Impact of Sodium Butyrate on the Network of Adhesion/Growth-regulatory Galectins in Human Colon Cancer *In Vitro*

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Abstract. *Background/Aim: The physiological compound sodium butyrate can induce differentiation in colon cancer cells in vitro. Due to the role of galectins in growth control we explored its effect on this network beyond galectins-1 and -3, with deliberate consideration of the status of microsatellite stability, for nine cell lines. Materials and Methods: Microscopical monitoring and measurement of alkaline phosphatase activity ascertained butyrate's impact on cells. Monitoring by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting with galectin-type-specific probes characterized galectin expression. Results: Controlled by expectable strong up-regulation of galectin-1 and comparatively small effects on galectin-3 regulation for galectins-4, -7, -8 and -9 were reported with no obvious association to microsatellite stability status. Neoexpression of the GAL-12 gene was observed in eight out of nine tested lines. Conclusion: Butyrate affects the galectin network beyond galectins-1 and -3, warranting further cell biological and histochemical studies.*

Control experiments in the course of examining the effect of cyclic adenosine monophosphate and its N^6, $O^{2'}$-dibutyryl derivative on induction of alkaline phosphatase (in HeLa cells) or of differentiation (in erythroleukemia cells) led to the discovery of the unsuspected potency of butyrate in this respect (1, 2). Consideration of its physiological occurrence at sites of tumor development and progression prompted further study, especially on colon cancer cells (3, 4). Intriguingly, gross morphological changes including transient or permanent acquisition of typical enterocytic features, increase in doubling times and up-regulation of expression of markers for a differentiated phenotype, such as alkaline phosphatase or carcinoembryonic antigen, are characteristics associated with exposure to butyrate, with dependence of extent of effect on the type of cell line (5-15). Butyrate, thus, is a means to re-program the cells' gene expression profile so that respective monitoring before and after its application can help to identify effectors for the reduction of degree of malignancy. The emerging insights into the role of tissue lectins and their interplay with cell surface glycans for regulating diverse aspects of cell sociology, *e.g.* cell attachment and growth control (16-20), provide incentive to profile alterations in expression with this approach. In the present study, we focused on members of the family of galectins (β-galactoside-specific β-sandwich proteins showing a distinct sequence signature), which are multi-functional, *e.g.* acting as potent growth modulators extra- and intra-cellularly (16, 21-26).

Following initial observations that sodium butyrate (NaBut; tested at 1-2 mM) affects glycan-binding capacity (monitored with neoglycoproteins) and induces galectin-1 (GAL-1) expression in colon carcinoma cells (COLO205; KM12P) (27, 28), its special ability to induce GAL-1 in KM12 cells, when compared to 10 other differentiating compounds, was delineated and extended to seven other colon cancer lines (DLD-1, MIP101, HT-29, LoVo, LS174T, SW403, HCT15), while galectin-3 (GAL-3) was only modestly affected (29). Further work confirmed the strong butyrate effect on HT-29 cells and added LS180 to the list of responsive lines (30, 31). A Sp1 site around -57 appears to be crucial for the transcriptional regulation of the *GAL-1* gene in murine embryonal carcinoma cells (32). On the side of binding partners, the glycoproteins carcinoembryonic antigen and lysosome-associated membrane glycoproteins-1/2 were defined as ligands for GAL-1 and -3 and found to be similarly up-regulated (29, 33). Gene expression for the surface

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glycoprotein β1-integrin, also a galectin ligand when properly glycosylated, was also found to be up-regulated (4.8-fold) in murine colon epithelial cells, along with galectin-9 (GAL-9; 4.7-fold) (34). When moving on from this experimental basis, two parameters deserve attention: (i) the presence of a network of galectins in colon cancer beyond GAL-1 and -3 with documented prognostic relevance of combinations (35-39) and (ii) the stratification of tumor line origin according to status of microsatellite stability/instability (MSS/MSI), which may be reflected in galectin expression (40). Consequently, we monitored the effect of NaBut on gene expression for a panel of nine human galectins, comparatively testing four lines with MSI status and five lines with MSS status.

