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Serglycin and secretion in human monocytes

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Abstract The human monocytic cell line U-937 has been widely used as a model system for human monocytes. The subclone U-937-B has been adapted to serum-free conditions. This particular U-937 clone and its parent clone U-937-1 were used to investigate the role of the proteoglycan serglycin in human monocytes. For this purpose cells were treated with hexyl-β-D-thioxyloside to abrogate proteoglycan expression. U-937-B cells expressed and secreted exclusively chondroitin sulphate proteoglycans, and after treatment with this xyloside they only expressed and released free chondroitin sulphate chains. Western blotting showed that serglycin core protein was present in conditioned medium of control cells, but absent in medium from xyloside-treated cells. Also, serglycin core protein could be detected in the cell fractions of control cells, but not in the cell fractions from xyloside-treated cells. Furthermore, less proteoglycan-associated proteins could be detected in medium from cells incubated with xyloside, suggesting that the absence of secreted sergycin affects the secretion of such proteins. Cells incubated in the presence of xyloside were analyzed by transmission electron microscopy and shown to contain numerous large empty vesicles. The lack of serglycin, the dominant proteoglycan in U-937 monocyte-like cells, consequently, leads to effects on vesicle formation and secretion of some low molecular weight proteins, suggesting that this particular proteoglycan is of importance for secretory processes in human monocytes.

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Introduction

Proteoglycans (PGs) are highly diverse molecules with a series of functions, both on cell surfaces and in the extracellular matrix [4, 7]. Furthermore, PGs are also located in intracellular compartments and have been shown to have important functions [11] (and references therein), which have been shown for the PG serglycin in mast cells and cytotoxicT-lymphocytes (CTLs). In these cells the maturation and functions of the storage granules depend on the expression of serglycin [1, 5]. In human macrophages it has been demonstrated that serglycin is the major secreted PG [22, 24]. Furthermore, serglycin secreted from macrophages has the ability to interact with other secretory products of macrophages, such as macrophage inflammatory protein-1α and lysozyme [10]. The secretion of PGs from macrophages is linked to inflammatory processes, as it has been shown to increase when the cells are exposed to activating agents such as interferon-γ, lipopolysaccharide and platelet factor 4 [20, 21].

In hematopoietic cells with storage granules, such as mast cells and CTLs, serglycin is important both for the maturation of the granules and for the interaction with and regulation of partner molecules before and after secretion [1, 5]. Monocytes and macrophages do not contain storage granules. Using histochemistry and transmission electron microscopy with X-ray analyses it was possible to demonstrate that mast cells, but not monocytes, harboured heparan sulphate and sulphur-containing intracellular granule, respectively [9, 19]. PGs expressed by monocytes, including serglycin, are for the most part constitutively

secreted. Furthermore, secretion is increased after inflammatory stimuli [11, 20]. To gain further understanding of the role(s) of serglycin in monocytes and macrophages, one approach would be to investigate if and how serglycin could be involved in secretion and vesicle formation.

One widely used set of tools in PG research has been the β-D-xylosides. With these compounds, which can be used with different aglycone groups, it is possible to "uncouple" PG biosynthesis by interfering with the glycosaminoglycan (GAG) polymerization onto xylosylated core proteins [12] (and references therein). The effects of xylosides depend on cell type, aglycone groups of the xyloside and the concentrations used. In some cell systems the result can be a complete abrogation of PG biosynthesis and generation of free GAG chains. Most xylosides available, are efficient as abrogators of chondroitin sulphate biosynthesis [12], whereas heparan sulphate biosynthesis is less affected, although there are some reports to that effect [2, 14].

To investigate the possible role of serglycin in secretory processes in U-937 cells we have used hexyl-β-D-thioxyloside (HX-xyl), previously used to show that PGs expressed by U-937 cells are important for the regulation of proliferation. We have, furthermore, made use of a clone of U-937 cells adapted to serum-free conditions, U-937-B. The use of this cell line made it possible to identify, by N-terminal sequencing, serglycin as the major secreted PG in these cells [22]. It is further a useful system to study secreted products from the cells, without the interference of serum proteins. In this report we show that serglycin secretion is inhibited after xyloside treatment and that vesicle formation and secretion of several proteins are also affected.

