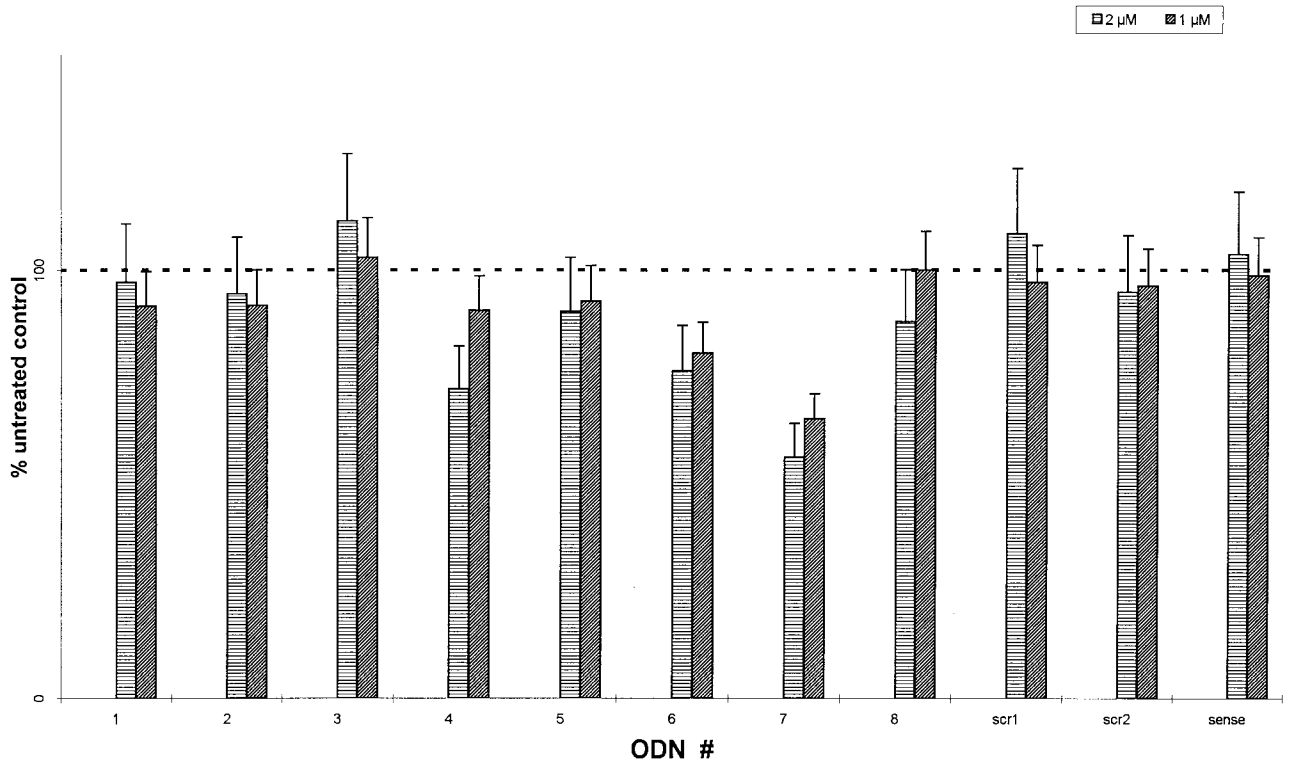

Fig. 3. Effect of antisense-ODN treatment on sialyltransferase activity. HT29 cells were incubated with 2 µM, 1 µM antisense ODN or remain untreated (control). Enzyme activity was determined by fluorometric sialyltransferase assay using asialo-α1GP as acceptor. Enzyme activity is expressed as percentage of total activity of untreated controls (100%). One hundred percent was 0.096 ± 0.012 µU. Each value represents the mean ± SD of at least three determinations.

96 ± 6% of that of untreated cells. Treatment with a 8 µM solution of *Scramble 2* decreased cell number to 78 ± 7% of that of untreated controls.

3.2. *Effects of ODN treatment on α2,6ST-mRNA*

RT-PCR of RNA extracted from HT29 cells treated with antisense ODN AS 7 produced an α2,6ST band displaying less fluorescence compared with untreated controls (Fig. 1). Co-amplification of β-actin within the same tubes produced bands with a similar density in untreated controls and antisense-treated cells.

