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Glycosaminoglycan secretion in xyloside treated polarized human colon carcinoma Caco-2 cells

Kristian Prydz · Tram T. Vuong · Svein O. Kolset

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Abstract Polarized epithelial cells like Madin-Darby canine kidney (MDCK) and CaCo-2 cells synthesize and secrete proteoglycans (PGs), mostly of heparan sulphate (HS) type in direction of the basal extracellular matrix, but also some in the apical direction. MDCK cells possess the capacity to synthesize chondroitin sulphate (CS) PGs that are mainly secreted into the apical medium, a process that is enhanced in the presence of hexyl-β-D-xyloside. We have now tested the capacity of several xylosides to enhance glycosaminoglycan (GAG) chain secretion from the human colon carcinoma cell line CaCo-2 in the differentiated and non-differentiated state. In these cells, benzyl-β-D-xyloside was a potent initiator of CS chains, which for these cells were predominantly secreted into the basolateral medium. Xylosides with other aglycone groups mediated only minor changes in GAG secretion. Although benzyl-β-D-xyloside stimulated the basolateral CS-GAG secretion in both differentiated and undifferentiated CaCo-2 cells, basolateral secretion of trypsin-like activity was dramatically enhanced in undifferentiated cells, but not significantly altered in differentiated cells.

K. Prydz
Department of Molecular Biosciences, University of Oslo,
Box 1041, Blindern,
0316 Oslo, Norway

T. T. Vuong
Department of Pathology, Rikshospitalet University Hospital,
0027 Oslo, Norway

S. O. Kolset (✉)
Department of Nutrition, University of Oslo,
Box 1046, Blindern,
0316 Oslo, Norway
e-mail: s.o.kolset@medisin.uio.no

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Introduction

Human Caco-2 colon carcinoma cells grown on filters are frequently used as a model system for studies of polarized human intestinal epithelial cells. Appropriate seeding densities allow confluent epithelial sheets to form after 3–4 days. Further cultivation for 10–12 days allow the cells to acquire morphology and functional abilities similar to normal enterocytes [21]. This cell system can be used to study aspects of differentiation of intestinal epithelial cells, particularly in relation to cellular polarity and sorting and transport of newly synthesized molecules to the apical and basolateral surface domains of an established epithelium.

Proteoglycans (PGs) play important roles at cell surfaces, in extracellular matrices, including the basement membrane underlying epithelial cells, and in intracellular granules [3, 8]. The most common types of glycosaminoglycan (GAG) chains that are coupled to PGs in vertebrate cells are chondroitin sulphate (CS) and heparan sulphate (HS). PG expression and the polymerization and sulphation of their GAG chains, have been shown to change during differentiation, transformation, and activation of various cell types [3, 16]. CaCo-2 cells displayed an altered HS sulphation pattern upon differentiation, changes that affected the binding to platelet derived growth factor, but not to fibroblast growth factor [19].

Polarized transport of newly synthesized proteins in direction of the apical and basolateral surfaces of epithelial cells is according to the prevailing view channeled through the endoplasmic reticulum (ER) and Golgi apparatus along

a common pathway, until the Golgi terminus, the *trans*-Golgi network, where sorting into distinct apical and basolateral routes is thought to occur. Recently, however, it has been demonstrated that a single recombinant PG is modified by different mechanism in the apical and basolateral secretory pathway in epithelial MDCK cells [22, 23]. These, and other studies, suggest that the apical and basolateral pathways diverge earlier than previously suggested in MDCK cells [17]. When the GAG synthesis and secretion capacity was probed with xylosides in these cells, the most potent xyloside primer was hexyl-β-D-xyloside, which gave rise to CS chains secreted apically [9]. We now demonstrate that another xyloside, benzyl-β-D-xyloside is the most potent initiator of CS chains in CaCo-2 cells, and that these chains are secreted mainly basolaterally. Thus, two commonly used model systems for epithelial cell polarity differ with respect to xyloside specificity and direction of CS-GAG synthesis and secretion. The use of benzyl-xyloside induced a large increase in the release of free CS chains and some reduction in the secretion of sulphated HSPGs.

