The effect of β -D-xylosides on the proliferation and proteoglycan biosynthesis of monoblastic U-937 cells

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The monoblastic cell line U-937 was cultured in the presence of *C*-ethyl β -D-xyloside (E-xyl), hexyl β -D-thioxyloside (HX-xyl), *p*-nitrophenyl β -D-xyloside, phenyl β -D-xyloside or phenyl α -D-xyloside. All of the β -D-xylosides inhibited proliferation, but HX-xyl was by far the most efficient, and had a maximum effect at 1 mM concentration. The inhibitory effect of HX-xyl could be reversed; after washing, the HX-xyl-treated cells proliferated with a pattern similar to that of control cells. For more detailed analysis of the effects of β -D-xylosides on cell proliferation and chondroitin sulphate (CS)/chondroitin sulphate proteoglycan (CSPG) structure, a comparison between the effects of E-xyl and HX-xyl was made. Treating the cells with 1 mM-HX-xyl resulted in a large increase in CS synthesis, whereas 1 mM-E-xyl had only minor effects on the rate of PG/glycosaminoglycan synthesis. Sepharose CL-6B gel chromatography of medium and cell fractions from 35 S-labelled cells revealed that HX-xyl treatment resulted in the expression of only free CS chains, whereas E-xyl exposure leads to the synthesis of both large and small CSPGs, as well as some free CS chains. The expression of elevated levels of free CS chains was clearly correlated to the inhibition of proliferation. The proliferation of U-937-4, a clone of U-937 synthesizing ten times more CSPG/CS than the parent line, was equally inhibited by HX-xyl treatment. With this clone, however, there was no stimulation of CS synthesis after xyloside exposure, indicating that the elevated level of CS evident after xyloside treatment of the parent cell line is not causing the inhibition of proliferation. Furthermore, the biosynthesis of hyaluronate was shown not to be implicated in the xyloside-induced decrease in proliferation. The inhibition of proliferation observed in the presence of 1 mM-HX-xyl did not lead to differentiation of the cells into macrophage-like cells, as is observed when the cells are cultured in the presence of phorbol esters, agents also known to inhibit proliferation of U-937 cells.

INTRODUCTION

In a large number of studies, the biosynthesis (Okayama *et al.*, 1973; Schwartz *et al.*, 1974; Robinson *et al.*, 1975) and functions of proteoglycans (PGs) in various biological processes (Gibson *et al.*, 1978, 1979; Kinoshita & Saiga, 1979; Spooner *et al.*, 1983) have been studied using β -D-xylosides. These monosaccharides, when attached to various hydrophobic groups, penetrate plasma membranes on exposure to cells and interfere with PG biosynthesis by competing with the xylosylated endogenous peptide core as an acceptor for glycosaminoglycan (GAG) polymerization. When the xylosides are added at appropriate concentrations, endogenous PG biosynthesis can be completely inhibited and only free GAG chains are synthesized. The extent of the effect of β -D-xylosides on PG biosynthesis has been shown to differ depending upon the aglycone group attached to the xylose residues (Robinson *et al.*, 1975; Sobue *et al.*, 1987). In the latter study, some β -D-xylosides did not affect PG biosynthesis and were, furthermore, demonstrated to have no inhibitory effect on the development of chicken embryos, unlike those xylosides which inhibited PG biosynthesis.

When studying the effects of xylosides on cellular

processes, possible non-specific effects cannot be entirely excluded. The demonstration, however, that xylosides with different aglycone groups vary to a considerable extent in their effects on PG biosynthesis has provided us with a tool to exclude possible non-specific effects of xylosides on the parameters tested. In the present report, we have investigated the implications of endogenous PG biosynthesis for the proliferation of the monoblastic cell line U-937, originally derived from a patient with a histiocytic lymphoma (Sundström & Nilsson, 1976). This cell line has been shown previously to synthesize exclusively chondroitin sulphate proteoglycan (CSPG) (Kolset, 1987; Kolset *et al.*, 1988a). The CSPG biosynthesis and the proliferation of U-937 cells were studied in the presence of *C*-ethyl β -D-xyloside (E-xyl), a xyloside shown to have a relatively small effect on PG biosynthesis, and hexyl β -D-thioxyloside (HX-xyl), a potent inhibitor of PG biosynthesis (Sobue *et al.*, 1987).

EXPERIMENTAL PROCEDURES

Proliferation studies

U-937 GTB cells and U-937 clone 4 (U-937-4) were cultured in RPMI medium with 10% fetal calf serum,

Abbreviations used: E-xyl, *C*-ethyl β -D-xyloside; HX-xyl, hexyl β -D-thioxyloside; CS, chondroitin sulphate; CSPG, chondroitin sulphate proteoglycan; PG, proteoglycan; GAG, glycosaminoglycan; PAGE, polyacrylamide-gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.