Materials and methods

Cells

U-937-B cells and the parent clone U-937-1 were provided by Dr. Kenneth Nilsson, Department of Pathology, Uppsala University, Uppsala, Sweden. U-937-B cells were cultured in RPMI 1640 medium (Sigma, St. Louis, USA) and split in a ratio of 1:10 twice a week. Before experiments the cells were established in 75 cm^2 flasks at a density of 0.2×10^6 cells/ml and cultured with and without 0.5 mM HX-xyl for one to 3 days. Thereafter the conditioned media were harvested, centrifuged at 1,000 rpm (350×*g*) for 10 min and frozen before further analyses. U-937-1 cells were cultured in the same medium, but with 10% fetal calf serum included. At the onset of experiments these cells were washed three times with serum-free medium by centrifugation and thereafter cultured in RPMI 1640 medium without serum, and with or without 0.5 mM HX-xyl for 24 and 48 h. Medium fractions were then harvested after centrifugation at 350×*g* for 10 min and frozen. Cell pellets were solubilzed in 50 mM Hepes buffer, pH 7.5, containing 0.15 M NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2 mM EDTA, 1 mM EGTA, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mM NaF and 1 mM DTT. Protease inhibitor cocktail (Roche, Mannheim, Germany) was added fresh before each experiment, according to instructions from the supplier. The obtained cell fractions were frozen before further analysis.

Proteoglycans

To measure PG biosynthesis and secretion U-937-B cells were established at a starting density 0.5×10^6 cells/ml and cultured for 24 h in the presence of 100 μCi/ml of [^{35}S]-sulphate. U-937-B cells grow and proliferate in suspension and do not adhere to the culture dish. Pilot studies revealed that [^{35}S]-sulphate was incorporated almost exclusively into PGs, both in the recovered cell and the medium fractions (Kolset, unpublished observation). After labelling the medium fractions were centrifuged at 350×*g* for 10 min and transferred to separate tubes. The cell pellet was either frozen directly or solubilized in 4 M guanidine with 1% Triton X-100. [^{35}S]-macromolecules from medium and cell fractions were isolated by Sephadex G-50 Fine (Amersham Biosciences, Uppsala, Sweden) gel chromatography and analyzed further as previously described [22, 25].

[^{35}S]-labelled PGs purified with the same method as described in the "Proteoglycan binding proteins" section were subjected to sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) using 5–13% gradient gels with 4.5% in the stacking gel. Medium samples were run both prior to and after treatment with chondroitinase ABC (C-ABC, Seikagaku Kogyo Co, Tokyo, Japan). [^{14}C]-radiolabelled molecular weight markers were from Amersham Biosciences.

U-937-B cells were labelled with [^{35}S]-sulphate both in the absence and presence of 0.5 mM HX-xyl, a concentration previously shown to lead to complete abrogation of PG biosynthesis [12].

Proliferation

Cell proliferation was assayed by measuring the incorporation of [^{3}H]-thymidine, as has been described [12].

Proteoglycan binding proteins

To analyze for proteins expressed by U-937-B cells with ability to interact with PGs, conditioned media from both control and HX-xyl- treated cells were collected every 2 days from large culture flasks. The media were centrifuged, frozen at −20°C and stored before further

analyses. In each experiment volumes of 1,500–3,000 ml conditioned media were subjected to DEAE (Amersham Biosciences) ion exchange chromatography. The columns were run in Tris-HCl, pH 8.0, with 0.2 M NaCl and 0.1% Triton X-100. After the samples had been applied the column was washed extensively in this buffer, whereafter it was washed in 0.05 M acetate-buffer, pH 4.0, with 0.35 M NaCl and 0.1% Triton X-100. To elute bound material a linear gradient of 0.35 to 1.5 M NaCl (in the same acetate buffer) was applied. Fractions of 1 ml were collected and the content of PG/GAGs in the different fractions was determined by the carbazole reaction [3]. In some experiments purified [^{35}S]PG was added as tracer, and aliquots of fractions from both ion exchange and gel chromatography were analyzed for radioactivity. Fractions containing radioactivity were then pooled for further analysis. PG and GAG containing fractions were pooled and subjected to Superose 6 (Amersham Biosciences) gel chromatography in 4 M guanidine. Fractions containing PGs and GAGs were pooled and dialysed against distilled water with a cocktail of protease inhibitors. Finally, control and xyloside medium material was freeze-dried and resuspended in PBS with protease inhibitors and used for SDS-PAGE.

