JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 197–204

Received August 14, 2007
Accepted October 12, 2007
DOI 10.1211/jpp.60.2.0008
ISSN 0022-3573

N-Acetyl-D-glucosamine oligosaccharides induce mucin secretion from colonic tissue and induce differentiation of human keratinocytes

Alexandra Deters, Frank Petereit, Jörg Schmidgall and Andreas Hensel

Abstract

Chitin oligosaccharides (DP2, DP3, DP4, DP5 and DP7) were investigated for their effects on epithelial cells and tissue (skin keratinocytes in-vitro and ex-vivo, and gastrointestinal epithelial membranes ex-vivo). Oligomers DP2, DP3 and DP5 at 10 μg mL^{-1} significantly stimulated the mitochondrial activity of cultured keratinocytes in-vitro (primary cells and HaCaT cell line), with highest activity observed for the pentamer (150% of untreated control). The effects were dose dependent. This higher energy status of primary cells was triggered into a higher differentiation status, as determined by the early and late differentiation markers keratins K1/K10 and involucrin, respectively. In contrast, increased mitogenic cell proliferation was not induced by the oligosaccharides. Toxic effects on keratinocytes were absent. Additionally for the first time a mucin-stimulating effect of chitin oligosaccharides DP3 and DP5 was observed in an ex-vivo model based on intestinal epithelial mucosa tissue. Mucin secretion was time dependent, leading to the secretion of polymers comparable to those normally secreted under physiological conditions. Mucin induction was observed from colonic tissue isolated from humans and pigs. Also, porcine stomach mucosa was stimulated by DP5, while ileum tissue reacted to only a minor extent. Potential developments towards products with wound-healing capacity and activity against chronic bowel disease are discussed.

Introduction

In recent years chitin and its deacetylated form chitosan have been receiving increasing focus in the development of new functional biopolymers. In particular, a number of biological activities have opened new perspectives for biotechnological products; these include film- and fibre-forming properties, strong bioadhesion to mucosal membranes, adsorption of metal ions and coagulation of suspensions. The chemistry and functionality of chitin and chitosan have recently been reviewed by Kurita (2006). In particular, haemostatic activity, induction of wound healing, antimicrobial activity, immune-stimulating properties via macrophage activation, use for overweight or obesity (Mhurchu et al 2005), for tissue engineering (DiMartino et al 2005) and use of chitosan as a non-viral vector for effective gene transfer (Hashimoto et al 2006) have stimulated biomedical research concentrating on chitin and chitosan in the development of innovative products.

Because of their better solubility and absence of swelling properties, chitosan oligomers are preferred over chitin for biotechnical applications (e.g. elicitation). These oligosaccharides have greater functionality in cell and tissue physiology compared with the high-molecular-weight chitosan. For example, chitin oligosaccharides (DP5 to DP8) prevent the growth of tumour cells and act as inhibitors of angiogenesis in-vitro (Harish Prashanth & Tharanatha 2005). In-vivo, chito- oligosaccharide (DP about 19) increases interleukin (IL)-12 and interferon-alpha levels, while levels of IL-1 and tumour necrosis factor are reduced (Kim et al 2006). The strong bioadhesion of high-molecular-weight chitosan and the permeation-enhancing capacities of the oligomers indicate that the glycan interacts actively with cell surface structures. This interaction with the glycocalyx and membrane

University of Münster, Institute for Pharmaceutical Biology and Phytochemistry, Hittorfstraße 56, D-48149 Münster, Germany

Alexandra Deters, Frank Petereit, Jörg Schmidgall, Andreas Hensel

Correspondence: A. Hensel, University of Münster, Institute for Pharmaceutical Biology and Phytochemistry, Hittorfstraße 56, D-48149 Münster, Germany.
E-mail: ahensel@uni-muenster.de

Acknowledgements: The study was financed by federal grant BMBF 0311827A/B/C from the German Ministry for Education and Research to A.H.

structure seems to depend on the fine structure of the glycan. Despite the work published on the deacetylated glucosamine oligomers, preclinical and pharmacological studies (Kurita 2006) on the low-solubility chitins are scarce. The present study investigates the influence of homologous chitin oligosaccharides DP2, DP3, DP4, DP5 and DP7 on the induction of physiological processes in two different test systems. First, the induction of physiological effects (mitochondrial activity, proliferation, toxicity and differentiation) was investigated on human keratinocytes in-vitro. Testing in these highly sensitive epithelial cells provides information on the potential toxic or stimulatory effects of these exogenous compounds on cell physiology (Zuang et al 2002).

Secondly, an ex-vivo model based on porcine and human colonic tissue (Schmidgall & Hensel 2002) was used to investigate the potential tissue-protecting effects of the chitin oligosaccharides, which have been shown to cause significant induction of endogenous mucin secretion by mucosal cells. The results obtained from both test systems indicated that the chitin oligosaccharides increase the barrier function of skin cells by induction of cell differentiation and also induces secretion of mucin from intestinal epithelial cells.

Materials and Methods

General experimentation procedure

All chemicals were purchased from Sigma (Deisenhofen, Germany) unless stated otherwise. Waymouth's medium was purchased from Life Technologies GmbH (Karslruhe, Germany), chitin oligosaccharides DP 2 to 7 were obtained from Südzucker AG (Obrigheim, Germany).