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50 µg of streptomycin/ml and 100 units of penicillin/ml (all from Gibco Laboratories, Glasgow, Scotland). E-xyl, HX-xyl, phenyl β-D-xyloside and phenyl α-D-xyloside were prepared as previously described (Sobue *et al.*, 1987). *p*-Nitrophenyl β-D-xyloside was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All xylosides were dissolved in culture medium at a stock concentration of 50 mM and stored at -20 °C. U-937 and U-937-4 cells were established in 16 mm Nunclon wells (A/S Nunc, Roskilde, Denmark) at a density of 0.1×10^6 /ml in a total volume of 1 ml per well. At the same time, xylosides were added, and duplicate cultures were counted each day after the addition of Trypan Blue. The viability in all cultures was > 95 % as judged by the Trypan Blue exclusion test.

Alternatively, cell proliferation was assayed by measuring the incorporation of [³H]thymidine. U-937 cells were set up in 6 mm Linbro wells (Linbro microtitration plate; Flow Laboratories Inc., McLean, VA, U.S.A.). A total of 20 000 cells in a volume of 200 µl of culture medium were established in the presence of 1 mM-E-xyl, 1 mM-HX-xyl or 50 ng of phorbol 12-myristate 13-acetate (PMA)/ml. The cells were labelled with [³H]thymidine (Amersham International, Amersham, Bucks., U.K.) (0.4 µCi per well, specific radioactivity 47 Ci/mmol) for 60 min every day. The cells were harvested on a Titertek cell harvester (Flow Laboratories/Skatron A/S Lier, Norway), precipitated on filters with distilled water and washed. The radioactivity on the filters was counted in a Packard Tri-Carb scintillation counter after the addition of scintillation fluid. The amount of [³H]thymidine incorporated in the various cultures is expressed as a percentage of control values (no β-D-xylosides added). All assays were run in triplicate, and the S.E.M. values were 10–15 %.

Proteoglycan structure

U-937 cells were labelled with ³⁵SO₄ (50 µCi/ml; Amersham) during either days 0–4 or days 3–4. Xylosides were added on day 0. In these experiments the starting cell density was normally 0.2×10^6 /ml. Cell and medium fractions were subjected to proteolytic digestion and gel chromatography as previously described (Kolset *et al.*, 1983). The isolated ³⁵S-labelled macromolecules were degraded with chondroitin ABC lyase (EC 4.2.2.4) (Sigma) and the degradation products were analysed by Sephadex G-50 gel chromatography (Pharmacia, Uppsala, Sweden) (Kolset *et al.*, 1983).

Medium and cell fractions were also solubilized in 1 % SDS, boiled for 3 min and subjected to Sepharose CL-6B gel chromatography (Pharmacia) in 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl and 0.1 % SDS, after Sephadex G-50 gel chromatography to remove free ³⁵SO₄. Fractions were collected and counted for radioactivity. Markers for void volume and total volume of the column were Dextran Blue and dinitrophenyl alanine respectively (both from Sigma).

³⁵SO₄ incorporation studies

The incorporation of ³⁵SO₄ into CS and CSPG was measured in both medium and cell fractions after SDS solubilization of both fractions. Disposable syringes (2 ml) (Gillette, U.K. Ltd., Isleworth, Middx., U.K.) with glass wool in the bottom were filled with Sephadex G-50 Superfine gel. The syringes were placed in 10 ml tubes

and centrifuged at 1000 *g* for 5 min. SDS-solubilized samples (50 or 100 µl) were applied to the top of the dehydrated gels, whereafter the G-50 columns were subjected to a second centrifugation at 1000 *g* for 5 min. Control samples containing only free ³⁵SO₄ were routinely run in parallel, and were found not to elute from the gel after the centrifugation. Radiolabelled material eluting after the second centrifugation was counted for radioactivity in a scintillation counter after the addition of scintillation fluid.