Materials and Methods

Cell lines and NaBut treatment. The nine human colon adenocarcinoma cell lines (details given in Table I) were cultured in RPMI 1640 medium (GE Healthcare, Freiburg, Germany) supplemented with 10% fetal calf serum (GE Healthcare), 1% streptomycin and 1% penicillin (GE Healthcare) at 37˚C in a humidified atmosphere containing 5% CO_2. The medium was changed three times per week. Prior to treatment, cells were seeded on six-well plates or 10-cm dishes (Greiner, Frickenhausen, Germany) and allowed to attach to the plastic surface for three days. NaBut (Sigma-Aldrich, Munich, Germany) was brought into solution with water; the solution was sterile filtrated through a 0.2 μM syringe filter (Merck Millipore, Darmstadt, Germany) and directly added to the growth medium. NaBut treatment routinely was for seven days at the following concentrations: 1.5 mM (LS180), 2 mM (SW480, SW707) and 5 mM (HCT15, KM12, TC7, CX1, HT-29, LS513). The medium was changed on days 2, 4 and 6 during treatment. For cell harvest, after the treatment period, medium was removed, cells were washed twice with phosphate-buffered saline, collected by scraping and pelleted by centrifugation at 1,200 rpm for 10 min. Subsequently, cell pellets were stored at –20˚C for biochemical assays or at –80˚C for RNA isolation. Morphological alterations upon NaBut treatment were documented by light microscopy (CK40; Olympus Europa Holding, Hamburg, Germany).

Assay for alkaline phosphatase activity. As a measure of cellular differentiation, enzyme activity was assessed. Cells ($1\text{-}3\times10^5$/well) were grown in triplicates on six-well plates and cultured with or without NaBut as described before. Immediately after harvesting, cell pellets were resuspended in 200 μl extraction buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA and 0.5% Triton X-100, pH 8). To disrupt the cell membrane, samples were sonicated for 30 s on ice. Subsequently, 450 μl assay buffer (5 mM $MgCl_2$, 100 mM $NaHCO_3$, pH 10) and 50 μl 40 mM p-nitrophenylphosphate (stored in solution at –80˚C; Sigma-Aldrich) were added. After incubation at 37˚C for 15 min, the conversion of p-nitrophenylphosphate into p-nitrophenolate was stopped by adding 1 M NaOH. Extent of enzyme activity was determined in triplicates at an absorbance value of 405 nm using a spectrophotometer (Ultrospec 3000; Amersham Pharmacia, Freiburg, Germany). Protein concentrations were analyzed by the method of Lowry. Enzyme activity was expressed in units: 1 unit is equivalent to 1 micromole of substrate hydrolyzed per minute.

Analysis by reverse transcriptase-polymerase chain reaction (RT-PCR). Cells (1.3×10^5/well) were grown on six-well plates and treated as described above. Cell pellets were immediately stored at –80˚C to prevent RNA degradation. Total RNA was extracted from cells using the RNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. RNA yield and purity were assessed using a spectrophotometer. One μg of total RNA was converted into cDNA using SuperScript™ II reverse transcriptase (Invitrogen, Dreieich, Germany) and oligo dT Primer (Invitrogen) according to the manufacturer's protocol. Production was controlled by amplifying the transcript of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*). Twenty ng of cDNA were amplified by RT-PCR using a Robo Cycler Gradient 96 (Stratagene, Bremen, Germany). The cycling program was performed as follows: activation of DNA polymerase (Hot-MolPol DNA-Polymerase; Molegene, Butzbach; Germany) at 95˚C for 15 min followed by 40 cycles of 95˚C for 53 s, 60˚C for 1 min 5 s and 72˚C for 52 s with a final extension at 72˚C for 2 min. Sets of primer sequences for each galectin are given in Table II. Amplified DNA was separated by electrophoresis in a 2% agarose gel, stained with Midori Green Advance (Biozym Biotech Trading GmbH, Oldendorf, Germany) at 140 V for 20-40 min. As a positive control, cDNA samples from cell lines documented to be positive were amplified.

Analysis by western blotting. Using aliquots of cell pellets prepared from bulk cultures as described above, they were re-suspended in 100-200 μl radioimmunoprecipitation assay (RIPA) cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.01 mM $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) and sonicated for 30 sec on ice. To reach completion of cell lysis, samples were subsequently incubated for 1 h at 4˚C. Afterwards, samples were centrifuged at 15,000 rpm and 4˚C for 20 min. The supernatant was collected for Western blot analysis and total protein concentration was determined by the Bradford reagent (BioRad, Munich, Germany). Equal amounts of proteins were separated by electrophoresis in 4-20% polyacrylamide gradient gels (RunBlue SDS-Page precast gels; Expedeon, Harston, UK) for 40-50 min at 200 V, 250 mA and 50 W. After semidry blotting onto a nitrocellulose membrane (0.45 μm), protein binding to the membrane was blocked by incubation in a solution of 5% milk in Tris-buffered saline (TBST) containing 20 mM Tris-HCl, 0.5 mM NaCl and 0.1% Tween-20 overnight at 4˚C. Then the galectin-type-specific primary antibody was added to the blocking buffer and allowed to bind to its antigen on the nitrocellulose for 24-72 h at 4˚C. Optimized antibody concentrations were: GAL-1 and -2, 0.5 ng/μl; GAL-3, 0.16 ng/μl; GAL-4 and -8, 0.25 ng/μl; GAL-7, 2 ng/μl. All antibody preparations had been rigorously put through in-house cycles of analysis for cross-reactivity and removal of cross-reactive fractions by affinity depletion to ensure specificity.