Analytical characterization of oligosaccharides was performed by NMR and mass spectroscopy (MS): ^{1}H NMR (400 MHz, D_2O, δ 4.65 ppm) spectra were measured on a Varian AS 400 (Darmstadt, Germany). Electronspray ionzation (ESI) MS data were measured on a Finnigan LCQ (ThermoElectron, Bremen, Germany), in positive ion mode. Chitin oligosaccharides were identified by signal integration (Ishiguro et al 1992; Sørbotten et al 2005) of the reducing end of the α- and β-anomers of the acetylated glucosamine unit at 5.0–5.1 ppm and 4.5–4.6 ppm, respectively; the anomeric signals of extender units at 4.4–4.5 ppm; the remaining sugar protons in the range 3.2–3.9 ppm; and the acetyl protons near 2.0 ppm. The integration of these signals ranges from a relative intensity of 1:1:12:6 for the dimer, up to 1:4:30:15 for the pentamer. DP7 was not investigated by NMR because of low solubility. ESI-MS indicated pseudomolecular ion peaks $[M + Na]^+ m/z$ 447 (dimer), m/z 650 (trimer), m/z 853 (tetramer), m/z 1056 (pentamer). The molecular weights corresponded with the proposed structures of fully acetylated chitin oligosaccharides.

Testing on human keratinocytes

HaCaT keratinocytes were kindly provided by Professor Fusenig (German Cancer Research Institute, Heidelberg, Germany). HaCaT keratinocytes were cultured in high-glucose Dulbecco's modified Eagle medium supplemented with fetal calf serum (10%), penicillin/streptomycin (1%), glutamine (1%) and non-essential amino acids (1%). Before incubation with test polysaccharides, HaCaTs were adapted to serum- and bovine pituitary extract-free EpiLife medium (Invitrogen, Karlsruhe, Germany). Test compounds were investigated with respect to mitochondrial activity (Mosmann 1983), 5-bromo-2-deoxyuridine (BrdU) incorporation assay (Porstman et al 1985) and necrosis by lactate dehydrogenase assay (Martin & Clynes 1993). Culture conditions, assays and procedures were as reported by Deters et al (2005a, b).

Natural human keratinocytes (NHK) were isolated as primary cells from human skin, obtained by surgical resection from Caucasian subjects. Ethical committee approval was obtained from the University of Münster, Germany (acceptance number 2006-117-f-S). Investigations were carried out with cells from the second to the sixth passages. For quantification of the terminal differentiation-specific proteins involucrin and keratins K1 and K10, 5×10^4 NHK were plated into each well of 24-well cell culture plates (Greiner, Frickenhausen, Germany). After incubation with polysaccharides for 9 days, cells were harvested with a cell scraper. The calcium ionophor A23187, 13 μg mL^{-1}, in medium supplemented with 2 mM calcium was used as a positive control. Cytokeratins were quantified as described by Deters et al (2005a). Involucrin was extracted as follows: cells were lysed by ultrasonic irradiation for 3×5 min in 50 mM sodium phosphate buffer, pH 6.8 (5% SDS, 40 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 15% glycerin) and centrifuged for 15 min at 7000 g. The RC/DC protein assay (BioRad, München) was used to determine total protein in the supernatants. After standardization of protein contents, involucrin amounts were determined semi-quantitatively by the immuno-dot-blotting technique with monoclonal mouse antibodies against involucrin (Sigma) and detected with a monoclonal rabbit anti mouse IgG1 peroxidase–digitonin-coupled antibody (Chemikon Int., Hofheim, Germany).

Ex-vivo assay with gastrointestinal tissue

The test system described for ex-vivo cultivation of buccal mucous membranes was modified for the cultivation of tissue from the colon, ileum, jejunum and stomach, with the approval of the ethical committee of the University of Erlangen, Germany (approval number he-414-2) according to the methods of Schmidgall et al (2000) and Schmidgall & Hensel (2002). For the colon assay, pieces of colon, approximately 10×10 cm, were taken from freshly killed pigs from the local abattoir, cleaned with tap water and transferred into Waymouth's medium supplemented with chloramphenicol and sodium benzyl penicillin (50 μg mL^{-1}). A biopsy punch (Ø 8 mm) was used to cut pieces from the same area, which were incubated with 500 μL of the test solution (test compounds dissolved in Waymouth medium), or Waymouth medium without supplementation (control), under oxygen atmosphere (95% O_2/5% CO_2) at 37°C for 3 h. For each test solution, three independent samples were incubated with and without the test oligosaccharides.

Levels of lactate dehydrogenase (LDH) were determined using an LDH quantification kit (Sigma). LDH values measured in the incubation assay were about 27 ± 5 U mL^{-1} at

the beginning, and increased to about $58 \pm 7\,U\,mL^{-1}$ after 3 h incubation. Values were < 5% of the maximum LDH release ($1108 \pm 13\,U\,mL^{-1}$) induced by incubation under toxic conditions with 0.1% Triton X100.

Determination of total carbohydrate content

Testing was performed in 96-well microtitre plates using the method of Monsigny et al (1988). To 20 μL of the test solution was added 20 μL resorcin ($6\,mg\,mL^{-1}$) and 100 μL 75% sulphuric acid, and the mixture incubated for 60 min at 80°C. Absorption was determined at 450 nm. Absolute carbohydrate content was calculated from a calibration curve.

Quantification of acidic glycoconjugates by alcian blue staining

Basic alcian blue was used for mucin quantification (Whitehead 1978); this dye binds to positively-charged groups of acidic carbohydrates at low pH. At pH 2.5, alcian blue stains both sulphated and carboxylated acid mucopolysaccharides and sialomucins. Sulphate esters react at a lower pH than do the carboxyl groups. For the test, 200 μL samples of incubation supernatant from ex-vivo cultures were incubated with 1 mL alcian blue reagent (100 mg alcian blue in 100 mL 0.2 M sodium acetate, 50 mM $MgCl_2$, pH 2.5) with shaking for 2 h at room temperature. The mixture was centrifuged (10 min at 12 500 *g*) and the absorption of 200 μL of the clear supernatant measured at 595 nm. Calibration curves were constructed using commercial porcine mucin. The residual alcian blue in the supernatant correlates inversely with the amount of precipitated mucin.