Trypsin-like enzymes were secreted mainly to the apical medium in the absence of xyloside. However, xyloside treatment enhanced the secretion of trypsin-like enzymes to the basolateral medium in undifferentiated cells, but not in differentiated cells.

Materials and methods

Cells

CaCo-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) (4.5 g/l glucose) obtained from Bio-Whittaker (Verviers, Belgium). The medium was supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 1% non-essential amino acids (all from Bio-Whittaker). Finally, insulin Sigma Aldrich St. Louis, MO, USA,) was added to the medium to a final concentration of 10 μg/ml. Stock cultures were kept in tissue culture flasks. Before experiments the cells were detached with 0.25% trypsin in Ca^{2+} and Mg^{2+} -free phosphate-buffered saline containing 0.2 g/l of EDTA. Medium with serum was added to inhibit the trypsin after detachment. The cells were pelleted and resuspended in complete DMEM as described above. The cells were then seeded onto polycarbonate filters (Costar Transwell) with a pore size of 0.4 μm and a diameter of 24.5 mm (10^6cells/filter). The cells establish tight monolayers after 3–4 days of cultivation on filters. After further incubation for 10–12 days the cells have differentiated into enterocyte-like cells [21].

Metabolic labeling

Filter-grown CaCo-2 cells were labeled with (^{35}S)-sulphate at two different time points. Firstly, the cells were labeled after reaching confluency. These cells are referred to as undifferentiated cells. Secondly, cells were labeled after 14 days of cultivation on filters. These cells are referred to as differentiated cells. Before metabolic labeling the cells were washed with serum-free medium and transferred to sulphate-depleted RPMI 1640 (Invitrogen, Paisley, UK) with 10% FCS and other supplements as described above apart from penicillin and streptomycin. The cells on filters were labeled in 1 ml of apical medium and 2 ml of basolateral medium. The labeling was for 24 h with 100 μCi/ml of (^{35}S)-sulphate (Amersham Biosciences/GE-Healthcare, Chalfont St. Giles, UK) in each medium.

Xylosides

Both undifferentiated and differentiated CaCo-2 cells were labeled with (^{35}S)-sulphate for 24 h in absence or presence of three different β-D-xylosides; hexyl-β-D-thioxylopyranoside (referred to as HX-xyl), 2-*O*-(2,6-naphtalene)-β-D-xylopyranoside (referred to as naphtyl-xyloside or N-xyl) and benzyl-β-D-xyloside (referred to as BX-xyl). All three xylosides were kindly provided by the Seikagaku Kogyo, Tokyo, Japan.

Isolation of (^{35}S)-labeled macromolecules

At the end of the metabolic labeling period the apical and basolateral media were harvested separately, centrifuged to remove loose cells and 8 M guanidine-HCl with 4% Triton X-100 in 0.05 M sodium acetate buffer (pH 6.0) was added to each of the fractions in 1:1 volume. Cells were solubilized in 4 M guanidine with 2% Triton X-100 in 0.05 M sodium acetate buffer pH 6.0. All three fractions were subjected to Sephadex G-50 Fine (Amersham Biosciences/GE-Healthcare) gel chromatography in 0.05 M Tris–HCl, pH 8.0 with 0.15 M NaCl. The amount of (^{35}S)-labeled macromolecules was determined by scintillation counting of aliquots of the material eluted in the void volume.

For some experiments (^{35}S)-labeled macromolecules were further purified by DEAE-Sephacel (Amersham Biosciences/GE-Healthcare) ion exchange chromatography. Samples were applied and washed in 0.05 M Tris–HCl pH 8.0 with 0.15 M NaCl. Material bound to the column was eluted in 1 M NaCl and diluted with water before further use (see below).