Conditioned media from control cells and cells treated with HX-xyl were subjected to serglycin affinity chromatography, as previously described [10]. The volume of conditioned medium used for affinity chromatography was adjusted for the difference in cell number in the two culture systems. Material (approximately 50–70 ml) was loaded to the column and the column was washed with PBS, thereafter with PBS with 0.25 M NaCl. Finally, material was eluted with 1 M NaCl. All solutions used contained 1 mM PMSF. After elution, control and xyloside material (1 M NaCl fractions) were dialyzed against distilled water at 4°C, subjected to concentration by speedvac centrifugation and thereafter directly used for SDS-PAGE. The dialysis tubing used had a molecular weight cut-off of 3 kDa. The concentrated material was solubilized in loading buffer and subjected to 4–20% SDS-PAGE gradient gel electrophoresis. After electrophoresis the gels were silver stained, dried and photographed.

Western blotting

Cell fractions and conditioned media from cells incubated with and without xyloside were desalted, freeze-dried and treated with C-ABC for 2 h to remove CS chains in the presence of protease inhibitors [25]. Samples were thereafter treated with 2-mercaptoethanol and separated on SDS-PAGE using 12.5% polyacrylamide gels. The separated proteins on the gels were transferred to polyvinylidene fluoride (PVDF) membranes by Western blotting and probed with a rabbit polyclonal antibody to human serglycin, which was a kind gift from Dr. N. Borregaard and Dr. C.U. Niemann, Rigshospitalet, Department of Haematology, Copenhagen, Denmark. Bound antibody was detected using a peroxidase-linked secondary antibody followed by chemiluminescence detection. Protein molecular weight standards were from Amersham Biosciences.

In some experiments PG-associated proteins from U-937-B conditioned media were analyzed by SDS-PAGE after having been subjected to ion exchange chromatography and gel chromatography, as described above. After electrophoresis the gels were silver stained (Bio-Rad). Protein bands were scanned and quantified using Epson 1680 PRO scanner (Epson Norway, Oslo, Norway) and Scion Image (Scion, Frederick, MD, USA) quantification program.

Transmission electron microscopy

U-937-B cells were cultured for 24 h with and without 0.5 mM HX-xyl. The cells were pelletted by centrifugation at 350×*g* for 10 min. Both cell preparations were thereafter fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, and postfixed in 1% OsO_4 in the same buffer as above. The cells were further prepared as previously described [9] and finally viewed in a JEM 1200 EX transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Results

U-937-B cells were labeled with [^{35}S]-sulphate and [^{35}S]-macromolecules were recovered after gel chromatography of the medium fraction. After SDS-PAGE both in the absence and presence of C-ABC, an enzyme depolymerizing chondroitin sulphate (CS) it was evident that only CSPG was released from the cells, as can be seen in Fig. 1. This is in accordance with what has been shown in the parent cell line U-937-1 [8, 9]. Cells exposed to HX-xyl secreted only free CS chains (result not shown). In addition, cell fractions from HX-xyl treated cells contained only free CS chains, whereas control cells contained CSPG and CS, also in accordance with previous results from parent U-937-1 cells [12].

It has previously been demonstrated that the predominant PG in the culture medium of U-937-B cells is serglycin, as shown by N-terminal sequencing of medium PG purified from this clone [22]. To further investigate this, U-937-B cells were cultured both in the absence and presence of HX-xyl for 3 days. In addition, the parent clone U-937-1 cells were cultured 24 or 48 h under serum-free conditions with and without HX-xyl. The medium and cell fractions were recovered, and subjected to Western

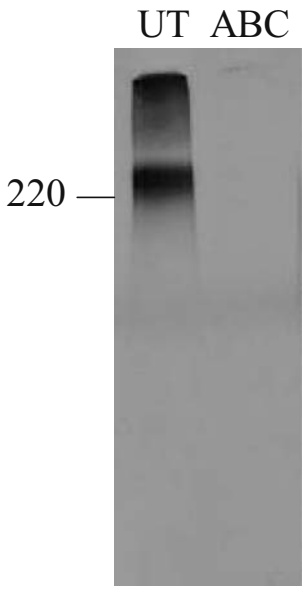

Fig. 1 SDS-PAGE of [^{35}S]-PG from U-937-B cells. [^{35}S]PGs were isolated from medium of U-937-B cells after labelling with [^{35}S]-sulphate and subjected to SDS-PAGE gel electrophoresis on a 5–13% gradient gel. Samples were run untreated (*UT*) and after C-ABC treatment (*ABC*). The migration of the 220 kDa high molecular weight marker is indicated on the left side