Selective quantification of mucins by enzyme-linked lectin assay

This was measured using a commercially available DIG glycan ELISA kit (Boehringer Mannheim, Germany). Test solutions from the incubation assay were diluted (1:1000) with 0.05 M sodium carbonate buffer, pH 9.25, and transferred to MaxiSorb plates (Nunc, Wiesbaden, Germany); non-adsorbed material was removed after 14 h at 4°C. Wells were washed with 300 μL phosphate-buffered saline (PBS), blocked for 90 min with 200 μL block buffer (0.5% bovine serum albumin in PBS) and washed three times with 0.05 M Tris-buffered saline (TBS), pH 7.5. To each well was added 150 μL lectin solution containing *Sambucus nigra* (SNA) agglutinin ($10\,\mu g\,mL^{-1}$ in TBS). Plates were incubated for 1 h then washed three times with TBS containing 0.1% Tween 20. Then 150 μL anti-DIG-peroxidase-coupled digitonin ($0.3\,U\,mL^{-1}$ in TBST (Tris HCl in NaCl plus 0.1% Tween 20)), was added and the samples incubated for 1 h. They were washed three times with TBS; 150 μL ABTS solution ($1\,mg\,mL^{-1}$) was added, and the absorption at 492 nm determined after 5 min. All measurements were performed in triplicate, with calibration with transferrin as reference and creatinase as negative control.

Determination of carbohydrate content by SEC-HPLC

Glycoproteins from the ex-vivo assay were quantified by size-exclusion (SEC)-HPLC using the Shimadzu (Düsseldorf, Germany) LC 10 A system with a UV detector (10A) and a Bio-Sil SEC 250 7.8 × 300 mm column (Biorad, Munich, Germany). The eluent was 0.1 M K_2HPO_4/KH_2PO_4, pH 6.0, at a flow rate of $1\,mL\,min^{-1}$; the injection volume was 10 μL; the detection wavelength was 280 nm. Data acquisition and processing were accomplished with Class-VP software (Shimadzu). If necessary, solutions from the ex-vivo assay were diluted with eluent in order to achieve a suitable concentration range for the detection range of the fluorescence detector.

Identification of mucins from assay incubation solutions

Incubation solutions were separated by 8% SDS-PAGE (Laemmli 1970) and the polymers were blotted electrophoretically onto polyvinylidenfluoride (PVDF) membranes (Biorad Trans-blot SD transfer cell). PVDF membranes were blocked for 12 h with a solution of Tween 20 (0.5% in 50 mM TBS), washed twice with TBS, then with buffer (TBS with $MgCl_2$, $MnCl_2$ and $CaCl_2$, each 1 mM) and then incubated for 1 h with lectin buffer containing *Maackia amurensis* (MAA) or SNA agglutinin (DIG glycan differentiation kit, Boehringer Mannheim) (Becker et al 1993).

Statistical analysis

The influence of test compounds on keratinocyte physiology was evaluated using a Lèvene variance calculation. *P* values below 0.05 were considered significant. All data presented are the means of 12–30 random samples (errors bars represent the s.e.). To analyse the influence of test compounds on intestinal tissue, each test substance was tested in at least two independent series; values from both test series were combined in cases where the results did not differ statistically (Dunnett's post-hoc analysis of variance, Student's *t*-test). In cases of higher discrepancies, two more test series were performed and analysed statistically for differences.

Results

Effects of chitin oligosaccharides on human keratinocytes

Chitin oligosaccharides (DP2, DP3, DP5 and DP7) were investigated for their influence on epidermal keratinocytes (HaCaT cell line and NHK). Physiological parameters of oligosaccharide-treated cells were determined, such as mitochondrial activity by MTT test, mitotic cell proliferation by BrdU incorporation ELISA, necrotic cell toxicity by LDH release and quantification of differentiation-specific keratins K1/K10 and involucrin as specific markers for cell differentiation towards barrier cells.

While *N*-acetyl-D-glucosamine (GluNAc; DP1) was inactive, the respective β-(1 → 4)-linked oligomers increased the mitochondrial enzymatic metabolism at $10\,\mu g\,mL^{-1}$, as

shown by the MTT-reducing activity of cellular dehydrogenases. As indicated in Figure 1, the stimulation was in the range 50–150% relative to untreated control cells. The highest effects were observed for DP5. No effects were observed at $1\,\mu g\,mL^{-1}$.

To investigate whether this higher metabolic activity was due to an increase in cell proliferation or an induction of cell differentiation, both parameters were quantified. Cell proliferation, determined by the BrdU incorporation assay, was not increased significantly by the respective oligosaccharides at $10\,\mu g\,mL^{-1}$ (data not shown). The differentiation behaviour of NHK was investigated on the basis of terminal differentiation-specific protein expression, with keratin K1 and K10 as late differentiation markers and involucrin specific for earlier stages. Cytokeratin expression was significantly elevated in NHK incubated with the pentamer, while GluNAc, the dimer, trimer and the respective heptamer showed slight, but not significant increases in keratin formation (data not shown). Involucrin expression (Figure 2A) was not influenced by GluNAc or the respective β-(1 → 4)-linked dimer, while the trimer and the pentamer resulted in increased involucrin formation at $10\,\mu g\,mL^{-1}$, although the effect was significant compared with untreated control cells only in the case of the pentamer (Figure 2B). However, differentiation was not induced at concentrations below $1\,\mu gmL^{-1}$. Membrane degeneration – a marker of necrotic cell toxicity – was not observed after treatment of cells with the different GluNAc oligosaccharides. LDH was monitored as a marker of necrosis. While no signs of necrosis were observed, slightly reduced LDH values (30–40% reductions compared with untreated control groups) were evident, indicating that the chitin oligomer has slight cell protecting effects.

