Oligosaccharides from human milk induce growth arrest via G2/M by influencing growth-related cell cycle genes in intestinal epithelial cells

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Oligosaccharides are present in human milk in large amounts and in a high variety. We have previously shown that these oligosaccharides are strong inhibitors of proliferation and inducers of differentiation in intestinal cell lines. To elucidate the molecular mechanism, we investigated the influence on cell cycle events via flow cytometry and expression levels by using quantitative real-time RT–PCR. Human intestinal cells, i.e. HT-29, HIEC and Caco-2 cells, were exposed to neutral or acidic human milk oligosaccharides. Both fractions induced a concentration-dependent G2/M arrest. Cell cycle analysis for HT-29 revealed 37 % of cells in G1 and 35 % in G2/M (neutral oligosaccharides) and incubation with acidic oligosaccharides led to 42 % cells in G1 and 40 % in G2/M. In control experiments without oligosaccharides we found 71 % of cells to be in G1 and 17 % in G2/M. This G2/M arrest was associated with changes in mRNA expression of cyclin A and B. A G2/M arrest with concomitant alterations of cell cycle gene expression could also be shown for HIEC and Caco-2 cells. Analysing the expression of cyclin-dependent kinase inhibitors p21kip1 and p27kip1 and the tumour suppressor p53 we observed that the expression of p21kip1 was p53-independent and necessary for arresting cells in the G2/M phase, while p27kip1 was associated with differentiation effects. Both neutral and acidic human milk oligosaccharides were able to induce epidermal growth factor receptor, extracellular signal-regulated kinase 1/2 and p38 phosphorylation. These results suggest that oligosaccharides from human milk inhibited intestinal cell proliferation and altered cell cycle dynamics by affecting corresponding regulator genes and mitogen-activated protein kinase signalling.

Oligosaccharides: G2/M arrest: Cyclins: Cyclin-dependent kinase inhibitors: Mitogen-activated protein kinase signalling

Human milk oligosaccharides (HMO) are currently discussed as participating in the development of a normal microflora in neonates, as soluble receptors for pathogens preventing their adhesion to the epithelial cells and as modulators of inflammatory processes(1–4). The total oligosaccharide content in human milk ranges from 5 to 15 g/l compared to about 1 g/l in mature bovine milk(5–8). Neutral oligosaccharides comprise about 70 % of the total oligosaccharide content in human milk, whereas acidic oligosaccharides account for 30 % (9). As the intestine of the breast-fed infant is continuously exposed to such high concentrations of oligosaccharides for up to several months, it is intriguing to consider whether ingested oligosaccharides may modulate neonatal intestinal development. Recent studies using the intestinal cell line Caco-2 revealed that specific neutral oligosaccharides are minimally digested, could in part be taken up and get enriched by intestinal cells(10–12). Hence, they might have the potential to affect enterocyte function. Renewal of the intestinal villi is a well-regulated sequential process of proliferation, differentiation and apoptosis. We have recently shown that neutral and acidic HMO were able to reduce cell proliferation and enhance alkaline phosphatase activity particularly in undifferentiated cell lines(13). However, up to now, no information is available regarding a direct effect of HMO on intestinal cell integrity and the mechanisms involved. Processes affecting cell fate are highly regulated and cell cycle genes like cyclins, cyclin-dependent kinases (CDK) and inhibitors play an important role in that context(14,15). Dependent on their level of expression, cyclins regulate cell progression and are responsible for arresting cells at a defined state. Various molecular studies showed that a G1 arrest is associated with a decrease in cyclin D and cyclin E(16,17). This decrease was found to be associated with changes in CDK protein levels and activity. Concomitant to these changes CDK inhibitors such as p21kip1 and p27kip1 were up-regulated and arrested cells in cell cycle phase G1 or G2(18,19).

Although most of the in vitro studies on p21kip1 regulation have concentrated on p53-dependent and p53-independent transcriptional regulation, a series of new studies has shown that p21kip1 can be regulated by post-translational mechanisms. Phosphorylation of p21kip1 modulates not only interaction and binding with target proteins but also its stabilization(20). These phosphorylation events in cell cycle

Abbreviations: CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitors; Ct, cycle threshold; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HMO, human milk oligosaccharides; MAPK, mitogen-activated protein kinase.