blotting, using an antibody against the core protein of human serglycin. Prior to Western blotting the medium fractions from both control cells and HX-xyl treated cells were incubated with C-ABC, to expose core protein epitopes. From Fig. 2 it is evident that U-937-1 cells secreted serglycin, as the core protein could be detected in the conditioned medium of control cells. In contrast, the conditioned medium of U-937-1 cells treated with HX-xyl did not contain serglycin, showing that xyloside treatment had abrogated the expression and secretion of this particular PG. The data presented show that the serglycin core protein could be detected in two forms, with molecular weights of approximately 35 and 20 kDa, most probably due to differences in intracellular processing. In conditioned medium from the serum-free clone U-937-B only the 35 kDa form could be detected (result not shown). U-937-1 cells were used to investigate if xyloside treatment would result in accumulation of serglycin core protein in the cell fractions of HX-xyl treated cells, as serglycin could not be detected in the medium of cells subjected to this treatment.

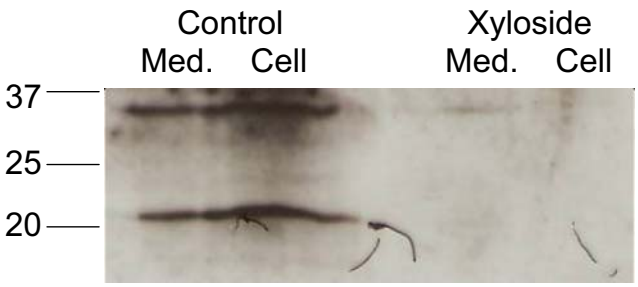

Fig. 2 Western blotting of conditioned media and cell fractions from U-937-1 cells. Conditioned media and cell fractions from control cells and HX-xyl treated U-937-1 cells were subjected to Western blotting after treatment with C-ABC, using an antibody against human serglycin. The migration positions of molecular weight markers (in kDa) are shown on the *left side of the panel*

Surprisingly, this was not the case. In cell fractions from HX-xyl treated U-937-1 cells it was not possible to detect any serglycin core protein, whereas the cell fraction from control cells clearly contained detectable amounts of the same protein, as can be seen in Fig. 2.

We have previously shown that the parent U-937-1 cells decreased their proliferation after treatment with the same xyloside as used in this study [12]. To investigate if the absence of secreted serglycin in this serum-free adapted clone would affect the proliferation we used [^{3}H]-thymidine incorporation assay in both control and HX-xyl treated cells. As can be seen in Table 1 the proliferation of U-937-B cells decreased to 32% of that of control cells. The expression and secretion of serglycin may, therefore, possibly be of importance for the autocrine regulation of proliferation in this U-937 clone adapted to serum-free conditions.

Monocytes and macrophages have for long been known to have a large capacity for secretion, of *e.g.* cytokines, enzymes and protease inhibitors [15]. We have previously shown that secretion of urokinase and matrix metalloproteinase-9 in macrophages is dependent on PGs [18]. Here, we wanted to make use of the serum-free U-937-B cells to analyze the secretory repertoire by focusing on the secreted PGs. Our aim was to investigate whether the lack of secreted PGs after HX-xyl treatment would affect the secretion of other proteins, with emphasis on the proteins binding to PGs. For this purpose we purified PGs and GAGs from conditioned media of control and HX-xyl treated cells. Thereafter, the obtained material was subjected to SDS-PAGE prior to and after C-ABC treatment. As can be seen in Fig. 3 it is evident that there are more proteins binding to the PGs recovered from control cells, than to the xyloside initiated GAGs released to the medium of HX-xyl treated cells, in particular those with molecular weights below 45 kDa. The gels were analyzed by scanning densitometry and the amount of stained proteins below 45 kDa was approximately four times higher in the medium fractions from control cells compared to medium from xyloside treated cells. The expression and secretion of PGs, is accordingly, linked to secretion of several more proteins, compared to when PG biosynthesis is abrogated. To further investigate this we subjected conditioned media from control and HX-xyl treated U-937-B cells to affinity chromatography on serglycin-Sepharose. Material eluting

Table 1 Proliferation in U-937-B cells as measured by [^{3}H]-thymidine incorporation (shown is one of three representative experiments)

Cells	[^{3}H]-thymidine incorporation—cpm (% of control)		
Control	8092	+/−33	(100%)
HX-Xyl	2623	+/−126	(32%)