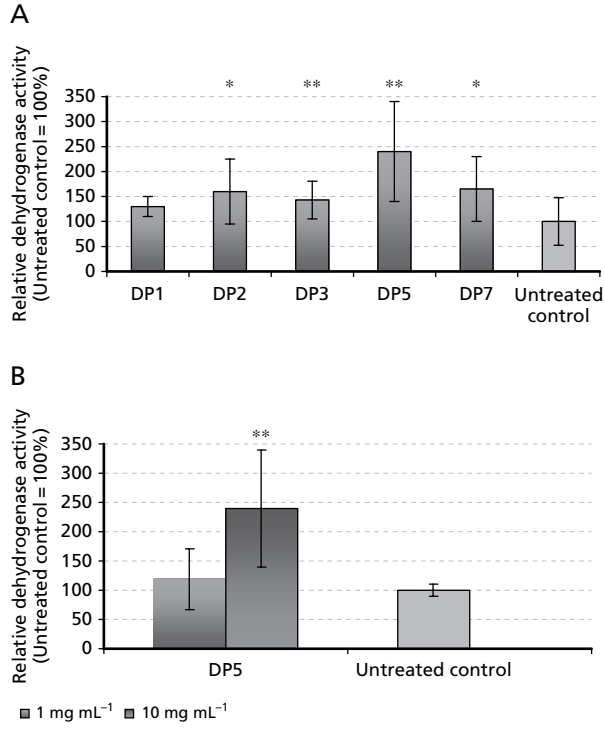

Figure 1 Mitochondrial activity (MTT test) of HaCaT keratinocytes incubated with different chitin oligosaccharides. A. Incubation of cells with *N*-acetyl-glucosamine (DP1) and oligosaccharides DP2 to DP7 at $10\,\mu g\,mL^{-1}$ for 72 h. B. Incubation of cells with DP5 at 1 and $10\,\mu g\,mL^{-1}$ for 72 h. Bars represent s.d. (n = 10 replicates). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the untreated control group (taken as 100%).

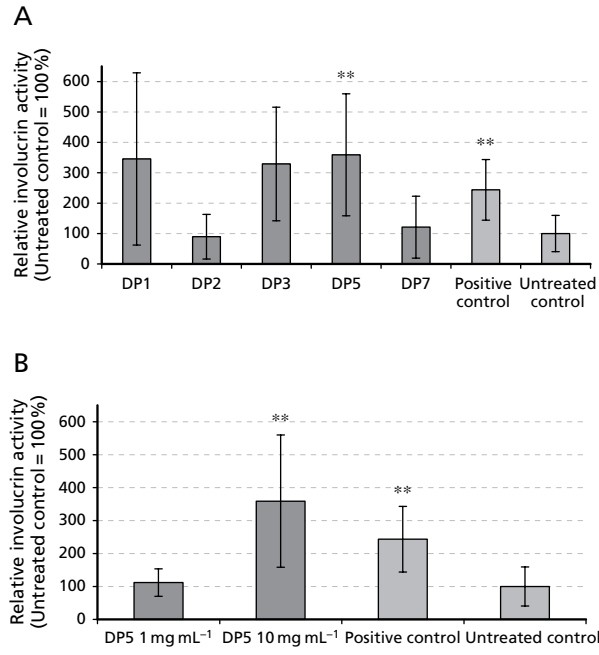

Figure 2 Influence of different chitin oligosaccharides on differentiation of natural human keratinocytes after 9 days' incubation. Involucrin content in cell lysates after incubation with (A) different chitin oligomers ($10\,\mu g\,mL^{-1}$) and (B) pentamer DP5 (1 and $10\,\mu g\,mL^{-1}$). Bars represent s.d. (n = 10 replicates). $^{*}P < 0.05$ compared with the untreated control group (taken as 100%). Positive controls were treated with the Ca^{2+} ionophor A23187 ($13\,\mu g\,mL^{-1}$).

Effects of oligosaccharides on intestinal tissue

A standard ex-vivo system using porcine colonic membranes was used to investigate the bioadhesive properties of GluNAc oligosaccharides in forming artificial mucin-like layers on gastrointestinal epithelial membranes (Schmidgall et al 2000). In the case of adsorption of the test compounds to the tissue surface or absorption into cells, the content of carbohydrates in the incubation medium decreases in a time-dependent way (Schmidgall et al 2000).

There were no signs of positive adhesion of the chitin oligosaccharides to the epithelial tissue. In contrast, the resorcinol assay showed that the total amount of carbohydrates in the incubation media increased significantly, particularly when the chitin pentamer DP3 was added (Figure 3A). Using the alcian blue colorimetric method to quantify acidic polysaccharides (Whitehead 1978) at low pH, we found that incubation of colonic tissue with GluNAc oligomers led to an increase in acidic polysaccharides, probably endogenous mucins (Figure 3B). Protein content also increased slightly in line with this, but not significantly (protein content $546 \pm 122\,\mu g\,mL^{-1}$ vs $496 \pm 61\,\mu g\,mL^{-1}$ in the control group).