After three washing steps in TBST, the membrane was incubated with a solution of horseradish peroxidase (HRP)-labeled anti-rabbit antibody (diluted 1:2,500 in blocking buffer; Promega, Mannheim, Germany) for at least 1 h at room temperature. Finally, the membrane was washed three times in TBST before HRP activity was visualized using a chemiluminescence detection system (Western Lightning *Plus* ECL; Perkin Elmer, Rodgau, Germany). Actin detection, using a mouse anti-actin monoclonal antibody (diluted 1:20,000-1:60,000 in blocking buffer, 30 min at room temperature; MP Biomedicals, Heidelberg, Germany), served as loading control. HRP-labeled sheep anti-mouse antibody (diluted 1:5,000 in blocking buffer, 1 h room

Table I. *Human colon adenocarcinoma cell lines used for analysis.*

Cell line	Status	Origin	Gender, age (years)[a]
HCT15	MSI-high	Colorectal adenocarcinoma	Male
KM12	MSI -high	Colorectal adenocarcinoma	-
LS180	MSI -high	Colorectal adenocarcinoma	Female, 29
TC7	MSI -high	Colorectal adenocarcinoma	Female, 58
CX1	MSS	Colorectal adenocarcinoma	Female, 44
HT-29	MSS	Colorectal adenocarcinoma	Female, 44
LS513	MSS	Colorectal carcinoma	Male, 54
SW480	MSS	Colorectal adenocarcinoma	Male, 50
SW707	MSS	Rectal adenocarcinoma	Male, 81

[a]Patients' characteristics; MSI, microsatellite instability, MSS, microsatellite stability.

Table II. *Primer sequences used for RT-PCR analysis.*

Gene name	Sequence (5'-3')	Product size
*GAL-1*_forward	AACCTGGAGAGTGCCTTCGA	322 bp[a]
*GAL-1*_reverse	GTAGTTGATGGCCTCCAGGT	
*GAL-2*_for	ATGACGGGGGAACTTGAGGTT	357 bp
*GAL-2*_rev	TTACGCTCAGGTAGCTCAGGT	
*GAL-3*_for	CTGCCTCGCATGCTGATAAC	308 bp
*GAL-3*_rev	CATTGAGTTTTTTAACCCGATGATTG	
*GAL-4*_for	TGGTAAATGGAAATCCCTTCTATG	289 bp
*GAL-4*_rev	GAGCTGTGAGCCCTCCTT	
*GAL-7*_for	CAGACGACGGCTTCAAGG	127 bp
*GAL-7*_rev	AAGATCCTCACGGAGTCCAG	
*GAL-8*_for	TCTGGGCATTTATGGCAAAGTG	*GAL-8S*: 174 bp[b] *GAL-8L*: 300 bp
*GAL-8*_rev	CATGGGGGTGTTCAACCTTG	
*GAL-9*_for	ACCTCTGCTTCCTGGTGCA	*GAL-9S*: 188 bp *GAL-9L*: 284 bp
*GAL-9*_rev	GCACTGTGTGGATGACTGTC	
*GAL-12*_for	TGCTCTTCCCCAGGGTCT	*GAL-12S*: 306 bp *GAL-12L*: 316 bp
*GAL-12*_rev	GGCCTGCTGGTTCATGCT	
*GAL-13*_for	GCAAACAATTTGAGCTGTG	148 bp
*GAL-13*_rev	CACTGAGGTCAGGGAGA	
*GAPDH*_for	CCTGCACCACCAACTGCTTA	233 bp
*GAPDH*_rev	TTCAGCTCAGGGATGACCTT	

[a]Base pair: bp; [b]alternative splicing of primary transcript generates two isoforms based on length: S (for short) and L (for long).

temperature; GE Healthcare) facilitated signal visualization based on chemiluminiscence. Positive controls were performed using the following quantity of recombinant galectins from in-house production: 5 ng GAL-2, -4, -7, -8S and -8L and 10 ng GAL-1 and -3.