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progression occur through different signal cascades such as receptor-mediated MAPK activation which could result in both G1 and G2 arrest.

Thus, the objective of the present study was to investigate how oligosaccharides induce inhibition of cell proliferation and which mechanism participates in these intestinal cell dynamics.

Materials and methods

Isolation of human oligosaccharides from human milk and human milk oligosaccharide preparation

Oligosaccharides were isolated from human milk as described previously\(^7,13,21\). Briefly, after centrifugation, the lipid layer was removed, and the proteins were precipitated from the aqueous phase with ice-cold ethanol. Lactose was removed by gel filtration on Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden). The total oligosaccharide fraction was further separated into an acidic fraction (acidic HMO), consisting of compounds with N-acetylnεuraminic acid residues, and a neutral fraction (neutral HMO) without N-acetylnεuraminic acid residues, using HPLC anion-exchange on a Resource Q column (Pharmacia Biotech) at the following conditions: 100 % eluent A (H₂O) from 0 to 7.5 min, a linear gradient to 55 % eluent B (0.6 M-NaCl) for 42.5 min, a linear gradient to 100 % eluent B for 2 min, and a constant flow with 100 % eluent B for 8 min. A flow rate of 2 ml/min was used. The eluting fractions were monitored at 214 nm. Afterwards, pooled HPLC fractions were desalted by gel filtration on Sephadex G-25 (Pharmacia Biotech).

Cell culture

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Rockville, MD, USA); Caco-2 cells were a gift from R. K. Kinne (Max Planck Institute of Molecular Physiology, Dortmund, Germany). The fetal intestinal colon cell line HIEC was generously donated by J. F. Beaulieu (Department of Anatomy and Cell Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada\(^22\)). Cells were cultured in 75 cm² tissue culture flasks (Renner, Darmstadt, Germany) in RPMI 1640 (HT-29 and HIEC) or Dulbecco’s modified Eagle’s medium (Caco-2) supplemented with 10 % fetal calf serum, 2 mM-glutamine (Invitrogen, Karlsruhe, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The samples were incubated at 37°C for 60 min, followed by an incubation at 95°C for 15 min.

Analysis of mRNA expression (real-time quantitative PCR)

Total RNA from intestinal cells was isolated using TRIZol reagent (Invitrogen) according to the manufacturer’s instruction with slight modifications. Total RNA was quantified by measuring the absorbance at 260 nm, and the purity was checked by the 260/280 nm absorbance ratio. cDNA synthesis was carried out in a reaction volume of 50 μl containing 500 ng RNA, 50 nm-2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (pH 8.3), 75 mM-KCl, 3 mM-MgCl₂, 10 mM-dithiothreitol, 100 ng oligodeoxythymidine 15 primer, 500 μM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 10 units ribonuclease inhibitor and 200 μl Moloney murine leukemia virus RT; all reagents were obtained from Invitrogen. The samples were incubated at 37°C for 60 min, followed by an incubation at 95°C for 15 min.
\( \Delta \text{Ct} \) is obtained by subtracting the \( \Delta \text{Ct} \) of each experimental sample from the \( \Delta \text{Ct} \) of the calibrator sample.

**Detection of receptor phosphorylation**

Nearly (70–80 %) confluent HT-29 cultures were incubated in Dulbecco’s modified Eagle’s medium with 10 % fetal calf serum for 24 h. Subsequently, reagents were added in concentrations indicated in the legends of the figures. After incubation, cells were trypsinized, washed and lysed with extraction buffer. Protein concentration was measured using the BioRad protein assay kit (Richmond, CA, USA). The Proteome Profiler™ array, human phospho-receptor tyrosine kinase array kit to identify the phosphorylation of forty-two different receptor tyrosine kinases, was used according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA) loading 250 \( \mu \)g protein/membrane. Blots were developed by applying the chemiluminescence detection kit ECL-Plus (Amersham Biosciences, Arlington Heights, IL, USA). Spot intensity was then analysed with the software Biocat Software (Köln) and density is expressed as area under the curve.