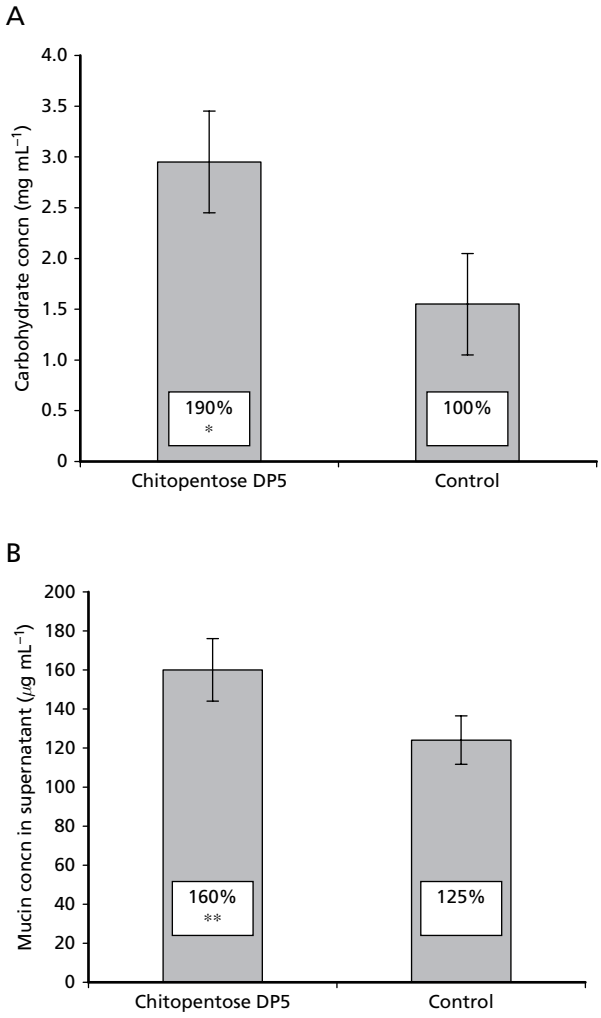

Figure 3 Influence of the chitin oligosaccharide DP5 (10 mg mL^{-1}) on the secretion of carbohydrate-containing material from porcine colon mucosa after 2 h incubation. A. carbohydrate content, determined by resorcinol test, in the incubation supernatant of untreated (taken as 100%) and treated tissue. B. Amount of polysaccharide material, measured by alcian blue, in the incubation supernatant of untreated and treated tissue. $^{*}P < 0.05$.

These additional glycoproteins in the incubation media were found by SEC-HPLC to originate from the carbohydrate moieties of high-molecular-weight, non-dialysable (cut-off 3.5 kDa) protein-containing compounds.

To prove that the secreted carbohydrate polymers were mucin-like compounds, the occurrence of sialic acids, typical for intestinal mucins, was demonstrated. The high-molecular-weight compounds secreted into the incubation medium were blotted by Western analysis onto a PVDF membrane, oxidized by periodate under conditions that oxidize sialic acids (Oshannessy et al 1987) and labelled with digoxigenin via succinylhydrazon (Kniep & Muhlradt 1990): the detection of sialic acids was obvious (Figure 4A).

In a further experiment to evaluate the linkage characteristics of the sialic acid within the secreted polymer, different digoxigeninated lectins were used in Western blotting (Figure 4B). SNA agglutinins (detecting NeuAc-α-(2–6)-GalNac and NeuAc-α(2–6)-Gal), MAA agglutinins (detecting NeuAc-α-(2–3)-Gal), and agglutins from *Galanthus nivalis* (GNA, detecting terminal mannose) were used; only SNA showed positive binding to the glycoproteins, while MAA and GNA did not label any compound. This demonstrated that the glycoproteins induced by DP5 are mucin-type compounds with the typical characteristics of neuraminic acid, being 2 → 6-linked; terminal mannose residues were absent. All of the described evidence (molecular weight, glycosylation, positive alcian blue reaction, 2 → 6-linked sialic acid) is consistent with the secreted compounds being mucin-type glycoproteins.

The stimulatory effect on mucin secretion is dependent on the molecular weight of the oligosaccharides: the GluNAc trimer DP3 and tetramer DP4 had no stimulating effect, while a significant increase in mucin secretion was obtained with the pentamer DP5 (Figure 5). Chitin and chitosan had no effects on the tissue (data not shown). The hexamers and heptamers were not tested because of limited solubility in the test media. The untreated control groups did not show an increased secretion of glycoprotein into the incubation supernatant,

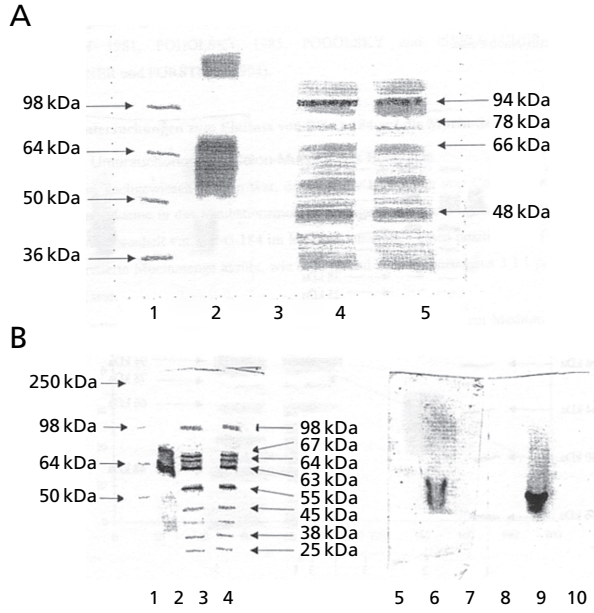

Figure 4 Detection of endogenous mucins in the incubation media of porcine colonic tissue after blotting. A. Detection of sialyl acid containing polymers after SDS-PAGE, followed by sialic-acid-specific periodate oxidation and digoxigenin labelling. Lane 1 = molecular weight marker; 2 = control fetuin, containing sialic acid; 3 = negative control asialofetuin, sialic acid absent; 4 = untreated colonic tissue; 5 = colonic tissue treated with DP5 10 mg mL^{-1}. B. Western blot of incubation supernatant after SDS-PAGE and detection with *Sambucus nigra* (SNA) (lanes 2–4), *Maackia amurensis* (MAA) (lanes 5–7) and *Galanthus nivalis* (GNA) (lanes 8–10). Lane 1 = molecular weight marker; 2, 6 and 9 = fetuin; 3, 5 and 8 = untreated colonic tissue; 4, 7 and 10 = colonic tissue treated with DP5 10 mg mL^{-1}. No labelling with MAA and GAA, but detection of NeuAc-α-(2,6)-GalNac with SNA.