Density of each α2,6ST band was compared with the appropriate β-actin band amplified within the same tube by calculating the ratio: (Fluorescence units of α2,6ST/Fluorescence units of β-actin) ×100. The detection limit of the fluorometric measurements was about 1 ng double-stranded DNA. The linear portion of this assay ranges up to 25 ng. Standard derivation between triplicate measurements was on an average 24%. The results show that incubation of HT29 cells with antisense-ODN AS 7 in a concentration of 2 µM reduced α2,6ST mRNA level down to 16 ± 8% of the mRNA level of untreated controls (Fig. 2).

3.3. *Effects of ODN treatment on α2,6ST enzyme activity*

Incubation of HT29 cells with antisense-ODNs in a concentration of 2 µM reduced fluorometrically determined sialyltransferase enzyme activity down to 56 ± 7% in the case of AS 7 (Fig. 3). No such inhibition of enzyme activity was

Table 1
Design of oligodeoxynucleotides synthesized for inhibition of α2,6ST expression

AS	Sequence										
1	AG GUU	GGU	GUG	AAU	**CAU**						
2	UU	GGU	GUG	AAU	**CAU**	AAU	GAA	G			
3	U	GGU	GUG	AAU	**CAU**	AAU	GAA	GA			
4			GUG	AAU	**CAU**	AAU	G				
5				AAU	**CAU**	AAU	GAA	GA			
6					**CAU**	AAU	GAA	GAU	GUG		
7					**CAU**	AAU	GAA	GAU	GUG	UUC	
8					**CAU**	AAU	GAA	GAU	GUG	UUC	AGG
Scramble 1			AGU	GAU	CUA	UAG	A				
Scramble 2			GAG	UGU	AAC	UAU	AAG	CUG	UGA		
Sense			ACA	UCU	UCA	UUA	*UG*A	UUC	ACA		

In bold letters the codon complementary to the initiation codon is depicted. U means C-5 propynyl-2′-deoxyuridin. *Scramble 1* contains the same content in A, C, G, U as AS 4 but in a random order. *Scramble 2* contains the same content in A, C, G, U as AS 8 but in a random order. *Sense* is a sense version with *AUG* in the middle of the sequence.

found after incubation of cells with AS 1, AS 2, AS 3, scrambled or sense sequences. Radiometric measurements, which allow to differentiate between α2,3ST and α2,6ST activity, showed that α2,6ST was decreased to 62 ± 5% after treatment of cells with 2 μM AS 7, but that α2,3ST activity was similar to that of untreated controls.

4. Discussion

Incubation of HT29 cells with 2 μM antisense-ODN led to a striking decrease of α2,6ST activity on the mRNA level as well as on the level of protein expression. Antisense treatment had no effect on cell viability or cell growth up to a concentration of 4 μM. ODNs which were complementary to the region upstream of the initiation codon of the α2,6ST mRNA (AS 6, AS 7, AS 8) were particularly effective in inhibiting enzyme expression. No inhibition on the level of the mRNA as well as on the level of protein expression was found by treatment of cells with ODNs complementary to the region downstream of the initiation codon, or by treatment of cells with scrambled or sense ODNs. Length of ODNs seems to influence their inhibitory potential, since the 18-mer AS 7 inhibits enzyme expression stronger than AS 8 (21-mer) or AS 6 (15-mer) with similar sequences. Inhibition of α2,6ST was stronger on the mRNA level than on the level of enzyme activity, presumably due to the longer half-life of enzyme proteins vs. RNA.

This is the first time that the expression of a glycoprotein-specific glycosyltransferase has been inhibited with antisense-ODNs. Similar treatment of human promyelocytic line HL-60 with antisense-ODNs to ganglioside glycosyltransferases has effectively down-regulated ganglioside biosynthesis [12]. Future experiments will show whether it is possible to reduce tumor cell surface sialylation in this way. Then functional studies will be possible with the aim to obtain antisense-ODNs suitable for the clinical treatment of metastasis.

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