**Detection of mitogen-activated protein kinase phosphorylation**

After incubation with oligosaccharides as indicated in the figures, cells were washed with PBS, solubilized in lysis buffer and 300 \( \mu \)g total protein were used for the human phosho-MAP array (R&D Systems, Heidelberg, Germany) according to the manufacturer’s protocol. Briefly, arrays were incubated with whole-cell lysates overnight at 4°C and washed with the supplied washing buffer. Arrays were then incubated with anti-phosphotyrosine-horseradish peroxidase antibodies for 2 h at room temperature before incubation with a chemiluminescent reagent and film exposure; spot intensity was then analysed applying the software Biocat Software and expressed as area under the curve.

**Statistical analysis**

Data were evaluated by ANOVA and statistical differences were tested by Bonferroni’s test. For each variable at least three independent experiments were carried out. Results are expressed as means and their standard errors. All analyses were done using the GraphPad Software Prism 3 (San Diego, CA, USA); differences were considered significant at \( P < 0.05 \).

**Results**

**Cell cycle analysis**

As a consequence of growth inhibition and differentiation cells were fixed to determine their specific cell cycle phase by using flow cytometry. The cell cycle histogram plots and distribution of cell populations in different cell cycle stages for HT-29 are shown in Figs. 1 and 2. It was observed that neutral as well as acidic HMO induced a cell cycle arrest in the G2/M phase which was moderate at a concentration of 7.5 mg/ml but pronounced at 15 mg/ml. Cell cycle analysis of controls without oligosaccharide exposure revealed that 73 % of the HT-29 population was in the G0/G1 phase and 16 % in the G2/M phase (Figs. 1(A) and 2(A)). HT-29 cells exposed to 7.5 mg/ml neutral HMO exhibited a similar distribution of cells among the different cell cycle phases (Fig. 1(B)). The higher dose of oligosaccharides (15 mg/ml) altered the cell population in a way that 42 % of the cell population was in the G0/G1 phase and 44 % in the G2/M phase.
(Fig. 2(A)). After a 24 h treatment with the lower dose of acidic HMO (Figs. 1(D) and 2(B)), 72% of the cells were in the G0/G1 phase. This was balanced by a lower percentage of cells in the G2/M stages (16%). Exposure of HT-29 for 24 h to the high dose of oligosaccharides resulted in a further accumulation of cells in the G2/M stage, 37% of the cell population were found to be in the G0/G1 stage and 35% accumulated in the G2/M stage (Fig. 2(B)).

In comparison to control cells, the proportion of cells in the G0/G1 phase significantly decreased in HIEC and Caco-2 cells after incubation with neutral and acidic HMO at a concentration of 15 mg/ml and thus accumulated in the G2/M phase (44 and 33% in HIEC and Caco-2 cells, respectively). The number of cells in the S phase did not change significantly in both cell lines after incubation with oligosaccharides when compared to controls (Table 2).

Transcriptional regulation of cyclins, cyclin-dependent kinase inhibitors and tumour suppressors with quantitative real-time PCR

In order to relate cell cycle arrest to important cell cycle regulator genes such as cyclins and CDK1 and tumour suppressor p53, we performed real-time PCR to quantify differences in the expression level of selected target genes. In Fig. 3, the data for the expression levels of cyclins are summarized for HT-29, HIEC and Caco-2 cells. After incubation of the cells with 15 mg/ml neutral or acidic HMO, mRNA expression

![Fig. 1. Representative histograms of flow cytometric analysis of the cellular DNA content in control and oligosaccharide-treated HT-29 cells, stained with the fluorescence dye 7-aminoactinomycin. The cells were untreated (A) or treated with 7.5 mg/ml (B, D) and 15 mg/ml (C, E) neutral (B, C) or acidic (D, E) human milk oligosaccharides for 24 h. Cell cycle distribution was performed as described in the Materials and methods. FL3-A, fluorescence intensity.](https://www.cambridge.org/core/asset/eml/cellcycle4732f87b7833d7d3d3e4d570a011a111.png)

![Fig. 2. Distribution of cell cycle phases based on histogram plots for HT-29 cells. The distribution of cell cycle phases is expressed as the percentage of gated cells in the G0/G1, S and G2/M phase of the cell cycle. Cells were treated with (A) neutral human milk oligosaccharides (7.5 mg/ml; 15 mg/ml) or (B) acidic human milk oligosaccharides (7.5 mg/ml; 15 mg/ml). Values are means with their standard errors depicted by vertical bars (n = 2). Mean values were significantly different from those of the control group: *P < 0.05, **P < 0.01. □, Control.](https://www.cambridge.org/core/asset/eml/cellcycle4732f87b7833d7d3d3e4d570a011a111.png)
HMO markedly increased cyclin B expression (221 (SEM 12)

cip1

Similar observations were made for HIEC cells. p21
charide treatment.