Isolation and characterization of ³H-labelled GAGs

Both U-937 and U-937-4 cells were labelled with [³H]glucosamine (Amersham) for 10 h after the cells had been exposed to 1 mM-E-xyl or 1 mM-HX-xyl for 3 days. The labelling was performed using RPMI 1640 medium with the concentration of glucose reduced to 50 % of the standard concentration and fortified as described earlier. The cells were labelled in 35 mm Nunclon wells at a concentration of 2×10^6 cells/ml with 50 µCi of [³H]glucosamine/ml. Control cells cultured for 3 days before labelling were treated identically. The respective medium and cell fractions were subjected to proteolytic digestion and gel chromatography as previously described (Kolset *et al.*, 1983). The void volume fractions containing radiolabelled material were dialysed against water and subjected to preparative DEAE-Sephacel ion-exchange chromatography (Pharmacia). Material binding to the column was eluted in sodium acetate buffer, pH 4.0, with a NaCl gradient extending from 0.2 to 1.5 M. Fractions were collected and aliquots were counted for radioactivity. Fractions containing radiolabelled material were pooled (see the Results section), dialysed against water at 4 °C, lyophilized and stored in small volumes of phosphate-buffered saline (0.14 M-NaCl/2 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-KH₂PO₄), pH 7.4. Samples were treated with chondroitin ABC lyase and *Streptomyces* hyaluronidase (Seikagaku Kogyo Ltd., Tokyo, Japan).

[³⁵S]Methionine labelling and SDS/polyacrylamide-gel electrophoresis

U-937 cells were cultured for 1 day in the absence or the presence of 1 mM-HX-xyl. The cells were washed three times with methionine-free RPMI medium and then incubated for 6 h in this medium containing 10 % (v/v) fetal calf serum and 400 µCi of [³⁵S]methionine/ml. The cell fractions were harvested, treated with sample buffer and subjected to SDS/PAGE (7–15 % gradient gel). ¹⁴C-labelled molecular mass standards were obtained from Amersham.

Immunofluorescence

The expression of antigens detected by the murine monoclonal antibodies OKM-1 and OKT-4 (Ortho Diagnostics, Sollentuna, Sweden) was performed with U-937 cells treated with β-D-xylosides and PMA. OKM-1 is directed against the receptor for complement factor C3b, whereas OKT-4 reacts with the T4 antigen shown to be expressed only by immature U-937 cells (Mosciacki *et al.*, 1983). Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Dakopatt A/S, Glostrup, Denmark) was used as second antibody, and the specimens were inspected in a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Fig. 1. Effect of β -D-xylosides on the proliferation of U-937 cells

U-937 cells were seeded at a density of 0.1×10^6 /ml and cultured in the absence and presence of β -D-xylosides for 4 days. The cells were counted each day. Viability was $> 95\%$ by the Trypan Blue exclusion test in all cultures. Xylosides used: $\triangle$, 0.1 mM-E-xy; $\square$, 1.0 mM-E-xy; $\blacktriangle$, 0.1 mM-HX-xy; $\blacksquare$, 1.0 mM-HX-xy; $\circ$, control.

RESULTS

Effect of xylosides on the proliferation of U-937 cells

U-937 cells were cultured in the presence of 0.1 and 1.0 mM-E-xy and HX-xy. The proliferation of the cells was only inhibited in the presence of 1.0 mM-HX-xy; after 4 days incubation, the cell number was 27% of that recovered from control cultures, compared with 73% (0.1 mM-HX-xy), 83% (0.1 mM-E-xy) and 88% (1.0 mM-E-xy) in the other cultures (see Fig. 1). These cell counting experiments were further supported by [3 H]thymidine incorporation studies on U-937 cells cultured in the presence of 1.0 mM-E-xy or 1.0 mM-HX-xy. After 1 day of incubation, DNA synthesis was 30% of the control in HX-xy-treated cultures, and after 2, 3 and 4 days these values were 39, 18 and 43% respectively. In contrast, E-xy was found to have either a small stimulatory or a small inhibitory effect on the [3 H]thymidine incorporation (Table 1). It is further evident that 1.0 mM-HX-xy was as effective as PMA (50 ng/ml) in inhibiting DNA synthesis (Table 1).

Table 1. Effect of β -D-xylosides and PMA on [3 H]thymidine incorporation into cultured U-937 cells

Treatment	[3 H]Thymidine incorporation (% of control)			
	Day 1	Day 2	Day 3	Day 4
1 mM-E-xy	77	103	100	128
1 mM-HX-xy	30	39	18	43
PMA (50 ng/ml)	38	26	37	43