Results

Changes in morphology and marker expression upon exposure to NaBut. The cell lines were kept in culture medium without or with NaBut (at different concentrations) for up to seven days and monitored for changes in morphological appearance and activity of alkaline phosphatase. Respective light microscopical monitoring confirmed an impact, leading to a shift in cellular features, *e.g.* elongation, increase of cytoplasmic volume and enlargement of the nucleus. All tested cell lines displayed such changes. The extent of susceptibility toward NaBut, as determined by studying gross cytological features, varied among the nine tested lines. According to the cells' individual sensitivity, this part of the comparative analysis documented responses when applying the following concentration over the given culture period: LS180, 1.5 mM/two days; HCT15, KM12, TC7, CX1 and LS513, 5 mM/two days; SW480, 2 mM/four days; SW707, 2 mM/four days; HT-29; 5 mM/six days). Representative examples of cell morphology are given in Figure 1.

At the indicated concentrations no signs of cytotoxicity were observed. In order to cover both major pathways of colon cancer development we tested both MSS, as well as MSI cancer colon cell lines (Table I). As a biochemical measure for a cellular response to NaBut, we next quantitated the specific activity of alkaline phosphatase. As documented in Figure 2, this parameter was consistently increased, albeit to different extents, in line with morphological alterations. Intra-group variability was observed in both sets of lines separated by the microsatellite status. Having herewith ascertained reactivity of human colon cancer lines to NaBut by two criteria, we proceeded to galectin profiling, by RT-PCR analysis using a set of specific primer pairs (and primers for *GAPDH* as loading control) to detect transcriptional regulation and by western blotting using non-crossreactive antibodies to compare levels of protein presence.

Galectin fingerprinting. All analytical experiments were performed on samples obtained after a culture period of seven days using the following NaBut concentrations: 1.5 mM (LS180); 2 mM (SW480, SW707); 5 mM (HCT15, KM12, TC7, CX1, LS513 and HT 29), with controls from the same passage kept parallel under otherwise identical conditions. Where literature data were available (for example for RT-PCR analysis on HCT15, HT-29, LS513 and SW480; (36)), the obtained data on expression of galectin genes without treatment were mostly in agreement. These profiles of cells without NaBut treatment clearly attested that