cell cycle remained unchanged in all cell lines after oligosac-

iments. p27

expression increased 3·2-fold (15 mg/ml neutral HMO) and

HT-29 (146 (SEM 6) %) and HIEC cells (132 (SEM 6) %),

level of cyclin A, a regulator for S/G2 transition, remained

remained unaffected (Fig. 3). The mRNA expression levels

of cyclin B, which is responsible for the regulation of the

G2/M cell cycle transition, were markedly increased in all

cell lines after incubation with neutral or acidic oligosacchar-

ides. After incubation with neutral HMO, the expression level

of cyclin B in HT-29 was increased to 198 (SEM 12) % and to

214 (SEM 8) %, respectively). Also, in Caco-2 cells

kip1

receptor phosphorylation was observed.

The effect of HMO was EGFR-specific because no other

cytokines were able to induce cell cycle arrest and/or differentia-
tion. To screen the phosphorylation of several receptors and MAPK in HT-29 cells, we used the protein profiling array to detect different phosphorylation events. Results are shown in Fig. 4 for receptor phosphorylation.

Incubation with both neutral and acidic HMO induced a
dose-dependent phosphorylation of the ERF receptor within

10 min indicating that oligosaccharides could interact with

the epidermal growth factor receptor (EGFR). The effects of

acridic oligosaccharides were more pronounced than the neu-

tral oligosaccharide effects. Acidic HMO concentrations of

7.5 and 15 mg/ml induced an EGFR phosphorylation up to

247 (SEM 3) and 447 (SEM 34) % compared to untreated

cells (100 %). Neutral HMO were also able to affect EGFR

phosphorylation and enhanced its phosphorylation to 168

(SEM 6) and 284 (SEM 8) % at 7.5 and 15 mg/ml, respectively.

The effect of HMO was EGFR-specific because no other receptor phosphorylation was observed.

<table>
<thead>
<tr>
<th>HMO</th>
<th>Sub G0/G1</th>
<th>7.5 mg/ml</th>
<th>15 mg/ml</th>
<th>Neutral HMO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.54</td>
<td>1.21</td>
<td>6.76*</td>
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<tr>
<td></td>
<td></td>
<td>0.90</td>
<td>2.1</td>
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</tr>
</tbody>
</table>

Table 2. Effects of oligosaccharides on cell cycle progression of HIEC and Caco-2 cells†
(Mean values for two independent experiments)

Neutral HMO Acidic HMO
7.5 mg/ml 15 mg/ml 7.5 mg/ml 15 mg/ml

Control 0.54 1.21 6.76* 0.90 2.1

Sub G0/G1 0.33 7.5 2.5 1.9

G0/G1 63.4 63.5 53.2* 60.4 50.3*

S 8.9 9.4 7.1 5.8

G2/M 20.4 27.3 31.2* 26.5 33.2*

HMO, human milk oligosaccharides.

Mean values were significantly different from those of the control group: *P<0.05, **P<0.01.

† For details of procedures, see Materials and methods. Intestinal cells were seeded at a density of 10⁶ cells and were allowed to attach for 24h. Finally, they were cultured without (control) or with 7.5 or 15 mg/ml neutral and acidic HMO for 24h. The distribution of cell cycle phases is expressed as percentage of gated cells in the G0/G1, S and G2/M phase of the cell cycle.

Levels were determined using the target gene/housekeeping

gene ratio by setting the control to 100 %.

expression of the undifferentiated cell lines HT-29 and HIEC with

HMO; treatment with the same amount of acidic HMO

resulted in an enhanced expression of p21

cip1

with an increased p21
kip1

mRNA expression 2·5- and 8·3-

fold in HT-29 and HIEC cells compared to controls. In con-

trast to HT-29 and HIEC cells, Caco-2 cells responded only

with an increased p21
kip1

mRNA level but p27
kip1

levels remained unchanged after supplementation with HMO.