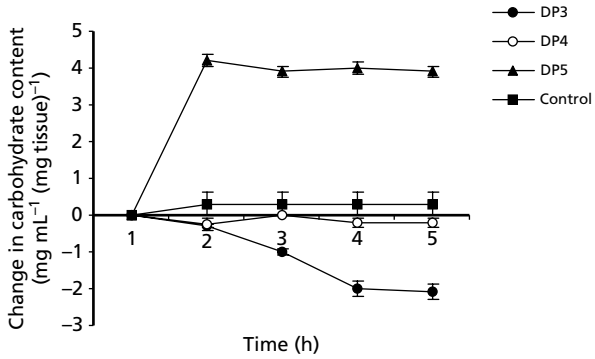

Figure 5 Temporal changes in carbohydrate content in the incubation supernatants of porcine colon mucosa treated with different oligosaccharides (10 mg mL^{-1}) compared with untreated control tissue (taken as 100%).

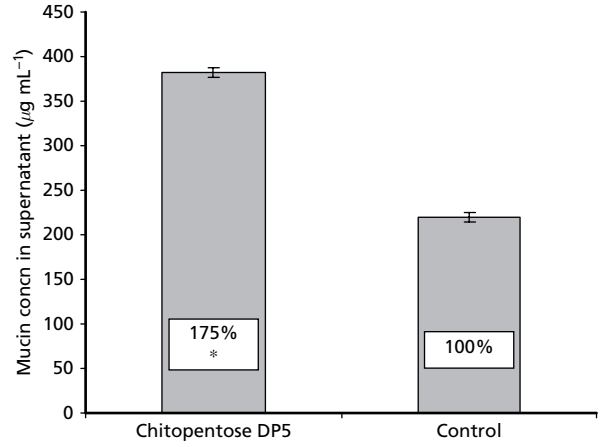

Figure 6 Mucin content, determined by sialic-acid-specific enzyme-linked lectin assay with *Sambucus nigra* agglutinin, after 2 h incubation of untreated (control, taken as 100%) human colon mucosa and tissue treated with the chitin pentose oligosaccharide DP5 (10 mg mL^{-1}); $^{*}P < 0.05$.

which clearly shows that the secretion of mucus within the oligosaccharide-treated groups is not an agonal response of necrotic or apoptotic tissue, but is specifically induced by the exogenous chitin oligosaccharides. This finding is in congruence with data obtained by LDH determination (see above) in the incubation media, which clearly showed that the titres of this necrosis marker were not changed compared with untreated controls.

In order to investigate whether the mucins secreted by chitin oligosaccharide stimulation were comparable to the polymers secreted under normal control conditions, the incubation medium was investigated by HPLC fingerprinting on Biosil SEC gel permeation chromatography and SDS-PAGE with lectin-staining by SAN agglutinin. No qualitative differences were detectable for the untreated control groups and groups treated with pentose oligosaccharide DP5, indicating that this chitin oligomer induces the secretion of native mucin polymers.

These stimulating effects of the pentamer on porcine colonic tissue were time dependent over about 1 h, with typical saturation effects (Figure 5). Mucin stimulation was also observed when assays were conducted with tissue from porcine stomach, but not when porcine ileum or jejunum were used. This result is consistent with the minor capacity of the small intestine for mucin synthesis. Using human colonic resections from two patients, clear stimulation of mucins was observed by the alcian blue colorimetric method and enzyme-linked lectin assay with SAN agglutinin (Figure 6). Whilst tissue from one patient secreted 175% mucin compared with the untreated control, the other tissue sample reacted to a lesser extent (109%). This clearly indicates that the chitopentose also stimulates mucin secretion in human tissue.

Discussion

Chitin oligosaccharides exhibited significant influences on the cell physiology of primary keratinocytes and of an established cell line. The energy status of the cells was up-regulated because of induction of the differentiation process. Significant increases in both early and late differentiation markers were observed. The higher mitochondrial activity was not correlated to an increased proliferation rate, but was directed to a metabolic switch towards protein- and lipid-rich barrier cells. This indicates that GluNAc oligosaccharides are quite strong inducers of differentiation. Because the effects on involucrin formation were seen only for the DP3 and DP5 oligosaccharides but not for glucosamine and the chitobiose dimer DP2 (DP4 oligosaccharide was not available for this test) in a dose-dependent manner, it is assumed that this is a specific receptor-mediated process, related to distinct structural features of the oligosaccharides rather than to an increased nutrition effect resulting from catabolic degradation of the sugar moieties. Longer chitin oligosaccharides, such as DP7, failed to induce cell differentiation, probably because of decreased absorption into and through the cell membrane. Triggering of differentiation as result of a contact inhibition due to a high cell density, which is typical for skin keratinocytes, was excluded because of the low proliferation of the cells.

From our experiments it became clear that DP5 stimulated the cellular activity, while other oligomers (e.g. DP2, DP3 and DP7) were less active. The mechanism by which the oligomers act on the physiology of keratinocytes, and which receptor proteins are influenced by the carbohydrates, is not yet clear, and any explanation for the observed effects is speculative. However, it is known that hepatocyte growth factor/scatter factor (HGF/SF) stimulates keratinocyte proliferation and can be modulated by exogenous sulphated heparin-derived oligosaccharides. As shown by Delehedde et al (2002), activity of homologous oligomers was observed only at a minimum degree of polymerization of DP4. Longer chain oligomers such as DP5 were more active, with DP8–14 showing clear increases in potency in association with the increasing length of the oligosaccharide. Structural elements of these sulphated oligosaccharides are known to interact with HGF/SF. It can also be speculated that the chitin oligomers

act in a similar way. In contrast to the heparin-derived carbohydrates, the chitin oligosaccharides have significantly decreased activity in the case of chain elongation. We assume that this is due to the decreased solubility of the long-chain derivatives. Using the DP7 oligomer, we observed limited solubility, which was enough for preparation of the test solution but may be further reduced under physiological conditions, leading to precipitation on the cell membrane and reduced availability into the cell.