SDS-PAGE

(^{35}S)-labeled macromolecules from CaCo-2 cells were analyzed by SDS-PAGE on precast 4–20% gradient gels (BioRad Laboratories, Hercules, CA, USA). (^{14}C)-labelled rainbow standards (Amersham Biosciences/GE-Healthcare) were used for molecular weight determinations. Samples analyzed were from the apical and basolateral media and cell fractions of both differentiated and undifferentiated cells. All samples were run on SDS-PAGE, either untreated, after chondroitinase ABC (E.C. 4.2.2.4; Seikagaku Kogyo, Japan) treatment, or nitrous acid treatment. Chondroitinase ABC treatment, used to degrade chondroitin/dermatan sulphate GAGs, was carried out over night at 37°C with 0.01 unit of enzyme per sample in 0.05 M Tris–HCl pH 8.0 with 0.025 M sodium acetate and 25 μg/ml of bovine serum albumin. Nitrous acid treatment at pH 1.5 was used to depolymerize heparin and heparan sulphate, according to published procedure [20]. All samples were boiled in sample buffer with 1% SDS and dithiothreitol as reducing agent. After completed electrophoresis the gels were treated with Amplify (Amersham Biosciences/GE-Healthcare), dried and subjected to autoradiography using Fuji Medical X-ray film (Fuji, Japan).

Immune precipitation

(^{35}S)-labeled macromolecules were recovered from apical and basolateral media of CaCo-2 cells. Cell fractions were solubilized in lysis buffer containing 0.05 M Tris–HCl, pH 7.5 with 1% Nonidet P-40, 2 mM EDTA, 0.15 M NaCl and 35 μg/ml PMSF. The fractions were incubated with two different antibodies: A rabbit antiserum against mouse perlecan (a gift from Dr. J.R. Hassell, University of Pittsburgh, PA, USA) and a mouse monoclonal antibody against human CD44 (provided by Dr. S. Jalkanen, University of Turku, Finland). The three different fractions were incubated with the respective antibodies at 4°C over night in lysis buffer. 5 mM $MgSO_4$ was added to reduce unspecific binding of free (^{35}S)-sulphate. Thereafter, the fractions were incubated with Protein A Sepharose (Amersham Biosciences/GE-Healthcare), which had been pre-washed with PBS containing 1% BSA, to reduce unspecific binding to the beads. Finally, the beads were washed, boiled in running buffer, centrifuged and the recovered samples were run on SDS-PAGE as described above.

Enzyme assays

Differentiated and undifferentiated CaCo-2 cells cultured on polycarbonate filters were washed three times in serum-free medium (supplemented DMEM without FCS) to remove serum proteins. The cells were thereafter incubated in serum-free medium in absence or presence of 0.1 mM BX-xyl. After 24 h of incubation apical and basolateral conditioned media were harvested and centrifuged to remove non-adherent cells. The cells adherent to filters were washed 3 times with PBS and then solubilized in 0.05 M Tris–HCl, pH 8,0 with 1% Triton X-100. Enzyme activities in the three fractions were measured by adding 20 μl of either substrate S-2288 or S-2444 to 200 μl samples in a 96 well microtiter plate. The S-2288 substrate is cleaved by trypsin-like enzymes, whereas S-2244 is regarded as a relatively specific plasminogen activator substrate [15]. Both substrates were obtained from Chromogenix, Mølndal, Sweden. The substrates were solubilized in distilled water at stock concentration of 1.25 mg/ml. The enzyme activities were recorded by reading the absorbance at 405 nm using a Titertek Multiscan spectrophotometer (Flow laboratories, Irvine, Scotland). The enzymatic determinations were performed in triplicates.

Serum-free media were also subjected to zymography, using SDS-PAGE with 0.1% (*w*/*v*) gelatin in both the stacking and separating gel. The conditions were as previously described [15]. Briefly, different fractions of conditioned media were subjected to SDS-PAGE before the gels were washed and incubated in assay buffer containing 50 mM Tris–HCl, pH 7.5 with 5 mM $CaCl_2$, 0.2 M NaCl and 0.02% Brij-35 for approximately 20 h. To visualize possible enzyme activities the gels were stained with 0.2% Coomassie brilliant blue in 30% methanol and destained in 30% methanol/10% acetic acid. Gelatinase activity was evident as clear zones on blue background. Gels were dried and analyzed using a computer program from Ultra Violet Products (Cambridge, UK).