Hence, neutral as well as acidic HMO induced an increase of

p21
kip1

levels which reached a maximum of 210 (SEM 12)

% (15 mg/ml neutral HMO) and 196 (SEM 10) % (15 mg/ml

acidic HMO) in comparison to control cells (100 %).

A well-known regulator of different checkpoints during the

cell cycle is the tumour suppressor p53 which is able to regu-
late G1 or G2 transition. To get insight into the role of p53 in

the observed G2/M arrest, we determined p53 mRNA levels in

cell lines after incubation with HMO, but no changes in

p53 mRNA levels were observed in any cell line (Table 3).

Influence of signal transduction pathways

Changes in expression levels of cyclins or CDKI are a conse-
quence of activation or inactivation of different signal cascades. Thus, we further investigated whether the effects of oligosaccharides are a result of influencing signalling pathways in the cell line HT-29, representing phenotypical undif-
fferentiated cells. To screen the phosphorylation of several

receptors and MAPK in HT-29 cells, we used the protein pro-
file array to detect different phosphorylation events. Results

are shown in Fig. 4 for receptor phosphorylation.

We then investigated whether the block in cell cycle pro-

gression was accompanied by increased levels of CDKI such as

p21
kip1

and p27
kip1

. It is well known that both CDKI are able to induce cell cycle arrest and/or differentia-
tion. Treatment of the undifferentiated cell lines HT-29 and HIEC with neutral and acidic HMO resulted in an enhanced expression of p21
kip1

and p27
kip1

. In HT-29 cells, 7.5 and 15 mg/ml neutral HMO induced expression of p21
kip1

3.7-

and 6.6-fold, respectively; acidic HMO were able to enhance

mRNA levels of p21
kip1

2·8- and 4·1-fold, respectively.

Similar observations were made for HIEC cells. p21
kip1

expression increased 3·2-fold (15 mg/ml neutral HMO) and

3·6-fold (15 mg/ml acidic HMO) compared to control exper-
iments. p27
kip1

mRNA expression was enhanced 2·8- and

4·3-fold in HT-29 and HIEC cells when exposed to neutral

HMO; treatment with the same amount of acidic HMO

(15 mg/ml) increased p27
kip1

mRNA expression 2·5- and 8·3-

fold in HT-29 and HIEC cells compared to controls. In con-

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(SEM 6) and 284 (SEM 8) % at 7.5 and 15 mg/ml, respectively.

The effect of HMO was EGFR-specific because no other receptor phosphorylation was observed.
Human milk oligosaccharides and growth arrest

As a consequence of receptor phosphorylation different signal pathways could be involved. To determine whether different MAPK were further activated we used the MAPK array to investigate how and to which extent oligosaccharides are able to induce downstream events from EGFR signalling. As shown in Fig. 5, the analysis of these signalling pathways revealed a role for p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2 which were most significant on p38 MAPK. Phosphorylation and activation of different p38 MAPK subtypes, especially p38α and p38δ in HT-29 cells, was induced by acidic HMO (Fig. 5(A)) and to a lesser extent, but still significantly, by neutral HMO (Fig. 5(B)). Furthermore, the activation of p38α was stronger than that of p38δ in HMO-treated cells within 30 min. In addition, PKB-β/Akt (Akt2), a growth factor-regulated protein kinase, and the downstream kinase ERK1 have emerged as critical enzymes in signal transduction pathways involved in cell proliferation and apoptosis. We have shown that both were activated by HMO at concentrations of 7.5 and 15 mg/ml.