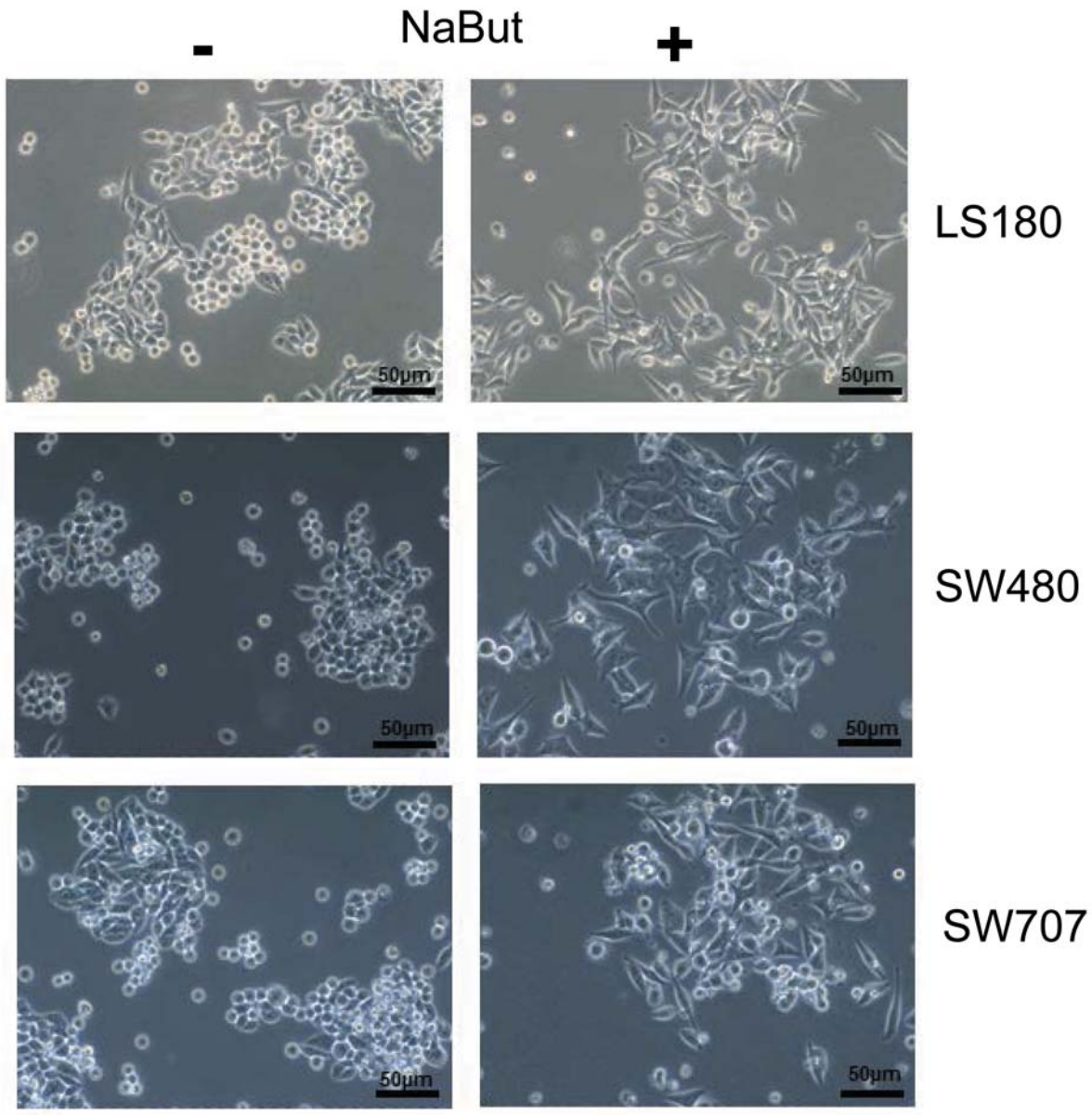

Figure 1. *Light microscopical analysis of effect of NaBut on colon carcinoma cells. Cells were incubated in the absence (–) or presence (+) of NaBut: LS180 with 1.5 mM NaBut for two days; SW480 and SW 707 with 2 mM NaBut for four days. Microphotographs were taken with an Olympus CK40 light microscope.*

presence of transcripts for several galectin genes is a common feature of colon cancer cells, underscoring the network concept. When examining expression of NaBut-treated cells, the densitometric quantitation of signal intensity revealed an overall trend for increases in *GAL-1* gene transcription, less so for GAL-3 (Table III). As shown exemplarily in Figure 3, cases of galectin genes were detected with differential regulation (for *GAL-2* and *-7*; Figure 3A-F) and with nearly invariable induction of expression (for *GAL-12*; Figure 3G, H) among the tested cell lines. In the two cases with known occurrence of isoforms by alternative splicing, *i.e. GAL-8* and *-9*, the effect of butyrate depended on the cell line (Figure 3I, J) (Table III).

When taking the analysis to the level of protein presence with non-crossreactive antibodies against GAL-1, -2, -3, -4, -7 and -8, any influence of post-transcriptional mechanisms could be assessed. The differences obtained by comparing results from extracts of control and NaBut-treated cells could

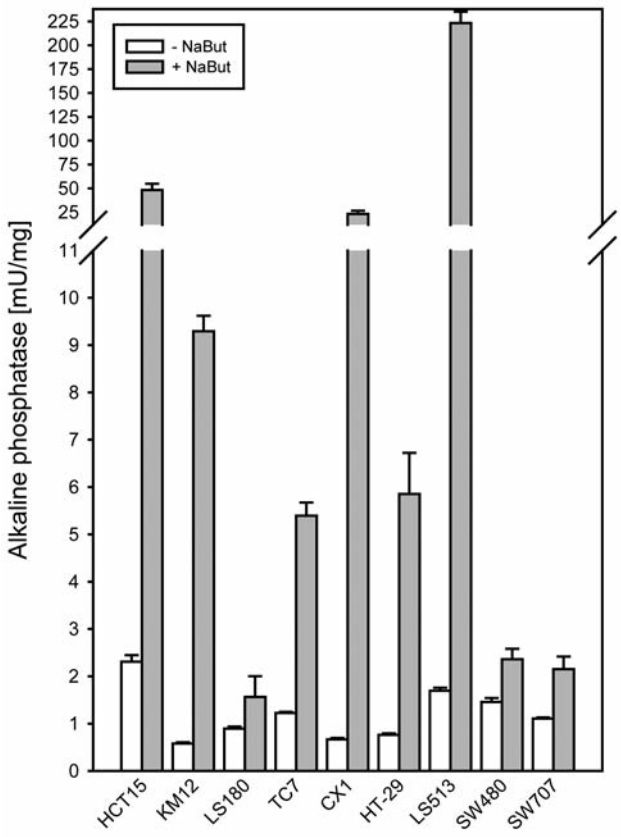

Figure 2. *Induction of activity of alkaline phosphatase in colon carcinoma cell lines. The cells were cultured in the absence (–) and presence (+) of NaBut for seven days before cells were harvested, the pellets homogenized and enzyme activity determined by a colorimetric assay. NaBut concentrations were 1.5 mM for LS180; 2 mM for SW480, SW707 and 5 mM for HCT15, KM12, TC7, CX1, HT-29 and LS513. Results are the mean of three independent observations +/- SD (p-values by t-test always <0.01).*