The observed differences in effect between HX-xy and E-xy could possibly be a question of the different doses used. Higher concentrations of E-xy were therefore tested for their ability to affect cellular proliferation. Furthermore, it might be that the inhibition of proliferation by HX-xy is mediated through the aglycone group. Other β -D-xylosides were therefore tested for their ability to influence cellular proliferation. U-937 cells were cultured in the presence of 5 or 10 mM concentrations of E-xy. After 4 days' incubation, the cell number was 97% and 74% of controls respectively. Thus proliferation is only minimally affected, even at such a high dose as 10 mM-E-xy (Table 2). Three other xylosides were also tested for their effects on proliferation, namely *p*-nitrophenyl β -D-xyloside, phenyl β -D-xyloside and phenyl α -D-xyloside. Both of the β -species inhibited cellular proliferation after 4 days' incubation to a large extent at concentrations of 5 mM, nitrophenyl β -D-xyloside being the most efficient. However, none of these β -D-xylosides exhibited the same anti-proliferative effects as HX-xy at 1 mM concentrations (see Table 2). In contrast, the phenyl α -D-xyloside had a slight effect on proliferation at 1 mM and caused extensive cell death at 5 mM. The corresponding β -D-xyloside at 5 mM inhibited cellular proliferation by 65%, without affecting cell viability (Table 2). The nitrophenyl β -D-xyloside (5 mM) exhibited the greatest inhibitory effect on proliferation during a 4 day incubation. It was noted, however, that 5 mM-nitrophenyl β -D-xyloside in some experiments caused extensive cell death. Reproducible results were therefore somewhat difficult to obtain with this dose of this particular β -D-xyloside. In contrast, 1 mM-HX-xy was invariably found to inhibit cellular proliferation to a large extent without affecting the viability of the cells. Furthermore, the inhibitory effect was found to depend on the continuous presence of HX-xy. If the cells were washed after 4 days of incubation in the presence of

Table 2. Effects of xylosides on the proliferation of U-937 cells

The cells were counted after 4 days incubation with the respective xylosides.

Xyloside	Cell number (% of control)
0.01 mM-HX-xy	83
0.1 mM-HX-xy	73
1.0 mM-HX-xy	25
0.1 mM-E-xy	82
1.0 mM-E-xy	89
5.0 mM-E-xy	86
10.0 mM-E-xy	74
0.1 mM-Nitrophenyl β -D-xyloside	100
1.0 mM-Nitrophenyl β -D-xyloside	73
5.0 mM-Nitrophenyl β -D-xyloside	15
0.1 mM-Phenyl β -D-xyloside	91
1.0 mM-Phenyl β -D-xyloside	61
5.0 mM-Phenyl β -D-xyloside	35
0.1 mM-Phenyl α -D-xyloside	90
1.0 mM-Phenyl α -D-xyloside	71
5.0 mM-Phenyl α -D-xyloside	—*

* Extensive cell death, less than 60–70% viable cells.

Table 3. Effects of xylosides on ^{35}S incorporation into U-937 cells

The cells were cultured for 3 days in the presence of xylosides and labelled with $^{35}\text{SO}_4$ from days 3–4. Cell and medium fractions were analysed for the content of ^{35}S -labelled macromolecules. The total incorporation into cell and medium fractions per 10^6 cells was used as a basis for calculating the ratios.

Xyloside	^{35}S incorporation (xyloside-treated/ control cells)
0.01 mM-HX-xyl	2.3
0.1 mM-HX-xyl	5.2
1.0 mM-HX-xyl	17.0
0.1 mM-E-xyl	0.8
1.0 mM-E-xyl	1.4
5.0 mM-E-xyl	2.6
10.0 mM-E-xyl	2.3
0.1 mM-Nitrophenyl β -D-xyloside	2.7
1.0 mM-Nitrophenyl β -D-xyloside	8.5
5.0 mM-Nitrophenyl β -D-xyloside	21.8
0.1 mM-Phenyl β -D-xyloside	2.2
1.0 mM-Phenyl β -D-xyloside	2.8
5.0 mM-Phenyl β -D-xyloside	19.0
0.1 mM-Phenyl α -D-xyloside	1.2
1.0 mM-Phenyl α -D-xyloside	2.8
5.0 mM-Phenyl α -D-xyloside	—*

* Extensive cell death.

1 mM-HX-xyl, they proliferated during an additional 4 day period with a pattern closely similar to that of untreated control cells at the same starting cell density.

Effect of β -D-xylosides on the biosynthesis of CSPG

Cells treated with 1 mM-E-xyl or 1 mM-HX-xyl and labelled with $^{35}\text{SO}_4$ were found to incorporate the radioisotope exclusively into CS, i.e. into material susceptible to degradation with chondroitin ABC lyase, in both the cell and medium fractions. This is in accordance with what has been shown previously for untreated U-937 cells (Kolset, 1987) and PMA-differentiated cells (Kolset *et al.*, 1988a). The incorporation of $^{35}\text{SO}_4$ into macromolecules is, accordingly, a measure of CS/CSPG synthesis *de novo*.