**Discussion**

The intestine is a continuously renewing tissue that constantly undergoes proliferation, differentiation, and apoptosis. Recently, we reported that the exposure of the intestinal cell lines HT-29, HIEC and Caco-2 to HMO significantly inhibited cell proliferation. Whereas neutral oligosaccharides exerted an anti-proliferative effect along with a significant induction of apoptosis and a slight increase in differentiation, acidic oligosaccharides inhibited intestinal cell proliferation only by an induction of alkaline phosphatase activity as a marker of differentiation (13). To understand the effects of oligosaccharides on growth-associated events of intestinal cells, flow cytometric analyses were carried out. Cytometric analyses showed significant changes of the cell cycle pattern in HT-29, HIEC and Caco-2 cells following oligosaccharide treatment for 24 h (Figs. 1 and 2; Table 2). In control experiments, the major proportion of cells was arrested in the G0/G1 phase. After treatment with neutral or acidic HMO, cells accumulated in the G2/M phase of the cell cycle (44, 30 and 31 of HT-29, HIEC and Caco-2 cells, respectively, after exposure to neutral oligosaccharides; 37, 44 and 33 % of HT-29, HIEC and Caco-2 cells, respectively, after treatment with acidic HMO). This leads to the conclusion that the potent anti-proliferative effect of acidic and neutral oligosaccharides is coupled to an extensive G2/M arrest in intestinal epithelial cells. We further investigated whether the G2/M cell cycle arrest due to the exposure to oligosaccharides was associated with changes in mRNA levels for key proteins regulating the cell cycle. Progression through the cell cycle is regulated by CDK, a family of serine/threonine protein kinases which phosphorylate a variety of proteins for cell cycle control (23,24). The cellular concentrations of cyclins vary depending on the cell cycle stage, whereas the levels of CDK remain relatively stable, but must bind to the appropriate cyclin in order to be activated. Therefore, cyclin mRNA expression levels were determined and correlated to flow cytometric events.

After 24 h of oligosaccharide treatment, there was no significant change in cyclin D and E mRNA levels suggesting that cells enter the cell cycle and proceed to G2. Previous studies in intestinal cells demonstrated that cyclin D1 is essential for the progression through the cell cycle (25,26). The present data also indicate that additional cyclins may be responsible for transition and arrest, e.g. cyclin B1, a cell cycle regulatory protein that is generally induced at the G2/M transition and becomes deactivated as the cell exits mitosis. We found that cyclin B1 expression increased after HMO treatment (Fig. 3). This may prevent the cells from entering the G2/M stage, thus corroborating the observed cell cycle arrest at G2/M and the reduced cell proliferation when oligosaccharides were applied. This is in accordance with several other studies which had shown that blocking
cell cycle progression at G2/M was due to increasing cyclin B1 expression in different cancer cell lines (27).

In addition, the G2/M arrest seen in HT-29 and HIEC cells, but not in Caco-2 cells, was associated with a slightly increased mRNA expression level of cyclin A after incubation with acidic oligosaccharides. However, there was no response to an exposure to neutral HMO. If cyclin A were necessary for S/G2 phase transition one might speculate that the G2/M arrest in HT-29 and HIEC cells treated with acidic oligosaccharides is coupled to an increase in both cyclin A and B (27, 28).

The transition through the cell cycle is mediated not only by different time-dependent expression of cyclins but also by the activation of CDK. The interaction of cyclins with their corresponding CDK promotes further activation of target proteins responsible for cell cycle transition. On the other hand, CDKI of the CIP/KIP family are negative regulators of cell cycle progression and CDKI such as p21cip1 and p27kip1 were able to inhibit the formation of cyclin–CDK complexes. The inactivation of the CDK–cyclin B complex by its subsequent binding to p21cip1 is one of the probable mechanisms of G2/M arrest. The present data demonstrate (Table 3) a significant up-regulation of p21cip1 mRNA expression, indicating its role in oligosaccharide-mediated G2/M arrest in all intestinal cell lines. It has recently been shown that p21cip1 interacts with cyclin D throughout the cell cycle, whereas interaction between p21cip1 and cyclin A or cyclin B occurs in the later part of the cell cycle (29, 30). Thus, the G2/M growth arrest we observed may well be a p21-dependent mechanism. Several lines of evidence suggested that p21cip1 and p27kip1 exert similar effects on cyclin–CDK complexes and cell cycle progression but other observations indicate that these CDKI have no overlapping functions and are not biologically equivalent, because of their differentially regulated expression (31, 32).

In comparison to control cells HMO were able to enhance mRNA levels of p27kip1.