reach a fairly high extent (Figure 4A) so that visual scoring on a semi-quantitative scale was performed, its result given in Table IV. Rather similarly intense RT-PCR signals and markedly different staining for galectins in western blots can occur, as documented for a second case of GAL-1 positivity (Figure 4B). GAL-1 and -3 were nearly consistently present in higher amounts after treatment, GAL-7 (product of p53-induced gene 1) was found up-regulated in five out of nine cases and GAL-8 presence was regulatable on the level of both forms with disparity in linker length, with a tendency into opposite directions depending on the status of microsatellite stability (Figure 4C, D) (Table IV). In comparison, protein presence for GAL-2 appeared to be much less responsive to NaBut than for GAL-1, -3, -7 and -8, and level of GAL-4 was up-regulated in three out of nine lines (Table IV).

Discussion

The present study broadened the data basis on the effects of butyrate on human colon carcinoma cells. By deliberately considering the status of microsatellite stability, it is clarified that the tested lines, irrespective of this parameter, were responsive to exposure to NaBut, albeit to a variable degree in both groups. When monitoring galectin expression beyond the rather common focus on GAL-1 and -3, the results substantiated the concept of a network. Overall, the observed NaBut-induced up-regulation makes more capacity for binding β-galactosides available. This may be exploited to dock bi-functional reagents with galectin-sensitive headgroups, preferably in clustered presentation to enhance avidity and enable selectivity (41-43), at such sites, with the aim to increase delivery of therapeutic agents such as inhibitors of matrix-degrading proteases to the tumor (44, 45). In greater detail, regulation of protein expression of GAL-4, -7 and -8 (but not GAL-2) can now be added to the known impact on GAL-1 and -3. Since the effect on GAL-7, the product of the p53-induced gene 1 coded by a two-gene constellation in the human genome (46, 47), depended on the cell line, an involvement of p53 in mediating responses to butyrate is rather unlike, as noted before (48, 49). Most conspicuously, transcription of the *GAL-12* gene, not seen in untreated cells, is induced in eight of nine lines, LS513 being the exception. As described for GAL-1 and carcinoma cells exerting growth control *via* p21/p27 (50), GAL-12 is able to arrest the cell cycle at the G_1 stage (in HeLa cells), and the lectin's expression is up-regulated by cycle synchronization (in Jurkat cells) (51). Onset of apoptosis was observed after transfection in COS-1 cells, GAL-12 presence was correlated with frequency of apoptosis in adipose tissue, herewith strengthening the assumption for its growth-regulatory role (52).

In view of these gained insights, the following issues deserve to be a topic of further work: (i) it is an open question to what extent individual galectins (and especially combinations thereof) can effect colon cancer cell growth when added to the medium or expressed intra-cellularly, except for initial studies on LS-180 cells/GAL-1 and also recently on four lines (Colo6OH, HCT116, SW480, SW707)/GAL-8 and its natural variant (31, 53). (ii) Obviously, the immunohistochemical galectin fingerprinting in colon cancer should be extended to GAL-12, with parallel application of the labeled lectin as cyto- and histochemical probe to map presence of accessible binding sites, on cells as done for the COLO205/SW480/SW620 lines and GAL-1 and -3 (54) and on tissue sections as done with labeled GAL-3 (39). (iii) as the functional consequences of co-regulation of galectin and its counterreceptor by the tumor suppressor $p16^{INK4a}$ and in inter-T-cell communication after activation of effector/regulatory T-cells attest (55-58), the glycome profile in butyrate-treated cells may similarly be reprogrammed for roles underlying the phenotypical changes. These can concern general properties of glycoproteins, *e.g.*