All xylosides shown in Table 2 to be efficient inhibitors of proliferation were demonstrated to be potent stimulators of incorporation of ^{35}S into CSPG/CS, as shown in Table 3. Among those xylosides inhibiting proliferation and stimulating PG/GAG synthesis, only HX-xyl was highly efficient at a 1 mM concentration, whereas both nitrophenyl and phenyl β -D-xylosides were required at 5 mM concentrations in order to exert approximately the same effect. Both E-xyl and phenyl α -D-xyloside had only small stimulatory effects on ^{35}S incorporation at 10 and 1 mM concentrations respectively.

Effect of β -D-xylosides on the macromolecular properties of [^{35}S]CS and [^{35}S]CSPG synthesized by U-937 cells

In order to analyse further whether the anti-proliferative effect observed in U-937 cells under the influence

Fig. 2. Sepharose CL-6B gel chromatography of cell fractions from U-937 cultures

U-937 cells were labelled with $^{35}\text{SO}_4$ (200 $\mu\text{Ci}/\text{ml}$) for 60 min in the absence or the presence of 1 mM- β -D-xylosides. The xylosides were added concomitantly with the isotope, and the cell fractions were processed as described in the Experimental procedures section, and thereafter subjected to Sepharose CL-6B gel chromatography under dissociative conditions. (a) Control (no xylosides), (b) E-xyl, (c) HX-xyl.

of HX-xyl could be related to changes in the structure of the CS/CSPG synthesized, both cell and medium fractions were analysed by Sepharose CL-6B gel chromatography. After a 1 h pulse with $^{35}\text{SO}_4$ and 1 mM-E-xyl or 1 mM-HX-xyl, large changes in the macromolecular properties of the cell-associated PG/GAG were apparent. The control cells contained two dominant peaks, one eluting in the void volume of the gel and a second eluting with a peak K_{av} of 0.16 (Fig. 2). The cell fraction of E-xyl-treated cells was eluted with one dominant peak at a K_{av} of approx. 0.3, but also with some material eluting in the void volume. The HX-xyl-treated cell fraction, in contrast, contained only one peak with a K_{av} of 0.5, and no material in the void volume (Fig. 2).

Fig. 3. Sepharose CL-6B gel chromatography of medium fractions from U-937 cultures

U-937 cells were labelled with $^{35}\text{SO}_4$ (50 $\mu\text{Ci}/\text{ml}$) for 24 h in the absence and presence of β -D-xylosides. The xylosides were added to the cells at the same time as the isotope. After processing, the medium fractions were subjected to Sepharose CL-6B gel chromatography. (a) Control without (—) and with (---) alkali treatment. (b) E-xyl-treated cells. Pooled material from the first (····) and second (----) peaks of medium from E-xyl-treated cells was treated with alkali and re-run on the column. (c) HX-xyl-treated cells.

^{35}S -labelled material recovered from U-937 cultures and eluting in the void volume after Sepharose CL-6B gel chromatography is of PG nature, as has been demonstrated previously (Kolset, 1987). The second dominant peak in the control cell fraction represents partially degraded PG (S. O. Kolset, unpublished work), and is not released, in accordance with previous results (Kolset *et al.*, 1988a).

After a 24 h incubation of the cells in the presence of $^{35}\text{SO}_4$, the control cells released only CSPG, whereas the E-xyl-treated cells released both a small portion of intact CSPG and a large portion of smaller CSPG and CS (see below). In contrast, the HX-xyl-treated cells released only free GAG chains, with a smaller molecular size than those in the E-xyl-treated cultures, most probably due to

the higher rate of synthesis in the former cultures (Fig. 3). In order to investigate further the nature of the ^{35}S -labelled macromolecules expressed by E-xyl-treated cells, the first and the second peaks (Fig. 3) were separated by preparative Sepharose CL-6B gel chromatography and subjected to alkali treatment. In this separate experiment the first peak eluted in the V_0 fractions and the second peak eluted with a K_{av} of 0.35. After alkali treatment, the V_0 material was shifted to an elution position almost identical with that of the second peak. The second peak, however, was also shifted somewhat in elution position, from a K_{av} of 0.35 to one of 0.40 (see Fig. 3b), showing that these molecules are still attached to protein. Consequently, E-xyl-treated cells synthesize large and small PGs, and presumably some free GAG chains. The elution position of the ^{35}S -labelled macromolecules synthesized by U-937 cells after HX-xyl treatment (Fig. 3c) were not shifted after alkali treatment. The molecular mass of these CS chains would be approx. 18 kDa, whereas those synthesized in the presence of E-xyl would be in the range 35–46 kDa (Kolset *et al.*, 1986). In contrast, the molecular mass of the chains in the control CSPG, eluting with a peak K_{av} of 0.27, is approx. 60 kDa (Fig. 3a). Consequently, it is possible that the treatment of the cells with E-xyl leads to a partial shift in biosynthesis from CSPG to a mixture of intact and small CSPG and free CS chains. However, exposure of U-937 cells to 1 mM-HX-xyl leads to an efficient abrogation of CSPG biosynthesis, resulting in the exclusive expression of CS chains during the entire 4 days' incubation period. It is only when this latter distinct effect on the PG biosynthesis was prevailing that the anti-proliferative effect of the β -D-xylosides was observed.