Table 3. Effects of oligosaccharides on cell cycle progression of HT-29, HIEC and Caco-2 cells†

(Mean values with their standard errors for three independent experiments)

<table>
<thead>
<tr>
<th></th>
<th>Neutral HMO</th>
<th></th>
<th>Acidic HMO</th>
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<tr>
<td></td>
<td>Control</td>
<td>7.5 mg/ml</td>
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<td>7.5 mg/ml</td>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
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<tr>
<td>HT-29 p21cip1</td>
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<td>0.21</td>
<td>8.65</td>
<td>1.20***</td>
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<td>0.10</td>
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<td>0.33</td>
<td>2.65</td>
<td>0.41</td>
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</table>

HMO, human milk oligosaccharides.
Mean values were significantly different from those of the control group: *P<0.05, **P<0.01, ***P<0.001.
† For details of procedures, see Materials and methods. Intestinal cells were seeded at a density of 10^6 cells and were allowed to attach for 24 h. Finally, they were cultured without (control) or with 7.5 or 15 mg/ml neutral and acidic HMO for 24 h. Real-time PCR was carried out using cell cycle-specific primer and probes (see Table 1). Data were analysed as 2^-ΔΔCT (CT, cycle threshold) and given as means of the percentage of controls.

Fig. 4. Receptor tyrosine kinase antibody array analysis. Cells were treated with neutral human milk oligosaccharides (nHMO; □) or acidic human milk oligosaccharides (aHMO; □) (7.5 and 15 mg/ml) for 10 min. Lysate was prepared according to the manufacturer's instructions. Phospho-receptor tyrosine kinase array was used to detect phosphorylation of these receptor tyrosine kinases in HT-29. The signal was detected by chemiluminescence and the spot intensity is shown. Values are means of the percentage of controls with their standard errors depicted by vertical bars (n=3). Mean values were significantly different from those of the control group: **P<0.01, ***P<0.001. AUC, area under the curve; EGFR, epidermal growth factor receptor.
significantly in HIEC and HT-29 cells whereas in the more
differentiated Caco-2 cells oligosaccharides failed to further
increase p21<sup>cip1</sup> mRNA levels (Table 3). The present results
indicate distinct roles of these CDKI, i.e. p21<sup>cip1</sup> promotes
cell cycle arrest and p27<sup>kip1</sup> induces cell differentiation. Cells
arrested in the G2/M phase concomitant with enhanced
p21<sup>cip1</sup> mRNA and protein levels are often discussed in the con-
text of a p53-dependent mechanism<sup>(33-35)</sup>. This p53-indepen-
dent G2/M regulation was also seen in the present
experiments; however, p53 was not changed in the intestinal
cell lines tested in comparison to the control cells (Table 3).

The activation of EGFR and transmission of the extracellular
signal through different signalling pathways (the ERK pathway,
the stress-activated c-Jun N-terminal kinase and the p38/HOG
kinase pathways) in order to provoke cell progression is a
well-known and accepted process<sup>(36,37)</sup>. However, recent studies
suggest that the EGFR pathway is not simply a growth-promot-
ing signalling pathway, but phosphorylated EGFR also mediates
p21<sup>kip1</sup> expression and growth arrest or apoptosis via modulation
of signalling pathways<sup>(38,39)</sup>. From the present study two inter-
esting findings have emerged: firstly, we observed that oligosac-
charides were able to induce EGFR phosphorylation and,
secondly, we have confirmed that the EGFR/Ras/Raf/ERK pathway is involved. As shown in Figs. 4 and 5 neutral HMO as well as acidic HMO induce a potent phosphorylation of the EGFR within 10 min and, in addition, multiple downstream kinases such as p38α, p38β, ERK1 and Akt-2/PKB were activated within 30 min. As the EGFR is a glycoprotein and interactions of oligosaccharides with glycoprotein receptors are well documented it is conceivable that oligosaccharide fractions possess a general motif inducing the effects we observed. The cross-talk between EGFR phosphorylation, p38 activation and p21^{cip1} expression is observed in a variety of in vitro studies. For example, the p38 MAPK pathway can activate the Sp1/Sp3 transcription factors and this seems necessary for EGFR-dependent transactivation of the p21^{cip1} promoter.

Based upon the present observations we conclude that oligosaccharides are able to activate p21^{cip1} expression and stabilization via EGFR and p38 kinase phosphorylation. Fig. 6 summarizes a mechanism how oligosaccharides induce cell cycle arrest by possibly interacting with EGFR.

In conclusion, we identify HMO as ingredients which were able to induce growth arrest of intestinal cells by modulating EGFR signal pathways and cell cycle-associated gene expression. However, whether the ability of oligosaccharides to inhibit intestinal cell proliferation has implications for a different effect on the intestinal growth regulation in human milk-fed v. formula-fed infants is not known yet and requires further investigation.

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