Results

GAG synthesis and secretion from CaCo-2 cells in the presence of xylosides

To probe the glycosaminoglycan (GAG) synthesis capacity in polarized CaCo-2 cells, we made use of xylosides with different aglycone groups. Metabolic labeling and biochemical analysis allows determination of which xyloside has the greater potential in these cells. Initial comparative experiments were performed with 0.1 mM concentrations of HX-xyl, N-xyl and BX-xyl. This concentration has previously been shown to initiate substantial CS-GAG synthesis and affect endogenous PG expression in both monocytes [7] and kidney epithelial cells [9]. BX-xyl was shown to be by far the most efficient stimulator of (^{35}S)-incorporation into

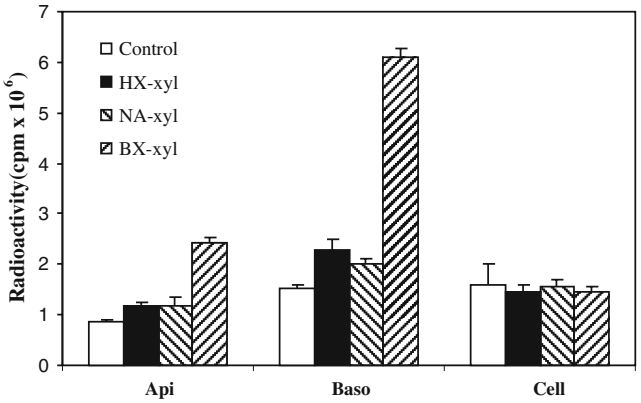

Fig. 1 The effect of β-D-xylosides on synthesis and secretion of (^{35}S)-labeled macromolecules in CaCo-2 cells. CaCo-2 cells were metabolically labeled with (^{35}S)-sulphate for 24 h in absence or presence of 0.1 mM of the indicated xylosides. Apical and basolateral media and cell fractions were harvested and subjected to Sephadex G-50 Fine gel filtration to separate (^{35}S)-labeled macromolecules from unincorporated label. The amounts of macromolecules were determined in a scintillation counter. The figure shows one representative experiment with three parallels from differentiated cells

secreted macromolecules in differentiated CaCo-2 cells, as can be seen in Fig. 1. The same trend was observed for undifferentiated CaCo-2 cells (not shown). No stimulation was observed for the cell fractions.

Evidently, the aglycone group of a xyloside is crucial for the ability to trigger GAG synthesis in CaCo-2 cells, as previously shown for other cell types. However, while BX-xyl was the most potent xyloside in CaCo-2 cells, HX-xyl has been shown to be the most efficient initiator of CS-GAGs in monocytes and epithelial MDCK cells [7, 9]. Further experiments with CaCo-2 cells were performed with BX-xyl.

Differentiated and undifferentiated CaCo-2 cells were exposed to different concentrations of BX-xyl during metabolic labeling with (^{35}S)-sulphate. Macromolecules were purified by gel filtration and the amount of incorporated (^{35}S)-sulphate determined by scintillation counting. Clearly, 0.1 mM BX-xyl was an optimal concentration for priming of GAG chain synthesis (Fig. 2). The higher concentration, 1 mM, does not give additional stimulatory effect. The data presented are generated with undifferentiated cells, but the same trend was observed for differentiated cells (not shown). In subsequent studies 0.1 mM, and in some cases 0.01 mM BX-xyl, was used to study GAG synthesis in polarized CaCo-2 cells.

PG and GAG secretion in the presence of xyloside

To investigate synthesis and secretion of PGs in undifferentiated and differentiated CaCo-2 cells, (^{35}S)-labelled macromolecules were recovered from apical and basolateral media and cell fractions and analyzed by SDS-PAGE. Material was recovered from control cells and cells exposed to 0.01 or 0.1 mM BX-xyl for 20 h. Furthermore, SDS-PAGE analysis was also performed after the respective fractions had been treated with either HNO_2 to degrade HS chains or chondroitinase-ABC (c-ABC) to degrade CS/DS chains. Evidently, most of the PGs synthesized are secreted into the basolateral medium from both differentiated and undifferentiated CaCo-2 cells (Fig. 3, panel A, control lanes) as seen in the upper region of the gel lanes. Lesser amounts of PGs were observed in the apical media and cell fractions.