It is known that glucosamine oligomers can influence skin cells. Deacetylated chitosans with a low degree of polymerization can enhance the proliferation of skin fibroblasts (depending on the fibroblast strain) and can decrease keratinocyte proliferation (Howling et al 2001). No influence on differentiation behaviour was observed in this study, however.

Because the chitin pentamer (DP5) turned out to be the most potent inducer of differentiation, further investigations will be performed. There seems to be a strong need for the development of such inducers of differentiation: many cells are known to lose rapidly their ability for differentiation when cultured in-vitro (Li et al 2007). In clinical practice, inducers of differentiation could be valuable tools as future therapeutics for wound healing. At the moment, only high-calcium wound dressings (e.g. calcium-alginates) are used to trigger keratinocytes into the final epidermal barrier cells. Other differentiation inductors, such as 1,25-dihydroxy-vitamin D3 cannot be used in clinical practice because of high toxicity (Gibbs et al 1996). From that point of view, use of the easily obtained chitin pentose DP5 with its good solubility, absence of swelling properties, absence of necrotic activity on skin cells and strong induction of the differentiation process seems to be a promising tool for further development as a skin-active compound for local wound healing and skin regeneration. We assume that bioavailability to the lower epidermis is possible in the case of damaged skin when using the oligomers for wound healing.

While skin cells are embedded into an extracellular matrix, mainly characterized as a lipid barrier, most other epithelial cells are shielded by a mucin layer on the luminal cell side. The interaction of the chitin oligosaccharides with gastrointestinal epithelial tissue (e.g. colonic) was investigated. In previous studies exogenous highly acidic galacturonic acid-containing polysaccharides were shown to exhibit strong adhesive interactions with such mucosal cells by binding to cell-secreted mucins via cross-linking of calcium-bridges (Schmidgall & Hensel 2002). Within our screening it was clearly shown that the chitin oligosaccharides lack these effects, but are capable of inducing the secretion of endogenous mucins by 2–4 fold compared with the native mucin secretion. At the moment it is not clear whether the tissue up-regulates the biosynthesis of mucins or if pools of stored mucins are depleted more quickly. The main secretion occurred during the first hour (Figure 5), suggesting the secretion of stored mucins by the induction of release systems. Secreted mucins were comparable to the polymers normally secreted, but to a lower extent, into the medium, as was shown by SDS-PAGE fingerprinting. The data obtained from porcine colon and human colon were comparable. Other intestinal tissues reacted differently, however. For example, mucosal tissue from ileum and jejunum was stimulated by the oligosaccharides only to a limited extent, explained by the low mucin content in these tissues, whereas tissue from the stomach was stimulated by chitin oligosaccharide DP5 to secrete elevated amounts of mucin.

Mucin secretion is an essential mechanism for the functionality of intestinal tissue. The polymer shields the epithelial tissue from mechanical, chemical and microbiological stress factors. An intact mucin barrier and normal secretion, regulated effectively under physiological conditions by bile salts (Klinkspoor et al 1999), is essential for activation of macrophages (Inoue et al 1999). Conversely, deficiencies of the mucin barrier, as is dramatically observed in the course of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, are associated with marked inflammation and destruction of mucosal tissue. From this perspective, the development of chitin oligosaccharides as mucin stimulants for treatment of colitis-like inflammations is plausible. Review of the literature revealed that few such stimulants have been described until recently; 3-isobutyl-methylxanthine and forskolin have been used experimentally (Barcelo et al 2001), while nicotine and hydrocortisone are used in clinical practice. Hydrocortisone is used mainly because of its anti-inflammatory capacity, although mucin-stimulating activity is also described (Finnie et al 1996). Thus, the development of chitin DP5 oligosaccharides suitable for oral or rectal administration may be a promising tool towards new strategies in the treatment of chronic bowel diseases. It is known from the oral administration of chitin that no or very limited hydrolysis of the polysaccharide occurs during intestinal passage. If β-glucosamine-degrading enzymes are not present, availability in colonic areas should be possible and therapeutic doses of the oligomers would be delivered to the site of inflammation.

Conclusions

Low-molecular-weight chitin oligosaccharides, particularly the GluNAc pentamer DP5, strongly influence the physiology of epithelial cells. In contrast to the poorly soluble chitin polysaccharide, the respective oligosaccharides seem to have the capacity to penetrate into cells and to trigger the target cell into an increased barrier status. Induction of the differentiation of skin cells and marked induction of mucin secretion from intestinal epithelial cells indicate that these oligosaccharides have considerable potential within pharmacy and medicine, particularly in the areas of wound healing and chronic bowel diseases.

References

Barcelo, A., Claustre, J., Abello, J., Chayvialle, J. A., Plaisancie, P. (2001) Selective involvement of calcium and calcium channels in stimulated mucin secretion from rat colon. *Scand. J. Gastroenterol.* **36**: 1339–1343

Becker, B., Salzburg, M., Melkonian, M. (1993) Blot analysis of glycoconjugates using digoxigenin-labeled lectins: an optimized procedure. *BioTechniques* **15**: 232–235

Delehedde, M., Lyon, M., Vidyasagar, R., McDonnel, T. J., Fernig, D. G. (2002) Hepatocytes growth factor/scatter factor

binds to small heparin-derived oligosaccharides and stimulates the proliferation of human HaCaT keratinocytes. *J. Biol. Chem*. **14**: 12456–12462

Deters, A., Schröder, K. R., Hensel, A. (2005a) Kiwi fruit (*Actinida chinnisis* L.) polysaccharides exert stimulating effects on cell proliferation via enhanced growth factors receptors, energy production and collagen synthesis of human keratinocytes, fibroblasts and skin equivalents, *J. Cell Physiol*. **202**: 717–722