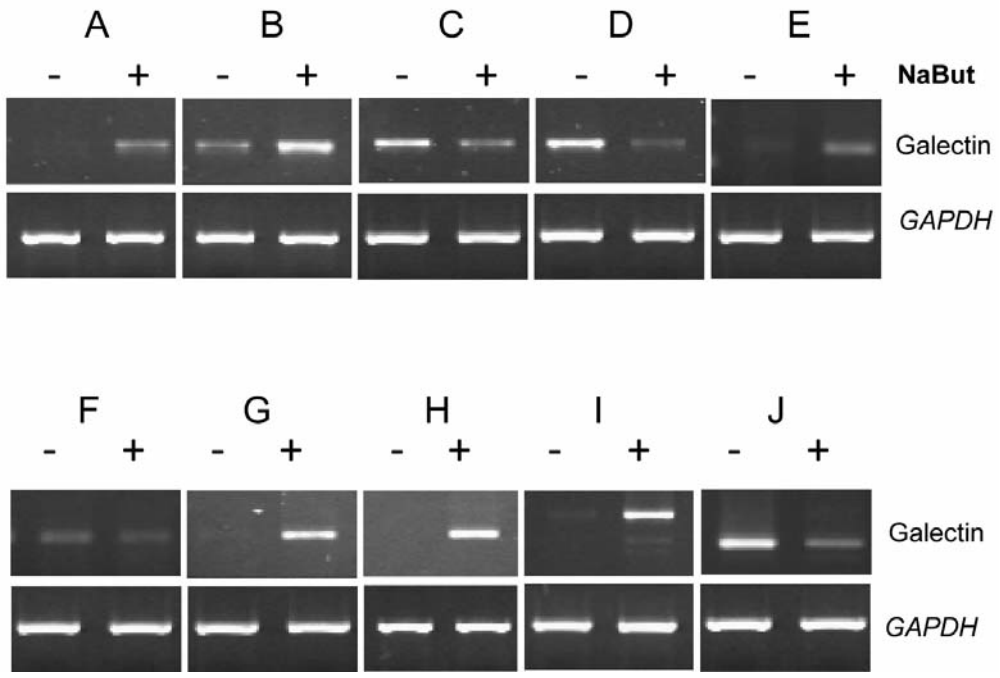

Figure 3. *Expression of galectin (mRNA) monitored by RT-PCR. Colon carcinoma cells were cultured for seven days in the presence (+) or absence (–) of NaBut. Conditions were as described in Figure 2. Presence of galectin-specific mRNA was analyzed by RT-PCR using detection of GAPDH-specific mRNA as control as detailed in Materials and Methods. A-D: GAL-2, KM12/TC7/CX1/HT-29; E, F: GAL-7, CX1/HT-29; G, H: GAL-12, CX1/SW480; I, J: GAL-9, HCT15 (GAL-9L)/SW707 (GAL-9S).*

stability and activity (59) but also ligand properties for cognate tissue lectins, leading to recognition and signaling induction (60). Fittingly, the activity of α2,6-sialyltransferase I, which produces a switch-off signal for galectin binding to N-glycans, was found to be drastically reduced in butyrate-treated T84 (colonic) cells, whereas the level of its mRNA was decreased by 90% post-transcriptionally in HepG2 hepatocellular carcinoma cells (61, 62). Of note, cell surface α2,6-sialylation was found to be down-regulated after reconstituting tumor suppressors in cells of the microsatellite-instable HCT116 colon carcinoma line (63) and in cells of the SW620 colon carcinoma line after reducing the expression of the Rho GTPase Rac1 (64). In contrast, oncogenic Ras increased α2,6-sialylation, leading to shifts in β1-integrin functionality towards increased motility in HD3 colonocytes (65). Alterations in galectin gene expression/presence, as noted to be regulated on different levels by $p16^{INK4a}$ in pancreas carcinoma cells (66), as well as glycosylation of galectin counterreceptors can additionally be flanked by changes in the presence of the carrier protein for the glycans, to make cells susceptible for galectin-dependent growth regulation, as is the case for the fibronectin receptor as downstream effector of Gal-1-dependent anoikis induction (67). (iv) Together with the presented results, observations that butyrate affects GAL-1 expression in prostate (LNCaP) and head and neck squamous carcinoma (five out of

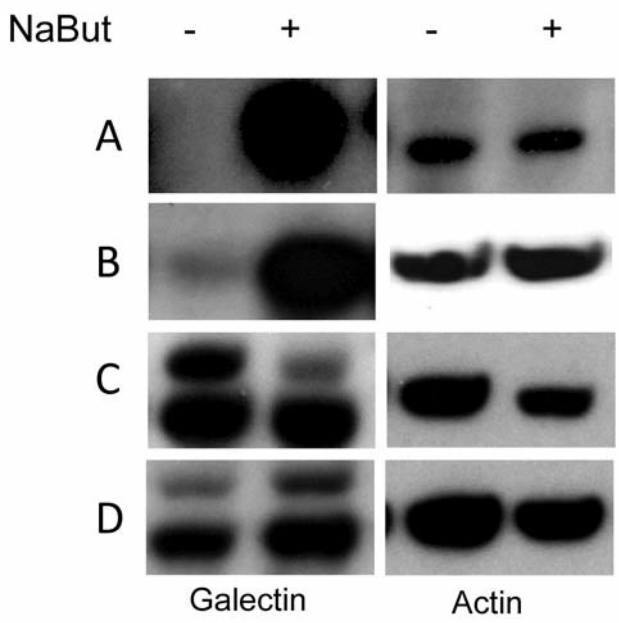

Figure 4. *Expression of galectins (protein) monitored by western blotting. Colon carcinoma cells were cultured for seven days in the presence (+) or absence (–) of NaBut. Conditions were as described in* Figure 2. *Presence of galectin proteins was analyzed by western blotting together with that of actin as loading control as detailed in Materials and Methods. A: GAL-1, KM12; B: GAL-1, TC7; C, D: GAL-8, LS180/LS513.*

Table III. *Densitometric scoring of RT-PCR analysis of galectin expression*[a].