The high molecular weight PGs were of the HS type, since these were degraded by HNO_2 treatment (Fig. 3, panel B). The PG band appearing at approximately

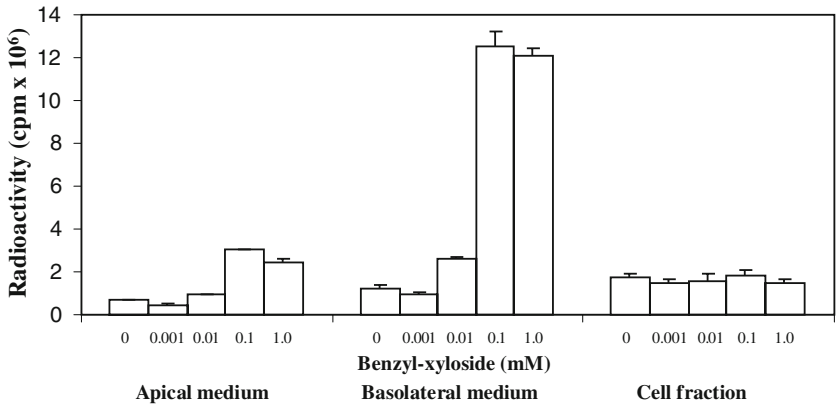

Fig. 2 The effect of different concentrations of benzyl-xyloside on synthesis and secretion of (^{35}S)-labeled macromolecules in CaCo-2 cells. Filter-grown CaCo-2 cells were treated at different concentrations of BX-xyl during the labeling period with (^{35}S)-sulphate. Apical and basolateral media and cell fractions were harvested and subjected to Sephadex G-50 Fine gel filtration to separate (^{35}S)-labeled macromolecules from unincorporated label. The figure shows one representative experiment with three parallels from undifferentiated cells