High levels of PG/GAG and proliferation

Several clones of the U-937 cell line are now available, and PG biosynthesis has recently been studied in one of these, U-937-4 (Kolset *et al.*, 1988b). This clone also expresses CSPG only, and it has been noted that U-937-4 cells express CSPG at a higher level than the parent U-937 line (S. O. Kolset, unpublished work). We have made use of this particular phenomenon, and compared the effect of β -D-xylosides on the expression of PG and proliferation in U-937 and U-937-4 cells.

Both U-937 and U-937-4 cells were labelled with $^{35}\text{SO}_4$ in short-term (60 min) and long-term (96 h) experiments in the presence of 1.0 mM-E-xyl or 1.0 mM-HX-xyl. Fig. 4 shows that the baseline synthesis of PG/GAG in U-937-4 cells was approx. 10 times higher (cell and medium total) than in U-937 cells. However, the level of PG/GAG synthesis is not particularly affected by xyloside treatment in the former, unlike the latter cell type. Although U-937-4 cells have a higher basal level of PG/GAG synthesis, the cells proliferate at approximately the same rate as U-937 cells and are equally inhibited by xyloside treatment, as is shown in Table 4. The levels of PG/GAG recovered from xyloside-treated U-937 cells, and from control and xyloside-treated U-937-4 cells are approximately the same, but proliferation is only inhibited in the xyloside-treated cells. This should argue against the notion that a high level of PG/GAG *per se* should form the basis of the decrease in proliferation observed after exposing both U-937 and U-937-4 cells to HX-xyl.

The possibility that a high level of GAG in the extracellular environment might influence the prolifer-

Fig. 4. Effect of β -D-xylosides on the incorporation of $^{35}\text{SO}_4$ into cell and medium fractions of U-937 and U-937-4 cells

Both cell lines were incubated without (C) or with 1 mM-E-xyl (E) or 1 mM-HX-xyl (HX). The incubation and labelling time was 96 h. ▨, Cell fractions; □, medium fractions.

ation of U-937 cells was also investigated by incubating the cells for 4 days in the presence of heparin or CS in concentrations ranging from 1 $\mu\text{g}/\text{ml}$ to 1 mg/ml . The proliferation of the cells was not affected by the presence of any of the concentrations of either of the two GAG preparations. A high level of GAG in the extracellular environment does not, therefore, in itself form the basis for the observed anti-proliferative effect of xylosides.

^3H GAGs synthesized by U-937 cells

It has been shown that the synthesis of hyaluronate is required in human fibroblasts during mitosis (Brecht *et al.*, 1986; Matuoka *et al.*, 1987). Such a regulatory role for hyaluronate in the U-937 cell system cannot be excluded, and stimulation of CSPG biosynthesis could affect the availability of precursors for such synthesis, with a possible decrease in hyaluronate synthesis followed by a decrease in proliferation. The biosynthesis of GAG was therefore investigated using ^3H glucosamine rather than $^{35}\text{SO}_4$ as a precursor in cells cultured in the presence of HX-xyl.

U-937 cells were labelled with ^3H glucosamine and the

Fig. 5. DEAE-Sephacel ion-exchange chromatography of medium and cell fractions from U-937 cells

U-937 cells were labelled with ^3H glucosamine, and the medium (M) and cell (C) fractions were processed as described in the Experimental Procedures section and separated by DEAE-Sephacel ion-exchange chromatography. The brackets indicate the column fractions pooled for further analyses. Broken line, cell fraction; solid line, medium fraction. Fraction no. 1 is the start of the NaCl gradient.

labelled macromolecules were subjected to preparative DEAE-Sephacel ion-exchange chromatography after proteolytic digestion of both medium and cell fractions from both control cultures and cells treated with 1 mM-HX-xyl. The ^3H -labelled material from control cells was eluted in two prominent peaks (C1 and C2) as can be seen in Fig. 5. The control medium fraction also contained two peaks, the later-eluting peak (M2) containing most of the material. When medium and cell fractions from HX-xyl-treated cells were subjected to the same protocol, almost identical elution profiles could be seen, except that most of the material was eluted in the M2 and C2 peaks (results not shown). Fractions indicated by the brackets in Fig. 5 were pooled. Corresponding fractions from HX-xyl-treated cells were also pooled (results not shown).