	GAL-1	*GAL-2*	*GAL-3*	*GAL-4*	*GAL-7*	*GAL-8S*	*GAL-8L*	*GAL-9S*	*GAL-9L*	*GAL-12*	*GAL-13*
HCT15	1.9	2.7	1.3	1.5	NEO	0.7	2.2	NEO	14	NEO	NEO
KM12	2.4	NEO	1.3	1.3	2.0	0.9	1.5	1.5	1.2	NEO	-
LS180	1.1	NEO	0.7	6.4	1.3	0.6	1.4	0.7	-	NEO	3.5
TC7	1.9	3.1	1.2	1.0	NEO	0.9	1.9	61.3	0.9	NEO	NEO
CX1	3.8	0.5	0.7	1.2	6.1	1.2	1.4	1.4	0.8	NEO	-
HT-29	5.1	0.2	1.0	0.8	0.5	0.6	6.0	1.7	1.4	NEO	-
LS513	6.1	-	1.3	0.9	1.2	0.9	2.1	1.2	0.6	-	-
SW480	1.1	NEO	0.8	0.9	1.3	0.7	1.3	0.7	-	NEO	NEO
SW707	1.6	NEO	0.9	0.8	1.0	1.1	0.7	0.3	-	NEO	3.1

[a]The optical density of each band was quantified by commercial Quantity One software (BioRad). Background was substracted using the rolling disc method, then the Gaussian trace quantity of each band, defined as area under its Gaussian-fitted profile (intensity ×mm), was determined. The fold change given in the table was defined as follows: Gaussian trace quantity of treated cells divided by this parameter of untreated cells; NEO, neo-expression; - , no signal detected. Following initial systematic testing cases of up- or down-regulation were ascertained in up to three independent experimental series.

Table IV. *NaBut-induced changes of presence of galectin protein*[a].

Galectin (GAL)	Cell line								
	HCT15	KM 12	LS180	TC7	CX1	HT-29	LS513	SW480	SW707
1	+3	NEO (+3)	+2	+3	+3	+3	NEO (+3)	+2	+2
2	0	0	0	0	-1	0	0	0	0
3	+2	+3	+2	0	+2	+3	0	+1	+2
4	0	+2	0	0	+2	+2	-1	0	0
7	NEO (+2)	+3	0	+3	0	+1	+2	0	0
8S	0	0	-1	0	+2	+2	+1	0	0
8L	+1	-2	-2	0	0	0	+1	0	0

[a]Presence of galectin protein was rated by visual scoring of bands in Western blot analysis in direct comparison of results obtained from extracts of NaBut-treated and control cells and any difference graded semiquantitatively as follows: 0, No detectable change of expression; 1, low extent of change (2-5-fold); 2, medium extent of change (5-10-fold); 3, high extent of change (<10-fold); NEO, Neo-expression (in these cases, the number is a measure of signal intensity); +, increase after NaBut treatment; –, decrease after NaBut treatment. Following initial systematic testing cases of up- or down-regulation were ascertained in up to three independent experimental series.

eight lines, with evidence for transcriptional up-regulation in MDA-886LN cells, to which increased histone acetylation may contribute) cells and on GAL-1 and -8 expression (GAL-8 is widely present in tumors (68, 69)) in lung cancer (squamous, SK-MES-1; adenocarcinoma, A549) cells (70-73) give direction to examine other carcinoma types in this respect, *e.g.* for GAL-12 induction. (v) With increasing attention being given to the tumor microenvironment and also galectin expression in the stroma, *e.g.* as prognostic factor or cellular effector (74-77), studying the impact of butyrate on stromal and immune cells should complement monitoring cancer cell properties.

Gaining control on tumor cell growth by physiological substances is an attractive aim worth to strive for by further efforts, as outlined here for butyrate and the protein side of galectin-counterreceptor interplay, an initially phenomenologically defined pathway now dissected in molecular terms (78, 79). Besides contributing to clarify butyrate's potential for clinical application, respective work will add to our understanding over the functionality of the galectin network with potential for additive activities and in connection to counterreceptor generation.

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