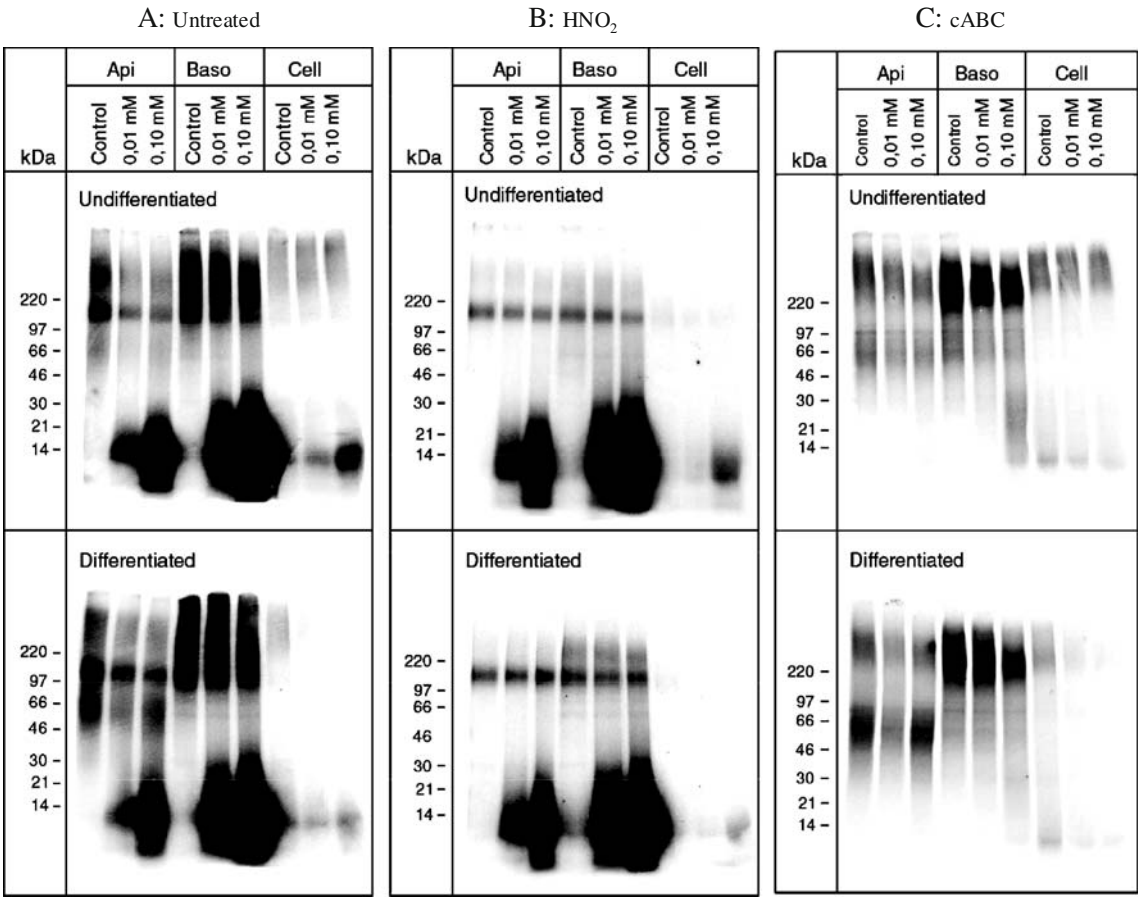

Fig. 3 Secretion of xyloside-based chondroitin sulphate chains from CaCo-2 cells. Aliquots of (^{35}S)-labeled PG fractions obtained from DEAE-ion exchange preparative chromatography of cells treated with or without BX-xyl were analyzed by SDS-PAGE gel. **a** Control samples of BX-xyl treated differentiated and undifferentiated cells, **b** HNO2-treated samples, and **c** cABC treated samples. Dried gels were subjected to autoradiography. The figure shows results from one representative experiment

200 kDa was a CS/DSPG resistant to HNO_2 treatment, but disappears with c-ABC treatment (Fig. 3, panel C).

BX-xyl treatment of both undifferentiated and differentiated cells resulted in the formation and secretion of free GAG chains, that were sensitive to c-ABC treatment (Fig. 3, panel C). We therefore conclude that BX-xyl treatment in these cells promotes the synthesis of CS and/or DS chains on the xyloside primer, since both GAG types are degraded by c-ABC. It is furthermore interesting to note that synthesis and secretion of the 200 kDa CS/DSPG is not significantly affected in BX-xyl treated cells (panel B), where PGs that are resistant to HS degrading HNO_2 treatment appear.

BX-xyl treatment does not prime detectable amounts of HS chains. The large amount of radiolabelled material appearing after xyloside treatment (molecular weight ranging from 30 kDa to less than 14 kDa) was completely degraded by c-ABC treatment. However, secreted HSPGs decreased somewhat in their average size and intensity, which could be most clearly observed when CS/DS had been removed (Fig. 3, panel C). This result suggests that, although BX-xyl did not initiate the synthesis of HS chains, the large stimulation of CS synthesis lead to reduced metabolic labeling of HSPG. An alternative explanation could be that PGs of the HSPG type may also carry CS/DS chains, and that these are affected by xyloside treatment. Stimulation of CS can be observed in both the apical and basolateral fractions (Fig. 3, panel A and C; 0.01 and 0.1 mM BX-xyl). Finally, an HSPG secreted into the apical medium of CaCo-2 cells (molecular mass ranging from 97–46 kDa) was much more pronounced for differentiated cells. This might represent an HSPG that has been shed from the cell surface.

Immune precipitation was performed from the different fractions of (^{35}S)-labelled macromolecules in an attempt to identify some of the PGs expressed. Both perlecan and CD44 could be identified (Fig. 4). The PG form of CD44 was detected in the cell fractions of both differentiated and undifferentiated cells, but not in the media. CD44 has been shown to be mainly cell-associated.[18]. Perlecan, in

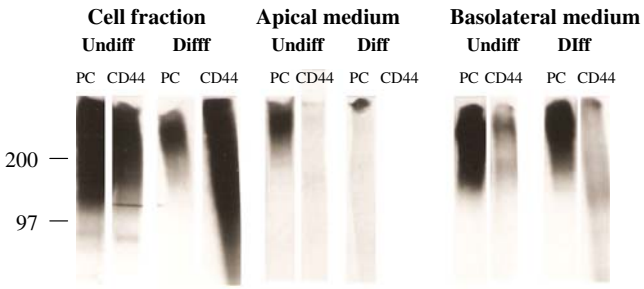

Fig. 4 Immunoprecipitation of proteoglycans from CaCo-2 cells. Aliquots of (^{35}S)-labeled PG fractions from control cells, obtained from DEAE-ion exchange preparative chromatography were subjected to immunoprecipitation with antibodies against perlecan and CD44 and thereafter subjected to SDS-PAGE, followed by autoradiography. The migration positions of the 200 kDa and 97 kDa standards are shown at the *left side of the panel*