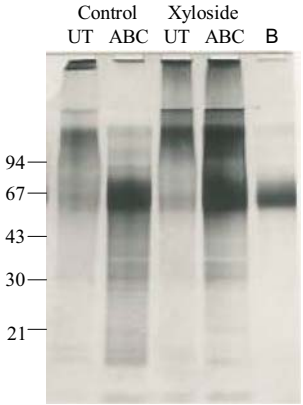

Fig. 3 SDS-PAGE of conditioned media purified by ion exchange chromatography. Conditioned media from control and HX-xyl treated U-937-B cells were purified by DEAE ion exchange chromatography and subjected to SDS-PAGE, both prior to (*UT*) and after C-ABC treatment (*ABC*). B denotes the enzyme buffer with enzyme included, but without test sample. The migration positions of the molecular weight markers (in kDa) are shown on the *left side of the panel*

at high ionic strength was subjected to SDS-PAGE and silver staining. Medium from control cells contained several more serglycin-binding proteins than corresponding medium from HX-xyl treated cells, as can be seen in Fig. 4. This is particularly evident for proteins with molecular weights lower than 45 kDa.

Monocytes and macrophages do not belong to the category of hematopoietic cells that contain storage granules [9, 19], which is the case for *e.g.* mast cells [9, 11]. The lack of serglycin in the latter cell type, had dramatic effects on formation and morphology of these granules [1]. To investigate whether the lack of secreted serglycin, which was evident after xyloside treatment (see Fig. 2), would have effects on vesicles or other intracellular compartments in human monocytes, both control and HX-xyl treated cells were analyzed by transmission electron microscopy. From Fig. 5a–c it is evident that HX-xyl treatment of U-937-B cells led to accumulation of vesicles that are not electron dense, both in the perinuclear area and closer to the plasma membrane (indicated by large and small arrows, respectively, for HX-xyl treated cells in Fig. 5b). It is therefore possible that the lack of serglycin affects the formation of secretory vesicles, and that the lack of serglycin leads to formation of enlarged intracellular vesicles.

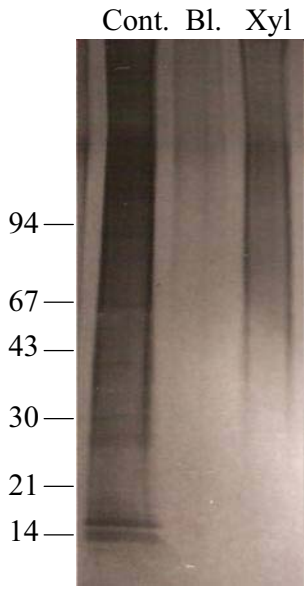

Fig. 4 SDS-PAGE of conditioned media after serglycin affinity chromatography. Conditioned media from control (*Cont.*) and HX-xyl (Xyl) treated U-937-B cells were subjected to serglycin affinity chromatography. Material eluting with 1 M NaCl was subjected to SDS-PAGE and silver staining. *Bl.* is 1 M NaCl fraction of a blank run on the column The migration positions of molecular weight markers are shown on the *left side of the panel*