All of the fractions were subjected to chondroitin ABC lyase and *Streptomyces* hyaluronidase treatment. The C2 and M2 fractions from both control and HX-xyl-treated cells contained almost exclusively ^3H CS, as shown by their susceptibility to chondroitin ABC lyase treatment. None of the C2, M1 or M2 fractions were affected by hyaluronidase treatment (results not shown). Approx. 10–15% of both control and HX-xyl material from the C1 fractions was depolymerized, as shown in Table 5. Accordingly, the synthesis of hyaluronate in the U-937 cells is very low. Furthermore, the low percentage of

Table 4. Effect of β -D-xylosides on the proliferation of two different U-937 cell lines

The two cell lines were seeded at a density of $0.1 \times 10^6/\text{ml}$ and exposed to 1 mM-E-xyl or 1 mM-Hx-xyl for 4 days, and then counted. The numbers of control cells after 4 days incubation were $1.1 \times 10^6/\text{ml}$ and $1.3 \times 10^6/\text{ml}$ in U-937-4 and U-937 cell lines respectively.

Cell line	Xyloside...	Cell number (% of control)	
		E-xyl	HX-xyl
U-937-4		98	26
U-937		84	25

Table 5. Degradation of [3 H]glucosamine-labelled material from U-937 cultures with *Streptomyces* hyaluronidase

3 H-labelled material was separated by DEAE-Sephacel ion-exchange chromatography and the various fractions (see Fig. 5) were subjected to enzymic treatment and gel chromatography. Degradation was calculated from the amount of label eluting in the included volume of the Sephadex G-50 gel as the % of total label (excluded fractions + included fractions). Values in parentheses are c.p.m. eluted.

Fraction	Degradation (%) (c.p.m.)
Control C1	10–15 (9764–14645)
Control M1	5 (445)
HX-xyl C1	10–15 (4670–7004)
HX-xyl M1	5 (642)

Fig. 6. SDS/PAGE of cell fractions from control and HX-xylose-treated U-937 cells

Cells were cultured in the absence and presence of 1 mM-HX-xylose for 1 day, and then labelled with [3 S]methionine for 4 h. The cell fractions were analysed by SDS/PAGE. Lanes a and b, control cells; lanes c and d, HX-xylose-treated cells. Lanes a and c are unreduced samples, and lanes b and d are reduced samples.

hyaluronate in the cell fractions (C1) does not seem to be influenced by xyloside treatment of the cells.

Effect of HX-xylose on protein synthesis

In order to investigate whether the exposure of U-937 to 1 mM-HX-xylose for 1 day would affect parameters other than PG biosynthesis, the cells were labelled with [3 S]methionine and the labelled products were analysed by SDS/PAGE. As shown in Fig. 6, the proteins synthesized by U-937 cells both in the absence (lanes a and b) and presence (lanes c and d) of H-xylose were almost identical, with some labelled proteins being more strongly expressed after xyloside treatment.

Effect of β -D-xylosides on the differentiation of U-937 cells

PMA has been demonstrated to inhibit cell proliferation and concomitantly to induce differentiation of U-937 cells into macrophage-like cells, by such criteria as adherence to culture dishes, spreading, display of macrophage-like morphology and new cell-surface antigen (Nilsson *et al.*, 1980; Gidlund *et al.*, 1981; Kolset *et al.*, 1988b). Cells exposed to E-xylose or HX-xylose did not adhere to or spread on the culture dish, even after 4 days' incubation *in vitro*. If, however, the cells cultured in the presence of E-xylose or HX-xylose for 4 days were exposed to 50 ng of PMA/ml, they were found to adhere and acquire macrophage-like morphology in a similar fashion to control cells not exposed to xylosides. Xyloside-treated cells do, therefore, have the capacity to differentiate into macrophage-like cells in the presence of PMA. Furthermore, in immunofluorescence studies, strongly OKM-1-positive cells could only be detected after treatment with PMA. Neither E-xylose nor HX-xylose promoted the expression of this particular antigen (see Table 6) which is normally present on monocytes and macrophages. The differentiation of U-937 cells into macrophage-like cells in the presence of PMA was further corroborated by demonstrating the disappearance of the T4 antigen on the cell surface. In contrast, exposure of the cells to HX-xylose and E-xylose did not lead to such a change; the cells still expressed this antigen after 4 days' incubation with either xyloside (Table 6). The decrease in cellular proliferation observed in the presence of 1 mM-

Table 6. Effect of β -D-xylosides on the expression of cell-surface antigens in U-937 cells

Approx. 100 cells were counted on each cover slip and the percentage of positive cells is given. The relative fluorescence-intensity is given in parentheses, from non-existent (–) to strongly fluorescence-positive cells (+++). The monoclonal antibody OKM-1 is directed against the C3b receptor, and OKT-4 against the T4 antigen.