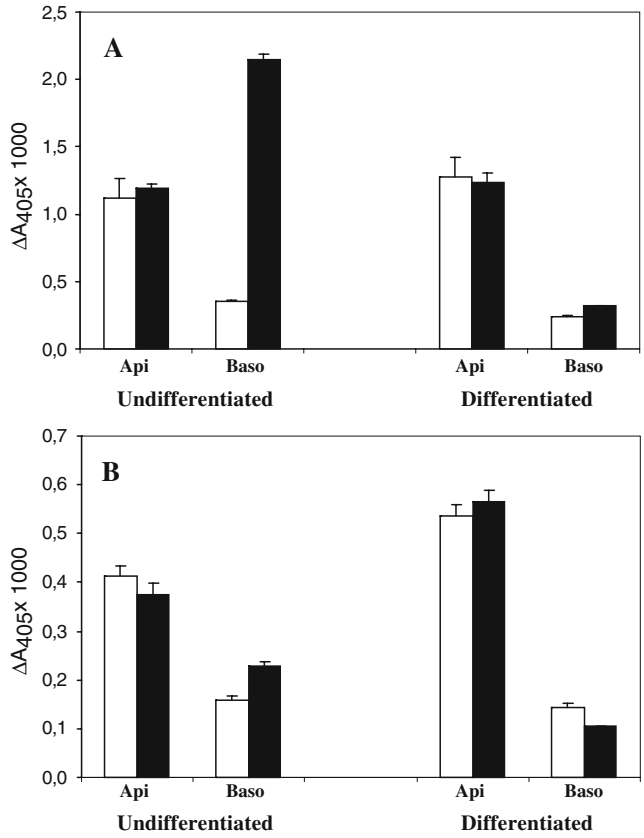

Fig. 5 The effect of benzyl-xyloside on enzyme secretion in CaCo-2 cells. Filter-grown CaCo-2 cells were incubated with serum-free medium in absence (*open bars*) or presence of 0.1 mM BX-xyl for 24 h (*filled bars*) before apical and basolateral medium were separately collected. The trypsin-like enzyme activities and PA activities in apical and basolateral media were measured by adding substrate S-2288 (**a**) or S-2444 (**b**), respectively to 200 µl samples in a 96 microtiter plate. The enzyme activities were recorded by reading absorbance in a spectrophotometer at 405 nm. The figure shows one representative experiment with triplicates

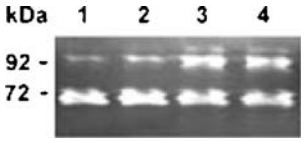

Fig. 6 Zymography of conditioned medium from undifferentiated CaCo-2 cells. Apical and basolateral conditioned medium were collected from undifferentiated cells and subjected to zymography. Basolateral medium from control cells (*lane 1*) and BX-xyl treated cells (*lane 2*). Apical medium from control cells (*lane 3*) and BX-xyl treated cells (*lane 4*). ProMMP-9 runs at 92 kDa and proMMP2 runs at 72 kDa

contrast, could be detected in the basolateral medium of both differentiated and undifferentiated cells, and with a stronger signal in the cell fraction of undifferentiated than differentiated cells. In this cell system perlecan would be expected to be deposited in the filter matrix and secreted to the basolateral medium, as it is an important component of basement membranes [6]. However, the higher level of perlecan found in cell fractions of undifferentiated cells suggest that differentiation of CaCo-2 cells affect secretion of perlecan. Finally, as a major portion of PGs are secreted in polarized CaCo-2 cells, these species must consist of other PGs than those detected here.