Deters, A. M., Schröder, K. R., Smiatek, T., Hensel A. (2005b) Ispaghula (*Plantago ovata*) seed husk polysaccharides promote proliferation of human epithelial cells (skin keratinocytes and fibroblasts) via enhanced growth factor receptors and energy production. *Planta Med.* **71**: 33–39

DiMartino, A., Sittinger, M., Risbud, M. V. (2005) Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* **26**: 5983–5990

Finnie, I. A., Campbell, B. J., Taylor, B. A., Milton J. D., Sadek, S. K., Yu, L. G., Rhodes, J. M. (1996) Stimulation of colonic mucin synthesis by corticosteroids and nicotin. *Clin. Sci. (Lond.)* **91**: 359–364

Gibbs, S., Backemdorf, C., Ponec, M. (1996) Regulation of keratinocyte proliferation and differentiation by all-trans-retinoic acid, 9-cis-retinoic acid and 1,25-dihydroxy vitamin D3. *Arch. Dermatol. Res*. **288**: 729–738

Harish Prashanth K. V., Tharanathan, R. N. (2005) Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. *Biochim. Biophys. Acta* **1722**: 22–29

Hashimoto, M., Morimoto, M., Saimoto, H., Shigemasa, Y., Sato, T. (2006) Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physiochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjug. Chem*. **17**: 309–316

Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M., Wood, E. J. (2001) The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. *Biomaterials* **22**: 2959–2966

Inoue, M., Fujii, H., Kaseyama, H., Yamashina, I., Nakada, H. (1999) Stimulation of macrophages by mucins through a macrophage scavenger receptor. *Biochem. Biophys. Res. Commun*. **264**: 276–280

Ishiguro, K., Yoshie, N., Sakurai, M., Inoue, Y. (1992) A ^{1}H NMR study of a fragment of partially N-deacetylated chitin produced by lysozyme degradation. *Carbohydr. Res*. **237**: 333–338

Kim, H. M., Hong, S. H., Yoo, S. J., Baek, K. S., Jeon, Y. J., Choung, S. Y. (2006) Differential effects of chitooligosaccharides on serum cytokine levels in aged subjects. *J. Med. Food* **9**: 427–430

Klinkspoor, J. H., Mok, K. S., Van Klinken, B. J., Tytgat, G. N., Lee, S. P., Groen, A. K. (1999) Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**: 13–19

Kniep, B., Muhlradt, P. F. (1990) Immunochemical detection of glycosphingolipids on thin layer chromatograms. *Anal. Biochem*. **88**: 5–8

Kurita, K. (2006) Chitin and chitosan: functional biopolymers from marine crustaceans. *Marine Biotechnol*. **8**: 203–226

Laemmli U. K. (1970) Cleavage of structural proteins of the head of bacteriophage T4. *Nature* **227**: 680–684

Li, W. C., Ralphs, K. L., Slack, J. M., Tosh, D. (2007) Keratinocyte serum-free medium maintains long-term liver gene expression and function in cultured rat hepatocytes by preventing the loss of liver-enriched transcription factors. *Int. J. Biochem. Cell Biol*. **39**: 541–554

Martin, A., Clynes, M. (1993) Comparison of 5 microplate colorimetric assays for in vitro cytotoxicity testing and cell proliferation assays. *Cytotechnology* **11**: 49–56

Mhurchu, N., Dunshea-Mooij, C. A., Bennet, D., Rodgers, A. (2005) Chitosan for overweight or obesity. *Cochrane Database Syst. Rev*. **20**: CD003892

Monsigny, M., Petit, C., Roche, A. C. (1988) Colorimetric determination of neutral sugars by a resorcinol sulphuric acid micromethod. *Anal. Biochem*. **175**: 525–530

Mosmann, M., (1983) Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55–63

Oshannessy, D. J., Voorstad, P. J., Quarles, R. H. (1987) Quantitation of glycoproteins on electroblots using the biotin-streptavidin complex. *Anal. Biochem*. **163**: 204–209

Porstmann, T., Ternyk, T., Avrameas, S. (1985) Quantitation of 5-bromo-2-deoxyuridine into DNA an enzyme immunoassay for the assessment of lymphoid cell proliferation response. *J. Immunol. Methods* **82**: 169–79

Schmidgall, J., Hensel, A. (2002) Bioadhesive properties of polygalacturonides against colonic epithelial membranes. *Int. J. Biol. Macromol*. **30**: 217–225

Schmidgall, J., Schnetz, E., Hensel, A. (2000) Evidence for bioadhesive effects of polysaccharides and polysaccharide-containing herbs in an ex vivo bioadhesion assay on buccal membranes. *Planta Med*. **66**: 48–52

Sørbotten, A., Horn, S., Eijsink, V., Vårum, K. (2005) Degradation of chitosans with chitinase B from *Serratia marcescens* – production of chito-oligosaccharides and insight into enzyme prcessivity. *FEBS J*. **272**: 538–549

Whitehead, D. (1978) Application of a rapid method for determining glycosaminoglycans in mucus secreted by an aquatic pulmonate biomphalaria glabrata. *Comp. Biochem. Physiol*. **59**: 27–29

Zuang, V., Balls, M., Botham, P. A., Coquette, A., Corsini, E., Curren, R. D., Elliott, G. R., Fentem, J. H., Heylings, J. R., Liebsch, M., Medina, J., Roguet, R., van de Sandt, J. J., Wiemann, C., Worth, A. P. (2002) Follow-up to the ECVAM prevalidation study on in vitro tests for acute skin irritation. The European Centre for the Validation of Alternative Methods Skin Irritation Task Force report 2. *Altern. Lab. Anim*. **30**: 109–129