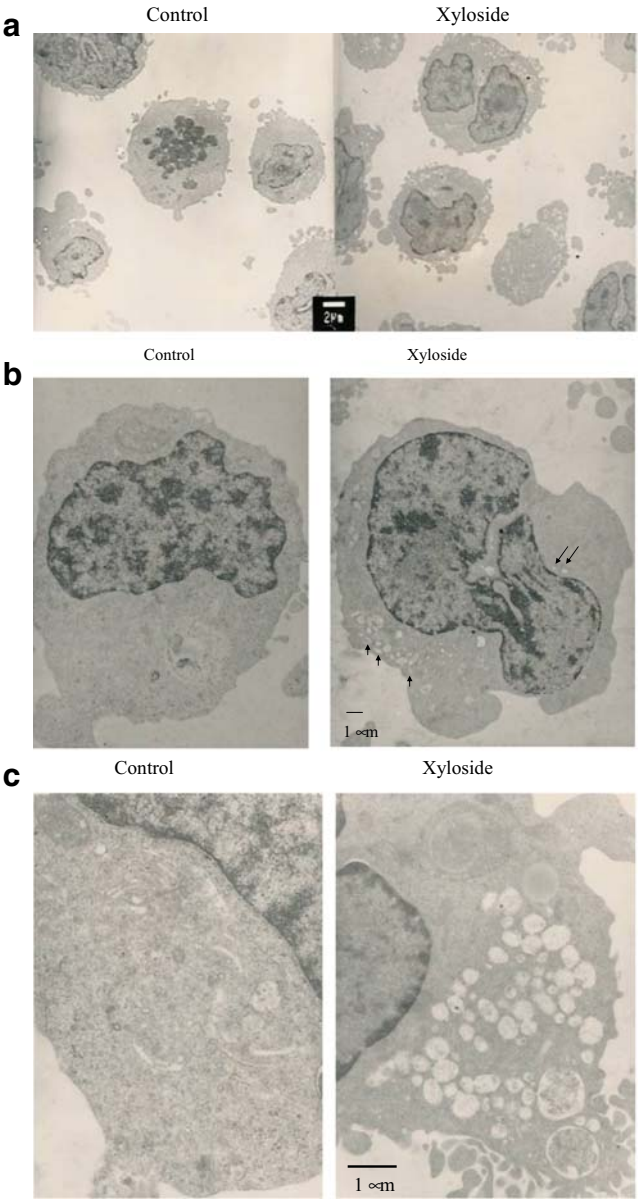

Fig. 5 a–c Transmission electron microscopy. U-937-B cells were cultured for 3 days in the absence and presence of 0.5 mM HX-xyl and thereafter fixed and prepared for electron microscopy. Pictures were taken at different magnifications. In the xyloside treated cells perinuclear vesicles (*large arrows*) and vesicles close to the plasma membrane (*small arrows*) are shown in b

Discussion

This investigation has confirmed previous observations that the human monocytic cell line U-937 secretes serglycin. The previous observation was based on N-terminal sequencing of purified PGs from the conditioned medium of U-937-B cells [22], suggesting that serglycin is the major secretory PG from monocytes. Here, we have made use of an antibody raised against the core protein of human serglycin [16, 25] and were able to detect the serglycin in conditioned medium from the serum-free adapted U-937-B cells and the parent cell line U-937-1. Interestingly, we were further able to show that serglycin could no longer be detected in the harvested conditioned medium when the PG biosynthesis had been abrogated with xyloside. Furthermore, we could not detect serglycin core protein in the cell fractions of HX-xyl treated cells. This result may suggest that serglycin core protein is subject to rapid degradation when the biosynthetic polymerization of GAG chains is inhibited with exogenously added xyloside, in this particular cell system. This is an interesting issue with relevance to regulation of PG biosynthesis in general, and should be the subject of more detailed studies.

In addition, fewer proteins were recovered from GAGs released from cells treated with xyloside than from PGs released by control cells, and less secreted proteins were able to bind to immobilized serglycin. These results suggest that after xyloside treatment there is no synthesis and secretion of serglycin PG with GAG chains attached. This again affects the secretion of several proteins that are normally associated with serglycin. Judging from the electron microscopy pictures the lack of serglycin also affected the formation of intracellular vesicles. The number and size of vesicles increase in xyloside treated cells, suggesting that the normal route of constitutive secretion is affected.

Monocytes and macrophages do not form storage granules [9, 19] as do the mast cells [9]. However, in mast cells lacking serglycin the maturation of storage granules is affected [1], leading to the formation of numerous empty vesicles. It therefore seems as if the generation of both storage granules in mast cells and secretory vesicles in monocytes depend on the expression of serglycin. In monocytes there may exist several types of secretory vesicles, some of which must be independent of serglycin, as secretion of a wide range of proteins is not inhibited by the lack of this PG. However, it will be of great interest in future experiments to identify proteins associated with serglycin. From the data presented it is evident that some of the proteins secreted from these cells can be involved in the regulation of autocrine cell growth, as xyloside treatment inhibited cell proliferation, measured by [^{3}H]-thymidine incorporation.

From a previous investigation it has been established that serglycin binds chemokines secreted from macrophages [10]. Several proteins with molecular weights below 45 kDa were absent in the medium from xyloside treated cells (Fig. 3). Both growth factors, cytokines and chemokines are possible candidates, as several of these are also PG-bindings proteins [6, 13]. A second class of potential candidates is the proteases. We have shown that the secretion of urokinase and matrix metalloproteinase-9 depend on PG biosynthesis in macrophages [18]. Such molecules have been shown to be of importance in inflammatory reactions [17, 23].

Identifying molecules interacting with serglycin will be of importance both for a further understanding of serglycin in relation to macrophage functions and formation of secretory vesicles. At present there is limited information on the role of serglycin in monocytes and macrophages. The data presented show that further studies using the monocytic cell lines U-937-B and U-937-1 and xylosides can be useful tools for such investigations.

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