Glycosaminoglycans and enzyme secretion

Serum-free media from control and BX-xyl treated cells were analyzed with respect to trypsin-like enzymes and plasminogen activator activity. Both trypsin-like enzymes (Fig. 5, panel A) and plasminogen activator activity (Fig. 5, panel B) were mainly secreted to the apical medium. However, only the secretion of trypsin-like activity was affected by BX-xyl treatment, which shifted the secretion of this activity towards the basolateral medium, the direction taken by the major part of the xyloside-initiated GAGs. This phenomenon was only observed in undifferentiated cells. Only very low enzymatic activities were observed in the cell fractions.

We also addressed the presence of gelatinase activity in the medium fractions. Secretion of proMMP-2 was very similar between basolateral and apical media, whereas proMMP-9 was predominantly secreted into the apical medium. BX-xyl treatment did not affect the secretion of MMP-2 and MMP-9 (Fig. 6).

Discussion

In this study we show that colon carcinoma CaCo-2 cells are stimulated to increase their production of glycosaminoglycan (GAG) chains dramatically by the addition of

benzyl-xyloside to the culture medium. Xylosides with other aglycone units did not have similar stimulatory effects, although proven to induce GAG synthesis in other cell types. Although untreated CaCo-2 cells synthesize mainly HSPGs and very little CSPGs, our studies with BX-xyloside demonstrate that these cells have a large capacity to synthesize CS/DS chains. Other studies have shown that it is possible to initiate HS GAGs on xyloside [10], but we could not observe this phenomenon in CaCo-2 cells with the xylosides tested here. This capacity seems independent of the differentiation state, although undifferentiated cells can be regarded as an earlier developmental stage, where CSPG synthesis normally is more prominent [11]. The fact that the same xyloside (or related glycan-priming) structure does not have the same potential across numerous cell systems is becoming an established fact [1, 5, 7, 9, 12].

However, an explanation to the phenomenon at the cellular level has not been presented. The hydrophobic moiety is required to cross membranes, but the ability of a xyloside to pass the plasma membrane is unlikely to differ substantially in different cell types. Rather, the association with the enzymatic machinery that polymerizes GAG chains is critical, since β-D-xylosides initiate GAG synthesis, while α-D-xylosides do not. Both isoforms may, however, inhibit glycolipid synthesis, presumably after being galactosylated [4]. Differences in glycolipid synthesis and membrane composition might be the reason why different xylosides take the lead in different cell types and affect membrane pathways differently [9, 23]. Since xylosides seem to compete with PG core proteins in the biosynthetic route only after addition of two galactose-units [13] it is likely that the differences reported reflect differences in properties of the Golgi-apparatus or the ER-Golgi-intermediate compartment (ERGIC) in the different cell types.

In another epithelial cell line (epithelial kidney –MDCK cells), it has been demonstrated that the same PG protein core obtains GAG chains of different sulfate densities in the apical and the basolateral secretory pathways, where the basolateral pathway always displays the highest capacity for sulphation [2, 22, 23].

The possible implications of stimulated CS/DS chain synthesis in CaCo-2 cells were investigated by measuring the release of different types of enzymes. Both trypsin-like enzymes and plasminogen activator type of enzymes were predominantly secreted to the apical medium, suggesting functions related to digestion or other intestinal processes. The release of MMPs did not follow the same pattern, as MMP-2 was secreted to both apical and basolateral media in approximately equal amounts. However, MMP-9 was secreted predominantly to the apical medium. BX-xyl stimulated the secretion of trypsin-like enzymes in undifferentiated cells, but not in differentiated CaCo-2 cells. The undifferentiated cells bear resemblance to transformed cells, whereas the differentiated cells resemble normal enterocytes [21].

Several studies have shown that PGs are involved in the regulation of secretion, transport and activation of enzymes in mast cells [14] and xyloside studies suggest that PGs can have similar functions in macrophages [15]. The relation between GAGs and enzyme secretion has not been investigated to any significant extent in CaCo-2 cells. The results presented demonstrate that xylosides may be used to study the GAG dependence of enzyme secretion. The difference in sensitivity among cell lines to different xylosides, and the variability in direction of secretion are interesting, but demand further experiments to increase our understanding of these phenomenona.

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