Treatment	Antibody...	Immunofluorescence (% of cells positive)	
		OKM-1	OKT-4
Control		67 (+)	50 (++)
1 mM-E-xylose		38 (+)	57 (++)
1 mM-HX-xylose		45 (+)	64 (++)
PMA (50 ng/ml)		94 (++++)	– (–)

HX-xyl did not, accordingly, lead to differentiation of the monoblastic cells by the above-established criteria.

DISCUSSION

The present study shows that HX-xyl is effective as an inhibitor of cell proliferation at much lower concentrations than the other xylosides employed, and without causing any effects on cellular viability. It was also possible to demonstrate that the inhibitory effect was reversible, and, furthermore, that HX-xyl-treated cells were able to differentiate into macrophage-like cells after exposure to PMA. Accordingly, the data suggest that the anti-proliferative effect of 1 mM-HX-xyl is not due to toxic or other non-specific effects. In addition to the data presented here, a good control of possible non-specific effects would be to use hexyl α -thioxyloside, but we have so far not been able to synthesize such a xyloside. However, another substituted monosaccharide, phenylethyl β -D-galactoside, one of the few galactosides found to stimulate GAG biosynthesis to a significant extent (Robinson & Robinson, 1981) has been tested, and did not affect cell proliferation or PG biosynthesis in concentrations up to 1 mM (S. O. Kolset, unpublished work).

Treatment of U-937 cells with 1 mM-HX-xyl led to highly elevated levels of CS in both the cell and the medium fractions. Addition of 1 mg of CS or heparin/ml to the cell cultures did not affect proliferation, and should indicate that an elevated level of CS, at least in the extracellular compartment, is not an important factor in the events leading to decreased proliferation. Furthermore, the U-937-4 cell line, synthesizing 10 times more CSPG/CS than U-937 cells, proliferated at the same rate, and was equally inhibited after HX-xyl treatment. The difference in effect between HX-xyl and E-xyl, on both cellular proliferation and PG biosynthesis, could indicate that the synthesis of intact CSPG molecules is important for the continuous proliferation of the U-937 cells. The biological basis for such an assumption is not known, and we cannot at present offer an explanation as to the mechanism with which the abrogation of PG biosynthesis might possibly be related to the inhibition of cellular proliferation. PGs are known to interact with a large number of macromolecules (Lindahl & Höök, 1978), and the presence of free CS chains only, along with the absence of intact CSPG in this particular cell system, may inhibit interactions between factors that are fundamental for the regulation of proliferation, as has been shown for heparan-sulphate-binding factors promoting the growth of haematopoietic cells (Roberts *et al.*, 1988). Furthermore, GAG chains attached to exogenous xyloside may be sorted to other intracellular compartments in this particular cell than when attached to endogenous xylose and protein, and might thereby interfere with processes affecting the regulation of proliferation. Such an assumption would indicate a biological effect, and not a function of endogenous PG/GAG. If this were the case, it would consequently grant us the opportunity to use β -D-xylosides as an experimental tool to investigate the nature of regulatory factors other than PG/GAG which are involved in the regulation of proliferation.

Xylosides have been used in various experimental systems to investigate the possible biological function(s) of PGs. Branching morphogenesis of em-

bryonic salivary glands was reversibly inhibited by β - and not α -xylosides (Thompson & Spooner, 1983). Furthermore, abnormal development of sea-urchin embryos (Kinoshita & Saiga, 1979), and chick embryos (Gibson *et al.*, 1978) was observed after exposure to xylosides. The latter experimental system (Sobue *et al.*, 1987) was also used when studying the effects of varying the aglycone groups attached to the β -D-xylosides, and clearly demonstrated that the inhibitory effects observed on chick embryogenesis were related to the specific effect of xylosides on PG biosynthesis. β -D-Xylosides with relatively small effects on PG biosynthesis had virtually no effect on embryogenesis (Sobue *et al.*, 1987). Relevant also to the results presented here is the demonstration that human T-cells are growth-inhibited when cultured in the presence of xylosides (Christmas *et al.*, 1988). Furthermore, a factor that stimulates B-cell proliferation and differentiation has been shown to co-purify with a T-cell CSPG (Levitt & Olmstead, 1986). The present investigation shows that β -D-xylosides cause effects leading ultimately to a decrease in the capacity of cells to proliferate. The availability of β -D-xylosides with large differences in their effects on cellular proliferation has provided us with tools to study possible functions or effects of endogenous PG/GAG in relation to the regulation of proliferation.

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